THE PALMAR SWEAT GLANDS OF THE RHESUS MONKEY

by

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The eccrine sweat glands on the palm of the rhesus are like those of man. During sweating the clear cells of the sweat glands of the rhesus show histochemically detectable decreases in glycogen synthetase I activity and glycogen and increases in phosphorylase a and phosphoglucomutase activity. The outer cells of the coiled duct show an increase in the proportion of phosphorylase in the a form during sweating. Severing the nerves to the sweat glands causes gradual loss of both spontaneous sweating and sweating in response to local injection of methacholine.

ACKNOWLEDGEMENTS

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INTRODUCTION

The skin of mammals has developed many highly differentiated adnexae. Hair follicles produce a polyamide fiber for insulation, sebaceous glands produce a lipid weatherproofing agent (cf. Washburn, 1962) and sweat glands secrete fluids for a variety of purposes. In primates, apocrine sweat glands produce a culture medium for bacteria that provide scents (Hurley and Shelley, 1960) for territorial marking (Buettner-Janusch, 1966) and sexual attraction (Ellis, 1911). The eccrine sweat glands secrete water to increase the coefficient of friction between the volar surfaces of the cheiridia and the substratum and to maintain the horny layer pliable.

In civilized man, hair, if not removed, is largely ornamental; sebum is removed with soaps and replaced by a fraction of ovine sebum (lanolin) or vegetable oils; and the scent-producing bacteria fed by the apocrine sweat glands are suppressed with aluminum salts (Hurley and Shelley, 1960). The eccrine sweat glands, however, not only continue to serve their original purpose but are found all over the body where they serve a thermoregulatory function.

Eccrine sweat glands are thus the most important of man's cutaneous adnexae. This importance is underlined by the fact that eccrine sweat gland dysfunction may cause serious, or even fatal, electrolyte imbalance (di Sant'Agnese and Powell, 1962).

Sweat glands were discovered by Niels Stensen (1662). The inadequacy of their equipment prevented Stensen and Malpighi (1687) from

doing more than noting the existence of sweat glands (Nordenskiöld, 1936; Cole, 1949).

Adequate microscopes became available early in the nineteenth century and sweat glands were rediscovered by Purkinje in 1833 and briefly described by Wendt (1834) and by Breschet and Roussel de Vouzeme in 1834 (Montagna, 1962). The gross structure of the glands was first clearly worked out by Todd and Bowman in 1845 (Robin, 1883). With a few minor emendations (Henle, 1866; Kölliker, 1879; Horn, 1935) their work stands today.

Although Leeuwenhoek seems to have counted the "pores" in a fingertip around 1719 (François-Franck, 1884), the first reliable counts of sweat glands are those made by Krause in 1844 (Kuno, 1956).

Although others had mentioned it (Leidy, 1861; Henle, 1866), Schiefferdecker (1917) made clear the distinction between eccrine and apocrine sweat glands and published a detailed account of both. All palmar sweat glands are eccrine, although the converse is not true.

Ito (1943), Montagna, Chase, and Lobitz (1953), and Lee (1960) distinguished two types of eccrine secretory cell. Each emphasized different criteria, and each proposed a different nomenclature. I shall follow the American custom and use Montagna et al's (1953) nomenclature.

Hibbs (1958) and Charles (1960) made the first serious electron microscope studies of sweat glands.

Around the end of the sixteenth century Sanctorius measured

perspiration under various conditions (Singer and Underwood, 1962). This may be considered the first attempt to study sweating scientifically. Some 200 years later Seguin and Lavoisier separated perspiration into respiratory and cutaneous perspiration (Kuno, 1956).

On the basis of indirect evidence Krause (1844, cited by Loewy and Wechselmann, 1911) and Milne-Edwards (1858, cited by François-Franck, 1884) distinguished between sweating and transcutaneous perspiration. Adolf Loewy and W. Wechselmann (1911) conclusively proved the existence of transcutaneous perspiration and quantitated it. They were thus able to make the first accurate measurements of sweating.

The hypothesis that the duct selectively resorbs sodium was gradually accepted on histochemical and teleological grounds. The hypothesis was finally proven by Brusilow (1963), and the resorption was measured by Cage and Dobson (1965).

Kuno's (1934) monograph, The Physiology of Human Sweating, and its (1956) revision, Human Perspiration, concentrated on physiology. The third volume of Advances in the Biology of Skin (Montagna Ellis, and Silver, 1962) was almost equally divided between anatomical and physiological papers.

Golz, Freusberg, and Gergens (1875) discovered fortuitously that sweating is controlled by nerves. Within a few years it was shown that sweat nerves follow sympathetic pathways (Kendall and Luchsinger, 1876; Luchsinger, 1876; Langley, 1891) although the

glands are stimulated by parasympathomimetic drugs (Weber, 1876, cited by Luchsinger, 1877). Langley (1922) appears to have been the first to understand the long-term effects of denervation on sweat glands.

Although most of the work on sweat nerves has been done on the cat, several studies have been made on man (Hyndman and Wolkin, 1941; Janowitz and Grossman, 1950) and other primates (Sakurai and Montagna, 1964, 1965).

The first experimental study of the sweat glands themselves was made by Nicolas, Regaud, and Favre in 1912 (Dobson, 1963).

For the next 35 years experimental studies of the eccrine sweat glands were rare and often published in journals of only local circulation. In Japan, Yuyama (1935) found that sweating decreases the glycogen content of the secretory coil and Ogata (1935, cited by Kuno, 1956) demonstrated that some histologically normal sweat glands never sweated. In the United States, Way and Memmesheimer (1936) found flattening and vacuolation in cells of the secretory coil of exhausted glands. In Germany, Deme (1940) reported dispersion of the Golgi apparatus in active sweat glands. (This paucity of activity may have been due to political factors. War, chauvinism, and depression decreased the mobility of scientists and publications.)

After the end of the Second World War a number of experimental studies of eccrine sweat glands appeared. Ring and Randall (1947) extended Way and Memmesheimer's findings to the rat. Sperling and

Koppanyi (1949) found some histochemical evidence that oxygen consumption increases during secretion. Ohara (1951) proved this by placing isolated sweat glands in drugged media in a Warburg apparatus.

Shelley and Mescon (1952) rediscovered and confirmed Yuyama's (1935) demonstration of the loss of glycogen from secretory cells during sweating. Several subsequent studies extended this result to the duct cells (Lobitz, Holyoke, and Brophy, 1955; Dobson, Formisano, Lobitz, and Brophy, 1958; Sargent and Dobson, 1962) and demonstrated its relation to salt intake (Dobson, Abele, and Hale, 1961; Dobson, 1963). Dobson and Abele (1962) further showed that the loss of glycogen from the duct occurs only if the duct is resorbing sodium. Histological acclimatization (i.e., the decrease or absence of histological and histochemical responses to sweating on the second and subsequent days in subjects used repeatedly) was observed but not explained (Dobson, 1960; Dobson, Abele, and Hale, 1961; Dobson and Abele, 1962).

In an electron microscope study of sweat secretion in man, Munger (1961) showed extrusion of granules from the dark secretory cells and mitochondrial vacuolization in the clear secretory cells during sweating.

Recent anatomical studies of sweating have focused on animals. Matsumoto and Ohkura (1960) used the Japanese monkey, Macaca fuscata (Blyth 1875); Smith and Dobson (1966) used the rhesus, Macaca mulatta (Zimmermann 1797); and Wechsler and Fisher (1968) used

the white rat, Rattus norvegicus (Berkenhout 1769), for histochemical studies. Munger and Brusilow (1961) used the cat for an electron microscope study of sweating.

These animals differ from man in some respects. Neither the cat nor the rat has eccrine sweat glands on the general body surface. More important, their eccrine sweat ducts are less well developed than those of man. Although eccrine sweat glands are present over the general body surface in the Cercopithecoidea (Old World monkeys), they appear to be only minimally functional (Montagna, 1963). Monkeys sweat visibly only on the palms and soles. The eccrine sweat ducts of the Cercopithecoidea are, however, histologically (Montagna and Yun, 1962; Montagna, Yun, and Machida, 1964; Machida, Perkins and Montagna, 1964) and cytologically (Ellis and Montagna, 1961; Terzakis, 1964) very like those of man (Munger, 1961).

The eccrine sweat glands of some Platyrrhini (New World monkeys) approximate those of man in appearance and distribution (Perkins and Machida, 1967). Immunological evidence, however, suggests that the Hominoidea and the Ceropithecoidea form a taxonomic unit, Catarrhini, that excludes the Platyrrhini and that, of the Platyrrhini, Aotes is their closest relative (Goodman, 1962; Hafleigh and Williams, 1966; Weiner, Moor-Jankowski, and Gordon, 1966; Sarich and Wilson, 1967). The owl or night monkey, Aotes trivirgatus Humboldt 1811, is the most primitive Platyrrhine (Hill, 1957; Hanson and Montagna, 1962; Perkins, Smith and Ford, 1969). The sweat glands of Aotes

are more like those of the Lorisidae (Hanson and Montagna, 1962) than like those of man. This suggests that the highly developed eccrine sweat ducts of the "higher" Platyrrhini evolved independently of those of the Catarrhini.

A Catarrhine is thus the best model of man with respect to sweat glands. Supply and habit have made the rhesus the standard laboratory Catarrhine (Montagna, Yun and Perkins, 1964).

In the rhesus, as in man (Szabo, 1962), eccrine sweat gland density is highest in the palms and soles. Over the rest of the body, eccrine sweat glands are so sparse as to make study difficult. I have often found no eccrine sweat glands in a 1.2 mm^2 area (the amount that can be studied easily on one slide) of skin from the general body surface of the rhesus. The fact that the palms and soles sweat visibly while the rest of the body does not is not a function of sweat gland density. A single highly active palmar sweat gland can produce a sweat droplet visible to the naked eye.

The palmar sweat glands are certainly the easiest and probably the most profitable eccrine sweat glands to study in rhesus monkeys.

This research was begun as part of a search for a model. The thesis problem is: What is the structure of the palmar sweat glands of the rhesus and, so far as one can infer from anatomical data, how does it function?

Fig. 1

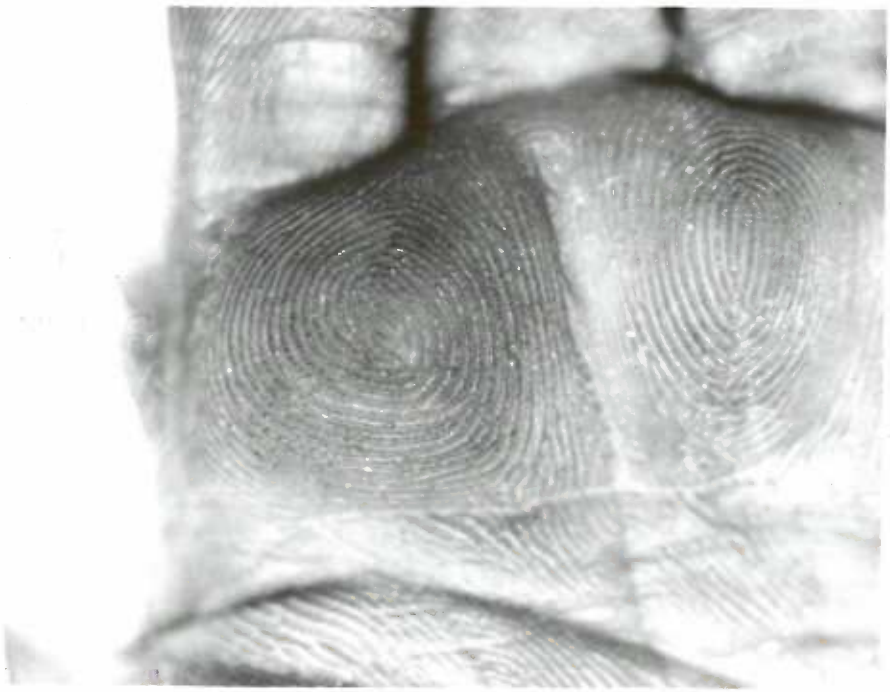

Fig. 2

Fig. 1

Rhesus monkey, Macaca mulatta (Zimmermann 1797). This is an adult female, about 2 ft. high at the shoulder. X 1/10.

Fig. 2

Beads of sweat appearing at the orifices of the sweat ducts atop the papillary ridges of the index eminence (2nd palmar torus) in response to an injection of methacholine. X 3.

MATERIALS AND METHODS

Biopsies were taken from untreated digital pads of each of 12 adult rhesus monkeys just before sacrifice. In 5 cases the biopsy specimen was cut into 1 x 1 x 2 mm pieces and fixed for 2 hours at 4°c in a 4.2% solution of glutaraldehyde (Eastman practical grade) in 0.08 M pH 7.2 cacodylate buffer, washed overnight in 0.1 M pH 7.2 cacodylate buffer at 4°c, cleared for 1 hour in propylene oxide at room temperature, left overnight in a 1:1 mixture of propylene oxide and araldite at 4°c, infiltrated with araldite for 3 hours at room temperature and embedded in araldite by polymerizing for 5 to 7 days at 50° C. 1 μ sections were cut from these blocks with a glass knife on a Porter-Blum MT-1 microtome. These sections were mounted on glass slides over a Bunsen burner, stained by heating a drop of stain on the slide, and coverslipped with "DPX" (Edward Gurr, Ltd., London). Stains used were 1% solutions of toluidine blue or safranin in 1% aqueous solution of "Boraxo" (U.S. Borax & Chemical Corp., Los Angeles), Harris' hematoxylin followed by a 0.3% solution of eosin Y in 25% ethanol, and Heidenhain's iron hematoxylin.

Tissues from 6 animals were fixed for 4 hours in Helly's fluid, washed overnight in tap water and dehydrargyrified for 2 hours in 0.5% iodine in 80% ethanol; tissue from 1 animal was fixed for 2 hours in Carnoy's fluid.

The Helly and Carnoy-fixed tissue was dehydrated in alcohol, cleared in xylene, infiltrated overnight with paraffin at 57° C, and embedded in "Paraplast" (a modified paraffin - Sherwood Medical Industries, St. Louis). Sections were cut from ice-cooled blocks at 4 or 7 μ with a steel blade on a rotary microtome; one section (Fig. 5) was cut at 150 μ. The sections were spread on a 0.01% aqueous solution of gelatine in a bath at 45° C, mounted on glass slides, and cooked at 45° C for 2 days to ensure adherence to the slides. The Helly-fixed sections were stained with Harris' or Ehrlich's hematoxylin (Lillie, 1958) followed by eosin Y, 0.05% toluidine blue in pH 4.5 McIlvaine buffer, Mowry's (1958) and Rinehart and Abul-Haj's (1951) colloidal iron, Heidenhain's iron hematoxylin (McManus & Mowry, 1960), the periodic acid-Schiff (PAS) reaction (Lillie, 1958) and Montagna, Chase and Lobitz's (1953) modification of it with controls incubated in 0.1 diastase in 0.02 M pH 6.0 phosphate buffer, Gomori's paraldehyde-fuchsin (U.S. Armed Forces Institute of Pathology, 1960), and a brazillin solution made by substituting brazillin for hematoxylin in the Delafield formula (Lillie, 1958) and adding 40 mg $NaIO_3$ and 40 mg $FeCl_3 \cdot 6 H_2O$ per gram of brazillin. The Carnoy-fixed sections were stained in a solution of 0.1 g chloroform-washed methyl green and 0.2 g pyronin G in 0.8 ml ethanol, 6.7 ml glycerol, and 33 ml pH 4.66 Michaelis buffer*.

*This is an unpublished method of J. Mulnard that has been passed from hand to hand.

Digital pads from 8 animals were frozen in liquid nitrogen or dry ice-ligroin and frozen sections were cut in a cryostat between -30° and -20° C. Four to 10 μ sections from 3 animals were incubated for adenosine triphosphatase by the lead method (Wachstein and Meisel, 1957); 20 to 40 μ sections from 3 animals were incubated for acid phosphatase by the method of Burton (1954) and for aminopeptidase by the L-leucyl-β-naphthylamide method (Nachlas, Crawford, and Seligman, 1957); 40 to 300 μ sections from 3 animals were incubated for alkaline phosphatase by Gomori's (1952) method; 20 to 40 μ sections from 2 animals were incubated for nonspecific carboxylic esterase by the α-naphthyl acetate method (Pearse, 1961); and 10 to 80 μ sections from 2 animals were stained for elastic fibers by Roman's "AOV" method (Roman, Perkins, Perkins, and Dolnick, 1967). A 300 μ section was incubated for succinate dehydrogenase by the method of Nachlas, Tsou, de Souza, Cheng, and Seligman (1957), and an eccrine sweat gland was dissected out by the method of Bartman and Dixon (1966); lengths of parts of this gland were measured in a photograph.

Cholinergic nerves were displayed in 10 to 300 μ frozen sections from 4 animals and in 1 x 1 x 2 mm blocks from 2 animals. After incubation a few of these frozen sections were "silvered" for photography* by fixing for 1 month in Winkelmann's (1960) fixative and applying his (1960) "simple silver method". The blocks were fixed in Brenner's (1966)

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*N. A. Roman, personal communication.

"broth" before and embedded in araldite after incubation. Five μ araldite sections were stained by Roman et al's (1967) "AOV" method to show the relationship of elastic fibers to the nerves.

One juvenile and 6 adult rhesus monkeys were used to study enzyme changes during sweating; 5 other animals were used to study changes in glycogen content.

Each animal was anesthetized with "Sernylan" (phencyclidine, Parke-Davis). Dosage varied from 1-1/2 to 3 mg/kg. Sweating was induced by injection of 40-120 μg of methacholine (Merck) into each of the experimental digital pads. Sweating was inhibited in the control digital pads with 4-12 μg atropine (as sulfate, Lilly). The drugs were in solution in 50-300 μl physiological saline to which 1-1/2 to 15 units/ml of hyaluronidase (Wyeth) had been added. Seven to 30 minutes after injection, the digital pads were removed and frozen in liquid nitrogen or a mixture of dry ice and ligroin for enzyme studies or fixed in Helly's fluid for the glycogen study.

Frozen sections were cut at 20 μ between -30° and -20° C. The sections were transferred to warm glass slides--except for glycogen synthetase studies, where cold slides were used. Sections were incubated for phosphoglucomutase by the method of Meijer (1967), for glycogen synthetases by the method of Sasse (1966), adding 10^{-2} M NaF and 2% gelatine to the medium for the I form, for phosphorylase b by the methods of Takeuchi and Kuriaki (1955) and of Eränkö and Palkama

(1961), for phosphorylase a by the methods of Godlewski (1962) and of Eränkö and Palkama (1961), for fructoaldolase by the method of Abe and Shimizu (1964), for glucose-6-phosphate dehydrogenase by the method of Hess, Scarpelli, and Pearse (1958), for succinate dehydrogenase by the method of Nachlas et al (1957), and for cytochrome oxidase by the method of Burstone (1960, 1961). Twenty μ sections from these animals were also incubated for branching enzyme by the method of Takeuchi and Kuriaki (1955), for TPN-linked isocitrate dehydrogenase by the method of Hess et al (1958), and for 6-phosphogluconate dehydrogenase by the method of Im (1965), but were not included in the study of changes in enzyme activity during sweating. All of these enzymes were also observed in at least 2 untreated digital pads. The preparations of glycogen synthetases, branching enzyme, and phosphorylases were mounted according to Smith, Perkins, and Machida (1966); those of cytochrome oxidase were mounted with glycerol gel; the rest were dehydrated through alcohols and mounted in "Diaphane" (Will Corporation, Rochester, New York).

The tissue fixed in Helly's fluid was embedded in "Paraplast", cut at 7 μ, stained for glycogen by the modified PAS procedure of Montagna et al (1953) and mounted in Permount (Fisher).

Reactions in stimulated sweat glands were compared visually with the reactions in inhibited glands from the same animal. The stain intensity produced by the reaction was graded subjectively on a six

point scale. The significance of differences between stimulated and inhibited glands was evaluated with the Kolmogorov test (Birnbaum 1952).*

Three adult rhesus monkeys were used to study effects of denervation on sweating. The radial, median, and ulnar nerves of one arm of each animal were severed in the upper arm; the control arm was not operated on. Sweating was induced by injections of 20 µg of methacholine (Sigma) in 0.05 ml physiological saline and detected visually or with bromphenol blue paper (Sakurai & Montagna, 1964). Biopsies from these animals were frozen in liquid nitrogen, cut at 50 µ, and incubated for cholinesterases (Gomori, 1952), phosphorylases (Takeuchi & Kuriaki, 1953; Godlewski, 1962; Eränkö & Palkama, 1961) and cytochrome oxidase (Burstone 1960, 1961).

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*A simple explanation of the test is given by Siegel (1956).

OBSERVATIONS

Numbers given as a ± b are an average ± standard deviation of measurements in araldite sections. Average is used in the common sense, viz. arithmetic mean (Webster's Third New International Dictionary, 1961). The counts of glands per unit are from paraffin sections. Widths of elastic fibers and all lengths are from frozen sections.

I. The Eccrine Sweat Gland.

An eccrine sweat gland is a thick-walled tube 0.02 to 0.07 mm in diameter (araldite sections) and 7 to 9 mm long. One end opens on the skin surface; the other is blind. The part of the gland nearest the blind end (~2-1/2 mm) is the secretory coil; the rest is duct. In the secretory coil and proximal duct region the gland is coiled upon itself in no apparent order to form a large irregular knot 0.2 to 0.4 mm across (paraffin sections). The interstices of the knot contain collagen and a few thin (0.4 to 1.2μ) elastic fibers. The knot, usually called the coil, lies in or just above the panniculus adiposus (subcutaneous fat). Each sweat gland is set off from its neighbors by collagen and thick (up to 7μ) elastic fibers and often by clusters of adipose cells.

There are 30 ± 5 glands/mm^2 in the fingertips (6 animals) and 20 ± 2 in the thenar eminence (4 animals).

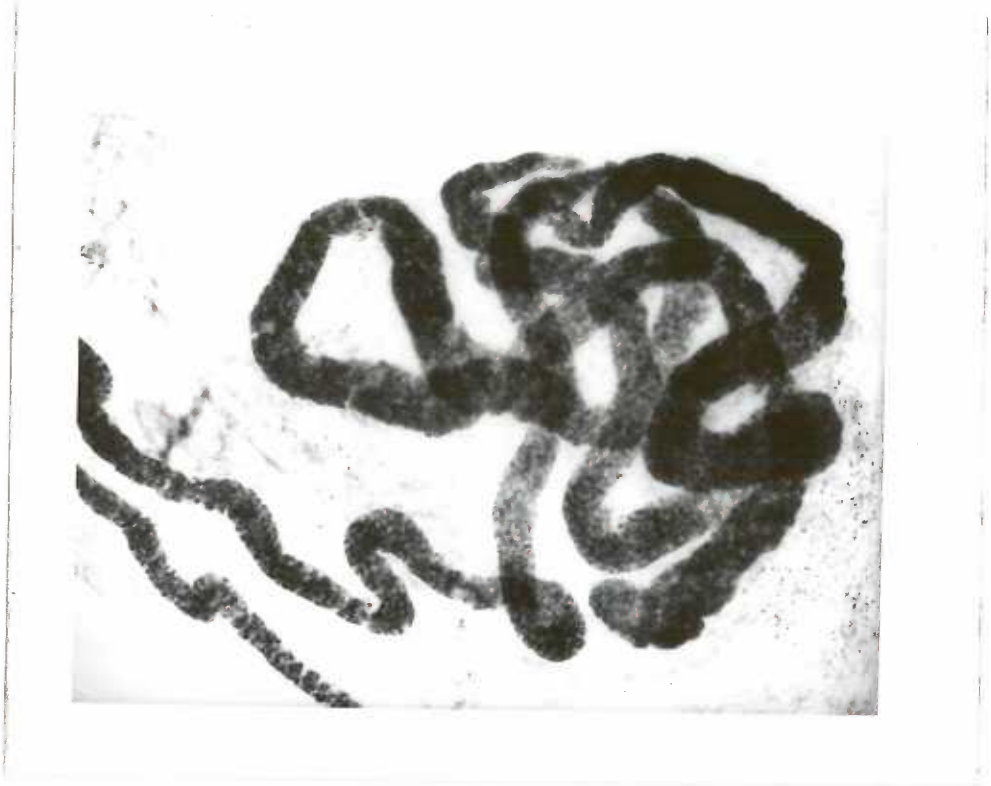


Fig. 3

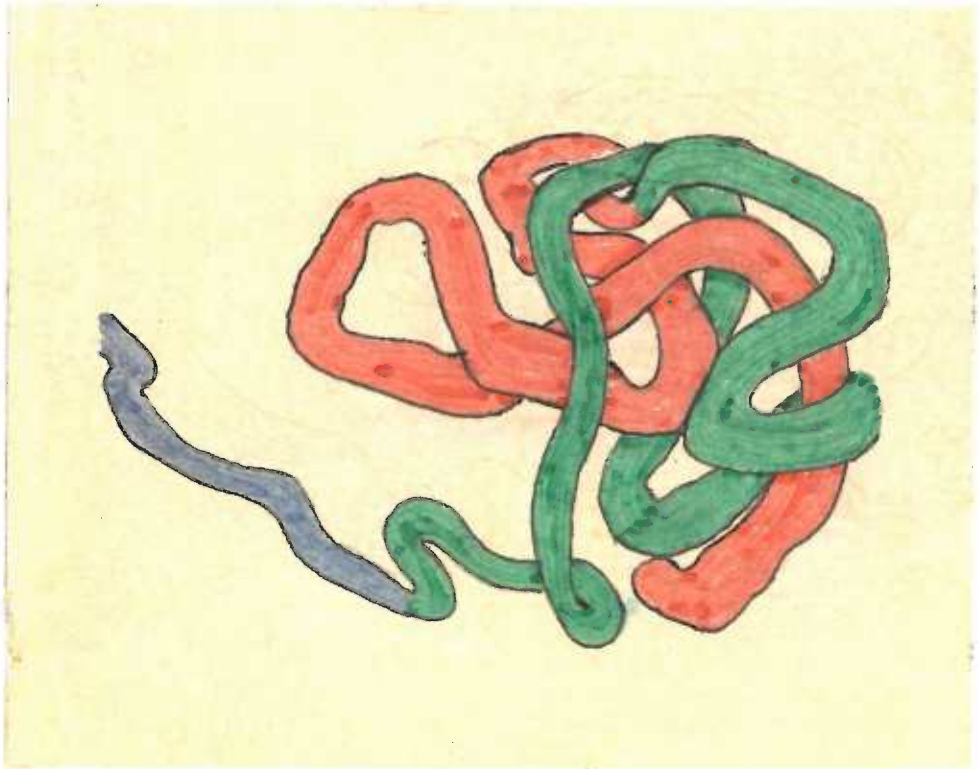


Fig. 4

Fig. 3

Palmar sweat gland stained for succinate dehydrogenase and dissected free of most of the surrounding tissue. X 100.

Fig. 4

Key to figure 3: straight duct is shown in blue; coiled duct, green; secretory coil, orange.

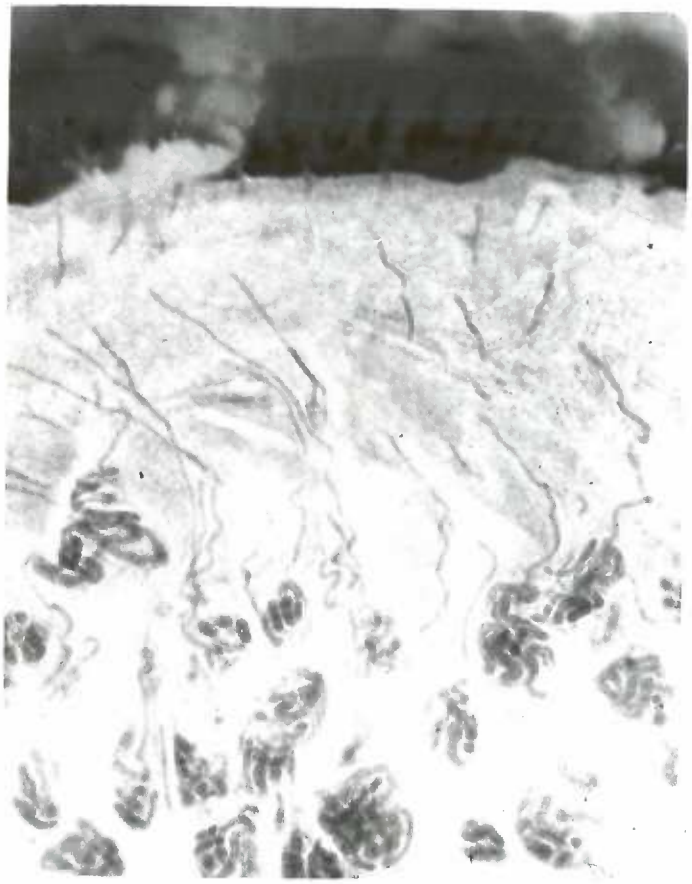


Fig. 5

Fig. 5

Sweat glands in the digital pad of a rhesus. Epidermis is at top of picture. This section is about 150 μ thick. Brazillin, X 10, enlarged to X 35.

II. The Secretory Coil.

The secretory coil is roughly circular in cross section with a diameter of 60 ± 8 μ (10 measurements in each of 5 animals). It is surrounded by a prominent basement membrane, the outside of which is wrapped in a layer of collagen, laced with elastic fibers 0.1 to 0.4 μ thick. The elastic fibers run perpendicular to the axis of the secretory coil.

Myoepithelial cells line most of the inner surface of the basement membrane. These cells are elongated, tapering at both ends, and roughly triangular in cross section; their nuclei are also fusiform but can be triangular, round, or elliptical in cross section. The nucleus is usually as far from the basement membrane as the cell's dimensions permit; a definite nucleolus is present near the center. The long axis of the nucleus is parallel to the axis of the cell, which is circa 30° oblique to the axis of the secretory coil. The cytoplasm of the myoepithelial cells is slightly basophilic--but is never stained metachromatically. Fibrils can sometimes be seen in araldite sections and their presence can be inferred from the linear distribution of enzyme reaction products in frozen sections.

A narrow, irregular lumen is on or near the axis of the secretory coil; the average width is 7 μ with a standard deviation of 4 μ (10 observations in each of 5 animals).

Dark cells usually lie around the lumen of the secretory coil, clear cells between the myoepithelial cells and the dark cells. A few

clear cells are adjacent to the lumen and some dark cells are not. Each clear cell has one or more processes contacting the basement membrane. A thin process connecting it to the basement membrane can be seen on an occasional dark cell. It is my opinion that all dark cells have such processes. The processes are so thin that if each cell had only one, very few would be present in a 1 μ section and very few could be seen in thicker sections. Clear and dark cells are found in small groups of cells of one type. A few cells seem to be intermediate between dark and clear cells.

Dark cells are smaller than clear cells and have more affinity for basic stains. They are irregular, almost stellate in shape. The nucleus contains a diffuse basophilic material and a few small clumps of chromatin. Usually a large number of basophilic granules are present in the cytoplasm. These granules give a positive PAS reaction that is unaffected by diastase; they are not notably metachromatic when stained with toluidine blue.

The nucleus of a clear cell has more and larger clumps of chromatin than a dark cell nucleus but shows no diffuse staining; the nucleolus is prominent. In thiazine-stained araldite sections, the cytoplasm of clear cells is, indeed, clear. In paraffin sections, it stains weakly with thiazines but has greater affinity for eosin than for alum hematoxylin; some glycogen is often present. Glycogen is abundant after the sweat glands have gone through a period of atropine-induced rest; it is hard to say what the "normal" state is. The stress of capture

and immobilization certainly affects the physiological state of many organs. Sweat glands are probably strongly affected since emotional stress usually induces palmar sweating in man (Kuno, 1956).

Between the clear cells are intercellular canaliculi, which appear as thin crescentic slits. These canaliculi are strongly ferrophilic. One often sees wide intercellular canaliculi, but I am uncertain whether these are normal variations, physiological alterations, or artifacts.

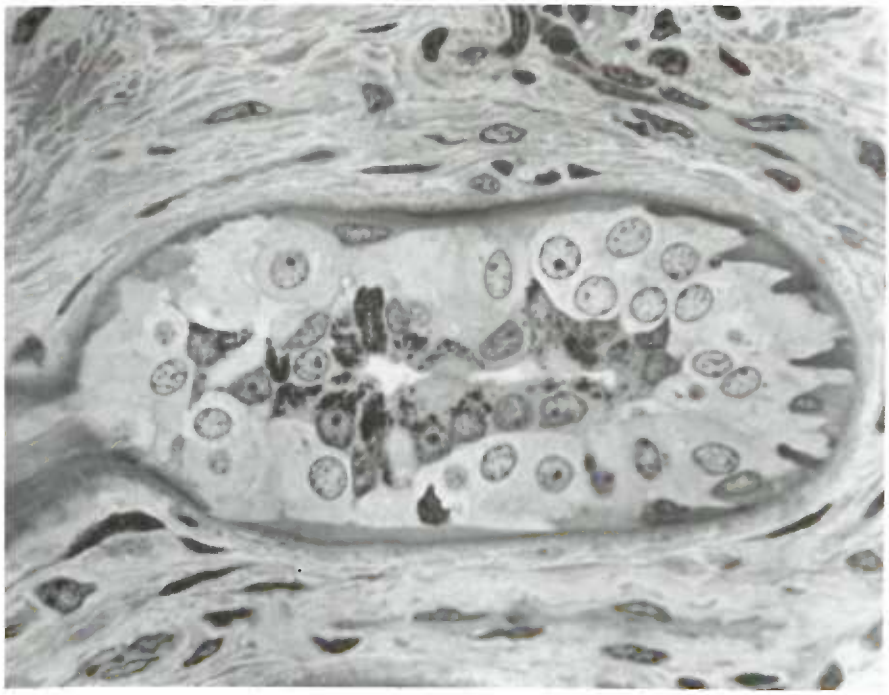


Fig. 6

Fig. 7

Fig. 6

Section through an "elbow" of the secretory coil. Toluidine blue-borax, X160 enlarged to X 680.

Fig. 7

Key to Fig. 6.

Dark cells - green

Clear cells - orange

Myoepithelial cells - brown

Lumen - yellow

Intercellular canaliculi - red

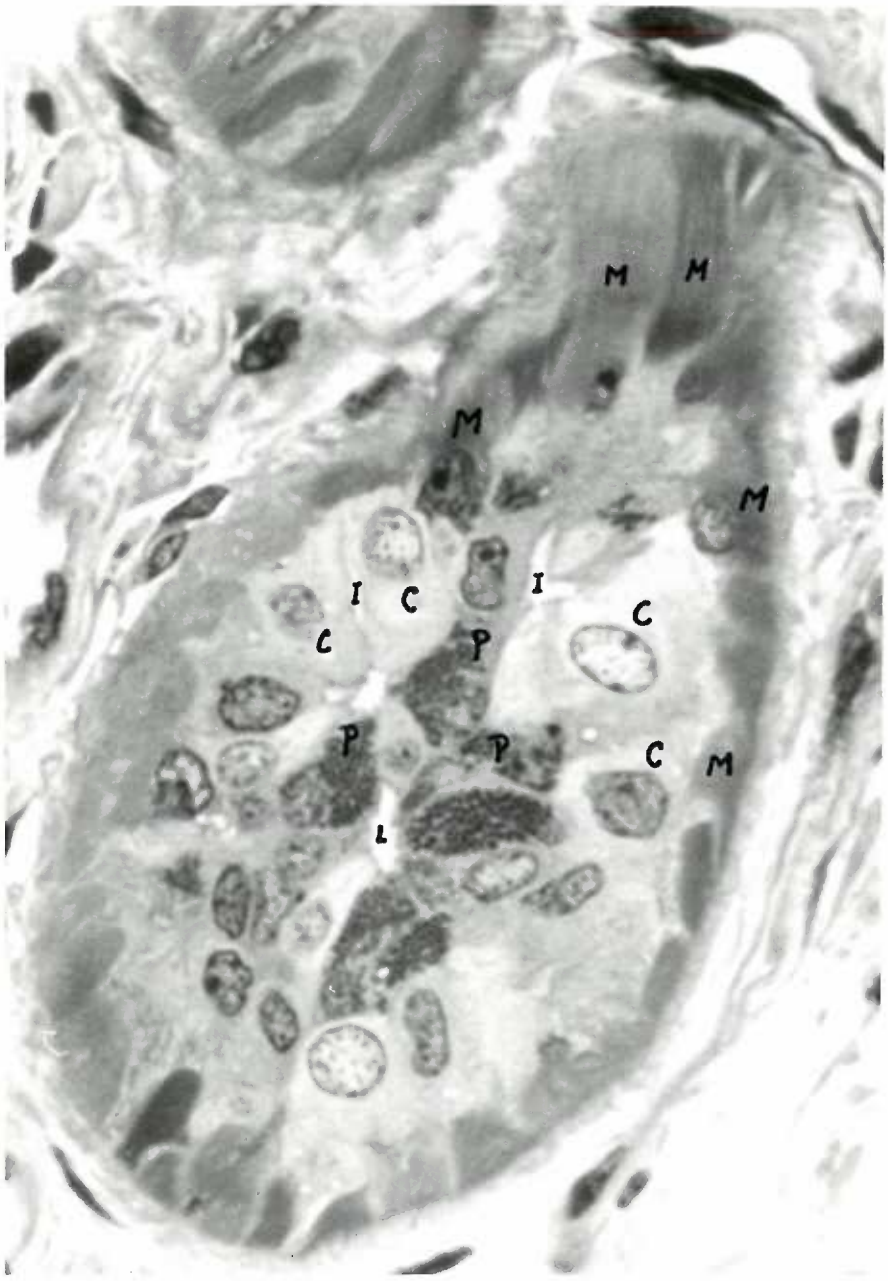


Fig. 8

Fig. 8

Secretory coil of an eccrine sweat gland. Oblique section. Toluidine blue-borax, X 160 enlarged to X 1500.

