THE CONTRACTILE SYSTEM OF HYDRA, AS STUDIED WITH SAPONIN-EXTRACTED MODELS AND ELECTRON MICROSCOPY

Barbara R. Beitch, A. B.

1968

Samuel P. Maroney
Major Professor

Dietrich Bodenstein

Starling Reed
Prof. of Psych.

Robert L. Searls

Gerald Robison, Jr.

P. D. McKinney

A Dissertation

Presented to the Graduate Faculty

of the

University of Virginia

in

Candidacy for the Degree of Doctor of Philosophy

TABLE OF CONTENTS

page

LIST OF ABBREVIATIONS AND SYMBOLS APPEARING IN TEXT

ATP	adenosine triphosphate
C I	a type of contraction, in which the cell has become round or spherical
C II	a type of contraction, in which the basal processes have constricted
χ^2	Chi-square (see Snedecor, 1956)
EDTA	disodium ethylenediaminetetraacetate
EGTA	ethylene glycol bis (amino ethylether) tetraacetic acid
pX	$-\log_{10} [X]$, where X is either an ion or a non-ionized molecule, the concentration of which is expressed in moles per liter
pSal	$-\log_{10}$ [Salyrgan]
Salyrgan	Mersalyl, sodium salicyl-(α -hydroxymercuri-β -methoxypropyl)- amide-O-acetate
SD	Standard deviation (see Snedecor, 1956)
SE	Standard error (see Snedecor, 1956)
SR	sarcoplasmic reticulum
T-system	transverse system
$\bar{X}$	mean (see Snedecor, 1956)

The Contractile System of Hydra, as Studied with Saponin-Extracted Models and Electron Microscopy

INTRODUCTION

Motile systems have been categorized by Hoffmann-Berling (1960) and H.H. Weber (1958) on the basis of the action of ATP on various types of movement. The means by which ATP manifests its effect on motility is usually, but not always, by stimulation of contraction. Although this is the nature of its action on movement of most cells, including higher muscle fibers (see Szent-Gyorgyi, 1953), ameba (see Hoffmann-Berling, 1960; Simard-Duquesne and Couillard, 1962), and dividing fibroblast cells (Hoffmann-Berling, 1953, 1954; Hoffmann-Berling and H.H. Weber, 1953), there exist systems in which ATP stimulates elongation (e.g., the spindle during mitosis; Hoffmann-Berling and Portzehl, 1955). Still other movements are inhibited or reversed by ATP, including the contraction of vorticella stalks (Hoffmann-Berling, 1958) and the elongation of trichocysts of certain ciliates (see Hoffmann-Berling, 1960; H.H. Weber, 1958). Finally, rhythmic movements of flagella and cilia are activated by ATP (Hoffmann-Berling, 1954, 1959, 1960); here it cannot be determined whether ATP is activating the contractile or the relaxing phase of the movement.

Contraction in hydra, a member of the most primitive group of multicellular organisms (the Coelenterates) capable of coordinated movement, is the subject of this investigation. Hydra bears functional similarities in its rapid, nerve-conducted epidermal shortening to vertebrate striated, skeletal

muscle, whereas its slow, peristaltic gastrodermal elongation is functionally similar to movement of smooth, visceral muscles (Mueller, 1950). On the other hand, the periodic, spontaneous contractions of hydra superficially resemble those of vorticella (Jennings, 1906, p. 189 to 190). The question of whether hydra movement should be classed with higher types of muscle or rather with more primitive contractile systems was the original impetus for this investigation. Another feature of this organism which stimulated the present study is its epidermal and gastrodermal myo-epithelial (or epithelio-muscular) cells, ectodermally and entodermally derived, respectively. Each such myo-epithelial cell consists of a basal contractile region, contiguous with the remaining epithelial portion of the cell. These myo-epithelial cells are in striking contrast to the unifunctional, mesodermally-derived muscle cells of most other metazoa.

In this study of the contractile system of hydra two major techniques have been employed, namely, extraction of cells to obtain cell models, and electron microscopy. This former, experimental means of analyzing mechanisms of contraction depends upon a permanent alteration of the functional organization of the plasma membrane of the contractile cell in order to make it freely permeable to the constituents of the external medium. At the same time, all soluble substrates, coenzymes, intracellular ions, etc. are washed out of the cell, leaving behind only its structural components, including large organelles such as the nucleus and mitochondria, and most important, the contractile system. The ionic environment of this system can then

be controlled experimentally, and movements resembling those of the corresponding living systems can be chemically stimulated. Such extracted cells are called fiber models, cell models, flagella models, etc. (Hoffmann-Berling, 1959, 1960; Hoffmann-Berling and H.H. Weber, 1953; Szent-Györgyi, 1949).

Most fiber and cell models are produced by extraction in 40 or 50% glycerol, the technique first developed by Szent-Györgyi (1949) with striated rabbit psoas and frog sartorious muscle fibers. Various modifications of his procedure were explored here, none of which yielded a satisfactory model of intact hydra or of hydra cells. The first phase of this study, then, was the development of a successful extraction technique, using either saponin or digitonin, surface-active agents first suggested by Hoffmann-Berling (1958, 1959) for use in making models. No mention was made by him of the rationale for using these agents, nor was any evidence presented that saponin- or digitonin- extracted systems met his criteria for a model. Such evidence will be presented here for the acceptability of these models with respect to basic physiological and morphological criteria.

A mechanism by which saponin (or digitonin) reacts with the cell membrane has been proposed by Lucy and Glauert (1964) and by Bangham and Horne (1962, 1964). The dimensions of the configurations they observed in electron micrographs of cell membranes and of phospholipid and cholesterol suspensions treated with saponin agree with their thesis that saponin interacts with cholesterol, thus stabilizing the "open"

(permeable, nonresistent, globular) configuration of the plasma membrane (see Kavanau, 1963, 1965; Lucy, 1964; Luzzati and Husson, 1962; Stoeckenius, 1962) rather than the otherwise stable "closed" (impermeable, resistent, lamellar) membrane configuration (Davson and Danielli, 1952). Taking into consideration these membrane alterations which saponin and digitonin are thought to cause, and also the criteria for models, the use of these agents to extract contractile cells is quite reasonable.

Once a contractile model of hydra was obtained, factors and parameters influencing motility could be considered, in particular, a system of interacting structural proteins (see Gergely, 1966) which are thought to generate movement (see Davies, 1963) by a combination of sliding (Huxley, 1957, 1960; Huxley and Hanson, 1960) and folding (Morales, 1959) in the presence of ATP and combinations of Mg^{2+} and Ca^{2+} (see Hasselbach, 1964; Hoffmann-Berling, 1959, 1960; Perry, 1956, 1960). This interaction is also influenced by pH and ionic strength and usually requires free sulfhydryl (-SH) sites on the proteins (Bailey and Perry, 1947). Thus, the second phase of the present study involves the roles of ATP, divalent ions, pH, ionic strength, and -SH groups in the contraction of hydra.

The final phase of this study is an electron-microscope study of features related to contraction in hydra. The suggested contractile apparatus of most flagella and cilia (Afzelius, 1959; Gibbons and Grimstone, 1960; Roth, 1958; Slautterback, 1963) and also the mitotic apparatus of dividing cells (see Roth and Daniels,

1962) is microtubular, while in many protists (see Nagai and Rebhun, 1966; Yagai and Shigenaka, 1963) and in vertebrate myo-epithelial cells (see Ellis, 1965; Tamarin, 1966) and smooth muscle (see Caeser, Edwards, and Ruska, 1957; Csapo, 1962; Schoenberg et al., 1966) it is characterized by thin filaments. A third morphological class includes metazoan striated muscle; here, a regular array of thick and thin filaments is revealed in electron micrographs (Huxley, 1957, 1960). Hydra's previously-mentioned functional resemblances to these morphologically heterogeneous systems stimulated the ultrastructural analysis in the present study.

MATERIALS AND METHODS

I. Routine for hydra stocks

Cultures of Hydra littoralis (Carolina Biological Supply Company) and Hydra viridis (strains from Carolina Biological Supply Company and from the laboratories of Drs. Robert P. Davis and Allison L. Burnett) were raised at 18°C in finger bowls, in hydra medium. This solution contained 1.3×10^{-4} M disodium ethylenediaminetetraacetate (EDTA), 1.2×10^{-3} M $NaHCO_3$ (pH 7.8), and 1.5×10^{-3} M $CaCl_2 \cdot 2H_2O$, and was prepared in distilled water as needed, from two 200x stocks: (1) 1.0% w/v EDTA plus 2.0% w/v $NaHCO_3$, and (2) 4.54% w/v $CaCl_2 \cdot 2H_2O$ (Davis, personal communication).

Animals were fed brine shrimp nauplii (Artemia salina) three times a week (see Appendix, I, for method of hatching nauplii). Bowls were changed twice on feeding days (one hour after feeding and again six hours later) and once a day otherwise.

II. Anatomy of hydra

The drawing by W.A. Kepner, reproduced in Fig. 10, represents a typical hydra. Its cylindrical stalk, or column, is usually between 5 and 15 mm long; its tentacles, 6 to 8 in number, are usually about the same length as the column. The organism is a diploblastic polyp, with longitudinal contractile fibers in its outer, epidermal layer, and circular fibers in its inner, gastrodermal layer, separated from each other by a thin (ca. 1μ), acellular, fibrous mesogleal layer. Several

regions of the column (see Burnett, 1961) will be referred to throughout this study: (1) the hypostome (the oral end of the animal, where the tentacles originate), (2) the gastric region (between the hypostome and the buds), (3) the budding zone (below the gastric region, where buds develop and later separate from the parent), (4) the peduncle (between the budding region and the base), and (5) the basal disc (the adhesive, aboral end of the hydra). The contractile cells of both layers will be called gastrodermal and epidermal myo-epithelial (epithelio-muscular) cells.

III. Solutions for contractile models.

A list of stock solutions may be found in Appendix, II. All experimental solutions were buffered with potassium phosphate, tris-HCl, or imidazole-HCl (see Appendix, III); the pH of each solution as determined with a Beckman Model 76 pH Meter never varied among replicates by more than $\pm$ 0.05 pH unit. Ionic strength (expressed in μ and defined as $I = \frac{1}{2} \sum_i c_i x_i^2$, where c_i = concentration and x_i = valence of each ion) was kept constant by adjusting the concentration of KCl (see Appendix, IV). Extraction agents used were saponin, digitonin, and glycerol, all in buffered KCl solutions. Wash solutions, in addition to buffer, KCl, and sometimes $CaCl_2$, usually contained urea.

Buffered solutions used to measure contraction (contraction solutions) included combinations of ATP, $MgCl_2$, and $CaCl_2$, with ionic strength adjusted with KCl. Calcium- and magnesium-ion concentrations were controlled with EDTA and ethylene glycol bis(amino ethylether)tetraacetic acid (EGTA) buffers (see Appendix, V) when less than 10^{-4} M of the free ion was desired.

Sodium salicyl-(α-hydroxymercuri-β-methoxypropyl)-amide-O-acetate (Salyrgan), a mercury-containing agent which binds with sulfhydryl groups, was added to the contraction solutions in the inhibitor experiments.

A modification of Ringer's (1883) amphibian saline solution, containing 0.11 M NaCl, 2 x 10^{-3} M KCl, 8 x 10^{-4} M CaCl, and 1 x 10^{-3} M sodium phosphate buffer (pH 6.8 or 7.0, total ionic strength = 0.12 μ), was utilized in the experiments involving frog muscle.

IV. Preparation and testing of contractile models

One- to three-day-starved H. littoralis or H. viridis which had neither visible reproductive organs nor visible buds (or one young bud in later experiments; see Lenhoff and Bovaird, 1961, "uniform hydra") were selected. In a few experiments, one hydra per deep-well depression slide was observed throughout the experiment; in most experiments, however, 6 to 12 animals per slide were treated. Occasionally several hydra were strung on each of several 2 cm pieces of clean baby hair. The orientation and number of hydra on each hair made possible observations of change in body length of each animal throughout the preparation and testing of the contractile models (i.e., during extraction, washing, and contraction). In some cases H. viridis (having one young bud) were narcotized in 5% v/v ethanol in hydra medium for 10 minutes, after which their gastric regions were removed, using tungsten needles (made from tungsten-NS-wire courtesy of Sylvania Electric Products, Inc., Towanda, Pennsylvania), for treatment with saponin or digitonin.

All solutions for preparation and testing of models were kept in an ice-water bath until ready to use. Three rapid changes of solution were carried out within 1 minute, at the beginning of each phase of the experiment (extraction, washing, contraction).

In the standard procedure for preparation of cell or whole-animal models, entire hydra or gastric regions were extracted for 8 minutes with saponin or digitonin, then washed for 10 to 30 minutes in a solution which in most cases contained 1.5 M urea. Whole hydra which remained intact throughout this treatment were called whole-animal models. Isolated cells from gastric regions or whole animals which dissociated during treatment were called cell models.

In the testing of whole-animal models, column and tentacle lengths were measured with an ocular micrometer, before and after the addition of contraction solution, using a dissecting microscope. In the cell-model experiments, contraction solutions were introduced in two ways: sometimes they were added while the extracted, washed cells were still in a deep-well depression slide, after which a sample was transferred with a pipette to a microscope slide. Using the other method, tissue practically dissociated into cells by the end of the wash treatment was transferred in a drop of wash solution to a slide, and a coverslip was added. These cells were sometimes held in place on a slide with silk bolting cloth and/or glass wool. This preparation could then be observed with phase-contrast microscopy. Contraction solutions were introduced by diffusing several drops at a time under the coverslip at one end and were

removed at the other end by blotting with small rectangles of no. 1 Whatman filter paper. New solutions were assumed to cross the preparation rapidly, based on observations of the moving front of a solution of 0.05% w/v methylene blue.

Quantitative data for mean percentages of contracted cells were obtained by counting 100 epidermal or 100 gastrodermal myo-epithelial cells per preparation, scoring cells which were completely spherical (C I cells), cells which were contracted in the basal region only (C II cells), and relaxed cells (see Fig. 11 to 17). When not otherwise stated, references to contraction of hydra cells will be to the C I type. Myo-epithelial cells, the most numerous of all cells observed, were distinguished from the others by size and shape. Gastrodermal cells contained symbiotic algae, Zoochlorella, which served as a convenient marker to distinguish them from epidermal cells. Three replicates of each experiment were performed.

The notation pX was used for $-\log_{10}[X]$, where X is an ion or a nonionized molecule. Calculations of concentrations of free ions and metal-ATP complexes were based on Martell and Schwarzenbach's (1956) values; see Appendix, VI.

V. Techniques for determining the physiological acceptability of saponin models

A. Oxygen consumption (H. littoralis)

Oxygen consumption was measured by standard manometric techniques (Umbreit, Burris, and Stauffer, 1959). Pretreatment consisted of 8 minutes in 0.1% w/v saponin (pH 7.0, I = 0.05μ),

followed by 30 minutes of washing (same pH, I). Each five-ml flask contained several hundred H. littoralis (starved 24 hours). Two flasks (S1 and S2) contained saponin-treated animals, and two (C1 and C2) contained normal hydra. Readings were made every 15 minutes for 150 minutes, followed by one last reading at 225 minutes. Results were expressed in μl O_2 consumed/mg dry wt/min.

B. Transmembrane resting potential (frog sartorious fibers)

Sartorious muscles from freshly-killed frogs (Rana pipiens) were transferred to amphibian saline solution and teased apart into bundles about 5 mm long and 1.5 to 4 mm wide, each containing several hundred fibers. They were treated with 0.1% w/v saponin in Ringer's solution, pH 6.8, for 12 minutes, then transferred back to Ringer's solution to wash for 30 minutes. Resting potentials were measured by the standard technique of Ling and Gerard (1949), using machine-pulled glass electrodes, tip diameter 0.5 μ, filled with 3 M KCl, and having a resistence of 20 to 50 megohms.

The only indication that a cell has beem impaled by the electrode is a change in potential. Potential differences of less than 10 mv were neither easily discerned nor recorded with confidence; and therefore, fibers having little or no transmembrane potential after saponin treatment were not scored. Their omission introduced an unavoidable positive sampling bias.

C. Contraction (frog sartorious fibers)

Sartorious muscles removed from freshly-killed frogs were transferred to Ringer's solution and stored overnight at 4°C. They were teased apart the next day into fibers, and into bundles containing 10 to 100 fibers, several millimeters long. These fibers or bundles were extracted in deep-well depression

slides at room temperature in 0.1% w/v saponin solution containing 0.05 M phosphate buffer (pH 6.8, I = 0.15μ) for 8 minutes, then washed (same pH, I) for 30 minutes. Before and after the addition of contraction solution, fiber lengths were measured, using an ocular micrometer on a dissecting microscope.

The shortening of saponin-treated frog fibers was carried out under conditions known to stimulate contraction of well-washed, glycerinated fibers: 1×10^{-3} M $CaCl_2$ (Marsh, 1952), 2×10^{-3} M ATP, and 5×10^{-3} M $MgCl_2$ (see Hoffmann-Berling, 1960), 5×10^{-2} M phosphate buffer (pH 6.8), and 7.2×10^{-2} M KCl (total ionic strength 0.15μ).

VI. Microscopy

Extended H. littoralis or H. viridis (narcotized in 5% v/v ethanol) were flooded with ice-cold 0.25 M glutaraldehyde fixative (containing 0.25 M sucrose to adjust the tonicity to a level yielding no discernible shrinkage, and buffered with 0.05 M sodium cacodylate-HCl, pH 7.4), transferred to a 10 ml container of fresh fixative, and left at 4°C for 30 minutes. They were washed overnight at 4°C in a solution containing 0.05 M sodium cacodylate-HCl buffer (pH 7.4) and 0.50 M sucrose, then transferred to 0.04 M (1% w/v) OsO_4 (in 0.5 M sucrose and 0.05 M sodium barbitol - sodium acetate - HCl buffer, pH 7.4) for 1 hour at 4°C. (The above procedure for fixation, washing, and OsO_4 post-fixation was modified from Sabatini, 1963).

The tissue was dehydrated in an ethanol series, carried through several 10 minute changes of propylene oxide, and then left in a hood overnight in an uncapped bottle of 1:1 propylene oxide:English Araldite. The latter was actually a mixture of

48.3% v/v Araldite CY 212, 48.3% v/v hardener HY 964 (dodecenyl succinic anhydride), 1.5% v/v dibutyl phthalate, and 1.9% v/v accelerator (these ratios were modified slightly from those of Glauert and Glauert, 1958). The following day, when the propylene oxide had all evaporated (odor gone), the tissue was embedded in fresh Araldite and left to polymerize in the oven at 60°C for 36 hours.

Thick (0.50 to 0.75μ) and thin (silver to pale gold) sections were cut with a glass knife on a Porter Blum MT-2 microtome. Thick sections were stained with 1% w/v methylene blue in a 1% w/v borax solution, and mounted in Araldite for light microscopical observations. Thin sections were collected on parlodion-coated, lightly carboned, copper grids and either stained 30 minutes with Reynolds (1963) lead citrate or double stained with a saturated solution of uranyl acetate in 50% ethanol (see Gibbons and Grimstone, 1960) for 30 minutes, followed by lead citrate (3 minutes). They were examined with a Phillips 200 EM, with an accelerating voltage of 60 kv.

H. viridis starved 72 hours and having neither visible buds nor reproductive organs were pretreated with 0.1% w/v saponin in 3×10^{-4} M $CaCl_2$ and 5×10^{-3} M potassium phosphate buffer (pH 6.8, I = 0.02μ) for 10 minutes, then washed 30 minutes in 1.5 M urea (same pH, ionic strength), before being prepared for electron microscopical observations.

For preliminary light microscopical observations, 48-hour-starved H. littoralis were fixed in extended form by flooding with hot Bouin's fluid (Gray, 1954, p. 116). They were then dehydrated in a graded ethanol series, cleared in xylene, and embedded in Tissuemat (mp 56°C, Fisher Scientific Company).

Serial sections were cut at 5μ, stained with Delafield's haematoxylin and eosin Y (Gray, 1954, p. 57 to 58), and mounted in Harleco Synthetic Resin (H.S.R., Hartman-Leddon Company).

VII. Statistical methods

The "Student" t ratio was used to test the null hypothesis that two means were from the same population. The 5% confidence level was selected for the rejection of this hypothesis.

Means, standard deviations, and standard errors were calculated directly from raw data that was normally distributed. However, for nonparametric, percentage data (e.g. $p = \frac{x}{n} \times 100\%$, where x = number of cells contracted, and n = total number of cells scored in one preparation), the values p first had to be transformed to $\sin^{-1}\sqrt{p}$ (n's equal for all preparations) or $\sqrt{n}\sin^{-1}\sqrt{p}$ (n's different), before statistical calculations could be made (see Snedecor, 1956; Southwood, 1966). Such trigonometrictransformations have been shown to yield normally distributed data, as was the case in the present study.

The means and standard errors calculated from the transformed data were converted back to percentages ($\bar{X} \longrightarrow \sin^2 \bar{X}$ for the first transformation, and $\bar{X} \longrightarrow \frac{1}{\bar{n}} \sin^2 \bar{X}$ for the other transformation, where $\bar{n}$ = the mean of the n's), for purposes of communication with the reader.

A χ^2 (Chi-square) analysis was employed in the calculations of relative frequencies of thin and thick myofilaments in the basal regions of the epidermal myo-epithelial cells.

VIII. Calculations of areas and linear dimensions in electron microscopical results

Cross-sectional areas of epidermal muscle tails were measured with an optical planimeter (Filotecnica Salmoiraghi, Model 236/A). Linear measurements of diameters and lengths of particles and filaments were made with dial calipers (Helios), calibrated in tenths of a mm. All measurements were made on electron micrographs enlarged ca. three times from the original plates.

RESULTS

I. Preparation of models

A. Criteria

In attempts to obtain an adequate contractile model of hydra, the following criteria were used: (1) The treatment must render cells incapable of metabolic activity (i.e., none of the ATP supplied to the contractile system must be produced endogeneously); (2) The ability to maintain a transmembrane resting potential must be lost during the treatment (i.e., there must no longer be an ionic diffusion barrier); (3) Motility must be stimulated by some combination of ATP and divalent ions; and (4) The contractile system must remain structurally intact.

The fourth criterion, structural integrity, was considered for whole-animal and cell models. For whole-animal models the following conditions were tested, based on observations of hydra during and after extraction and wash treatment: a. that the two layers, epidermis and gastrodermis, remain united by the mesoglea; b. that the outline of the animal be sharp and well defined; c. that the length of the column of the animal after extraction and wash treatment be essentially unchanged from its length at rest before treatment; d. that the animal be intact, i.e., that intercellular connections remain as extensive as in the untreated hydra. The conditions at the cellular level were that individual myo-epithelial cells remain unchanged in size and shape after treatment, and that the gross structural organization of the myofilaments and other essential contractile apparatus be undiscernibly altered by the treatment.

B. Glycerination

The above structural criteria were tested in H. littoralis and H. viridis which had been subjected to various modifications of Szent-Györgyi's (1949) glycerol-extraction procedure. This technique is now widely employed for preparation of models of muscle and of primitive contractile systems.

The bilayered organization typical of normal hydra was usually disrupted by glycerination. In many cases a mass of tissue was extruded through the hypostome. This tissue was presumably gastrodermal and appeared sometimes diffuse, sometimes distinct and columnar. This alteration occurred during storage in 40 or 50% glycerol (at -10°C), and occasionally during deglycerination. It was observed in one case after only 1 hour of treatment at room temperature in 50% glycerol. During this change in organization of the animal, the two cell layers did not separate completely; some pigmented gastrodermis remained within the overlying white epidermis, particularly near the base of the column. Moreover, the tentacles always remained intact. The tendency of the two layers to separate was correlated with the thickness of the mesoglea in different regions of the animal. Thus, according to Mueller (1950), the mesoglea is thickest in the column (where separation was extensive), thinner toward the basal disc (where gastrodermis remained attached) and near the hypostome, and "reduced to a mere basement membrane" in the tentacles (where no separation occurred).

Hydra failed to maintain a distinct outline after glycerination. The first regions to become fuzzy were the hypostome

and the basal disc. In animals treated with an ascending glycerol series (after Simard-Duquesne and Couillard, 1962) this fuzziness usually became evident by the 20% stage of the series. The outline of the column usually remained quite distinct during extraction with glycerol in the presence of 1.5×10^{-4} M EDTA; in its absence, however, the cells of the column dispersed after several minutes in 1 or 5% glycerol. When EDTA was included in the glycerol solutions, the columns usually remained intact during storage at -10°C (even after many months), but sometimes during the deglycerination series (1 to 5%), the columns became wholly disorganized and the cells dispersed. Bairati and Lehmann (1954) have suggested that EDTA may stabilize the cytoplasmic components; they found it necessary to treat ameba with this agent before fixation for electron microscope observations.

The above descriptions based on observations of hydra with a dissecting microscope during and after glycerination, together with phase microscopical observations of such animals, support the likelihood that intercellular adhesion was not maintained throughout this treatment. Thus, after glycerination, hydra fails to meet the structural criteria for whole-animal models, set down earlier (I A).

Attempts to obtain hydra cell models with glycerol extraction were equally unsuccessful. Relaxed, "T-shaped" myo-epithelial cells (see Fig. 11, for example, consistent with the diagrammatic representation of L. Hyman, 1940, p. 374)

were rarely seen after glycerol treatment. Only in one case were several such cells seen after hydra had been stored 15 months at -10°C in 40% glycerol. These cells remained relaxed during deglycerination at room temperature, as a solution containing 0.01 M imidazole buffer and KCl (I = 0.10μ) was diffused over them. Subsequent attempts to repeat these results were unsuccessful.

In several experiments H. viridis were introduced to glycerol of continuously increasing concentration by a dialysis method. For this technique they were transferred in hydra medium to a dialysis bag, which was then immersed in 40% glycerol and left for 8 hours at 8°C. The bag was then transferred to wash solution and left for 12 hours to deglycerinate gradually. During this treatment the cells apparently dissociated. Most of these dissociated cells were contracted, but several myo-epithelial cells appeared relaxed. However, the zoochlorellae were removed by this treatment, and thus positive identification of epidermal and gastrodermal myo-epithelial cells was prevented. (Albino H. viridis are routinely produced with glycerol; see Whitney, 1907).

Thus, glycerol-treated hydra failed to meet the structural criteria for contractile models. Moreover, they seldom responded to contraction solutions, as the following experiments demonstrate.

The columns of briefly glycerinated H. littoralis showed no change in length during a 10-minute period in 1×10^{-3} M ATP, 5×10^{-3} M $MgCl_2$, and 1×10^{-4} M $CaCl_2$. The results in

Table 1 are based on two such replicates and two wash-solution controls.

In a single experiment contractile cells were obtained from H. viridis treated in 40% glycerol (in 0.01 M imidazole-HCl buffer, pH 7.0, and KCl; I = 0.05μ) at 24°C for 45 minutes, then washed for 30 minutes. Squash preparations were made, and isolated cells and small bits of tissue were observed while a buffered contraction solution containing 1 x 10^{-3} M ATP, 1 x 10^{-3} M $CaCl_2$, and 5 x 10^{-3} M $MgCl_2$ was introduced. Instantaneously, several gastrodermal and epidermal columnar myo-epithelial cells, attached by mesoglea, became squamous as their apical portions shortened suddenly. A few minutes later both layers appeared to separate from the mesoglea. These results, however, were not repeatable.

Contraction was never observed in whole animals treated by an ascending and descending glycerination series, even after months in the freezer in 40 or 50% glycerol. Several dissociated cells from such animals were observed to contract; however, attempts to repeat these results were unsuccessful.

Relaxed, T-shaped myo-epithelial cells of H. viridis observed after gradual deglycerination by dialysis did not change in shape in a solution containing 3 x 10^{-4} M $CaCl_2$ and 5 x 10^{-4} M ATP in 5 x 10^{-3} M imidazole-HCl buffer (pH 6.8) and KCl (I = 0.02μ).

In conclusion, glycerol-extraction procedures which are customarily used to produce models of contractile systems were unsatisfactory with hydra.

C. The Standard Procedure for Model Preparation (Saponin Extraction and Urea Washing)

At Hoffmann-Berling's (1958, 1959) suggestion that models of vorticella can be made with saponin, a procedure was developed for hydra models, using the surface-active agent saponin (or digitonin in some cases) for extraction. The following experiments were conducted to determine optimum values for the parameters of treatment time for extraction and washing, ionic strength, saponin (or digitonin) concentration, etc., for this procedure.

1. Extraction treatment

a. Concentration of extraction agents

H. littoralis were immersed in 0.5, 0.1, and 0.05% saponin for up to 30 minutes at room temperature and analyzed with respect to the structural criteria described above (I A). In addition to saponin, solutions contained 0.01 M phosphate buffer (pH 7.0) and 2.6 x 10^{-2} M KCl (I = 0.05μ). The results are summarized in Table 2.

It was concluded that 0.05 to 0.1% w/v saponin produces the best results, with respect to size and shape, and lack of response to mechanical stimuli.

The basis of the choice of the concentration of digitonin (0.025%, compared with 0.1% saponin) was the fact that the time curves obtained by Ponder (1953) for hemolysis of red blood cells with saponin were comparable to his results with one-fourth as much digitonin.

b. Time of saponin treatment

Ten animals placed in 0.1% saponin in 0.01 M phosphate buffer (pH 7.0) and 0.13 M KCl (I = 0.15μ) contracted immedi-

ately. The column and tentacles re-elongated gradually; the column reached its initial length by 2 minutes. At this time the tentacles were still fully shortened; they did not begin to lengthen for 3 more minutes. By the end of 7 minutes in saponin, the tentacles and column were fully extended (to their initial length).

In another experiment body and tentacle lengths of 3 groups of animals were measured at 7- to 8-minute intervals after immersion in saponin (see Table 3). No significant changes in column or tentacle lengths with time were found (over a 30 minute period). However, by 15 minutes the animals appeared distorted, and by 23 minutes they were quite fuzzy in appearance.

The treatment chosen on the basis of the above results was 0.1% saponin for 8 minutes; under these conditions, hydra consistently appeared to remain relatively intact and extended (i.e., relaxed).

c. Ionic strength of the extraction medium

Steinbach (1963) has reported slight differences between H. viridis and H. littoralis with respect to intracellular concentrations of Na^+, Cl^-, and K^+. The mean ionic strength contributed by these ions, based on his two isotopic determinations for each species, is 0.023μ for H. viridis and 0.036μ for H. littoralis. The results which follow are consistent with Steinbach's (1963) work in that the optimum ionic strength found was higher for H. littoralis than for H. viridis. The decision to use I = 0.05μ for H. littoralis in extraction, wash, and contraction solutions was based on two experiments.

Four groups of H. littoralis were subjected to ionic strengths of 0.20, 0.15, 0.10, and 0.05μ respectively, throughout extraction with saponin, washing, and immersion in contraction solutions. The mean column length and overall shapes of the animals after saponin and wash treatment at 0.05μ most nearly approximated those before treatment. Column measurements made before and after the addition of contraction solution (see Table 4) revealed a trend of increasing relative length (mean final length/mean initial length) with increasing ionic strength.

In an experiment to measure the effects of different osmotic concentrations, four groups of extracted H. littoralis were immersed in wash solutions of ionic strengths 0.20, 0.15, 0.10, and 0.05μ respectively. Column and tentacle lengths decreased (see Table 5), and animals seemed to shrink, with increasing ionic strength. They were significantly longer at I = 0.05μ than at the other ionic strengths. Furthermore, when placed in solutions of ionic strength greater than 0.05μ, animals were soon unresponsive to probing, while those in 0.05μ moved actively for over 5 minutes and still responded to mechanical stimuli after 7 minutes.

Shapes of cells of H. viridis were compared with those of H. littoralis after squashing with a coverslip in solutions containing 0.01 M imidazole buffer and KCl to bring up the ionic strength to 0.02, 0.05, or 0.10μ. T-shaped myo-epithelial cells were obtained most often for H. littoralis at I = 0.05μ and for H. viridis at I = 0.02μ. Shrinkage of H. viridis cells was apparent in solutions of higher ionic

strength. Similar results have been reported by Lilly (1955), who exposed H. viridis to different concentrations of sucrose. She found that the volumes of the epidermis, mesoglea, and gastrodermis each decreased greatly upon immersion of whole animals or isolated tentacles in 0.05, 0.10, or 0.20 M sucrose (osmotically equivalent to half the corresponding molarity of KCl); but she observed no shrinkage in 0.04 M sucrose. From all the above information, it was decided to use I = 0.05μ for H. littoralis and I = 0.02μ for H. viridis.

2. Wash treatment

The same buffer, pH, and ionic strength were used here as described above for the extraction process. The only two features of the washing treatment which remain to be considered are its duration and the role of 1.5 M urea.

a. Duration of wash treatment

It was found that after saponin treatment, H. viridis and H. littoralis usually elongated in wash solution for about 15 minutes, then remained about the same shape and size over the next 15 minutes. At this time they began to disperse and lose their integrity. Each of the two curves in Fig. 1 is based on measurements of a single animal (H. littoralis). It can be seen that in wash solution they increase in length for approximately 10 minutes, reaching an equilibrium length of ca. three-fourths that before treatment.

H. viridis strung on segments of hair (see Materials and methods) were extracted with saponin, then washed for 45 minutes (see Table 6). Measurements of column length were made every 5 minutes up to 25 minutes in wash solution, then at 32 minutes. The results appear in Table 6 (also graphically

in Fig. 2). Column length decreased initially (between 5 and 10 minutes), then increased up to 15 minutes, reaching a plateau, length about 3 mm. Shortening of the column $\left(\frac{\text{change in column length}}{\text{initial column length}} = 20\%\right)$ was later observed upon addition of ATP and Ca^{2+} (discussed more fully in Section II A, below). When the wash time was increased beyond a half hour, no contraction could be induced by contraction solution. This failure may have been due to the separation of the cells (see structural criterion discussed earlier in Section I A).

b. Role of urea in the wash solution

For preparations of consistently relaxed myo-epithelial cells, urea had to be included in the wash solution. The minimum concentration (1.0 M) with which Bozler (1952) produced relaxation in glycerinated psoas muscle fibers, and with which Bárány, Bárány, and Trautwein (1960) inhibited the interaction of actin and L-myosin in the presence of ATP, proved too low to relax hydra cells. When the concentration of urea was increased to 1.5 M, however, T-shaped (relaxed) epidermal and gastrodermal myo-epithelial cells were observed. Glycerol (1.5 M) used in place of urea did not have the same relaxing effect, indicating that the action was not osmotic.

As a further assessment of the relaxing effect of urea, measurements of column lengths of 3 groups of saponin-extracted H. viridis were made every 5 minutes during wash treatment in solutions containing 1.5 M urea, 1.5 M glycerol, or neither (see Table 7). The mean column length of the urea group decreased very little in the first 5 minutes, then increased in

length up to 97% of the initial length, by 20 minutes. The group washed in 1.5 M glycerol contracted down to approximately two-thirds the initial length and remained in this condition for the duration of the experiment; likewise for the control group. Thus, 1.5 M urea relaxes not only individual myo-epithelial cells, but also whole animals.

In summary, a standard extraction and wash procedure for preparation of whole-animal and cell models of hydra was defined, based largely on the results of the above experiments. In this procedure gastric regions of H. viridis or H. littoralis (or occasionally whole animals) are treated for 8 minutes in 0.1% saponin plus 5×10^{-3} M potassium phosphate buffer (pH 6.8) and KCl ($I = 0.02\mu$). They are then washed for 30 minutes in buffered 1.5 M urea and KCl solution (same buffer, pH, ionic strength). After this treatment, they are called models.

With this procedure contractile models could now be tested with respect to the physiological and morphological criteria outlined earlier (1 A).

D. Physiological acceptability of saponin models

Hydra cells were used to measure oxygen consumption after saponin extraction and urea wash. In the measurement of resting potentials and of shortening in contraction solution saponin-treated frog sartorious fibers were employed in order to compare the physiological properties of these models with those reported in the literature for glycerinated skeletal muscle fibers.

1. Oxygen consumption

Oxygen consumption of H. littoralis was not completely inhibited but was diminished greatly by saponin treatment (see Fig. 3). Mean ratios of O_2 consumed/mg dry wt were calculated;

the results are summarized in Table 8. There was no significant difference in rate between the two saponin curves, S1 and S2. The overall rate of oxygen consumption for the saponin-treated hydra was concluded to be ca. 40% of that for one control (C1) and ca. 20% of that for the other (C2).

The slopes of the curves S1 and S2 decreased with time, while the curves C1 and C2 were almost linear (see Fig. 3). The ratio of the slopes of these two control curves was inversely proportional to the ratio of the corresponding dry wts (see table 8). This relationship will be discussed later.

2. Transmembrane resting potential

The mean of the transmembrane resting potentials recorded for saponin-treated fibers (see Table 9) was 22.5 mv (the true potential is probably lower; see Materials and Methods, V B), with a standard error of ± 2.7 mv. Potentials recorded for controls were on the order of 70 mv, with no discernible differences among the many (over 15) impalements made.

3. Contraction

Frog sartorious fibers, which had been subjected to saponin and wash treatment (Materials and Methods, V C) similar to that used successfully with hydra, shortened to approximately 4/5 of their initial length upon the addition of contraction solution (see Table 10). The presence of 1.5 M urea in the wash solution for two of the four replicates had no significant effect on initial or final fiber length.

It was concluded that models produced by the standard saponin-extraction and urea-wash treatment meet the physiological criteria which have been demanded of contractile systems. This will be discussed later in greater detail.

In order to test the structural criteria for hydra cell models, one must be familiar with the normal contractile system. Such a comparative study was conducted; the results follow below.

E. Contractile morphology of hydra

1. Normal morphology

The general cylindrical organization of hydra can be seen in Fig. 18, a cross-section through the gastric region of H. littoralis. Note the thinner outer epidermal layer (E, height 20 to 25μ), and the thicker inner gastrodermal layer (G, of variable height, between 35 and 75μ), bounding the very narrow acellular mesogleal layer (Mes, 1 to 2μ thick), and the wide, central gastrovascular cavity (GVC). Intercellular space (*) is evident throughout both layers but is most pronounced near the mesoglea, i.e., between the basal regions of the cells. These features may be seen more clearly at higher magnification, in both paraffin-embedded (Fig. 19) and araldite-embedded (Fig. 20) sections. Note that the cytoplasmic details are much more evident in the thinner, araldite-embedded section; this greater definition is also a function of the different fixation (see Materials and Methods). A long, meandering, necklike cytoplasmic process (Nk) connects the apex of each myo-epithelial cell (MEC) to its basal process (EBP and GBP, Fig. 20).

Light microscopy does not reveal the fibrous nature of the mesoglea, the size and arrangement of myofilaments in the basal regions of the myo-epithelial cells, or the internal organization of various organelles such as mitochondria; these are readily observed at low magnification in electron micrographs (see Fig. 21 and 22). Again, as in the light

micrographs, extensive regions of extra-cellular space (*) can be seen, broken up by the attenuated neck portion (Nk) of the myo-epithelial cells. Also note nuclei (N), each having one nucleolus (Nc); chromatin (C) may also be seen (Fig. 22).

a. Filaments

Three regions containing filament bundles were found in hydra cells: in the basal processes, or muscle tails, of the gastrodermal and epidermal myo-epithelial cells, and in the radially-oriented, epithelial portion of the epidermal myo-epithelial cell.

The epidermal muscle tails are packed with thick (about 160 A in diameter) and thin (diameter about 55 A) filaments (see, for example, Fig. 23 and 24, Tk and Tn), apparently unbranched, and randomly distributed. They are parallel to one another and run the length of the spindle-shaped base of the myo-epithelial cell, vertically oriented in the animal. Thin filaments far outnumber the thick ones; a ratio of 8:1 was obtained from counts of cross-sections of various body regions (see Table 11). There is considerable variation in filament density among different cells (note the large standard errors, Table 11). No correlation was found between density and either body region or distance from the plasma membrane.

In the gastrodermal basal processes only thin (ca. 45 A diameter) filaments, about 80 to 100 A apart, were seen in the cross sections of H. littoralis observed. Each such muscle tail contains one thin-filament bundle, about 0.1 to 0.2 μ in diameter, which runs the length of the basal process, oriented perpendicular to the long axis of the animal. The entire

muscle tail sometimes appears to be packed with these filaments (see Fig. 25), while in other sections cytoplasmic particles of uniform size and density (described below) share this space (see Fig. 26 and 27, Th, P). In several gastrodermal basal processes examined, in longitudinal sections of H. viridis, thick (about 140 Å diameter) filaments as well as thin (25 to 45 Å diameter) filaments were observed (see Fig. 28, Tn, Tk). Here the filaments are randomly and sparsely arranged.

In many of the epidermal cells are groups of parallel filaments, each of approximate diameter 40 Å and separated from each other by about 50 Å. They seem to originate at the edge of the muscle tail and extend the length of the neck of the cell. These bundles are up to 0.2 μ in diameter and several micra in length (see Fig. 27, 29 and 30, B). No such filaments were observed in the corresponding regions of the gastrodermal myo-epithelial cells.

b. Cytoplasmic organelles and granules in the muscle tails

Elliptical profiles of mitochondria (length 0.5 to 1.1 μ, width 0.3 to 0.5 μ) are often seen in close proximity to the basal filaments of the epidermis (Fig. 31 and 32, M) and gastrodermis (Fig. 26, 33, and 34, M), and occasionally in nearby cells (Fig. 35). Mitochondria are frequently pushed over to the edge of the cell, sometimes less than 120 A from the plasma membrane (see arrow, Fig. 24). In such cases the protruding mitochondria are usually associated with a prominent bulge in the cell (Fig. 24 and 32).

Near the gastrodermal thin filaments and also in adjacent cells and extracellular spaces are particles (P, Fig. 23, 26,

27, 35, and 36) having a diameter of ca. 270 A. Each such particle appears homogeneous, but variation in density among particles is common. They occur in discrete form in some cases, but usually in clusters or chains over 0.5μ long (see Fig. 36). Several such particles were occasionally observed within mitochondria (see arrow, Fig. 26). From all these observations it can be concluded (see Fawcett, 1966; Lentz, 1966; Revel, 1964) that they are probably glycogen.

In the gastrodermal cells is another population of discrete particles, each of approximate diameter 150 A. They are all uniformly dense and are usually in the vicinity of rough endoplasmic reticulum, which bears particles also about 150 A diameter. Thus, all these particles are probably ribosomes (see ER, R in Fig. 25 and 26).

Near and among the epidermal basal filaments are particles of similar density and size (ca. 160 A diameter) as the gastrodermal particles just described. They are always single, associated neither with each other nor with any discernible membrane system. Because endoplasmic reticulum is absent from these regions, the identification of these particles as free ribosomes is only tentative, and they are labeled simply as particles (see P, Fig. 27, 28, 31, and 37).

c. Size of the basal processes

The mean cross-sectional area of the epidermal muscle tails was found to be $0.71 \pm 0.09 \mu^2$ (N = 23). Such direct measurements of the corresponding cross-sectional areas of the gastrodermal muscle tails were not practical, because most of these basal processes were viewed in longitudinal section

(in electron micrographs of transverse sections of hydra). The range in width (d) of these gastrodermal bases was recorded (0.16 to 0.20μ) from such micrographs; and the cross-sectional area, assumed to approximate a circle or square ($\pi \times d^2/4$ or d^2, respectively), was estimated to be on the order of 0.02 to 0.04μ^2. The range in length of the muscle tails, measured in phase preparations of dissociated cells, was 20 to 50μ for epidermal cells, and 20 to 40μ for gastrodermal cells.

d. Membrane system

Few intracellular smooth membranes were observed in the basal region of the myo-epithelial cell; however, several scattered, irregular profiles of intracellular vesicles (see V, Fig. 24, 26, and 27) were noted. The plasma membrane is for the most part unconvoluted, with the exception of an occasional invagination (see I, Fig. 29 and 32). The basal process is always continuous with the rest of the cell; no interposed membranes were seen. Only one unit membrane (70 A thick) separates the muscle-like process of the myo-epithelial cell from the mesoglea and surrounding extracellular space. The basal filaments are not separated by any discernible membranes from the rest of the cell (see Fig. 22).

In the epidermal myo-epithelial cells are tubules having an outer diameter of ca. 200 A, usually in groups of 4 to 6, parallel to and near the thick and thin basal filaments. The usual position for these microtubules is the periphery of the muscle tail (see Mt, Fig. 23 and 25), but they are occasionally situated near the center, surrounded by filaments (Fig. 26).

In longitudinal sections, microtubules up to 0.9μ long may be seen (see Fig. 31, Mt).

There is a conspicuous absence, in the epidermal and gastrodermal myo-epithelial cells, of dense bodies, such as those frequently found in both vertebrate and invertebrate smooth muscle (see Hanson and Lowy, 1960; Lane, 1965; MacRae, 1965; Oosaki and Ishii, 1964; Rhodin, 1962) and also in vertebrate myo-epithelial cells (Ellis, 1965; Tamarin, 1966). These bodies are thought by most of these authors to correspond to the Z lines of striated muscle (Huxley, 1957).

e. Intercellular relationships

The interdigitating epidermal muscle bases (see Fig. 24 and 27) seem to be in intimate relationship with each other, usually separated by less than 200 A, with many thin strands running between them. This proximity is occasionally violated (see *, Fig. 24) by spaces (ca. 1μ wide, and up to 2μ long) in which such strands are absent. In regions in which there is no discernible space between two adjacent cells, membrane fusion is never observed; individual membranes maintain their trilaminar appearance (see Fig. 38).

Another type of epidermal junctional specialization may be observed in the region of the hypostome. Here again the cells are separated by less than 200 A, and intercellular strand-like material is evident. In addition, however, a homogeneous, dense layer of varying thickness (200 to 300 A) lines the inner layer of the cell membranes (see Fig. 39). This junction is rather extensive, up to 1μ long in cross-section,

and usually terminates near the mesoglea.

The gastrodermal basal processes are evidently very long; few endings were observed (but see Fig. 35, arrow), although the investigation was not exhaustive. There seems to be no unusual intercellular junction in this region; unit membranes of the two adjacent processes are 140 A apart.

Adjacent gastrodermal muscle tails are usually separated from each other. In one case, however, a wide cytoplasmic connection (0.1 μ) was observed between two adjacent muscle tails seen in cross section (see region between two arrows in Fig. 28). This might be anomalous, however; these basal processes are flanked by mesoglea (labeled Mes) on two opposite sides.

f. The mesoglea, and associations between epidermis and gastrodermis

The width of the acellular mesoglea varies; it is as thin as 0.2 μ in the peduncle region and up to 1.7 μ in the gastric region, with an overall average thickness of ca. 1 μ (see Mes, Fig. 24, 26, 27, and 29). Filaments (ca. 40 A diameter) in the mesoglea are arranged randomly, or in a complex pattern. Both longitudinal and transverse sectional views of these filaments may be observed in transverse sections of H. littoralis. In one particular longitudinal section of H. viridis (see Fig. 28, Mes) longitudinal profiles are seen in one region and perpendicular views in nearby areas (*).

In sections characterized by accumulations of glycogen in the cytoplasm, particles of similar size (about 300 A diameter) are

scattered throughout the mesoglea (P, Fig. 36). There are also small, dense particles (100 A diameter), particularly conspicuous near longitudinally arranged mesogleal filaments (see arrows, Fig. 25 and 30).

The surface of the epidermal and gastrodermal basal processes abutting the mesoglea are usually smooth (see Fig. 26 and 27) but occasionally convoluted (Fig. 28, 35, and 36). No direct contact was found between the epidermal and the gastrodermal cells, but there are many instances of cytoplasmic, rootlike insertions deep into the mesoglea (see epidermal RI, Fig. 21, 24, 25, 32, and 39; gastrodermal RI, Fig. 21). Fig. 30 shows a pair of processes, one from the epidermis and one from the gastrodermis. Such rootlike processes may even be seen, in some instances, in phase micrographs (Fig. 11, 12, RI) of dissociated myo-epithelial cells.

Small, faintly-staining, spherical bodies (ca. 400 A in diameter) are frequently observed between epidermal muscle processes (labeled S in Fig. 27, 30, and 32). They are homogeneous and not membrane bound. Some seem to be tangent to the outer surface of the cells. Similar bodies may be seen in the mesoglea and also in a gastrodermal muscle tail in Fig. 27 (S). A possible function of these bodies will be discussed later.

2. Morphology after treatment with saponin

Keeping in mind the above description of the normal contractile system and intercellular relationships in the contractile regions of hydra, we may now consider the models produced by saponin treatment, with respect to the structural criteria set down earlier (I A).

a. Maintenance of a bilayered organization

There was no evidence from observations made with a dissecting microscope for any extensive separation of the gastrodermis from the epidermis during extraction with saponin or digitonin. However, electron micrographs of these animals (see Fig. 40) reveal the separation of the bases of the myo-epithelial cells from the mesoglea. In contrast are the close associations of these basal processes with the mesoglea of normal hydra, described above, and also observed by Drs. Julian F. Haynes, Lowell E. Davis, and Allison L. Burnett (personal communication).

b. Maintenance of a distinct outline of hydra

After saponin or digitonin treatment, hydra never showed as complete breakdown of the gross organization of the column as that which occurred during glycerination. The fuzziness at the base still occurred, although the hypostome remained more distinct after saponin treatment than after glycerination.

c. Column length

Since the epidermal and gastrodermal layers oppose each other in maintaining the hydra's shape, it is very difficult to distinguish a state in which both layers are relaxed from a state in which the opposing layers are partially contracted; each state could define a "rest length". It was observed that animals in 5% ethanol for 10 minutes (suggested by Dr. Fred A. Diehl as a relaxant for both layers, personal communication) remained extended but did not respond to mechanical stimuli. This, the length of an animal narcotized in this way was defined as its rest length (for H. viridis, 4.3 ± 0.1 mm). Column

lengths, after saponin extraction and wash treatment, approached but never reached this narcotized length (see, for example, Tables 6 and 7).

In addition, the relaxed state of the column could be assessed qualitatively by observations of its overall shape: the more nearly it approximated a sphere (with width equal to length), the more contracted it was; the more nearly cylindrical (with parallel sides, and width much less than length), the more relaxed. However, this was not completely satisfactory, because it really described the state of contraction of the gastrodermis; the column elongates and the sides become parallel as the gastrodermal layer contracts. Data for changes in column lengths as a function of time of extraction and wash treatment have already been presented (Section I C).

d. Maintenance of intercellular adhesion

It is clear that this condition is critical if a change in column length produced by ATP and divalent ions is to be a valid measure of the combined contraction of all the myoepithelial cells; if the cells do not maintain the intercellular connections prevalent in live animals, then they contract only as independent units and not as two interacting sheets of cells. This property was the most difficult to achieve; in fact, after all extraction procedures tried, the cells were held together loosely, if at all.

Septate desmosomes and other intercellular junctions which are so pronounced in normal hydra (see, for example, Fig. 24, 27, and 39) apparently break down during treatment with saponin. Remnants of a tight junction (J) and nearby

what probably were previously three points of contact between two basal processes (arrows) may be seen in Fig. 43, an electron micrograph of a saponin model. Similar regions showing the breakdown of intercellular contact are seen in Fig. 42.

It was found necessary to keep the animals completely immersed during solution changes throughout extraction and washing; when most of the solution was removed, the resulting surface tension caused immediate dispersion of the cells. Furthermore, when these treated animals were transferred to a slide and covered with a coverslip, the cells dissociated immediately, without application of additional pressure. This was not the case with untreated animals.

In conclusion, the four conditions for whole animals were never completely attained under any of the extraction techniques tried; this fact must be borne in mind in interpreting the results (in Section II A which follows later) based on changes in column length of such treated hydra upon introduction of contraction solution.

e. Structural organization at the cellular level

The cytological organization of the contractile system, on the other hand, was well preserved throughout the standard saponin-extraction and urea-wash treatment. Myo-epithelial cells remained relaxed; many T-shaped cells were observed in phase preparations. Thick and thin myofilaments are still present in the epidermal myo-epithelial cells, as observed in electron micrographs (Fig. 41, Tk, Tn). Mitochondria appear slightly damaged in some areas (note swollen mitochondria, Fig. 44),

although the double-membraned organization is still evident (Fig. 42, M). In other areas (Fig. 45, M) the mitochondria appear completely normal after saponin and urea treatment. Plasma membranes (Fig. 41, UM) are still visible, but seem fainter.

Thus, the condition that the contractile apparatus remain intact, while the plasma membrane is altered, was met in cell models produced by saponin extraction.

Summarizing, a standard extraction and wash procedure was developed for the preparation of models which satisfied the physiological and morphological criteria; the chemical mechanism of contraction could now be investigated.

II. Contraction of models

A. Models of whole animals

Very few experiments were conducted on whole animals, due to the complications outlined above (Section I E 2) in keeping them intact. Moreover, even in intact animals, a change in column length represented the interaction of epidermal shortening and gastrodermal lengthening, the relative effects of which could not be assessed without the corresponding data for single cells of both layers. In spite of these complications, however, the results were useful in establishing time curves and concentration ranges of ATP and divalent ions for later experiments.

The saponin-extracted H. viridis from the time-of-wash experiment described above (see Table 6) were immersed in contraction solution (see Table 12) and measured every 5 minutes. The mean column length decreased for 10 to 15 minutes, then

reached a plateau at about 80% of the initial length (i.e., before contraction solution; see Table 6). The results are summarized graphically in Fig. 2.

The concentration range of ATP in which contraction of H. littoralis occurs was investigated by immersing 5 groups of saponin-extracted animals (see Table 13) in solutions containing $CaCl_2$, $MgCl_2$, and 0, 1, 5, 10, or 20 mM ATP, respectively. Column lengths were measured 15 minutes later. The results in Table 13 are based on the grouped data from 3 replicates done on consecutive days. An increase of about 20% of the initial column length was found in the absence of ATP (i.e., upon addition of just 1 mM $CaCl_2$ and 5 mM $MgCl_2$; see Table 13). Shortening of about 25% of the initial length was observed in 1 to 10 mM ATP (plus Mg^{2+} and Ca^{2+} as above), and of slightly less (about 15%) in 20 mM ATP. These results will be discussed later with respect to concentration ratios of ATP and divalent ions.

B. Cell models

Most observations and other results presented in this section were based on experiments in which H. viridis were subjected to the standard extraction and wash procedure described earlier, with occasional exceptions as indicated.

1. Contraction as a function of time in contraction solution

The temporal endpoint for observable changes in cell shape was determined by counts, at 1 minute intervals, of the percentage of gastrodermal and epidermal cells contracted after the introduction of contraction solution. It can be

seen in Fig. 4 that the epidermal curve levels off by 2 minutes, at approximately 55%, and the gastrodermal curve reaches a plateau by 3.5 minutes, at about 50%.

Cell counts in future experiments were made 4 minutes after the introduction of contraction solution.

2. Variation among replicates

Ten replicates of the same experimental procedure were carried out on two different days, in which the percentages of epidermal and gastrodermal cells contracted (out of a total of ca. 100 cells scored) were calculated, based on counts made 4 minutes after addition of contraction solution. The variation (see SD in Table 14) among replicates was found to be quite low.

3. Contraction as a function of pH

The optimum pH for epidermal contraction was found to be different from that for gastrodermis (see Fig. 5 and Table 15). Epidermal contraction was maximal at pH 6.8 (48 ± 1%); while the highest point on the gastrodermal curve was at pH 7.6 (58 ± 2%), with a possible second maximum at pH 7.0 (41 ± 0.2%). The $MgCl_2$ concentration was 1×10^{-4} M in some replicates and 5×10^{-4} M in others. As the data obtained were not significantly different in those two cases, they were all treated together.

4. Roles of ATP, Ca^{2+}, and Mg^{2+} in the contraction of cell models

a. Qualitative observations

(1) Reversibility of contraction

In a preliminary experiment epidermal cell models changed in shape from a "T" to a rectangle, i.e., to type C II, in

solution containing 5×10^{-4} M ATP, 3×10^{-4} M $CaCl_2$, 5×10^{-3} M imidazole-HCl buffer (pH 6.9), and KCl (I = 0.02μ). Washing out with urea solution reversed this effect. Gastrodermal cell shape remained unchanged throughout this time. The same contraction solution caused irreversible contraction of the C I type in both epidermal and gastrodermal myo-epithelial cells of another preparation (i.e., these cells became spherical). No contraction was observed if either $CaCl_2$ or ATP was omitted from the solution. Cells contracted (C I) in ATP, Ca^{2+}, KCl, and buffer (as above) plus 1.5 M urea; thus, the contractile response was to ATP and Ca^{2+}, since the ionic strength, pH, and urea concentration remained the same as in the wash solution.

(2) Epidermal and gastrodermal differences in contraction as a function of length of wash treatment

Saponin-treated hydra were exposed to 30, 60, or 90 minutes in urea-free solution (see Table 16) before being washed for 30 minutes in the usual urea solution. The difference in contractile response was greatest between gastrodermal and epidermal cells which had little or no extra wash treatment (see Table 16); very few gastrodermal cells were scored as C I, while almost all the epidermal cells contracted in contraction solution. When gastrodermal cells had been washed for at least 60 minutes beyond the normal treatment, they, too, contracted. However, cells began to lose definition by this time; as the period of wash treatment increased, progressively fewer intact cells were observed.

(3) The significance of the ATP:Mg^{2+} ratio and the influence of Ca^{2+} on contraction

Contraction of epidermal and gastrodermal cell models was

tested in the presence of various combinations of ATP (5×10^{-4} M), $CaCl_2$ (3×10^{-4} M), and $MgCl_2$ (3×10^{-4} or 1×10^{-3} M). It can be seen from the summary of the results (Table 17) that when ATP was present, the concentration ratio of ATP to Mg^{2+} was inversely related to the extent of the contractile response observed; i.e., as $[ATP]/[Mg^{2+}]$ increased, the contractile response diminished (compare prepns 1 and 8, 4 and 9). Furthermore, Ca ions seemed to stimulate contraction in the presence of ATP (compare prepns 1 and 4, 8 and 9). Finally, ATP is required for contraction. The only evidence to the contrary (prepn 5, gastrodermal cells) was unrepeatable (prepn 6). Few observable differences in contractile response between epidermal and gastrodermal cells were noted in this experiment.

The enhancing effect of Ca^{2+} may further be noted in Table 18; contraction occurred in the presence of 5×10^{-4} M ATP only when $CaCl_2$ concentration was greater than 5×10^{-6} M. Contraction of both epidermal and gastrodermal myo-epithelial cells was observed in the presence of $CaCl_2$ in the contraction range 5×10^{-5} through 5×10^{-4}M. At 1×10^{-3} M $CaCl_2$, however, few C I cells were seen.

In another experiment the role of Mg ion concentration was investigated, in particular with reference to ATP concentration (Table 19). Contraction (C I) of gastrodermal cells was observed for $MgCl_2$ concentration greater than 5×10^{-4} M (the concentration of ATP also present in each solution). As in the case of Ca^{2+}, an upper concentration limit was found above which no contraction occurred. Only a few cells contracted at 15×10^{-4} M, and none at 20×10^{-4}M $MgCl_2$.

b. Quantitative data: Contraction as a function of equal concentrations of ATP and $MgCl_2$ at different Ca levels

The remainder of the results on cell models are quantitative; mean percentages of cells contracted obtained in these experiments were based on counts of 100 cells per replicate, three replicates per experiment.

Contraction of cell models was measured as a function of equal concentrations of ATP and $MgCl_2$ (varied from 1×10^{-7} to 3×10^{-4} M), with total Ca concentration (after EGTA chelation; see Appendix, V) equal to 10^{-8}, 10^{-6}, and 10^{-4} M in three successive experiments. The results are summarized for epidermal and gastrodermal contraction in Tables 20 and 21, respectively, and compared graphically in Fig. 6. No differences in contractile response were found between saponin-extracted and digitonin-extracted cells, so these treatments are considered together. A slightly basic pH (7.2) was chosen for these experiments, since no differences in contractile response had been found previously between gastrodermal and epidermal cells at this pH value (see Fig. 5). However, as will be brought out, this relationship between epidermal and gastrodermal contractile responses does not hold under other ionic conditions. The results for the two types of contractile cells will therefore be considered first separately, then together.

The epidermal curves for 10^{-6} M and 10^{-4} M Ca each displayed two maxima, at p(total Mg) and p(total ATP) between 6.5 and 7.0, and between 4.0 and 4.5, respectively (see arrows Fig. 6 b and c). The 10^{-8} M curve (Fig. 6 a) had only one

real maximum (indicated by an arrow), between 4.5 and 5.0. At the two maxima for the 10^{-4} curve the percentages of epidermal cells contracted were greater than at the respective maxima for the 10^{-6} M curve (Table 20, *). Likewise, the minimum value of the former curve was greater than that of the latter (Table 20, +). Another trend was the shift to the right of points of inflection (i.e., minima and maxima) as Ca concentration increased (Table 20, Fig. 6). The 10^{-8} M curve differed most strikingly from the other two curves in the lower range of Mg and ATP concentration; little contraction was observed from Mg and ATP concentrations equal to 0 up to 10^{-6} M for the 10^{-8} M curve, whereas each of the other curves exhibited a maximum in this region.

The gastrodermal Mg-ATP curves for 10^{-8} M and 10^{-6} M Ca (see Fig. 6 a and b) did not differ significantly from each other (based on t ratios for each Mg-ATP level). Both curves showed a plateau (about 25 to 35% of the gastrodermal cells contracted) between 7.0 or 6.5 and 5.0, and relatively fewer cells contracted (about 10 to 20%) between 4.5 and 3.5. A questionable drop (to about 15%) appeared in both curves at 6.0. The 10^{-4} M curve (Fig. 6 c) seemed to be shifted to the right, in comparison with the other two gastrodermal curves; it was characterized by a wide plateau of maximal contraction (30 to 35%) between 6.0 and 4.0, possibly decreasing at 3.5. Up to 3×10^{-7} M total Mg and ATP concentration the mean percentage of cells contracted was between 8 and 13.

The area under each epidermal curve was greater than for the corresponding gastrodermal curve (see Fig. 6); i.e.,

relatively more epidermal cells were contracted. The difference between the heights of the two curves at each Ca level was greatest at the points (see arrows, Fig. 6) of maximal epidermal contraction.

5. Type C II contraction of cell models

The above results (II B 1 to 4) were all for contracted cells of type C I. C II cells were also scored (see Table 22) in the Mg-ATP experiment described above (II B 4 b). The results for such contracted epidermal and gastrodermal cells for 10^{-8} M and 10^{-4} M Ca were bizarre (see Fig. 7).

When Ca concentration equalled 10^{-8} M, varying the concentration of ATP and $MgCl_2$ between 1×10^{-7} and 3×10^{-4} had no effect on epidermal contraction; ca. 20% of the cells were contracted over this range and also in the absence of ATP and $MgCl_2$ (see Fig.7 a). The 10^{-4} M curve (see Fig. 7 b) decreased from 43% in the absence of ATP and $MgCl_2$ down to 18% at p(total Mg) and p(total ATP) equal to 5.0. At 4.5 there was a high epidermal peak (56%); the curve dropped down below 20% for higher $MgCl_2$ and ATP concentrations.

The curves for gastrodermal cells were considerably higher than the corresponding epidermal curves. The 10^{-8} M curve (Fig. 7 c) had a slight minimum (48%) at p(total Mg) and p(total ATP) equal to 5.5. The 10^{-4} M curve (Fig. 7 d) consisted of two plateaus, one below 6.5 and the other above 6.0, at approximately 80 and 60% respectively.

6. Inhibition of contraction of cell models by Salyrgan

Salyrgan in concentrations of 1×10^{-5}, 3×10^{-5}, 1×10^{-4}, and 3×10^{-4} M was added to contraction solutions (see Fig. 8)

which normally produced over 50% contracted epidermal cells and over 30% contracted gastrodermal cells of the type C I. Inhibition resulted, which did not vary in extent over the concentration range tested (see Table 23). The mean percentages of cells of the C I type were 54 and 38 respectively for the epidermal and gastrodermal controls, and about 22 and 12 respectively with Salyrgan (see Fig. 8 a). Salyrgan had no observable effect on C II contraction (see Fig. 8 b and Table 24).

DISCUSSION

I. Preparation of models

A. The standard procedure (saponin extraction and urea washing) for preparation of models

The optimum concentration of saponin and time of extraction, derived experimentally, are similar to those conditions used by Hoffmann-Berling (1958) in producing a model of Vorticella, and to the treatment which yields uniformly-spaced pits or holes, 80 A in diameter, over the surface of cell membranes (Dourmashkin, Dougherty, and Harris, 1963; Muir, 1962).

The total extraction and wash time (42 minutes) is less than that used to produce most glycerinated models (Szent-Györgyi, 1949). However, it is comparable to the period of glycerination and wash treatment used by Townes and Brown (1965) to make Vorticella models and by Kinoshita, Andoh, and Hoffmann-Berling (1964; see also Hoffmann-Berling, 1964) to extract relaxing factor from fibroblast cells. Thus, the conditions for the preparation of models are quite reasonable.

B. Physiological acceptability of models

The standard procedure for the preparation of contractile models of hydra met reasonably well the criteria for an acceptable model set down in the Results, Section I A. Each of the criteria will be evaluated separately here.

1. Respiration

The concern in this phase of the investigation was with the energy sources in hydra cells after extraction; as was brought out earlier (Results, I A), it is desirable that contractile models be no longer capable of endogenous respiration.

In this light, a great decrease in the ability to produce chemical energy has been demonstrated for glycerol models (see review by Perry, 1956), and also, in the present study, for saponin models (see Fig. 3, for example). There is much evidence that most of the substrates and coenzymes required for respiration are removed by extraction, particularly by saponin. McNairy (1966) showed that saponin treatment arrested oxygen consumption of rat liver, kidney, and diaphragm muscle; these tissues exhibited partial recovery upon the introduction of excess glucose. Liver and kidney mitochondrial oxidation of α-ketoglutarate was inhibited to a greater extent than was oxidation of succinate after saponin treatment. This inhibition of α-ketoglutarate oxidation was partially reversed by the addition of nicotinamide-adenine dinucleotide or Coenzyme A to the incubation medium. His contention that saponin might inhibit respiration by causing a leakage of respiratory co-factors from the system is supported by the results presented here, i.e., by the decrease in the slope of the saponin curves with increased wash time (Fig. 3). No such change in rate of oxygen consumption was noted for the controls. Differences between the two control flasks in rate of oxygen consumption might have been related to overcrowding in the flasks and a resulting competition for oxygen (see Umbreit et al., 1959); the control flask with more animals (i.e., greater total dry weight) consumed less oxygen per mg dry wt than did the other control.

It is not as evident from the literature that glycerol treatment causes a leakage of substrate, coenzymes, and/or

soluble enzymes. On the contrary, most of the investigators of metabolic activity in glycerinated models have added either substrate and/or coenzymes to a homogenate or have assayed for enzymes which are known to be structurally bound to membrane systems in the cell. Wilson et al. (1959) found no difference in oxygen consumption between fresh and well-glycerinated rabbit psoas muscle fibers in media containing succinate; the rate of oxygen consumption in fresh fibers decreased by one-third when succinate was omitted. Unfortunately, they did not measure oxygen consumption by glycerinated fibers in the absence of succinate. Naylor and Merrillees (1964), in demonstrating that 40 to 60% cytochrome oxidase and succinate dehydrogenase activity remain in toad ventricular muscle after storage for 10 months in 50% glycerol, included cytochrome c and succinate, respectively, in the medium. Thus, they showed that these enzymes, which are known to be tightly bound to the mitochondrial cristae (see Singer, 1963), are not removed by glycerination; because they used the technique for homogenates (Umbreit et al., 1959), they were unable to carry out the measurement of enzymatic activity due to endogenous substrate alone. On the other hand, Perry (1956) reports that the activity of many other enzymes present in fresh muscle has been shown to be greatly reduced or abolished by glycerination.

It is clear that extraction by either glycerol or saponin interferes with cellular respiration; the extent of the interference should be investigated before one can assume that he has isolated the contractile system.

2. Transmembrane resting potential

Muscle-fiber models were used to compare resting potentials after saponin extraction with those which have been measured (Naylor and Merrillees, 1964) after glycerination. The reduction in potential after saponin treatment to, at most, one-third the transmembrane resting potential of normal fibers (see Table 9), compares favorably with the results of Naylor and Merrillees (1964) for glycerinated muscle (30 to 35 mv after 6 weeks in glycerol, 92 to 95 mv for freshly-excised muscle). As was mentioned earlier (Materials and Methods, V B), only cells with a potential significantly greater than zero were recorded; it is likely that in many fibers the transmembrane resting potential after saponin treatment was too low to be detected. Furthermore, saponin and other surface-active agents have been shown to alter other related properties of plasma membranes, as will be discussed later (I D). Thus, the criterion that the membrane must no longer act as a diffusion barrier to ions, i.e., that it must no longer maintain a potential, can be considered to be met by saponin extraction.

3. Motility

To test the criterion of motility, saponin models of sartorious muscle fibers were tested so that comparisons could be made with known and thoroughly-studied glycerol models. It was found that saponin models shorten upon the addition of ATP and divalent ions; the extent of the contraction (down to ca. 80% of their initial, equilibrium length) was less, however, than that which has been reported for glycerinated

fibers (down to ca. 20% of their rest length; see, for example, Hoffmann-Berling, 1960; H.H. Weber, 1958). Several variations in technique can explain this difference. First of all, the fiber length measured after the addition of contraction solution (see Table 10) was compared with the equilibrium length attained after extraction. Because fibers shorten somewhat after removal from the animal (i.e., they decrease from their "rest length"; see Szent-Györgyi, 1949) and because saponin causes further shortening (Bárány et al., 1960), it follows that the final length expressed as a percentage of the equilibrium length was greater than if it had been compared with the rest length. Furthermore, relaxing factor (see Bendall, 1953; Marsh, 1952; also review paper by Hasselbach, 1964) may have remained after the short (38 minutes) extraction and wash treatment. Contraction of such briefly-washed fibers is favored by lower ATP concentration (Bozler, 1951; Hasselbach and A. Weber, 1955) and lower ionic strength (about 0.04 μ); higher values (0.15 μ or more) promote dissociation of actomyosin (Hasselbach, 1964) and inhibit contraction (Bozler, 1951). Finally, fiber bundles, rather than single fibers, were used; time for diffusion of molecules to the center was correspondingly greater (see Edman, 1957; Hasselbach, 1952; Hasselbach and A. Weber, 1955). It is likely that saponin models made from smaller fiber bundles would shorten in solution containing less ATP and of lower ionic strength, to the same extent as do glycerinated models, especially if this shortening is expressed as a percentage of rest length, rather than equilibrium length.

C. Contractile morphology after treatment with saponin

Models produced by saponin treatment are compatible morphologically as well as physiologically with contractile systems extracted with glycerol. Both extraction methods leave the ultrastructural appearance of the myofilaments unchanged. The decreased integrity of membranes and the various degrees of mitochondrial damage observed in electron micrographs in this work are similar to the changes in muscle produced by glycerination (Bergman, 1958; Naylor and Merrillees, 1964).

D. Relationship between increased permeability and dissociation of cells.

Hydra cells separated readily after saponin extraction and washing in Ca-free solution. The associated decrease in intercellular association, while hindering the maintenance of models of intact organisms, facilitated the development of isolated cell models.

The agents employed in this and similar experimental procedures to increase permeability of the cell, in doing so very likely alter the surface properties of the membrane which are necessary for the maintenance of intercellular adhesion. Such a correlation between increased permeability and decreased intercellular adhesion has been found in many types of cancer cells (see review by Abercrombie and Ambrose, 1962).