P - dark cell

C - clear cell

M - myoepithelial cell

L - lumen

I - intercellular canaliculus

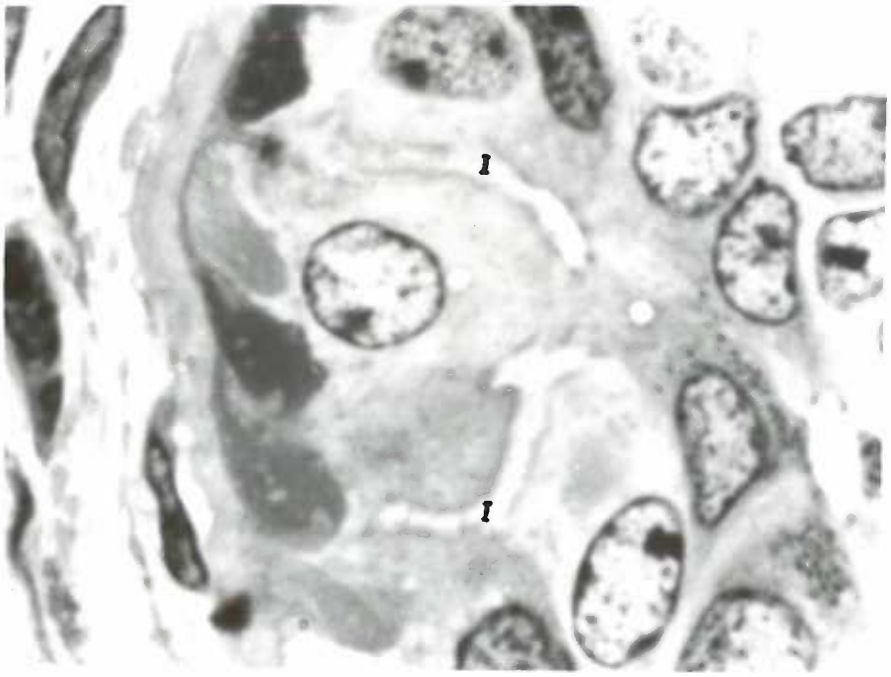


Fig. 9

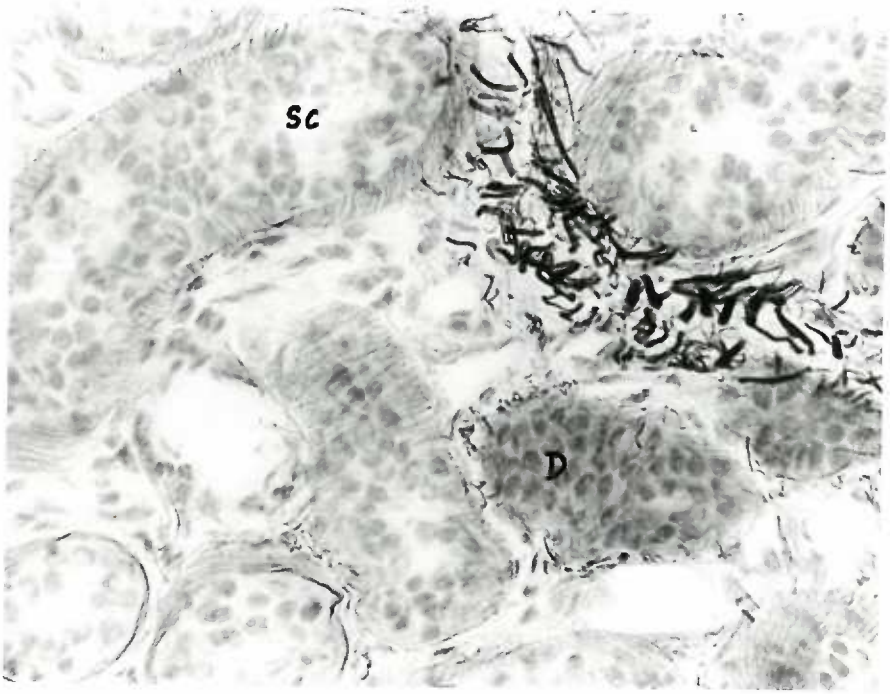


Fig. 10

Fig. 9

Intercellular caniliculi (I). Microvilli are barely visible in the lower canaliculus. Toluidine blue -borax, X 2250.

Fig. 10

Elastic fibers. The fibers around the secretory coil (sc) are more regularly oriented and much finer than those around the duct (D). Roman's AOV, X 100 enlarged to X 350.

III. The Transition Zone.

Where it connects to the coiled duct, the secretory coil is constricted. There are no dark cells in this region; myoepithelial cells, clear cells, and the prominent basement membrane are still present. The surrounding connective tissue still contains the fine, oriented elastic fibers that surround the rest of the secretory coil, but a few thicker, unoriented fibers are also present just outside these.

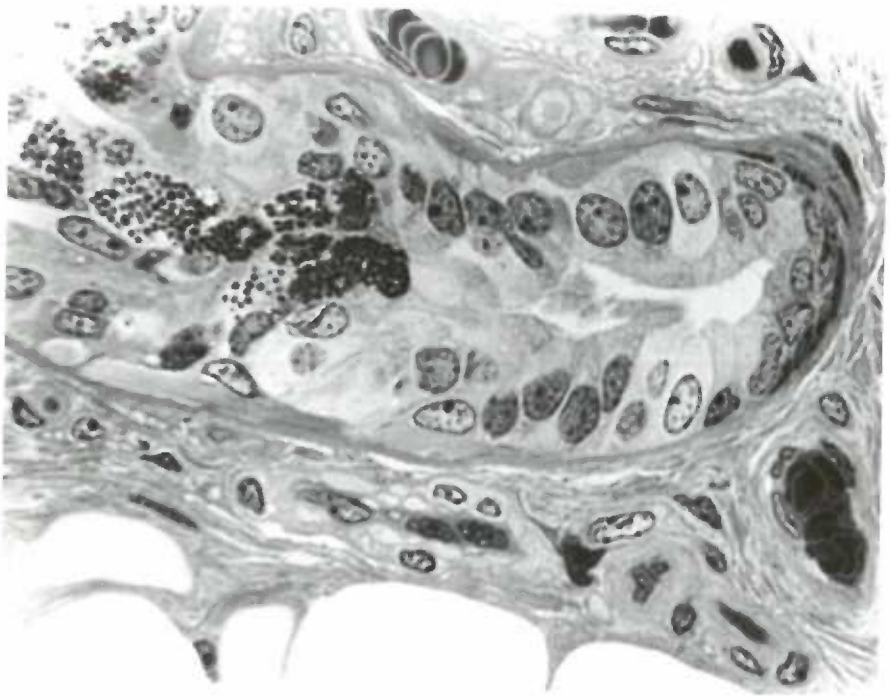


Fig. 11

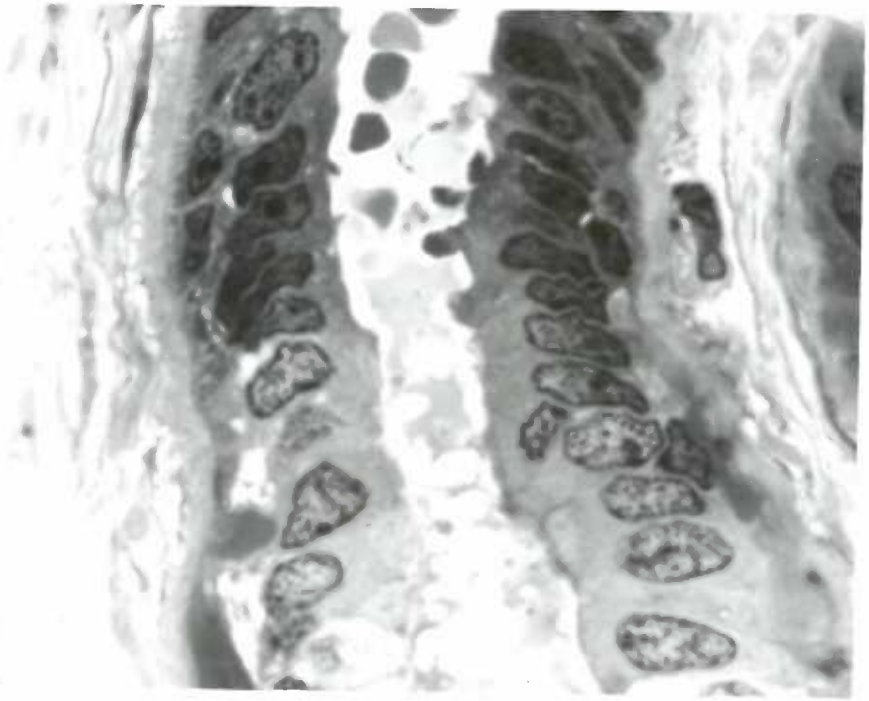


Fig. 12

The transition zone.

Fig. 11

The secretory coil is at the left; the transition zone, at right; the beginning of the coiled duct, at extreme right. Toluidine blue-borax, X 200 enlarged to X 700.

Fig. 12

Longitudinal section of the transition zone. The beginning of the coiled duct is at the top of the picture. Toluidine blue-borax, X 2550.

IV. The Duct.

Slightly more than half the length of the coiled portion of the sweat gland is coiled duct. In the one specimen that could be measured, the coiled duct was 3 mm long. The coiled duct is considerably more narrow than the secretory coil, having a diameter of $35 \pm 10\mu$ (10 measurements in each of 5 animals).

The lumen appears as either an ellipse or a triskelion (three-pointed star) from 0 to 11μ across (araldite sections). (A lumen width of 0 μ corresponds to the situation of a clamped hose or closed semilunar valve).

The basement membrane of the duct is very thin. Indeed, I trust my own observation of its existence only because Ellis and Montagna's (1961) electron micrographs confirm it. Around the collagen envelope of the coiled duct are fewer and thicker elastic fibers than are found in the envelope of the secretory coil. These elastic fibers can run in any direction, but most are either parallel or perpendicular to the axis of the coiled duct.

The duct has two layers of cells and some cells between the layers. The cells of both layers have a finely granular cytoplasm and irregular nuclei, with many small flecks of chromatin and a prominent nucleolus. The luminal, or "cuticular," border of the inner cells of the duct is denser than the rest of their cytoplasm and is agranular (in the light microscope). Unlike the nuclei and the rest of the cytoplasm, the cuticular border shows no trace of

metachromasia when stained with toluidine blue. In the coiled duct, the cuticular border occupies only a quarter of each inner cell.

The straight duct is long enough to reach from the coiled portion of the gland to a rete ridge of the epidermis with a few undulations. Since the glands do not lie at a uniform depth, this distance varies from 1-1/2 to 3 mm (paraffin sections).

The cells of the straight duct differ from those of the coiled duct only in being smaller; their nuclei and the cuticular border are, however, the same (absolute) size. In the dermis the straight duct has a diameter of 27 $\pm$ 3 μ (10 measurements in each of 3 animals). Longitudinal fibers predominate among the elastic fibers in the connective tissue around the straight duct.

On entering the epidermis, the duct loses its basement membrane and connective tissue sheath.

The epidermal cells next to the duct are organized around it and, like the duct cells, are flattened perpendicular to the axis of the duct. These epidermal cells still have the spaces and bridges between them that characterize the stratum spinosum.

The cells of the intraepidermal duct have no apparent spaces between them (at the highest power of the light microscope). The outer cells are flatter than the inner. The cuticular border takes up most of the cytoplasm of the inner cells. Except for the cuticular border, the cytoplasm of the duct cells appears less dense, more granular, and more metachromatic than that of the other cells

of the stratum Malpighii.

Where the duct has traversed 2/3 of its course through the stratum Malpighii, keratohyalin granules begin to appear in its cells. The granules increase in size until, at the level of the stratum granulosum, they occupy all but the cuticular border. Keratohyalin granules never appear in the cuticular border.

The granules disappear in the stratum lucidum, which begins 1-1/2 cells deeper around the eccrine sweat ducts than it does elsewhere. The erstwhile outer cells of the duct are indistinguishable from the other cells of the stratum lucidum, but in the stratum corneum they are less eosinophilic than their neighbors. The remains of the cuticular border persist through the strata lucidum and corneum, often appearing as concentric lamellae.

The ducts of palmar sweat glands always open onto the surface atop a papillary ridge.

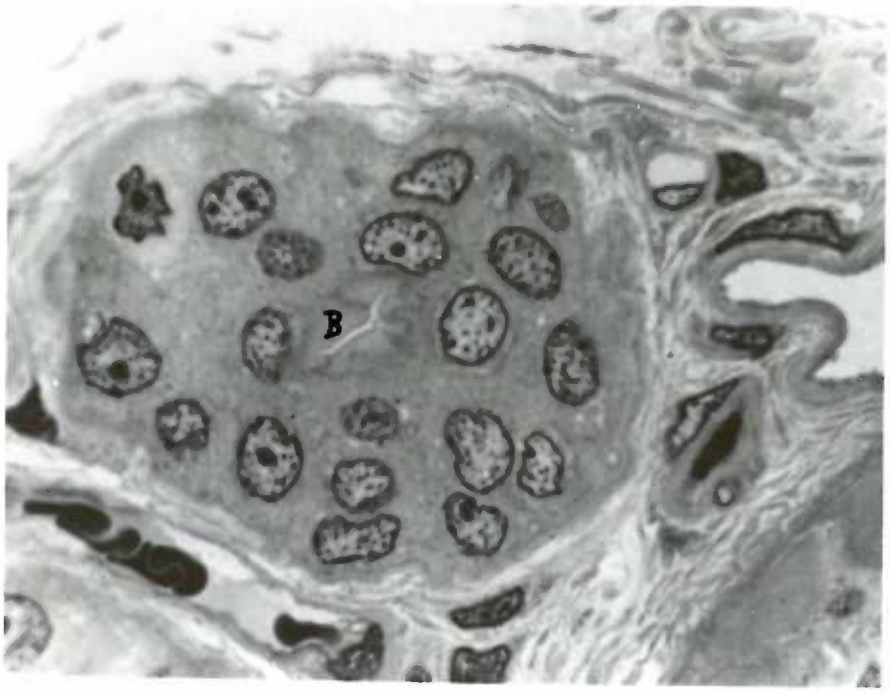


Fig. 13

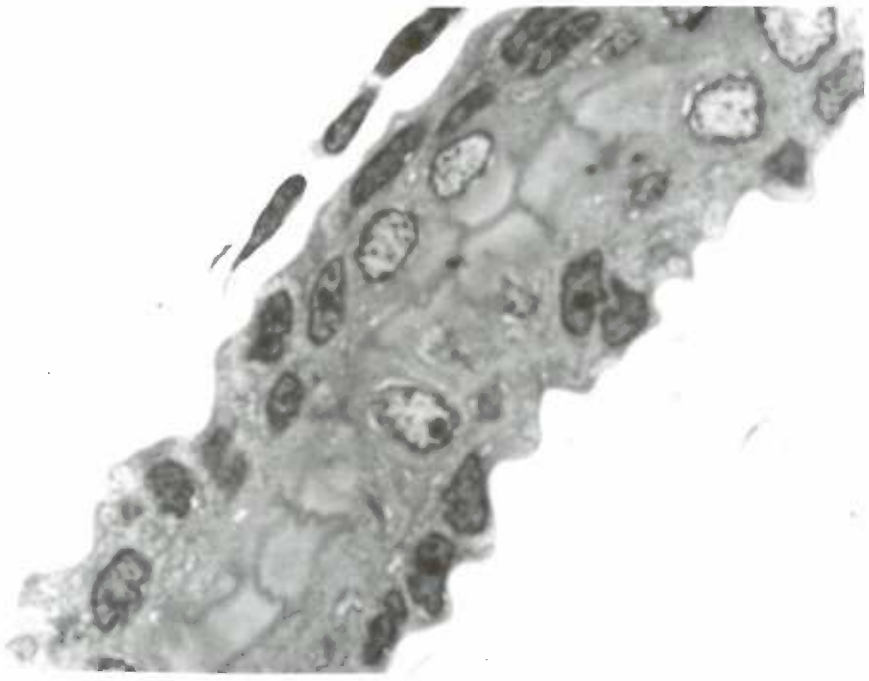


Fig. 14

Fig. 13

Coiled duct. Cross section. Toluidine blue-borax, X 400 enlarged to X 1500.

B - cuticular border

Fig. 14

Duct. Parasaggital section through the cuticular border. Toluidine blue-borax, X 1600.

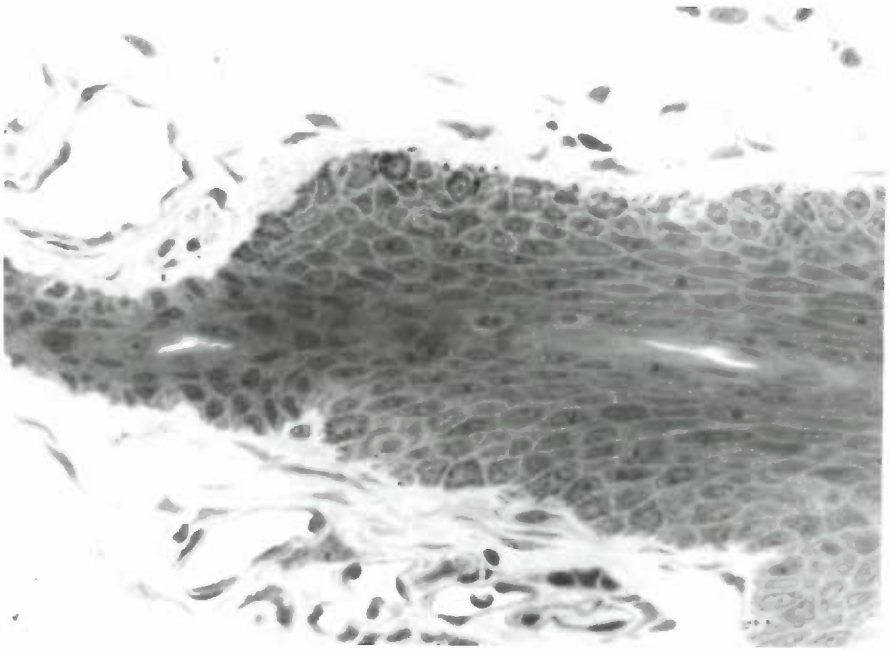


Fig. 15

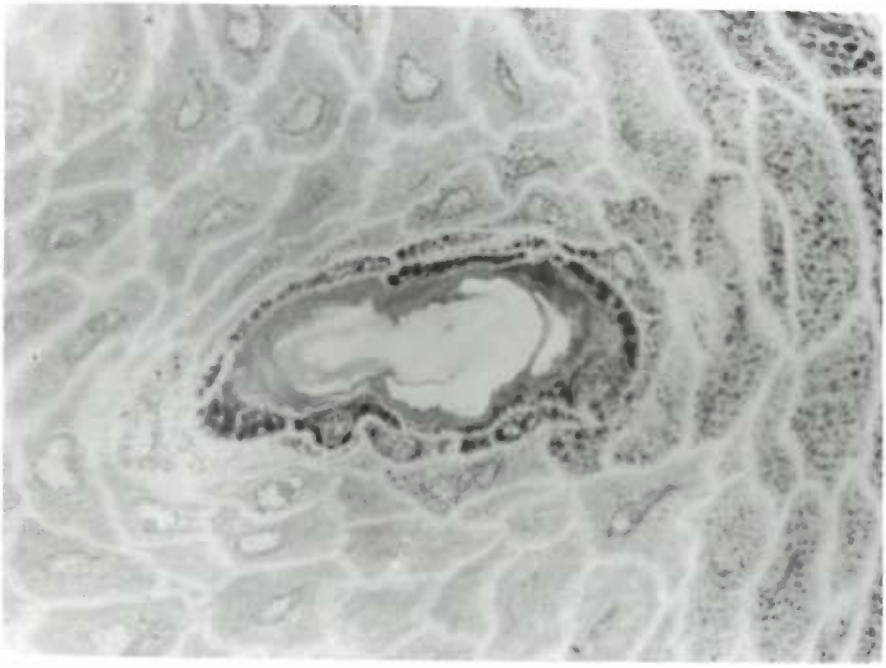


Fig. 16

Fig. 15

Straight duct entering the epidermis. Toluidine blue-borax, X 500.

Fig. 16

The duct in the upper epidermis. The stratum granulosum occupies the right third of the picture; the (deeper) stratum spinosum, the left two-thirds. Note the keratohyalin granules in the duct cells at the level of the stratum spinosum. H+E, X 1000.

V. Blood Supply.

The circulatory pattern of the eccrine sweat glands varies. Usually the coiled portion of a gland depends, for its blood supply, on two branches of a single arteriole, which often serves nothing but the one sweat gland. It or one of its branches may, however, send a branch toward the epidermis or to another sweat gland which it serves wholly or partially. Capillaries arise from the branches (metarterioles) and run around or along the secretory coil and coiled duct 8 to 10μ from it and 30 to 80 μ apart. A capillary usually follows the first 10 to 20 μ of the straight duct. The rest of the straight duct appears to be avascular.

Veins could not be seen since they lack the strong alkaline phosphatase activity that makes capillaries and arterioles demonstrable.

Fig. 17

Fig. 17

Blood supplies of two palmar sweat glands shown by Gomori's alkaline phosphatase reaction. Since the sweat gland also contains alkaline phosphatase, many of the capillaries are obscured. Cobalt sulfide, X 40 enlarged to X 140.

VI. Innervation.

It is generally believed that eccrine sweat glands are innervated exclusively by cholinergic fibers travelling through sympathetic pathways (Rothman, 1954; Kuno, 1956).

I have not looked for adrenergic fibers around the eccrine sweat glands of the rhesus and cannot guarantee their absence. Cholinergic fibers are abundant and show both acetylcholinesterase and non-specific cholinesterase activity. The secretory coil is surrounded by a lattice of these fibers, most of which run nearly perpendicular to the axis of the secretory coil. All the fibers lie on a single surface of revolution about the axis. The nerve fibers lie outside the network of elastica.

The rare nerve fibers around the duct show no particular orientation.

The nerves around the sweat gland are connected to the subpapillary nerve plexus, which lies beneath the epidermis.

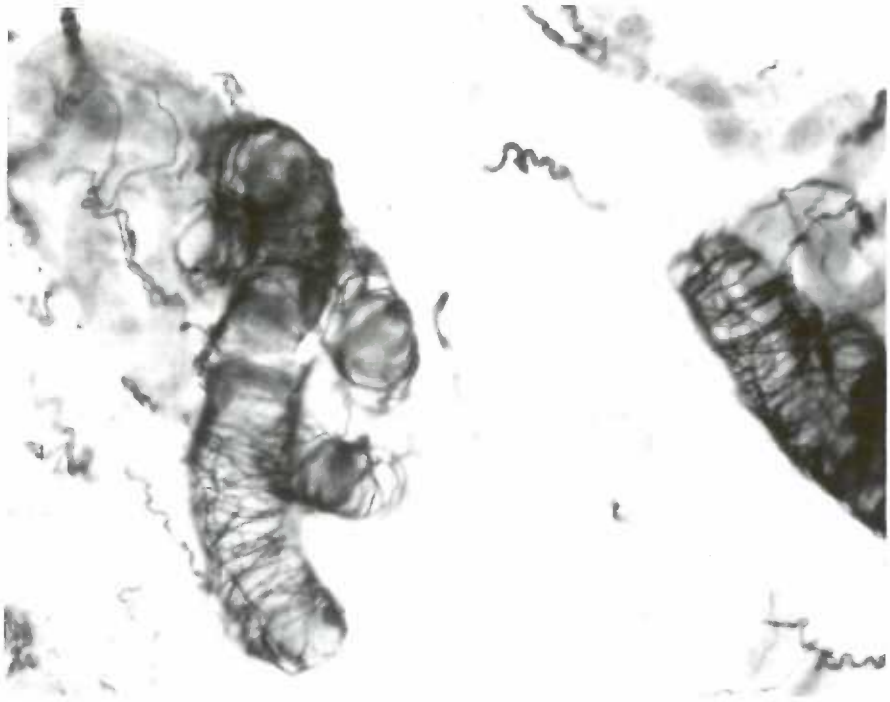


Fig. 18

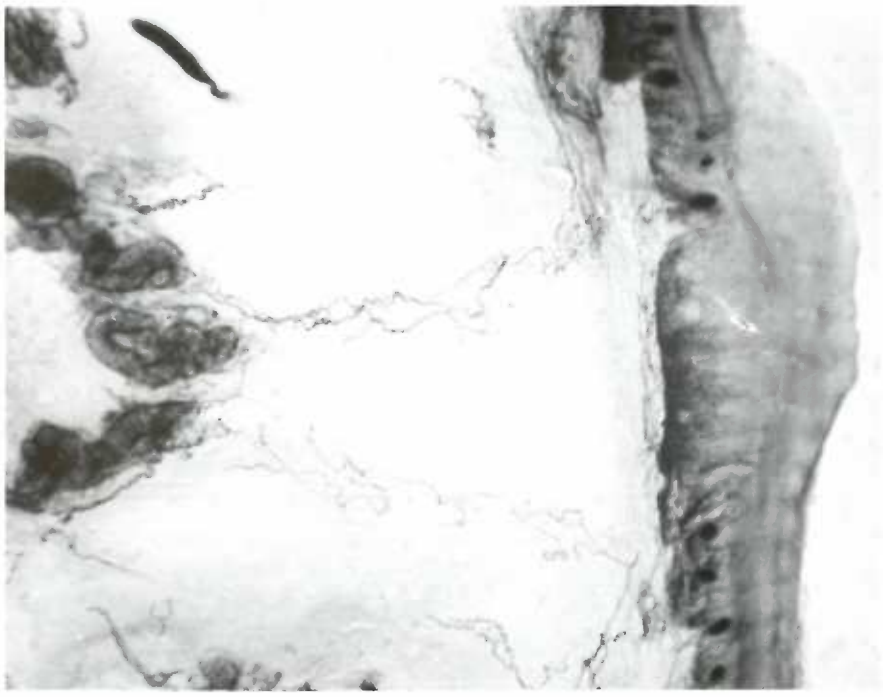


Fig. 19

Fig. 18

Innervation of palmar sweat glands. The secretory coils surrounded by lattices of nerves. Only a few nerves are associated with the coiled ducts (the pale gray structures in the upper corners of the picture). Silvered cholinesterase technique, X 165.

Fig. 19

Nerve connection to the subpapillary plexus. Copper method for acetylcholinesterase, X 10 enlarged to X 35.

VII. Enzymes.

Glycogen synthetase (uridine diphosphoglucose-glycogen glucosyltransferase) was detectable in the clear cells and in the outer cell layer of both parts of the duct and occasionally in the dark cells and the inner cell of the duct.

Branching enzyme was found in all but the myoepithelial cells.

All cell types contained phosphorylase, activity, which was strongest in the clear cells and in the duct.

Phosphoglucomutase activity was weak in the myoepithelial cells and moderate to strong elsewhere.

Fructoaldolase activity was strong in both clear and dark cells, weak in the myoepithelial cells, very strong in the coiled duct and moderate in the straight duct.

Glucose-6-phosphate dehydrogenase activity was strong in the clear cells and the outer cells of the coiled duct, moderate in the straight duct and the inner cells of the coiled duct and weak in the dark cells. I occasionally found glucose-6-phosphate dehydrogenase activity in the myoepithelial cells.

I always found 6-phosphogluconate dehydrogenase activity in the clear and dark cells and in the coiled duct. 6-phosphogluconate dehydrogenase activity was usually found in the straight duct and rarely detectable in the myoepithelial cells.

TPN-linked isocitrate dehydrogenase activity was strong in the coiled duct, moderate in the straight duct and the clear cells, and

weak in the dark cells and myoepithelial cells.

Succinate dehydrogenase activity was very strong in the coiled duct, strong in the straight duct and in the clear cells, and weak in the dark cells and myoepithelial cells.

Cytochrome oxidase activity was very strong in the coiled duct, strong in the straight duct and in the clear cells, moderate in the dark cells, and weak in the myoepithelial cells. Exogenous cytochrome c was necessary to produce a strong histochemical reaction.

All of the above enzymes were studied in 9 or more animals. All of the enzymes below were observed in 2 or more animals.

The dark cells contained acid phosphatase.

Alkaline phosphatase activity was confined to the clear cells and was strongest at their interfaces with the myoepithelial cells and along the intercellular canaliculi.

"Adenosine triphosphatase" activity was concentrated in the clear cells and in the outer cells of the coiled duct.

With α-naphthyl acetate as the substrate, non-specific carboxylic esterase activity was strong in the dark cells, moderate in the coiled duct and in the clear cells and weak in the straight duct.

Using l-leucyl-β-napthylamide as a substrate, aminopeptidase activity appeared strong in the clear cells, moderate in the dark cells and coiled duct, weak in the straight duct, and absent from the myoepithelial cells.

The activities of all enzymes found in the coiled duct varied

along its length. In the two cases (a slide of phosphorylase and a slide of 6-phosphogluconate dehydrogenase) where a sufficient length of duct could be followed, the point of maximal activity was near the junction with the secretory coil.

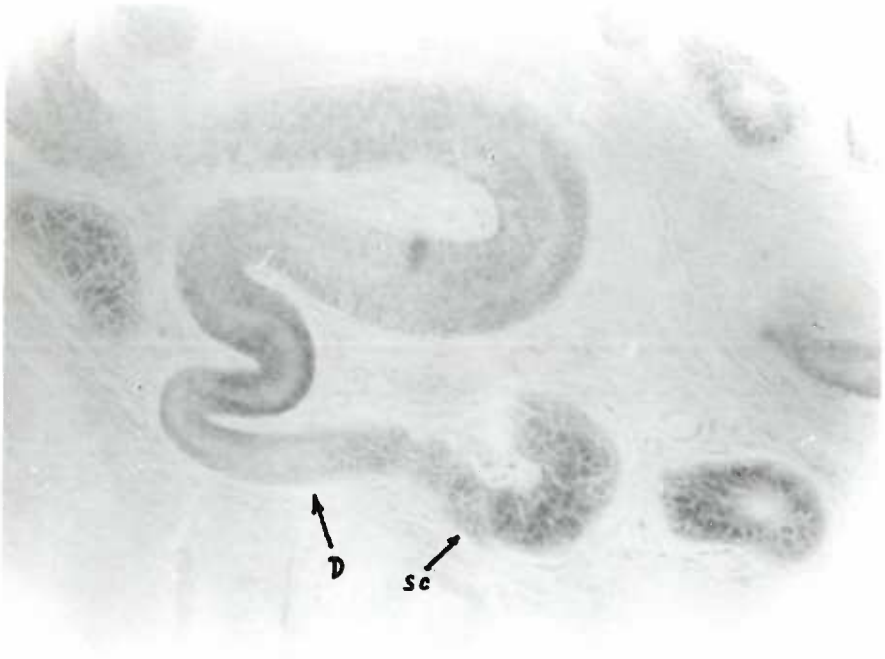


Fig. 20

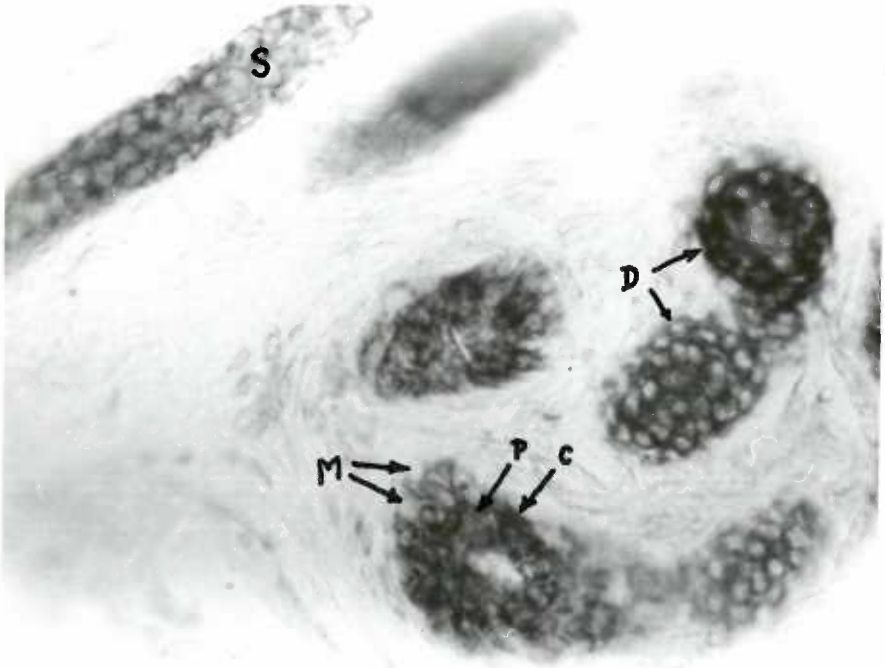


Fig. 21

Fig. 20

Weak 6-phosphogluconate dehydrogenase activity in the clear cells and coiled duct. Note the variations in activity along the length of the duct. Nitro-BT, X 64 enlarged to X 225.

D - coiled duct

sc - secretory coil

Fig. 21

TPN-linked isocitrate dehydrogenase activity. Nitro-BT, X 100 enlarged to X 350.

C - clear cells

P - dark cells

M - myoepithelial cells

D - coiled duct

S - straight duct

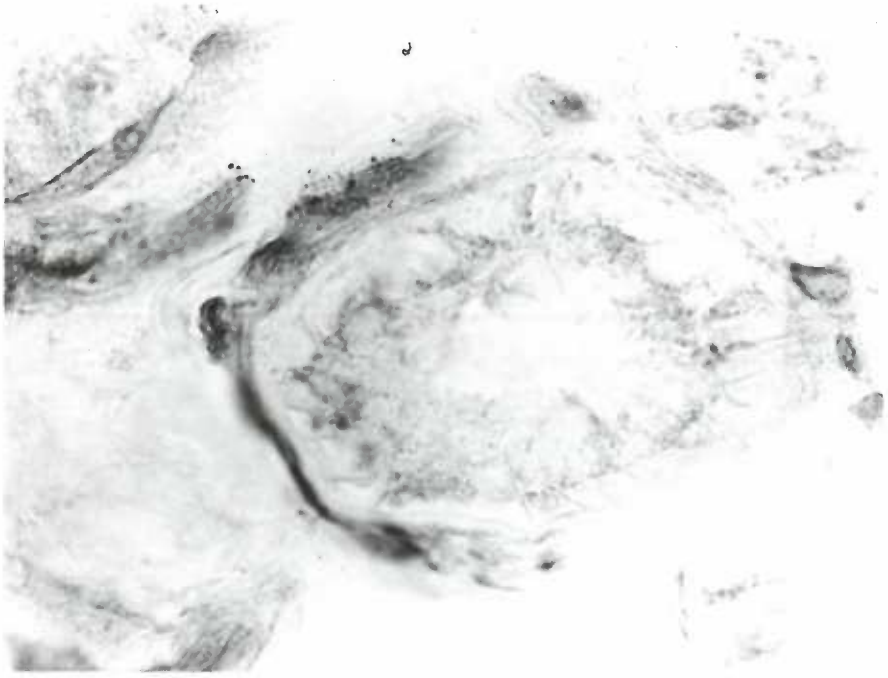


Fig. 22

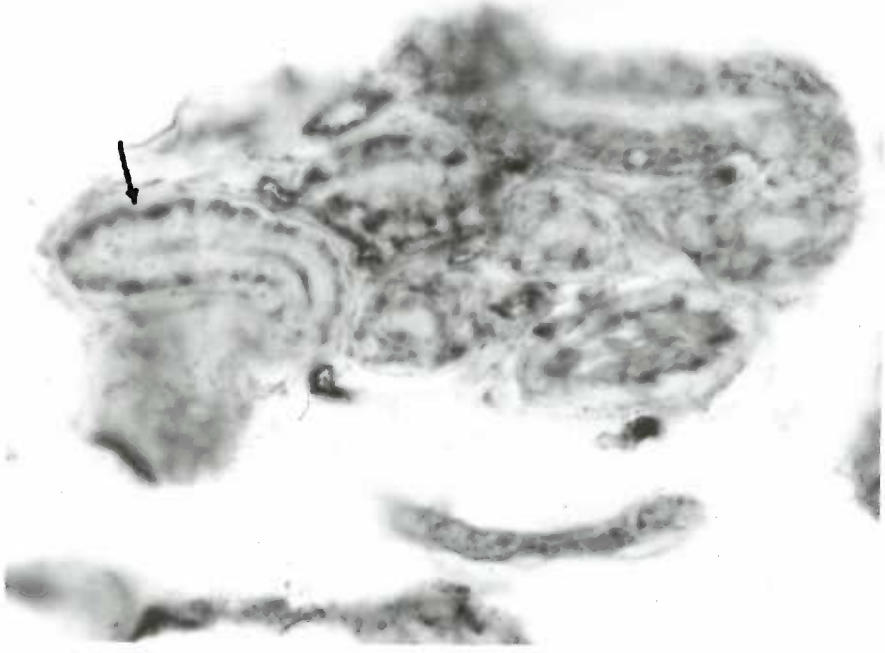


Fig. 23

Fig. 22

Adenosine triphosphatase activity in the secretory coil. In this 4 μ section activity is barely visible in the clear cells, but not in the other cells. The strongest reaction is in the capillaries around the coil. Lead sulfide, X 200 enlarged to X 800.

Fig. 23

Adenosine triphosphatase activity. Note the strong reaction in the outer cells of one section of the coiled duct (arrow). 10 μ section, lead sulfide, X 250 enlarged to X 400.

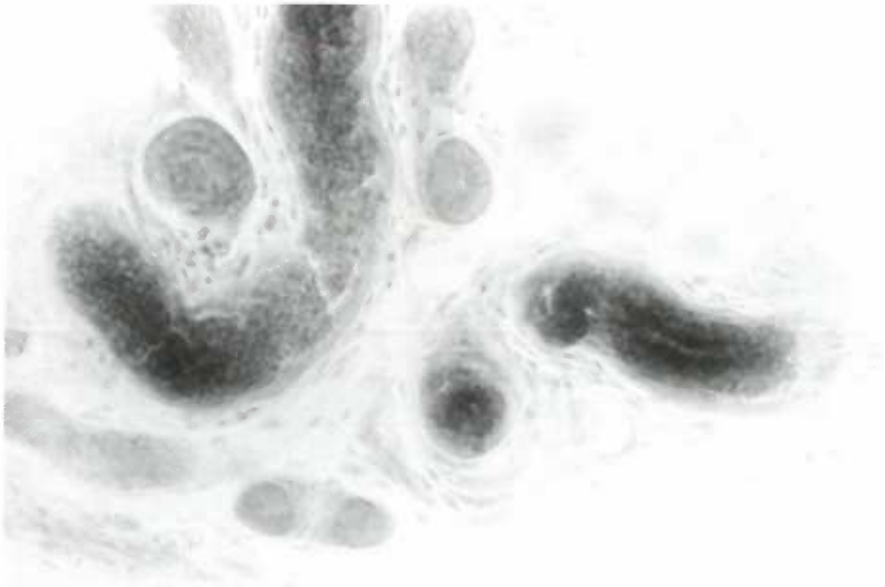


Fig. 24

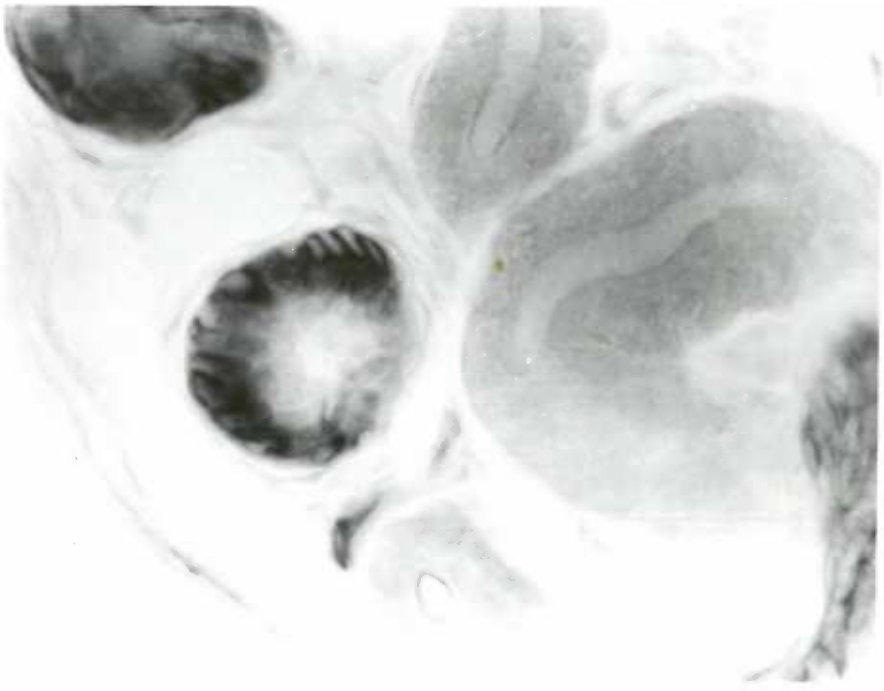


Fig. 25

Fig. 24

Acid phosphatase. The strong staining toward the center of the secretory coil indicates localization of acid phosphatase activity in the dark cells. o-Dianisidine-bis-diazonium chloride ("naphthanil diazo blue B"), X 64 enlarged to X 225.

Fig. 25

Strong alkaline phosphatase reaction in the clear cells -- especially at their junction with the myoepithelial cells. Cobalt sulfide, X160 enlarged to X 560.

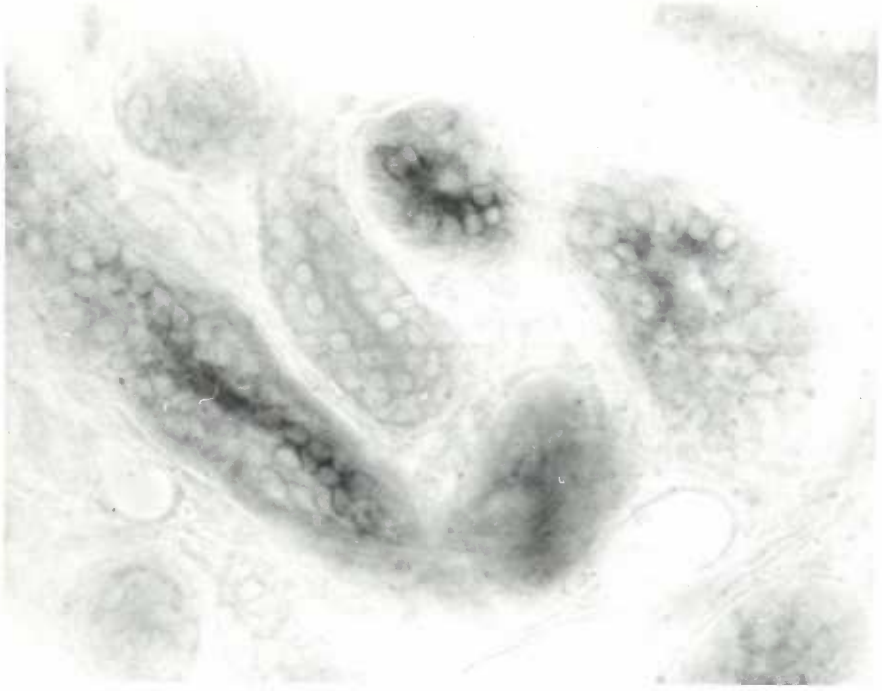


Fig. 26

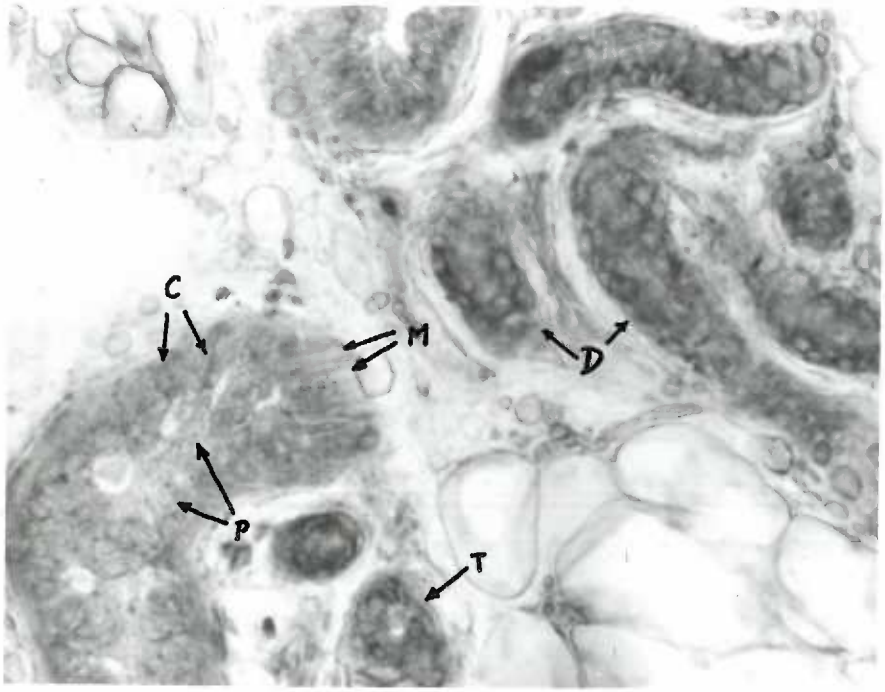


Fig. 27

Fig. 26

Nonspecific carboxylic esterase activity. The strongest reaction is in the dark cells. o-Dianisidine-bis-diazonium chloride, X 100 enlarged to X 350.

Fig. 27

Aminopeptidase activity. L-leucyl-β-naphthylamide, o-dianisidine-bis-diazonium chloride, and copper(II), X 100 enlarged to X 350.

C - clear cells

P - dark cells

M - myoepithelial cells

T - transition zone

D - coiled duct

VIII. Functional Changes

Except for the decreased glycogen content of the clear cells, I noticed no constant differences between stimulated and inhibited sweat glands in paraffin sections. I made no measurements in these specimens.

In araldite sections from 1 animal there were no significant differences in the diameters of the secretory coil or coiled duct or their lumina between stimulated and inhibited glands (at the 0.1 level; t test, 9 degrees of freedom).

There were some histochemical changes. The levels of significance (Kolmogorov test) of the changes or lack of change are listed in Tables 1 and 2. In the following description, definite statements have a statistical probability of error of 0.04 (=1/23) or less.

In the clear cells of stimulated glands, the I (active) form of glycogen synthetase decreased, both absolutely and relative to total demonstrable glycogen synthetase. The a (active) form of phosphorylase increased both absolutely and relative to total phosphorylase, which showed no change. The glycogen content of the clear cells decreased. Demonstrable phosphoglucomutase activity increased, but fructoaldolase and glucose-6-phosphate dehydrogenase activities showed no change. Nothing can be said about changes in total glycogen synthetase, succinate dehydrogenase, or cytochrome oxidase activities in the clear cells.

TABLE 1

HISTOCHEMICAL CHANGES - Secretory Coil

d = difference: + indicates stronger reactions in stimulated glands than in inhibited glands. (7 pairs of digital pads from 7 animals)

- indicates weaker reactions in stimulated glands than in inhibited glands.

0 indicates little or no difference in location of central tendency.

p = probability that difference is due to chance. Underlined probabilities are one-sided; all other probabilities are two-sided. (p = .00 is a rounding off of p .005, and p = 1.0 is a rounding off of p .995.) The text calls p .05 significant. Where a difference of 0 is listed, p is an indicator of differences in variability rather than differences in central tendency.

	Clear cells		Dark cells		Myoepithelial cells	
	d	p	d	p	d	p
Total glycogen synthetase	0	.53	0	.53	0	1.0
Glycogen synthetase I	-	.00	0	.53	0	1.0
Ratio of 1/total	-	.00	0	.99	0	1.0
Glycogen	-	.00	0	.96	0	1.0
Total phosphorylase	0	.99	0	.99	-	.53
Phosphorylase a	+	.00	+	.53	0	1.0
Ratio of a/total	+	.00	0	.99	+	.53
Phosphoglucomutase	+	.02	0	.99	0	1.0
Fructoaldolase	0	.99	+	.53	0	.99
Glucose-6-phosphate dehydrogenase	0	.99	0	1.0	-	.53
Succinate dehydrogenase	+	.53	0	.99	0	.99
Cytochrome oxidase	+	.53	0	.99	0	1.0

TABLE 2

HISTOCHEMICAL CHANGES - Duct

d = difference: + indicates stronger reactions in stimulated glands than in inhibited glands. (7 pairs of digital pads from 7 animals)

- indicates weaker reactions in stimulated glands than in inhibited glands.

0 indicates little or no difference in location of central tendency.

p = probability that difference is due to chance. Underlined probabilities are one sided; all other probabilities are two-sided. (p=.00 is a rounding off of p .005, and p=1.0 is a rounding off of p .995.) The text calls p .05 significant.

Where a difference of 0 is listed, p is an indicator of differences in variability rather than differences in central tendency.

	Coiled Duct				Straight Duct			
	outer cells		inner cells		outer cells		inner cells	
	d	p	d	p	d	p	d	p
Total glycogen synthetase	-	.11	0	.99	0	.53	+	.53
Glycogen synthetase I	-	.11	0	.99	0	.53	0	.99
Ratio of I/total	0	.53	0	.99	0	.99	0	.99
Glycogen	-	.48	0	.48	0	.48	0	.50
Total phosphorylase	0	.99	0	.53	0	1.0	0	1.0
Phosphorylase a	+	.26	0	.50	+	.50	+	.26
Ratio of a/total	+	.01	+	.06	+	.26	+	.26
Phosphoglucomutase	0	.99	0	.99	+	.53	+	.53
Fructoaldolase	-	.53	-	.53	-	.53	0	.53
Glucose-6-phosphate dehydrogenase	0	.99	0	1.0	0	.99	0	.53
Succinate dehydrogenase	0	.99	0	1.0	0	.99	0	.99
Cytochrome oxidase	0	1.0	0	1.0	0	1.0	0	1.0

Neither the dark cells nor the myoepithelial cells showed significant changes in enzyme activities or glycogen content.

The increased proportion of phosphorylase in the active form in the outer cells of the coiled duct was significant. No other significant histochemical changes were found in the coiled duct. The increase in the proportion of phosphorylase in the inner cells and the decreases in glycogen synthetases in the outer cells of the coiled duct were not significant, but did suggest that functional changes had occurred. None of the apparent histochemical differences between the straight ducts of inhibited glands and those of stimulated glands were significant.

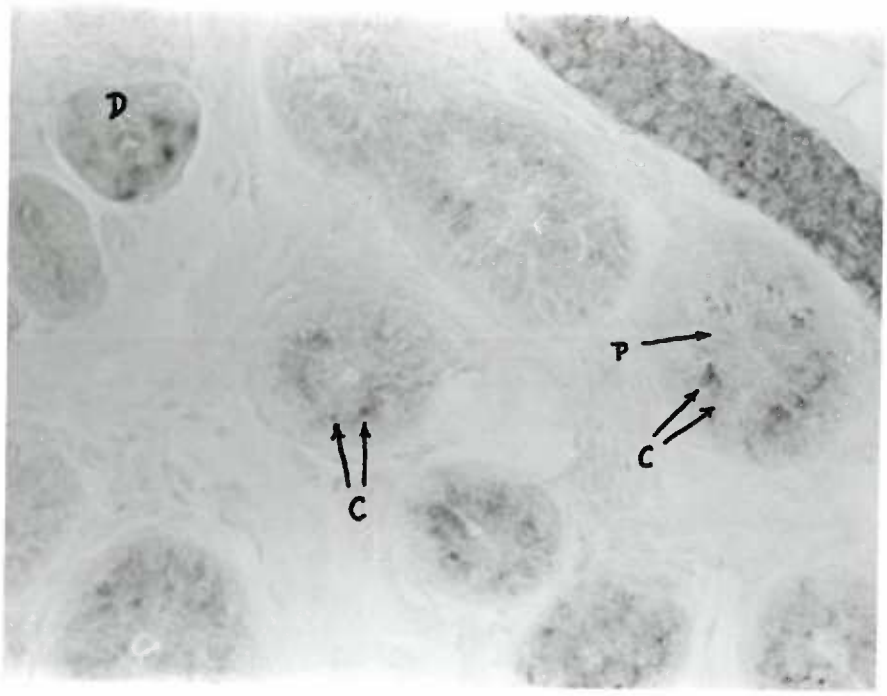


Fig. 28

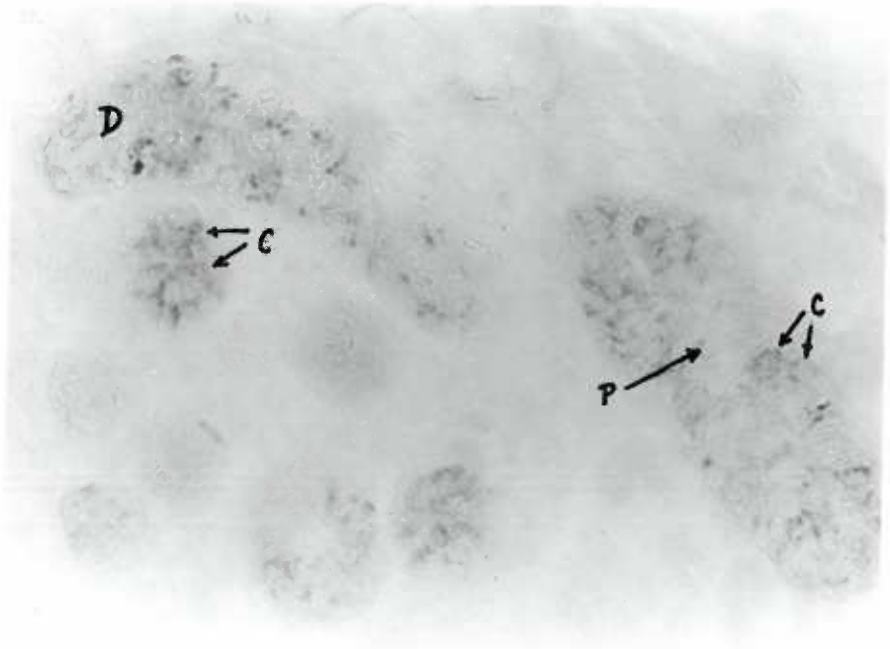


Fig. 29

Fig. 28

Total glycogen synthetase, inhibited glands.
Iodine, X 100 enlarged to X 350.

Fig. 29

Total glycogen synthetase, stimulated gland.
There is no significant difference in activity
between this and the inhibited glands (Fig. 28).
Iodine, X 100 enlarged to X 350.
C - clear cells
P - dark cell
D - coiled duct

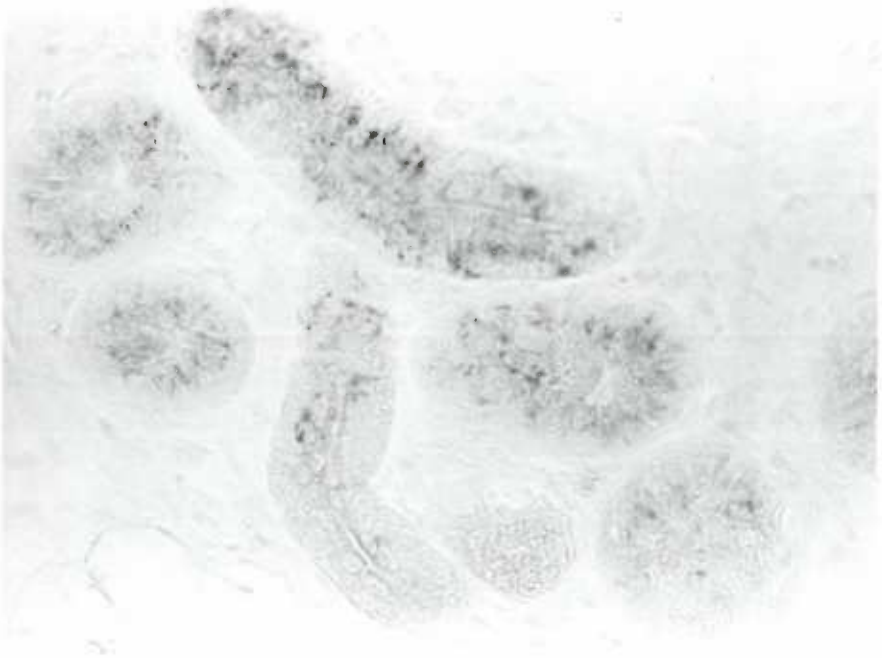


Fig. 30

Fig. 31

Fig. 30

Glycogen synthetase I, inhibited gland. This is strong for a glycogen synthetase reaction in skin. Iodine, X 100 enlarged to X 350.

Fig. 31

Glycogen synthetase I, stimulated gland. No reaction. Iodine, X 100 enlarged to X 350.

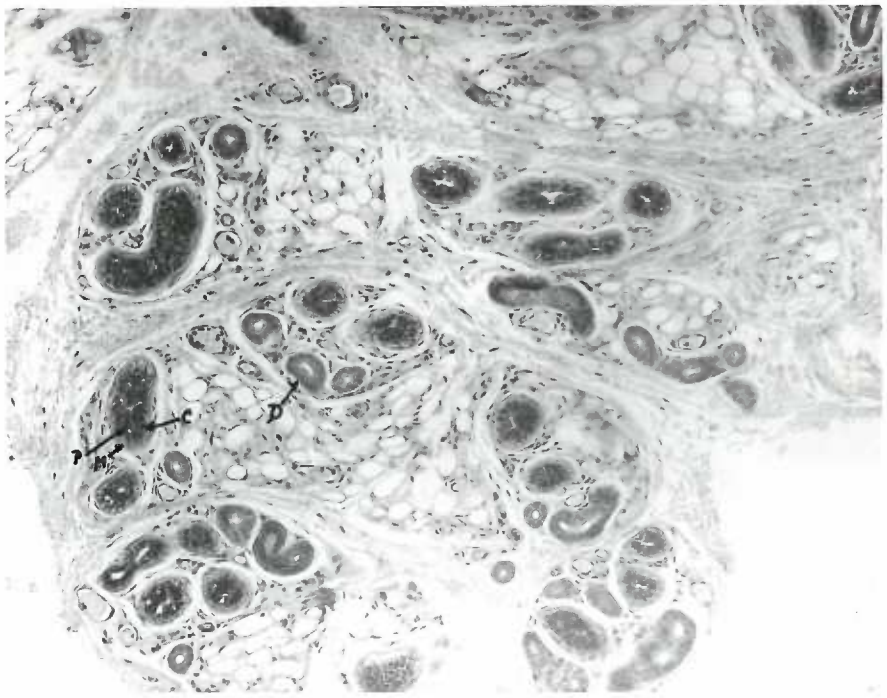


Fig. 32

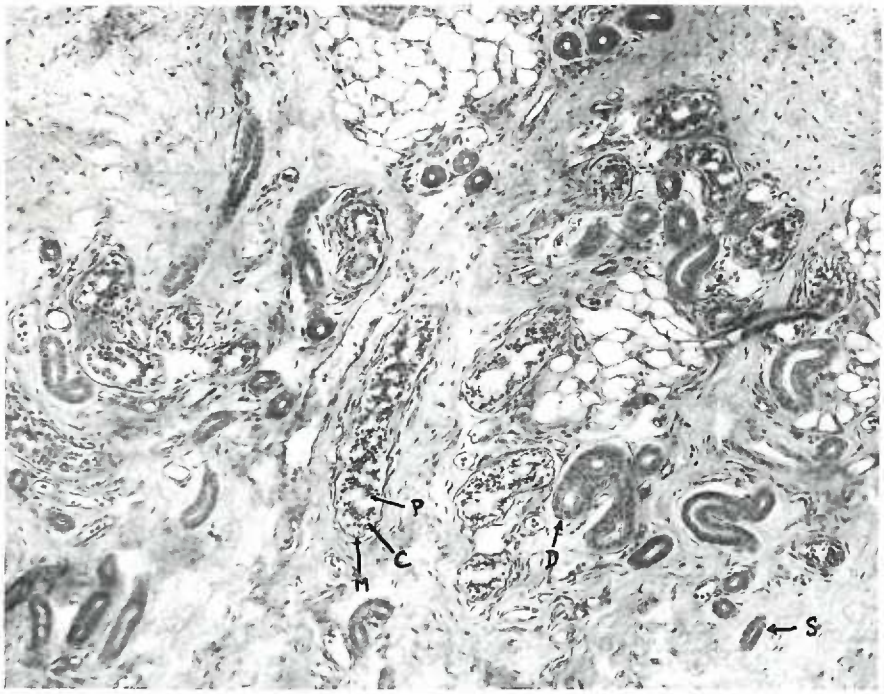


Fig. 33

Fig. 32

There is much glycogen in the clear cells and coiled ducts of the inhibited glands. Most of their dark cells contain mucin ("Schiff-positive-diastase-resistant material"). PAS-hematoxylin, X 100.

Fig. 33

The clear cells of the stimulated glands have lost their glycogen. In this specimen the coiled ducts have lost most of their glycogen and the dark cells have lost much of their mucin. (The latter two events were found in only two out of five cases.) PAS-hematoxylin, X 100.

C - clear cell

P - dark cell

M - myoepithelial cell

D - coiled duct

S - straight duct

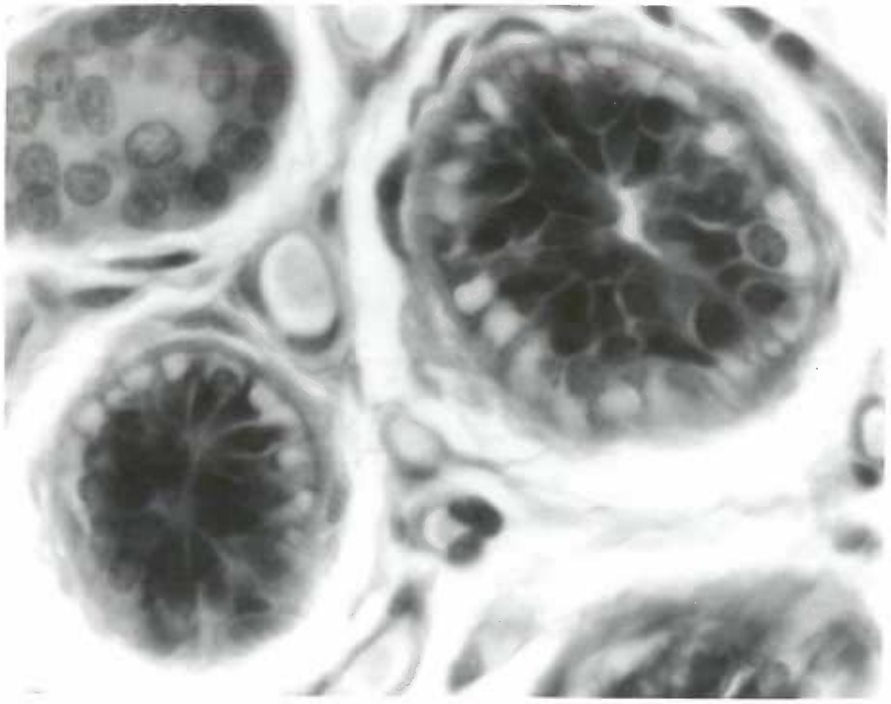


Fig. 34

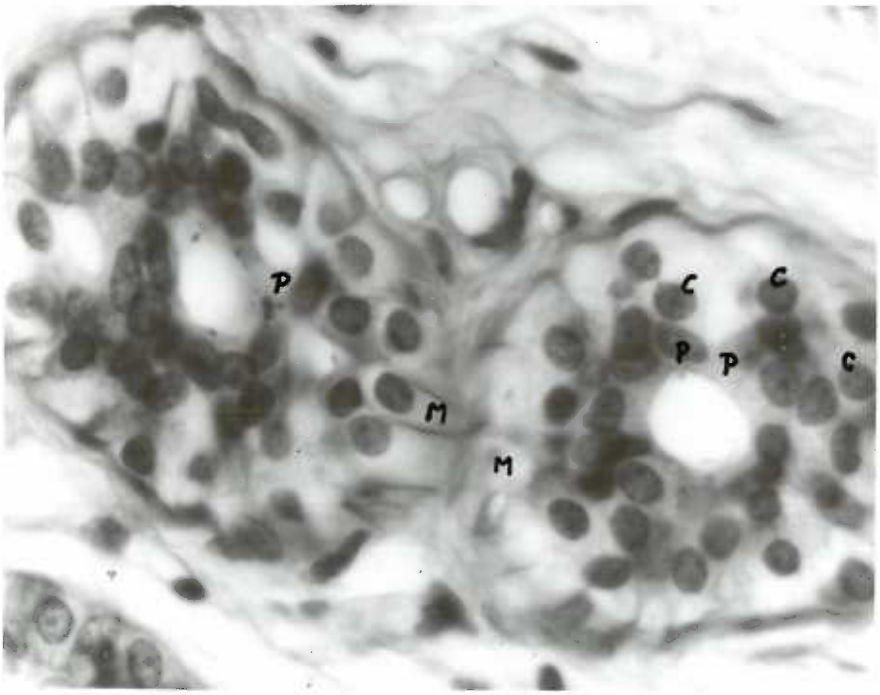


Fig. 35

Fig. 34

In the inhibited gland the clear cells contain much glycogen and the dark cells contain mucin. PAS-hematoxylin, X 1000.

Fig. 35

In the stimulated gland the clear cells contain no glycogen and only a few of the dark cells contain mucin. PAS-hematoxylin, X 1000.

C - clear cells

P - dark cells

M - myoepithelial cells

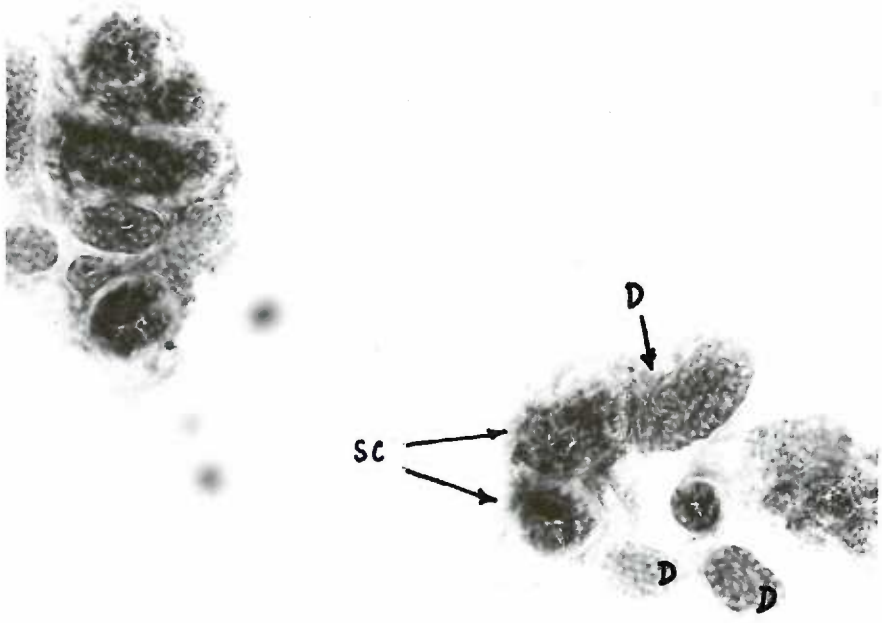


Fig. 36

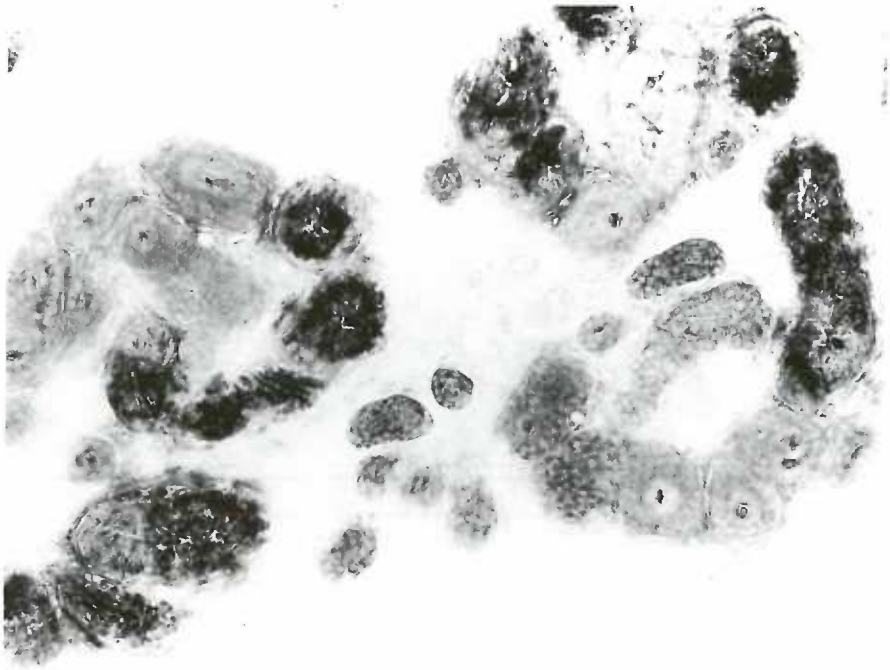


Fig. 37

Fig. 36

Total phosphorylase activity, inhibited glands. Activity is strong in the secretory coil (sc) and varies from place to place in the coiled duct (D). Method of Eränkö and Palkäma, iodine stain, X 40 enlarged to X 140.

Fig. 37

Total phosphorylase activity, stimulated glands. Activity is the same as in the inhibited glands. Method of Eränkö and Palkäma, iodine, X 40 enlarged to X 140.

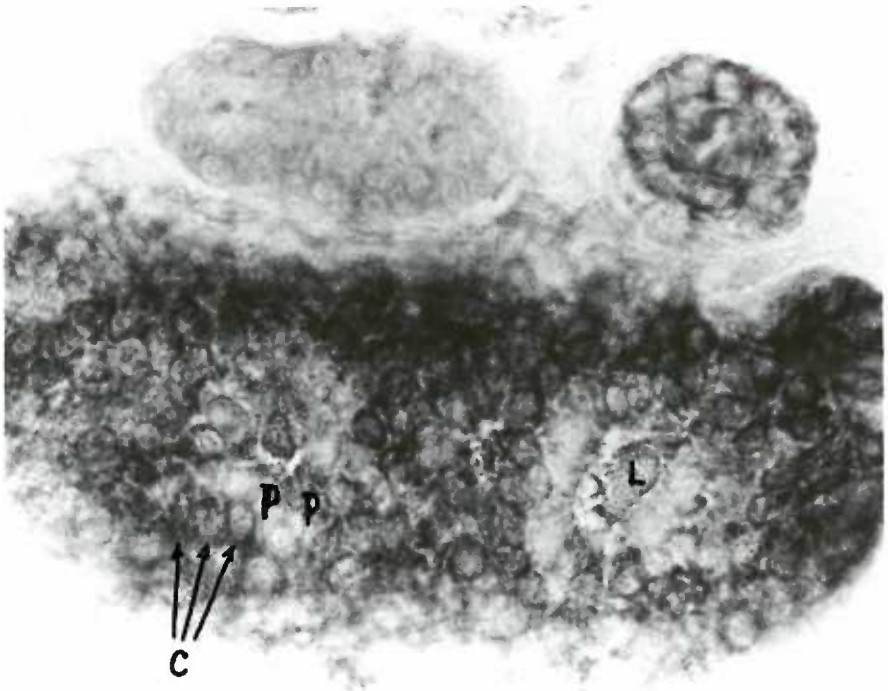


Fig. 38

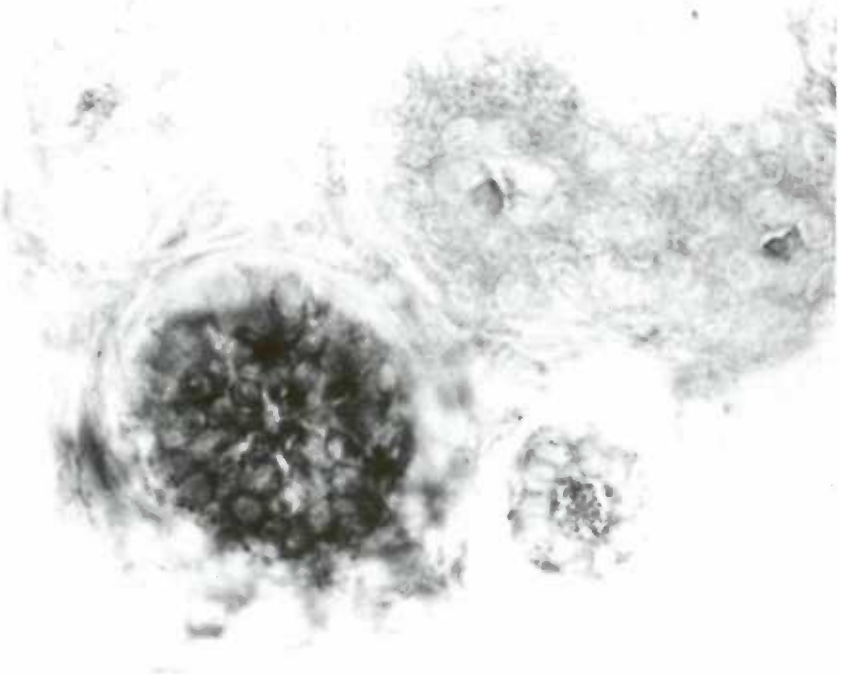


Fig. 39

Fig. 38

Total phosphorylase activity, inhibited gland. The staining in the lumen is a diffusion artifact. Method of Eränkö and Palkäma, iodine, X 160 enlarged to X 560.

C - clear cells

P - dark cells

L - lumen

Fig.39

Total phosphorylase activity in the clear cells of the stimulated gland is the same as in those of the inhibited gland (Fig. 38). The single-layered structure at the lower right is the transition zone. Method of Eränkö and Palkäma, iodine, X 560.

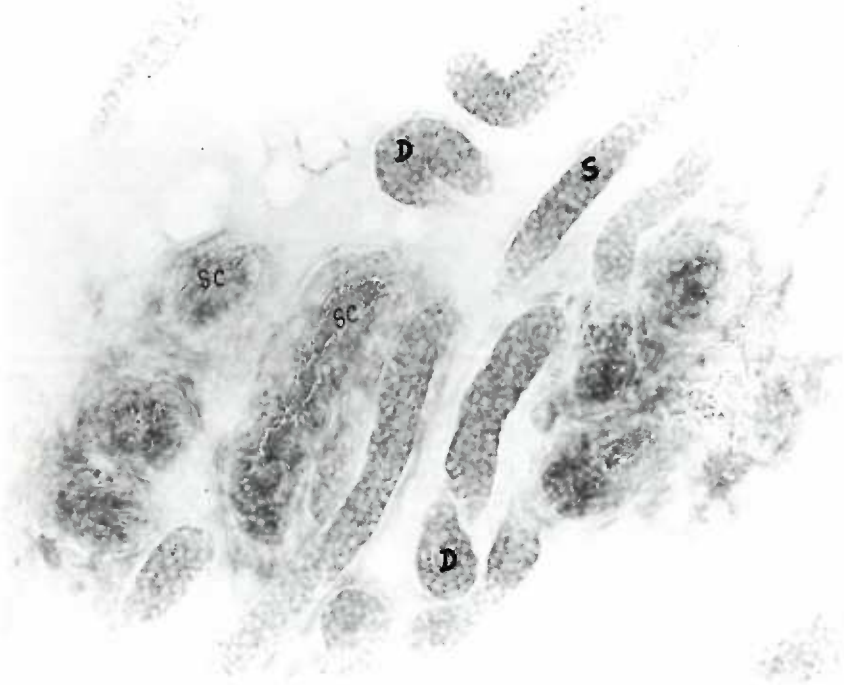


Fig. 40

Fig. 41

Fig. 40

Phosphorylase a activity, inhibited glands. Method of Eränkö and Palkäma, iodine, X 40 enlarged to X 140.

sc - secretory coil

D - coiled duct

S - straight duct

Fig. 41

Stimulated glands. Phosphorylase a activity in the secretory coil has increased greatly. Method of Eränkö and Palkäma, iodine, X 40 enlarged to X 140.

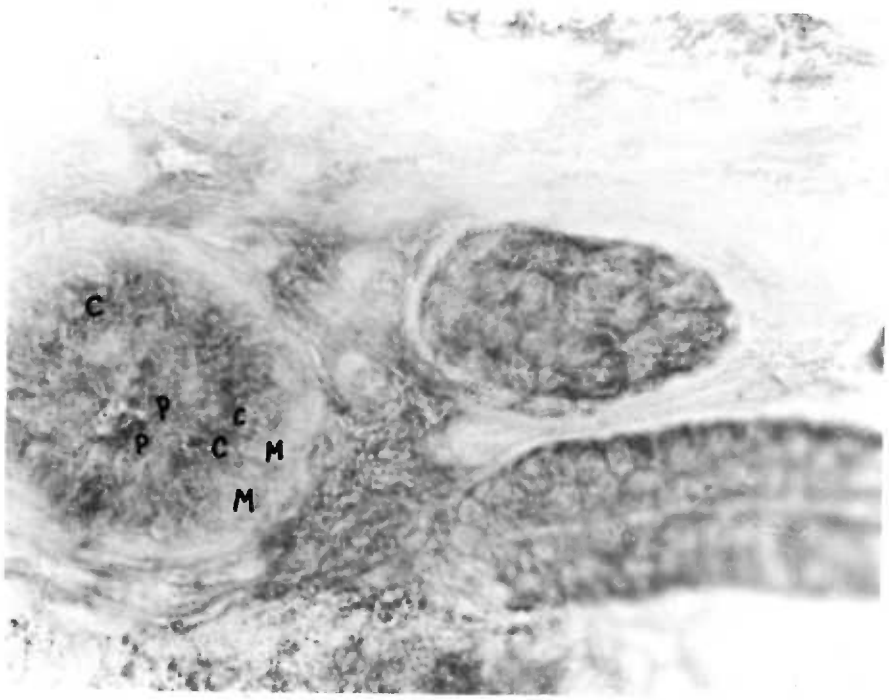


Fig. 42

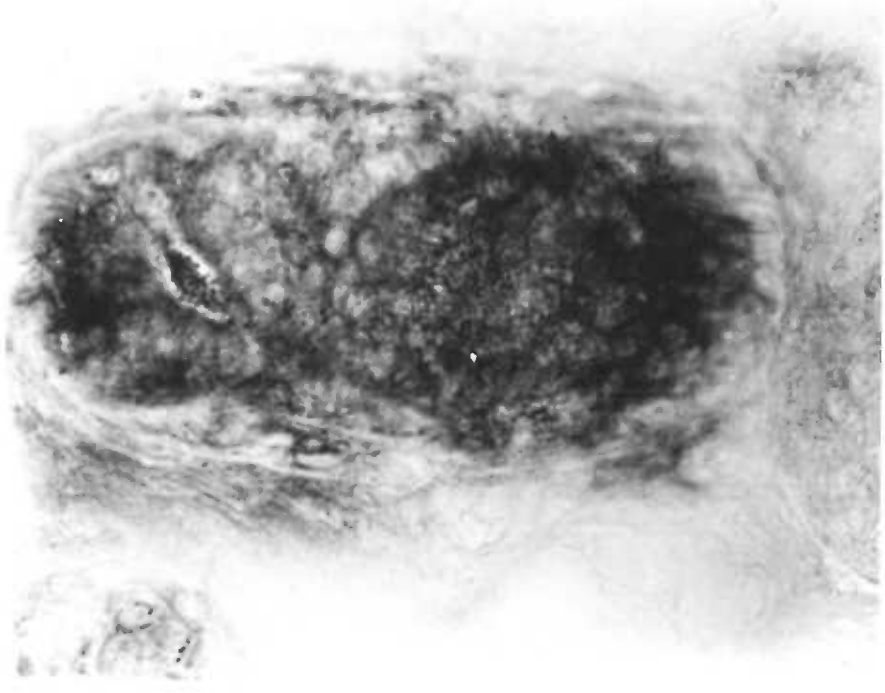


Fig. 43

Fig. 42

Phosphorylase a activity, inhibited gland. Method of Eränkö and Palkäma, iodine, X 160 enlarged to X 560.