The extraction and wash treatment of hydra probably affected permeability and cellular interaction in three non-related phases: (1) alteration of the membrane by saponin, followed by (2) the action of urea, in (3) a Ca-free solution.

There is ample evidence that surface-active agents, in addition to increasing permeability, also alter cell shape and cellular interactions. With respect to their first action, lytic agents such as saponin and digitonin lower transmembrane potentials (measured in saponin-treated muscle fibers; see Table 9) and increase permeability, both to ions (Bangham, Standish, and Weissmann, 1965; Davson and Danielli, 1938) and to larger molecules; saponin treatment increases insulin uptake by leucocytes (Karnovsky et al., 1964), and the detergent sodium lauryl sulfate causes uptake of the dye nigrosin by Ehrlich ascites cells (Palmer, Hodes, and Warren, 1961). The second action of saponin and digitonin is evidenced by their stimulation of phagocytosis in leucocytes (Karnovsky et al., 1964) and bulging of erythrocyte cell membranes (Seeman, 1967). A similar effect on cellular interaction by detergents was reported by Cahn (1967), who showed that failure to remove the detergent present on new Millipore filters resulted in a great decrease in aggregation and differentiation of chick cartilage cells. Thus, it is not surprising that saponin and digitonin decrease intercellular adhesion in hydra.

Treatment with Ca-free urea solution further contributed to the decrease in intercellular association. The importance of Ca and other divalent ions in the maintenance of adhesion between cells is well established (see review, Steinberg, 1958); in fact, a Ca- and Mg-free medium is commonly used to separate cells. This separation is a gradual process; Zeidman (1947) found that the force required to separate two buccal epithelial

cells decreased with time, over a half-hour period. Negatively-charged sites on the surfaces of two adjacent cells are thought to be linked by one divalent Ca ion, and weaker H bonds and van der Waals forces probably increase the intercellular attractions (Pethica, 1962). The configurations of the surface proteins contributing to these forces and charges may have been altered by the mild urea treatment (see Hamaguchi, 1955 a, 1955 b; von Hippel and Harrington, 1960; Klotz, 1960).

In summary, the saponin-extraction, urea-wash treatment of contractile cells was shown to yield models which satisfy the criteria which have been set forth. Namely, these cell models contract upon the addition of ATP and divalent ions; their transmembrane potentials are greatly reduced (in sartorious fibers), perhaps abolished (in hydra cells); their endogenous respiration is greatly reduced and decreases with time; their contractile systems are structurally intact. Furthermore, evidence has been presented from the literature that after such a treatment these models are highly permeable to soluble ions and larger molecules. Saponin models compare favorably with those produced with glycerol, with respect to the above criteria. Thus, a high degree of confidence can be placed in the isolation within the cell of the contractile system, the chemical environment of which can be altered experimentally and systematically to produce movement comparable to that of the living system. The effects on contraction by such systematic alterations of pH, ionic strength, ATP and divalent-ion concentrations, have already been presented (Results, II); their interpretations and various significances will now be discussed.

II. The contractile system of hydra

A. The chemical mechanism of contraction (as studied with cell models)

1. Changes in cell shape during contraction

A transformation in the myo-epithelial cell model from a relaxed, T shape to a round or spherical appearance was employed as a direct indication of its contraction. Such changes in cell shape have been observed in fibroblast models (Hasselbach and A. Weber, 1955; Hoffmann-Berling, 1959, 1960, 1964; Hoffmann-Berling and H.H. Weber, 1953; H.H. Weber, 1958) and models of ameba (Hoffmann-Berling, 1960; Simard-Duquesne and Couillard, 1962). The conversion in the hydra-cell model to a spherical shape seems to involve retraction of the attenuated basal processes, accompanied by shortening of the columnar portion of the cell, although these two separate local contractions are usually masked by the uniform contraction of the entire cell. Both of these alterations probably occur in hydra cells in vivo. The retraction of the basal processes of the gastrodermal or epidermal myo-epithelial cells would lead to the contraction of the respective layer (i.e., elongation of the animal in the case of gastrodermis, and shortening for the epidermal layer). The conversion from columnar to cuboidal epithelium has been correlated with changes in the contracted state of hydra; Gauthier (1963), Greenwood (1888), and Semal-van Gansen (1952) all observed a decrease in height of these cells during contraction.

An attempt to separate the contraction of the basal processes from uniform contraction of the cell led to ambiguous results (see, for example, Fig. 7). Hoffmann-Berling (see

review article, 1964) has expressed a similar difficulty in obtaining equatorial constriction (cytokinesis) of glycerinated models of fibroblast cells in anaphase; they became spherical in the presence of ATP and divalent ions. Kinoshita et al. (1964) recently reported that relaxing factor, normally present and probably unevenly distributed in these fibroblast cells, was removed and/or inactivated in less than an hour of glycerol extraction. When these cells were incubated in a medium containing "relaxing grana" isolated either from rabbit skeletal muscle or from homogenized fibroblast cells, their ability to exhibit regional constriction was restored. Possibly, the extraction of hydra inactivated some similar, unevenly-distributed factor partially responsible for asymmetrical, local contractions of the cell. In this event, the gastrodermal and epidermal inactivation rates would probably differ; then, after extraction more active relaxing factor would remain in, say, the gastrodermal than the epidermal cells. Thus, relatively more gastrodermal cells would be capable of type C II contraction. This was found to be the case (compare Fig. 7 a and c, b and d).

However, there exists another possible interpretation of the high percentage of type C II contracted gastrodermal cells always obtained, based on a scoring artefact. There are two major types of large, green gastrodermal cells in H. viridis: (1) digestive cells, having basal muscle processes (and thus, the true "gastrodermal myo-epithelial" cell) and (2) gland cells, lacking them (Burnett, 1959; Hyman, 1940, p. 374). The gland cells could easily have been mistaken for

digestive cells in type C II form. Furthermore, the gastrodermal basal processes are rather short and are much less robust than those of the epidermal myo-epithelial cells (see Fig. 11 and 15, also Mueller, 1950); thus, it is difficult to distinguish a relaxed gastrodermal myo-epithelial cell from one of type C II.

2. Interactions among ATP, Ca, and Mg

At nearly neutral pH and low ionic strength, contraction of epidermal myo-epithelial cells of hydra requires ATP (see, for example, Tables 13 and 17) plus at least one divalent ion. In the presence of ATP, either Ca (Table 18) or Mg (Table 17, prepn 8; Table 20; Fig. 6a) is necessary but not sufficient to stimulate contraction. In this respect not only the absolute concentrations of these divalent ions, but more important, their relative concentrations with respect to ATP are critical, as will be discussed here.

When ATP, Ca, and Mg are in buffered solution (at approximately neutral pH), the following ionic species will be present in equilibrium concentrations: ATP^{3-}, ATP^{4-}, Ca^{2+}, Mg^{2+}, $CaATP^{2-}$, and $MgATP^{2-}$ (see Appendix, VI, for stability constants). Contraction, particularly of epidermal cells, will be analyzed, then, as a function of the relative concentrations of all these substrates. In this light, two broad questions may be posed: (1) What causes the peak of contraction (Fig. 6 a, b, c) that occurs at high concentrations of total Mg and ATP, affected only slightly by changes in total Ca concentration; and (2) Which substrate or substrates are responsible for the peak that occurs at lower Mg and ATP concentrations and increases in amplitude with increasing

concentration of total Ca. One way of approaching these problems is to examine the concentration ratios for all possible combinations of ions and ion complexes, as functions of total ATP, Mg, and Ca concentrations, looking for correlations between contraction peaks and maximum activator-to-inhibitor concentration ratios. In this approach ATP^{4-} and ATP^{3-} may be combined and their sum denoted simply by "ATP" or "free ATP," because at a given pH their concentration ratio is constant (see Appendix, VI, equation [1]), that is, invariant under changes in total ATP, Mg, and Ca concentrations. Negative logarithms of absolute concentrations of ATP ions, Ca^{2+}, Mg^{2+}, $CaATP^{2-}$, and $MgATP^{2-}$, as evaluated at pH 7.2 for the solutions corresponding to Figure 6 a, b, and c, are listed in Table 25; and curves showing concentration ratios for all possible combinations of these five substrates versus total Mg and ATP concentrations, at each total Ca level, may be seen in Fig. 9.

There is much evidence from studies of higher muscle (Geske, Ulbrecht, and H.H. Weber, 1957; Hasselbach, 1964; Maruyama and Watanabe, 1962; Seidel and Gergely, 1963; Watanabe, Sergeant, and Angleton, 1964; A. Weber, 1959; and A. Weber and Winicur, 1961) that all of these components bind, with different affinities, to the contractile protein, with various degrees of stimulation or inhibition among the enzyme-substrate complexes so formed. Support will be presented for the following three theses: (1) Each of the above-mentioned substrates forms a complex with the contractile system in the following decreasing order of affinity: ATP^{4-}, ATP^{3-}, $Mg^{2+} > MgATP^{2-} > CaATP^{2-}$. Furthermore, (2) The true

activators of contraction are $MgATP^{2-}$ and $CaATP^{2-}$; while free ATP ions and possibly Mg ions inhibit. Finally, (3) $MgATP^{2-}$, competes with $CaATP^{2-}$ - stimulated contraction; that is, both metal-ATP complexes are competetive inhibitors for contraction. A similar relationship between Ca and Mg has been demonstrated by Prosser (1967) in sponges and by Filo, Bohr, and Ruegg (1965) in smooth muscle. Furthermore, Ca and Mg are mutually antagonistic in stimulating myosin ATPase activity of striated muscle (Hasselbach, 1964; Perry, 1955). However, the conditions for contraction in hydra are not completely identical to those for shortening of striated muscle fibers. In the former, Ca promotes contraction even in the absence of Mg; in the latter, Mg is an absolute requirement for contraction (see Hasselbach, 1964; Watanabe et al., 1964).

The epidermal contraction peak in Fig. 6 at high total Mg and ATP concentration occurs even in the absence of Ca (Fig. 6 a). Furthermore, the peak is affected only to a minor extent by the addition of Ca; as the total concentration of Ca increases, this peak shifts slightly to the right. Activation by $MgATP^{2-}$ and inhibition by free ATP would satisfy the first condition; i.e., the concentration ratio of $MgATP^{2-}$: free ATP increases with increasing total concentrations of Mg and ATP and is only slightly altered by changes in Ca concentration (see Fig. 9 a). Restating this relationship, it follows from the equilibrium equation (see Appendix, VI, [3]) for $Mg^{2+} + ATP^{4-} \rightleftharpoons MgATP^{2-}$ that if the total concentration of Mg equals that of ATP, and they are simultaneously varied, that $MgATP^{2-}$ concentration will vary directly with the square of the free ATP concentration. Furthermore, the

absolute concentration of $MgATP^{2-}$ is independent of Ca concentration. The drop in the curve which occurs at high total Mg and ATP concentration might be caused by the correspondingly high absolute concentration of $MgATP^{2-}$ or Mg^{2+}, either by a form of substrate inhibition by $MgATP^{2-}$ or by inhibitory alteration by Mg^{2+} of the configuration of the structural contractile system (Bozler, 1952; A. Weber, 1959). Because this decrease in the curve is shifted to the right with higher Ca concentrations (see the arrow in Fig. 6 a, and the right-hand arrows in Fig. 6 b and 6 c), it is possible that Ca^{2+} or $CaATP^{2-}$ reverses this inhibition to a slight extent. It might be argued (Fig. 9 c) that free ATP is the stimulating substrate causing this peak, and that Ca^{2+} is inhibitory. First of all, this contention is not borne out by other experiments (see Tables 17, 18). Second, as the total Ca concentration increases in Fig. 6, the concentration ratio of free ATP to Ca^{2+} decreases, but there is no corresponding decrease in the height of the curve. Thus, it is most likely that the stimulatory substrate for the high peak of contraction is $MgATP^{2-}$, inhibited by free ATP.

The peak of contraction at low ATP and Mg concentration seems, first of all, to be directly related to total Ca concentration, and, second, to decrease with increasing total concentrations of Mg and ATP. Because of the first property, the substrate inducing contraction in this region is probably Ca^{2+} or $CaATP^{2-}$. Since Ca^{2+} alone cannot stimulate contraction (see Fig. 6 c, for example; less than 15% of the epidermal cells contracted in the absence of ATP), the activator is probably $CaATP^{2-}$. If $CaATP^{2-}$ had less affinity than $MgATP^{2-}$ for the contractile system, then as the concentration ratio $MgATP^{2-}$: $CaATP^{2-}$ decreased, $CaATP^{2-}$ -stimulated contraction

would increase. This ratio does indeed decrease both with increasing total Ca concentration and also with decreasing total Mg and ATP concentrations (see Fig. 9 c), corresponding to the behavior of the lower contraction peak.

The curve decreases to the right of the lower peak as the $MgATP^{2-}$:$CaATP^{2-}$ concentration ratio increases, while at the same time the $MgATP^{2-}$:ATP ratio is still low enough for the inhibitory effect of free ATP to be expressed. The concentration ratio ATP:$CaATP^{2-}$ does not contribute to this drop in the curve, as this ratio is invariant under changes in total ATP and Mg concentration (Fig. 9 b). This statement follows readily from the equilibrium equation (Appendix, VI, [2]) for the reaction $Ca^{2+} + ATP^{4-} \rightleftharpoons CaATP^{2-}$; when the total Ca concentration is constant, the Ca^{2+} concentration (much greater than the $CaATP^{2-}$ concentration) will remain almost constant, and $CaATP^{2-}$ concentration will vary directly with ATP concentration.

As was noted in the legend for Fig. 9, the Mg^{2+} concentration remained at all times approximately equal to that of free ATP; thus, it is impossible in Fig. 6 to separate the inhibitory effect of one from the other. If contraction in hydra is like that in higher muscle, then inhibition by free ATP is more likely (Geske et al., 1957; Perry and Grey, 1956; Seidel and Gergely, 1963), although inhibition by Mg^{2+} has been suggested in some situations (Bozler, 1954; A. Weber, 1959). Mitochondrial $MgATP^{2-}$-stimulated ATPase activity has been shown to be inhibited by either free Mg^{2+} or free ATP (Ulrich, 1964).

The order of binding affinity to the contractile system proposed above, namely ATP, $Mg^{2+} > MgATP^{2-} > CaATP^{2-}$, explains the ability of free ATP to compete with $MgATP^{2-}$, and of $MgATP^{2-}$ to compete with $CaATP^{2-}$. The relationship $Mg^{2+} > MgATP^{2-}$ is implied from the results for contraction of cell models in Table 19. Here, the concentration ratio Mg^{2+}:$MgATP^{2-}$ increased with total Mg concentration; thus, the inhibitory effect of Mg^{2+} on $MgATP^{2-}$-stimulated contraction was greatest at high total Mg concentration in this experiment. At low total Mg concentration the ratio of free ATP to $MgATP^{2-}$ concentration was greatest, and free ATP inhibited the $MgATP^{2-}$-activated contraction.

Although the data for gastrodermal contraction are more ambiguous than for epidermal contraction, similar trends are evident. For example (see Fig. 6), contraction seems to be inhibited at high concentrations of total Mg and ATP (Fig. 6 a and b), and this inhibition is reversed to some extent (Fig. 6 c) by the addition of Ca. A similar enhancing effect by Ca addition may be noted in Table 17. As was the case for epidermal cells, contraction is stimulated either by Ca and ATP (Table 18) or Mg and ATP (Table 19).

Thus, the evidence presented in this work sustains the thesis that the substrates $MgATP^{2-}$ and $CaATP^{2-}$ both stimulate contraction of hydra cells (from the gastric region of the column) in a competitive manner, and that free ATP and possibly Mg ions inhibit the contraction stimulated by either metal-ATP complex. It is entirely possible that $MgATP^{2-}$ binds with the contractile system in preference to $CaATP^{2-}$, while the latter substrate may promote greater ATPase activity. Such ATP splitting is a universal component of contractile systems (see,

for example, Davies, 1963; Hoffmann-Berling, 1960; Perry, 1960).

Further experiments are planned to determine if the cells of different regions of hydra, especially those of the tentacles and basal disc, obey the same mechanism. The regions are of particular interest because the contractions of their respective cells are manifested in the coiling of the tentacles during the trapping of prey, and in the gliding of the organism along a substrate; while contractions of the cells along the column are observed as relatively one-dimensional alterations in the total column length.

3. pH

It can be seen from the equilibrium equation for ATP^{4-} and ATP^{3-} (Appendix, VI, equation [1]) that as the pH increases, the concentration ratio of ATP^{4-}:ATP^{3-} will increase, and therefore (see Appendix, equations [4] , [5]), the concentration ratios Ca^{2+}:$CaATP^{2-}$ and Mg^{2+}:$MgATP^{2-}$ will decrease. Thus, it is difficult to separate the direct effect of pH on the contractile system, e.g., alteration of the structural and enzymatic protein configuration (see Davies, 1963; Giese, 1962, p. 318; Scheraga, 1967), from the effect on the equilibrium concentrations of the various ATP and divalent ion substrates described above. However, regardless of whether the first (direct) or the second (indirect) effect on contraction was being measured in the pH experiment, the curve for epidermis differed from that for gastrodermis (Fig. 5); in particular, the pH maximum for epidermal cells was 6.8, and the maximum for gastrodermal cells was 7.6 (plus another possible maximum

at 7.0). At other total ATP, Mg, and Ca concentrations the maxima might shift. Mommaerts and Seraidarian (1947) pointed out that the optimum pH is a function of ionic conditions; they found two peaks of actomyosin ATPase activity, one between 8 and 9 which disappeared in the absence of Mg or Ca, and another at about 6.5 which remained relatively unchanged when divalent ions were removed. Contraction (of over 30% of the cells) was obtained over the pH range 6.6 to 7.6, comparable with the optimal range for contraction of higher muscle (see Kielley, 1955; Sarkar, Szent-Györgyi, and Varga, 1950; also review articles by H.E. Huxley, 1960; and Szent-Györgyi, 1953) and also of fibroblast-cell models (Hoffmann-Berling, 1954; Hoffmann-Berling and H.H. Weber, 1953).

4. Ionic strength

The ionic strength used to achieve contraction of cell models throughout this work was unusually low (0.02μ for H. viridis and 0.05μ for H. littoralis). Up to 0.20μ has been used to stimulate activity of myosin ATPase from rabbit psoas muscle (Perry, 1955), and about 0.1μ is most commonly employed to produce tension or shortening in glycerinated fibers of skeletal muscle (see Hasselbach, 1964; Sarkar et al., 1950; H.H. Weber, 1958). Furthermore, an internal potassium-ion concentration of on the order of 150 millimolar seems to characterize most animal cells (Steinbach, 1963); the total ionic strength in frog muscle is about 0.10μ (see Davson, 1964, p. 392) and in crab muscle, about 0.15μ (see Davson, 1964, p. 404). It must be recalled, however, that the intracellular concentrations of inorganic ions is quite low

in hydra (Steinbach, 1963; see also Results, I C 1 c). Furthermore, several other contractile systems have been reported which are activated at low ionic strength; Simard-Duquesne and Couillard (1962) found it necessary to extract and reactivate ameba with 0.05μ; and Maruyama (1966) obtained ATPase activity of insect actomyosin only in the presence of low ionic strength (less than 0.05μ). Therefore, while such a low optimum ionic strength as was found for hydra is unusual, it is not unique among contractile systems.

5. Relaxation: effects of urea and Salyrgan

Urea treatment was necessary for the relaxation of cells which had contracted (see Bárány et al., 1960) during extraction with saponin. The urea concentration used here (1.5 M) was found by Bárány et al. (1960) to be sufficient to affect a reversible dissociation of actomyosin into actin and myosin, and a reversible relaxation or decrease in tension (see also Bozler, 1951) in contracted glycerinated psoas fibers and in fibroblast-cell models (Hoffmann-Berling, 1954). Thus, it is likely that urea exerted a similar action on hydra-cell models.

Salyrgan, which was mentioned in Materials and Methods as a known mercury-containing, sulfhydryl (-SH) binder that inhibits contraction and actomyosin ATPase activity of skeletal muscle, also inhibited epidermal and gastrodermal cell contraction of type C I, but was ineffective for type C II (see Fig. 8). The C II results are difficult to interpret, especially in light of the scoring difficulties already discussed. The inhibition of C I contraction, on the other

hand, is evidence that sites involving free -SH groups on a contractile-protein system, which have been shown to be necessary for contraction of higher muscle (see Perry, 1960; H.H. Weber, 1958) and for motility of many other systems, including fibroblast cells (Hoffmann-Berling, 1953, 1954, 1960, 1964), vorticella (Hoffmann-Berling, 1958), flagella (Hoffmann-Berling, 1960), and ameba (Simard-Duquesne and Couillard, 1962), are also required for contraction in hydra.

6. Speed of contraction

Differences between time to complete shortening and re-elongation of living hydra (see, for example, Mueller, 1950; Passano and McCullough, 1965) were not reflected in the cell models; the period of time required to reach a contraction plateau for the gastrodermal myo-epithelial cell models (i.e., after which no more cells contracted) did not differ greatly from that of epidermal cell models (see Fig. 4). Casual observations have indicated that hydra usually shorten within seconds or fractions of seconds and require up to several minutes for complete re-elongation (see also Passano and McCullough, 1965). The rapid epidermal contraction *in vivo* is correlated with its efficient communication system, manifested in extensive innervation (Burnett and N. Diehl, 1964; Kepner and Looper, 1926; Marshall, 1923; Spangenberg and Ham, 1960) and elaborate intercellular connections, including the tight junctions observed here and also described by Wood (1959; see also Loewenstein and Kanno, 1964; Passano, 1963; Wiener, Spiro, and Loewenstein, 1964); while the slower gastrodermal contraction exhibited by live hydra is correlated

with the absence or sparsity of gastrodermal neurons (Hyman, 1940; Kepner and Looper, 1926; Lentz and Barrnett, 1965; Mueller, 1950) and the relatively poorly developed gastrodermal musculature (Mueller, 1950).

Each of the two cell populations (epidermis and gastrodermis) was found to be heterogeneous with respect to contraction speed and threshold for response (see Bullock, 1965, p. 485); that is, synchronous contraction of an entire field of epidermal or gastrodermal cells was never observed. This contractile heterogeneity is probably related to the general nature of hydra; unlike a homogeneous population of striated muscle cells or vertebrate fibroblast cells, the cells along the column of hydra display a gradient of metabolic activity (Burnett, 1961; Child, 1947, 1951), age (Burnett, 1959, 1961), and state of differentiation with respect to histochemical criteria (Burnett, 1959); thus, a contractile gradient would not be surprising. As a corollary to the contractile heterogeneity of hydra cells, the state of contraction of each layer is directly proportional to the ratio of active to inactive cells. Csapo (1962) has described an analogous situation in uterine muscle. There, individual fibers shorten independently of one another; and total contraction of the uterus is a reflection of the number of contracted fibers.

In brief, the contraction of hydra myo-epithelial cells, as studied with saponin models, resembles that of muscle in higher animals in its stimulation by ATP, its optimum pH range, and its inhibition by urea and Salyrgan. It differs, on the other hand, in several vital aspects: first, either

$CaATP^{2-}$ or $MgATP^{2-}$ is sufficient to stimulate contraction; second, the optimum ionic strength for contraction is comparatively low; and third, unlike muscle models, the hydra cell model shortens not just unidimensionally, but rather uniformly, often becoming spherical in the presence of ATP plus Mg or Ca.

B. The morphology of the contractile system (as studied with electron microscopy)

As has already been discussed, models of both gastrodermal and epidermal myo-epithelial cells of hydra change from a T shape to a spherical appearance when activated with ATP and divalent ions. However, not only do they differ in the details of their requirements for optimum contraction, but they also diverge in several morphological aspects, including types and arrangement of myofilaments, and, to a lesser extent, size and shape of the basal processes and of the over-all cell. These differences will be pointed out here, in the morphological comparisons of hydra myo-epithelial cells with other contractile systems.

1. Myofilaments

Regions filled with uniformly-oriented contractile filaments were found in the basal processes of the myo-epithelial cells of both layers, as well as in the vertical, epithelial portions of the epidermal cells. Similar vertically-oriented filaments probably exist in the gastrodermal cells, although they were not found in the electron micrographs examined in this study.

The randomly arranged, parallel thick (160 A) and thin (55 A) filaments in the base of each epidermal myo-epithelial cell bear resemblances, with respect to size and disposition, to those of many invertebrate smooth muscles (see Lowy and

Hanson, 1962; Hanson and Lowy, 1957, 1961, and also review article, 1960). They found that many molluscan and annelid muscles, which had previously been shown with light microscopy to lack striations, possessed both thick and thin filaments, frequently interconnected by transverse bridges not unlike those of vertebrate skeletal muscle (Huxley, 1957, 1960). These filaments were randomly arranged in some organisms, helically ordered in others. In all cases the filaments were apparently longitudinally staggered, so as to obscure any regular, repeating pattern. In all the tissues that they examined, the thin filaments were about 50 A in diameter, while the thick ones covered a wide spectrum, from 130 A in Loligo (squid) mantle muscle, up to 1500 A in the opaque adductor muscle of Crassostrea. Recently, similar arrays of thick and thin filaments have been reported in many other invertebrate muscles, including Microciona (Porifera) myocytes (150 to 250 A, 50 to 70 A diameters; Bagby, 1965, 1966), Dugesia (Platyhelminthes) vertical head muscle (250 A, 50 to 60 A, in a ratio of about 6 to 1, thin to thick filaments; Morita, 1965), Tubellaria (Platyhelminthes) body muscle (200 A and 50 A; MacRae, 1965), and Ascaris (Nematoda) body muscle (175 A and 90 A; Reger, 1964) and esophageal myoepithelium (140 to 175 A, 35 to 75 A; Reger, 1966).

The diameters of the thick and thin filaments, about 160 A and 55 A respectively, found in the present study for H. littoralis are close to the corresponding dimensions recently reported by Slautterback (1967) for Hydra oligactis, 140 A and 55 A. There were also interspecific similarities in thin: thick filament ratios, about 7:1 for H. oligactis and close to

8.1 for H. littoralis. These ratios are unusually high compared with most muscle, particularly with vertebrate skeletal muscle, where the hexagonally-arranged thin and thick filaments are in 2:1 ratio (Huxley, 1957). However, 6:1 ratios have recently been found in striated muscle of arthropods, molluscs, nematodes, and annelids (see Anderson and Ellis, 1967; Hagopian, 1966; D.S. Smith, Gupta, and Una Smith, 1966). In these organisms, the myofibrillar arrangement is highly regular (about 12 thin filaments surround each thick filament, and two thick filaments share each thin filament), in contrast to the random organization in hydra.

Striation in hydra muscle was observed neither in the present work nor by Slautterback (1967). However, the dissimilarity between the epidermal muscle fibers in hydra and those of striated coelenterates (see Horridge, 1954; Chapman, Pantin, and Robson, 1962) is likely only a reflection of different arrangements of the thick and thin myofilaments (see Lowy and Hanson, 1962).

The vertical bundles of thin filaments seen in electron micrographs of the epidermal myo-epithelial cells are probably the same filaments seen by early light microscopists; von Gelei (1925) thought that they were supporting fibrils, and the name "Stützfibrillen" remained in the literature for quite some time (see Hyman, 1940). However, later light microscopists suggested that these vertical fibrils might be myonemes (Mueller, 1950; Semal-van Gansen, 1952); Slautterback (personal communication) also feels that they may be contractile. Lentz (1965) has seen

vertical myofilament bundles, similar to those described here, in differentiating myo-epithelial cells of hydra; and filament bundles of similar orientation appear in the micrographs of Hess, Cohen, and Robson (1957) and Zeikus and Steinhaus (1966), although the authors made no mention of them.

The contention that these filaments are contractile, greatly supported by observations of cell-model contraction in the present study, is in accord with the similarity of hydra myo-epithelial cells to vertebrate myo-epithelial cells. These vertebrate cells are ectodermal in origin; and their contraction is associated with various secretory activities (Ham and Leeson, 1961, p. 239 to 240, 417, 552). Their fine structure has been examined (see Ellis, 1965; Tamarin, 1966); in all cases a bundle containing filaments all of the same diameter, usually between 50 and 70 A, was found, oriented in a baso-apical direction. The analogy with respect to filament orientation and diameter in hydra epidermal cells is striking.

The thin-filament bundles present in these hydra cells are also reminiscent of similar structural elaborations associated with movement in numerous protists, including Nitella (Nagai and Rebhun, 1966), Physarum (Wohlfarth-Bottermann, 1964), Chaos chaos (Nachmias, 1964), Ameba (Wolpert, Thompson, and O'Neill, 1964), Stentor (Randall and Jackson, 1958), Spirostomum (Yagai and Shigenaka, 1963), and Vorticella (Sotelo and Trujillo-Cenóz, 1959; Yagai and Shigenaka, 1963).

Finally, the epidermal vertical filaments bear morphological and physiological resemblances to vertebrate smooth

muscle; such comparisons with this smooth muscle have been made for vertebrate myo-epithelial cells (Hurley and Shelley, 1954; Tamarin, 1966). Most electron micrographs of sectioned vertebrate smooth muscle have revealed only one type of myofilament, 50 A in diameter, which is probably actin (see Csapo, 1962; Rhodin, 1962; Schoenberg et al., 1966). However, there is a growing feeling that another component is normally present in vivo, corresponding to a thicker, myosin-containing filament, and that this component is labile, readily breaking down to much smaller monomers (see Kaminer and Bell, 1966; Schoenberg et al., 1966). Needham (1962) has prepared purified actin and myosin separately from uterine smooth muscle (where only one type of filament is seen in electron micrographs) which cross-react with the complementary components extracted from rabbit skeletal muscle (where two components are seen in electron micrographs). Thus, it is possible that while only one type of filament can be observed in electron micrographs of the vertical protion of the epidermal myo-epithelial cell and also the base of the gastrodermal myo-epithelial cell, there could nevertheless be an actomyosin-like system in each region. Further evidence for such a system in hydra is the inhibitory action of Salyrgan on contraction of cell models, already discussed (II A 5).

2. Organelles and granules associated with active contraction

Mitochondria have been found in association with every type of contractile system just discussed (see Fawcett, 1966); their role in ATP production is well known (Lehninger, 1965).

Their usual peripheral position in the myofibril (see D.S. Smith, 1964) was shown here to be greatly exaggerated in the basal muscle processes in hydra cells; they appear as large protrusions in the outer surface of the cell (also noted by Hess et al., 1957). This disposition is unusual for smooth muscle, in which mitochondria are usually scattered throughout the cell and are frequently surrounded by myofilaments (see Rhodin, 1962). However, MacRae (1963) has described a mitochondrial arrangement in planarian smooth muscle similar to that in hydra.

Both α- and β- forms of glycogen (see Revel, 1964) are in abundance near the myofilaments in hydra; their probable role as a source of chemical energy for contraction is well established (Fawcett, 1966; Fawcett and Selby, 1958; Perry, 1956).

An unusual relationship between mitochondria and glycogen has been demonstrated by Lentz (1966). In well-fed hydra an intramitochondrial glycogen-synthesizing pathway is apparently activated; when the intracristae region is full of glycogen granules, the outer membrane seems to burst on one side of the mitochondrion, releasing glycogen to the cell for further cytoplasmic metabolism. Such mitochondria in the process of synthesizing glycogen, near a basal gastrodermal myofibril in the gastric region of hydra, may be seen in Fig. 26.

3. Comparison of the plasma membrane of myo-epithelial cells with the sarcoplasmic reticulum and T-system of other contractile cells

Rapid conduction of a chemical and/or electrical contractile

impulse to the center of a muscle fiber can present serious problems, especially in the case of very thick (greater than 10μ) fibers. To solve these problems, such muscles with large diameters have evolved an extensive extracellular transverse membrane system, or T-system, and an associated intracellular longitudinal system of tubular and vesicular membranes, the sarcoplasmic reticulum, or SR (see Porter and Bonneville, 1964; D.S. Smith, 1966). The extensiveness of these systems has been positively correlated with fiber diameter (Peachey and Porter, 1959, Reger, 1964); the T-system and SR are quite pronounced in vertebrate skeletal muscle (Porter, 1961) and insect flight muscle (D.S. Smith, 1962), where long fibers up to 100μ in diameter are common. The contractile fibril along the base of hydra is not only short (length not greater than 50μ), but it is also unusually thin (less than 1μ in diameter). An impulse from an adjacent nerve or another myo-epithelial cell would rapidly reach the central myofilaments; thus, the sparsity of membranes possibly associated with impulse conduction is not at all surprising. The fast epidermal muscle of hydra, then, is another example of the rule (see Peachey, 1959; Peachey and Porter, 1959) that the structural elaborations of the SR and T-system are not correlated with rapid muscle contraction, but rather with fiber size.

The few microtubules present amongst and parallel to the basal epidermal myofilaments might possibly play a minor role in the transport of ions associated with contraction and/or communication between the basal and vertical myofilaments;

similar roles of ion transport have been ascribed to microtubules in various types of cells (see Slautterback, 1963). They might, however, be associated with motility (see Ledbetter and Porter, 1963; Nagai and Rebhun, 1966; Porter and Tilney, 1965; Robison, 1966; Roth and Daniels, 1962), or have cytoskeletal function (see Fawcett, 1966).

Pinocytotic vesicles, commonly seen on the surface of large smooth muscle cells (Caeser, et al., 1957; Rhodin, 1962) and myo-epithelial cells (Ellis, 1965), were not observed in cells of hydra. If, as Rhodin (1962) suggests, their major function is to increase the surface-to-volume ratio, thus increasing excitation efficiency, then such membrane elaborations would not be advantageous to the small cells of hydra.

4. Intercellular associations and impulse conduction

The desmosome-like close associations which were frequently seen between two adjacent myo-epithelial cells are similar to the septate desmosomes found by Overton (1963) in the hydroid Cordylophora and by Wood (1959) in Hydra. Such cell-to-cell membrane apposition has been shown conclusively to be directly related to intercellular ion transport and lower intercellular resistence in many cell types (Loewenstein, 1967; Loewenstein et al., 1964; Penn, 1966; Wiener et al., 1964). Since conduction of contractile impulses via epithelial cells has been demonstrated in other coelenterates (Mackie, 1965), and direct muscle conduction has been postulated in this phylum by others (Barnes, 1964; Batham and Pantin, 1954; Chapman et al., 1962;

Josephson and Macklin, 1967; Passano, 1963; Passano and McCullough, 1965; Pantin, 1965), it is quite expected that these intercellular junctions are responsible for myoid conduction in hydra.

This argument becomes even more convincing when one notes the similarities of hydra septate desmosomes to the intercalated discs of cardiac muscle, which are postulated to function in the spread of the excitation over the muscle (Sjöstrand and Andersson-Cedergren, 1960). Furthermore, the contractile system of hydra has been likened to heart muscle in its rhythmic behavior; Passano and McCullough (1962, 1963, 1964, 1965; Passano 1963) have found three interrelated pacemaker systems in hydra. They postulate that two of these systems, the rhythmic potentials (RP) and prelocomotor bursts (PLB), are dependent upon myoid conduction via the gastrodermis and epidermis, respectively, and the third, the contraction burst (CB), is conducted by the epidermal nerve net. Contractions associated with the myoid-conducted gastrodermal RP and epidermal PLB are frequent but slow, while nerve-transmitted epidermal contraction bursts are infrequent and rapid.

If hydra does indeed have several overlapping conducting systems, all using the same two sets of muscle, then it is unique among smooth muscles. Bozler (1948) classified all smooth muscle as falling into one of two categories: (1) multiunit or (2) visceral. Multiunit smooth muscle is not spontaneously active and is dependent upon abundant innervation for electrical activation; in visceral muscle excitation

is transmitted via the muscles, fiber to fiber, and is independent of nerves for activation or transmission. The gastrodermis presents no real problem; it falls readily into the visceral category. The epidermis, on the other hand, has some of the features of both smooth muscle types; it displays both myoid conduction (PLB), independent of nerves, and also multiunit behavior in its rapid, nerve-conducted CB.