C - clear cells

P - dark cells

M - myoepithelial cells

Fig. 43

Stimulated gland. Phosphorylase a activity has increased greatly in most of the clear cells. Method of Eränkö and Palkäma, iodine, X 160 enlarged to X 560.

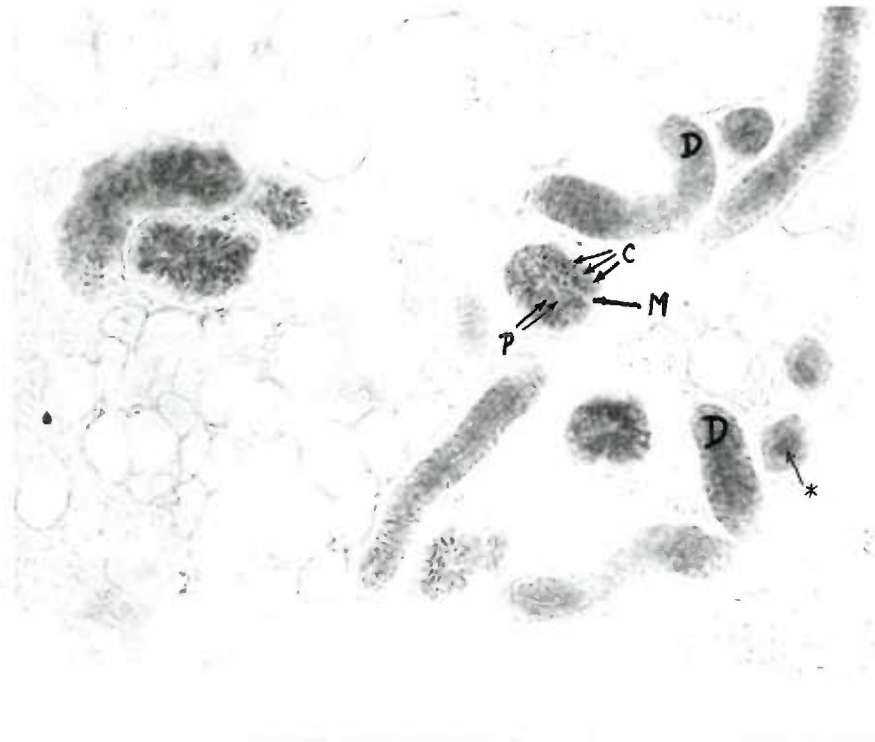


Fig. 44

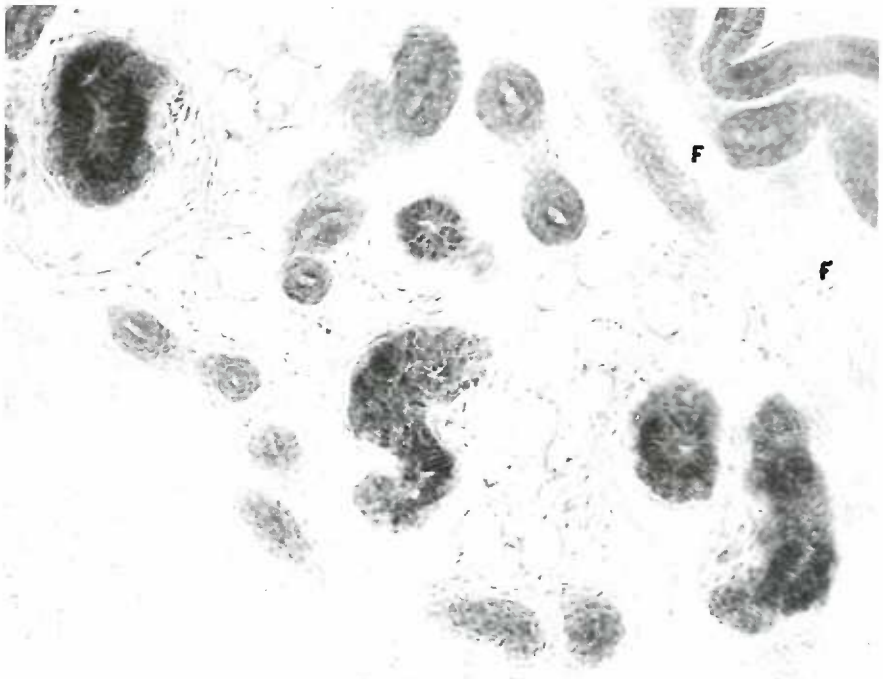


Fig. 45

Fig. 44

Phosphoglucomutase activity, inhibited glands. The strong reaction in the cuticular border of the duct (*) is probably an intracellular diffusion artifact. Nitro-BT, X 100 enlarged to X 200.

C - clear cells

P - dark cells

M - myoepithelial cells

D - coiled duct

Fig. 45

Phosphoglucomutase activity, stimulated glands. The clear cells show slightly greater activity than those of inhibited glands (Fig. 44). The refringence patterns around collagen fibers (F) are characteristic of "Diaphane"-mounted sections. Nitro-BT, X 100 enlarged to X 200.

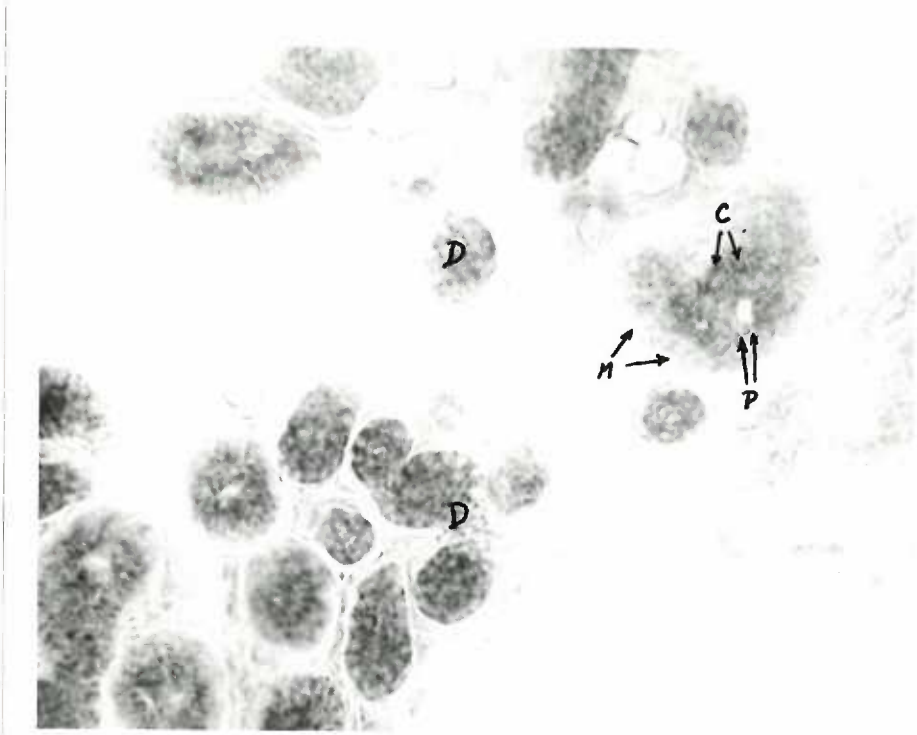


Fig. 46

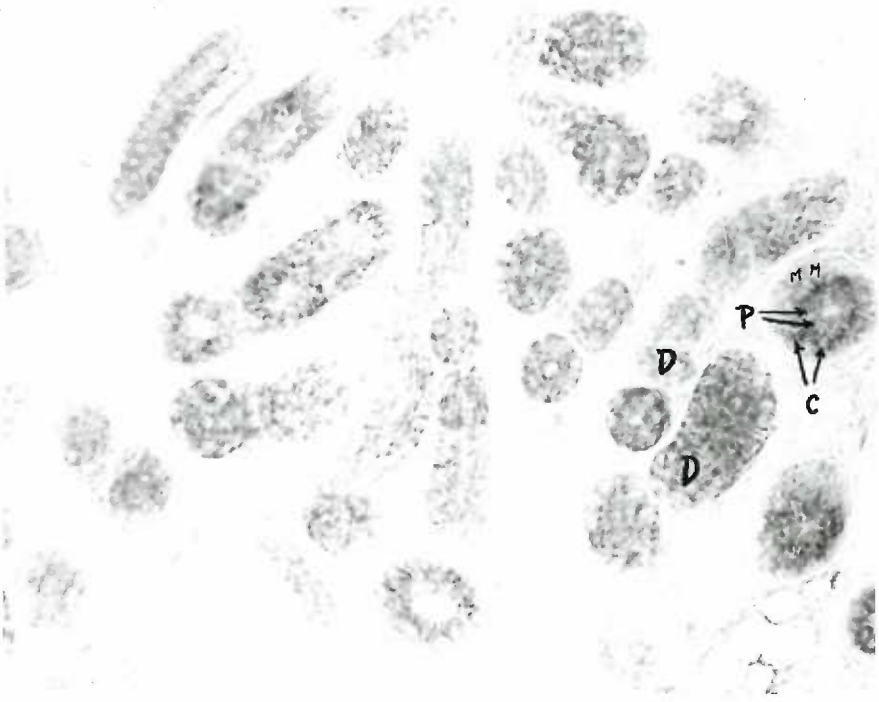


Fig. 47

Fig. 46

Fructoaldolase activity, inhibited glands.
Nitro-BT, X 100 enlarged to X 200.

C - clear cells
P - dark cells
M - myoepithelial cells
D - coiled duct

Fig. 47

Fructoaldolase activity, stimulated glands.
No difference in activity in clear cells
compared to inhibited glands (Fig. 46);
decreased activity in ducts was not repated
often enough to be significant.

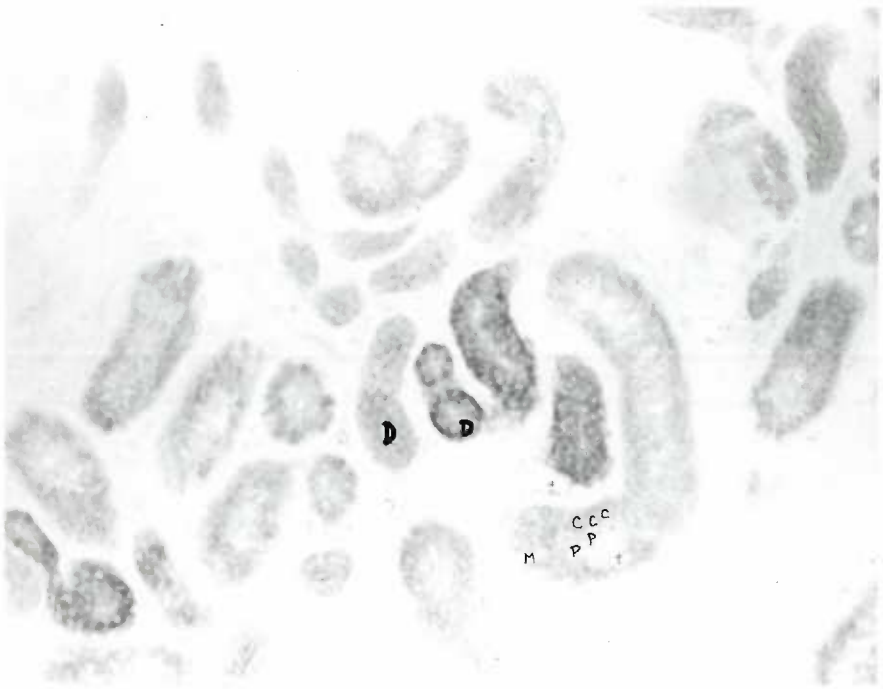


Fig. 48

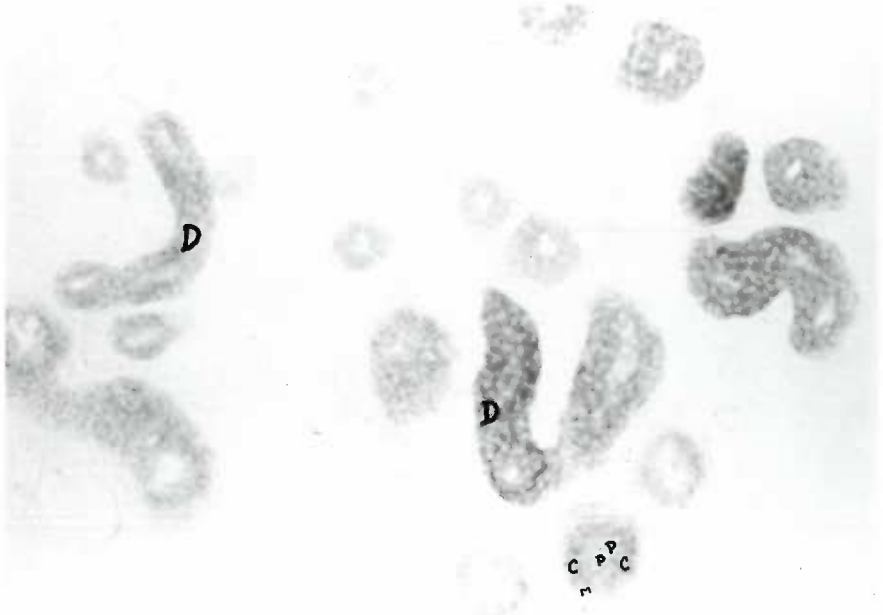


Fig. 49

Fig. 48

Glucose-6-phosphate dehydrogenase activity, inhibited glands. Nitro-BT, X 40 enlarged to X 140.

C - clear cells
P - dark cells
M - myoepithelial cells
D - coiled duct

Fig. 49

Glucose-6-phosphate dehydrogenase activity, stimulated glands. Activity is the same as in inhibited glands (Fig. 48). Nitro-BT, X 40 enlarged to X 140.

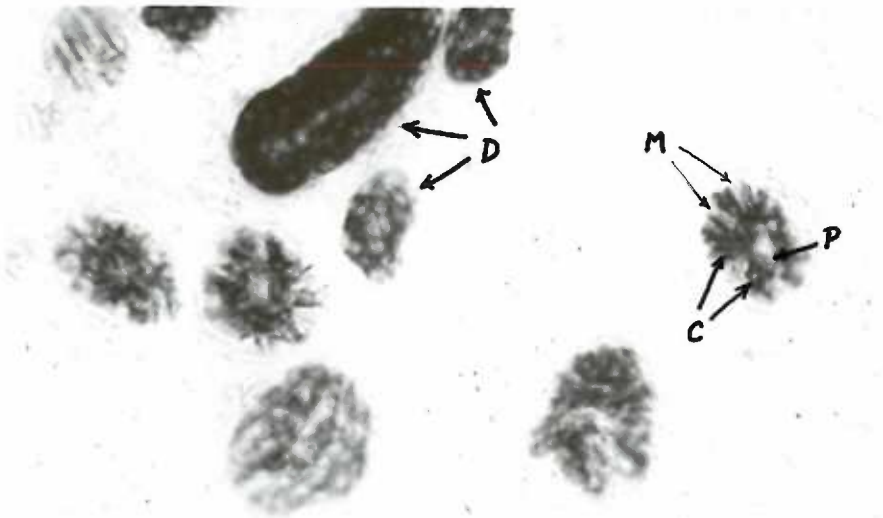


Fig. 50

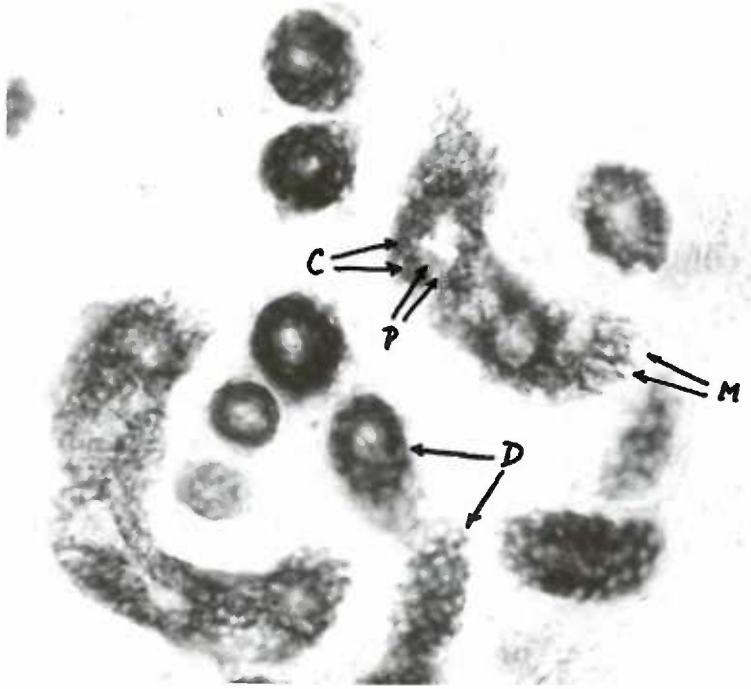


Fig. 51

Fig. 50

Succinate dehydrogenase activity, inhibited glands. Nitro-BT, X 100 enlarged to X 300.

C - clear cells
P - dark cells
M - myoepithelial cells
D - coiled duct

Fig. 51

Stimulated gland. Succinate dehydrogenase activity is the same here as in inhibited glands (Fig. 50), a typical but not invariable finding. Nitro-BT, X 100 enlarged to X 300.

Figs. 52 and 53

(overleaf)

Cytochrome oxidase activity is the same in stimulated glands (Fig. 53) as in inhibited glands (Fig. 52). The fat globules (G) are characteristic of glycerol-gel-mounted sections. N-phenyl-p-phenylenediamine and 8-amino-1,2,3,4-tetrahydroquinoline, X 100 enlarged to X 300.

C - clear cells

P - dark cells

M - myoepithelial cells

D - coiled duct

S - straight duct

G - fat globules

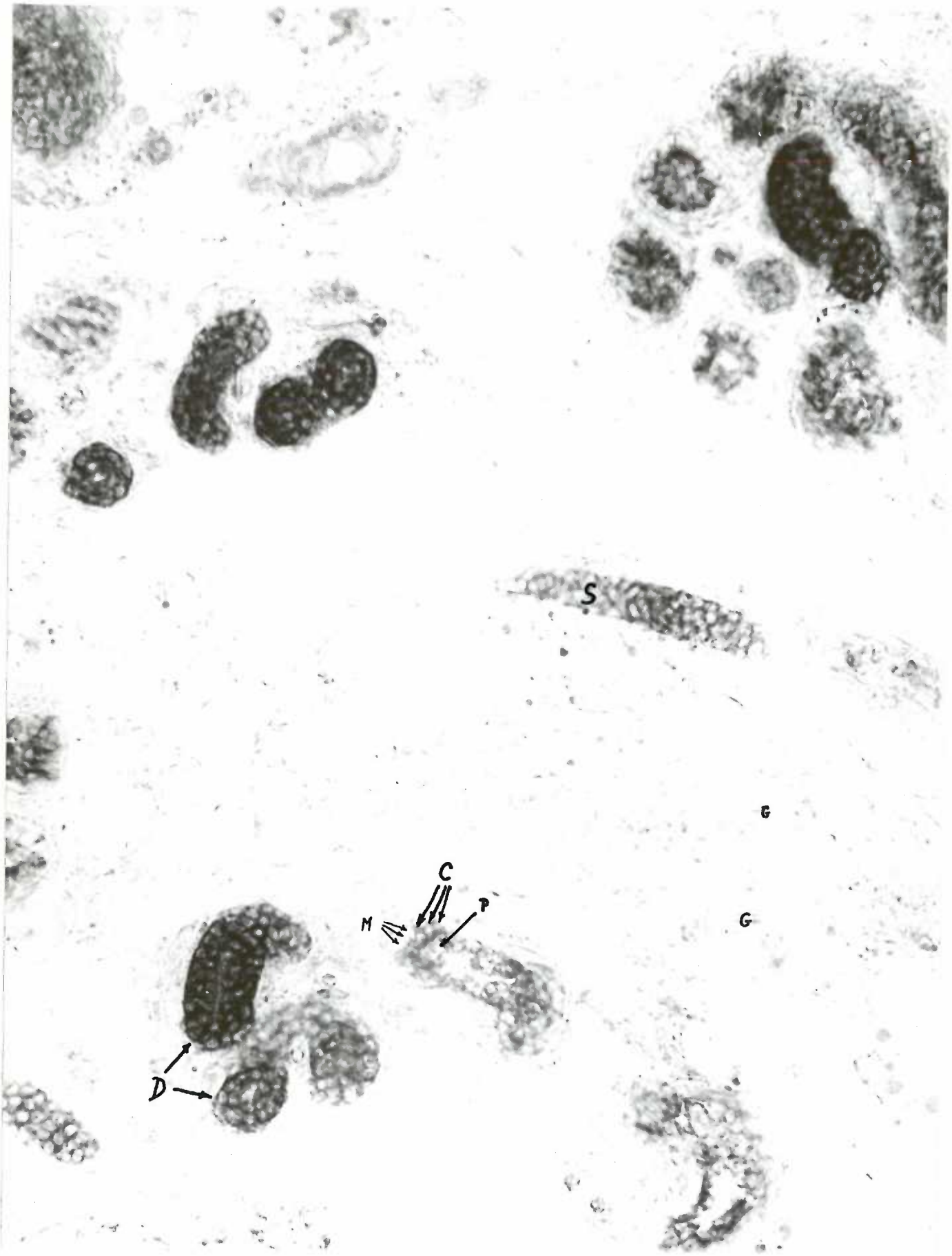


Fig. 52

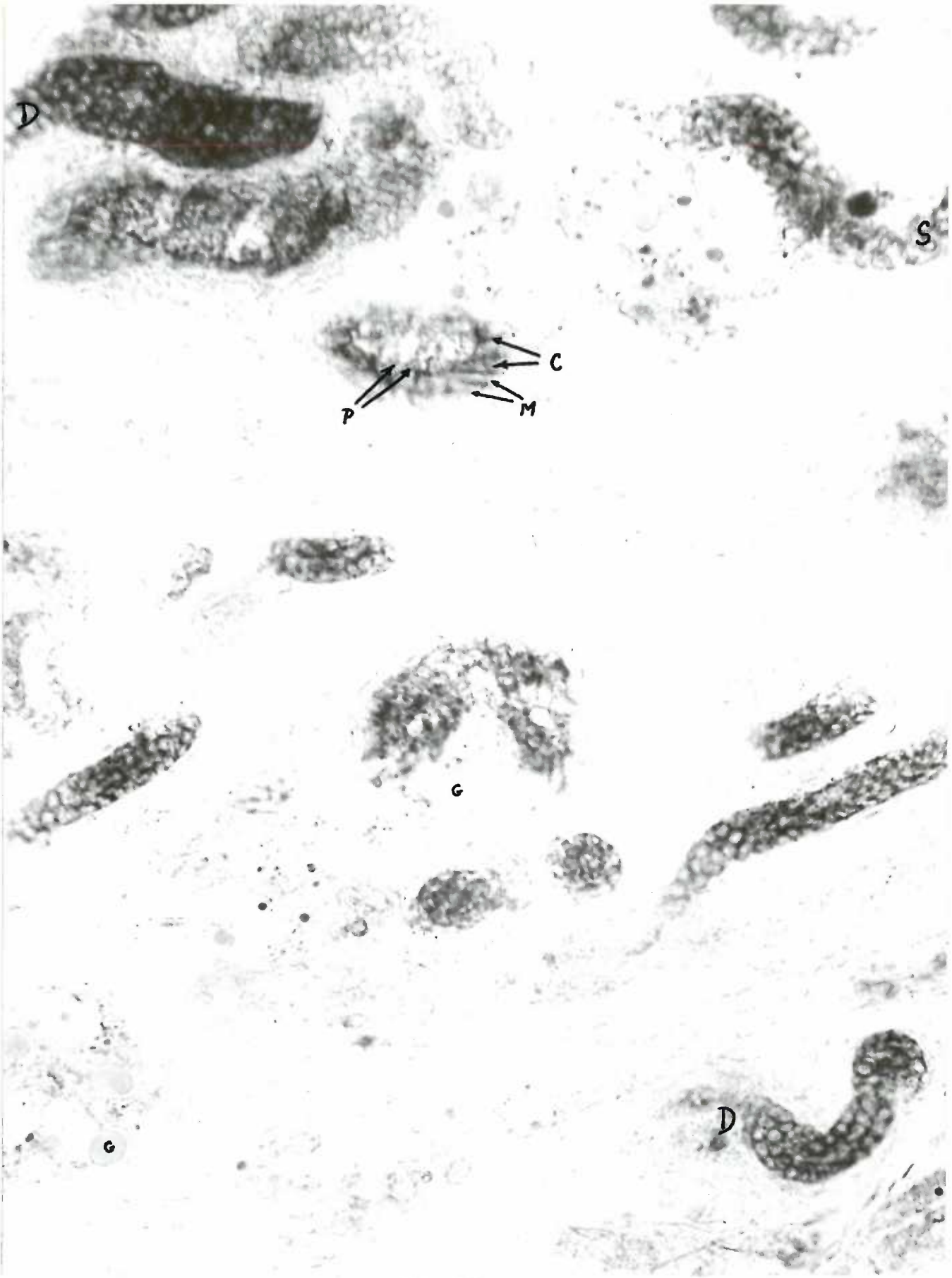


Fig. 53

Two of the animals used in the nerve study were sweating "spontaneously" when brought to the operating table. Methacholine elicited visible sweating in both in 25 to 30 seconds. One hour after neurotmesis, neither sweated spontaneously. Methacholine elicited visible sweating in 2 minutes in one animal and in 3 minutes in another. Two other injections in different sites had no effect; response in the control hand was the same as before neurotmesis. Spontaneous sweating reappeared in both on subsequent days and disappeared again, 11 days after neurotmesis in the first animal and 6 days after in the second. Three days after neurotmesis, the response of the first animal to methacholine was so weak that it could not be timed; it disappeared altogether 11 days after the operation. In the second animal, the response to methacholine was printable but not visible on the day after the operation; 2 days after the operation, methacholine elicited visible sweating in 1-3/4 minutes; on the sixth day after the operation, the response was again printable but not visible; on the 14th day there was no response to mecholyl.

The third animal was not sweating spontaneously just before the operation, nor on the third and fourth days after. On the eighth day after neurotmesis, spontaneous sweating appeared in the control hand but not in the operated hand. In this animal, methacholine elicited visible sweating in 1-1/2 minutes before the operation and in 6 minutes 3 days after it; the response was printable but not visible on the fourth day and disappeared entirely on the eighth day.

In biopsies taken from all three animals immediately after neurotmesis, the nerves gave normal cholinesterase reactions. In the first animal, cholinesterase activities were normal on the third day after neurotmesis; 7 days after neurotmesis the acetylcholinesterase reaction showed the nerves as dotted lines, whereas non-specific cholinesterase activity was weak but normally distributed. On the thirty-fifth day acetyl- but not non-specific cholinesterase activity was completely absent. In the second animal, the activities of both cholinesterases were normal on the first and second days after neurotmesis; by the sixth day acetyl- but non-specific cholinesterase activity had fallen off; on the fourteenth day acetylcholinesterase activity was absent from the nerves and non-specific cholinesterase activity was patchy. In the third animal, acetyl- but not non-specific cholinesterase activity was weak 3 days after neurotmesis; on the eighth day acetylcholinesterase activity was patchy and non-specific cholinesterase activity was weak but normally distributed; on the thirty-fifth day small clumps of activities of both cholinesterases still outlined the courses of some of the nerves around the sweat glands.

Early in the second week after neurotmesis, acetyl- but not non-specific cholinesterase activity was found in the lumina of the eccrine sweat ducts of the first and third animals. No biopsies were taken from the second animal during this period.

To summarize: spontaneous sweating ceased a week after neurotmesis; responsiveness to methacholine lasted only a few days longer.

Acetylcholinesterase activity weakened in a few days, became patchy in one week, and usually disappeared within a month of neurotmesis. Non-specific cholinesterase activity weakened in one week, but was still present at the end of the first month.

In all 3 animals the glycogen content of all cells of the coiled duct increased dramatically after neurotmesis. In 2 of the 3, the glycogen content of the clear cells also increased. These effects became visible 1 day after neurotmesis. In the third animal, the glycogen content of the clear cells was unusually high before neurotmesis and remained at the same level.

Neurotmesis had no visible effect on cytochrome oxidase activity or phosphorylase activities in the sweat glands.

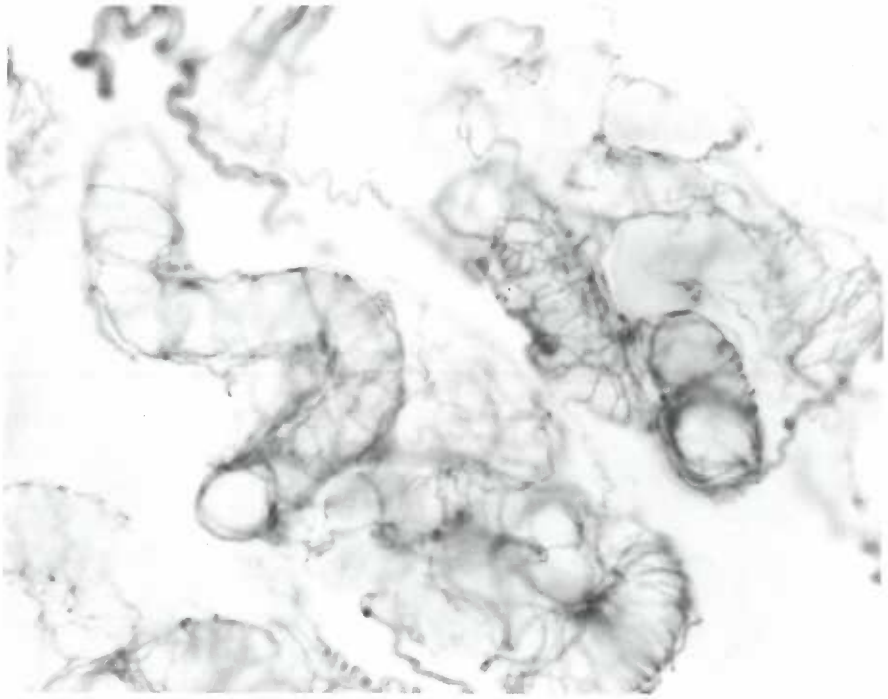


Fig. 54

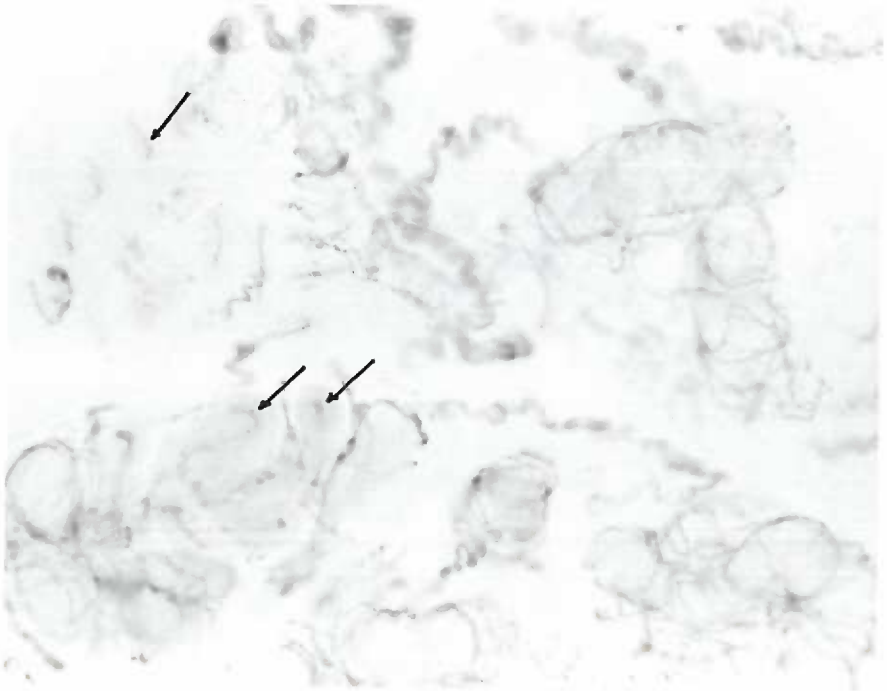


Fig. 55

Fig. 54

Nerves around sweat glands 3 days after neurotmesis. No degeneration is evident yet. Copper method for acetylcholinesterase, X 40 enlarged to X 140.

Fig. 55

Nerves around sweat glands 7 days after neurotmesis. Some degeneration is apparent, and a few duct lumina (arrows) have collected cholinesterase. Copper method for acetylcholinesterase, X 40 enlarged to X 140.

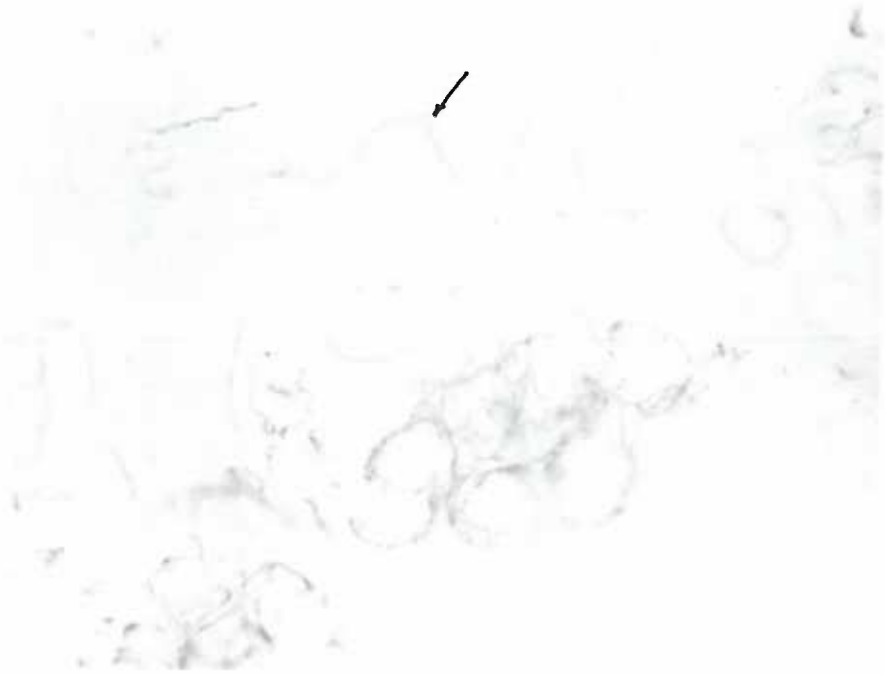


Fig. 56


DRUG INDUCED
SWEATING
M+3 ring Dec 16
15 MIN.
3 DAYS
1 WEEK
11 DAYS
AFTER NERVE SECTION

Fig. 57

Fig. 56

Degenerating nerves around sweat glands 14 days after neurotmesis. The lumen of a duct (arrow) has acquired much cholinesterase. Copper method for acetylcholinesterase, X 40 enlarged to X 140.

Fig. 57

Dependence of the capacity to sweat on innervation. (There are still two active sweat glands in the ring fingertip one week after neurotmesis.) Prints on bromphenol blue paper of the same animal used for figs. 54 through 56, X 6/7.

DISCUSSION

I. Methodology

In studies of induced changes in microscopic anatomy, one usually expects that the preparation of the tissue for observation will affect the experimental and control tissues similarly. On the other hand, in studies of normal microanatomy, serious questions arise about the relation of the observations to the living tissue.

Dehydrating in alcohols, clearing in xylene and infiltrating with hot paraffin cause tissue shrinkage (King, 1910; Allen, 1916; Galigher and Kozloff, 1964). Further shrinkage probably occurs as the paraffin solidifies. Distortion of the tissue in cutting and the efforts to correct it by floating the tissue on warm water are familiar to every histologist. Certainly paraffin sections cannot be expected to show the true dimenstions of the living material.

Very few structures can be measured in frozen sections. Dimensions of what can be measured vary according to the stain and the mounting medium. Thick frozen sections are flattened by the coverslip. In the present study, the gland used to measure length (Fig. C) was incubated in a hypotonic medium and mounted in glycerol-gel. Since both factors tend to cause swelling, the lengths given here are probably overestimated. The freezing and the acidity of the stain in the AOV technique would be likely to cause swelling (cf. Galigher and Kozloff, 1964) and the dehydration and clearing would tend to cause shrinkage. However, the fixative effect of the stain would tend to

minimize both latter two effects and to preserve the real dimensions fairly well.

For araldite sections, tissues were fixed in glutaraldehyde which causes much less shrinkage than Helly's fluid. (Secretory coil diameters in an araldite section of Helly-fixed skin averaged 25% less than those in glutaraldehyde-fixed skin.) Tissues were infiltrated at room temperature and the block was polymerized with no apparent shrinkage at 45°. Araldite sections were not visibly distorted during the cutting. One may therefore hope that the dimensions seen in araldite sections are close to the true dimensions.

Measurements in araldite sections and the AOV stained frozen sections are at least consistent with each other: the diameter of the secretory coil is 60 ± 8 μ in the former and 60 ± 9 μ in the latter. Together, these two techniques, which I think yield the best available subjects for microscopic measurement, cover the major structural features.

Glycogen is poorly preserved in glutaraldehyde-fixed araldite sections (Terzakis, 1964). Since even with buffered osmium tetroxide fixation, I had trouble staining glycogen in araldite sections, (probably because of poor penetration by the Schiff's reagent), my descriptions of "normal" and drug-affected glycogen distributions are based on paraffin sections, in which the PAS technique is reliable.

Figs. 58 - 61

The effects of preparation on sections.

Fig. 58

1 μ araldite section, toluidine blue-borax, X 400.

Fig. 59

4 μ paraffin section, H+E, X 400

Fig. 60

10 μ frozen section, Roman's AOV, X 400.

Fig. 61

20 μ frozen section, incubated for aminopeptidase (L-leucyl-β-naphthylamide and o-dianisidine-bis-diazonium chloride), X 400.

sc - secretory coil

D - coiled duct

T - transition zone

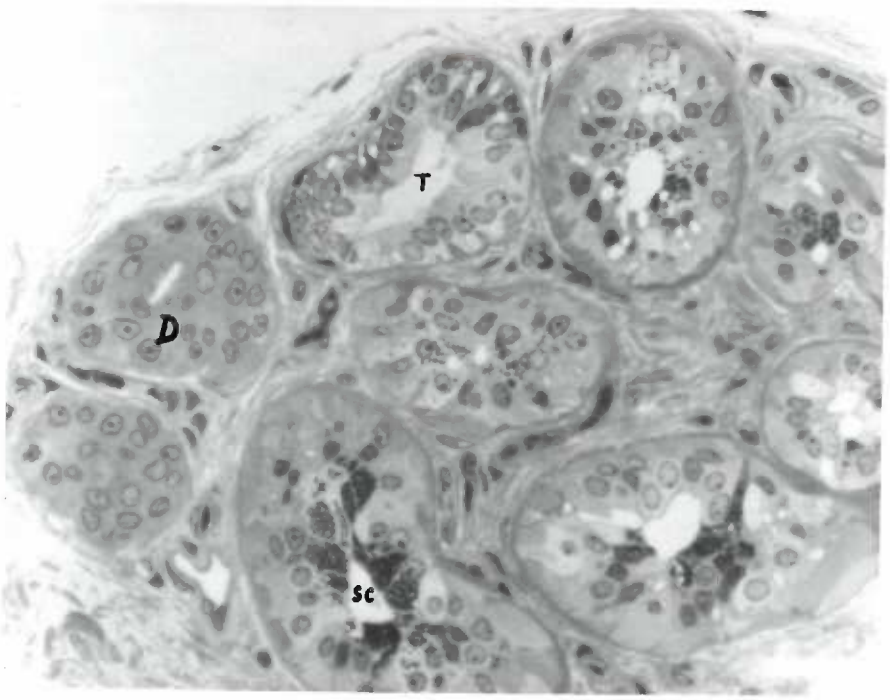


Fig. 58

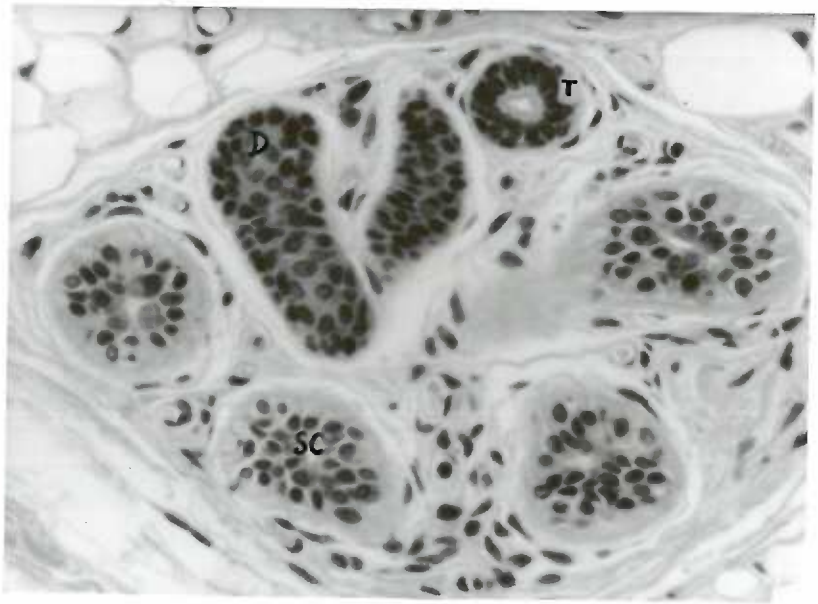


Fig. 59

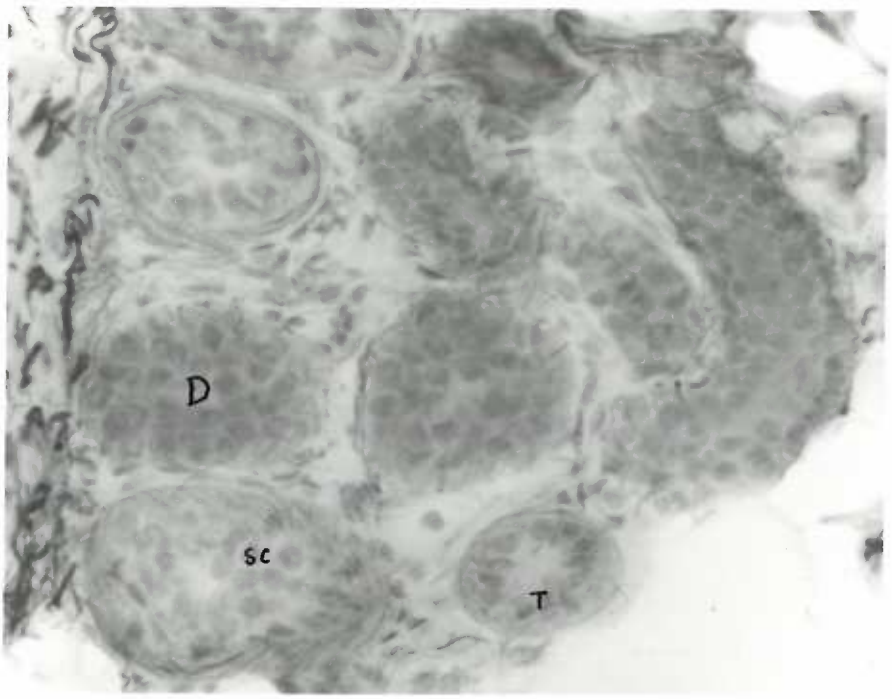


Fig. 60

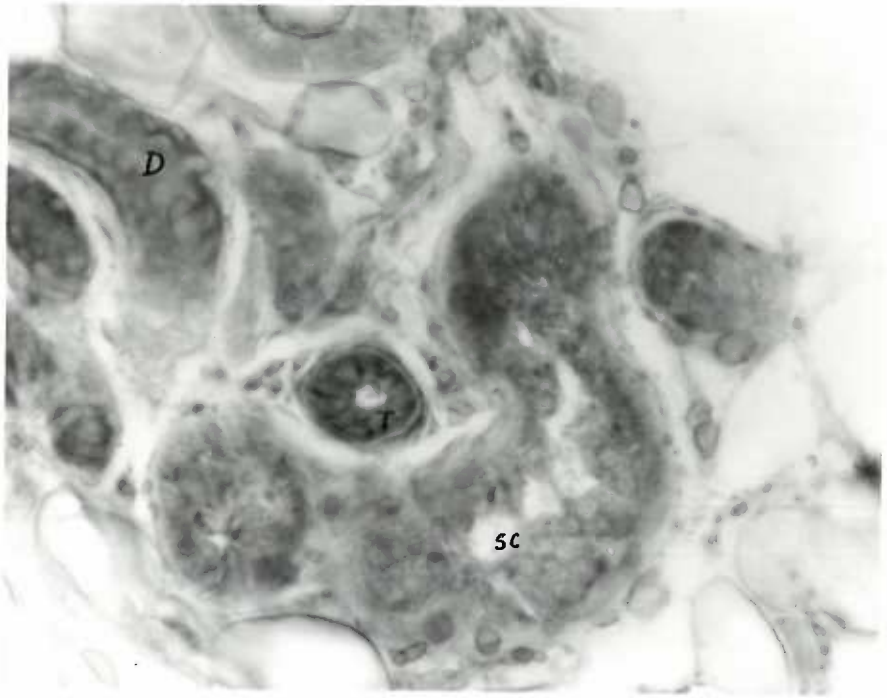


Fig 61

It was necessary to compare stimulated sweat glands to inhibited sweat glands rather than to unstimulated sweat glands because the latter may be active. In the African green monkey, Cercopithecus aethiops (Linnaeus 1758), and the mongoose lemur, Lemur mongoz Linnaeus 1766, phencyclidine anesthesia usually prevents spontaneous sweating (Sakurai & Montagna, 1964, 1965). In the rhesus monkey, however, spontaneous sweating often occurred in animals anesthetized with phencyclidine alone. A small local or systemic dose of atropine will suppress spontaneous sweating. The local dose recommended by Collins, Sargent, and Weiner (1959) to prevent methacholine-induced fatigue was satisfactory for inhibition.

The mecholyl and hyaluronidase solution recommended for sweat stimulation by Dobson and Ratner (1963) was satisfactory. Hyaluronidase was added to the atropine solution to ensure diffusion of the drug. Both drugs seemed to work best in the larger volumes of solution (e.g. 40 µg in 100 µl was bettern than 40 µg in 50 µl). Hydrostatic pressure may be important in getting the drug through connective tissue barriers.

In order to minimize disruption of structure, I omitted hyaluronidase in the animal used for the araldite sections of stimulated and inhibited glands. The response to the drugs, with or without hyaluronidase appeared to the naked eye to be the same. Since the data on the lack of structural change are from only 3 glands of one animal and are not fully comparable to the data on changes in enzyme activity, they have only heuristic value.

Enzyme histochemistry is full of problems. If the reaction

localizes a soluble enzyme, the enzyme may diffuse from its in vivo site to another locale. If fixation would destroy the enzyme, the usual solution is to ignore unreasonable and inconstant localizations. The reaction product is often soluble enough to leave its place of origin and settle on some tissue component for which it has greater "substantivity" (i.e., affinity). One learns to recognize characteristic substantivity artifacts for each type of stain. Both types of artifact can be detected by incubating live and killed tissues in contact with each other. The enzyme sought may not be the only thing in the cell that will produce the stain; whether or not it is can be detected by adding a specific inhibitor to the medium or, in some cases, by leaving out the specific substrate. The usual practice is to run the reaction with a control once or twice, remember the false positives found, and discount similar localizations in subsequent experiments.

If the section is too thin, there will be no intact cells, some enzymes will be lost, and weak reactions will be hard to see. If the section is too thick, it will be impossible to recognize individual cells and, in some cases, the substrate may not diffuse into the tissue. I found 20 μ to be a good compromise in sweat glands.

Dark cells are recognized in fixed tissue by their basophilia; they lost this property and became unrecognizable in frozen sections. Since the dark cells of the rhesus are generally more central than the clear cells, one can infer different enzyme activity in dark and

clear cells from the differences in stain intensity in central and peripheral areas of the secretory coil. Since nuclei usually do not show enzyme reactions, cells can usually be identified as the reactive area around anunreactive nucleus. Myoepithelial cells can be recognized by their position and shape.

The usual practice is to record differences in enzyme activity on an ordinal scale based on one's visual impression of the stained section. Reactions are graded strong, weak etc., in relation to the expected intensity of the particular reaction. Paired slides can be compared well with this system. (After recording the direction and magnitude of differences in seven pairs, I reread them after a colleague had covered the labels with dummy labels and shuffled the slides: grade differences within the pairs remained the same, but grade differences between members of different pairs were not repeatable). Differences in the activity of glands in the same area (cf. Kuno, 1956) make counts of cells at each level of visible staining of little use in the study of sweat glands (e.g., I found in classifications of 50 clear cells in each member of a pair that the first trial showed a difference with p=.001 and the second trial showed a difference with p = .43 on the Kolmogorov test).

The traditional method of recording enzyme activities gives many ties and a strongly leptokurtic, and often skewed, distribution. In order to apply a statistical test to the comparisions, one needs a non-parametric test. The unmatched distribution of null classes

rules out the chi-square test. The large number of ties makes a cumulative distribution function test the easiest to apply. These tests have the added advantage of being able to accept as well as to reject the null hypothesis (Birnbaum, 1952; Miller, 1956; Noether, 1967). The number of comparisons made the computationally simple Kolmogorov test the most practical choice.

An exact table makes the assessment of "probably significant" results easier than a table of percentage points does (e.g., $p = .06$ means more than $.10 > p > .05$.). The most accurate available exact table (Birnbaum, 1952) gives the probability that the null hypothesis is false. Since biologists customarily give the probability that it is true, I converted the relevant columns of Birnbaum's table to this form.

Since I expected to duplicate the decrease of glycogen found in human clear cells (Shelley & Mescon, 1952) and ducts (Lobitz et al, 1955), I used one-tailed tests for these comparisons. I also used one-tailed tests (v. McNemar, 1955) to compare the relative and absolute levels of phosphorylase a activity in these locations since previous experience (Smith & Dobson, 1966) and theoretical considerations (infra) indicated a decrease during sweating. One-half of the two-tailed probability is a good enough approximation of the one-tailed probability "for most practical purposes" (Noether, 1967) and was used here. Miller (1956) claims

that this approximation is accurate to four significant figures.

Given the nature of the data, two significant figures are sufficient. $p = .00$ suggests 1 chance in 200 of error; $p = .000$ suggests 1 chance in 2000 of error. I do not have the latter degree of confidence in any of my results. Statistics should be an aid to judgment, not a substitute for it (Feller, 1969).

The Kolmogorov test is inherently conservative (Lehmann, 1951) and ties increase its conservative bias (Slakter, 1965). Therefore, the probabilities that the samples come from like populations are actually upper limits; conservatism strengthens confidence in the significance of differences and weakens confidence in the significance of similarities. In any event, "significant lack of change" says only that histochemically detectable change is unlikely to occur in the population. This implies that the more sensitive biochemical methods would not find large changes but says nothing about smaller changes. It is hard to say what "large" means in this context: Raekallo and Makinen's (1967) data suggest 20% for aminopeptidase; Im and Adachi's data suggest 30% to 100% for dehydrogenases (Im, 1965; Im and Adachi, 1966 a & b).

Biochemical methods require that the cell or tissue under study be dissected free of any surrounding material that could interfere. The components of the coil of eccrine sweat glands cannot be reliably separated in unstained tissue (Adachi and Yamasawa, 1966). In this study, I felt that the technical problems involved in dissecting out the

components of stained sweat glands and dissolving and measuring the enzyme reaction product outweighed the advantages accruing from such a procedure.

II. Biochemistry

In the absence of specific biochemical studies, one must resort to analogy for an understanding of the metabolism of the eccrine sweat gland. Fortunately, most metabolic pathways are common to all mammalian cells (West, Todd, Mason, & Van Bruggen, 1966). Thus the risk of error in outlining pathways is slight. The control of the pathways, however, differs greatly. For example, heart and liver phosphorylase are not subject to control by the same factors (Rall, Wosilait, & Sutherland, 1956), and liver and mammary gland glucose-6-phosphate dehydrogenases have different velocity curves (Noltmann & Kuby, 1963). The following speculations on the control of metabolism in the cells of eccrine sweat glands are based on what is known of the metabolism of other organs. Muscle and mammary gland data, when available, have been preferred to data from liver. This preference is supported by the fact that the inactive forms of the phosphorylases of eccrine sweat glands (Smith & Dobson, 1956) and muscle (Rall et al, 1956) are active in the presence of adenylic acid, whereas that of liver is not (Rall et al, 1956).

Stored Energy-Glycogen

Glycogen is a polymer of α-D-glucose. The metabolism of glucose begins through phosphorylation of the primary alcohol group on

the sixth carbon.

glucose + ATP $\xrightarrow{\text{hexokinase}}$ glucose-6-phosphate + ADP

The glucose may then be broken down via the Embden-Meyerhof or pentose pathways (infra) or may be incorporated into glycogen (Hassid, 1962):

glucose-6-phosphate $\xrightleftharpoons{\text{phosphoglucomutase}}$ glucose-1-phosphate

glucose-1-phosphate + UTP + H^+ + glucose-1-phosphate uridyl-transferase $\longrightarrow$ UDPG + $HP_2O_7^{\equiv}$

UTP = uridine triphosphate

UDPG = uridine diphosphoglucose

(The apparent electronic imbalance of the above reaction is due to the convention that the 2 negative charge on glucose-1-phosphate, the 4 on UTP, and the 2 on UDPG are not written.)

Not all of the UDPG produced is used to make glycogen; some of it is turned into the uridine nucleotides of other sugars used in making lactose, mucopolysaccharides, and glycoproteins.

Glycogen is produced by the addition of glucose units to glycogen already present. These are added by linking the first, or phospho-hemiacetal carbon of the glucose moiety of UDPG to glycogen synthetase. The uridine diphosphate breaks off and the first carbon of the enzyme-bound glucose is attached to the oxygen of the fourth carbon of a terminal glucose of the glycogen chain; water is split out and the enzyme breaks off (Hassid, 1962). Pieces of these 1, 4 chains from 11 to 21 units long are broken off between the first carbon of one glucose unit

and the oxygen of the fourth carbon of the unit remaining in place and are attached to the oxygen of the sixth carbon of the second glucose unit from the end of the remaining chain, displacing hydrogen (Larner, 1953). Thus, glycogen synthesis means an increase in the size of the glycogen molecules rather than in the number of molecules.

The reaction can, for convenience, be imagined as:

$$n \text{ glucose-1-phosphate} \xrightarrow{\text{glycogen synthetase}} \underset{(n \text{ units})}{\text{amylose}} + n\,\textcircled{P}$$

$$\underset{(n)}{\text{amylose}} \xrightarrow{\text{branching enzyme}} \underset{(n)}{\text{glycogen}}$$

Ⓟ = inorganic phosphate, partly as $HPO_4^{=}$, partly as $H_2PO^-_4$ [the ionization constant of $H_2PO_4^-$ is 7.5×10^{-8} (Handbook of Chemistry and Physics, 1956)].

The actual reaction is:

$$n \text{ glucose-1-phosphate} + \underset{(x \text{ units})}{\text{glycogen}} \xrightarrow[\text{branching enzyme}]{\text{glycogen synthetase}} \underset{(x+n)}{\text{glycogen}} + n\,\textcircled{P}$$

Glycogen synthetase has two forms. The activity of the phosphorylated, or D, form depends on the concentration of glucose-6-phosphate. The unphosphorylated, or I, form is fully active regardless of glucose-6-phosphate concentration (Friedman & Larner, 1963). Interconversion of the two forms is accomplished by a specific phosphatase and a specific kinase (Danforth, 1965; Mazur and Harrow; 1968). Since physiologic concentrations of glucose-6-phosphate will activate

glycogen synthetase D (Leloir & Cardini, 1962), control of glycogen synthesis resides in both this and the I - D interconversion.

The breakdown of glycogen is also accomplished by two enzymes: phosphorylase (amylo-1, 4'-phosphorylase), which breaks 1, 4' bonds; and amylo-1, 6'-glucosidase, which breaks 1, 6' bonds. Both enzymes remove terminal glucose units only. Their action can be imagined as:

$$\text{glycogen}_{(x)} + n\,\text{Ⓟ} \xrightarrow{\text{phosphorylase}} n\ \text{glucose-1-phosphate} + \text{dextrin}_{(x-n)} + nH^+$$

$$\text{dextrin}_{(x)} + n\ H_2O \xrightarrow{\text{amylo-1,6'-glucosidase}} n\ \text{glucose} + \text{glycogen}_{(x-n)}$$

Since both enzymes act together no dextrin ever appears and the actual reaction is:

$$\text{glycogen}_{(x)} + n\,\text{Ⓟ} + m\ H_2O \xrightarrow[\text{amylo-1,6'-glucosidase}]{\text{phosphorylase}} n\ \text{glucose-1-phosphate} + m\ \text{glucose} + \text{glycogen}_{(x-n-m)}$$

Amylo-1, 6'-glucosidase, like branching enzyme, is known in only one form; phosphorylase, like glycogen synthetase, is known in two forms. Here, however, the phosphorylated, or a, form is the active one, and the dephosphorylated, or b, form is active only in the presence of a high concentration of adenylic acid. The phosphorylases are also interconverted by a specific kinase and a specific phosphatase (Brown & Cori, 1961). Both enzyme kinases are stimulated by conditions that inhibit both enzyme phosphatases and vice versa (Larner, Rosell-Perez, Friedman, & Craig, 1964). Under extreme conditions (e. g. , anoxia), the intracellular concentration of adenylic acid may be high enough to affect

glycogen metabolism (Morgan & Parmeggiani, 1963).

Phosphoglucomutase (supra) also converts the glucose-1-phosphate from glycogen catbolism to glucose-6-phosphate for oxidation. The equilibrium of the reaction lies strongly (19:1) in the direction of glucose-6-phosphate (Jagannathan & Luck, 1949).

Histochemical methods are available for phosphoglucomutase, both forms of glycogen synthetase, branching enzyme, glycogen phosphorylase a, and phosphorylase b.

The Meijer (1967) method for phosphoglucomutase is complicated, depending on the following series of reactions:

1) glucose-1-phosphate + ATP $\xrightarrow{\text{phosphoglucokinase}}$ glucose-1, 6-diphosphate + ADP

2) phosphoglucomutase + glucose-1, 6-diphosphate $\longrightarrow$ phosphoglucomutase phosphate + glucose-6-phosphate

3) phosphoglucomutase phosphate + glucose-1-phosphate $\longrightarrow$ phosphoglucomutase + glucose-1, 6-diphosphate

4) glucose-6-phosphate + TPN $\xrightarrow{\text{glucose-6-phosphate dehydrogenase}}$ 6-phosphogluconolactone + TPNH + H^+

TPNH = reduced TPN

5) TPNH + ditetrazolium salt $\xrightarrow{\text{TPN diaphorase}}$ TPN + monoformazan

6) TPNH + monoformazan $\xrightarrow{\text{TPN diaphorase}}$ TPN + diformazan

Diformazan is a deep violet, water and alcohol insoluble precipitate. ATP, TPN, and glucose-6-phosphate dehydrogenase are added to the incubating medium. Sufficient phosphoglucokinase to produce catalytic

amounts of glucose-1, 6-diphosphate is present, since the reaction works. If the amount of phosphoglucokinase were rate-limiting in tela it would also have been rate-limiting in vivo. Therefore, although the amount of formazan deposited may not reflect the amount of phosphoglucomutase present, it does reflect phosphoglucomutase activity.

Diaphorase is a histochemical concept covering anything capable of passing hydride ions to an indicator. A number of flavoprotein enzymes can function as diaphorases. TPNH-cytochrome reductase appears to be an important TPN diaphorase (Mahler, 1955). The reoxidation of TPNH appears to be rate-limiting for TPN-linked reactions in liver cells in vivo (Navazio, Ernster, & Ernster, 1957). Pearse (1961) holds that diaphorase activity is not rate-limiting for histochemical reactions because the product (formazan) is immediately removed from solution. The intensity of staining will depend, at least in part, on the activity of the enzyme studied, unless sufficient TPNH is produced to saturate all of the cell's TPN diaphorase. The problem of saturation of the cell's diaphorase can arise only in the case of the reaction giving the highest rate of formazan production. Among the TPN-linked enzymes with which I have worked, isocitrate dehydrogenase gives the highest rate of formazan production. Thus, for all other TPN-linked enzymes studied variations in enzyme activity will be reflected by variations in the amount of formazan deposited. If incubation times are held to 5 minutes so that exhaustion of the

substrate is not a factor, TPN diaphorase activity (TPNH as substrate) is somewhat greater than TPN-linked isocitrate dehydrogenase activity in sweat glands. This suggests that even in the case of TPN-linked isocitrate dehydrogenase the need for a diaphorase may not prevent detection of changes in dehydrogenase activity. The possibility that some of the apparent changes in dehydrogenase activities were actually due to changes in diaphorase activity should be investigated. The lack of change in detectable glucose-6-phosphate dehydrogenase activity in the clear cells suggests that the changes in detectable phosphoglucomutase activity in the clear cells were not due to changes in diaphorase activity.

The intracellular localization of the reaction product is, as in all dehydrogenase reactions, that of the last enzyme in the sequence, the diaphorase. I suspect that the freezing and thawing in histochemical reactions makes extramitochondrial TPNH available to mitochondrial transhydrogenase and diaphorases. Thus, the reaction is accurate only to the cellular level, which is as far as this discussion is carried (cf. Novikoff, 1961a).

The reactions for glycogen synthetases depend on the deposition and staining of glycogen. The uncertainties in this reaction are those that are due to the extreme sensitivity of the enzyme to small differences in the handling of the tissue. Branching enzyme activity is sufficient so that all of the deposit is glycogen. The glycogen deposits

produced in this method are sufficiently large to be easily stained with Lugol's iodine.

I chose a periodic acid-Schiff reaction (Montagna et al, 1953) for the smaller amounts of native glycogen because I have found it to show greater sensitivity than Lugol's iodine method and a broader gradation of intensities than the Best's carmine procedure. With the diastase control, it is eminently reliable (Pearse, 1961).

The reactions for phosphorylases (Takeuchi & Kuriaki, 1955; Godlewski, 1962; Eränkö & Palkama, 1961) depend on the reversal of the reaction by a high concentration of glucose-1-phosphate and a low concentration of inorganic phosphate (Leloir, 1963). The phosphorylases appear to be relatively resistant to dessication and freezing and thawing. The specificity of the reaction is excellent, but occasional reactions in lumina attest to the presence of diffusion artifacts.

The iodine-glycogen clathrate is henna-colored, whereas the iodine-amylose clathrate is violet. Since branching enzyme is required to form glycogen from the amylose produced by the reversal of the phosphorylase reaction, branching enzyme activity can be estimated from the color of the deposit, provided enthanol (which sharpens localization but inhibits branching enzyme) is omitted from the incubating medium (Takeuchi & Kuriaki, 1955). The method is a bit crude.

Data show that glycogen decreases during sweating, just as it does in man. Matsumoto and Ohkura's data (1960) suggest a similar

result in the Japanese monkey, Macaca fuscata (Blyth 1875). Although the change was slight, the dose of pilocarpine given to stimulate sweating was also small.

Since glycogen is used up in sweating, it is not surprising that the enzyme largely responsible for its initial breakdown increases in sweating. The decrease in the proportion of glycogen synthetase in the I form shows that in the clear cells of sweat glands the conditions favoring phosphorylation of the enzymes of glycogen metabolism (Larner et al, 1964) during sweating outweigh the influence of glycogen depletion on the dephosphorylation (activation) of glycogen synthetase (cf. Danforth, 1965). The total amount of each enzyme seems to remain relatively constant and the greatest change is in the proportion of the enzyme in the active form. Short-term control of glycogen production and use is thus a matter of conversion of the enzymes from one form to the other. This is consonant with data suggesting that enzyme synthesis in mammalian cells requires a minimum of 2 hours (Peraino, Blake, & Pitot, 1965; Mishkin & Shore, 1967), 4 times the duration of my longest experiment.

Branching enzyme, known in only one form, does not show changes in activity -- in so far as changes can be detected by the Takeuchi method.

Phosphoglucomutase activity in the clear cells increases with heavy sweating. This indicates that glycogen utilization is a more rapid process than glycogen synthesis, a conclusion that has been reached before (Leloir, 1964). The increase is most likely in the amount of phosphoglucomutase or phosphoglucokinase in an active form, rather

than in total enzyme concentration. This could be an increase in phosphoglucomutase phosphate because of the law of mass action, or pH, or small molecule concentration in vivo. It could also be a membrane-binding effect (Siekevitz 1958).

Oxidation

Most biological oxidation is mediated by the Krebs cycle (West et al, 1966). Glucose is fed into this cycle by the Embden-Meyerhof pathway and the monophosphate shunt, both of which contain oxidative steps. The hydrogen and electrons produced by oxidation pass successively through several carriers and finally reduce molecular oxygen to water.

The Embden-Meyerhof pathway consists of the following steps (Umbreit 1960):

1) glucose-6-phosphate $\xrightarrow{\text{phosphoglucose isomerase}}$ fructose-6-phosphate

2) fructose-6-phosphate + ATP $\xrightarrow{\text{phosphofructokinase}}$ fructose-1, 6-diphosphate + ADP

3) fructose-1, 6-diphosphate $\xrightarrow{\text{fructoaldolase}}$ dihydroxyacetone phosphate + glyceraldehyde-3-phosphate

4) dihydroxyacetone phosphate $\xrightarrow{\text{triose isomerase}}$ glyceraldehyde-3-phosphate

5) glyceraldehyde-3-phosphate + DPN + Ⓟ + $\xrightarrow{\text{glyceraldehyde-3-phosphate dehydrogenase}}$ 1, 3-diphosphoglycerate + DPNH + H^+

Ⓟ = inorganic phosphate

6) 1, 3-diphosphoglycerate + ADP $\xrightarrow{\text{phosphoglycerate kinase}}$ 3-phospho-glycerate + ATP

7) 3-phosphoglycerate $\xrightarrow{\text{phosphoglycerate mutase}}$ 2-phosphoglycerate

8) 2-phosphoglycerate $\xrightarrow{\text{enolase}}$ 2-phospho-enol - pyruvate

9) 2-phospho-enol-pyruvate + ADP $\xrightarrow{\text{pyruvate kinase}}$ pyruvate + ATP

The monophosphate shunt is an alternate pathway from glucose-6-phosphate to glyceraldehyde-3-phosphate. West et al's (1966) map can be reduced to a series of reactions:

1) glucose-6-phosphate + TPN $\xrightarrow{\text{glucose-6-phosphate dehydrogenase}}$ 6-phosphogluconolactone + TPNH + H^+

2) 6-phosphogluconolactone + H_2O $\xrightarrow{\text{lactonase}}$ 6-phosphogluconate

3) 6-phosphogluconate + TPN $\xrightarrow{\text{6-phosphogluconate dehydrogenase}}$ ribulose-5-phosphate + TPNH + CO_2 + H^+

4) ribulose-5-phosphate $\xrightarrow{\text{phosphoribose isomerase}}$ ribose-5-phosphate

5) ribulose-5-phosphate $\xrightarrow{\text{phosphopentose epimerase}}$ xylulose-5-phosphate

6) ribose-5-phosphate + xylulose-5-phosphate $\xrightarrow{\text{transketolase}}$ glyceraldehyde-3-phosphate + sedoheptulose-7-phosphate

7) sedoheptulose-7-phosphate + glyceraldehyde-3-phosphate $\xrightarrow{\text{transaldolase}}$ erythrose-4-phosphate + fructose-6-phosphate

8) erythrose-4-phosphate + xylulose-5-phosphate $\xrightarrow{\text{transketolase}}$ glyceraldehyde-3-phosphate + fructose-6-phosphate

A little counting shows that for reactions 6), 7), and 8) to take place once, reactions 1), 2), and 3) must take place three times, reaction 5) must take place twice, and reaction 4) must take place once.

(This stoichiometry is not maintained *in vivo* since the products of several of the reactions are used in other ways as well.) The overall reaction may be represented as:

3 glucose-6-phosphate + $3H_2O$ + 6TPN $\longrightarrow$ 2 fructose-6-phosphate + glyceraldehyde-3-phosphate + $3CO_2$ + $6H^+$ + 6 TPNH

The glyceraldehyde-3-phosphate and fructose-6-phosphate may be catabolized via the Embden-Meyerhof pathway. The fructose-6-phosphate may be returned to the monophosphate pathway by phosphoglucose isomerase. The glyceraldehyde-3-phosphate may also be recycled by triose isomerase and reversal of the fructoaldolase reaction.

Phosphorylase and phosphoglucomutase activity supply glucose-6-phosphate. One mole of glucose-6-phosphate can yield 39 moles of ATP *via* the Embden-Meyerhof, pyruvate, Krebs cycle route or 12 moles of hydride via the monophosphate shunt if the end products are recycled or 32 2/3 moles of ATP and 2 moles of hydride if the end products of the shunt are metabolized via the Krebs cycle.

If ATP is the only proximate energy source for sweating, energy would be best supplied by the Embden-Meyerhof, Krebs cycle route. Siekevitz (1959) has suggested, however, that the energy required for transport across ergastoplasmic membranes ("rough surfaced endoplasmic reticulum") in secretion is supplied by TPNH or DPNH via the cytochrome(s) located in this membrane.

Judging from the relative activities of fructoaldolase (Adachi & Yamasawa, 1966b) and glucose-6-phosphate dehydrogenase (Im & Adachi, 1966a) of rhesus monkey sweat glands in vitro, the Embden-Meyerhof pathway in sweat glands seems capable of carrying twice as much glucose as the monophosphate shunt. Since the velocity curves for the enzymes are probably different, changes in substrate concentration would affect each enzyme differently. If the cell is as compartmentalized as Siekevitz (1958) suggests, local variations in substrate and product concentration and pH would also influence the actual route of metabolism.

Histochemical methods are available for 3 of the 16 enzymes catalyzing the above 17 reactions.

The Abe and Shimizu (1964) method for fructoaldolase resembles the method for phosphoglucomutase; the glyceraldehyde-3-phosphate produced by fructoaldolase is oxidized by endogenous glyceraldehyde-3-phosphate dehydrogenase. (Changing substrates would produce a reaction for glyceraldehyde-3-phosphate dehydrogenase.) DPN and its diaphorase mediate the formation of formazan deposits. A low DPN concentration and long fixation by ethanol and dessication prevent significant reduction of DPN by cysteine. The usefulness of the reaction is based on the probability that fructoaldolase rather than glyceraldehyde-3-phosphate dehydrogenase will be rate-limiting for the histochemical reaction. Adachi and his colleagues (Adachi & Yamasawa, 1966b; Im, Yamasawa, & Adachi, 1966) have shown this to be the case in the skin of the rhesus monkey or in any adnexa taken as a whole.

The methods for glucose-6-phosphate and 6-phosphogluconate dehydrogenases are relatively simple (Hess et al, 1958; Im, 1965). The TPNH is produced by the enzyme under study. The reaction for glucose-6-phosphate dehydrogenase is reliable; 6-phosphogluconate dehydrogenase is capricious, being very sensitive to storage, dehydration, and substrate concentration.

The increase in glucose-6-phosphate provided by the increased activities of phosphorylase and phosphoglucomutase should cause increased energy output via both the Embden-Meyerhof pathway and the monophosphate shunt. The data show no significant change in the activities of fructoaldolase and glucose-6-phosphate dehydrogenase. This suggests that these pathways may be adjusted to meet the increased demand for energy solely by increases in substrate concentrations. Adjustments made by changes in pH or concentration of loosely bound small molecules, however, could not be detected by current histochemical techniques. The data also suggest that the relative importance of the Embden-Meyerhof and monophosphate pathways does not change during sweating.

Pyruvate is fed into the Krebs cycle as follows (Koike, Reed & Carroll, 1963; Hayakawa & Koike, 1967):

pyruvate + H^+ + TPP $\xrightarrow{\text{pyruvate carboxylase}}$ TPP-acetaldehyde + CO_2

TPP = thiamine pyrophosphate

TPP-acetaldehyde + lipoate $\xrightarrow{\text{lipoate reductase-transacetylase}}$ acetyl hydrolipoate + TPP

acetyl hydrolipoate + coenzyme A $\xrightarrow{\text{lipoate reductase-transacetylase}}$ acetyl coenzyme A + dihydrolipoate

These enzymes and the enzyme that reoxidizes the lipoate are bound together in an ordered unit (Fernandez-Moran, Reed, Koike, & Willms, 1964). There are no published histochemical reactions for them.

The following reactions consitute the Krebs cycle (Sallach & McGilvery, 1963):

1) acetyl coenzyme A + oxaloacetate $\xrightarrow{\text{citrate condensing enzyme}}$ citrate + coenzyme A + H^+

2) citrate $\xrightarrow{\text{aconitase}}$ cis-aconitate + H_2O

3) cis-aconitate + H_2O $\xrightarrow{\text{aconitase}}$ d-isocitrate

4a) d-isocitrate + DPN $\xrightarrow{\text{DPN-linked isocitrate dehydrogenase}}$ α-ketoglutarate + DPNH + H^+ + CO_2

4b) d-isocitrate + TPN $\xrightarrow{\text{TPN-linked isocitrate dehydrogenase}}$ α-ketoglutarate + TPNH + H^+ + CO_2

5) α-ketoglutarate + TPP + H^+ $\xrightarrow{\alpha\text{-ketoglutarate carboxylase}}$ TPP-succinaldehydate + CO_2

TPP = thiamine pyrophosphate

6) TPP-succinaldehydate + lipoate $\xrightarrow{\text{lipoate reductase-transacylase}}$ succinyl hydrolipoate + TPP

7) succinyl hydrolipoate + coenzyme A $\xrightarrow{\text{lipoate reductase-transacylase}}$ succinyl coenzyme A + dihydrolipoate

8) dihydrolipoate + FAD $\xrightarrow{\text{lipoate dehydrogenase}}$ lipoate + FADH + H^+

FAD = flavin adenine dinucleotide

9) succinyl coenzyme A + GDP + Ⓟ $\xrightarrow{\text{succinate thiokinase}}$ succinate + GTP + coenzyme A

GDP = guanosine pyrophosphate Ⓟ = inorganic phosphate

GTP = guanosine triphosphate

10) succinate + FAD $\xrightarrow{\text{succinate dehydrogenase}}$ fumarate + FADH + H^+

11) fumarate + H_2O fumarase L-malate

12) L-malate + DPN malate dehydrogenase oxaloacetate + DPNH + H^+

The existence of 3 enzymes capable of catalyzing the oxidation of isocitrate to α-ketoglutarate has generated much confusion. Both TPN-linked and DPN-linked isocitrate dehydrogenases are found in mitochondria (Plaut & Aogaichi, 1967). There is also an extramitochondrial TPN-linked isocitrate dehydrogenase (Lowenstein & Smith, 1962). It has been suggested that the function of this last enzyme is to maintain the level of TPNH in the cell (Lowenstein, 1961). Its reaction (4b) can be easily reversed to produce isocitrate by coupling with another dehydrogenase reaction (Ochoa, 1948). Since the equilibrium constant of aconitase is strongly in favor of citrate production (Ochoa, 1948), the direction of enzyme activity should depend on the TPN/TPNH ratio. Mitochondrial membrane impermeability would prevent mitochondrial pyridine nucleotides from influencing the TPN/TPNH ratio *in vivo* (Lehninger, 1964), unless some mechanism for transfer across the membrane were active (*infra*).

The relative importance of the two mitochondrial enzymes varies from tissue to tissue (Stein, Stein, & Kirkman, 1967; Lowenstein, 1967).

Adachi* is of the opinion that the TPN-linked enzyme predominates in skin. Cruickshank, Hershey, and Lewis's (1958) observations support this view since their media contained sufficient to override the requirement of the DPN-linked dehydrogenase for adenosine diphosphate (Goebell & Klingenberg, 1964).

I studied reactions 4b) and 10) histochemically.