The ability of a unified group of fibers (e.g., the cylindrical, sheet-like hydra epidermis, analogous to a vertebrate muscle) to carry out both fast and slow contraction is not unique to coelenterates. Such "mixed" muscles have been described in several vertebrate classes, including amphibia (amplexus muscles; Sommerkamp, 1928), reptiles (muscle connecting the ribs to the skin in the garter snake; Hess, 1965; Hoyle et al., 1966), and mammals (extraocular muscles in the guinea pig; Hess, 1961b). Electron microscopic studies of all these tissues have revealed two kinds of fibers present in the same muscle: one type, thought to be the physiologically fast or "twitch" fiber (Hess, 1961 b; Hoyle et al., 1966) is composed of small fibrils separated by regular spaces, each surrounded by extensive SR, and each associated with single nerve endings; the other, which would then be the slow or "tonic" fiber, consists of larger fibrils, more irregular in shape and distribution, and on which are seen multiple, branched nerve endings. The fact that all of the many epidermal myo-epithelial basal fibers examined were similar in size, shape, and myofilament content supports the contention that in hydra, unlike

vertebrates, each epidermal myo-epithelial cell is capable of several types of contraction (e.g., corresponding to Passano and McCullough's CB and PLB, 1964, 1965). It is plausible and worthy of future investigation that one such kind of contraction might be stimulated by $MgATP^{2-}$, and the other mediated by $CaATP^{2-}$. If this were so, then regulation of the contractile state of the cell could be accomplished by the release at nerve endings (Lentz and Barrnett, 1965) of one of these two divalent ions and release of the other ion from the desmosome region between the basal processes of two adjacent myo-epithelial cells. A situation similar to this proposed scheme for hydra exists in the sea anemone; here the alteration of the Ca ion concentration in the sea-water medium has been shown to effect fast contraction in a manner profoundly different from that on slow contraction of the same tissue; similarly for Mg (Ross, 1960 a, 1960 b). On the other hand, it must be considered that instead of some cell being stimulated by either divalent ion, there might be two populations of epidermal myo-epithelial cells in hydra, one which contracts only in the presence of Ca and the other only with Mg. However, this is not likely; almost 60% of the cells contracted at each of the two peaks of the 10^{-4} M Ca curve (Fig. 6 c); i.e., the total fraction of Mg-stimulated cells and Ca-stimulated cells is greater than 100%.

The intercellular, cytoplasmic continuity observed in one instance between two adjacent gastrodermal muscle cells (Fig. 37) deserves a brief comment. It was already mentioned that this particular region is probably anomalous, since the mesogleal disposition is bizarre and unexplainable. Further-

more, muscles which were thought before the advent of electron microscopy to be syncytial, especially cardiac muscle and also many smooth muscles, have been since then shown to be composed of closely-opposed cells, each completely bound by a unit membrane (see Choi, 1962; Dewey and Barr, 1962, 1964; Sjöstrand and Andersonn-Cedergren, 1960). Cytoplasmic bridges between muscle cells are unusual (Wright, 1966) and are thought to be transitory in the rate instances in which they are seen (Thaemert, 1959).

5. Relationships and associations between epidermis and gastrodermis

Possible evidence for two general types of communication between the epidermal and gastrodermal layers of hydra has been presented here: (1) direct contact by meandering processes of epidermal and gastrodermal myo-epithelial cells across the mesoglea and (2) diffusion of large (400 A diameter) granules from the epidermal basal cell surfaces across the mesoglea into the region of the gastrodermal myofibrils. The first type of contact between the two layers across the mesoglea, i.e., via deep insertions and projections of cell processes, has also been demonstrated all along the length of hydra by Haynes, Davis and Burnett (personal communication), Hess (1961 a), Hess et al. (1957), and Wood (1961). The second suggested type, the production of granules by activated epidermal cells, followed by the shipment of these granules across the mesoglea to the gastrodermal myofilaments, has not been reported in the literature, apparently, either in hydra or in any other contractile system. It is conceivable that these granules are

involved in pumping calcium (see Hasselbach, 1964) or magnesium to regulate contraction in the two reciprocal muscle layers of hydra.

To recapitulate the salient features of the contractile system in hydra, changes in shape of the myo-epithelial cells in the interacting epidermal and gastrodermal layers are manifested in the co-ordinated movements of the animal. These changes are reflected both in the ultrastructural features of these cells and also in the behavior of saponin models in solutions of various concentrations and combinations of ATP, Ca and Mg ions, at different ionic strengths and pH values. Basically, contraction is stimulated by ATP and either Ca or Mg at nearly neutral pH and low ionic strength. It is likely that in hydra, as in almost every other contractile system, this contraction is mediated by two interacting structural proteins, actin- and myosin-like. As in higher muscle, active -SH sites on these proteins are probably essential for this interaction in hydra, as evidenced by the inhibitory effect of Salyrgan on contraction.

Two types of myofilaments, corresponding in diameter to actin and myosin, are present in the basal regions of the epidermal cells (longitudinally oriented in the animal); and bundles of thin filaments are situated in the corresponding regions of the gastrodermal myo-epithelial cells (of transverse, circular orientation) and also in the vertical portion of each epidermal cell (transversely and radially arranged with respect to the animal). The double filament array

resembles those of many invertebrate smooth muscles, while the two types of thin-filament bundles in hydra are similar to those present in the contractile regions of vertebrate myo-epithelial cells, vertebrate smooth muscle, and many protozoa. The unconvoluted plasma membrane and the sparsity of intracellular membranes associated with hydra myofilaments is unusual for muscular contractile systems and is probably associated with the small size of the contractile cells and the related speed of impulse conduction to the central myofilaments. Microtubules present among and parallel to the basal myofilaments might be implicated in movement of ions, possibly associated with cell-to-cell impulse conduction down the length of the animal. Desmosomes between the basal contractile regions of adjacent myo-epithelial cells are strikingly similar in appearance and probably in function to the intercellular, impulse-conducting intercalated discs of vertebrate cardiac tissue and smooth muscle. Finally, contraction-related communication between the epidermis and gastrodermis may be accomplished by at least two morphologically-evident means: close junctions between basal, root-like processes of both types of myo-epithelial cells, across the mesoglea; and possibly by the movement of large "signal" granules secreted by the epidermal cells, across the mesoglea, to the gastrodermal myofilaments.

SUMMARY

This study can be divided into three phases: A. the preparation and verification of contractile models of hydra, B. the chemical mechanism of contraction in hydra, based on the behavior of these models, and C. the fine structure of the contractile system.

A. Relaxed whole-animal and cell models of hydra were prepared by brief extraction in saponin followed by washing in urea. These models meet the following criteria: they respire much more slowly than do normal animals; and their contractile systems are morphologically intact. Frog sartorious fibers treated in the above manner are similar in the following physiological properties, to glycerinated fiber models: they no longer maintain a significant transmembrane potential; and they shorten in the presence of ATP and divalent ions.

Therefore, models prepared with saponin meet all the morphological and physiological criteria which are desirable for contractile models.

B. The roles of ATP and divalent ions, pH, ionic strength, and inhibitors of contraction were assessed in these models, as enumerated below:

1. Adenosine triphosphate is required for contraction of hydra cell models; moreover, at least one divalent ion must be present. It is proposed that $MgATP^{2-}$ and $CaATP^{2-}$ act as competitive inhibitors in the stimulation of contraction and that free ATP and probably free Mg ions act to inhibit contraction.

2. The pH optima for epidermal and gastrodermal myoepithelial cells are 6.8 and 7.6 respectively.

3. An unusually low ionic strength (0.05μ for H. littoralis, 0.02μ for H. viridis) is required for optimum preparation and activation of models.

4. Urea acts in hydra cells as a relaxing agent, as it is known to do in glycerinated muscle fibers.

5. Salyrgan inhibits contraction of hydra cell models. Thus, hydra resembles vertebrate muscle in its ATP requirement, response to inhibitors, and pH optima, but differs in its low ionic strength, and its contraction stimulated by Ca in the absence of Mg.

C. An electron microscopic investigation was conducted of the contractile system of hydra. Emphasis was placed on the contractile filaments and on the membrane system.

1. Hydra has at least three independent myofilament regions. In the basal regions of the epidermal myo-epithelial cells are random arrays of thick and thin filaments; in the corresponding gastrodermal regions only thin filaments are present. Each epidermal cell is characterized by an additional bundle of filaments, all thin, in its epithelial region.

2. Intracellular and extracellular membrane systems like the transverse membranes and sarcoplasmic reticulum of muscle were not observed in hydra myo-epithelial cells. This finding was correlated with their small size.

It was concluded that in their motility, hydra myo-epithelial cells have many morphological and physiological features in common with such diverse groups as the protists,

vertebrate myo-epithelial cells, and muscle cells of higher metazoa. Thus, hydra must be recognized as a functionally important intermediate.

ACKNOWLEDGMENTS

The unremitting encouragement of my major professor, Dr. S. P. Maroney, Jr., during my graduate training has been most gratifying. For suggesting many aspects of the study, for his overall support, and for his constructive criticism, I would like to extend my sincere appreciation.

Of particular benefit have been numerous discussions with Dr. Robert L. Searls, on biochemical questions, with Dr. J. J. Murray, Jr., concerning statistical techniques, and with Dr. W. Gerald Robison, Jr., regarding contractile morphology. Special thanks are extended to Dr. David S. Smith and my husband, Irwin, who taught me ultrastructural techniques and took the electron micrographs for this study. I should also like to acknowledge the advice and aid of Stanley B. Kater in the recording of the transmembrane potentials.

Mr. Lewis ("Sarge") Eaton deserves very special mention for going out of his way on countless occasions to find equipment and chemicals, no matter how unusual.

This work was supported in part by a training grant, 5-T1HD2902, from the National Institutes of Health, and in part by a teaching assistantship from the University of Virginia. I am grateful to the members of the Department of Biology, in particular to Dr. Dietrich Bodenstein, for making these awards possible.

My husband's understanding, ideas and assistance, and most of all moral support have been sustaining forces throughout my graduate work.

LITERATURE CITED

Abercrombie, M., and E. J. Ambrose. 1962. The surface properties of cancer cells: a review. Cancer Res. 22: 525-548.

Afzelius, Björn. 1959. Electron microscopy of the sperm tail. Results obtained with a new fixative. J. Biophys. Biochem. Cytol. 5(2): 269-278.

Anderson, W.A., and R.A. Ellis. 1967. A comparative electron microscope study of visceral muscle fibers in Cambarus, Drosophila, and Lumbricus. Z. Zellforsch. Mikroscop. Anat. 79(4):587-591.

Bagby, R.M. 1965. The contractile system of marine sponges. Diss. Abstr. 25(11):6721.

_____. 1966. The fine structure of myocytes in the sponges Microciona prolifera (Ellis and Solander) and Tedania ignis (Duchassaing and Michelotti). J. Morphol. 118: 167-182.

Bailey, K., and S.V. Perry. 1947. The role of sulphydryl groups in the interaction of myosin and actin. Biochim. Biophys. Acta. 1:506-516.

Bairati, A., and F.E. Lehmann. 1954. Partial disintegration of cytoplasmic structures of Amoeba proteus after fixation with osmium tetroxide. Experientia. 10:173-175.

Bangham, A.D., and R.W. Horne. 1962. Action of saponin on biological cell membranes. Nature. 196(4858):952-953.

_____. 1964. Negative staining of phospholipids and their structural modification by surface-active agents as observed in the electron microscope. J. Mol. Biol. 8: 660-668.

Bangham, A.D., M.M. Standish, and G. Weissman. 1965. The action of steroids and streptolysin S on the permeability of phospholipid structures to cations. J. Mol. Biol. 13:253-259.

Bárány, M., K. Bárány, und W. Trautwein. 1960. Die Hemmung der Aktin-L-Myosin Interaktion in Lebenden und extrahierten Muskeln durch Urea. Biochim. Biophys. Acta. 45: 317-335.

Barnes, W.J.P. 1964. Nervous, muscular, and chemical transmission in coelenterates. Biol. J. 4:12-24.

Batham, E.J., and C.F.A. Pantin. 1954. Slow contraction and its relation to spontaneous activity in the sea-anemone Metridium senile (L). J. Exp. Biol. 31:84-103.

Bendall, J.R. 1953. Further observations on a factor (the 'Marsh' factor) effecting relaxation of ATP-shortened muscle-fibre models and the effect of Ca and Mg upon it. J. Physiol. 121:232-254.

Bergman, R.A. 1958. An experimental study of the non-fibrillar components in frog striated muscle. Bull. Johns Hopkins Hosp. 103:267-280.

Bozler, E. 1948. Conduction, automaticity, and tonus of visceral muscles. Experientia. 4:213-218.

_____. 1951. Mechanism of relaxation in extracted muscle fibers. Amer. J. Physiol. 167:276-283.

_____. 1952. The effect of electrolytes on the contractile elements of muscle. J. Gen. Physiol. 35(5):703-710.

_____. 1954. Relaxation in extracted muscle fibers. J. Gen. Physiol. 38:149-159.

Bullock, Theodore Holmes. 1965. Coelenterata and Ctenophora, p. 459 to 534. In Theodore Holmes Bullock and G. Adrian Horridge (ed.) Structure and Function in the Nervous System of Invertebrates. W. H. Freeman & Co., San Fran. Two Vol. (Vol. I, xx + 798).

Burnett, Allison L. 1959. Histophysiology of growth in hydra. J. Exp. Zool. 140:281-342.

_____. 1961. The growth process in hydra. J. Exp. Zool. 146: 21-84.

Burnett, Allison L., and N.A. Diehl. 1964. The nervous system of hydra. I. Types, distribution, and origin of nervous elements. J. Exp. Zool. 157(2):217-226.

Caeser, R., G.A. Edwards, and H. Ruska. 1957. Architecture and nerve supply of mammalian smooth muscle. J. Biophys. Biochem. Cytol. 3:867-878.

Cahn, Robert D. 1967. Detergents in membrane filters. Science. 155(3759):195-196.

Cerame-Vivas, Maximo J. 1961. Separation of cell layers in Hydra. Wilhelm Roux' Arch. Entw. Mech. Organismen. 153: 213-216.

Chapman, D.M., C.F.A. Pantin, and E.A. Robson. 1962. Muscle in coelenterates. Rev. Can. Biol. 21(3/4):267-278.

Child, C.M. 1947. The effects of certain depressing agents on intracellular oxidation of indicators in Hydra. Physiol. Zool. 20:332-348.

_____. 1951. Oxidation-reduction patterns in the coelenterates. Physiol. Zool. 24:97-115.

Choi, Jae Kwan. 1962. Fine structure of the smooth muscle of the chicken's gizzard, p.M-9. Fifth Int. Congr. Electron Microscopy, Proc., Phil. Acad. Press, N.Y.

Csapo, Arpad I. 1962. Smooth muscle as a contractile unit. Physiol. Rev. (Suppl. 5, II) 42:7-33.

Davies, R.E. 1963. A molecular theory of muscle contraction: calcium-dependent contractions with hydrogen bond formation plus ATP-dependent extensions of part of the myosin-actin cross-bridges. Nature, 199(4898):1068-1074.

Davson, Hugh. 1964. A Textbook of General Physiology, 3rd Ed. Little, Brown, and Co., Boston. xvi + 1166.

Davson, Hugh, and J.F. Danielli. 1938. Studies on the permeability of erythrocytes. V. Factors in cation permeability. Biochem. J. 32:991-1001.

_____. 1952. Permeability of Natural Membranes, 2nd Ed. Cambridge Univ. Press. xii + 365.

Dawson, R.M.C., D.C. Elliot, W.H. Elliot, and K.M. Jones. 1959. Data for Biochemical Research. Oxford Press. xiii + 299.

Dewey, M.M., and L. Barr. 1962. Intercellular connection between smooth muscle cells: the nexus. Science. 137(3531): 670-672.

_____. 1964. A study of the structure and distribution of the nexus. J. Cell Biol. 23:553-585.

Dourmashkin, R.R., R.M. Dougherty, and R.J.C. Harris. 1963. The effect of saponin and digitonin on Rous sarcoma virus and cell membranes; an approach to the cytochemistry of cellular components by negative staining. J. Roy. Microscop. Soc. 81(3-4):215-218.

Edman, K.A.P. 1957. On the contraction of glycerol-extracted fiber bundles under highly isometric conditions. Acta. Physiol. Scand. 41:229-254.

Ellis, Richard A. 1965. Fine structure of the myoepithelium of the eccrine sweat glands in man. J. Cell Biol. 27: 551-563.

Fawcett, Don W. 1966. An Atlas of Fine Structure. The Cell, its Organelles and Inclusions. W.B. Saunders Co., Phil. viii + 448.

Fawcett, Don W., and C.C. Selby. 1958. Observations on the fine structure of the turtle atrium. J. Biophys. Biochem. Cytol. 4:63-72.

Filo, Ronald S., David F. Bohr, and J.C. Ruegg. 1965. Glycerinated skeletal and smooth muscle: calcium and magnesium dependence. Science. 147(3665):1581-1583.

Gauthier, Geraldine F. 1963. Cytological studies on the gastroderm of Hydra. J. Exp. Zool. 152:13-39.

Geigy Industrial Chemicals. 1958. Comparison of CHEL ME and CHEL DE with EDTA. Tech. Bull. tentative 2/58.

Gelei, J. von. 1925. Bemerkungen zu der "morphologischen" und physiologischen Gliederung des Körpers unserer Süsswasserpolypen. Zool. Anz. 64:117-125.

Gergely, J. 1966. Contractile proteins. Ann. Rev. Biochem. (II)35:691-722.

Geske, G., M. Ulbrecht, und H.H. Weber. 1957. Die Einfluss der Mg-Konzentration auf die Substrathemmung der ATP-Spaltung als angebliche ursache der Erschlaffung des Muskels. Naunyn-Schmiedeberg's Arch. Exp. Pathol. Pharm. 230:301-309.

Gibbons, I.R., and A.V. Grimstone. 1960. On flagellar structure in certain flagellates. J. Biophys. Biochem. Cytol. 7:697-716.

Giese, Arthur C. 1962. Cell Physiology, 2nd Ed. W. B. Saunders Co., Phil. xxi + 592.

Glauert, A.M., and R.H. Glauert. 1958. Araldite as an embedding medium for electron microscopy. J. Biophys. Biochem. Cytol. 4:191-194.

Gray, Peter. 1954. The Microtomist's Formulary and Guide. The Blakiston Co., Inc., N.Y. xiii + 794.

Greenwood, M. 1888. On digestion in Hydra, with some observations on the structure of the endoderm. J. Physiol. 9: 317-344.

Hagopian, Martin, 1966. The myofilament arrangement in the femoral muscle of the cockroach, Leucophaea maderae Fabricius. J. Cell Biol. 28:545-562.

Hale, L.J. 1958. Biological Laboratory Data. John Wiley and Sons, Inc., N.Y. x + 132.

Ham, Arthur W., and Thomas Sydney Leeson. 1961. Histology, 4th Ed. J. B. Lippincott Co., Phil. xv + 942.

Hamaguchi, Kozo. 1955a. Studies on protein denaturation by surface chemical method. I. The relationships between monolayer properties and urea denaturation of lysozyme. J. Biochem. 42(5):449-459.

_____. 1955b. Studies on protein denaturation by surface chemical method. II. On the mechanism of surface denaturation of lysozyme. J. Biochem. 42(6)705-714.

Hanson, J., and J. Lowy. 1957. The presence of a double array of myofilaments in certain invertebrate smooth muscles. J. Physiol. 137:42P-43P.

_____. 1960. Structure and Function of the contractile apparatus in the muscles of invertebrate animals, p. 265 to 336. In G. H. Bourne (ed.) The Structure and Function of Muscle, Vol. I, Structure. Acad. Press, N.Y. (xvi + 472.

_____. 1961. The structure of the muscle fibers in the translucent part of the adductor of the oyster, Crassostrea angulata. Roy. Soc. (London), Proc., B. 154:173-196.

Hasselbach, W. 1952. Die Diffusionkonstante des Adenosinetriphosphats im Inneren des Muskels. Z. Naturforsch. 7: 334-337.

_____. 1964. Relaxing factor and the relaxation of muscle. Prog. Biophys. Mol. Biol..14:167-222.

Hasselbach, W., and A. Weber. 1955. Models for the study of the contraction of muscle and cell protpolasm. Pharm. Rev. 7:97-117.

Hess, Arthur. 1961a. The fine structure of cells in Hydra, p. 1 to 50. In Howard M. Lenhoff and W. Farnsworth Loomis (ed.) The Biology of Hydra and of some other Coelenterates: 1961. Univ. Miami Press, Coral Gables, Fla. (xv + 467).

_____. 1961b. The structure of slow and fast extrafusal muscle fibers in the extraocular muscles and their nerve endings in guinea pigs. J. Cell. Comp. Physiol. 58: 63-80.

_____. 1965. The sarcoplasmic reticulum, the T system, and the motor terminals of slow and twitch muscle fibers in the garter snake. J. Cell.Biol. 26:467-476.

Hess, Arthur, A.I. Cohen, and Elaine A. Robson. 1957. Observations on the structure of hydra as seen with the electron and light microscopes. Quart. J. Microscop. Sci. 98(3):315-326.

Hippel, P. H. von, and W. F. Harrington. 1960. The structure and stabilization of the collagen macromolecule, p. 213 to 231. In Brookhaven Symp. in Biol., No. 13, Protein Structure and Function. Brookhaven Nat. Lab., Upton, N.Y. (ix + 226).

Hoffmann-Berling, Harmut. 1953. Die wasser-glyzerin-extrahierte Zelle als Modell der Zellmotilitat. Biochim. Biophys. Acta. 10:628.

_____. 1954. Adenosinetriphosphat als Betriebsstoff von Zellbewegungen. Biochim. Biophys. Acta. 14:182-195.

_____. 1958. Der Mechanismus eines neuen, von der Muskelkontraktion verschiedenen Kontraktionszyklus. Biochim. Biophys. Acta. 27:247-255.

_____. 1959. The role of cell structures in cell movements, p. 45 to 63. In D. Rudnick (ed.) Cell, Organism, and Milieu. The Seventeenth Symposium of the Society for the Study of Development and Growth, 1958. The Ronald Press Co., N.Y. (v + 326.)

_____. 1960. Other mechanisms producing movements, p. 341 to 370. In Marcel Florkin and Howard S. Mason (ed.) Comparative Biochemistry, Vol. II, Free Energy and Biological Function. Acad. Press, N.Y. (xix + 685.)

_____. 1964. Relaxation of fibroblast cells, p. 365 to 376. In Robert D. Allen and Noburô Kamiya (ed.) Primitive Motile Systems in Cell Biology. Acad. Press, N.Y. (xix + 642).

Hoffman-Berling, Harmut, und Hildegard Portzehl. 1955. Bewegungen von extrahierten Zellen und Muskelfibrillen ("Modellen") durch Adenosinetriphosphat (ATP). Institut für den Wissenschaftlichen Film C685/1954. Gottingen, 1955.

Hoffman-Berling, Harmut, und Hans Hermann Weber. 1953. Vergleich der Motilitat von Zellmodellen und Muskelmodellen. Biochim. Biophys. Acta. 10:629-630.

Horridge, Adrian. 1954. Observations on the nerve fibers of Aurellia aurita. Quart. J. Microscop. Sci. 95:85-92.

Hoyle, Graham, Patricia Ann McNeill, and Benjamin Walcott. 1966. Nature of invaginating tubules in Felderstruktur muscle fibers of the garter snake. J. Cell.Biol. 30(1): 197-201.

Hurley, Harry J., and Walter B. Shelley. 1954. The role of the myopithelium of the human apocrine sweat gland. J. Invest. Dermatol. 22:143-156.

Huxley, H.E. 1957. The double array of filaments in cross-striated muscle. J. Biophys. Biochem. Cytol. 3:631-648.

_____. 1960. Muscle cells, p.365 to481. In J. Brachet and A.E. Mirsky (ed.) The Cell; Biochemistry, Physiology, Morphology; Vol. IV, Specialized Cells: Part I. Acad. Press, N.Y. (xv + 511.)

Huxley, H.E., and H. Hanson. 1960. The molecular basis of contraction in cross-striated muscles, p. 183-229. In G. H. Bourne (ed.) The Structure and Function of Muscle, Vol. I, Structure. Acad. Press, N.Y. (xvi + 472).

Hyman, Libbie Henrietta. 1940. The Invertebrates; Protozoa through Ctenophora. McGraw-Hill Book. Co., N.Y. p. 365 to 662. (xii + 726).

Jennings, H.S. 1906. Behavior of the Lower Organisms. The Columbia Univ. Press, N.Y. (xiv + 366).

Josephson, Robert K., and Martin Macklin. 1967. Transepithelial potentials in Hydra. Science. 156(3782):1629-1631.

Kaminer, B., and A.L. Bell. 1966. Myosin filamentogenesis: Effects of pH and ionic concentration. J. Mol. Biol. 20(2):391-403.

Karnovsky, Manfred L., A. William Shafer, Helen Korshak, and K. Saito. 1964. Effects of surface-active substances on the behavior of phagocytic cells. Fed. Proc. 23(2): 270.

Kauzmann, W. 1959. Some factors in the interpretation of protein denaturation. Advances Prot. Chem. 14:1-63.

Kavanau, J. Lee. 1963. Structure and functions of biological membranes. Nature. 198:525-530.

_____. 1965. Structure and Function in Biological Membranes (Vol. I and II). Holden-Day, Inc., San Fran. (xxiv + 760).

Kepner, W.A., and J.B. Looper, 1926. The nutrition of the ovum of Hydra viridis. Biol. Bull. 50: 525-532.

Kielley, W. Wayne. 1955. Mg-activated muscle ATPases, p. 589 to 591; In S.P. Colowick and N.O. Kaplan (ed.) Methods in Enzymology, Vol. II, Preparation and Assay of Enzymes. Acad. Press, N.Y. (xx + 987).

Kinoshita, Seiichiro, Benjamin Andoh, und Harmut Hoffmann-Berling. 1964. Das Erschlaffungssystem von Fibroblastenzellen. Biochim. Biophys. Acta. 79(1):88-97.

Klotz, I.M. 1960. Non-covalent bonds in protein structure, p. 25 to 43. In Brookhaven Symp. in Biol., No. 13, Protein Structure and Function. Brookhaven Nat. Lab., Upton, N.Y. (ix + 226).

Lane, Bernard. 1965. Alterations in the cytologic detail of intestinal smooth muscle cells in various stages of contraction. J. Cell Biol. 27:199-213.

Ledbetter, M.C., and Porter, K.R. 1963. A "microtubule" in plant cell fine structure. J. Cell Biol. 19:239-250.

Lehninger, Albert L. 1965. The transfer of energy within cells, p. 173 to 202. In John A. Moore (ed.) Ideas in Modern Biology. The Natural History Press, N.Y. (ix + 202.)

Lenhoff, H.M., and J. Bovaird. 1961. A quantitative chemical approach to problems of nematocyst distribution and replacement in Hydra. Devel. Biol. 3:227-240.

Lentz, Thomas L. 1965. The fine structure of differentiating interstitial cells in Hydra. Z. Zellforsch. 67:547-560.

_____. 1966. Intramitochondrial glycogen granules in digestive cells of Hydra. J. Cell Biol. 29:162-167.

Lentz, Thomas L., and Russell J. Barrnett. 1965. Fine structure of the nervous system of Hydra. Amer. Zool. 5:341-356.

Lilly, S.J. 1955. Osmoregulation and ionic regulation in Hydra. J. Exp. Biol. 32:423-439.

Ling, G., and R.W. Gerard. 1949. The normal membrane potential of frog sartorious fibers. J. Cell. Comp. Physiol. 34: 383-396.

Loewenstein, Werner R. 1967. On the genesis of cellular communication. Devel. Biol. 15(6):503-520.

Lowenstein, Werner R., and Yoshinobu Kanno. 1964. Studies on an epithelial (gland) cell junction. I. Modifications on surface membrane permeability. J. Cell Biol. 22:565-586.

Lowy, Jack, and Jean Hanson. 1962. Ultrastructure of invertebrate smooth muscle. Physiol. Rev. (suppl. 5, II)42:34-47.

Lucy, J.A. 1964. Globular lipid micelles and cell membranes. J. Theor. Biol. 7:360-373.

Lucy, J.A., and Audrey M. Glauert. 1964. Structure and assembly of macromolecular lipid complexes composed of globular micelles. J. Mol. Biol. 8:727-748.

Luzzati, V., and F. Husson. 1962. The structure of the liquid-crystalline phases of lipid-water systems. J. Cell Biol. 12:207-219.

McNairy, Sidney Archie, Jr. 1966. Chemical and biological properties of alfalfa-top saponins and mineral aspects of bloat. Diss. Abstr. B 27(2):388-389.

Mackie, G.O. 1965. Conduction in the nerve-free epithelia of siphonophores. Amer. Zool. 5:439-453.

MacRae, Edith Krugelis. 1963. Observations on the fine structure of pharyngeal muscle in the planarian Dugesia tigrina. J. Cell Biol. 18:651-662.

_____. 1965. The fine structure of muscle in a marine Turbellarian. Z. Zellforsch. Mikroscop. Anat. 68:348-362.

Marsh, B.B. 1952. The effect of adenosine-triphosphate on the fibre volume of muscle homogenate. Biochim. Biophys. Acta. 9:247-260.

Marshall, S. 1923. Observations on the behaviour and structure of Hydra. Quart. J. Microscop. Sci. 67:593-616.

Martell, A.E., und Schwartzenbach, G. 1956. Adenosinphosphate und Triphosphat als Komplexbildner fürCalcium und Magnesium. Helv. Chim. Acta. 39:653-661.

Maruyama, Koscak. 1966. Effect of magnesium on the adenosine-triphosphatase activity of insect actomyosin at low ionic strength. Comp. Biochem. Physiol. 18(3):481-487.

Maruyama, K., and S. Watanabe. 1962. The role of magnesium in the superprecipitation of Myosin B. J. Biol. Chem. 237:3437-3442.

Mommaerts, W.F.H.M., and K. Seraidarian. 1947. A study of the adenosine triphosphatase activity of myosin and actomyosin. J. Gen. Physiol. 30:401-422.

Morales, Manual F. 1959. Mechanisms of muscle contraction, p. 426-433. In J.L. Oncley (ed.) Biophysical Science--A Study Program. John Wiley & Sons, Inc., N.Y. (viii + 568 + AI14 + SI27).

Morita, Michio. 1965. Electron microscopic studies on planaria. I. Fine structure of muscle fiber in the head of the planarian Dugesia dorotocephala. J. Ultrastr. Res. 13: 383-395.

Mueller, J.F. 1950. Some observations on the structure of hydra, with particular reference to the muscular system. Trans. Amer. Microsc. Soc. 69:133-147.

Muir, A.R. 1962. An electron microscopic demonstration of a surface pattern on the plasma membrane of sectioned intestinal epithelium after saponin treatment. Nature. 195 (4845):1023-1024.

Nachmias, Vivianne. 1964. Fibrillar structures in the cytoplasm of Chaos chaos. J. Cell Biol. 23:183-188.

Nagai, Reiko, and Lional I. Rebhun. 1966. Cytoplasmic microfilaments in streaming Nitella cells. J. Ultrastr. Res. 14(5/6):571-589.

Naylor, Winifred G., and Neil C.R. Merrillees. 1964. Some observations on the fine structure and metabolic activity of normal and glycerinated ventricular muscle of toad. J. Cell Biol. 22(3):533-551.

Needham, Dorothy M. 1962. Contractile proteins in smooth muscle of the uterus. Physiol. Rev. (Suppl. 5, II)42: 88-97.

Overton, J. 1963. Intercellular connections in the outgrowing stolon of Cordylophora. J. Cell Biol. 17: 661-671.

Palmer, C.G., M.E. Hodes, and A.K. Warren. 1961. The action of synthetic surfactants on membranes of tumor cells. I. Morphological observations. Exp. Cell. Res. 24:429-439.

Pantin, C.F.A. 1965. Capabilities of the coelenterate behavior machine. Amer. Zool. 5(3):581-589.

Passano, L.M. 1963. Primitive nervous systems. Nat. Acad. Sci., Proc. 50(2):306-313.

Passano, L.M., and C.B. McCullough. 1962. The light response and the rhythmic potentials of Hydra. Nat. Acad. Sci., Proc. 48(8):1376-1382.

_____. 1963. Pacemaker hierarchies controlling the behavior of hydras. Nature. 199(4899) :1174-1175.

_____. 1964. Co-ordinating systems and behavior in Hydra. I. Pacemaker system of the periodic contractions. J. Exp. Biol. 41:643-664.

_____. 1965. Co-ordinating systems and behavior in Hydra. II. The rhythmic potential system. J. Exp. Biol. 42:205-231.

Peachey, Lee. 1959. Structure of the longitudinal body muscles of Amphioxus. J. Biophys. Biochem. Cytol. 10(4): 159-176.

Peachey, Lee D., and Keith R. Porter. 1959. Intracellular impulse conduction in muscle cells. Science. 129:721-722.

Penn, Richard D. 1966. Ionic communication between liver cells. J. Cell Biol. 29:171-174.

Perry, S.V. 1955. Myosin adenosinetriphosphatase, p. 582 to 588. In S.P. Colowick and N.O. Kaplan (ed.) Methods in Enzymology, Vol. II, Preparation and Assay of Enzymes. Acad. Press, N.Y. (xx + 987).

_____. 1956. Relation between chemical and contractile function and structure of the skeletal muscle cells. Physiol. Rev. 36:1-76.

_____. 1960. Muscular contraction, p. 245 to 340. In Marcel Florkin and Howard S. Mason (ed.) Comparative Biochemistry, Vol. II, Free Energy and Biological Function. Acad. Press, N.Y. (xix + 685).

Perry, S.V. and T.C. Grey. 1956. A study of the effects of substrate concentration and certain relaxing factors on the magnesium-activated myofibrillar adenosine triphosphatase. Biochem. J. 64:184-192.

Pethica, B.A. 1962. The physical chemistry of cell adhesion. Exp. Cell Res. Suppl. 8:123-141.

Ponder, E. 1953. The kinetics of progressive reactions in systems containing saponin, digitonin, and sodium taurocholate. J. Gen. Physiol. 36(5):723-732.

Porter, Keith R. 1961. The sarcoplasmic reticulum. Its recent history and present status. J. Biophys. Biochem. Cytol. (4, suppl.)10:219-226.

Porter, Keith R., and Mary A. Bonneville. 1964. An Introduction to the Fine Structure of Cells and Tissues. Lea and Febiger, Phil. 32 plates.

Porter, Keith R., and Lewis G. Tilney. 1965. Microtubules and intracellular motility. Science. 150(3694):382.

Prosser, C. Ladd. 1967. Ionic analyses and effects of ions on contractions of sponge tissues. Z. Vergl. Physiol. 54:109-120.

Randall, J.T., and Sylvia Fitton Jackson. 1958. Fine structure and function in Stentor polymorphus. J. Biophys.Biochem. Cytol. 4(6):807-830.

Reger, James F. 1964. The fine structure of the fibrillar network and sarcoplasmic reticulum in smooth muscle cells of Ascaris lumbricoides (var. suum). J. Ultrastr. Res. 10(1-2):48-58.

_____. 1966. The fine structure of fibrillar components and plasma membrane contacts in esophageal myoepithelium of Ascaris lumbricoides (var. suum). J. Ultrastr. Res. 14(5/6):602-617.

Revel, J.P. 1964. Electron microscopy of glycogen. J. Histochem. Cytochem. 12:104-114.

Reynolds, E.S. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. 17:208-212.