A method for malate dehydrogenase is known (Hess et al, 1958), but it will give false positive results if "malic enzyme" is present.

Histochemical demonstration of DPN-linked isocitrate dehydrogenase has been claimed (Hess et al, 1958; Spector, Mutton, & Hamerton, 1964), but I am not convinced that the histochemical reaction in the presence of DPN is due to DPN-linked isocitrate dehydrogenase. The reaction is unaffected by 0.01 M cyanide; DPN-linked isocitrate dehydrogenase from yeast is seriously inhibited by this concentration of cyanide in vitro (Kornberg, 1955) and the enzyme from guinea pig heart is inhibited by much lower (0.0002 M) concentrations (Plaut & Sung, 1954). The histochemical reaction in sweat glands was also unaffected by 0.001 M ATP, 180 times the amount required for almost complete inhibition of the guinea pig heart enzyme in vitro (Plaut & Sung, 1954). The reaction might be due to the TPN-linked isocitrate dehydrogenase, endogenous TPN, and pyridine nucleotide transhydrogenase; but I found hardly any reaction in the absence of exogenous pyridine nucleotide. At pH 6.5 - 6.8 the isocitrate dehydrogenase reaction is stronger in the presence of DPN than of TPN. Although both reactions are equally inhibited by 0.0006 M PCMB, the reaction is more inhibited by 0.0003 M PCMB in the presence of TPN than of DPN.

* Kenji Adachi, personal communication, 1968.

The method for TPN-linked isocitrate dehydrogenase (Hess et al, 1958) is almost identical with the method for glucose-6-phosphate dehydrogenase. The method probably demonstrates both TPN-linked isocitrate dehydrogenases.

In the Nachlas (et al, 1957) method for succinate dehydrogenase, the hydrogen is probably passed directly to the tetrazolium salt by FADH. Since the FAD is bound to the enzyme, no exogenous coenzyme is required. It is one of the most reliable of all histochemical methods.

Succinate dehydrogenase shows occasional increases in activity in the clear cells during sweating. The Kolmogorov test, however, shows that data for the changes in succinate dehydrogenase activity are inconclusive ($p = .5$). Spearman's rank order correlation coefficient (Siegal, 1956) showed no significant correlation between the length of the sweating period and the changes in succinate dehydrogenase activity ($rho = .2$).

The final steps by which the hydride ions on the reduced pyridine nucleotides and the free protons produced with them are combined with atmospheric oxygen to produce water are not completely understood.

It is agreed that DPNH reduces the FAD prosthetic group of DPN dehydrogenase. The resulting FADH probably reduces the nonheme iron of the same enzyme molecule (Pullman & Schatz, 1967). There is some evidence that the nonheme ferrous iron then reduces ubiquinone (Bruni & Racker, 1968; Ziegler & Doeg, 1962), which picks up a proton to become "ubihydroquinone." The "ubihydroquinone" probably reduces

cytochrome b (Tisdale, Wharton, & Green, 1963), which accepts only one electron, rather than the whole hydride ion (which contains two electrons)(West & Todd, 1961). Cytochrome b reduces cytochrome c_1, possibly through another nonheme iron (Green & MacLennan, 1967). Cytochrome c oxidizes cytochrome c_1, and reduces cytochrome a. Cytochrome a is oxidized by cytochrome a_3 (King & Kuboyama, 1964), possibly via a copper ion held in common (Green & MacLennan, 1967). The electrons from four cytochrome a_3's are then accepted, along with four protons from solution, by molecular oxygen to form water (Mason, 1957).

Three molecules of ATP are produced per molecule of DPNH oxidized, probably one at a time at or near the points in the electron transport chain involving nonheme iron or copper (Green & MacLennan, 1967; Pullman & Schatz, 1967; Racker, 1968).

Succinate dehydrogenase transfers its hydride ion to ubiquinone without producing ATP (West et al, 1966). Mitochondrial TPNH is oxidized by DPN in the presence of pyridine nucleotide transhydrogenase (Mazur & Harrow, 1968). Extramitochondrial TPNH and DPNH are thought to be used in syntheses other than that of ATP (West et al, 1966). There are a cytochrome and oxidoreductases in the outer mitochondrial membrane (Sottocasa, Kuylenstierna, Ernster, & Bergstrand, 1967) which might, however, feed their reducing potential into the (inner membrane) mitochondrial electron transport system.

Cytochrome oxidase is a physiological polymer of cytochromes a and a_3 and their copper atoms. It can be demonstrated by virtue of its ability to oxidize reduced cytochrome c after the cytochrome c has oxidized an indicator (Burstone, 1960). Two indicators are used: N-phenyl-p-phenylendediamine and 8-amino tetrahydroquinoline. The ferriheme of cytochrome c takes one of the nonbonding electrons of an amine group of either reagent (Keilin & Hartree, 1938; Borei & Björklund, 1953). The resulting resonance - stabilized free radicals are highly reactive both at the amino group and at the aromatic carbon para to it. They condense--nitrogen to carbon--and split out two protons which combine with oxygen to form water (Waters, 1953). The resulting mixed indamines form a purple tar in the mitochondria. The process can continue to visibility only where cytochrome oxidase continually oxidizes the ferrocytochrome c to ferricytochrome c. The method is very reliable and variations of it have been in use for nearly a century (Keilin & Hartree, 1938). (Kodachrome, incidentally, involves a similar process). The absence of detectable response of cytochrome oxidase activity to stimulation indicates that this sytem remains "at ready" at all times rather than adjusting to meet the demands of the moment.

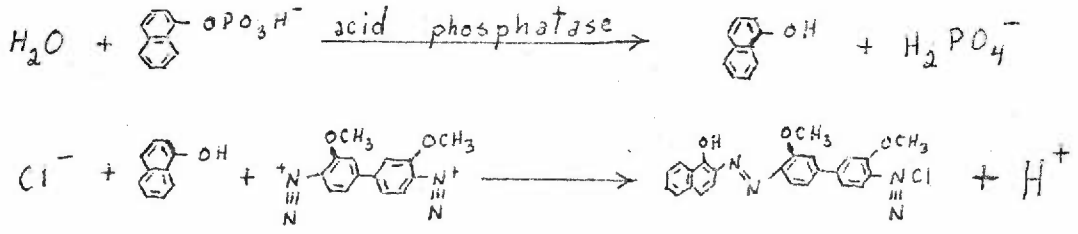
The histochemical phenomenon known as ATPase is, in part, related to the electron transport system. In mitochondria that have been damaged by freezing and thawing (inevitable in histochemistry),

the couplers of oxidative phosphorylation, which normally produce ATP, hydrolyze ATP to ADP and phosphate ions (Racker, 1968; Meyers & Slater, 1957). Unfortunately, almost every membrane (Meyers & Slater, 1957; Wachstein, Bradshaw, & Ortiz, 1962; Wachstein & Fernandez, 1964) and contractile protein (Mommaerts & Seraidarian, 1947; Green, 1964) in the cell has an ATP-ase activity. These "ATPases" cannot be differentiated without extensive inhibitor and activator studies and comparisons.

Other Enzymes

Acid and alkaline phosphatase are histochemical phenomena that have considerable value as markers (Novikoff, 1961; Ellis, 1961; Quevedo & Montagna, 1962; Kechijan, 1965). They probably represent several enzymes capable of catalyzing the same reactions. Acid phosphatase has a pH optimum < 7 (usually ~ 5); alkaline phosphatase has an optimum > 7 (usually 9 to 10) (Pearse, 1961). In vivo, they probably act as phosphotransferases (Meyerhof & Green, 1950; Morton, 1955).

The Burton (1954) method for acid phosphatase depends on the reaction of dephosphorylated naphthol with o-dianisidine bis-diazonium chloride to produce a red precipitate:



Specificity is good but the reaction is weak and localization is not sharp.

The Gomori (1952) method for alkaline phosphatase is the classic type for ionic histochemical reactions.

$$H_2O + (CH_2OH)_2\,CHOPO_3^{=} \xrightarrow{\text{alkaline phosphatase}} (CH_2OH)_2\,CHOH + HPO_4^{=}$$

$$HPO_4^{=} + Ca^{++} \longrightarrow CaHPO_4\downarrow$$

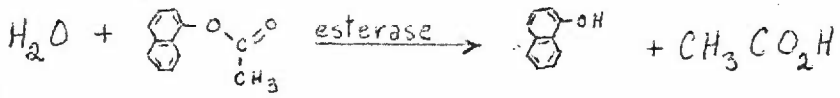
$$2\,\underline{CaHPO_4} + 3\,CoCl_2 \longrightarrow 2\,CaCl_2 + \underline{Co_3(PO_4)_2} + 2HCl$$

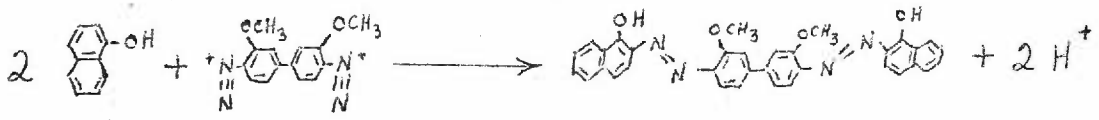
$$\underline{Co_3(PO_4)_2} + 3S = \longrightarrow 3\underline{CoS} + 2\,PO_4^{\equiv}$$

Specificity is poor since nucleic acids bind divalent ions, but localization is sharp.

"Nonspecific carboxylic esterase" includes a number of enzymes, with at least two different types of active center, whose biological function is unknown (Myers, 1960).

The Pearse (1961) method for nonspecific carboxylic esterase resembles the Burton (1954) method for acid phosphatase:





Localization is better here than in the acid phosphatase technique, probably because the higher pH allows both diazo groups to react (Pearse, 1961). The reaction is capricious in my hands, but failures are so obvious that they can be discounted.

Aminopeptidases remove the N-terminal amino acid from polypeptides. The number of enzymes demonstrated by the histochemical reaction is a matter of opinion (Fruton, Irving & Bergmann, 1941; Smith & Hill, 1960; Adachi & Montagna, 1961). This reaction (Nachlas, Crawford, and Seligman, 1957) also depends on the coupling of a substituted naphthalene released by the enzyme with o-dianisidine bis-diazonium chloride. The substituent on the naphthalene in this case is a β-amino group, which makes the end product capable of chelating copper and thus becoming insoluble in xylene. The resulting preparations are beautiful if the incubation time is not so long that crystals form. The reaction seems to be reliable.

III. Comparative Anatomy and Physiology

My description of the secretory coil of the eccrine sweat glands of the rhesus concurs with the briefer description of Montagna et al (1964). Matsumoto, Yoshida, and Tataneo (1960) reported a broader distribution of alkaline phosphatase in acetone-fixed sections from the Japanese monkey, Macaca fuscata, than I found in formalin-fixed sections from the rhesus, M. mulatta. The difference is probably due to fixation rather than to species differences. I agree with Pearse (1961) that formalin fixation gives the truer picture.

Terzakis' (1964) low-power electron micrographs of the secretory coils of the palmar sweat glands of the African green monkey, Cercopithecus aethiops Linnaeus 1766, show a structure similar to

what I found in the rhesus. As in the rhesus, there are a few cells intermediate between dark and clear types. The myoepithelial cells appear farther apart in the green monkey than in the rhesus.

Comparison of many pictures of the eccrine secretory coils of man and the rhesus suggests that the ratio of nucleus to cytoplasm in the dark and clear cells is higher in the rhesus than in man. The clear dichotomy between the dark and clear cells in man (Montagna et al, 1953; Munger, 1961; Ellis, 1968) distinguishes his sweat glands from those of the rhesus, although some sections of the rhesus secretory coil contain no intermediate cell types. In paraffin sections the dark cell granules of man usually stain metachromatically with toluidine blue (Montagna et al, 1953; Ellis, 1968) and bind colloidal iron (Constantine & Mowry, 1966); those of the rhesus did not. Human dark cell granules also stain less constantly with the PAS technique (Fusaro & Goltz, 1961). The dark cells of the rhesus often contain traces of stainable glycogen; human dark cells do not (Ellis, 1968). (Since human dark cells appear to contain phosphorylase, it is likely that they contain glycogen in amounts less than currently necessary for histochemical recognition.)

The spacing of the myoepithelial cells in man resembles that in the rhesus rather than that in the green monkey. Kuno (1956) reports a diameter for the human secretory coil similar to what I found in the rhesus.

The dark cell - clear cell distinction has been found in all of the Catarrhini whose skin has been studied (Parakkal, Montagna, & Ellis, 1962; Ellis & Montagna, 1962; Montagna & Yun, 1962; 1963; Machida, Perkins & Montagna, 1964; Machida, Perkins, Montagna & Giacometti, 1965; Montagna, Machida & Perkins, 1966; Perkins, Arao & Dolnick, 1968). It is also found in the African lorisoids, but not in the Asiatic lorisoids (Ellis & Montagna, 1963). Oddly, the relative positions of the dark and clear cells are reversed in one and only one of the African lorisoids (Montagna, Machida & Perkins, 1966).

Although the eccrine sweat glands of the uacari, Cacajao rubicundus, I. Geoffroy, 1848, contain only a single, intermediate type of secretory cell (Perkins, Arao & Uno, 1968), the glands of all other Platyrhini studied, including the primitive Aotes, contain both dark and clear cells (Hanson & Montagna, 1962; Perkins, 1966; Machida & Perkins, 1966; Perkins & Machida, 1967; Machida, Perkins & Hu, 1967; Perkins, 1968 a & b ; Machida & Giacometti, 1968).

Among the lemurs, Lemur mongoz has both dark and clear cells (Montagna & Yun, 1963) whereas L. catta has only clear cells (Montagna & Yun, 1962). A tree shrew, Tupaia glis (Diard 1820), has a single intermediate type of secretory cell (Montagna, Yun, Silver & Quevedo, 1962), and the sweat glands of the tarsier are so unique as to defy classification (Montagna & Machida, 1966).

The eccrine sweat glands on the snout of the pig (Montagna & Yun, 1964) and on the paws of the cat (Munger & Brusilow, 1961) and

dog (Machida, Giacometti, & Perkins, 1966) contain both dark and clear cells although the epithelium is not pseudostratified in the pig and cat as it is in the higher primates and the dog. Even the platypus, Ornithorhyncus paradoxus, has both dark and clear cells in the eccrine sweat glands of the bill (Montagna & Ellis, 1960). The eccrine sweat glands on the paws of the white rat (Wechsler & Fisher, 1968) and the opossum, Didelphis marsupialis Linnaeus 1758*, however, have no dark cells.

The apparent distributions of phosphorylase (Yasuda, Furosawa, & Ogata, 1958) succinate dehydrogenase (Braun-Falco & Rathjens, 1954), cytochrome oxidase (Ellis, 1968), alkaline and acid phosphatases (Shelly & Mescon, 1952), and aminopeptidase (Adachi & Montagna, 1961) are the same in the eccrine secretory coils of man as in those of the rhesus. The myoepithelial cells of the eccrine sweat glands of man contain more glycogen (Ellis, 1968) and nonspecific carboxylic esterase (Ellis, 1968) than do those of the rhesus. The distribution of these substances between dark and clear cells is the same in both animals.

The dark cells of the dog (Machida et al, 1966) and platypus (Montagna & Ellis, 1960) resemble those of the rhesus and man in containing more nonspecific esterase than the clear cells do, whereas those of the pig (Montagna & Yun, 1964) are distinguished by their nonspecific cholinesterase content.

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*Tatsuyoshi Arao, personal communication, 1968.

The lumina of the human eccrine sweat ducts usually appear wider than those of the rhesus (cf. Dobson et al, 1961; Shibasaki & Ito, 1967). The exterior diameter, however, is similar (Kuno, 1956). Other structural features are, so far as I can tell, the same.

The coiled ducts of the eccrine sweat glands of the green monkey are relatively shorter than the coiled ducts of man and the rhesus (Machida et al, 1964). Aotes is the only member of the Anthropoidea known to lack a coiled duct (Hanson & Montagna, 1962; Perkins 1968(b)). Although the eccrine sweat glands of lemurs and lorisoids have no coiled ducts those of Tupaia do (Montagna, Yun, Silver & Quevedo, 1962).

The eccrine sweat glands of the cat, rat, pig, and platypus have no coiled ducts. The straight duct of the cat is like that of man and the rhesus (Munger & Brusilow, 1961). The straight duct of the rat often lacks a cuticular border (Wechsler & Fisher, 1968).

Like the rhesus, man has more glycogen and phosphorylase in the outer cells of his eccrine sweat ducts than in the inner cells (Montagna & Ellis, 1963).

The rhesus appears to be unique in showing spontaneous sweating the first few days after neurotmesis. Spontaneous eccrine sweating ceases immediately after neurotmesis in the African green monkey (Sakurai & Montagna, 1964), Lemur mongoz (Sakurai & Montagna, 1965), and the cat (Luchsinger, 1876; Lloyd, 1960), and usually in man (Lewis & Landis, 1930).

After neurotmesis human eccrine sweat glands retain their ability to respond to methacholine for about 3 weeks (Kahn & Rothman, 1942), as opposed to ~11 days for the rhesus and one week for the green monkey (Sakurai & Montagna, 1964). Neurotmesis does not abolish the responsiveness of the eccrine sweat glands of L. mongoz (Sakurai & Montagna, 1965) or the cat (Langley, 1922) to cholinergic drugs.

The enormous variety of eccrine sweat apparati among the primates and the presence in other orders of glands more like those of the Anthropoidea than those of the lowest primates and the differences within a single genus strengthen the hypothesis of parallelism in the evolution of sweat glands.

The pig is the one ungulate or subungulate known to possess eccrine sweat glands; the goat (Sar & Calhoun, 1966) and the rabbit (Montagna, 1959) are known to have none. This suggests the possibility that eccrine sweat glands were lost before the development of hooves and that the similarities of the eccrine sweat glands of the pig to those of other mammals are due more to functional necessities than to a direct phylogenetic relationship.

IV. Histophysiology

On the basis of my own work and the work of others, I would suggest 6 conclusions:

1. Clear cells actively transport sodium ions into the lumen of the secretory coil, and water follows the osmotic gradient to produce a secretory fluid isotonic with the tissue fluid.

2. Dark cells secrete a mucous that protects the glabrous skin from dessication.

3. The myoepithelial cells provide structural support for the secretory coil.

4. The coiled duct recovers sodium from the secretory fluid by active transport to make sweat hypotonic.

5. Most of the work of the coiled duct in the rhesus is done by a short section near its proximal end.

6. The principal, if not the only, "target organ" of the secretomotor nerves of the eccrine sweat glands are the clear cells.

Except for the fifth and sixth and the teleology of the second, these ideas are not original; however, much of my evidence and reasoning is original. I shall explain my reasoning for each of the above.

1. Clear cells actively transport sodium ions into the lumen of the secretory coil and water follows the osmotic gradient to produce a secretory fluid isotonic with the tissue fluid.

Since sweat glands can produce sweat in the absence of circulation (Kendall and Luchsinger, 1876) and produce sweat pressures in excess of blood pressure (Best and Taylor, 1955), sweat must be a secretion rather than a filtrate. Despite numerous attempts, active transport of water has never been shown in an animal cell membrane (Csaky, 1965). Furthermore, active transport of water would require such enormous amounts of energy as to be grossly inexpedient (Andersen and Ussing,

1960). Pinocytosis and elimination of fluid is highly unlikely since pinocytotic vesicles are not found on the outer membranes of sweat gland cells (cf. Munger, 1961; Terzakis, 1964; Shibasaki and Ito, 1967). The only other way to get water into the lumen is by an osmotic gradient. The sweat glands contain ouabain-sensitive adenosine triphosphatase (Adachi and Yamasawa, 1966a), an enzyme believed to be involved in the active transport of sodium and potassium ions (Judah and Ahmed, 1964; Csaky 1965).

The fact that the nerves are concentrated around the secretory coil and that sweating stops when they are destroyed strongly suggests that secretion takes place in the secretory coil rather than in the duct. (If the secretory coil were a mere reservoir, destroying the nerves to it would not stop spontaneous sweating although it might end the response to methacholine.)

Active transport requires much energy (Junquiera 1965). The clear cells quickly shift from glycogen synthesis to glycogenolysis in sweating and store huge quantities of glycogen after neurotmesis. It is very likely that they are responsible for secretion of the secretory fluid.

2. Dark cells secrete a mucous that protects the glabrous skin from dessication.

Since salivary mucin is a mucoprotein (Blix 1963; Gottschalk 1963), it is reasonable to call the mucoprotein of human sweat

(Jirka and Kotas, 1957: Pallvicini, Gabriel, di Sant'Agnese and Buskirk, 1963) mucin. There is little doubt that the dark cells are the source of the mucin of human sweat (Lee, 1960; Munger 1961; Constantine and Mowry, 1966).

The similarity of the reactions of the dark cell granules of almost all animals to stains for polysaccharides suggests that the secretion of mucin is a universal function of the dark cells of eccrine sweat glands.

I doubt that the sole function of eccrine sweat gland mucin is to prevent the ducts from becoming a route of infection (Lee, 1960). Since mucin protects other body surfaces from dessication (Finerty and Cowdry, 1960; Bang and Bang, 1963) and irritation (Falk, Kotin, and Rowlette, 1963), it is probable that eccrine sweat mucin serves this purpose also. The glabrous skin, where most eccrine sweat glands are found, has no other glands to provide a protective film. As dark cells show less Krebs cycle activity than clear cells and do not appear to change their energy output during sweating, it is unlikely that they contribute to the active transport that produces the secretory fluid.

3. The myoepithelial cells provide structural support for the secretory coil.

The myoepithelial cells of salivary glands (Babkin, 1950; Burgen and Emmelin, 1961), mammary glands (Linzell, 1955) and apocrine sweat glands (Hurley and Shelley, 1960) are considered contractile. The smooth muscle stimulant, oxytocin, causes delivery

of saliva (Kay 1954), milk (Whittlestone, 1952; Munsick, 1965) and apocrine sweat (Hurley and Shelley, 1960). Adenosine triphosphatase activity is a notable feature of the myoepithelial cells of salivary glands (Shear, 1964; Smith, 1969). The myoepithelial cells of eccrine sweat glands do not contain adenosine triphosphatase (Im and Montagna, 1965), nor do eccrine sweat glands respond to oxytocin (Hurley and Witkowski, 1962).

Although clear cells and coiled duct cells show histochemical changes during sweating and after neurotmesis, the myoepithelial cells of the rhesus eccrine sweat gland show no histochemical response to either event.

It is thus unlikely that the myoepithelial cells of eccrine sweat glands express sweat.

Except for the sweat glands of the general body surface of primates, eccrine sweat glands are organs of the glabrous skin on paws, ischial callosities, prehensile tails, and rooting snouts which make frequent rough contact with solid parts of the environment. It is likely that the myoepithelial cells prevent the secretory coil from being squashed in these encounters. Ellis (1968) has suggested that the cuticular border serves to keep the duct patent.

4. The duct resorbs sodium from the secretory fluid.

This has been shown in man (Bulmer and Forwell, 1956; Cage and Dobson, 1965). Homology is the strongest argument for it in the rhesus, although the increase in the proportion of phosphorylase in

the active form during sweating and the increase in glycogen during the period of inactivity following neurotmesis strengthen the case.

5. Most of the work of the coiled duct in the rhesus is done by a short section near its proximal end.

The variation in enzyme activity along the length of the coiled duct makes this conclusion hard to avoid. (This variation appears in most of the enzyme pictures and is very clearly shown in Fig. 20.)

6. The principal, if not the only, "target organ" of the secretomotor nerves of the eccrine sweat glands are the clear cells.

Since the nerves show orientation only with respect to the secretory coil, it is likely that they act on and only on it. Since the myoepithelial cells show no evidence of contractility, the pulsed delivery of sweat (Kuno 1956) must be the result of intermittent secretion in response to intermittent neural stimulation (Wang 1964), rather than to waves of myoepithelial contraction. Finally, removal of the source of nervous stimulation halts not only sweat production but the capacity for sweat production. Since the clear cells produce most of the secretory fluid, they must be the ultimate site of secretomotor stimulation. (The data do not permit one to rule out a neurotransmitter function for the myoepithelium).

Since most of this evidence is from the Catarrhini, the conclusion is valid only for catarrhines. There is some evidence

of myoepithelial contractility in the eccrine sweat glands of the cat (Sperling and Koppanyi, 1949).

Concluding Note

Although I have indulged in considerable physiological speculation on the roles of the various parts of the palmar eccrine sweat glands of the rhesus, my anatomical and histochemical data are of more importance than my inferences from them. I have merely supplied an anatomical foundation which I hope will assist other disciplines in explaining the functioning of these organs. When the necessary physiological work has been done, it is likely that the eccrine sweat glands of the rhesus will prove to be excellent models for an understanding of those of man.

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APPENDICES

APPENDIX A

Systematic Names of Enzymes (from Enzyme nomenclature. Recommendations 1964 of the International Union of Biochemistry, Amsterdam, Elsevier, 1965).

Name in Thesis	I. U. B. No.	Systematic Name
acetylcholinesterase	3.1.1.7	acetylcholine acetyl-hydrolase
acid phosphatase	3.1.3.2	orthophosphoric monoester phosphohydrolase [probably more than 1 enzyme]
alkaline phosphatase	3.1.3.1	orthophosphoric monoester phosphohydrolase
aminopeptidase	3.4.1.1 and 3.5.1.13	L-leucyl-peptide hydrolase [possibly two enzymes] aryl-acylamide amino-hydrolase
amylo-1,6-glucosidase	3.2.1.33	dextrin 6-glucanohydrolase
ATP ase	3.6.1.3	ATP phosphohydrolase [several enzyme and non-enzyme catalysts]
branching enzyme	2.4.1.24	α-1, 4-glucan: α-1, 4-oligoglucan 6-glucosyltransferase

citrate condensing enzyme	4.1.3.7	citrate oxaloacetate-lyase (Co-A acetylating)
cytochrome oxidase	1.9.3.1	ferrocytochrome c: oxygen oxidoreductase
DPN diaphorase	1.6.4.3 and unknowns	reduced-NAD: lipoamide oxidoreductase
DPN-linked isocitrate dehydrogenase	1.1.1.41	threo-D_s-isocitrate: NAD oxidoreductase (decarboxylating)
enolase	4.2.1.11	2-phospho-D-glycerate hydro-lyase
fructoaldolase	4.1.2.13	fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate-lyase
fumarase	4.2.1.2	L-malate hydro-lyase
glucose-6-phosphate dehydrogenase	1.1.1.49	D-glucose-6-phosphate: NADP oxidoreductase

glucose-1-phosphate uridyltransferase	2.7.7.9	UTP: α-D-glucose-1-phosphate uridylyltransferase
glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	D-glyceraldehyde-3 phosphate: NAD oxidoreductase (phosphorylating)
glycogen synthetase	2.4.1.11	UDP glucose: glycogen α-4-glucosyltransferase
glcogen synthetase kinase	none assigned	ATP: UDP glucose-glycogen-glucosyltransferase phosphotransferase
glycogen synthetase phosphatase	none assigned	UDP glucose-glycogen-glucosyltransferase phosphohydrolase
hexokinase	2.7.1.1	ATP: D-hexose 6-phosphotransferase

α-ketoglutarate carboxylase	none assigned, part of 1.2.4.2	2-oxoglutarate carboxylyase (acceptor α-hydroxyalkylating), part of 2-oxoglutarate: lipoate oxidoreductase (acceptor acylating)
lactonase	3.1.1.17	D-glucono-δ-lactone hydrolase
lipoate dehydrogenase	none assigned, part of 1.2.4.2	dihydrolipoate: (acceptor) oxidoreductase, part of 2-oxoglutarate: lipoate oxidoreductase (acceptor acylating)
lipoate reductase-transacetylase	none assigned, part of 1.2.4.1	1-hydroxyethyl thiamine pyrophosphate: lipoate oxidoreductase (Co-A acetylating), part of pyruvate: lipoate oxidoreductase (acceptor-acetylating)

lipoate reductase-transacylase	none assigned, part of 1.2.4.2	1-hydroxyalkyl thiamine pyrophosphate: lipoate oxidoreductase (Co-A acylating), part of 2-oxoglutarate: lipoate oxidoreductase (acceptor acylating)
malate dehydrogenase	1.1.1.37	L-malate: NAD oxidoreductase
malic enzyme	1.1.1.40	L-malate: NADP oxidoreductase (decarboxylating)
nonspecific carboxylic esterase	3.1.1.1 and	carboxylic-ester hydrolase [probably more than 1 enzyme]
	3.1.1.2	aryl-ester hydrolase [probably more than 1 enzyme]
nonspecific cholinesterase	3.1.1.8	acylcholine acyl-hydrolase

phosphofructokinase	2.7.1.11	ATP: D-fructose-6-phosphate 1-phosphotransferase
phosphoglucokinase	2.7.1.10	ATP: D-glucose-1-phosphate 6-phosphotransferase
phosphoglucomutase	2.7.5.1	α-D-glucose-1, 6-diphosphate: α-D-glucose-1-phosphate phosphotransferase
6-phosphogluconate dehydrogenase	1.1.1.44	6-phospho-D-gluconate: NADP oxidoreductase (decarboxylating)
phosphoglucose isomerase	5.3.1.9	D-glucose-6-phosphate ketol-isomerase
phosphoglycerate kinase	2.7.2.3	ATP: 3-phospho-D-glycerate 1-phosphotransferase
phosphoglycerate mutase	5.4.2.1	D-phosphoglycerate 2, 3-phosphomutase

phosphopentose epimerase	5.1.3.1	D-ribulose-5-phosphate 3-epimerase
phosphoribose isomerase	5.3.1.6	D-ribose-5-phosphate ketol-isomerase
phosphorylase	2.4.1.1	α-1, 4-glucan: orthophosphate glucosyltransferase
phosphorylase kinase	2.7.1.38	ATP: phosphorylase phosphotransferase
phosphorylase phosphatase	3.1.3.17	phosphorylase phosphohydrolase
pyridine nucleotide transhydrogenase	1.6.1.1	reduced-NADP: NAD oxidoreductase
pyruvate carboxylase	none assigned	pyruvate carboxy-lyase (acceptor α-hydroxyethylating),
	part of 1.2.4.1	part of pyruvate: lipoate oxidoreductase (acceptor acetylating)

pyruvate kinase	2.7.1.40	ATP: pyruvate phosphotransferase
succinate dehydrogenase	1.3.99.1	succinate: (acceptor) oxidoreductase
succinate thiokinase	6.2.1.4	succinate: Co A ligase (GDP)
TPN diaphorase	1.6.99.2	reduced NAD (P): (acceptor) oxidoreductase [possibly more than 1 enzyme]
	and	
	1.6.2.3	[infra]
	and	
	unknowns	
TPNH-cytochrome reductase	1.6.2.3	reduced NADP: cytochrome c oxidoreductase [In Report of the enzyme commission. Oxford, Pergamon, 1961. Not in 1964 Recommendations. Physiologic existence disputed].

TPN-linked isocitrate dehydrogenase	1.1.1.42	threo-D_s-isocitrate: NADP oxidoreductase (decarboxylating) [2 enzymes]
transaldolase	2.2.1.2	sedoheptulose-7-phosphate: D-glyceraldehyde-3-phosphate dihydroxyacetonetransferase
transketolase	2.2.1.1	sedoheptulose-7-phosphate D-glyceraldehyde-3-phosphate glycolaldehyde-transferase
triose isomerase	5.3.1.1	D-glyceraldehyde-3-phosphate ketol-isomerase

APPENDIX B
RAW DATA

The last table (p. 153) is a compilation of numbers of changes from the first 8 tables. The numbers in the first 8 tables are grades of staining intensity. 0 means no visible stain; 1, barely visible; 2 to 5, increasing intensities of staining. There are a few intergrades indicated by + and - signs. $2 < 2+ < 3- < 3$. A slash (/) indicates that the condition of the slide was such that the information could not be obtained.

The grades are not completely consistent from table to table or from one stain to another. The grading is consistent for any stain in any one animal (i.e. for each column in each table). Comparisons of stimulated and inhibited glands are possible because each animal was its own control in reading the slides as well as in making them.

None of the 5 animals in the glycogen study were among the 7 in the enzyme study.

A stands for atropine inhibited.

M stands for methacholine stimulated.

Glycogen Data

		first animal	second animal	third animal	fourth animal	fifth animal
Clear Cells	A	2	2	3	3	2
	M	0	1	1	0	0
Dark Cells	A	0	0	1	2	0
	M	0	0	1	0	0
Myoepithelial Cells	A	0	0	0	0	0
	M	0	0	0	0	0
Coiled Duct Inner Cells	A	0	0	0	1	2
	M	0	0	0	1	1
Coiled Duct Outer Cells	A	1	0	0	1	2
	M	0	0	0	1	1
Straight Duct Inner Cells	A	0	0	0	0	0
	M	0	0	0	0	0
Straight Duct Outer Cells	A	0	0	0	2	1
	M	0	0	0	1	1

Enzyme Study

First Animal

		Total glycogen synthetase	Glycogen synthetase I	Total phosphorylase	Phosphorylase a	Phosphoglucomutase	Fructoaldolase	Glucose-6-phosphate dehydrogenase	Succinate dehydrogenase	Cytochrome oxidase
Clear Cells	A	3	2	4	3	3	3	1	2	3
	M	3	1	3	3	4	3	1	3	3
Dark Cells	A	1	1	2	2	2	3	1	1	2
	M	2	1	2	1	2	3	1	1	2
Myoepithelial	A	0	0	2	1	1	1	1	1	2
Cells	M	0	0	1	0	1	1	0	1	2
Coiled Duct	A	3	0	2	0	3	4	1	4	3
Inner Cells	M	3	0	0	0	3	4	1	4	3
Coiled Duct	A	3	0	3	2	2	4	3	4	3
Outer Cells	M	3	1	3	2	2	4	3	3	3
Straight Duct	A	1	1	2	2	2	3	1	1	2
Inner Cells	M	2	0	2	2	3	2	1	2	2
Straight Duct	A	2	2	2	2	0	3	1	2	2
Outer Cells	M	3	0	2	2	0	2	1	2	2

Enzyme Study

Second Animal

		Total glycogen synthetase	Glycogen synthetase I	Total phosphorylase	Phosphorylase a	Phosphoglucomutase	Fructoaldolase	Glucose-6-phosphate dehydrogenase	Succinate dehydrogenase	Cytochrome oxidase
Clear Cells	A	3	2	3	3	2	3	1	2	2
	M	2	2	3	4	3	3	2	3	3
Dark Cells	A	2	0	0	0	2	3	1	0	2
	M	1	1	0	2	2	3	1	1	2
Myoepithelial	A	0	0	1	1	/	1	0	1	1
Cells	M	0	0	1	1	/	1	0	1	1
Coiled Duct	A	0	0	2	0	2	4	3	3	3
Inner Cells	M	1	0	2	0	2	4	3	3	3
Coiled Duct	A	3	0	2	2	2	4	3	4	3
Outer Cells	M	1	0	2	2	2	4	3	4	3
Straight Duct	A	0	0	2	2	2	3	2	3	3
Inner Cells	M	0	0	2	2	2	3	2	3	2
Straight Duct	A	2	0	2	2	0	3	2	3	3
Outer Cells	M	0	0	2	2	0	3	2	3	2

Enzyme Study

Third Animal

		Total glycogen synthetase	Glycogen synthetase I	Total phosphorylase	Phosphorylase a	Phosphoglucomutase	Fructoaldolase	Glucose-6-phosphate dehydrogenase	Succinate dehydrogenase	Cytochrome oxidase
Clear Cells	A	2	0	3	2	2	3	3	3	3
	M	3	1	3	4	3	3	3	3	3
Dark Cells	A	2	0	2	2	1	2	1	1	2
	M	0	0	2	2	2	3	1	1	2
Myoepithelial	A	0	0	1	1	/	1	1	1	1
Cells	M	0	0	0	0	1	1	1	1	1
Coiled Duct	A	0	0	2	2	2	4	2	4	4
Inner Cells	M	0	0	3	3	3	3	2	4	4
Coiled Duct	A	1	2	3	2	2	4	4	4	4
Outer Cells	M	2	0	3	3	2	3	4	4	4
Straight Duct	A	0	0	3	3	2	3	3	2	3
Inner Cells	M	2	0	3	3	3	2	2	2	3
Straight Duct	A	1	0	3	3	0	3	2	2	3
Outer Cells	M	0	0	3	3	2	2	1	2	3

Enzyme Study

Fourth Animal

		Total glycogen synthetase	Glycogen synthetase I	Total phosphorylase	Phosphorylase a	Phosphoglucomutase	Fructoaldolase	Glucose-6-phosphate dehydrogenase	Succinate dehydrogenase	Cytochrome oxidase
Clear Cells	A	2+	2-	3	2	2	3	2	3	2
	M	2	0	4	3+	2+	3+	2	3	3
Dark Cells	A	1	0	2	2	1	2	1	1	2
	M	1	0	3	2+	1	2	1	1	2
Myoepithelial Cells	A	0	0	1	0	/	1-	1	1	1
	M	0	0	1-	1	/	1	0	1+	1
Coiled Duct Inner Cells	A	0	0	3	2	2	3	2	3	4
	M	0	0	0	0	2	3	2	3	4
Coiled Duct Outer Cells	A	3	2	3	2	3	3	3	4	4
	M	2	1	2	2	3	3	3	4	4
Straight Duct Inner Cells	A	0	0	3	2	1	2	3	3	3
	M	0	0	3	2	2	2	3-	3	3
Straight Duct Outer Cells	A	1	0	3	2	1	2	1	3	3
	M	1	0	3	2	2	2	1	3	3

Enzyme Study

Fifth Animal

		Total glycogen synthetase	Glycogen synthetase I	Total phosphorylase	Phosphorylase a	Phosphoglucomutase	Fructoaldolase	Glucose-6-phosphate dehydrogenase	Succinate dehydrogenase	Cytochrome oxidase
Clear Cells	A	2	2	4	3	3	3	3	3	4
	M	3	1	4	5	4	3	3	3	4
Dark Cells	A	2	1	2	2	2	2	2	1	2
	M	0	0	2	2	2	2	2	1	2
Myoepithelial	A	0	0	0	0	0	0	0	0	1
Cells	M	0	0	0	0	0	0	0	0	1
Coiled Duct	A	2	2	2	2-	2	4	2	2	4
Inner Cells	M	2	0	2	2	2	4	2	2	4
Coiled Duct	A	2	2	3	2	4	4	4	4	4
Outer Cells	M	2	2	4	2	4	4	4	4	4
Straight Duct	A	0	0	3	2	3	0	2	3	3
Inner Cells	M	0	0	3	2+	3	2	2	3	3
Straight Duct	A	0	0	3	2	2	2	2	3	3
Outer Cells	M	0	0	3	2+	2	2	2	3	3

		Total glycogen synthetase	Glycogen synthetase I	Total phosphorylase	Phosphorylase a	Phosphoglucomutase	Fructoaldolase	Glucose-6-phosphate dehydrogenase	Succinate dehydrogenase	Cytochrome oxidase
Clear Cells	A	3	2	5	3	3	3	3	3	4
	M	3	1	5	5	4	3	3	3	4
Dark Cells	A	1	0	2	2	3	2	2	2	2
	M	1	0	2	2	3	2	2	2	2
Myoepithelial	A	0	0	2	0	1	1	2	1	1
Cells	M	0	0	1	1	1	1	2	1	1
Coiled Duct	A	1	0	0	0	3	4	2	4	4
Inner Cells	M	0	0	0	0	3	3	2	4	4
Coiled Duct	A	4	2	2	1	3	4	4	4	4
Outer Cells	M	3	0	2	2	3	3	3	4	4
Straight Duct	A	2	0	2	1	3	2	3	3	3
Inner Cells	M	2	0	2	2	3	2	3	3	3
Straight Duct	A	2	0	2	1	2	2	3	3	3
Outer Cells	M	2	0	2	2	2	2	3	3	3

Enzyme Study
Seventh Animal

		Total glycogen synthetase	Glycogen synthetase I	Total phosphorylase	Phosphorylase a	Phosphoglucomutase	Fructoaldolase	Glucose-6-phosphate dehydrogenase	Succinate dehydrogenase	Cytochrome oxidase
Clear Cells	A	2	2	3	2	2	3	3	3	3
	M	3	1	4	4	3	3	3	4	3
Dark Cells	A	1	1	3	2	3	2	2	2	2
	M	2	0	3	3	3	3	2	2	1
Myoepithelial	A	0	0	0	0	0	0	0	1	0
Cells	M	0	0	0	0	0	0	0	0	0
Coiled Duct	A	0	1	3	1	3	4	4	5	4
Inner Cells	M	1	1	3	1	3	4	4	5	4
Coiled Duct	A	3	3	3	2	3	4	4	5	4
Outer Cells	M	2	2	3	3	3	4	4	5	4
Straight Duct	A	0	0	3	1	3	2	3	2	2
Inner Cells	M	0	2	3	2	3	2	3	2	2
Straight Duct	A	1	2	3	2	2	2	2	3	2
Outer Cells	M	1	1	3	2	2	2	3	4	2

Compilation

Number of animals in which cells of stimulated glands showed "changes" as compared to inhibited glands from the same animal.