Rhodin, Johannes A.G. 1962. Fine structure of vascular walls in mammals, with special reference to the smooth muscle component. Physiol. Rev. (Suppl. 5,II)42:48-81.

Ringer, S. 1883. A third contribution regarding the influence of the inorganic constituents of the blood on the ventricular contraction. J. Physiol. 4:222-225.

Robison, W. Gerald. 1966. Microtubules in relation to the motility of a sperm syncytium in an armored scale insect. J. Cell Biol. 29(2):251-266.

Ross, D.M. 1960a. The effects of ions and drugs on neuromuscular preparations of sea anemones. I. On preparations of the column of Calliactis and Metridium. J. Exp. Biol. 37:732-752.

_____. 1960b. The effects of ions and drugs on neuromuscular preparations of sea anemones. II. On sphincter preparations of Calliactis and Metridium. J. Exp. Biol. 37:753-774.

Roth, L.E. 1958. A filamentous component of fibrillar systems. J. Ultrastr. Res. 1:223-234.

Roth, L.E., and E.W. Daniels. 1962. Electron microscopic studies of mitosis in amebae. J. Cell Biol. 12:57-78.

Sabatini, D.D., K. Bensch, and R.J. Barrnett. 1963. Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. J. Cell Biol. 17:19-58.

Sarkar, Nirmal K., Andrew G. Szent-Györgyi, and Leslie Varga. 1950. Adenosintriphosphatase activity of the glycerol extracted muscle fibres. Enzymologia. 14:267-272.

Scheraga, Harold A. 1967. Contractility and conformation. J. Gen. Physiol. 50(6:2):5-27.

Schoenberg, C.F., J.C. Rüegg, D.M. Needham, R.H. Schirmer, and H. Nemetchek-Gansler. 1966. A biochemical and electron microscope study of the contractile proteins in vertebrate smooth muscle. Biochem. Z. 345:255-266.

Seeman, Phillip. 1967. Transcient holes in the erythrocyte membrane during hypotonic hemolysis and stable holes in the membrane after lysis by saponin and lysolecithin. J. Cell Biol. 32(1):55-70.

Seidel, J.C., and J. Gergely. 1963. Studies on myofibrillar adenosine triphosphatase with calcium-free adenosine triphosphate. I. The effect of EDTA, calcium, Mg, and ATP. J. Biol. Chem. 238:3648-3653.

Semal-van Gansen, P. 1952. Étude du systéme musculaire chez Hydra attenuata (Pallas). Acad. Roy. Belg. Bull., C. Sci. 38:642-651.

Simard-Duquesne, N., and P. Couillard. 1962. Amoeboid movement. I. Reactivation of glycerinated models of Amoeba proteus with adenosine triphosphate. Exp. Cell Res. 28: 85-91.

Singer, Thomas P. 1963. Flavoprotein dehydrogenases of the electron-transport chain (Survey), p. 345 to 382. In P.D. Boyer, H. Lardy, and K. Myrbäck (ed.) The Enzymes, Vol. VII, 2nd Ed., Completely Revised. Acad. Press, N.Y. (xxi + 726).

Sjöstrand, F.S., and E. Andersson-Cedergren. 1960. Intercalated discs of heart muscle, p. 421 to 445. In G.H. Bourne (ed.) the Structure and Function of Muscle, Vol. I, Structure. Acad. Press, N.Y. (xvi + 472.)

Slautterback, David. 1963. Cytoplasmic microtubules. I. Hydra. J. Cell Biol. 18(2):367-388.

_____. 1967. The cnidoblast-musculo-epithelial cell complex in the tentacles of Hydra. Z. Zellforsch. Mikroscop. Anat. 79(2):296-318.

Smith, D.S., 1962. The sarcoplasmic reticulum of insect muscles, p. TT3. Fifth Int. Congr. Electron Microscopy, Proc., Phil. Acad. Press, N.Y.

_____. 1964. Skeletal, cardiac, and smooth muscle, p. 267-293. In Stanley M. Kurtz (ed.) Electron Microscopic Anatomy. Acad. Press, N.Y. (xii + 425).

_____. 1966. The organization and function of the sarcoplasmic reticulum and T-system of muscle cells, p. 107 to 142. In Prog. Biophys. Mol. Biol., Vol. 16. Pergamon Press, N.Y.

Smith, D.S., B.L. Gupta, and Una Smith. 1966. The organization and myofilament array of insect visceral muscles. J. Cell Sci. 1:49-57.

Snedecor, George W. 1956. Statistical Methods. The Iowa State Univ. Press. xiii + 534.

Sommerkamp, H. 1928. Das Substrat der Dauerverkürzung am Froschmuskel. Naunyn-Schmiedeberg's Arch. Exp. Pathol. Pharm. 128:99-115.

Sotelo, J. Roberto, and O. Trujillo-Cenóz. 1959. The fine structure of an elementary contractile system. J. Biophys. Biochem. Cytol. 6:126-127.

Southwood, T.R.E. 1966. Ecological Methods, with Particular Reference to the Study of Insect Populations. Methuen & Co., Ltd., London. xviii + 391.

Spangenberg, D.B., and R.G. Ham. 1960. The epidermal nerve net of Hydra. J. Exp. Zool. 143:195-201.

Steinbach, H. Burr. 1963. Sodium, potassium, and chloride in selected hydroids. Biol. Bull. 124(3):322-336.

Steinberg, M.S. 1958. On the chemical bonds between animal cells. A mechanism for type-specific associations. Amer. Natur. 92:65-81.

Stoeckenius, W. 1962. Some electron microscopical observations on liquid-crystalline phases in lipid-water systems. J. Cell Biol. 12:221-229.

Szent-Györgyi, A. 1949. Free energy relations and contraction of actomyosin. Biol. Bull. 96:140-162.

_____. 1953. Chemical Physiology of Contraction in Body and Heart Muscle. Acad. Press, N.Y. xviii + 135.

Tamarin, Arnold. 1966. Myoepithelium of the rat submaxillary gland. J. Ultrastr. Res. 16(3-4):320-338.

Thaemert, J.C. 1959. Intercellular bridges as protoplasmic anastomoses between smooth muscle cells. J. Biophys. Biochem. Cytol. 6:67-70.

Townes, Mary M., and Dugald E.S. Brown. 1965. The involvement of pH, adenosine triphosphate, calcium, and magnesium in the contraction of the glycerinated stalks of Vorticella. J. Cell. Comp. Physiol. 65(2):261-271.

Ulrich, F. 1964. Kinetic studies of the activation of mitochondrial adenosine triphosphatase by Mg. J. Biol. Chem. 239:3532-3536.

Umbreit, W.W., R.H. Burris, and J.F. Stauffer. 1959. Manometric Techniques, 3rd Ed. Burgess Pub. Co., Minn. iii + 338.

Watanabe, Shizuo, Tania Sergeant, and Marita Angleton. 1964. Role of magnesium in contraction of glycerinated muscle fibers. Amer. J. Physiol. 207:800-808.

Weber, Annemarie. 1959. On the role of calcium in the activity of adenosine-5′- triphosphate hydrolysis by actomyosin. J. Biol. Chem. 234(10):2764-2769.

Weber, Annemarie, and S. Winicur. 1961. The role of calcium in the superprecipitation of actomyosin. J. Biol. Chem. 236(12):3198-3203.

Weber, Hans H. 1958. The Motility of Muscle and Cells. Harvard Univ. Press, Cambridge, Mass. 69 p.

Whitney, D.D. 1907. Artificial removal of the green bodies of Hydra viridis. Biol. Bull. 13:291-299.

Wiener, Joseph, David Spiro, and Werner R. Loewenstein. 1964. Studies on an epithelial (gland) cell junction. II. Surface structure. J. Cell Biol. 22:587-598.

Wilson, J.A., P.R. Elliott, K.F. Guthe, and D.G. Shapiro. 1959. Oxygen uptake of glycerol-extracted muscle fibres. Nature. 184:1947.

Wohlfarth-Botterman, K.E. 1964. Differentiations of the ground cytoplasm and their significance for the generation of the motive force of amoeboid movement, p. 79 to 110. In Robert D. Allen and Noburō Kamiya (ed.) Primitive Motile Systems in Cell Biology. Acad. Press, N.Y. (xix + 642).

Wood, Richard L. 1959. Intercellular attachment in the epithelium of Hydra as revealed by electron microscopy. J. Biophys. Biochem. Cytol. 6(3):343-351.

_____. 1961. The fine structure of intercellular and mesogleal attachments of epithelial cells in hydra, p. 51 to 68. In Howard H. Lenhoff and W. Farnsworth Loomis (ed.) The Biology of Hydra and of Some other Coelenterates: 1961. Univ. Miami Press, Coral Gables, Fla. (xv + 467.

Wright, K.A. 1966. Cytoplasmic bridges and muscle systems in some polymyarian nematodes. Can. J. Zool. 44(2):329-340.

Yagai, R., and Y. Shigenaka. 1963. Electron microscopy of the longitudinal fibrillar bundle and the contractile fibrillar system in Spirostomum ambiguum. J. Protozool. 10: 364-369.

Zeidman, I. 1947. Chemical factors in the mutual adhesiveness of epithelial cells. Cancer Res. 7:386-389.

Zeikus, Regina D., and E.A. Steinhaus. 1966. Observations on a previously undescribed type of cellular degeneration in Hydra. J. Invertebr. Pathol. 8(1):14-34.

LIST OF ABBREVIATIONS IN THE ILLUSTRATIONS

B	bundle of thin filaments in the neck of a myo-epithelial cell
C I	a cell which has become spherical during contraction
C II	a cell whose basal processes have contracted
E	epidermis
EBP	epidermal basal process
ER	endoplasmic reticulum
G	gastrodermis
GBP	gastrodermal basal process
GVC	gastrovascular cavity
I	invagination of plasma membrane
J	tight junction
M	mitochondrion
MEC	myo-epithelial cell
Mes	mesoglea
Mt	microtubule
N	nucleus
Nc	nucleolus
Nk	neck-like process connecting the apex of a myo-epithelial cell to the basal process
P	particle
R	ribosome
Rel	relaxed myo-epithelial cell
RI	rootlet-like insertion
S	secretory body

Tk thick filament

Tn thin filament

UM unit membrane

V vesicle

GRAPHS AND ILLUSTRATIONS

Fig. 1. Changes in column length of H. littoralis during saponin and wash treatment (each curve represents a single animal). ------ represents discontinuity in the curve (during changes of solution).

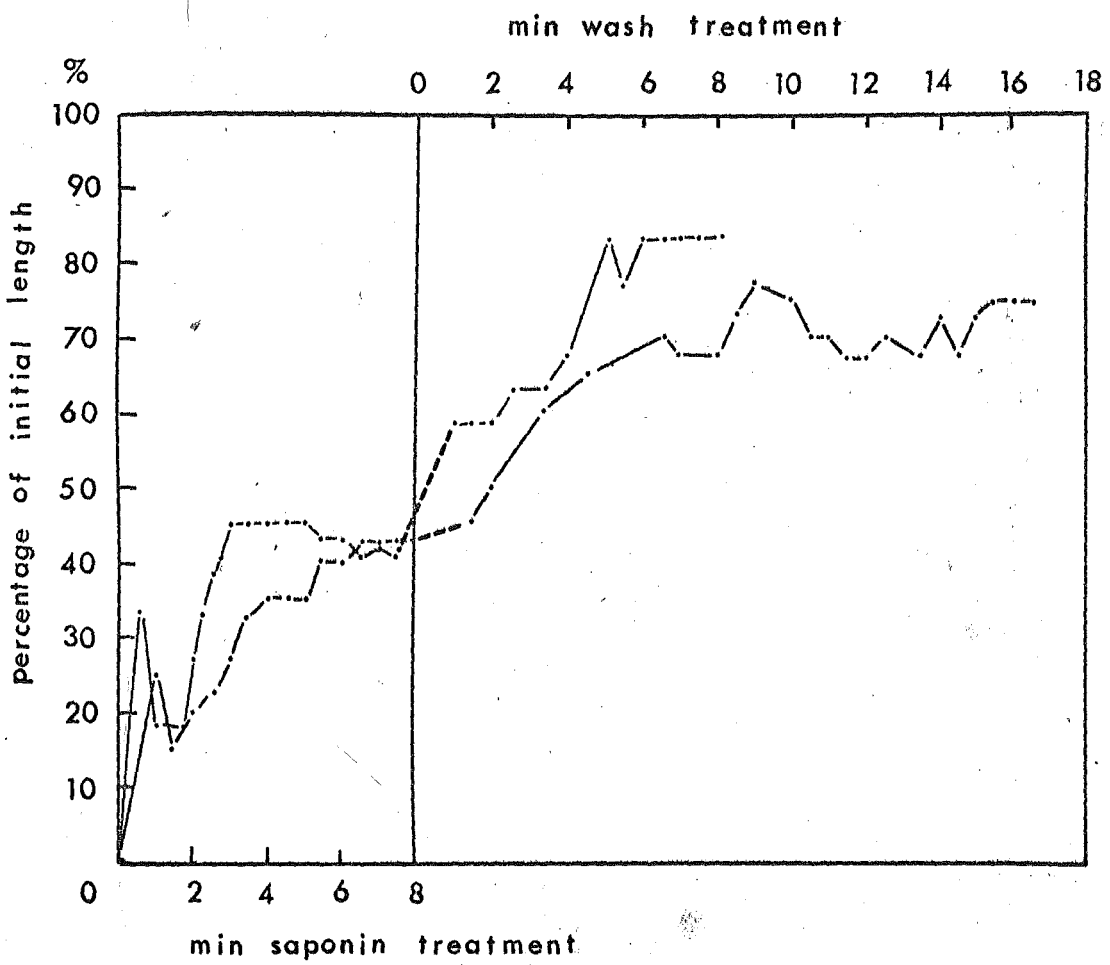

min wash treatment
%
0 2 4 6 8 10 12 14 16 18
100
90
80
70
60
50
40
30
20
10
0
percentage of initial length
0 2 4 6 8
min saponin treatment

Fig. 2. Effect of time in wash solution (5×10^{-3} M imidazole-HCl buffer, pH 6.8, and KCl; total I = 0.02 μ), then in contraction solution (3×10^{-4} M $CaCl_2$ and 5×10^{-4} M ATP, plus buffer and KCl as above), on column length of saponin-treated (8 minutes in solution containing 0.1% saponin and 3×10^{-4} M $CaCl_2$ plus buffer and KCl as above) H. viridis. Open circles represent means, vertical lines standard errors.

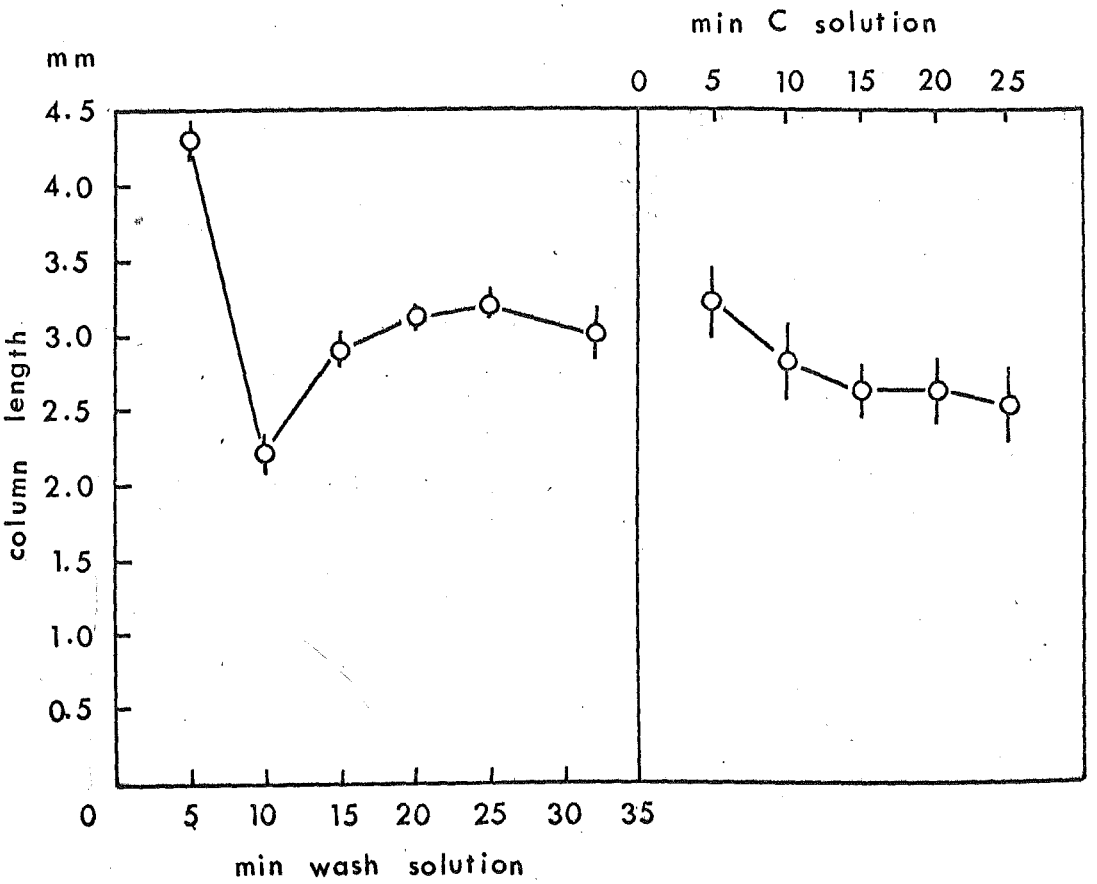

min C solution
mm
0 5 10 15 20 25
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
column length
0 5 10 15 20 25 30 35
min wash solution

Fig. 3. Oxygen consumption in saponin-treated H. littoralis. Curves C1 and C2 are control animals, total dry wt 7.7 and 4.7 mg, respectively; S1 and S2 are saponin-treated animals, total dry wt 6.5 and 9.8 mg, respectively.

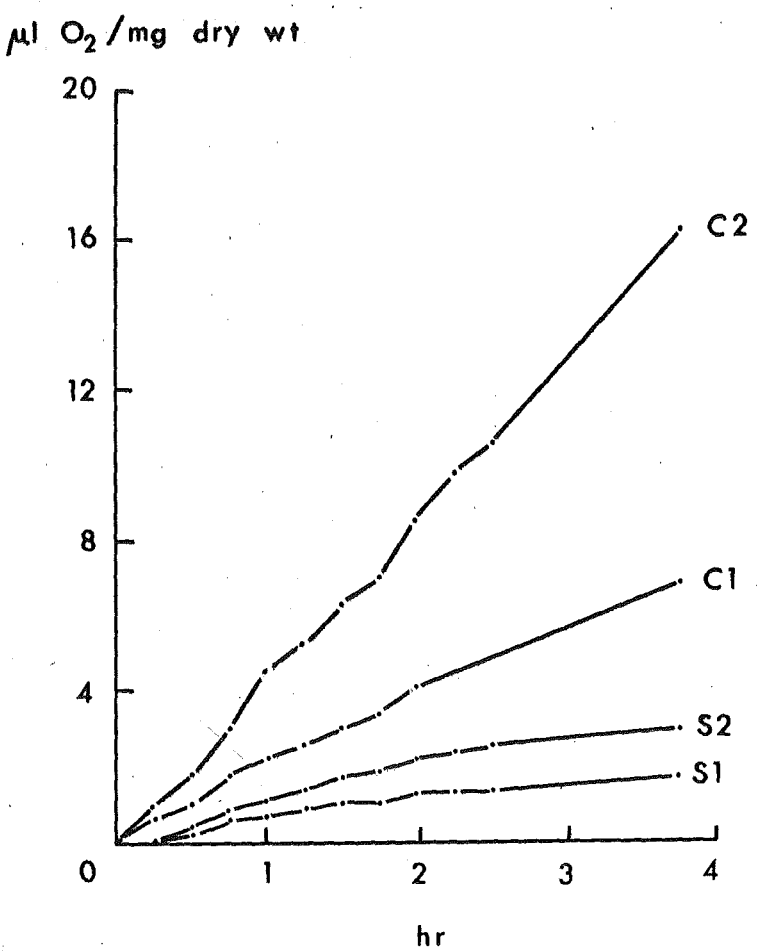

µl O2 /mg dry wt
20
16
12
8
4
0
1
2
3
4
hr
C2
C1
S2
S1

Fig. 4. Contraction of cell models of *H. viridis* versus time in contraction solution (1×10^{-4} M ATP, 5×10^{-4} M $MgCl_2$, 5×10^{-3} M phosphate buffer, pH 7.2, and 5.7×10^{-3} M KCl; I = 0.02 μ). Each point represents ca. 85 gastrodermal (O) or epidermal (●) cells scored.

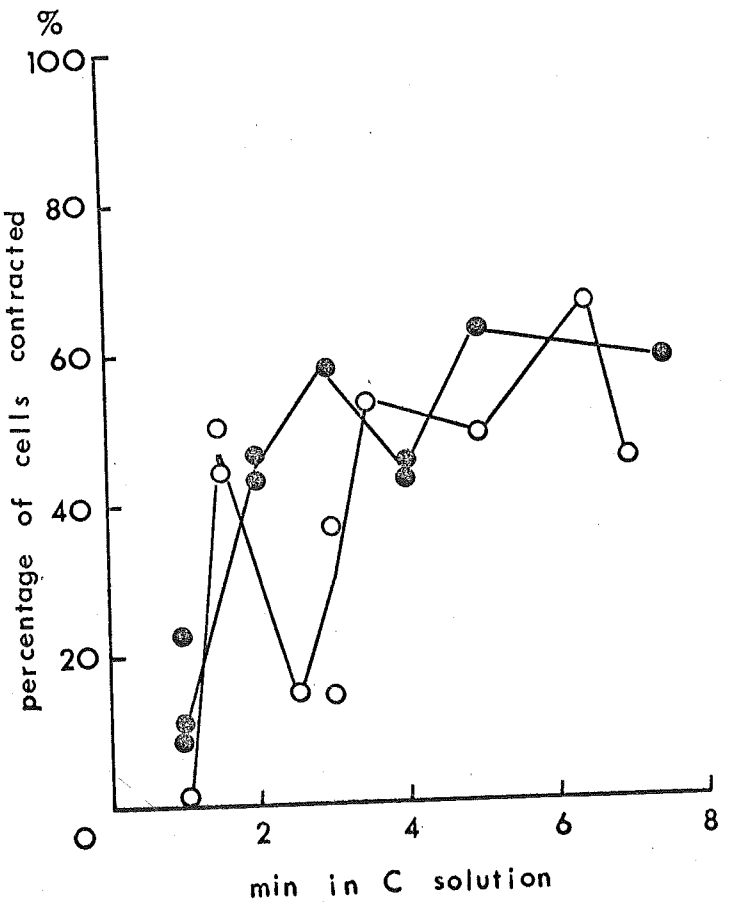

%
100
80
60
40
20
0
percentage of cells contracted
2
4
6
8
min in C solution

Fig. 5. Contraction of cell models of H. viridis as a function of pH. Contraction solutions contain 1×10^{-4} M ATP, 1×10^{-4} M or 5×10^{-4} M $MgCl_2$, 5×10^{-3} M phosphate buffer (pH varied), and KCl (I = 0.02μ). each point represents the mean of several replicates, 100 epidermal (●) or gastrodermal (O) cells per replicate. The ordinates are p (%) and $\sqrt{n}\ \sin^{-1}\sqrt{p}$ (degrees). (See also Table 15).

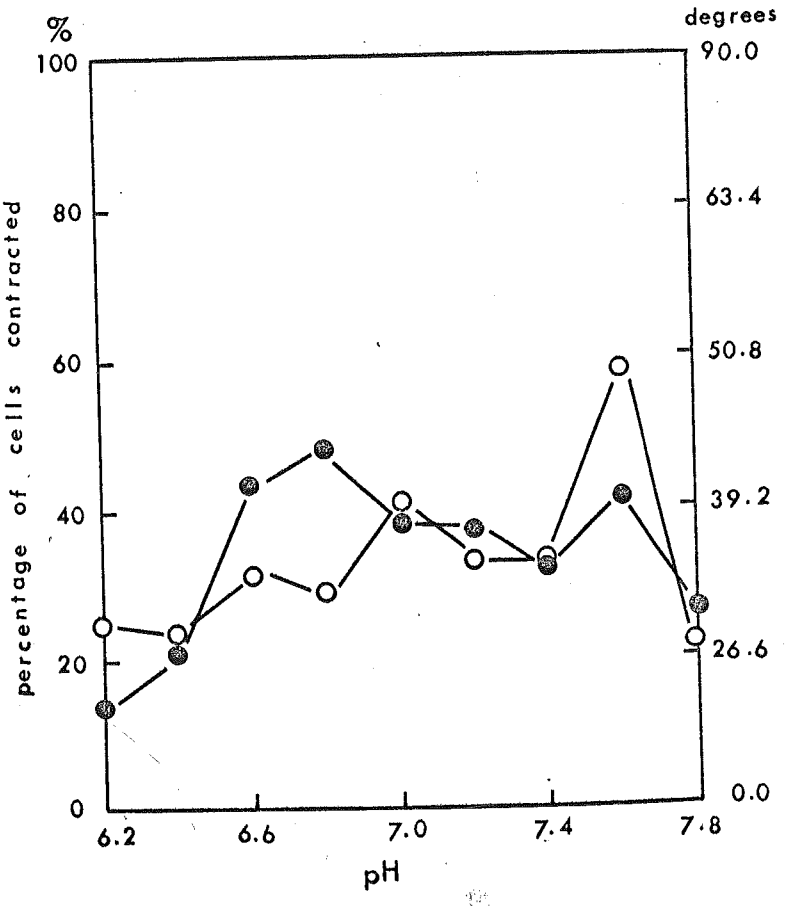
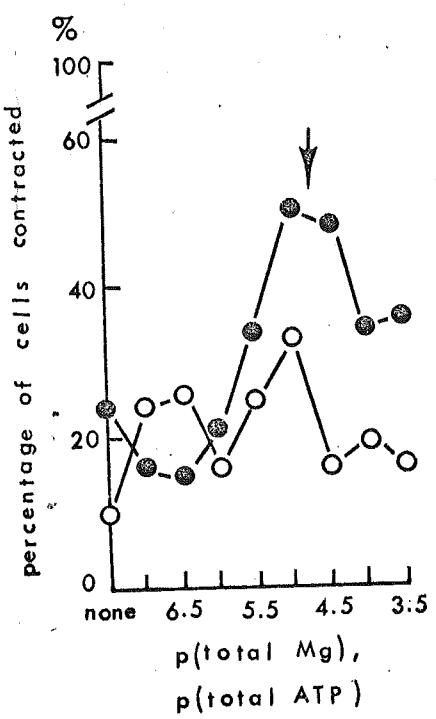
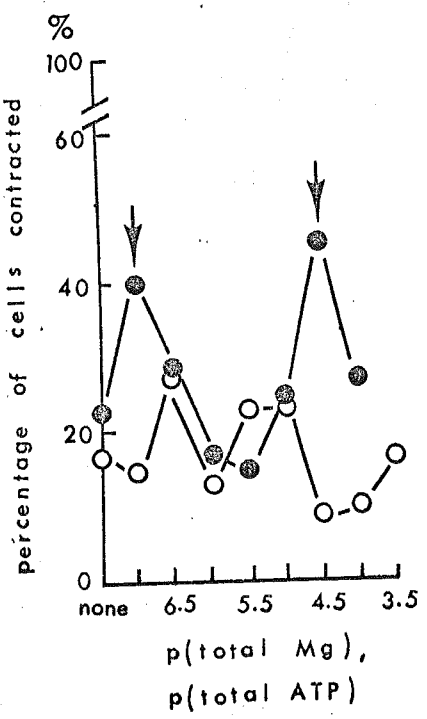

%
degrees
100
90.0
80
63.4
60
50.8
40
39.2
20
26.6
0
0.0
percentage of cells contracted
6.2
6.6
7.0
7.4
7.8
pH

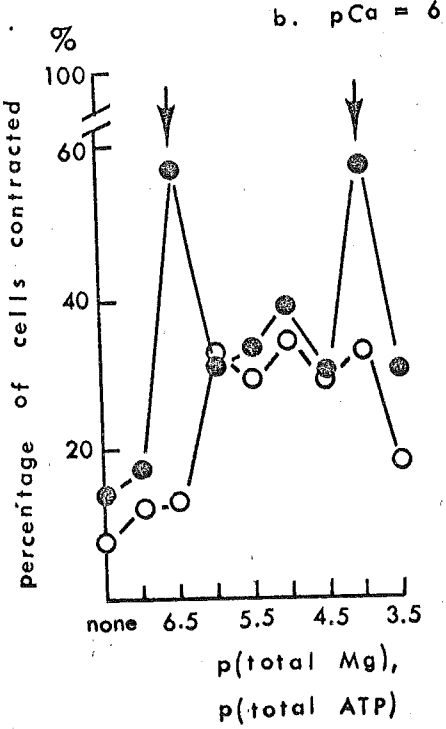
Fig. 6. Contraction (C I) of cell models versus $MgCl_2$ and ATP concentrations, at different Ca levels. Contraction solutions contain Ca, Mg, ATP, KCl, and 5×10^{-3} M phosphate buffer, plus 1×10^{-4} M EGTA in a. and b. (pH 7.2, I = 0.02μ). Each point represents the mean of three replicates, 100 epidermal (●) or gastrodermal (O) cells per replicate. Epidermal maxima are indicated by ↓. In a. and b. "pCa" refers to total Ca concentration after chelation by 1×10^{-4} M EGTA.



a. pCa = 8.

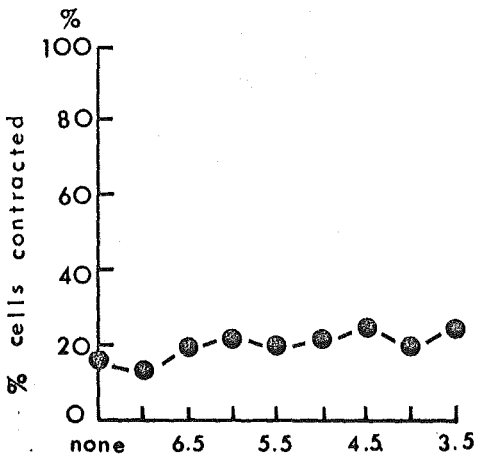


b. pCa = 6.

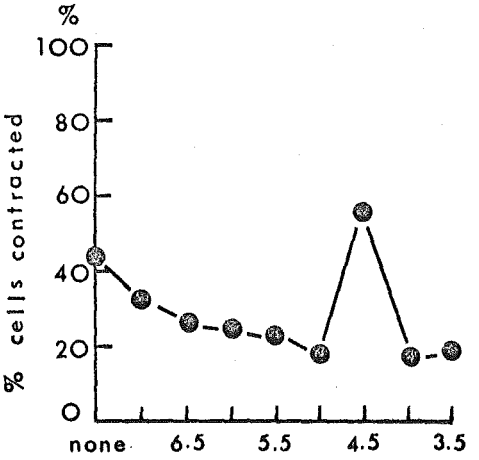


c. pCa = 4.

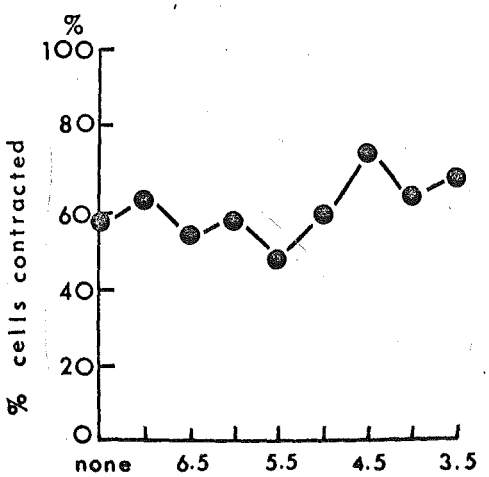
Fig. 7. C II contraction of cell models. Contraction solutions contain Ca, Mg, ATP, KCl, and 5 x 10^{-3} M phosphate buffer, plus 1 x 10^{-4} M EGTA in a. and c. (pH 7.2, I = 0.02μ). Each point represents the mean of three replicates, 100 cells per replicate. In a. and c. "pCa" refers to total Ca concentration after chelation by 1 x 10^{-4} M EGTA.



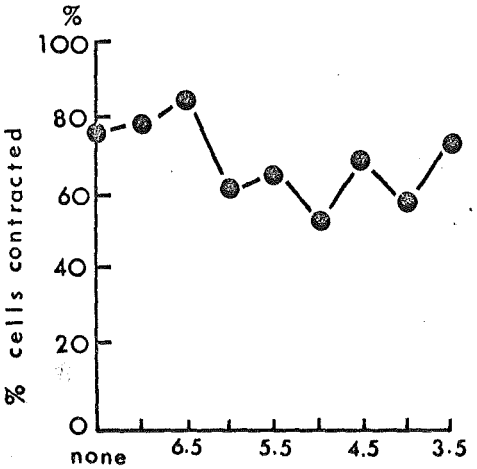
a. Epidermis, pCa = 8.



b. Epidermis, pCa = 4.

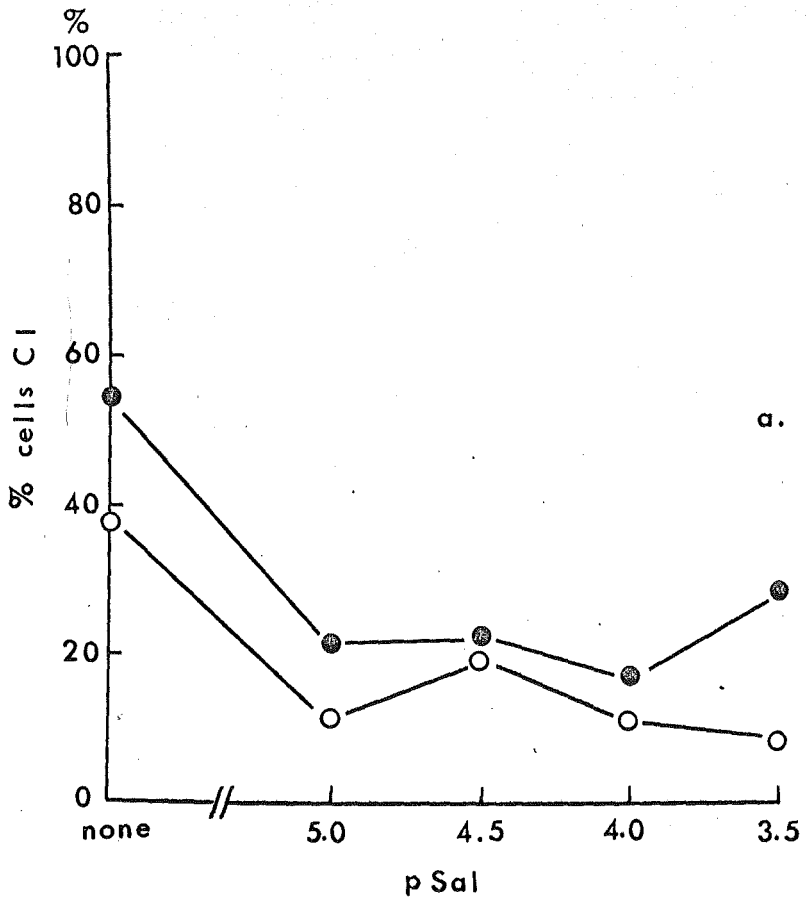


c. Gastrodermis, pCa = 8.