Cell Type	Direction of "change" (inhibited gland as baseline)	Total glycogen synthetase	Glycogen synthetase I	Proportion of synthetase in I form	Glycogen	Total phosphorylase	Phosphorylase a	Proportion of phosphorylase in a form	Phosphoglucomutase	Fructoaldolase	Glucose-6-phosphate dehydrogenase	Succinate dehydrogenase	Cytochrome oxidase
Clear Cells	up	3	1	2	0	2	5	7	7	1	1	3	2
	down	2	5	5	5	1	0	0	0	0	0	0	0
	no change	2	1	0	0	4	2	0	0	6	6	4	5
Dark Cells	up	2	1	2	0	1	3	2	1	2	0	1	0
	down	3	2	2	1	0	1	2	0	0	0	0	1
	no change	2	4	3	4	6	3	3	6	5	7	6	6
Myoepithelial Cells	up	0	0	0	0	2	2	2	0	1	0	1	0
	down	0	0	0	0	2	2	0	0	0	2	1	0
	no change	7	7	7	5	3	3	5	4	6	5	5	7
Coiled Duct -inner cells	up	2	0	1	0	1	2	3	1	0	0	0	0
	down	1	1	3	1	2	1	0	0	2	0	0	0
	no change	4	6	3	4	3	4	4	6	5	7	7	7
Coiled Duct -outer cells	up	1	1	2	0	1	3	4	0	0	0	0	0
	down	4	4	3	2	1	0	1	0	2	1	1	0
	no change	2	2	2	3	5	4	2	7	5	6	7	6
Straight Duct -inner cells	up	2	0	1	0	0	3	3	2	1	0	1	0
	down	0	2	2	0	0	0	0	0	2	1	0	1
	no change	5	5	4	5	7	4	4	5	4	6	6	6
Straight Duct -outer cells	up	1	0	2	0	0	2	2	2	0	1	1	0
	down	2	1	2	1	0	0	0	0	2	1	0	1
	no change	4	6	3	4	7	5	5	5	5	5	6	6

APPENDIX C
THE KOLMOGOROV TEST

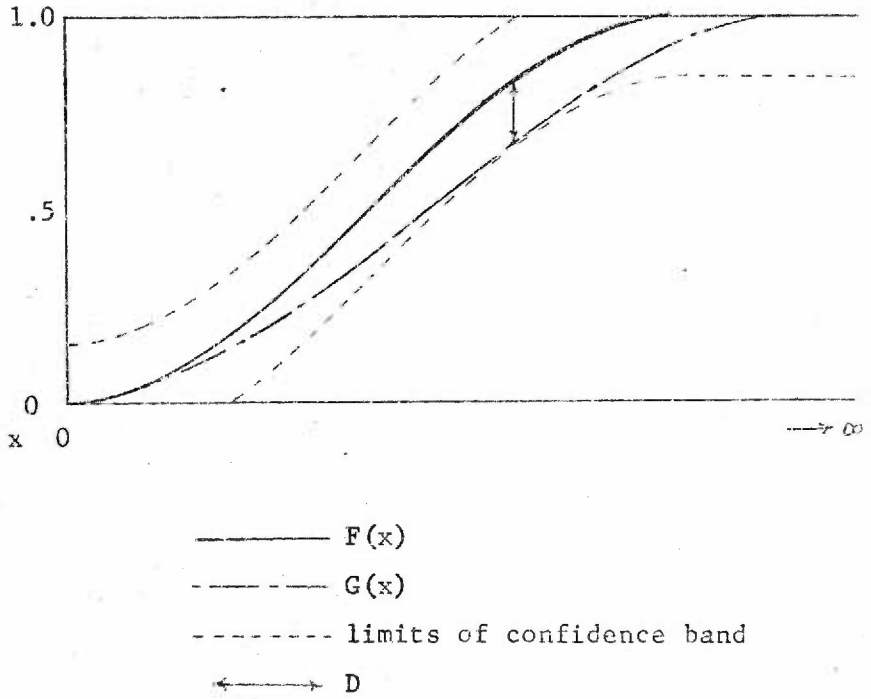
This is an unweighted cumulative distribution function test. The test answers the question, "How often would the cumulative distributions of random samples from the same population as sample 1 touch or cross the limits of the narrowest confidence band around the cumulative distribution of sample 1 containing all points of the cumulative distribution of sample 2?" (Since the test is unweighted, the confidence band is of uniform width.) This one way of asking the classical statistical question, "How probable is the null hypothesis that both samples are from the same population?"

Let $F(x) \equiv$ proportion of sample $1 \leq x$

and $G(x) \equiv$ proportion of sample $2 \leq x$

and $D = \max|F(x) - G(x)|$,

where x = any member of a monotonic series (i.e., $x_k \leq x_{k+1}$, k=1,2,3....)



Obviously the width of the confidence band necessary to contain all points of the distribution of sample 2 is D, the absolute value of the maximum difference between F(x) and G(x). For a one-tailed test D must be in the predicted direction and is called D^+ or D^-, depending on the direction predicted.

Since larger random samples tend to represent the populations from which they were drawn better than smaller ones do, the sample distributions will approach the population distributions as sample sizes increase. (This is the Glivenko-Cantelli theorem.) The probability that the cumulative distributions of samples from the same population as the first sample will touch or cross the limits of the confidence band based on the second sample will depend on D and the sizes of both samples, decreasing as D or sample size increases.

For samples of equal size the probability that the samples are from the same population is given by the formula of Gnedenko and Korolyuk:

$$p = 1 - \frac{(N!)^2}{(2N)!} \sum_{k=0}^{k=N} (-1)^k \left[\frac{(2N)!}{(N-kND)!\ (N+kND)!} \right]$$

where N = size of each sample

A class is all values of $x \geq x_k$ and $< x_{k+1}$. Since D is the difference between cumulative distribution functions of x, combining classes can reduce the size of D but not increase it. Any class that has real (i.e., not infinitesimal) width is a combination of an infinite number of infinitesimal classes. Therefore, for any finite number of classes the test is conservative.

Sample Problem

Let x = ranks

f(x)= number of observations in the first sample given rank x

g(x)= number of observations in the second sample given rank x

F'(x)= number of observations in first sample having a rank $\leq x$

G'(x)= number of observations in second sample having a rank $\leq x$

$c = ND = \max\left|F'(x) - G'(x)\right|$

x	0	1	2	3	4
f(x)	0	1	0	3	3
g(x)	0	1	5	0	1
F'(x)	0	1	1	4	7
G'(x)	0	1	6	6	7
F'(x)-G'(x)	0	0	-5	-2	0

$c = \max\left|F'(x) - G'(x)\right| = \left|-5\right| = 5$

Locking in the table under N=7 and c=5 one finds p=.000. For a one-tailed test, $c = \max\left[F'(x) - G'(x)\right]$ and p is $\sim \frac{1}{2}$ that given in the table.

Table of two-tailed p for the Kolmogorov test
(after Birnbaum)

N	c=0	1	2	3	4	5	6	7	≥8
3	1.00	.778	.074	.000					
4	1.00	.906	.188	.008	.000				
5	1.00	.962	.309	.030	.001	.000			
6	1.00	.985	.424	.066	.004	.000	.000		
7	1.00	.994	.526	.111	.011	.000	.000	.000	
8	1.00	.998	.614	.162	.023	.002	.000	.000	.000
9	1.00	.999	.687	.216	.039	.004	.000	.000	.000
10	1.00	1.00	.749	.271	.059	.008	.001	.000	.000
11	1.00	1.00	.799	.325	.083	.014	.001	.000	.000
12	1.00	1.00	.840	.378	.109	.021	.003	.000	.000
13	1.00	1.00	.873	.429	.137	.031	.005	.000	.000
14	1.00	1.00	.899	.477	.167	.042	.008	.001	.000
15	1.00	1.00	.920	.522	.197	.055	.011	.002	.000
16	1.00	1.00	.937	.564	.228	.069	.016	.003	.000

A Selected Bibliography on the Kolmogorov Test

Birnbaum, Z. W. Numerical tabulation of the distribution of Kolmogorov's statistic for finite sample size. J. Amer. Stat. Assoc., 1952. 47, 425-441. (A table for samples of equal size.)

Gnedenko, B. V. & Korolyuk, V. S. On the maximum discrepancy between two empirical distributions. Math. Rev., 1952. 13, 570-571. (A formula for p for samples of equal size.)

Goodman, L. A. Kolmogorov-Smirnov tests for psychological research.* Psychol. Bull., 1954. 51, 160-168. (A simple explanation of the basis of the test with directions and tables for its use.)

Massey, F. J., Jr. Distribution table for the deviation between two sample cumulatives. Ann. Math. Stat., 1952. 23, 435-441. (A table for samples of unequal sizes.)

Noether, G. E. Note on the Kolmogorov statistic in the discrete case. Metrika, 1963. 7, 115-116. (A mathematical discussion of the effects of discontinuities in the cumulative distribution function on the validity of the test.)

Noether, G. E. Elements of nonparametric statistics. New York, John Wiley & Sons, 1967. (pages 7-23) (A mathematical discussion of the basis of the test.)

Siegal, S. Nonparametric statistics for the behavioral sciences. New York, McGraw-Hill, 1956. (pages 127-136 & 278-279) (Cookbook directions and tables for carrying out the test.)

Slakter, M. J. A comparison of the Pearson chi-square and Kolmogorov goodness-of-fit tests with respect to validity. J. Amer. Stat. Assoc., 1965. 60, 854-858. (A Monte-Carlo simulation study of the effects of ties and sample size on validity.)

* In the non-mathematical literature the Kolmogorov test is sometimes referred to as the "Kolmogorov-Smirnov test" because N. Smirnov published the first table for the test. Smirnov's name should be dropped for the sake of clarity since it has come to be associated with weighted tests.

APPENDIX D

Histochemical Techniques

Fixatives (araldite sections)

BRENNER'S BROTH

Brenner, R. M. Fine structure of adrenocortical cells in adult male rhesus monkeys. Amer. J. Anat., 1966. 119, 429-453

Make under a hood!

1.5 g paraformaldehyde

49 ml water

Heat to 60° C and stir with "bug". Slowly add 0.5 M NaOH until paraformaldehyde dissloves,

Add:

.75 ml 50% (by weight) aqueous glutaraldehyde (must be "Fisher" biological grade)

2.25 g sucrose

.5 g sodium cacodylate trihydrate (K & K)

.025 g anhydrous magnesium sulfate

Chill to 4° C.

This solution can be stored

Just before use add:

.025 g anhydrous calcium chloride

Fix tissue blocks 30 minutes at 4° C.

Fixatives (araldite sections)

BUFFERED GLUTARALDEHYDE

Make and use under a hood!

41 ml 0.1M pH 7.2 cacodylate buffer

9 ml 25% aqueous glutaraldehyde

Store at 40° C.

Fix tissue 2 hr. at 4° C.

EMBEDDING AND SECTIONING (araldite sections)

Solutions

0.1 M pH 7.2 cacodylate buffer:

2.14 g sodium cacodylate trihydrate

50 ml water

4.2 ml 0.2 M HCI (or equivalent)

(.07 ml 37% HCI)

Add water to make 100 ml.

1% osmic acid solution:

Make and use in a hood!

Put 12.5 ml water in a 4 oz. bottle. Scratch a 250 mg ampoule of osmium tetroxide with a file and gently slide the ampoule into the bottle. Seal the bottle and shake until the ampoule breaks. Leave the sealed bottle under a hood for 24 hr. Store the bottle in a closed peanut butter jar in the refrigerator (4° C).

Disslove 0.8 g sucrose in 10 ml cacodylate buffer (supra). Store at 4° C. Immediately before use mix 1 ml of each solution to make 1% osmic acid.

EMBEDDING AND SECTIONING (araldite sections)

Araldite:

Components are reputedly carcinogenic. Measure in disposable graduate or syringe.

4 ml Araldite Resin 502 (Ladd Industries)

6 ml DDSA (dodecenylsuccinic anhydride-Ladd)

0.2 ml DMP-30 ((N, N-dimethylaminomethyl) phenol-Ladd)

Stir 10 minutes.

Use at once or store at 4° C not over 2 days.

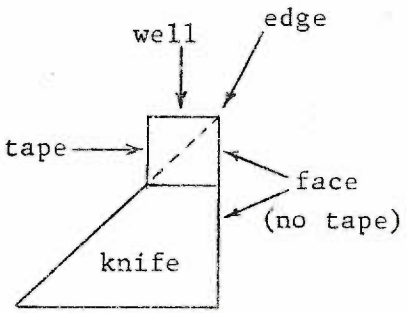
1. Fix.
2. 0.1 M pH 7.2 cacodylate buffer (supra), overnight.
3. 1% osmic acid (supra), 2 hours.
4. 30% aqueous ethanol at 4° C, 5 minutes.
5. 50% ethanol at 4° C, 5 minutes.
6. 70% ethanol at 4° C, 5 minutes.
7. 85% ethanol at 4° C, 10 minutes.
8. 95% ethanol at 4° C, 10 minutes.
9. 100% ethanol (drug grade) at 4° C, 2 changes, 15 minutes each.
10. Propylene oxide at 15-20° C, 2 changes, 30 minutes each.
11. 50-50 (by volume) propylene oxide-araldite at 4° C, overnight.
12. Araldite in labelled capsules at 25° C, 3 hours.
13. Position tissue in bottom of capsule with desired orientation.
14. 45° C, 5 days.

15. Slit capsule and remove block.

16. Trim block around tissue-a "Weck" single edge razor blade is the best tool for this.

17. Cut.

I cut sections with glass knives made with a "Messer" knifemaker (distributed by C. W. French). I made a 1/4 inch deep well at the back of the knife with masking tape and sealed the well by painting molten white beeswax onto the tape and the adjacent surfaces of the knife (not inside the well or on the face!) and then touching the waxed surfaces to a hotplate set at 100 watts (~ 110° C).



After mounting the knife at a 4° angle in the knife holder of a Porter-Blum MT-1 microtome (Ivan Sorvall), I filled the well with water to above the level of the tape, and drained off water until the meniscus was flat and level with the edge of the knife.

After facing the block with an old knife, I used a new knife to cut sections. Most sections were cut at 1 μ. A few thicker sections, up to 12 μ, were cut by holding down the leading edge of the section with a bamboo cake testing stick (Import Plaza) while cutting.

18. Transfer sections with bamboo stick to a drop of water on a microscope slide.
19. Slowly (less than 1 minute is too fast) evaporate water to dryness over the oxidizing flame (vents open) of a small Bunsen burner. The water must not boil.
20. Hold the slide in the flame, tissue side up, 5 seconds.
21. Set the slide on a dry towel to cool.

Stains (araldite sections)

TOLUIDINE BLUE or SAFRANIN

Traditional.

0.1 g toluidine blue O (C.I. 52040) or saffranin O (C.I. 50240)

0.1 g "Boraxo" (U.S. Borax & Chemical Corp.)

10 ml water

1. Cover sections with a few drops of stain ($\sim$ 0.1 ml).
2. Heat on a hotplate set at 100 watts ($\sim$ 110° C) until steam appears, $\sim$ 30 seconds. Do not boil stain.
3. Remove slide from hotplate and allow to cool, 2 minutes.
4. Wash off stain with running distilled water.
5. Wipe underside of slide dry.
6. Replace slide on hotplate until dry, 5 minutes.
7. Cool slide.
8. Coverslip with D.P.X. (Edward Gurr).

Stains (araldite sections)

H + E (Harris)

Smith, A.A., Unpublished.

Stain:

1. Cover sections with a few drops of Harris hematoxylin.
2. Heat on hotplate, 2 minutes, adding a drop of stain every 15 seconds.
3. Remove slide from hotplate and allow to cool.
4. Rinse with distilled water.
5. Distilled water, 5 minutes.
6. Tap water, 5 minutes.
7. Wipe underside of slide dry.
8. Heat on hotplate, 5 minutes.
9. Cool slide.
10. Cover sections with a few drops of eosin.
11. Heat on hotplate, 1 minute, adding a drop of stain every 10 seconds.
12. Cool slide.
13. Rinse in 80% ethanol, 5 seconds.
14. Wipe underside of slide dry.
15. Heat on hotplate, 5 minutes.
16. Cool slide.
17. Coverslip with Permount (Fisher).

Fixatives (paraffin sections)

CARNOY'S FLUID

6 ml absolute ethanol

3 ml chloroform

1 ml glacial acetic acid

1. Fix 2 hours.
2. Wash 1 hour in absolute ethanol.
3. Clear and embed (p. 172).

Fixatives (paraffin sections)

HELLY'S FLUID

5 g mercuric chloride

2.5 g potassium dichromate

1 g anhydrous sodium sulfate

100 ml water

Fixative (make no more than 30 minutes before use):

9.5 ml stock solution

.5 ml formalin (~ 37% aqueous formaldehyde)

1. Fix, 4 hours.
2. Wash overnight in several changes of water.
3. 70% ethanol, 1 hour.
4. Dehydrargyrify (remove mercury) in 0.5% iodine in 80% ethanol, 2 hours.
5. 80% ethanol, 1 hour.
6. 95% ethanol, 1 hour.
7. Absolute ethanol, 5 changes, 15 minutes each.
8. Clear and embed.

EMBEDDING AND SECTIONING (paraffin sections)

1. Fix and wash (p. 170-171).
2. Clear in xylene, 3 changes, 10 minutes each.
3. Paraplast (Sherwood--do not use Paraplast-Plus) at 60° C (molten), overnight.
4. Fill mold with molten Paraplast.
5. Heat forceps in Bunsen burner flame, 2 seconds.
6. Transfer tissue to mold with heated forceps.
7. Allow mold to cool, 3 hours.
8. Remove paraffin block by tapping the mold upside down on a hard surface.
9. Fill a tissue bath with distilled water to just above the brim, add gelatin in the gel form to make a 0.01% solution and bring to 45° C.
10. Position a steel knife 7 to 8° from the vertical in the knife holder of a rotary microtome. I sharpened my knife 1 hour on a Trident honing plate (Aloe) with American Optical fine abrasive and cut about 2,000 sections between sharpenings. I used a Leitz microtome, which has a fairly accurate screw advance as opposed to the erratic inclined plane advances of most microtomes.
11. Lock the microtome and clamp the block in the chuck.
12. Face block (i.e., cut until the desired region of the tissue is reached).

13. Allow a piece of ice to warm up until a film of water appears on it.
14. Hold the ice against the face of the block for 20 to 30 seconds. This step must be repeated for every group of 8 to 20 sections.
15. Cut sections.

    I cut at 7 μ most of the time. Four μ sections made the observation of fine detail easier. In order to cut 4 μ sections I had to produce a tissue of uniform hardness by removing the epidermis before embedding. (Epidermis is harder than dermis). I cut a few thicker sections, up to 150 μ. These had to be cut very slowly from an unchilled block to avoid breaking the paraffin.
16. Pick up loose end of ribbon of 8 to 20 sections with forceps and separate the other end of the ribbon from the knife with a camel's hair brush.
17. Touch what is now the loose end of the ribbon to the rim of the tissue bath and slowly lay out the rest of the ribbon on the surface of the water.
18. Break the ribbon into pieces of the desired length by touching the curve of a Von Graefe eye dressing forceps (Lawton Surgical) in the closed position against the seam between two sections and opening the forceps.
19. Immerse 3/4 of the length of a microscope slide held vertically in the water bath.

20. Touch the slide to the end of a piece of ribbon and withdraw it from the water bath.
21. Adjust the position of the sections with a dissecting needle.
22. Set the slide vertically on a towel to drain, 30 minutes.
23. Label the slide.
24. Lay the slide flat on a slide warmer at 45° C, 2 days.
25. Slide may then be stored.

Stains (paraffin sections)

GENERAL PROCEDURES

"Bring to water"

1. Xylene, 2 changes, 10 minutes each.
2. Absolute ethanol, 3 changes, 5 minutes each.
3. 95% ethanol, 2 changes, 5 minutes each.
4. 80% ethanol, rinse.

Helly fixed tissue	Carnoy fixed tissue
5. 0.5% iodine in 80% ethanol, 5 minutes.	5. 70% ethanol, rinse.
6. 70% ethanol, 2 changes, rinse in each.	6. 60% ethanol, rinse.
7. 60% ethanol, rinse.	7. 50% ethanol, rinse.
8. 50% ethanol, rinse.	8. 30% ethanol, rinse.
9. 30% ethanol, rinse.	9. Water, rinse.
10. Water, 5 minutes.	10. Water, 5 minutes.
11. 5% sodium thiosulfate in water, 1 minute.	
12. Running water, 5 minutes.	

"Dehydrate, clear, and mount"

1. 70% ethanol, 5 minutes.
2. 80% ethanol, 5 minutes.
3. 95% ethanol, rinse.
4. 95% ethanol, 5 minutes.
5. Absolute ethanol, 3 changes, 3 minutes each.
6. Xylene, 3 changes, 5 minutes each.
7. Coverslip with Permount (Fisher).

Stains (paraffin sections)

BRAZILIN

Smith, A. A., Unpublished.

Staining solution:

0.25 g brazilin (C.I. 75280) (Hartman-Leddon Co.).

2.4 g ammonium alum = $NH_4 Al (SO_4)_2 \cdot 12H_2O$

8 ml ethanol

6.5 ml glycerol

25 ml distilled water

Dissolve all ingredients.

Add:

10 mg sodium iodate

Filter.

Add:

0.1 ml 10% ferric chloride hexahydrate.

1. Bring to water.
2. Staining solution, 15 minutes.
3. Rinse in tap water.
4. If necessary, differentiate with a 1% solution of conc. (37%) HCl in 70% ethanol, checking constantly under a 40x microscope, and rinse again in tap water.
5. Tap water, 5 minutes.
6. Dehydrate, clear, and mount.

Stains (paraffin sections)

COLLOIDAL IRON (Mowry)

Mowry, R. W. Improved procedure for the staining of acidic polysaccharides by Muller's colloidal (hydrous) ferric oxide and its combination with the Feulgen and the periodic acid-Schiff reactions. J. Clin. Invest., 1958. 7, 566-576.

Stock "solution":

6.75 g $FeCl_3 \cdot 6H_2O$

6 ml distilled water

0.4 ml conc. ($\sim$ 37%) HCl

Stir until dissolved.

Bring 250 ml distilled water to a boil.

Add $FeCl_3$ solution to the boiling water.

Cool.

Colloidal iron:

15 ml water

5 ml glacial acetic acid

20 ml stock "solution"

Ferrocyanic acid:

15 ml 2% potassium ferrocyanide trihydrate.

15 ml 2% solution of conc. (37%) HCl.

Make ferrocyanide solution 30 minutes before use.

Mix solutions just before use.

1NHCl:

27.5 ml water.

0.5 ml conc. (37%) HCl.

Sulfurous acid:

0.5 g sodium pyrosulfite = $Na_2 S_2 O_5$.

100 ml water.

0.5 ml conc. (37%) HCl.

1. Bring to water.
2. 12% acetic acid, 30 seconds.
3. Colloidal iron, 1 hour.
4. 12% acetic acid, 4 changes, 3 minutes each.
5. Ferrocyanic acid, 20 minutes.
6. Running tap water, 5 minutes.
7. 1 N HCl at 60° C, 10 minutes.
8. Running tap water, 5 minutes.
9. Distilled water, rinse.
10. Schiff's reagent (Hartman-Leddon), 10 minutes.
11. Sulfurous acid, 3 changes, 2 minutes each.
12. Running water, 5 minutes.
13. Dehydrate, clear, and mount.

Stains (paraffin sections)

COLLOIDAL IRON (Rhinehart & Abul-Haj)

Rinehart, J. F. & Abul-Haj, S. K. An improved method for histologic demonstration of acidic mucopolysaccharides in tissues. Arch. Path., 1951. 52, 189-194.

Stock solution:

100 g ferric chloride hexahydrate

333 ml distilled water

Dissolve and add:

133 ml glycerol

Mix well and add:

33 ml conc. (28%) ammonium hydroxide

Stir until precipitate dissolves and add:

17 ml conc. ammonium hydroxide

Stir until precipitate dissolves and add:

10 ml conc. ammonium hydroxide

Stir until precipitate dissolves and add:

6.2 ml conc. ammonium hydroxide

Stir until precipitate dissolves.

Soak a 55 cm (22 in.) length of 47 mm (1-7/8 in.) circular diameter seamless cellulose 24 Å pore radius dialysis tubing (Scientific Products, 1968 cat. no. D1615-3) in distilled water, 5 minutes. Tie off one end. Hold the tubing 45 cm (18 in.) from the

tied end and pour all of the above "solution" into the tube. Tie the open end where you are holding it - 45 cm from the other end. Immerse the filled tubing in 15 liters distilled water for 3 days, stirring constantly and changing the water every hour of the working day (9 times a day).

Open the tubing and filter its contents through Whatman no. 1 (fast, medium porosity) filter paper. The filtrate is the stock "solution" and will keep for years if light and air are excluded.

Colloidal iron:

28 ml stock "solution"

7 ml glacial acetic acid

Cochineal stain:

5.2 g ammonium alum = $NH_4Al(SO_4)_2 \cdot 12H_2O$

90 ml water

Heat to 85° C and add:

6 g cochineal powder (C.I. 75470) (Matheson, Coleman & Bell)

Stirring at 85° add, a drop by drop:

1.2 ml conc. (28%) ammonium hydroxide

Boil, 35 minutes.

Cool and filter.

If not used at once add a crystal of phenol, store at 4° C, and decant before using.

Picrofuchsin:

32 ml saturated aqueous picric acid

2 ml 1% aqueous acid fuchsin (C.I. 42685)

Ferrocyanic acid:

10 ml 2% potassium ferrocyanide trihydrate

20 ml 1% conc. (37%) HCl

Make up ferrocyanide solution 30 minutes before use.

Mix solutions just before use.

1. Bring to water.
2. 3% acetic acid, 30 seconds.
3. Colloidal iron, 10 minutes.
4. Distilled water, rinse.
5. Distilled water, 2 changes, 5 minutes each.
6. Ferrocyanic acid, 10 minutes.
7. Distilled water, 2 changes, rinse in each.
8. Cochineal, 15 minutes.
9. Running tap water, 10 minutes.
10. Picrofuchsin, 7 minutes.
11. 95% ethanol, 2 changes, rinse in each.
12. 95% ethanol, 3 minutes.
13. Absolute ethanol, 3 changes, 5 minutes each.
14. Xylene, 3 changes, 5 minutes each.
15. Coverslip with Permount (Fisher).

Stains (paraffin sections)

H + E (Ehrlich)

Traditional.

Ehrlich's hematoxylin:

0.64 g hematoxylin (C.I. 75290)

32 ml absolute ethanol

Dissolve and add:

32 ml glycerol

32 ml water

4 g ammonium alum = $NH_4Al(SO_4)_2 \cdot 12H_2O$

3.2 ml glacial acetic acid

Stir well and add:

64 mg sodium iodate

1. Bring to water.
2. Ehrlich's hematoxylin, 5 minutes.
3. Distilled water, rinse.
4. Saturated aqueous lithium carbonate, 5 minutes.
5. Water, 5 minutes.
6. 0.5% aqueous eosin Y (C.I. 45380), 5 minutes.
7. Water, 2 seconds.
8. 95% ethanol, 2 minutes.
9. Absolute ethanol, rinse.
10. Absolute ethanol, 2 changes, 2 minutes each.

11. Xylene, 3 changes, 5 minutes each.

12. Coverslip with Permount (Fisher).

Stains (paraffin sections)

H + E (Harris)

Traditional.

Harris hematoxylin:

1 g hematoxylin (C.I. 75290)

10 ml 95% ethanol

Dissolve.

20 g ammonium alum = $NH_4Al(SO_4)_2 \cdot 12H_2O$

200 ml distilled water

Dissolve and add the hematoxylin in alcohol.

Heat to boiling and slowly add:

0.5 g mercuric oxide

Boil, 1 minute.

Cool at once by putting vessel in cold water.

Filter.

Eosin:

0.6 g eosin Y (C.I. 45380)

150 ml water

50 ml absolute ethanol

1 drop ($\sim$ 0.05 ml) glacial acetic acid

Filter.

1. Bring to water.
2. Harris hematoxylin, 7 minutes.
3. Distilled water, 5 minutes.
4. 1% conc. (37%) HCl in 70% ethanol, 2 seconds.
5. Distilled water, 5 minutes.
6. Tap water, 5 minutes.
7. 30% ethanol, 3 minutes.
8. 50% ethanol, 3 minutes.
9. 60% ethanol, 3 minutes.
10. 70% ethanol, 3 minutes.
11. 80% ethanol, 3 minutes.
12. Eosin, 4 seconds.
13. 80% ethanol, rinse.
14. 95% ethanol, rinse.
15. 95% ethanol, 5 minutes.
16. Absolute ethanol, 3 changes, 3 minutes each.
17. Xylene, 3 changes, 5 minutes each.
18. Coverslip with Permount (Fisher).

Stains (paraffin sections)

IRON HEMATOXYLIN (Heidenhain)

(Can also be used for araldite sections)

Traditional.

Mordant:

1.5 g ferric ammonium sulfate dodecahydrate

60 ml distilled water

Hematoxylin.

0.2 g hematoxylin (C.I. 75290)

2 ml absolute ethanol

Dissolve and add:

30 ml distilled water

1. Bring to water (omit for araldite sections).
2. Distilled water, 3 changes, 5 minutes each.
3. Mordant, 24 hours.
4. Running tap water, 5 minutes.
5. Distilled water, 2 changes, 5 minutes each.
6. Hematoxylin, 24 hours.
7. Distilled water, 5 minutes.
8. Wipe underside and edges of slide dry.
9. Put slide under 40 X microscope.
10. Cover sections with a few drops of mordant and wait until the sections look right. For most purposes wait until all

structures but the nuclei have lost their stain. It is sometimes desirable to leave secretion granules and even muscle fibers stained.

11. Running tap water, 10 minutes.

12. Dehydrate, clear, and mount. (Dehydrate araldite sections by heating; use no alcohol or xylene).

Stains (paraffin sections)

METHYL GREEN-PYRONIN (Mulnard)

Mulnard, J. Unpublished.

Tissue must be fixed in Carnoy's fluid.

Washed methyl green:

Wash under a hood.

Put 1 g methyl green (C.I. 42590) into a funnel lined with a retentive filter paper (e.g., Whatman no. 42) and slowly pour 3 liters of chloroform through it. Throw away the filtrate and allow the residue to dry. This residue is relatively pure methyl green. (Commercial "methyl green" may be 1/4 or more crystal violet, C.I. 42555).

pH 4.66 Michaelis buffer:

0.97 g sodium acetate trihydrate

1.47 g sodium diethylbarbiturate (= veronal)

1.7 g sodium chloride

240 ml distilled water

10 ml 1 N HCl (formula, p. 179)

Methyl green pyronin:

0.1 g washed methyl green

0.2 g "Fluka" pyronin G (C.I. 45005) (International Chemical and Nuclear Corp.)

33 ml pH 4.66 Michaelis buffer

0.8 ml absolute ethanol

6.7 ml glycerol

TBA:

30 ml tert-butyl alcohol (=2-methyl-2-propanol)

10 ml absolute ethanol

1. Bring to water.
2. Methyl green-pyronin, 30 minutes.
3. TBA, 90 seconds.
4. tert-butyl alcohol, 3 minutes.
5. Xylene, 3 changes, 5 minutes each.
6. Coverslip with Permount (Fisher).

Stains (paraffin sections)

PARALDEHYDE FUCHSIN

U. S. Armed Forces Institute of Pathology. Manual of histologic and special staining techniques. 2nd ed. New York, McGraw-Hill, 1960. (page 78).

Paraldehyde fuchsin:

0.2 g basic fuchsin (mine is labelled C.I. 42510, but is probably mostly C.I. 42500).

40 ml 70% ethanol

0.4 ml conc. (37%) HCl

0.4 ml paraldehyde (=trimer of acetaldehyde)

Ripen 2 days at 25° C.

If not used then, store not over 3 months at 4° C.

Picrofuchsin:

0.5 ml 1% aqueous acid fuchsin (C.I. 42685)

40 ml saturated aqueous picric acid

1. Bring to water.
2. 70% ethanol, 5 minutes.
3. Paraldehyde fuchsin, 30 minutes.
4. 95% ethanol, rinse.
5. Water, rinse.
6. Picrofuchsin, 5 minutes.
7. 95% ethanol, rinse.

8. 95% ethanol, 3 minutes.

9. Absolute ethanol, 3 changes, 3 minutes each.

10. Xylene, 3 changes, 5 minutes each.

11. Coverslip with Permount (Fisher).

Stains (paraffin sections)

P A S

Lillie, R. D. Histopathologic technic and practical histochemistry. 2nd ed. New York, McGraw-Hill, 1954. (pages 124-126)

Montagna, W., Chase, H. B., and Lobitz, W. Histology and cytochemistry of human skin. IV. The eccrine sweat glands. J. Invest. Derm., 1953. 20, 415-423.

Schiff (Lillie):

I prefer to buy mine ready-made (Hartman-Leddon, cat. no. 2818). At the current Federal minimum wage, the break-even point for making one's own is 1 gallon.

Stain 10 minutes.

Schiff (Montagna):

5 ml Schiff's reagent (Hartman-Leddon)

35 ml sulfurous acid

Stain 1 hour in the dark.

Sulfurous acid:

0.8 g sodium pyrosulfite $=Na_2S_2O_5$

151 ml water

0.8 ml conc. (37%) HCl

Hematoxylin:

10 ml Harris hematoxylin (p. 185)

30 ml water

Diastase:

40 mg diastase ("diastase malt analytical," Nutritional Biochemicals. Do not use α-amylase: it takes the sections off the slides).

40 ml 0.02 M pH 6.0 phosphate buffer

Heat to 37° C and filter

pH 6.0 phosphate buffer:

3.2 g sodium chloride

0.113 g Na_2HPO_4

0.790 g $NaH_2PO_4 \cdot H_2O$

100 ml distilled water

1. Bring to water.
2. Digestion controls. (If one does not wish to draw specific histochemical conclusions this step may be omitted).

   Glycogen slide:

   pH 6.0 phosphate buffer, 1 hour at 37° C.

   Control slide:

   Diastase, 1 hour at 37° C.
3. Water, 5 minutes.
4. 0.5% aqueous periodic acid, 10 minutes.
5. Running tap water, 5 minutes.
6. Distilled water, 5 minutes.
7. Stain in Schiff.

8. Sulfurous acid, 3 changes, 2 minutes each.

9. Running tap water, 5 minutes.

10. Distilled water, 5 minutes.

11. Hematoxylin, 5 minutes.

12. Distilled water, 5 minutes.

13. Tap water, 5 minutes.

14. Dehydrate, clear, and mount.

Stains (paraffin sections)

TOLUIDINE BLUE

Montagna, W., Chase, H. B., and Melaragno, H. P. Histology and cytochemistry of human skin. I. Metachromasia in the mons pubis. J. Nat. Cancer Inst., 1951. 12, 591-597.

M/10 citric acid:

1.1 g citric acid

18.75 ml distilled water

6.25 ml methanol

M/5 dibasic phosphate:

0.17 g Na_2HPO_4

18.75 ml distilled water

6.25 ml methanol

Buffer:

23 ml M/10 citric acid

17 ml M/5 dibasic phosphate

1000 ml distilled water

N. B. This yields 1040 ml of buffer, pH 4.3.

Stain:

20 mg toluidine blue O (C.I. 52040)

40 ml buffer

The pH of this solution should be 4.5

1. Bring to water.
2. Stain, 15 minutes.
3. Distilled water, rinse.
4. tert-Butyl alcohol, 2 changes, 5 minutes each.
5. Absolute ethanol, 2 changes, 3 minutes each.
6. Toluene, 3 changes, 5 minutes each.
7. Mount in D. P. X. (Edward Gurr).

SECTIONING (frozen)

1. Freeze fresh tissue in liquid nitrogen or ligroin cooled by dry ice. (The latter is preferable for study of non-specific esterase).

   The tissue may be stored up to 6 months in liquid nitrogen. (Longer storage is deleterious because one's tissue is brought to higher temperatures every time a drawer-mate uses the freezer).

2. Sharpen a steel blade on an automatic sharpener, 2-6 hours with coarse abrasive, 2-4 hours with fine abrasive.
3. Bring cryostat and blade to -20° C. (This takes 5 hours from 25° C).
4. Place an object disk (specimen holder) in the quick-freeze rack of the cryostat.
5. Cover the grooved surface of the disk with water.
6. Turn on the quick-freeze control.
7. Lay the (frozen) tissue on the quick-freeze rack.
8. As soon as the water on the disk begins to freeze hold the tissue in the water until the tissue is frozen in place.
9. Add water until half of the tissue is covered.
10. When all the water is solidified turn off quick-freeze.
11. Wait 30 minutes.