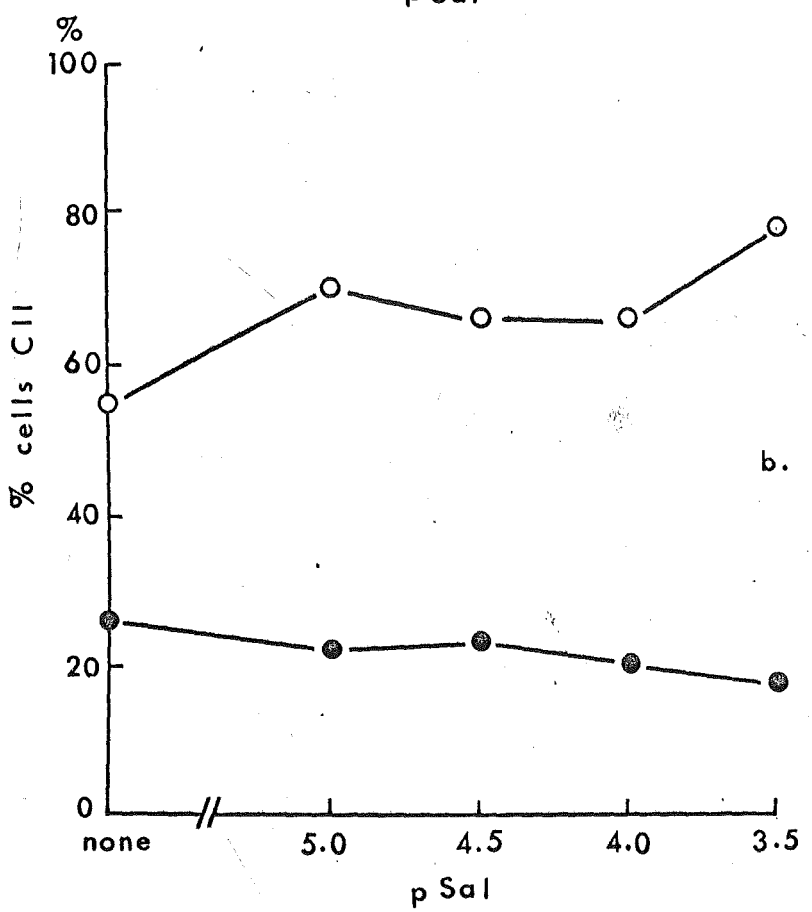


d. Gastrodermis, pCa = 4.

Fig. 8. Inhibitory effect of Salyrgan on C I and C II contraction of cell models, in contraction solutions also containing 1×10^{-4} M ATP, 1×10^{-4} M $MgCl_2$, 1×10^{-4} M $CaCl_2$, 5×10^{-3} M phosphate buffer (pH 7.2), and KCl (I = 0.02 μ). Each point represents the mean of three replicates, 100 epidermal (●) or gastrodermal (o) cells per replicate.

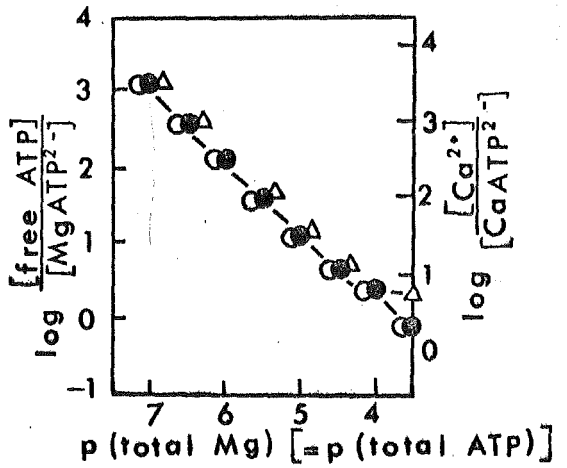


a. C I contraction

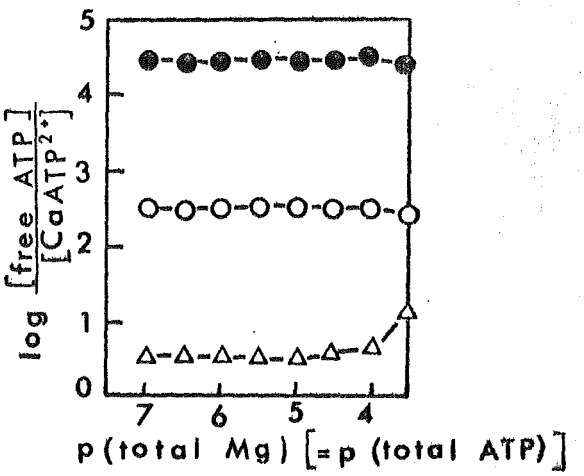


b. C II contraction

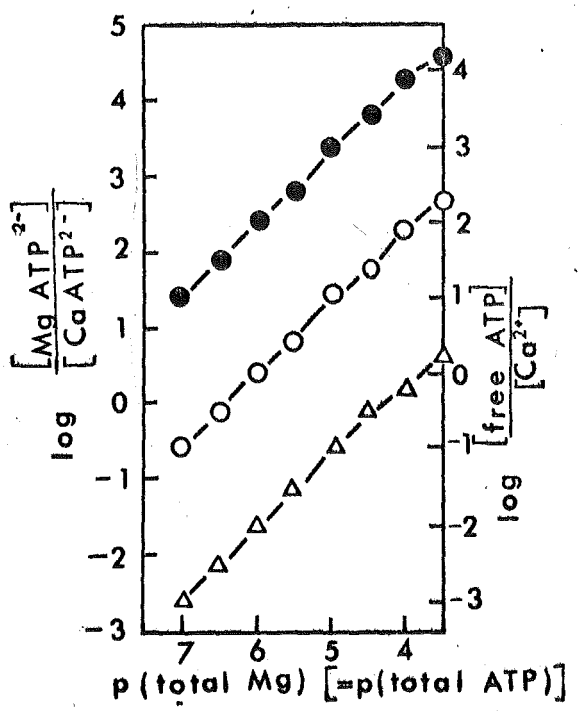
Fig. 9. Concentration ratios of all substrates (see Table 25) involved in contraction for Fig. 6 and 7. The values for pCa = 8 are represented by ● , for pCa = 6 by O , and for pCa = 4 by Δ . The concentrations of Mg^{2+} and free ATP are almost equal for all total concentrations of ATP, Mg, and Ca (see Table 25); therefore, the curves for $[Mg^{2+}] / [MgATP^{2-}]$, $[Mg^{2+}] / [CaATP^{2-}]$, and $[Mg^{2+}] / [Ca^{2+}]$, resembling the corresponding ATP curves (in a, b, and c, respectively) were omitted.


log [free ATP] / [MgATP2-]
log [Ca2+] / [CaATP2-]
p (total Mg) [= p (total ATP)]

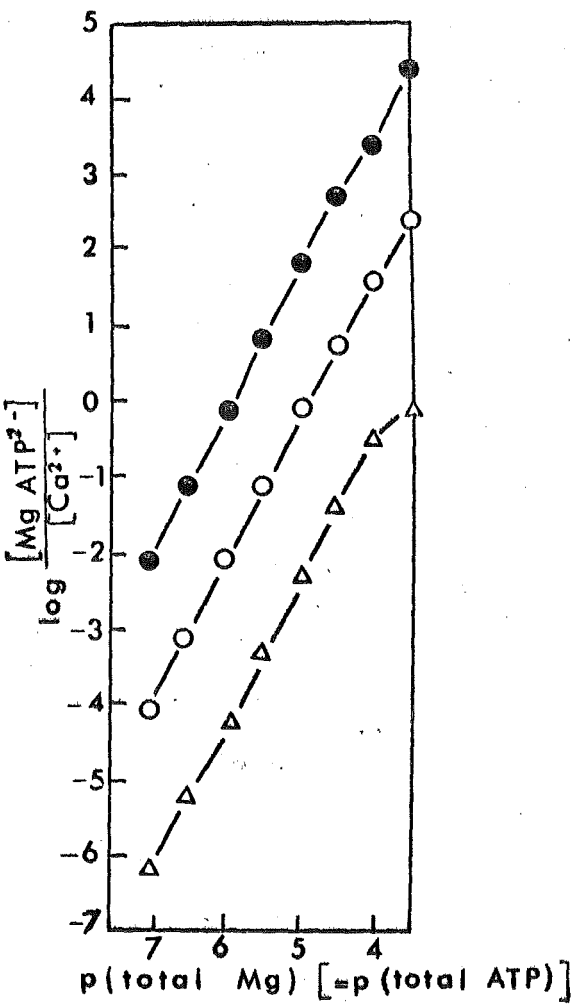
a


log [free ATP] / [CaATP2+]
p (total Mg) [= p (total ATP)]

b

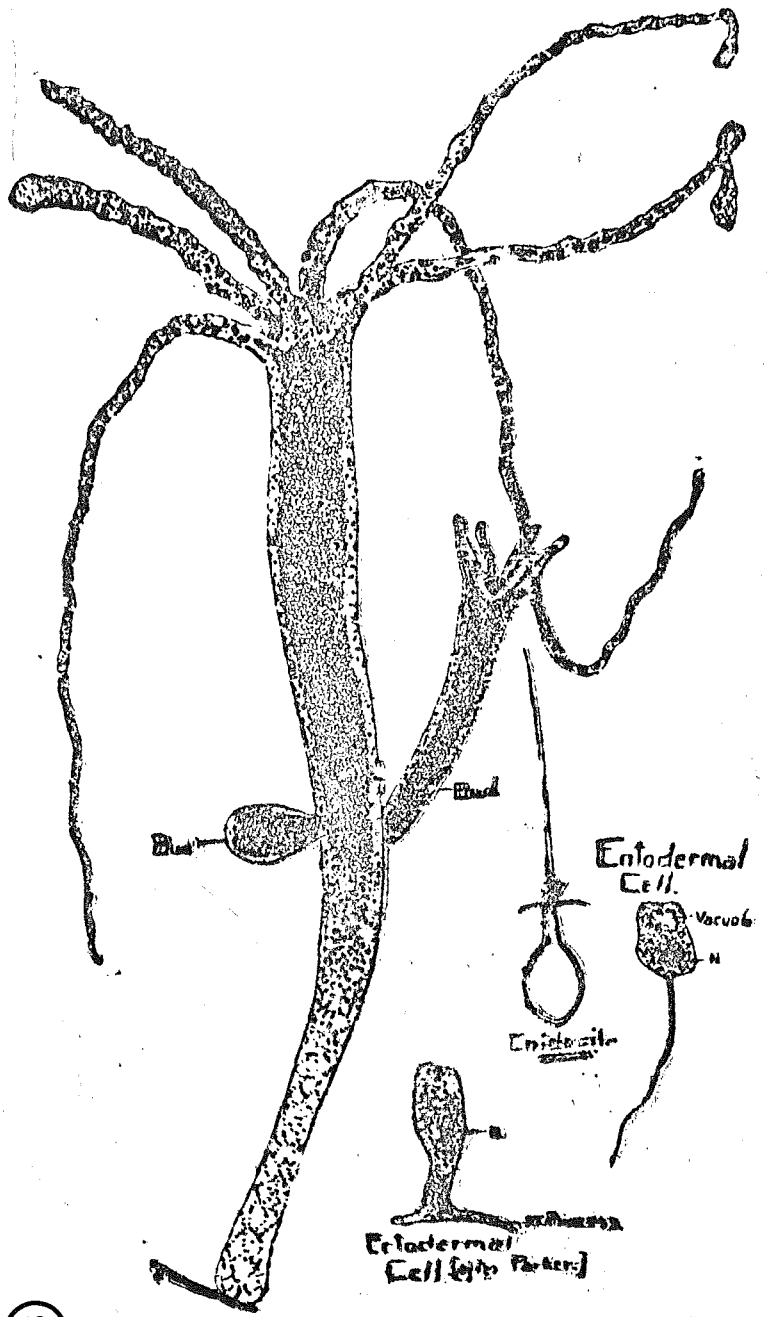

log [Mg ATP2-] / [CaATP2-]
log [free ATP] / [Ca2+]
p (total Mg) [= p (total ATP)]

c


log [Mg ATP2-] / [Ca2+]
p (total Mg) [= p (total ATP)]

d

Fig. 10. A drawing of a typical hydra, by W. A. Kepner, revealing its cylindrical column, several long tentacles, and two buds in different developmental stages. From the oral to the aboral end (top to bottom) are the following regions on the column: hypostome, gastric region, budding zone, peduncle, and basal disc.


Bud
Bud
Ectodermal
Cell
Vacuole
N
Endodermal
Cell (with Flagella)

10

Fig. 11 to 17 are phase-contrast micrographs of H. viridis, revealing epidermal (Fig. 11 to 14) and gastrodermal (Fig. 15 to 17) myo-epithelial cell models in various stages of contraction.

Fig. 11. Two relaxed epidermal cells. Note the robust basal processes (EBP), from which emanate small root-like projections (RI). In the intact hydra, these projections insert in the mesoglea. X 710.

Fig. 12. Several epidermal cells; three are relaxed (Rel) and bear root-like projections (RI), and one is spherical (i.e., contracted, C I). X 710.

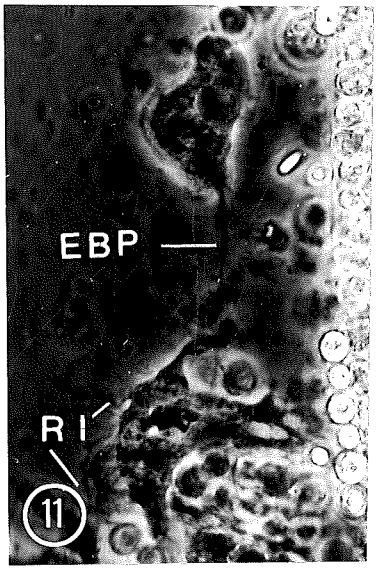
Fig. 13. Epidermal myo-epithelial cells. The large cell in the upper right-hand corner is relaxed (Rel), while the basal processes of the rectangular cell in the lower left-hand corner are contracted (C II). X 710.

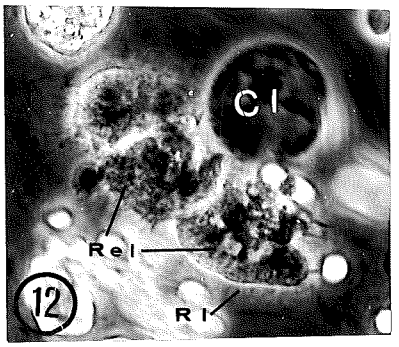
Fig. 14. Spherical epidermal cells (contracted, C I). X 710.

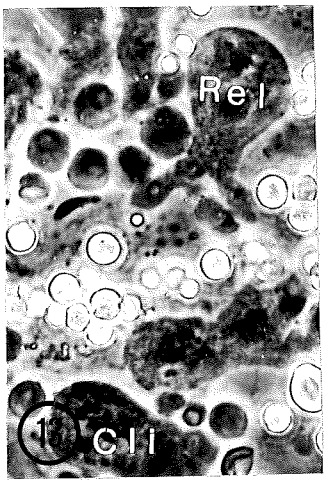
Fig. 15. A relaxed gastrodermal myo-epithelial cell model, in the center of the field. Note the small basal processes (GBP), and the numerous round zoochlorellae (Z) filling the rest of the cell.

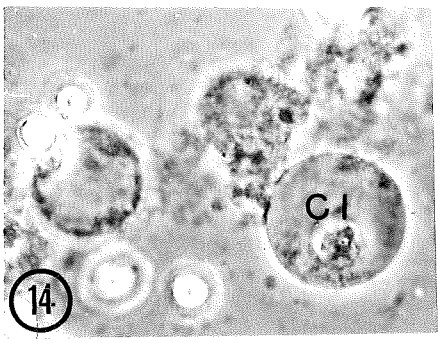
Fig. 16. A field of contracted (C I) gastrodermal cells, distinguished by their zoochlorellae (Z) from epidermal cells. X 710.

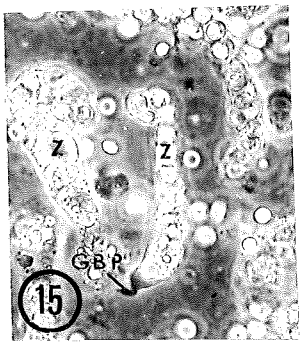
Fig. 17. A gastrodermal cell in a state of contraction of the basal processes (C II). X 710.

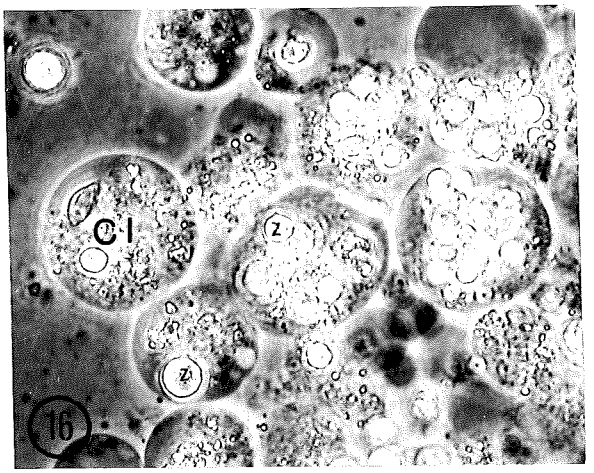

EBP
RI
11


CI
RelI
RI
12


RelI
CII


CI
14


Z
Z
GBP
15


CI
Z
Z
16

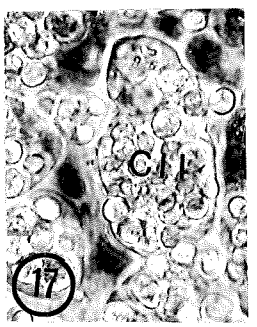
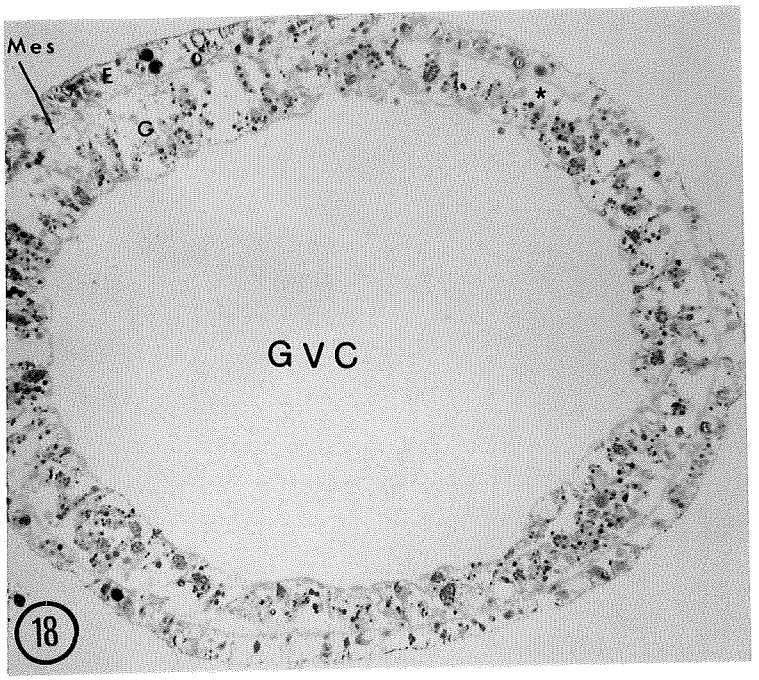

CII
17

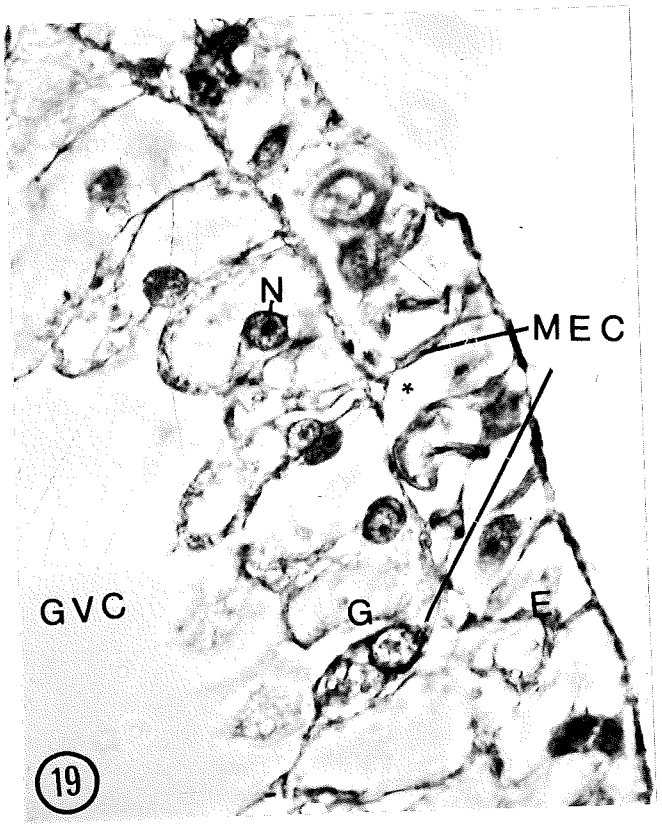
Fig. 18 to 20 are phase-contrast micrographs of transverse sections through the gastric region of H. littoralis.

Fig. 18. At low magnification the bilayered, cylindrical organization is apparent, consisting of an outer epidermis (E) and an inner gastrodermis (G), separated from each other by the thin acellular mesoglea (Mes). Note the wide space, the gastrovascular cavity (GVC) in the center. Extracellular space is indicated by *. (Araldite embedding). X 120.

Fig. 19. At higher magnification many of the above features become more evident. Individual myo-epithelial cells (MEC) may now be discerned readily. (Paraffin embedding). X 880.

Fig. 20. At the same magnification as that of Fig. 19, much more cytoplasmic detail may be seen after araldite embedding (also different fixative and staining; see Materials and Methods). Long, neck-like processes (Nk) connect the basal processes (GBP and EBP) of the gastrodermal and epidermal myo-epithelial cells (MEC), respectively, to their apical regions. The mesoglea (Mes) appears two dimensional here, in contrast to its linear appearance in Fig. 18 and 19. Again, the extent of extracellular space is striking (*). X 880.


Mes
E
G
*
GVC
18


N
MEC
*
GVC
G
E
19

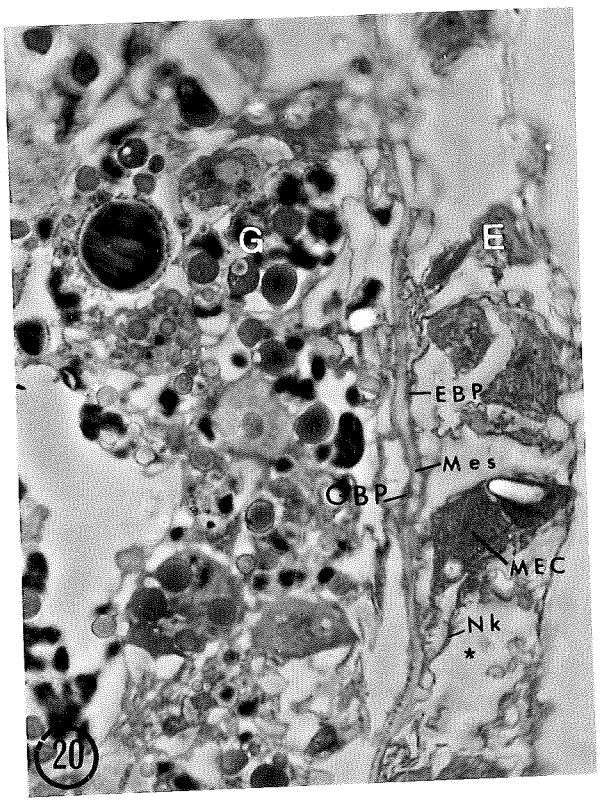
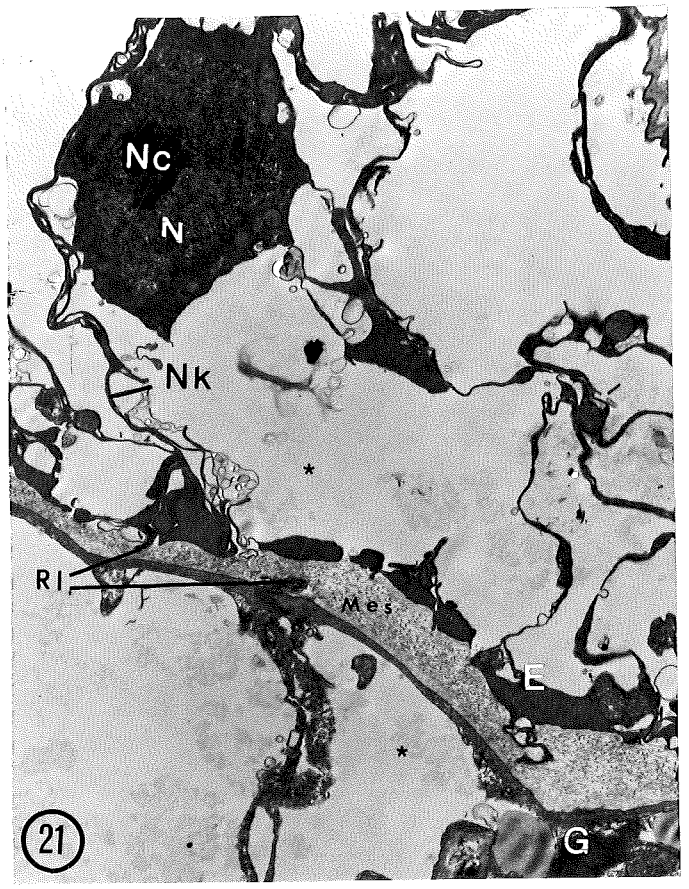

G
E
EBP
Mes
CBP
MEC
Nk
*
20

Fig. 21 through 45 are electron micrographs of transverse and longitudinal sections of normal hydra (Fig. 21 to 39) and whole-animal models (Fig. 40 to 45).

Fig. 21 and 22, on the facing page, were taken at low magnification; both are transverse sections of H. littoralis. The extensive extracellular space (*) is interrupted by the attenuated neck regions (Nk) of the epidermal and gastrodermal myo-epithelial cells. The nuclei (N) of these cells each contain one nucleolus (Nc) and occasionally partially-condensed chromatin (C). Meandering, root-like insertions (RI) into the mesoglea (Mes), from the myo-epithelial cells of one layer, may contact similar processes from the other layers. No discernible membranes separate the basal, filament-containing portion of the gastrodermal cell in Fig. 22 from its remaining epithelial region.


Nc
N
Nk
Rl
Mes
E
G
21

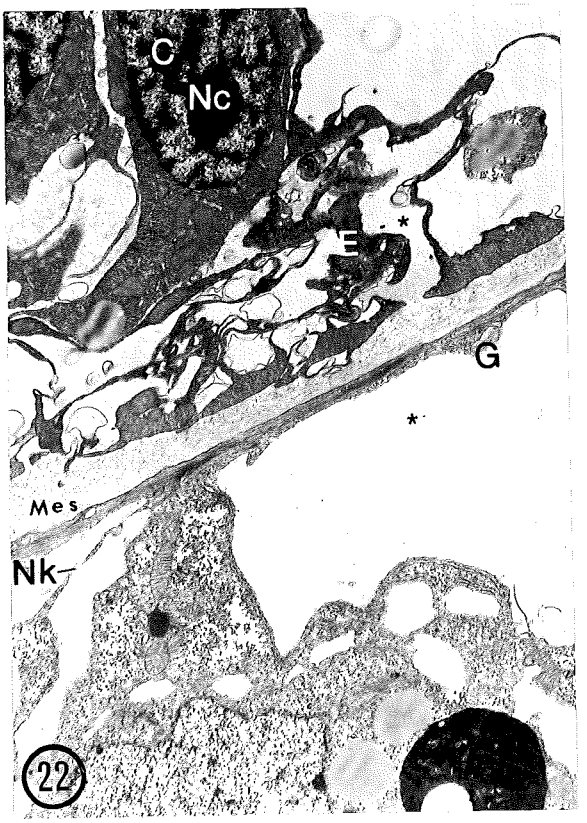

C
Nc
E
G
Mes
Nk
22

Fig. 23 through 26 are transverse sections of H. littoralis.

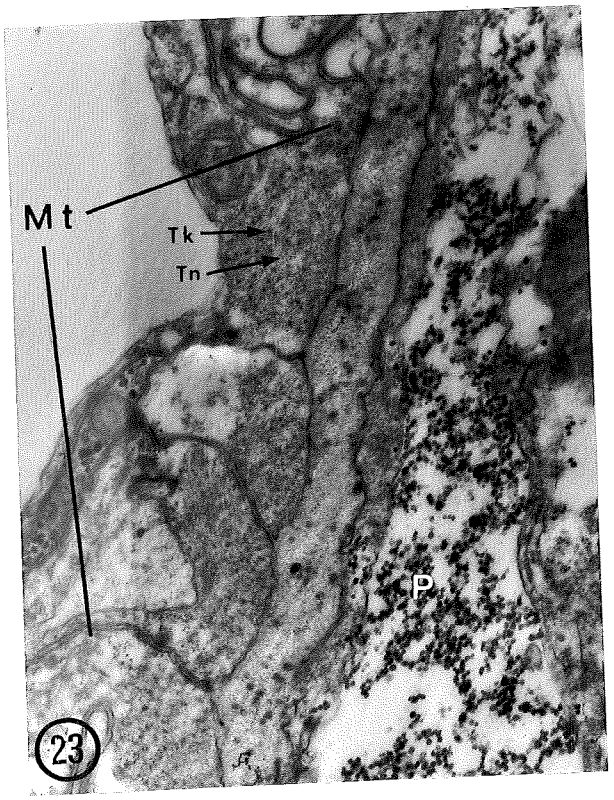
Fig. 23. In the epidermal basal "muscle tails" may be seen randomly arranged, apparently unbranched filaments, thick (Tk, about 160 A diameter) and thin (Tn, diameter ca. 55 A), in a ratio of about 8:1. In the peripheries of these basal processes are small groups of microtubules (Mt). Note the abundance of large particles (P, ca. 270 A diameter), probably glycogen, near the gastrodermal myofilaments. X 25,800.

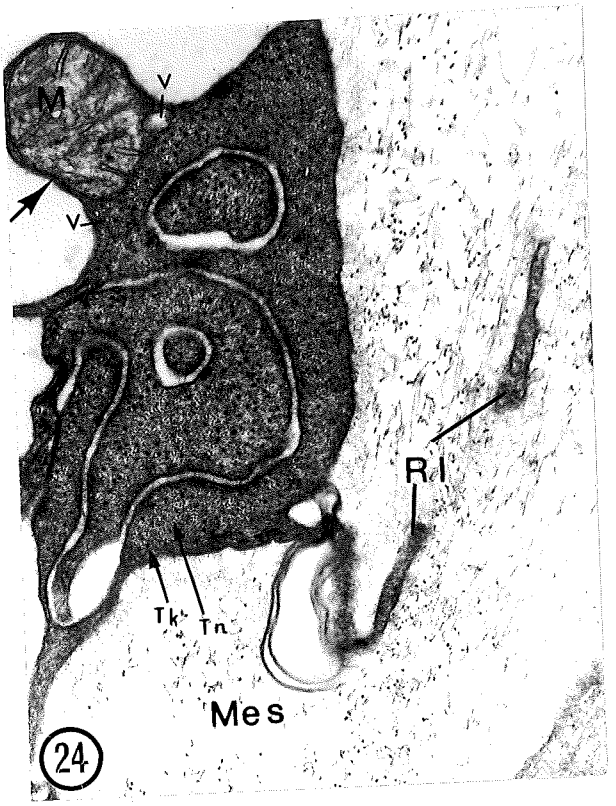
Fig. 24. Again note the epidermal thick (Tk) and thin (Tn) filaments in the interdigitating muscle processes. Faintly-staining material lies between two such nearby processes in regions of intercellular distance less than 200 A and is absent in other areas (*). A nearby mitochondrion (M) is associated with a large bulge in the cell and is very close (less than 200 A, see arrow) to the plasma membrane. Nearby are profiles of large vesicles (V). Inserting in the mesoglea (Mes) are long, rootlike extensions (RI) of the myo-epithelial cells. X 33,200.

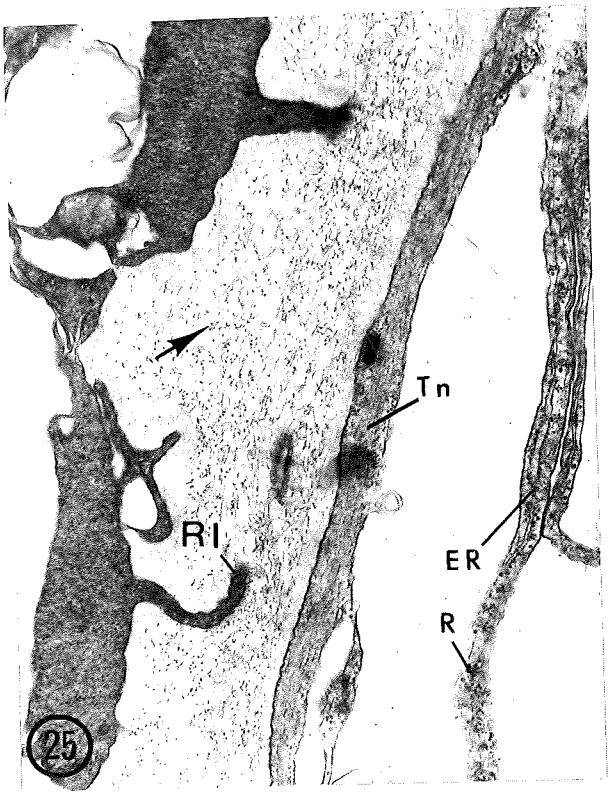
Fig. 25. The gastrodermal basal contractile process is seen here longitudinally; note the densely-packed thin filaments (Tn). In nearby gastrodermal cells may be seen endoplasmic reticulum (ER) and free ribosomes (R). The mesoglea is dense with thin filaments, associated with small particles (arrow). X 18,700.

Fig. 26. Particles in the mesoglea (Mes) are again indicated by an arrow. In the center of an epidermal basal process is a small group of microtubules (Mt). Adjoining the

gastrodermal myofilaments are many large particles (P) and several mitochondria which contain similar granules, probably glycogen. A vesicle (V) may also be seen in this region. Endoplasmic reticulum (ER) and ribosomes (R) are sparsely distributed. Note the smooth surface of the muscle tails abutting the mesoglea. X 18,700.


Mt
Tk
Tn
P
23


M
V
V
Rl
Tk
Tn
Mes
24


Tn
Rl
ER
R
25

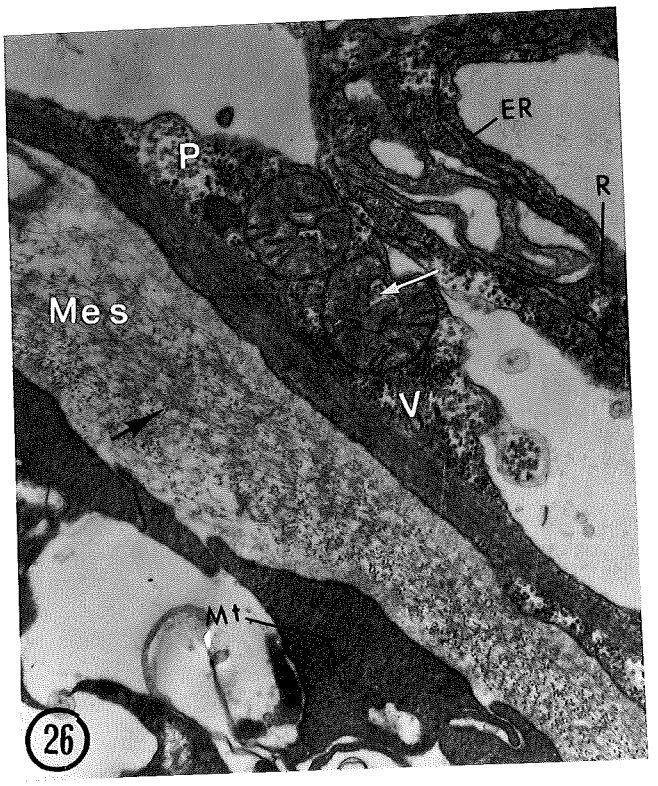
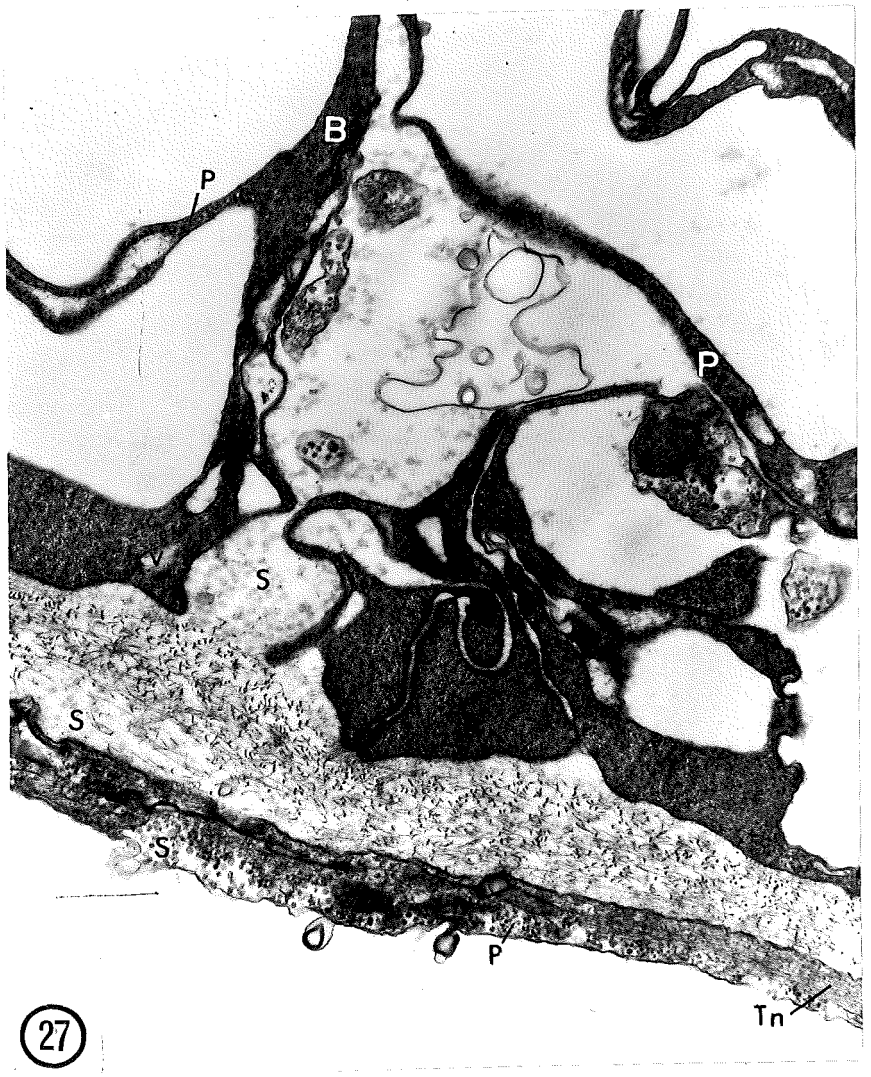
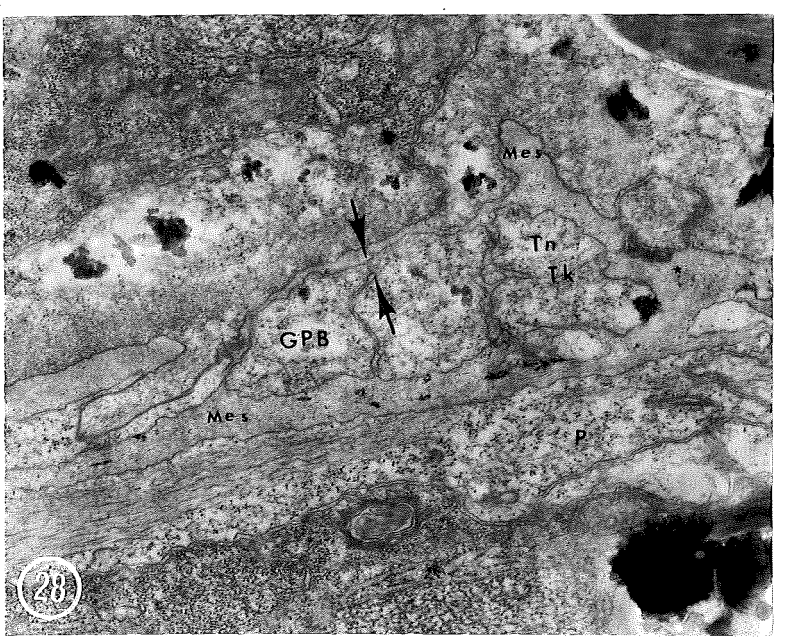

P
ER
R
Mes
V
Mt
26

Fig. 27. A transverse section of H. littoralis, revealing the three regions of myofilaments. Note the thin-filament bundle (B) in the neck region of the epidermal cell and also the nearby small (150 A diameter) particles (P). Between and tangent to the outside of the basal regions of the epidermal myo-epithelial cells are faintly-staining, large (ca. 400 A in diameter), spherical bodies (S). Similar bodies (S) also appear in the mesoglea and near the gastrodermal thin myofilaments (Tn). The interdigitation of the epidermal basal processes may again be observed, as well as an intracellular vesicle (V) in this region. Note the random arrangement of the mesoglea filaments and the smooth surfaces of the adjacent cells, each bounded by a single unit membrane. X 25,800.

Fig. 28. A longitudinal section through H. viridis, very likely anomalous, in light of the peculiar orientation of the mesoglea (Mes) with respect to the gastrodermal basal processes (GBP). Here, sparsely, randomly arranged thick (Tk) and thin (Tn) filaments are evident. Longitudinal profiles of mesogleal filaments may be seen in one region and transverse views nearby (*). An intercellular continuity between the gastrodermal basal processes is indicated by arrows. Note the small epidermal particles (P), about 150 A in diameter. X 18,000.



27

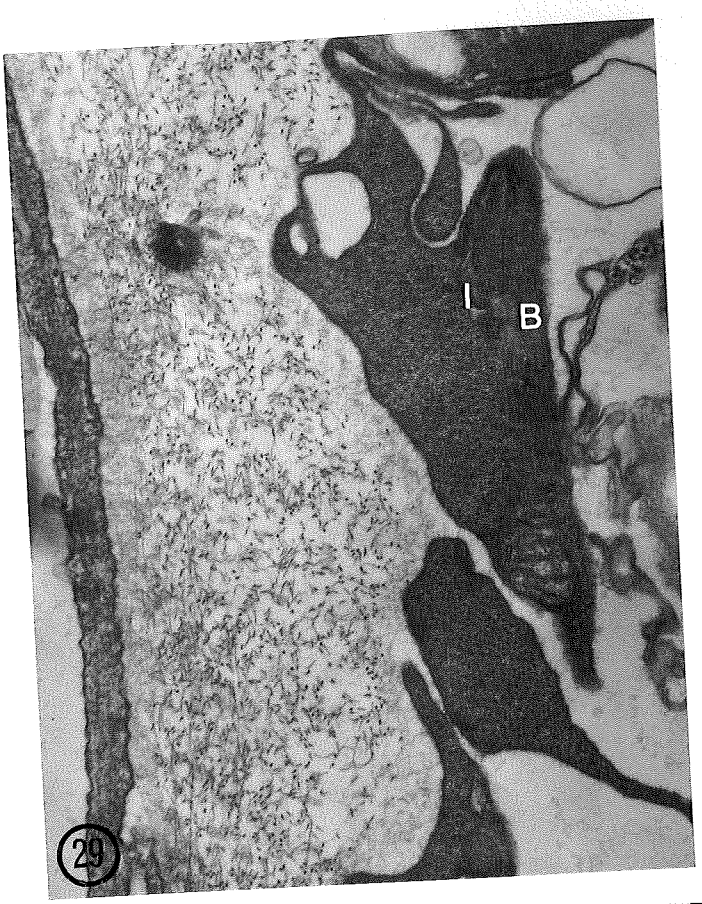


28

Fig. 29 and 30 are transverse sections through the gastric region of H. littoralis.

Fig. 29. The probable origination of the thin-filament bundle (B) near the epidermal basal myofilaments may be observed here. Note the membrane-lined invagination (I) of the basal processes, and also the random organization of the mesogleal filaments. The mesoglea in this region is about 1μ thick. X 27,000.

Fig. 30. Another view of the small mesogleal particles (arrow) in close proximity to filaments. Note the pair of epidermal and gastrodermal insertions (RI) transversing the mesoglea. Spherical bodies (S) may be seen, tangent to the outer surface of the epidermal muscle tail. X 36,000.


I
B
29

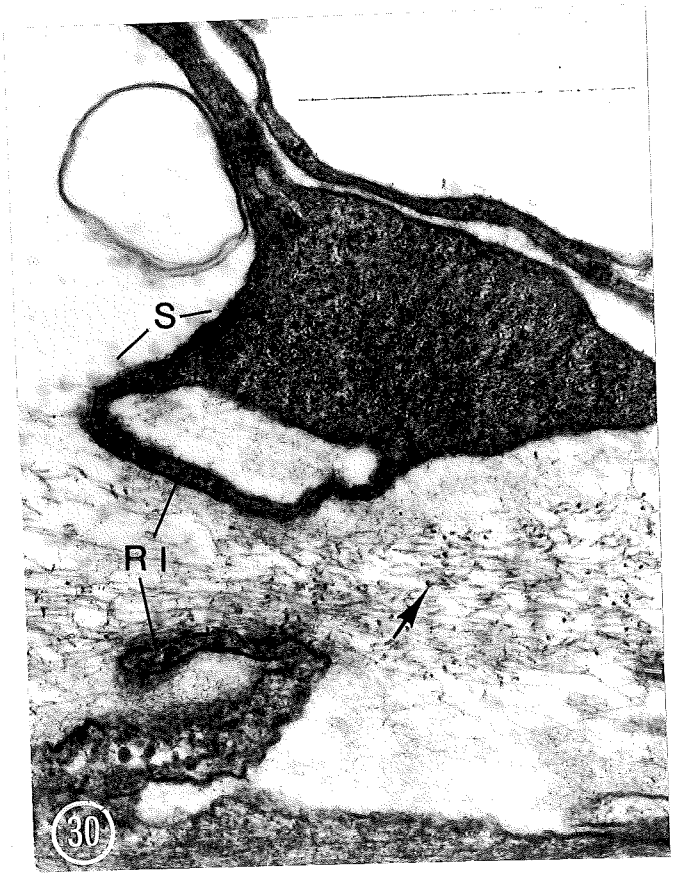
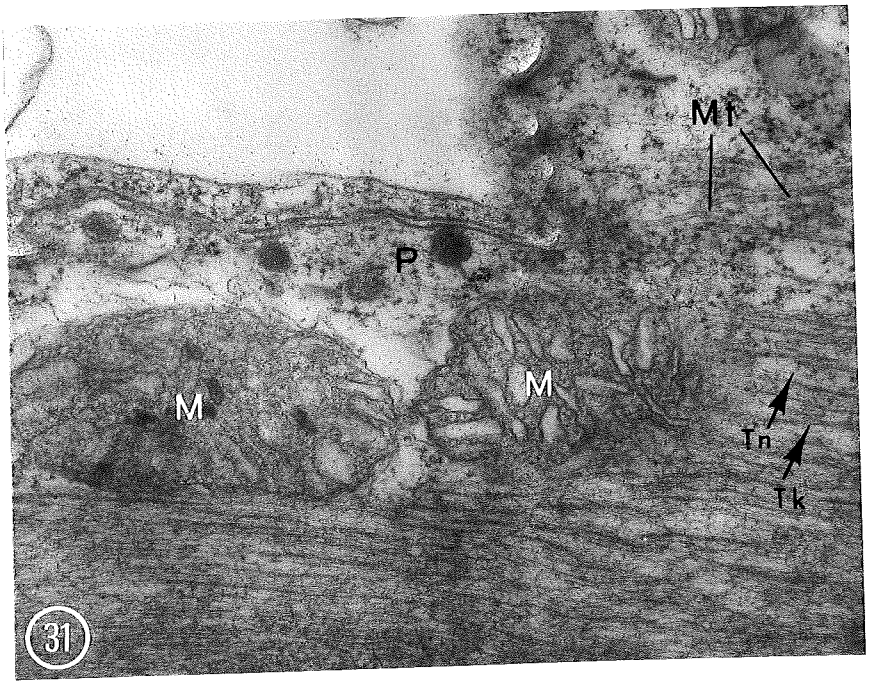

S
RI
30

Fig. 31. A longitudinal section of H. viridis, a region of thick (Tk) and thin (Tn) epidermal myofilaments. Nearby are elliptical profiles of mitochondria (M), many free ribosomes (P), and longitudinal views of microtubules (Mt). X 48,000.

Fig. 32. Mitochondria (M) near the epidermal myofilaments may also be seen in this transverse section of H. littoralis. Note the associated bulges in the outlines of the cells. A long invagination (I) may be seen nearby. Also note the epidermal rootlike insertion (RI) and many extracellular spherical bodies (S), some tangent to the outer epidermal surface. X 25,800.


Mt
P
M
M
Tn
Tk
31

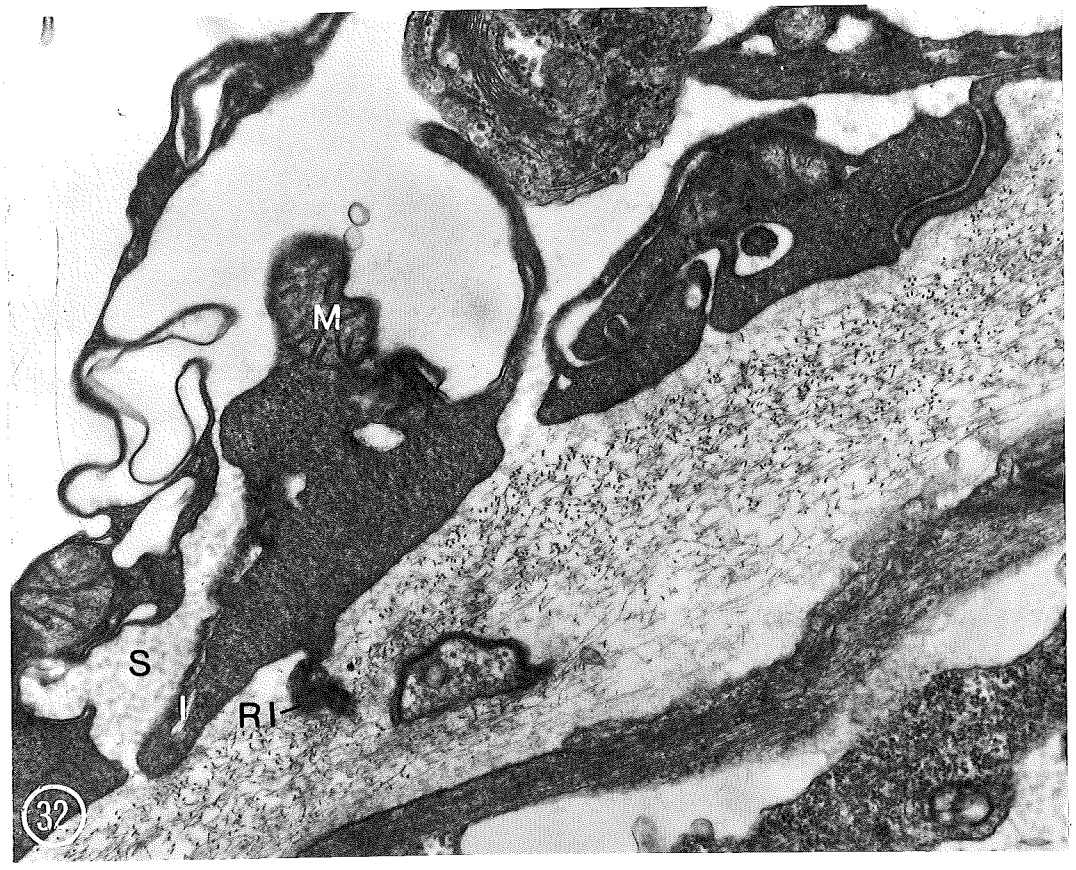

M
S
I
RI
32

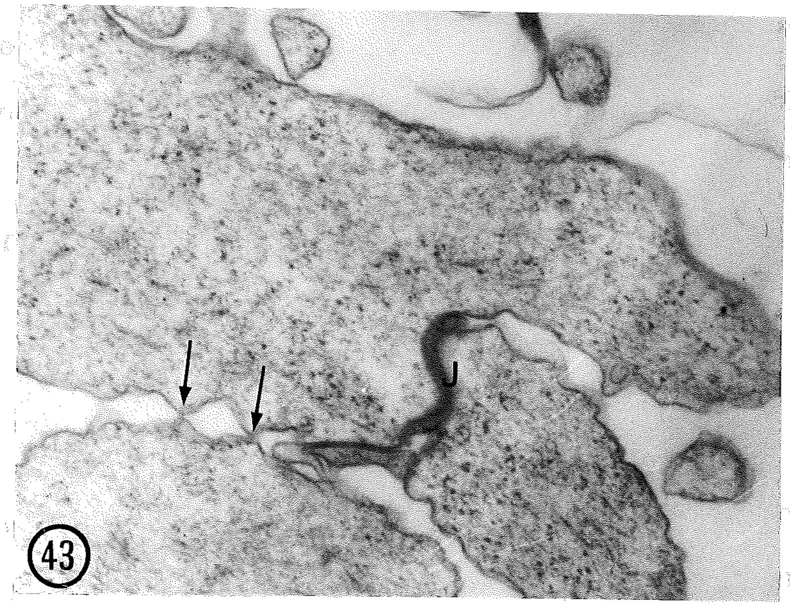
Fig. 33 to 36 are all transverse sections through the peduncle region of H. littoralis. Note the numerous mitochondria (M) near the gastrodermal (G) and epidermal (E) myofilaments. There is very little extracellular space here.

Fig. 33. X 13,000.

Fig. 34. X 19,500.

Fig. 35. An ending between two gastrodermal basal cell processes is indicated by an arrow. An overlying gastrodermal cell seems to be in direct continuity with the basal process, at the top of the micrograph. In the middle two-thirds of the field, an interrupted set of two closely-opposed unit membranes can be observed, separating the basal filaments from the overlying cytoplasm (see circles). Near the bottom the two unit membranes are each continuous and are separated from each other by about 140 A. X 18,700.

Fig. 36. Clusters of particles (P) can be seen in the gastrodermis near the myofilaments and also in the mesoglea. Note the convoluted surface of the gastrodermal cell abutting the mesoglea. Several microtubules (Mt) lie among the epidermal myofilaments. X 25,800.


J
43


44

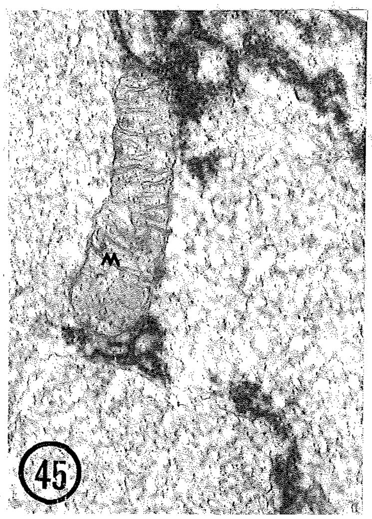
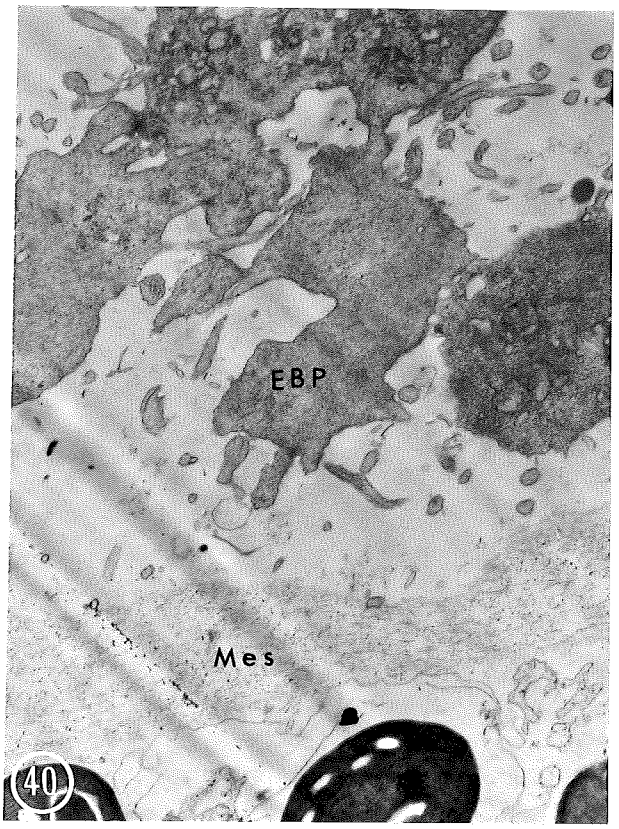
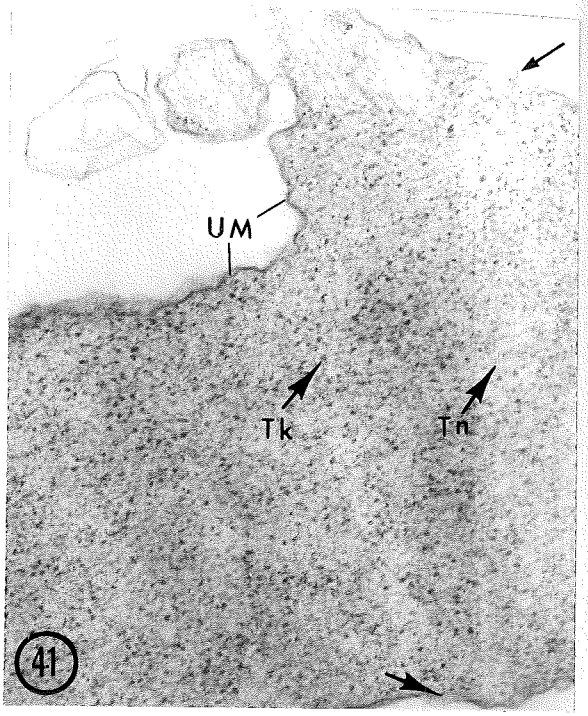

M
45

Fig. 43. Remnants of a tight junction (J) and nearby what may have been several points of contact (arrows) between two basal processes may be seen. X 48,000.

Some mitochondria are swollen (see Fig. 44, X 33,800), while others remain normal in appearance (see Fig. 45, X 27,300) after saponin treatment.


EBP
Mes
40


UM
Tk
Tn
41

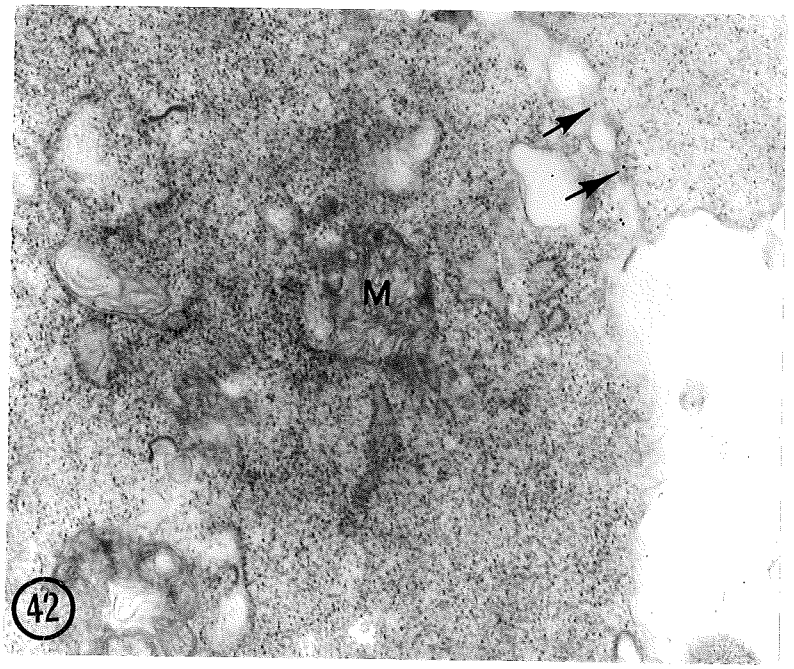

M
42

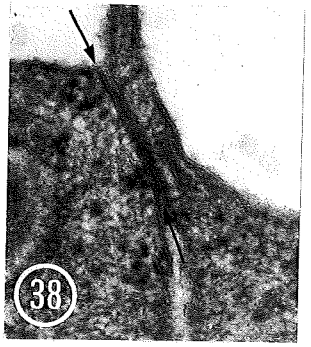
Fig. 40 through 45 are transverse sections of saponin-prepared models of *H. littoralis*.

Fig. 40. Note the separation of the basal contractile processes (EBP) from the mesoglea (Mes). X 7,500.

Fig. 41. Epidermal thick (Tk) and thin (Tn) myofilaments are still evident after extraction. The unit membrane (UM) seems fainter and is possibly disrupted in some regions (arrow). X 48,000.

Fig. 42. Note the onset of intercellular separation (arrows). The double-membraned structure of the mitochondria is still intact. X 33,000.


P
37


38

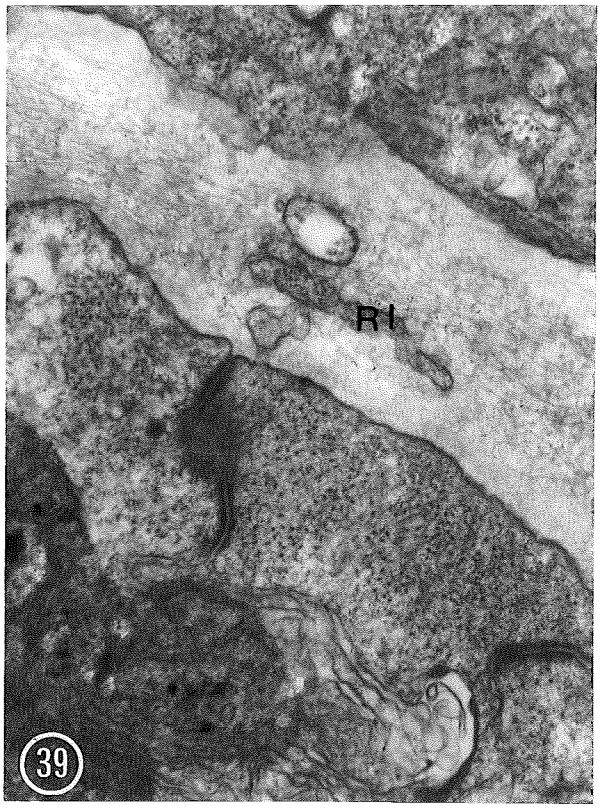
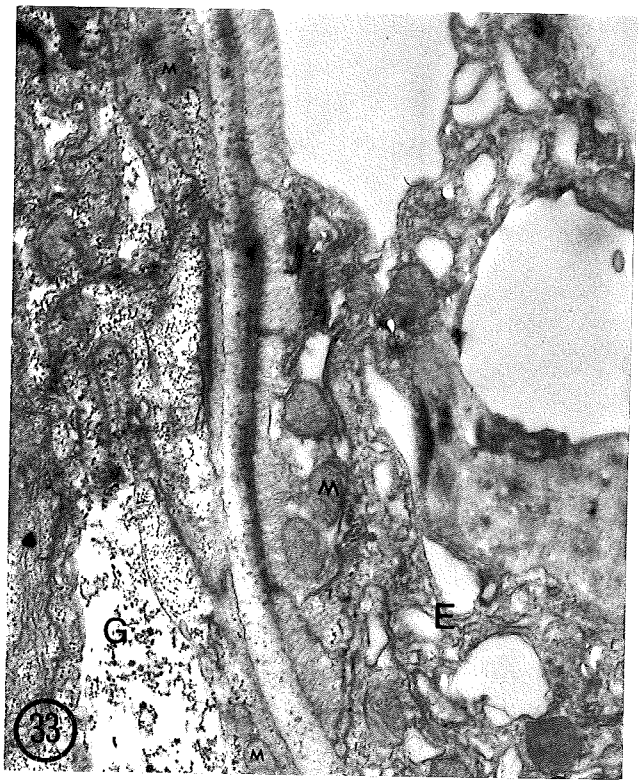
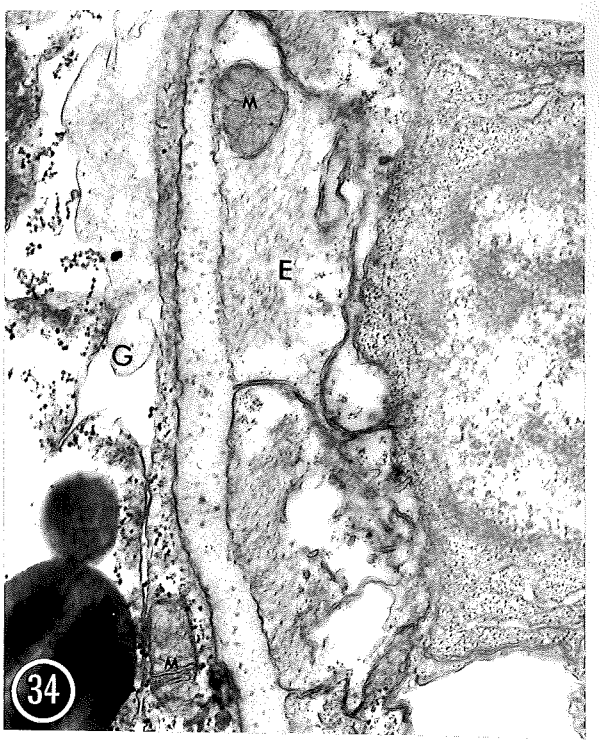

RI
39

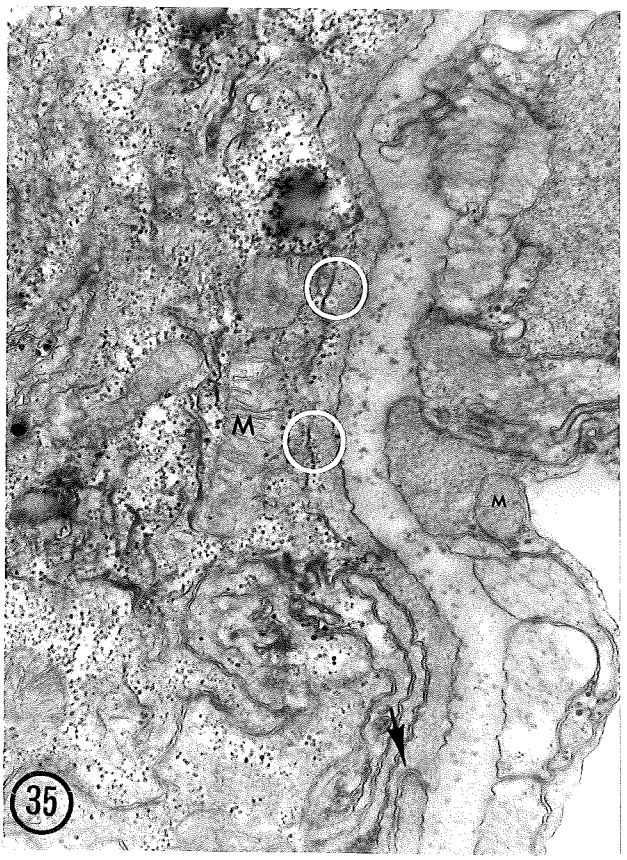
Fig. 37. A longitudinal section of H. viridis. Numerous particles (P) 150 A in diameter are seen near the epidermal myofilaments. X 34,000.

Fig. 38. When the membranes of two adjacent epidermal basal contractile processes are in close contact (between arrows), the trilaminar appearance of each membrane is maintained. X 83,100.

Fig. 39. Note the desmosomes (dark regions) at the junctions between epidermal muscle tails, and the meandering rootlike processes (RI) in the mesoglea. X 25,600.


M
M
G
E
M
33


M
E
G
M
34


M
M
35

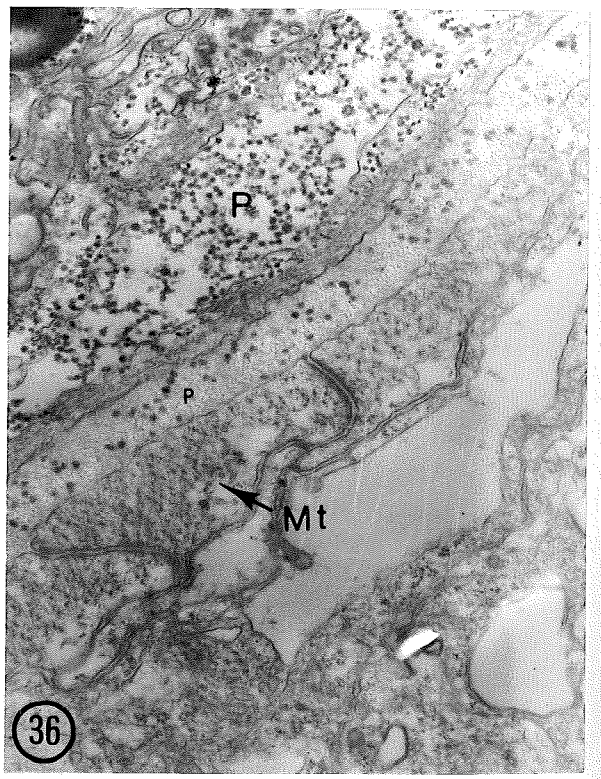

P
P
Mt
36

TABLE 1. Effect of contraction solution[1] on column lengths of briefly-glycerinated[2] H. littoralis

Treatment	Column length before treatment		Column length after treatment	
	$\bar{X} \pm SE$	N[4]	$\bar{X} \pm SE$	N[4]
	mm		mm	
Contraction solution[1]	3.4 ± 0.4	10	3.5 ± 0.6	8[5]
	4.0 ± 1.1	7	3.6 ± 0.8	7
Wash solution[3] (control)	3.3 ± 0.4	7	3.6 ± 1.2	7
	3.5 ± 0.4	8	4.1 ± 0.4	6[5]

[1] Containing $1_2 \times 10^{-3}$ M ATP, 5×10^{-3} M $MgCl_2$, 1×10^{-4} M $CaCl_2$, 1×10^{-2} M Tris-HCl buffer (pH 7.2), and KCl (I = 0.05 M).

[2] One hour in 50% glycerol in buffered KCl (see above) at -10°C, followed by brief washing (Townes and Brown, 1965, similar procedure with Vorticella stalks).

[3] Wash solution renewed as a control for manipulation.

[4] N = number of animals.

[5] Two animals lost during change of solution.

TABLE 2. Appearance and responsiveness of H. littoralis after treatment with saponin of various concentrations

Saponin concentration %	Number of animals	Immediate response to saponin	After 30 sec	After 1 min	At 16 min[5]	After 30 min wash	After several hrs wash
0.5	9	shortens →	→	→	disintegrating		
0.1	8	shortens →	→	begins recovery	normal, NR[3], OI[4]	→	cells dispersing
0.05	9	shortens	normal, →	→	→	→	slight dispersion of cells, NR[3]
0	7	normal[1], R[2]	→	→	→	outline distinct, NR[3]	

[1] N: Normal appearance (resembling live hydra)

[2] R: Response to mechanical stimulus (shortening, then gradual re-elongating)

[3] NR: No response to mechanical stimulus

[4] OI: Outline indistinct

[5] Transferred to buffered KCl wash solution after 16 minutes in saponin

TABLE 3. Effect of time of saponin treatment[1] on column and tentacle lengths of H. littoralis

Time in saponin solution	Columns		Tentacles	
min	$\bar{X} \pm SE$	N[2]	$\bar{X} \pm SE$	N[2]
	mm		mm	
8	5.0 ± 0.3	10	1.9 ± 0.2	8
15	5.5 ± 0.3	8	2.3 ± 0.2	7
23	5.1 ± 0.2	8	2.5 ± 0.6	7
30	5.5 ± 0.3	9	2.5 ± 0.1	8

[1] 0.1% saponin in 0.01 M phosphate buffer (pH 7.0) and 2.6×10^{-2} M KCl ($I = 0.05\ \mu$).