12. Put disk in microtome chuck with hardest part of tissue (epidermis) toward knife.
13. Set the edge of the anti-roll plate against the edge of the knife and lift the plate so that it rests gently on (instead of being pressed against) the knife edge.
14. Cut sections. 20 μ is a good general purpose setting. 6 μ is ideal for ATP ase. 150 μ is best for nerves in skin (cholinesterase techniques).
15. Free the last section cut from the knife edge with a cold dissecting needle. (All but the last section will have been freed by the section following).
16. Arrange sections on knife in the array you want on the slide.
17. Touch the sections with the face of a warm (25° C) slide. The sections will adhere to the slide. (This takes practice). For ATP ase and glycogen synthetase studies brush the sections onto a cold (-20° C) slide with a cold dissecting needle; then warm underside of slide with finger until sections melt.
18. Place the slide, face up, in a 37° C incubator, 1 minute. (30 seconds for glycogen synthetase).
19. Carry out reaction.

Stains (frozen sections)

GENERAL PROCEDURES

"Dehydrate and mount in Diaphane:"

1. 30% aqueous ethanol, 3 minutes.
2. 50% ethanol, 3 minutes.
3. 60% ethanol, rinse.
4. 70% ethanol, 3 minutes.
5. 80% ethanol, rinse.
6. 95% ethanol, 2 changes. 3 minutes each.
7. Absolute ethanol, 2 changes, 3 minutes each.
8. Coverslip with Diaphane (Will).

"Dehydrate, clear, and mount:"

1. 30% ethanol, 3 minutes.
2. 50% ethanol, 3 minutes.
3. 60% ethanol, 3 minutes.
4. 70% ethanol, 3 minutes.
5. 80% ethanol, 3 minutes.
6. 95% ethanol, 3 changes, 3 minutes each.
7. Absolute ethanol, 3 changes, 5 minutes each.
8. Xylene, 3 changes, 5 minutes each.
9. Coverslip with Permount (Fisher).

"Coverslip with glycerol-gel:"

Two products are available for this: Sigma "G G - 1" and Hartman-Leddon "glycerine jelly, Kaiser;" both are terrible, but polyvinyl alcohol is even worse. Both the slide and the glycerol-gel must be at 45° C while the coverslip is put in place. Glycerol-gel from a fresh bottle will often set when it cools (very desirable). Painting around the edges of the coverslip with Varniton "V-21B" label varnish sometimes helps preserve the sections.

Stains (frozen sections)

ACID PHOSPHATASES

Burton, J. F., Histochemical demonstration of acid phosphatase by an improved azo dye method. J. Histochem. Cytochem., 1954. 2, 88-94.

Tissue may be kept at -20° C for up to 10 days.

Incubating medium:

2 mg sodium alpha-naphthyl acid phosphate

30 mg naphthanil diazo blue B

10 ml 0.1 M acetate buffer, pH 5.0 (Buffer must be at 4° C.)

(p. 203)

Filter.

1. 10% formalin at 4° C, 2 hours.
2. Distilled water, 5 minutes.
3. Incubate at 4° C, 30 minutes.
4. Cold (4° C) water, three changes, 3 minutes each.
5. 10% formalin at 4° C, overnight.
6. Water, 5 minutes.
7. Coverslip with glycerol-gel.

Stains (frozen sections)

ACID PHOSPHATASES

Stock Solutions

0.1 M acetate buffer, pH 5.0

214 ml 0.1 M acetic acid

500 ml 0.1 M sodium acetate

0.1 M acetic acid

1.5 ml glacial acetic acid

248.5 ml water

0.1 M sodium acetate

6.8 g sodium acetate trihydrate in 500 ml of solution

Stains (frozen sections)

ALKALINE PHOSPHATASE

Gomori, G., Microscopic histochemistry, Chicago, University of Chicago Press, 1952. (page 184).

Tissue may be kept at -20° C for up to 10 days.

Incubating medium:

2.5 ml "2% calcium chloride" (p. 205)

2.5 ml 2% sodium glycerophosphate

1.3 ml 2% sodium veronal (barbital sodium, N.F.; sodium diethylbarbiturate)

0.1 ml "10% magnesium sulfate"

6.3 ml water

1. 10% formalin at 4° C, 2 hours.
2. Water, 5 minutes.
3. Incubate at 37° C, 1 hour.
4. Water, three changes, 3 minutes each.
5. "2% cobalt chloride", 5 minutes.
6. Water, three changes, 3 minutes each.
7. 10% ammonium sulfide, 3 minutes.
8. Water, three changes, 3 minutes each.
9. Neutral 10% formalin, overnight.
10. Water, 5 minutes.
11. Dehydrate, clear, and mount.

Stains (frozen sections)

ALKALINE PHOSPHATASE

Stock Solutions

"2% calcium chloride"

2 g calcium chloride dihydrate

100 ml water

2% sodium glycerophosphate

2 g disodium DL-beta-glycerophosphate pentahydrate

100 ml water

"10% magnesium sulfate"

10 g magnesium sulfate heptahydrate (epsom salts)

100 ml water

"2% cobalt chloride"

2 g cobaltous chloride hexahydrate

100 ml water

Stains (frozen sections)

AMINOPEPTIDASE

Nachlas, M. M., Crawford, D. T., & Seligman, A. M.

The histochemical demonstration of leucine aminopeptidase.

J. Histochem. Cytochem., 1957. 5, 264-278.

Tissue may be kept at -20° C up to 10 days.

Incubating Medium:

8 mg LNA (L-leucyl-β-naphthylamide hydrochloride)

10 mg naphthanil diazo blue B (tetrazotized o-dianisidine)

1 ml water

1 ml 0.02 M potassium cyanide (p. 207)

8 ml physiological saline (68 mg NaCl in 8 ml water)

10 ml 0.1 M acetate buffer, pH 6.0 ± 0.5 (p. 207)

1. Incubate 37° C, 20 minutes (10 minutes is better for photography).
2. Saline, 2 minutes.
3. 0.2 M cupric sulfate, 2 minutes.
4. Saline, 3 minutes.
5. 10% formalin, 3 minutes.
6. Rinse in saline.
7. Dehydrate, clear, and mount.

Stains (frozen sections)

AMINOPEPTIDASE

Stock Solutions

0.02 M potassium cyanide

130 mg KCN

100 ml water

Physiological saline

850 mg NaCl

100 ml water

0.1 M pH 6.0 acetate buffer

193 ml 0.1 M sodium acetate

7 ml 0.1 M acetic acid (0.3 ml glacial acetic acid + 49.7 ml water = 50 ml 0.1 M acetic acid)

0.2 M cupric sulfate

5 gm cupric sulfate pentahydrate

100 ml water

Stains (frozen sections)

A O V

(Can also be used on araldite or paraffin sections).

Roman, N., Perkins, S. F., Perkins, E. M., Jr, & Dolnick, E. H. Orcein-hematoxylin in iodized ferric chloride as a stain for elastic fibers, with metanil yellow counterstaining. Stain Technol., 1967. 42, 199-202.

Orcein solution:

50 mg "Chroma" synthetic orcein (Roboz Surgical)

25 ml 70% ethanol

0.2 ml conc. (37%) HCl

Filter.

Hematoxylin solution:

0.4 g "Chroma" (cat. no. 50835) hematoxylin (C.I. 75290)

8 ml absolute ethanol

Filter.

Iron solution:

0.576 g ferric chloride hexahydrate

6 ml distilled water

Set aside 1 ml of this solution.

Iodine solution:

50 mg iodine

100 mg potassium iodide

5 ml distilled water

Stain:

Add the hematoxylin, iron, and iodine solutions, in that order, to the orcein solution. The staining solution will keep for about 6 hours.

(0. Bring paraffin sections to water).

1. 70% ethanol, 5 minutes.
2. Stain, 2 hours.
3. Distilled water, rinse.
4. Distilled water, 30 minutes.
5. Wipe underside of slide dry and place under a 40 x microscope.
6. Cover sections with a few drops of a 1 to 5 (1 in 6) dilution of the iron solution and wait until sections look right (until collagen appears clear).
7. Running tap water, 5 minutes.
8. Dehydrate, clear, and mount.

Stains (frozen sections)

ATP ase ("ADENOSINE TRIPHOSPHATASES")

Wachstein, M., & Meisel, E. Histochemistry of hepatic phosphatases at a physiologic pH with special reference to the demonstration of bile canaliculi. Amer. J. Clin. Path., 1957. 27, 13-23.

Tissue may be stored up to 6 hours at -20° C.

Incubating medium:

12 mg adenosine-5'-triphosphate, disodium

11 ml water

10 ml M/10 pH 7.2 tris-maleate buffer (p. 211)

1.5 ml 1-1/2% plumbous nitrate

1.5 ml 0.1 M magnesium sulfate

Bring to 37° C and filter through slow paper (e.g., Whitman # 42)

1. Cut at 4 to 6 micra.
2. Transfer to cold slide.
3. Melt section to slide with finger under slide and dry briefly at 37° C.
4. Incubate at 37° C, 30 minutes.
5. Wash in 0.1% acetic acid, 5 seconds.
6. Wash in three changes of water, 5 minutes (total).
7. 0.1% ammonium sulphide, 3 minutes.

8. Fix in 10% formalin over marble chips at 4° C, overnight.

9. Dehydrate, clear, and mount.

Stains (frozen sections)

ATP ase

Stock Solutions

M/10 pH 7.2 tris-maleate buffer:

1.16 g maleic acid

1.21 g tris (hydroxymethyl) aminonethane

(=2-amino-2-hydroxymethyl-1, 3-propanediol)

99 ml water

1 ml 40% NaOH

1-1/2% plumbous nitrate:

1.5 g $Pb(NO_3)_2$

100 ml water

0.1 M magnesium sulfate

2.46 g $MgSO_4 \cdot 7H_2O$ (Epsom salts)

100 ml water

Stains (frozen sections)

BRANCHING ENZYME

Takeuchi, T. Histochemical demonstration of branching enzyme (amylo-1, 4 ⟶ 1, 6- transglucosidase) in animal tissues. J. Histochem. Cytochem., 1958. 6, 208-216.

Smith, A. A., Perkins, E. M., & Machida, H. Durable mounts of the iodine stain for the phosphorylase reaction. Stain Technol., 1966. 41, 346-348.

Tissue may be kept at -20° C for up to 36 hours.

Incubating medium:

50 mg glucose-1-phosphate (Na or K salt)

10 mg 5'-adenylic acid (free acid)

4 mg glycogen (soluble)

15 ml water

10 ml 0.1 M pH 5.8 acetate buffer (p. 214)

Iodine stain:

3 ml Weigert's ("Lugol's") iodine (p. 214)

27 ml water

1. Incubate at 25° C, 1 hour.
2. Iodine stain, 5 minutes.
3. 0.1% iodine in 70% ethanol, 3 minutes.
4. 0.1% iodine in 95% ethanol, 5 minutes.
5. 0.1% iodine in absolute ethanol, 2 changes, 5 minutes each.

6. 0.1% iodine in xylene, 2 changes, 5 minutes each.

7. Mount in iodized Histoclad (p. 213).

Henna (red-brown) deposits indicate strong enzyme activity; brown deposits, moderate activity; black, gray, or blue deposits, little or no activity.

Stains (frozen sections)

BRANCHING ENZYME

PHOSPHORYLASES

Stock Solutions

0.1 M pH 5.8 acetate buffer:

25 ml 0.1 M acetic acid

475 ml 0.1 M sodium acetate

OR

6.4 g sodium acetate trihydrate

500 ml water

0.15 ml acetic acid

Weigert's ("Lugol's") iodine:

1 g iodine

2 g potassium iodide

100 ml water

Iodized Histoclad:

10 ml Histoclad (Clay-Adams)

10 mg iodine

Make up 1 day in advance of use. It keeps about a week in a half-full stoppered bottle, longer in a full bottle.

Stains (frozen sections)

CHOLINESTERASES

Gomori, G. Microscopic histochemistry. Chicago, University of Chicago Press, 1952. (pages 210-212)

Tissue may be kept at -20° C for up to 1 week.

Incubating media:

10 g anhydrous sodium sulfate (Na_2SO_4)

34 ml water

Heat to 37° C, dissolve, and add:

60 mg cupric sulfate pentahydrate

(or 44 mg of the monohydrate)

Dissolve and add:

75 mg glycine (free base)

Dissolve and add:

200 mg magnesium chloride hexahydrate

Dissolve and keep solution at 37° C.

Dissolve 350 mg maleic anydride in 6 ml 1 N (=4%) NaOH.

Check the pH of the sulfate solution and add the sodium maleate solution until the pH comes down to 5.15 ± 0.5. This usually requires just over 2 ml of the sodium maleate solution.

Acetylcholinesterase:

Add 40 mg acetylthiocholine iodide to 20 ml of the above solution.

Nonspecific cholinesterase:

Dissolve 50 mg butyrylthiochine iodide in 0.3 ml of warm distilled water and add 20 ml of the above solution.

1. Fix in 10% formalin at 4° C, 1-1/2 hours.
2. Distilled water, 10 minutes.
3. Incubate at 37° C, 5 hours.
4. Saturated aqueous sodium sulfate, 5 changes, 5 minutes each.
5. 1% ammonium sulfide, 3 minutes.
6. Water, 5 minutes.
7. 10% formalin at 4° C, overnight.
8. Distilled water, 10 minutes.
9. Ehrlich's hematoxylin (p. 183), 5 seconds.
10. Distilled water, 3 minutes.
11. Saturated aqueous lithium carbonate, 5 minutes.
12. Water, 5 minutes.
13. Dehydrate, clear, and mount.

Stains (frozen sections)

CYTOCHROME OXIDASE

Burstone, M. S. Modifications of histochemical techniques for the demonstration of cytochrome oxidase. J. Histochem. Cytochem., 1961. 9, 59-65.

Tissues may be kept at -20° C for up to 1 week.

Incubating medium:

12 mg p-aminodiphenylamine (N-phenyl-p-phenylenediamine)

2 drops (12 mg) 8-amino-1, 2, 3, 4-tetrahydroquinoline

Dissolve in:

0.5 ml absolute ethanol

Add:

35 ml water

15 ml 0.2 M tris buffer, pH 8.0

20 mg cytochrome C

Filter.

1. Incubate at 25° C, 1 hour.
2. "10% cobaltous acetate", 1 hour.
3. Water, 5 minutes.
4. 10% formalin, overnight.
5. Water, 10 minutes.
6. Coverslip with glycerol-gel.

Stains (frozen sections)

CYTOCHROME OXIDASE

Stock Solutions

0.2 M tris buffer, pH 8.0

6.05 g tris-hydroxymethylaminomethane (2-hydroxymethyl-2-amino-1, 3-propanediol)

997.5 ml water

2.5 ml conc. (37%) HCl

"10% cobalt acetate"

10 g cobaltous acetate tetrahydrate

100 ml 10 % formalin

Stains (frozen sections)

FRUCTOALDOLASE

Abe, T. & Shimizu, N. Histochemical method for demonstrating aldolase. Histochemie, 1964. 4, 209-212.

Tissue may be kept at -20° C for up to 5 days.

Incubating medium:

5 mg DPN

110 mg fructose-1, 6-diphosphate (disodium, hexahydrate)

10 ml 0.1% aqueous nitro BT

10 ml 0.05 M pH 7.6 arsenate buffer

1. Dry sections at 25° C, 2 hours.
2. Fix in 80% ethanol at 4° C, 20 minutes.
3. Dry at 25° C, 30 minutes.
4. Incubate at 37° C, 30 minutes.
5. Fix in 10% formalin at 4° C, overnight.
6. Dehydrate and mount in Diaphane.

Stains (frozen sections)

FRUCTOALDOLASE

Stock Solutions

DPN = diphosphopyridine nucleotide = nicotinamide adenine dinucleotide

nitro BT = nitro blue tetrazolium = 2, 2' -di-p-nitrophenyl-5, 5' -diphenyl-3, 3' - (3, 3' -dimethoxy-4, 4' -diphenylene) - ditetrazolium chloride.

0.05 M pH 7.6 arsenate buffer:

25 ml 0.1 M disodium hydrogen arsenate

2 ml 0.1 M HCl

23 ml water

0.1 M disodium hydrogen arsenate:

780 mg $Na_2 H AsO_4 . 7H_2O$ (Fisher, sodium arsenate, secondary, dibasic)

25 ml water

0.1 M HCl:

248 ml water

2.1 ml conc. (37%) HCl

GLUCOSE-6-PHOSPHATE DEHYDROGENASE

Hess, R., Scarpelli, D. G., & Pearse, A. G. E. The cytochemical localization of oxidative enzymes. II. Pyridine nucleotide linked dehydrogenases. J. Biophys. Biochem. Cytol., 1958. 4, 753-760.

Tissues may be stored at -20° C for up to 5 days.

Incubating medium:

35 mg glucose-6-phosphate, disodium, trihydrate (minor variations on this are acceptable)

7 mg triphosphopyridine nucleotide, sodium salt

75 mg PVP-40 (polyvinylpyrrolidone, mol. wt. 40,000)

0.1 ml 0.1 M sodium azide

0.1 ml 0.05 M magnesium chloride

0.25 ml 0.1 M pH 7.5 tris-maleate buffer

0.25 ml 1 mg/ml Nitro BT

0.3 ml water

1. Cover sections with a few drops (0.2ml) of incubating medium.
2. Incubate 30 minutes at 37° C, adding two more drops (0.1 ml) of medium after 15 minutes.
3. Fix in 10% formalin at 4° C, overnight.
4. Water, 10 minutes.
5. Dehydrate and mount in Diaphane.

Stains (frozen sections)

GLUCOSE-6-PHOSPHATE DEHYDROGENASE

0.1 M sodium azide

650 mg NaN_3

100 ml water

0.05 M magnesium chloride

1.017 g magnesium chloride hexahydrate

100 ml water

1 mg/ml nitro BT

100 mg p-nitro blue tetrazolium

100 ml water

0.1 M pH 7.5 tris-maleate buffer

580 mg maleic acid

607 mg tris (hydroxymethyl) aminomethane

39.5 ml water

0.55 ml 40% NaOH

Alternative buffer formula:

5 ml 1.0 M maleic acid

5 ml 1.0 M tris

11 ml 0.5 M sodium hydroxide

29 ml water

Stains (frozen sections)

GLYCOGEN SYNTHETASE I

Smith, A. A. Unpublished.

Tissues may be kept at -20° C for up to 3 days.

Incubating medium:

25 mg uridine 5'-diphosphoglucose

14 mg disodium ethylenediaminetetraacetate dihydrate (EDTA)

5 mg glycogen

5 mg sodium fluoride

5 ml pH 7.5 tris-maleate buffer (p. 222)

Dissolve.

Dissolve 250 mg gelatin in 7 ml water and add to above solution.

Add 0.5 ml 100% ethanol (drug grade).

1. Cool slides and dissecting needle to -20° C.
2. Cut 20 μ sections in cryostat at -20° C.
3. Brush sections onto slide with dissecting needle.
4. Melt sections by placing finger against underside of slide.
5. Dry sections at 37° C, 30 seconds.
6. Fix in 100% ethanol (drug grade)* at 4° C, 10 minutes.
7. Incubate at 37° C, 3 hours.
8. Iodine stain (p. 214). 5 minutes.

9. 0.1% iodine in absolute ethanol, 2 changes, 5 minutes each.

10. Coverslip with iodized Histoclad (p.214)

*Industrial solvent "absolute alcohol" often contains a trace of benzene, which has adverse effects on some enzymes.

Stains (frozen sections)

NONSPECIFIC CARBOXYLIC ESTERASES

Pearse, A. G. E. Histochemistry, 2nd ed. Boston, Little, Brown and Co., 1961. (page 886).

Tissue should be frozen in dry ice-ligroin.

Tissue may be stored up to 5 days at -20° C.

1. Fix in 10% formalin at 4° C, 2 hours.
2. Make up and filter medium at 4° C:

   12 mg naphthanil diazo blue B

   30 ml water (4° C!)

   1.2 ml 0.2 M Na_2HPO_4

   0.3 ml 1% α-naphtyl acetate in 50% acetone (water)
3. Cut frozen sections and incubate at 4° C, 15 minutes.
4. Rinse in water at 4° C.
5. Cold 10% neutral formalin, overnight.
6. Rinse in water.
7. Mount in glycerol-gel.

Stains (frozen sections)

NONSPECIFIC CARBOXYLIC ESTERASES

Stock Solutions

Naphthanil diazo blue B = tetrazotized o-dianisidine

Usually sold as zinc chloride complex (Echtblausalz B)

0.2 M Na_2HPO_4

2.84 g anhydrous sodium monhydrogen phosphate

100 ml water

1% α-naphthyl acetate

1 g alpha- naphthyl acetate

50 ml acetone

50 ml water

Stains (frozen sections)

PHOSPHOGLUCOMUTASE

Meijer, A. E. F. H. Histochemical method for the demonstration of the activity of phosphoglucomutase. Histochemie, 1967. 8, 248-251.

Tissues may be kept at -20° C for up to 1 week.

Incubating medium:

Dissolve 2 mg nitro BT in 1.5 ml 0.04 M pH 7.4 imidazole buffer.

Add:

40 mg 95-98% glucose-1-phosphate (I use Sigma grade III. Glucose-1,6-diphosphate is a desirable impurity)

1.2 mg TPN

2.5 mg ATP

12.5 mg magnesium chloride hexahydrate

Dissolve 105 mg gelatin in 3.5 ml water (i.e. 3% solution) and add to above solution.

Add 0.02 ml of a 1 mg / ml solution of glucose-6-phosphate dehydrogenase. (I used Sigma type V).

1. Fix in acetone at -20° C, 30 minutes.
2. Cover sections with a few drops (~ 0.5 ml) of medium and incubate at 25° C, 30 minutes.
3. Hot (60° C) tap water, 2 changes, 2 minutes each.

4. Fix in 10% formalin, overnight.

5. Water, 5 minutes.

6. Dehydrate and mount in Diaphane.

Stains (frozen sections)

PHOSPHOGLUCOMUTASE

Stock Solutions

TPN = triphosphopyridine nucleotide = nicotinamide adenine dinucleolide phosphate

0.04 M pH 7.4 imidazole buffer:

99 ml water

273 mg imidazole

1.05 ml 1 M HCl

1 M HCl:

91.5 ml water

8.4 ml conc. (37%) HCl

(This formula was computed by Mr. Roman on the basis of a specific gravtiy of 1.180 and differs slightly from formulas based on 37%).

Stains (frozen sections)

6 - PHOSPHOGLUCONATE DEHYDROGENASE

Im, M. J. C. Distribution of dehydrogenases in the skin of the rhesus monkey (Macaca mulatta). J. Histochem. Cytochem., 1965. 13, 668-676.

Tissue may be stored at -20° C for up to 24 hours.

Incubating medium:

1 mg trisodium 6-phosphogluconate

2 mg TPN

75 mg PVP-40

0.3 ml water

0.25 ml 0.1 M pH 7.5 tris-maleate buffer (p. 222)

0.1 ml 0.05 M magnesium chloride (p. 222)

0.25 ml 0.1% nitro-BT

0.1 ml 0.1 M sodium azide (p. 222)

1. Cover sections with a few drops (0.2 ml) of incubating medium.
2. Incubate 30 minutes at 37° C, adding two more drops (0.1 ml) of medium after 15 minutes.
3. Fix in 10% formalin at 4° C, overnight.
4. Water, 10 minutes.
5. Dehydrate and mount in Diaphane.

Stains (frozen sections)

PHOSPHORYLASE A (Eränkö)

Eränkö, O. & Palkama, A. Improved localization of phosphorylase by the use of polyvinylpyrrolidone and high substrate concentration. J. Histochem. Cytochem., 1961. 9, 585.

Tissue may be kept at -20° C for up to 36 hours.

Incubating medium:

200 mg glucose-1-phosphate (Na or K salt)

4 mg glycogen

28 mg EDTA, disodium

10 mg sodium flouride

1.8 g PVP-40

10 ml 0.1 M pH 5.8 acetate buffer (p. 214)

10 ml water

5 ml 100% ethanol (drug grade)

Dissolve by heating to 37° C; then cool to 25° C.

This medium will keep up to 2 months at 4° C.

1. Incubate at 25° C, 1 hour.
2. Wipe underside and edges of slide dry.
3. Iodine stain (p. 212), 5 minutes.

   Since polyvinylpyrrolidone reacts with iodine, fresh stain must be used for each 3 or 4 slides.
4. Wipe underside and edges of slide dry.

5. 0.1% iodine in 70% ethanol, 3 minutes.

6. 0.1% iodine in 95% ethanol, 5 minutes.

7. 0.1% iodine in absolute ethanol, 2 changes, 5 minutes each.

8. 0.1% iodine in xylene, 2 changes, 5 minutes each.

9. Coverslip with iodized Histoclad (p. 214).

Stains (frozen sections)

PHOSPHORYLASE A (Godlewski)

Godlewski, H. G. Interprétation des réactions histochemiques sur les phosphorylases. Folia Morph., 1962. 21, 434-448.

Tissue may be kept at -20° C for up to 36 hours.

Incubating medium:

50 mg glucose-1-phosphate (dipotassium, dihydrate)

4 mg glycogen

28 mg EDTA, disodium

10 mg sodium flouride

10 ml 0.1 M pH 5.8 acetate buffer (p. 214)

10 ml water

5 ml 100% ethanol (drug grade)

1. Incubate at 25° C, 1 hour.
2. Iodine stain (p. 212), 5 minutes.
3. 0.1% iodine in 70% ethanol, 3 minutes.
4. 0.1% iodine in 95% ethanol, 5 minutes.
5. 0.1% iodine in absolute ethanol, 2 changes, 5 minutes each.
6. 0.1% iodine in xylene, 2 changes, 5 minutes each.
7. Coverslip with iodized Histoclad (p. 214)

Stains (frozen sections)

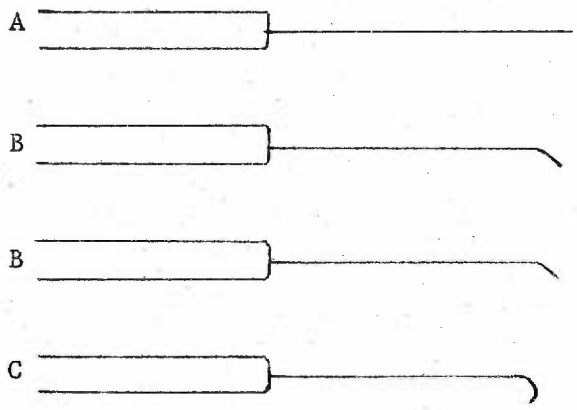
SEMIMICRO DISSECTION

Bartman, J. & Dixon, J. F. P. Enzyme histochemistry of microdissected sweat glands. J. Invest. Derm., 1966. 46, 484-487.

Equipment:

Single edge razor blade

4 dissecting needles



Type B and C needles are made by heating a type A needle in the oxidizing (vents open) flame of a standard Bunsen burner and bending the tip with pliers in the flame.

Collagenase:

10 mg collagenase (I used Sigma type I Clostridium histolyticum collagenase).

10 ml 0.1 M pH 7.4 phosphate buffer (p. 241)

1. Cut a 1 mm section from fresh tissue with a razor blade or scalpel.

2. Carry out succinate dehydrogenase procedure (p. 240). through step 2. (Do not fix).
3. Collagenase at 37° C, overnight.
4. Put tissue in a drop of water on a slide under a 20 x microscope.
5. Hold tissue in place with a bent (type B) needle held in the left hand and strip tissue away from a sweat gland with the corner of a single edge razor blade and the type C and A needles.
6. Transfer one or two glands to a drop of water on a clean slide.
7. Coverslip with glycerol-gel.

Stains (frozen sections)

SILVERED CHOLINESTERASE

Roman, N. Unpublished. (To be submitted to J. Invest. Derm.).

Winkelmann, R. K. Nerve endings in normal and pathologic skin. Springfield, Ill., Charles C. Thomas, 1960. (page 159).

This technique requires free floating sections which must be transferred from solution to solution with a bent glass rod.

1. Cut 50 to 100 μ sections in cryostat at -20° C.
2. Brush sections into 10% formalin at 4° C.
3. Fix in 10% formalin at 4° C, 1-1/2 hours.
4. Distilled water, 10 minutes.
5. Incubate in cholinesterase medium (p. 215), 4 hours.
6. Saturated sodium sulfate, 8 changes, 3 minutes each.
7. 1% ammonium sulfide, 3 minutes.
8. Distilled water, 6 changes, 5 minutes each.
9. Winkelmann's fixative (p. 239) at 4° C, 1 month.
10. Distilled water, 3 changes, 5 minutes each.
11. 30% aqueous ethanol, 3 minutes.
12. 50% ethanol, 3 minutes.
13. 60% ethanol, 3 minutes.
14. 70% ethanol, 3 minutes.
15. 80% ethanol, 3 minutes.

16. 95% ethanol, 2 changes, 3 minutes each.

17. Absolute ethanol, 2 changes, 5 minutes each.

18. Xylene, 30 minutes.

19. Xylene, 2 hours.

20. Absolute ethanol, 2 changes, 5 minutes each.

21. 95% ethanol, 5 minutes.

22. 80% ethanol, 3 minutes.

23. 70% ethanol, 3 minutes.

24. 60% ethanol, 3 minutes.

25. 50% ethanol, 3 minutes.

26. 30% ethanol, 3 minutes.

27. Distilled water, 2 changes, 3 minutes each.

28. 10% formalin at 4° C, overnight.

29. Distilled water, 6 changes, 10 minutes each.

30. 20% silver nitrate, 20 minutes.

(31. Make reducer while sections are in silver nitrate: dissolve 20 mg hydroquinone and 100 mg anhydrous sodium sulfite in 10 ml distilled water).

32. Distilled water, 3 changes, 4 seconds each.

33. Reduce until section takes on a greenish cast -- no longer. This takes 3 to 4 minutes for 100 μ sections, longer -- up to 10 minutes -- for 50 μ sections.

34. Distilled water, rinse.

35. Distilled water, 3 changes, 5 minutes each.

36. "0.2% gold chloride" (p. 239), 3 minutes.

37. Distilled water, 4 changes, 3 minutes each.

38. 5% sodium thiosulfate pentahydrate, 5 minutes.

39. Distilled water, 4 changes, 3 minutes each.

40. Dehydrate, clear, and mount.

Stains (frozen sections)

SILVERED CHOLINESTERASE

Stock Solutions

Winkelmann's fixative:

100 ml 10% formalin

15 g sucrose

1 ml conc. (28%) ammonium hydroxide

"0.2% gold chloride"

20 mg (0.3 grains) $HAuCl_4 \cdot 3H_2O$

10 ml distilled water

Stains (frozen sections)

SUCCINIC DEHYDROGENASE

Nachlas, M. M., Tsou, K. -C., de Souza, E., Cheng., C. -S. & Seligman, A. M. Cytochemical demonstration of succinic dehydrogenase by the use of a new p-nitrophenyl substituted ditetrazole. J. Histochem. Cytochem., 1957. 5, 420-436.

Tissues may be kept at -20° C for up to 1 week.

Incubating medium:

7.5 mg sodium bicarbonate

6.5 ml water

3 ml 0.5 M sodium succinate

10 ml 0,1 M phosphate buffer, pH 7.4

3 ml 0.004 M calcium chloride

7 ml 0.1% nitro blue tetrazolium

1. Warm medium to 37° C. (This requires half an hour).
2. Incubate at 37° C for 30 minutes.
3. 10% formalin at 4° C, overnight.
4. Water, 10 minutes.
5. Dehydrate and mount in Diaphane.

Stains (frozen sections)

SUCCINIC DEHYDROGENASE

Stock Solutions

0.1 M phosphate buffer, pH 7.4

404 ml 0.1 M Na_2HPO_4 (7.2 gm anhydrous sodium hydrogen phosphate in 500 ml of solution)

96 ml 0.1 M KH_2PO_4 (1.36 gm anhydrous potassium dihydrogen phosphate in 100 ml of solution)

0.5 M sodium succinate

13.5 gm sodium succinate hexahydrate in 100 ml of solution

0.004 M calcium chloride

59 mg calcium chloride dihydrate in 100 ml of solution

0.1% nitro blue tetrazolium

100 mg 2, 2' -di-p-nitrophenyl-5, 5' -diphenyl-3, 3' - (3, 3'-dimethoxy-4, 4' -diphenylene)-ditetrazolium chloride

100 ml water

Stains (frozen sections)

TOTAL PHOSPHORYLASE (Takeuchi)

Takeuchi, T., & Kuriaki, H. Histochemical detection of phosphorylase in animal tissues. J. Histochem. Cytochem., 1955. 3, 153-160.

Tissue may be kept at -20° C for up to 36 hours.

Incubating medium:

50 mg glucose-1-phosphate (dipotassium, dihydrate)

10 mg 5' -adenylic acid (free acid, sesquihydrate)

4 mg glycogen

10 ml water

10 ml pH 5.8 acetate buffer, 0.1 M (p. 214)

5 ml absolute ethanol

1. Incubate for 1 hour at 25° C.
2. Iodine stain (p. 212), 5 minutes.
3. 0.1% iodine in 70% ethanol, 3 minutes.
4. 0.1% iodine in 95% ethanol, 5 minutes.
5. 0.1% iodine in absolute ethanol, 2 changes, 5 minutes each.
6. 0.1% iodine in xylene, 2 changes, 5 minutes each.
7. Coverslip with iodized Histoclad (p. 214).

Stains (frozen sections)

TOTAL PHOSPHORYLASE (Eränkö)

Eränkö, O. & Palkama, A. Improved localization of phosphorylase by the use of polyvinylpyrrolidone and high substrate concentration. J. Histochem. Cytochem., 1961. 9. 585.

Tissue may be kept at -20° C for up to 36 hours.

Incubating medium:

200 mg glucose-1-phosphate (Na or K salt)

10 mg 5' -adenylic acid (free acid)

4 mg glycogen

1.8 g PVP-40

10 ml 0.1 M pH 5.8 acetate buffer (p. 214)

10 ml water

5 ml 100% ethanol (drug grade)

Dissolve ingredients by heating to 37° C.

Cool to 25° C.

1. Incubate at 25° C, 1 hour.
2. Wipe underside and edges of slide dry.
3. Iodine stain (p. 212), 5 minutes.
4. Since polyvinylpyrrolidone reacts with iodine, fresh stain must be used for each 3 or 4 slides.
4. Wipe underside and edges of slide dry.

5. 0.1% iodine in 70% ethanol, 3 minutes.

6. 0.1% iodine in 95% ethanol, 5 minutes.

7. 0.1% iodine in absolute ethanol, 2 changes, 5 minutes each.

8. 0.1% iodine in xylene, 2 changes, 5 minutes each.

9. Coverslip with iodized Histoclad (p. 214).

Stains (frozen sections)

TOTAL GLYGOGEN SYNTHETASE

Sasse, D. Untersuchen zur Nachweismethodik der Uridindiphosphoglukose - glykogentransferase. Histochemie, 1966. 7, 39-49.

Tissue may be kept at -20° C for up to 3 days.

Incubating medium:

5 mg glucose-6-phosphate (Na or K salt)

25 mg uridine-5'-diphosphoglucose

14 mg disodium ethylenediamine tetraacetate dihydrate (EDTA)

5 mg glycogen

5 mg sodium fluoride

5 ml pH 7.5 tris-maleate buffer (p. 222)

7 ml water

0.5 ml 100% ethanol (drug grade)

1. Cool slides and dissecting needle to -20° C.
2. Cut 20 μ sections in cryostat at -20° C.
3. Brush sections onto slide with dissecting needle.
4. Melt sections by placing finger against underside of slide.
5. Dry sections at 37° C, 30 seconds.
6. Fix in 100% ethanol (drug grade) at 4° C, 10 minutes.
7. Incubate at 37° C, 3 hours.

8. Iodine stain (p. 212), 5 minutes.

9. 0.1% iodine in absolute ethanol, 2 changes, 5 minutes each.

10. Coverslip with iodized Histoclad (p. 214).

Stains (frozen sections)

TPN-linked ISOCITRATE DEHYDROGENASE

Hess, R., Scarpelli, D.G. & Pearse, A.G.E. The cytochemical localization of oxidative enzymes. II. Pyridine nucleotide linked dehydrogenases. J. Biophys. Biochem. Cytol., 1958. 4, 753-760.

Tissue may be stored at -20° C for up to 1 week.

Incubating medium:

27 mg trisodium dl-isocitrate

7 mg triphosphopyridine nucleotide, sodium salt

75 mg PVP-40

0.1 ml 0.1 M sodium cyanide (p. 248)

0.1 ml 0.05 M magnesium chloride (p. 248)

0.25 ml 0.05 M pH 7.6 phosphate buffer (p. 248)

0.25 ml 0.1% Nitro-BT

0.3 ml water

1. Pick up ca. 20μ cryostat sections on room temperature slides.
2. Dry 1 minute at 37° C.
3. Incubate at 37° C in a few drops (0.2 ml) of medium, 15 minutes.
4. Fix formalin at 4° C, overnight.
5. Water, 5 minutes.
6. Dehydrate and mount in Diaphane.

Stains (frozen sections)

TPN-linked ISOCITRATE DEHYDROGENASE

Stock Solutions

0.1 M sodium cyanide

4.9 g NaCN

100 ml water

0.05 M magnesium chloride

1.015 g $MgCl_2 \cdot 6H_2O$

100 ml water

0.05 M pH 7.6 phosphate buffer

5 ml 0.1 M NaH_2PO_4 (1.38 g $NaH_2PO_4 \cdot H_2O$ in 100 ml water)

45 ml 0.1 M Na_2HPO_4 (1.42 g anh. Na_2HPO_4 in 100 ml water)

0.1% Nitro-BT

100 mg 2, 2'-di-p-nitrophenyl-5, 5'-diphenyl-3, 3'-(3, 3'-dimethoxy-4, 4'-diphenylene)-ditetrazolium chloride

100 ml water

SUPPLIERS' ADDRESSES

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American Optical

Buffalo, New York 14215

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Washington, D.C. 20006

Clay - Adams, Inc.

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Hollywood, California 90028

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East Rutherford, New Jersey 07073

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Redmond, Washington 98052

Sherwood Medical Industries

1831 Olive Street

St. Louis, Missouri 63103

Sigma Chemical Company

3500 DeKalb Street

St. Louis, Missouri 63118

Ivan Sorvall, Inc.

Norwalk, Connecticut 06852

U.S. Borax & Chemical Corp.

Los Angeles, California

The Varniton Company

416 North Varney Street

Burbank, California 91502

Will Scientific Inc.

Box 1050

Rochester, New York 14603

Fig. 6

Section through an "elbow" of the secretory coil. Toluidine blue-borax, X160 enlarged to X 680.

Fig. 7

Key to Fig. 6.

Dark cells - green

Clear cells - orange

Myoepithelial cells - brown

Lumen - yellow

Intercellular canaliculi - red