[2] N = number of animals.

TABLE 4. Effect of ionic strength on column lengths of saponin-treated[1] H. littoralis, measured just before and 5 minutes after introduction of contraction solution[2]

Ionic strength	Initial length		Final length		Relative length
μ	$\bar{X} \pm SE$	N[3]	$\bar{X} \pm SE$	N[3]	
	mm		mm		
0.20	5.2 ± 0.2	8	8.0 ± 0.5	8	1.54
0.15	6.1 ± 0.3	10	7.1 ± 0.4	10	1.16
0.10	13.9 ± 0.8	10	9.2 ± 0.3	11	0.66
0.05	8.8 ± 0.7	8	5.0 ± 0.5	9	0.57

1 Eight minutes in 0.1% saponin solution containing 1 x 10^{-2} M phosphate buffer (pH 7.0) plus sufficient KCl to maintain each respective ionic strength, followed by 1 hour in the corresponding buffered KCl solution.

2 1 x 10^{-3} M ATP, 1 x 10^{-3} M $CaCl_2$, 5 x 10^{-3} M $MgCl_2$, and 1 x 10^{-2} M phosphate buffer (pH 7.0), plus sufficient KCl to maintain each respective ionic strength.

3 N = number of animals.

TABLE 5. Effect of ionic strength on column and tentacle lengths of unextracted H. littoralis in wash solutions[1]

Ionic strength	Columns[2]		Tentacles[3]	
	$\bar{X} \pm SE$	N[4]	$\bar{X} \pm SE$	N[4]
μ	mm		mm	
0.20	5.7 ± 0.6	11	3.6 ± 0.3	11
0.15	5.0 ± 0.3	10	2.9 ± 0.2	9
0.10	6.0 ± 0.3	10	3.5 ± 0.2	10
0.05	9.6 ± 0.6	12	5.0 ± 0.4	12

1 Containing 0.01 M phosphate buffer (pH 7.0) and KCl to maintain each respective ionic strength.

2 Measured after 7 minutes in wash solution.

3 Measured after 11 minutes in wash solution.

4 N = number of animals.

TABLE 6. Changes in column length of saponin-extracted[1] H. viridis with time of wash treatment[2]

Time in wash solution	Column length $\bar{X} \pm SE$	N[3]
min	mm	
5	4.3 ± 0.1	9
10	2.2 ± 0.1	9
15	2.9 ± 0.1	9
20	3.1 ± 0.1	9
25	3.2 ± 0.1	9
32	3.0 ± 0.2	7
15 through 32	3.0 ± 0.1	34

1 Eight minutes in 0.1% saponin solution containing 3×10^{-4} M $CaCl_2$, 5×10^{-3} M imidazole-HCl buffer (pH 6.8), and 1.5×10^{-2} M KCl (I = 0.02 μ).

2 In buffered KCl (see above).

3 N = number of animals.

TABLE 7. Column lengths[1] of saponin-extracted[2] H. viridis in wash solutions containing urea, glycerol, or neither[3]

Time in wash solution	Urea wash		Glycerol wash		Control	
min	$\bar{X}$ ± SE	%[4]	$\bar{X}$ ± SE	%	$\bar{X}$ ± SE	%
0	3.9 ± 0.4	100	3.4 ± 0.4	100	3.3 ± 0.3	100
5	3.2 ± 0.3	82	2.1 ± 0.2	62	2.2 ± 0.2	67
10	3.4 ± 0.3	87	2.3 ± 0.2	68	1.9 ± 0.0	58
15	3.6 ± 0.3	92	2.5 ± 0.3	74		
17					1.8 ± 0.1	55
20	3.8 ± 0.3	97				
25			2.3 ± 0.3	68		

1 Each mean based on 6 animals.

2 Ten minutes in 0.1% saponin solution containing 5×10^{-3} M imidazole-HCl buffer (pH 7.0) and 1.4×10^{-2} M KCl (I = 0.02 μ).

3 Wash solutions contained, in addition to buffer and KCl as above, 1.5 M urea, 1.5 M glycerol, or neither (control).

4 Percentages of column length at 0 minutes.

TABLE 8. Rates of oxygen consumption in saponin-treated[1] H. littoralis

Flask[2] no.	µl O_2/mg dry wt/min $\bar{X} \pm SE$	N[3]	dry wt mg
S1	0.0090 ± 0.0023	10	6.5
S2	0.0167 ± 0.0022	10	9.8
S1 + S2	0.0128 ± 0.0020	20	
C1	0.0327 ± 0.0022	10	7.7
C2	0.0714 ± 0.0049	10	4.7

1 Eight minutes in 0.1% saponin solution containing 0.05 M phosphate buffer (pH 7.0, I = 0.05 µ), followed by 30 minutes in buffered KCl (same pH and I).

2 "S" indicates pretreatment with saponin; "C" indicates control.

3 N = number of 15-minute periods. (one reading every 15 minutes).

TABLE 9. Transmembrane resting potentials of saponin-treated frog sartorious fibers

Prepn	Transmembrane resting potential mv
1	17.6
2	27.2
3	12.8
4	41.6
5	19.2
6	44.0
7	9.6
8	15.2
9	13.6
10	17.6
11	22.4
12	27.2
13	25.6
14	23.2
15	20.0

$\bar{X} \pm SE = 22.5 \pm 2.7$ mv

TABLE 10. Contraction[1] of saponin-treated frog muscle fibers

Prepn	Initial length[2]		Final length[3]		Presence or absence of urea in wash solution
	$\bar{X} \pm SE$ mm	N[4]	$\bar{X} \pm SE$ mm	N[4]	
1	4.8 ± 0.3	5	4.3 ± 0.2	4[5]	+
2	4.6 ± 0.4	5	3.6 ± 0.2	5	+
3	5.0 ± 0.6	3	3.3 ± 0.3	3	-
4	5.5 ± 0.5	3	4.8 ± 0.5	3	-
1 to 4	4.9 ± 0.2	16	4.0 ± 0.6	15	+,-

1 In solution containing 1×10^{-3} M $CaCl_2$, 2×10^{-3} M ATP, 5×10^{-3} M $MgCl_2$, and 5×10^{-2} M phosphate buffer (pH 6.8, I = 0.15 /u).

2 Just before addition of contraction solution.

3 Fifteen minutes after addition of contraction solution.

4 N = number of fiber bundles.

5 One fiber bundle lost during change of solution.

TABLE 11. Thin and thick filaments in the basal regions of the epidermal myo-epithelial cells of H. littoralis

Fig.[1] number	Body region	Thin filaments		Thick filaments		Ratio of thin:thick
		Number counted	Density number/ μ^2	Number counted	Density number/ μ^2	
24	gastric	296	4933	31	517	9.54:1
30	gastric	776	3880	88	440	8.82:1
32	gastric	729	1519	104	217	6.99:1
23	peduncle	292	2433	33	275	8.85:1
23	peduncle	327	1022	52	163	6.29:1
		2420 (total)	2757 ± 730 ($\bar{X}$ ± SE)	308 (total)	322 ± 67 ($\bar{X}$ ± SE)	7.86:1[2] (total)

[1] All transverse sections.

[2] $\chi^2 = 6.078$.

TABLE 12. Effect of time in contraction solution[1] on column lengths of saponin-treated[2] H. viridis

Time in contraction solution	Column length ($\bar{X} \pm SE$)	N[3]
min	mm	
5	3.1 ± 0.2	7
10	2.7 ± 0.3	7
15	2.5 ± 0.2	7
20	2.5 ± 0.2	7
25	2.4 ± 0.3	7
15 through 25	2.5 ± 0.1	21

1 Containing 5×10^{-4} M ATP, 3×10^{-4} M $CaCl_2$, 5×10^{-3} M imidazole-HCl buffer (pH 6.8), and 1.26×10^{-2} M KCl ($I = 0.02\ \mu$).

2 See Table 6.

3 N = number of animals.

TABLE 13. Change in column length of saponin-extracted[1] H. littoralis as a function of ATP concentration[2]

ATP concentration	Initial length[3]		Final length[4]	
	$\bar{X}$ ± SE	N[5]	$\bar{X}$ ± SE	N[5]
mM	mm		mm	
0	5.7 ± 0.1	34	6.9 ± 0.2	35
1	5.9 ± 0.1	32	4.7 ± 0.1	31
5	5.6 ± 0.1	33	4.4 ± 0.1	33
10	5.7 ± 0.2	33	4.4 ± 0.1	34
1 through 10	5.8 ± 0.1	98	4.5 ± 0.1	98
20	5.8 ± 0.1	34	5.0 ± 0.2	34

1 Eight minutes treatment with 0.1% saponin in 10 mM Tris-HCl buffer (pH 7.2) and KCl (I = 0.05 μ), followed by 10 minutes in buffered KCl wash solution.

2 Contraction solutions also contained 1 mM $CaCl_2$, 5 mM $MgCl_2$, and buffer and KCl as above.

3 After wash treatment.

4 After 15 minutes in contraction solution.

5 N = number of animals.

TABLE 14. Variation among replicates in percentage of saponin-extracted cells of H. viridis contracted in the presence of ATP and divalent ions*

Type of cell	$\bar{X}$ %	SD %	Number of replicates
Epidermal	38.3	0.6	10
Gastrodermal	36.1	0.3	7

* 1×10^{-4} M ATP, 1×10^{-4} M $MgCl_2$, 5×10^{-3} M phosphate buffer (pH 7.2), and 6.9×10^{-3} M KCl ($I = 0.02\ \mu$).

TABLE 15. Contraction of cell models of H. viridis as a function of pH

pH	Epidermal cells			Gastrodermal cells		
	$\bar{X}/\sqrt{n} \pm SE$		N^3	$\bar{X}/\sqrt{n} \pm SE$		N^3
	Transformed data[2]	Corresponding percentage		Transformed data[2]	Corresponding percentage	
	degrees	%		degrees	%	
6.2	22.0 ± 0.5	14 ± 0.1	5	30.3 ± 1.4	25 ± 0.1	3
6.4	27.4 ± 6.3	21 ± 1	4	29.5 ± 3.7	24 ± 0.4	5
6.6	40.6 ± 3.5	42 ± 0.4	5	33.7 ± 5.9	31 ± 1	4
6.8	43.7 ± 6.5	48 ± 1	4	32.5 ± 6.6	29 ± 1	3
7.0	38.2 ± 5.0	38 ± 0.8	5	39.8 ± 2.3	41 ± 0.2	5
7.2	37.3 ± 2.6	37 ± 0.2	6	34.7 ± 6.8	33 ± 1	6
7.4	34.6 ± 4.1	32 ± 0.5	6	34.9 ± 7.2	33 ± 2	4
7.6	40.1 ± 5.3	41 ± 0.9	6	49.9 ± 8.1	58 ± 2	5
7.8	30.7 ± 3.8	26 ± 0.1	5	28.1 ± 5.4	22 ± 0.9	5

1 See Fig. 5 for description of contraction solutions.

2 The transformation used was $\sqrt{n} \sin^{-1}\sqrt{p}$ (see Materials and Methods, VII).

3 N = number of replicates.

TABLE 16. Effect of extra wash time[1] on contraction of cell models[3]

Extra wash period min	Replicate number	Gastrodermal cells[2]	Epidermal cells[2]
0	1	-	+
	2	±	+
	3	-	+
30	1	-	+
	2	-	-
	3	-	-
60	1	0	+
	2	±	0
	3	±	0
90	1	+	+
	2	+	+
	3	0	+

[1] In 3×10^{-4} M $CaCl_2$, 5×10^{-3} M phosphate buffer (pH 6.8), and 9.1×10^{-3} M KCl ($I = 0.02\ \mu$). This treatment was always followed by 30 minutes in a similarly buffered solution containing 1.5 M urea and 1×10^{-2} M KCl (same ionic strength).

[2] Most cells C I (+), no change in shape of any cells (-), a few cells C I (±), not observed (0).

[3] In solutions containing 1×10^{-4} M ATP, 1×10^{-3} M $MgCl_2$, 5×10^{-3} M $CaCl_2$, and KCl and buffer as above.

TABLE 17. The contractile response of cell models to various combinations of ATP, Ca, and Mg*

Prepn	Contraction solution	G	E
1	3×10^{-4} M $MgCl_2$, ATP	-	-
2	$MgCl_2$	-	-
3	$MgCl_2$, $CaCl_2$	-	-
4	$MgCl_2$, ATP, $CaCl_2$	±	±
5,6	1×10^{-3} M $MgCl_2$	+,-	-,-
7	$MgCl_2$, $CaCl_2$	-	-
8	$MgCl_2$, ATP	±,+	+
9	$MgCl_2$, ATP, $CaCl_2$	+	+

ATP 5×10^{-4} M

$CaCl_2$ 3×10^{-4} M

$MgCl_2$ concentrations indicated above

G gastrodermal cells

E epidermal cells

+ most cells C I; - no change in any cells; ± a few cells C I.

* 5×10^{-3} M imidazole buffer (pH 6.8) and KCl (I = 0.02 μ) were included in all contraction solutions.

TABLE 18. Contraction of cell models as a function of Ca ion concentration*

Concentration of $CaCl_2$ M	G	E
5×10^{-7}	-	-
5×10^{-6}	-	-
5×10^{-5}	+	±
1×10^{-4}	+	+
5×10^{-4}	+	+
1×10^{-3}	±	±

\+ Most cells C I

\- No change in any cells

± A few cells C I

G Gastrodermal cells

E Epidermal cells

* Contraction solutions all contained 5×10^{-4} M ATP, 5×10^{-3} M phosphate buffer (pH 6.8), and KCl (I = 0.02 μ), as well as $CaCl_2$.

TABLE 19. Contraction of gastrodermal cell models as a function of Mg ion concentration*

Concentration of $MgCl_2$ 10^{-4} M	Gastrodermal cells
5	-
7	+
9	+
10	+
15	±
20	-

\+ Most cells C I

\- No change in any cells

± A few cells C I

* Contraction solutions all contained 5×10^{-4} M ATP, 5×10^{-3} M phosphate buffer (pH 6.8), KCl (I = 0.02 μ), and $MgCl_2$ as above.

TABLE 20. Percentage of epidermal cells scored as C I as a function of equal concentrations of ATP and $MgCl_2$ at different Ca levels[4]

pMg = pATP	pCa = 8[1, 2]		pCa = 6[1, 3]		pCa = 4[2]	
	$\sin^{-1}\sqrt{p}$	p	$\sin^{-1}\sqrt{p}$	p	$\sin^{-1}\sqrt{p}$	p
	degrees	%	degrees	%	degrees	%
none	27.8 ± 4.3[5]	22 ± 0.6[5]	28.4 ± 1.6[5]	23 ± 0.1[5]	22.4 ± 2.0[5]	14 ± 0.1
7.0	23.8 ± 3.1	16 ± 0.3	39.1 ± 3.6	40 ± 0.4 *	24.1 ± 5.7	17 ± 1.0
6.5	23.0 ± 3.1	15 ± 0.3 +	32.4 ± 0.2	29 ± 0.0	48.9 ± 3.5	57 ± 0.4 *
6.0	27.4 ± 2.0	21 ± 0.1	24.4 ± 2.5	17 ± 0.2 +	33.8 ± 1.9	31 ± 0.1
5.5	35.4 ± 2.1	34 ± 0.1	23.1 ± 2.6	15 ± 0.2 +	35.0 ± 3.0	33 ± 0.3
5.0	44.9 ± 8.9	50 ± 2.4 *	29.9 ± 2.4	25 ± 0.2	38.5 ± 3.5	39 ± 0.4 +
4.5	44.0 ± 1.8	48 ± 0.1 *	42.9 ± 0.7	46 ± 0.0 *	33.3 ± 6.8	30 ± 1.4 +
4.0	35.7 ± 4.3	34 ± 0.6	31.4 ± 5.7	27 ± 1.0	49.0 ± 1.5	57 ± 0.1 *
3.5	37.1 ± 4.5	36 ± 0.6			33.2 ± 1.9	30 ± 0.1

* maxima as determined by t tests; + minima as determined by t tests.

1 Ca^{2+} buffered with 1 x 10^{-4} M EGTA.

2 Cells extracted with digitonin.

3 Cells extracted with saponin.

4 Contraction solutions all contained 5 x 10^{-3} M phosphate buffer (pH 7.2) and KCl (I = 0.02 μ).

5 $\bar{X}$ ± SE.

TABLE 21. Percentage of gastrodermal cells scored as C I as a function of equal concentrations of ATP and $MgCl_2$ at different Ca levels[4]

pMg = pATP	pCa = 8[1, 2]		pCa = 6[1, 3]		pCa = 4[2]	
	$\sin^{-1}\sqrt{p}$	p	$\sin^{-1}\sqrt{p}$	p	$\sin^{-1}\sqrt{p}$	p
	degrees	%	degrees	%	degrees	%
none	18.0 ± 2.9[5]	10 ± 0.2[5]	24.5 ± 5.6[5]	17 ± 1.0[5]	15.9 ± 3.0[5]	8 ± 0.3[5]
7.0	29.3 ± 1.4	24 ± 0.1	22.6 ± 3.8	15 ± 0.4	20.7 ± 3.3	12 ± 0.3
6.5	30.4 ± 0.2	26 ± 0.0	31.3 ± 1.0	27 ± 0.0	21.0 ± 1.7	13 ± 0.1
6.0	23.2 ± 1.6	16 ± 0.1	21.3 ± 1.7	13 ± 0.1	34.7 ± 3.1	32 ± 0.3
5.5	30.0 ± 2.9	25 ± 0.3	28.7 ± 4.4	23 ± 0.6	32.8 ± 4.8	29 ± 0.7
5.0	34.8 ± 6.5	33 ± 1.0	29.2 ± 2.3	24 ± 0.2	35.9 ± 3.5	34 ± 0.4
4.5	23.4 ± 2.7	16 ± 0.2	17.2 ± 2.0	9 ± 0.1	32.4 ± 3.4	29 ± 0.4
4.0	25.1 ± 3.1	18 ± 0.3	18.4 ± 2.6	10 ± 0.2	34.4 ± 2.1	32 ± 0.1
3.5	23.9 ± 2.7	16 ± 0.2	24.0 ± 3.4	17 ± 0.4	25.2 ± 3.6	18 ± 0.4

[1] Ca^{2+} buffered with 1 x 10^{-4} M EGTA. [2] Cells extracted with digitonin. [3] Cells extracted with saponin.

[4] Contraction solutions all contained 5 x 10^{-3} M phosphate buffer (pH 7.2) and KCl (I = 0.02 μ). [5] $\bar{X}$ ± SE.

TABLE 22. C II contraction[2] of cell models

	Epidermal cells				Gastrodermal cells			
	pCa = 8[1]		pCa = 4		pCa = 8[1]		pCa = 4	
	$\sin^{-1}\sqrt{p}$	p	$\sin^{-1}\sqrt{p}$	p	$\sin^{-1}\sqrt{p}$	p	$\sin^{-1}\sqrt{p}$	p
pMg = pATP	degrees	%	degrees	%	degrees	%	degrees	%
none	23.5 ± 1.7[3]	16 ± 0.1[3]	40.8 ± 0.7	43 ± 0.0	49.4 ± 0.5	58 ± 0.0	60.6 ± 2.6	76 ± 0.2
7.0	21.7 ± 0.1	14 ± 0.0	34.2 ± 1.4	32 ± 0.1	53.3 ± 0.8	64 ± 0.0	62.4 ± 3.7	79 ± 0.4
6.5	26.4 ± 2.8	20 ± 0.2	30.3 ± 2.3	26 ± 0.2	47.9 ± 0.3	55 ± 0.0	67.1 ± 2.4	85 ± 0.2
6.0	27.8 ± 2.3	22 ± 0.2	30.3 ± 2.6	25 ± 0.2	50.4 ± 6.0	59 ± 1.0	51.4 ± 3.3	61 ± 0.3
5.5	26.7 ± 1.6	20 ± 0.1	28.4 ± 1.3	23 ± 0.1	43.7 ± 0.8	48 ± 0.0	52.9 ± 3.3	64 ± 0.3
5.0	27.8 ± 6.1	22 ± 1.0	25.1 ± 4.2	18 ± 0.5	51.2 ± 7.5	60 ± 2.0	46.2 ± 3.7	52 ± 0.4
4.5	30.3 ± 3.0	25 ± 0.3	48.5 ± 4.6	56 ± 0.6	59.0 ± 1.9	73 ± 0.1	55.3 ± 3.1	68 ± 0.3
4.0	25.8 ± 1.4	19 ± 0.1	23.3 ± 1.3	15 ± 0.1	53.8 ± 2.4	65 ± 0.2	49.2 ± 1.0	57 ± 0.0
3.5	29.3 ± 1.7	24 ± 0.1	28.1 ± 0.2	19 ± 0.0	57.1 ± 2.0	70 ± 0.1	58.4 ± 3.1	73 ± 0.3

[1] Ca^{2+} buffered with 1×10^{-4} M EGTA.

[2] Contraction solutions all contained 5×10^{-3} M phosphate buffer (pH 7.2), KCl (I = 0.02 μ), and ATP, $CaCl_2$, and $MgCl_2$.

[3] $\bar{X}$ ± SE.

TABLE 23. Percentage of cells of C I type as a function of Salyrgan concentration*

	Epidermal cells		Gastrodermal cells	
	$\sin^{-1}\sqrt{p}$	p	$\sin^{-1}\sqrt{p}$	p
pSal	degrees	%	degrees	%
none	47.5 ± 1.0	54 ± 0.0	38.4 ± 2.0	38 ± 0.1
5.0	27.6 ± 2.4	21 ± 0.2	19.4 ± 2.6	11 ± 0.2
4.5	28.0 ± 5.1	22 ± 0.8	25.5 ± 5.2	19 ± 0.8
4.0	24.1 ± 2.7	17 ± 0.2	19.2 ± 2.0	11 ± 0.1
3.5	32.3 ± 1.5	28 ± 0.1	15.9 ± 1.7	8 ± 0.1

* In solutions containing ATP, $MgCl_2$, and $CaCl_2$ (all 1×10^{-4} M), 5×10^{-3} M phosphate buffer (pH 7.2), and KCl (I = 0.02 μ).

TABLE 24. Percentage of cells of type C II as a function of Salyrgan concentration*

	Epidermal cells		Gastrodermal cells	
	$\sin^{-1}\sqrt{p}$	p	$\sin^{-1}\sqrt{p}$	p
pSal	degrees	%	degrees	%
none	30.8 ± 1.8	26 ± 0.1	48.1 ± 2.5	55 ± 0.2
5.0	28.1 ± 1.5	22 ± 0.1	56.6 ± 2.1	70 ± 0.1
4.5	28.7 ± 4.2	23 ± 0.5	54.5 ± 4.9	66 ± 0.7
4.0	26.7 ± 1.4	20 ± 0.1	54.1 ± 0.9	66 ± 0.0
3.5	24.8 ± 3.3	18 ± 0.3	60.9 ± 1.4	76 ± 0.1

* See Table 23 for description of solutions.

TABLE 25. Concentrations of free ATP, Ca, and Mg, and of metal-ATP complexes, for Fig. 6 and 7

		Negative logarithms of concentrations of free ions and ion complexes				
total Ca	total Mg, total ATP	$MgATP^{2-}$	Mg^{2+}	$CaATP^{2-}$	Ca^{2+}	free ATP
8.00	7.00	10.10	7.00	11.50	8.00	7.00
	6.52	9.12	6.50	11.00	8.00	6.52
	6.00	8.09	6.00	10.48	8.00	6.00
	5.52	7.15	5.54	10.01	8.00	5.54
	5.00	6.16	5.03	9.54	8.01	5.03
	4.52	5.29	4.60	9.12	8.04	4.60
	4.00	4.58	4.13	8.91	8.06	4.13
	3.52	3.74	3.92	8.43	8.20	3.92
6.00	7.00	10.10	7.00	9.50	6.00	7.00
	6.52	9.12	6.50	9.00	6.00	6.52
	6.00	8.09	6.00	8.48	6.00	6.00
	5.52	7.15	5.54	8.01	6.00	5.54
	5.00	6.16	5.03	7.54	6.01	5.03
	4.52	5.29	4.60	7.12	6.04	4.60
	4.00	4.58	4.13	6.91	6.06	4.13
	3.52	3.74	3.92	6.43	6.20	3.92
4.00	7.00	10.24	7.00	7.64	4.00	7.11
	6.52	9.27	6.52	7.14	4.00	6.64
	6.00	8.23	6.00	6.64	4.00	6.12
	5.52	7.28	5.54	6.16	4.00	5.64
	5.00	6.28	5.02	5.67	4.01	5.14
	4.52	5.43	4.58	5.28	4.02	4.68
	4.00	4.62	4.11	4.85	4.05	4.19
	3.52	4.14	3.64	4.85	4.05	3.66

APPENDIX

I. Method of hatching nauplii of Artemia salina

Brine shrimp were raised at 24°C by seeding ½ teaspoon eggs (Longlife Fishfood Products) in 2 liters of well-aerated NaCl solution (2 tablespoons Fisher certified reagent-grade NaCl per liter of distilled water), all in a three-liter beaker. Newly hatched nauplii were harvested in the light 48 hours later and rinsed briefly in tap water before being fed to hydra.

II. Stock solutions for preparation and study of models

The following stock solutions, prepared in glass-distilled water (with one exception, noted below), were stored in 100-ml polyethylene containers in the refrigerator (4°C):

0.05 M KH_2PO_4 (monobasic)

0.05 M K_2HPO_4 (dibasic)

0.2 M tris (tris(hydroxymethyl)aminomethane)

0.2 M imidazole (glyoxaline, purchased from K and K laboratories)

$MgCl_2$ (1.0, 0.5, and 0.01 M)

$CaCl_2$ (0.1 and 0.01 M)

KCl (1.0 and 0.1 M)

0.01 M EGTA (trade name CHEL DE, donated by Geigy Industrial Chemicals)

0.04 M EDTA

1% w/v saponin (purchased from Fisher Scientific Co.)

0.001 M Salyrgan (Mersalyl, sodium salicyl-(γ-hydroxymercuri-β-methoxypropyl)-amide-O-acetate, purchased from Mann Research Laboratories)

2.5% w/v digitonin (purchased from Nutritional Biochemical Corporation) in absolute ethanol

A 0.025% digitonin solution was made by diluting the above stock 1:99 in glass-distilled water. Dipotassium adenosine-5′-triphosphate (ATP), purchased from General Biochemicals, was kept in a freezer at -10°C and weighed out just before using.

III. Buffers

Type of buffer	pK	Range	Reference
K_2HPO_4-KH_2PO_4	6.81[1,2]	5.29 - 8.04	Hale, 1958, p. 73
tris-HCl	8.08[1]	7.19 - 9.10	Hale, 1958, p. 76
imidazole-HCl	6.95	6.2 - 7.8	Dawson et al., 1959, p. 194

1 Calculated from Hale's (1958) tables.

2 From Sørensen's Na_2HPO_4-KH_2PO_4 buffer.

IV. Calculations of KCl concentration required to maintain a specified ionic strength

In the following example the desired ionic strength is 0.02 μ for a solution also containing 1.4×10^{-3} M KH_2PO_4, 3.6×10^{-3} M K_2HPO_4 (i.e., 5×10^{-3} M phosphate buffer, pH 7.2), 1.0×10^{-4} M K_2-ATP, and 1.0×10^{-4} M $MgCl_2$:

salt	ion	concentration ($= c_i$) M	valence ($= x_i$)	$\frac{1}{2} c_i x_i^2$ μ
K_2HPO_4	K^+	0.0072	1	0.0036
	HPO_4^{2-}	0.0036	2	0.0072
KH_2PO_4	K^+	0.0014	1	0.0007
	$H_2PO_4^-$	0.0014	1	0.0007
K_2-ATP	K^+	0.0002	1	0.0001
	ATP^{4-}	0.0001	4	0.0008
$MgCl_2$	Mg^{2+}	0.0001	2	0.0002
	Cl^-	0.0002	1	0.0001

$\sum \frac{1}{2} c_i x_i^2$ = ionic strength without KCl = 0.0134 μ.

Therefore, the contribution by KCl to the total ionic strength is 0.02 μ - 0.0134 μ = 0.0066 μ; and the required KCl concentration is 6.6×10^{-3} M.

V. EDTA and EGTA as Ca and Mg buffers

Calculations of final concentrations of free Ca and Mg ions were based on the following dissociation constants and solubility products (Geigy, 1958):

Acidity constants	EDTA	EGTA
pK_1	1.99	2
pK_2	2.67	2.68
pK_3	6.16	8.85
pK_4	10.26	9.46

Stability constants	EDTA	EGTA
Mg	8.7	5.21
Ca	10.7	11.00

If, for example, the initial concentration of EGTA is 1×10^{-4} M, and the free calcium ion concentration desired is 1×10^{-7} M, and the pH is 6.8, then the required initial concentration of $CaCl_2$ (i.e., concentration of total Ca) can be calculated as follows:

From K_3 and K_4 the following ratio can be derived:

$$\frac{[EGTA^{4-}]}{[EGTA^{2-}]} = \frac{10^{-8.85} \times 10^{-9.46}}{[H^+]^2} = \frac{10^{-18.31}}{(10^{-6.8})^2} = 10^{-4.71}.$$

Using this ratio, together with the stability constant for EGTA and calcium, we arrive at the following relationship:

$$10^{-11} = \frac{[Ca^{2+}] \times [EGTA^{4-}]}{[CaEGTA^{2-}]} = \frac{[Ca^{2+}] \times ([EGTA^{2-}] \times 10^{-4.71})}{[CaEGTA^{2-}]};$$

that is, $[CaEGTA^{2-}] = [Ca^{2+}] \times [EGTA^{2-}] \times 10^{6.29}$.

Since the total concentration of EGTA is 10^{-4} M, and since $[EGTA^{4-}]$ is negligible with respect to $[EGTA^{2-}]$, it follows that

$$[EGTA^{2-}] + [CaEGTA^{2-}] = 1 \times 10^{-4}.$$

Substituting,

$$[CaEGTA^{2-}] = [Ca^{2+}] \times (10^{-4} - [CaEGTA^{2-}]) \times 10^{6.29}.$$

If we assume that almost all the calcium present initially as $CaCl_2$ will end up complexed as $CaEGTA^{2-}$, that is,

$$[\text{total Ca}] = [CaEGTA^{2-}],$$

then it follows that

$$[\text{total Ca}] = [Ca^{2+}] \times (10^{-4} - [\text{total Ca}]) \times 10^{6.29}.$$

Rearranging terms,

$$[\text{total Ca}] = \frac{10^{-4} \times [Ca^{2+}]}{10^{-6.29} + [Ca^{2+}]}.$$

It was required that the free Ca^{2+} concentration be 1×10^{-7} M; therefore,

$$\begin{aligned}
[\text{total Ca}] &= \frac{10^{-4} \times 10^{-7}}{10^{-6.29} + Ca^{2+}} \\
&= \frac{10^{-11}}{10^{-7} \times (10^{0.71} + 1)} \\
&= 10^{-4} / 6.13 \\
&= 10^{-4} / 10^{0.79} \\
&= 10^{-4.79}.
\end{aligned}$$

That is,

$$[\text{total Ca}] = 1.62 \times 10^{-5} \text{ M}.$$

VI. Calculations of concentrations of Ca^{2+}, Mg^{2+}, $CaATP^{2-}$, $MgATP^{2-}$, ATP^{4-}, and ATP^{3-} when pH, total Ca, total Mg, and total ATP concentrations are known

These calculations were based on Martell and Schwarzenbach's (1956) stability products and dissociation constants, as follows:

$$[1] \quad K^{H}_{HZ} = \frac{[ATP^{4-}][H^{+}]}{[ATP^{3-}]} = 10^{-6.5}.$$

$$[2] \quad K^{Ca}_{CaZ} = \frac{[Ca^{2+}][ATP^{4-}]}{[CaATP^{2-}]} = 10^{-3.6}.$$

$$[3] \quad K^{Mg}_{MgZ} = \frac{[Mg^{2+}][ATP^{4-}]}{[MgATP^{2-}]} = 10^{-4.0}.$$

(where $Z = ATP^{4-}$)

If the pH is between 6 and 8, then almost all the ATP will exist as ATP^{4-} and ATP^{3-}, the relative concentrations of which can be calculated from [1].

Let [total Ca] = A,

[total Mg] = B,

and [total ATP] = C.

Also, let $[CaATP^{2-}]$ = a,

and $[MgATP^{2-}]$ = b.

Then $[Ca^{2+}]$ = [total Ca] - $[CaATP^{2-}]$ = A - a,

and $[Mg^{2+}]$ = [total Mg] - $[MgATP^{2-}]$ = B - b.

Furthermore, $[ATP^{4-}] + [ATP^{3-}]$ = [total ATP] - $[CaATP^{2-}]$ - $[MgATP^{2-}]$

= C - a - b.

From [1], $[ATP^{3-}] = 10^{6.5}[H^{+}][ATP^{4-}]$;

and $[ATP^{4-}] + [ATP^{3-}] = (1 + 10^{6.5} \times [H^{+}]) \times [ATP^{4-}] = C - a - b.$

Let $K = 1 + 10^{6.5} \times [H^+]$. Then, $[ATP^{4-}] = \frac{C - a - b}{K}$.

Equation [2] becomes [4] $10^{-3.6} = \frac{(A - a)(C - a - b)}{K a}$.

and equation [3] becomes [5] $10^{-4.0} = \frac{(B - b)(C - a - b)}{K b}$.

Dividing [4] by [5],

$$10^{0.4} = \frac{(A - a) b}{(B - b) a} .$$

Solving for a in terms of b,

$$a = \frac{Ab}{10^{0.4} B - 1.512 b} .$$

Substituting this expression for a in [5],

$$10^{-4.0} = \frac{(B - b)\left[C - \left(\frac{A b}{10^{0.4} B - 1.512 b}\right) - b\right]}{K b} .$$

Combining terms,

$$0 = -1.512 b^3 + (4.024 B + 1.512 C + A + 1.512 \times 10^{-4} K) b^2$$
$$+ (-4.024 B C - 10^{0.4} B^2 - A B - 10^{-3.6} K B) b + 10^{0.4} B^2 C.$$

Dividing all terms by -1.512,

$$b^3 + C_1 b^2 + C_2 b + C_3 = 0,$$

where $C_1 = -2.660 B - C - 0.6613 A - 10^{-4} K$,

$C_2 = 2.660 B C + 1.661 B^2 + 0.6613 A B + 1.661 \times 10^{-4} K B$,

and $C_3 = -1.661 B^2 C$.

When pH = 7.2, and [total Mg] = [total ATP], then K = 1.1995, and B = C. In this case,

$C_1 = -3.660 B - 0.6613 A - 1.1995 \times 10^{-4}$,

$C_2 = 4.321 B^2 + 0.6613 A B + 1.977 \times 10^{-4} B$,

and $C_3 = -1.661 B^3$.

When A and B are known, the cubic equation can be solved for b; a is then evaluated in terms of b, and all other ionic concentrations are evaluated in terms of a and b. These concentrations are listed in Table 25 for pH = 7.2; [total Ca] = 10^{-8}, 10^{-6}, and 10^{-4} M; and [total Mg] = [total ATP] = 1×10^{-n} and 3×10^{-n} M, where n = 7, 6, 5, and 4.