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THE LOCALIZATION OF ENDOGENOUS PEROXIDASE IN THE LACRIMAL GLAND OF THE RAT DURING POSTNATAL DEVELOPMENT

Electron Microscope Cytochemical and Biochemical Studies

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ABSTRACT

The distribution of endogenous peroxidase activity in the lacrimal gland of the rat during postnatal development was investigated by electron microscope cytochemistry Peroxidase activity is first found 6 hr after birth in only a few acinar cells At this stage, reaction product fills only localized segments of the scant rough endoplasmic reticulum and of the perinuclear cisternae. Peroxidase activity thus develops asynchronously in a given cell as well as in the secretory cell population as a whole 2 days after birth, all cisternae of the rough endoplasmic reticulum of a peroxidase-positive cell contain reaction product, but the majority of the acinar cells is still negative During the next days, the number of peroxidase-positive cells and the amount of the rough endoplasmic reticulum increase rapidly. By 15 days postparturition, all secretory cells are peroxidase-positive. Reaction product is then found in all cisternae of the rough endoplasmic reticulum including the perinuclear cisternae, in smooth surface vesicles located mainly between the rough endoplasmic reticulum and the Golgi stacks, in condensing vacuoles, and in all secretory granules The Golgi cisternae rarely contain reaction product In total homogenates and in fractions of glandular tissue of adult rats, peroxidatic and catalatic activities are demonstrable. The microsomal fractions and the postmicrosomal supernatants were used to separate peroxidase from catalase by precipitation with ammonium sulfate, and the following parameters were determined: substrate (H_2O_2) optimum ($\sim 2.0 \times 10^{-4}$ M), pH-optimum (pH 6 5), temperature-optimum (42°C), and the absorption maximum (415 nm before and 425 nm after addition of H_2O_2) The same parameters were obtained from lacrimal fluid peroxidase. Both peroxidase from lacrimal gland and that from lacrimal fluid are almost completely inhibited by 10^{-3} M aminotriazole and are possibly identical enzymes. Peroxidase is secreted into lacrimal fluid, which does not contain catalase.

INTRODUCTION

Bactericidal agents occur in the lacrimal fluid and in saliva of several animal species (1, 2, 3). Myelo- and lactoperoxidase also form a virucidal system with halides and hydrogen peroxide (4) and seem to inhibit certain bacteria under aerobic conditions (5). Lactoperoxidase has been isolated from bovine submaxillary and sublingual glands (6) and from Harderian and lacrimal glands (5). Endogenous peroxidase has been found by electron microscope cytochemistry in the submaxillary (7) and parotid (8) glands of the rat and demonstrated in the saliva (8).

The lacrimal fluid in the rat is secreted mainly by two glands opening with a common duct into

the conjunctival sac, the smaller glandula infraorbitalis near the external angle of the eye, and the larger (~1:0.6:0.5 cm) glandula orbitalis (or lacrimalis) externa located anterior to and under the ear and touching the parotid gland with its lower margin. The confusing nomenclature of the orbital glands of rodents and their topographic anatomy have been clarified by Kittel (9). The two lacrimal glands are compound tubuloacinar glands. The larger external orbital gland is easier to handle than the smaller infraorbital gland and we have therefore restricted our investigations to this gland.

We have used electron microscope cytochemistry to study the distribution of endogenous peroxidase within the secretory cells of the lacrimal gland and to determine the time point at which production of the enzyme begins. This technique can provide information on the topographic relation of various cell compartments during development of the gland and can answer the question whether enzyme production starts synchronously or at different time points and whether sites of enzyme activity occur evenly or localized in the rough endoplasmic reticulum (er) of a given cell.

In addition, we have separated peroxidase from catalase in tissue fractions in order to determine several biochemical parameters of the enzyme and to compare them with those obtained from the lacrimal fluid.

MATERIAL AND METHODS

General

The animals used were male Wistar rats, obtained from our own colony or from commercial breeders. Fetal rats were removed 1 or 3 days before birth under ether narcosis. The external orbital glands were dissected out under a stereomicroscope in fetal rats, and in postnatal rats after decapitation or under ether narcosis 2 and 6 hr, and 2, 4, 6, 10, 15, 21, and 28 days after birth, as well as in adult rats. Glands from fetal and postnatal rats were cut under a stereomicroscope into small strips in ice-cold fixative, tissues from adult rats were also embedded in 7% agar at 40°C and cut with a tissue sectioner (10) at ~ 40 μ.

Cytochemical Methods

Fixation was carried out in ice-cooled paraformaldehyde-glutaraldehyde[1] (11) diluted 1:1 with 0.1 M cacodylate buffer, pH 7.6, osmolality[2] 1000 mosmols. Fixation time was 6 hr for morphological and 2 hr for cytochemical studies. After fixation, the tissues were rinsed for 30 min at 4°C in 0.1 M cacodylate buffer, pH 7.6, containing 0.22 M sucrose.

Incubation was carried out in the medium of Graham and Karnovsky (12) at pH 7.6 with 0.01% H_2O_2 and 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Schuchardt, München) for 2 hr at room temperature, the incubation medium was replaced by a fresh one after 1 hr.

For controls, tissues were incubated in the peroxidase medium without H_2O_2, or after addition of 10^{-2} M 3-amino-1,2,4-triazole (14, 15). In order to differentiate the peroxidase reaction against catalase, an adult rat was injected intracardially with 4 ml of beef liver catalase (2 × crystallized, Sigma Chemical Co., St. Louis, Mo.), and another rat with 40 mg of horseradish peroxidase (type II, Sigma Chemical Co.). The glands were removed after 90 sec. Catalase was demonstrated cytochemically in the medium of Venkatachalam and Fahimi (15) at pH 8.5, peroxidase in the medium of Graham and Karnovsky (12) at pH 7.6. In both cases 10^{-2} M aminotriazole was also added to the incubation media for controls.

After incubation the tissues were rinsed, as described above, in cacodylate buffer containing sucrose, postfixed for 1 hr at 4°C in 1% OsO_4 in 0.1 M cacodylate buffer, pH 7.6, kept for 1 hr at room temperature in 0.5% uranyl acetate in Veronal-acetate buffer, pH 5.0 (16), dehydrated in ethanol, and embedded in Epon 812 (17). Thin sections were cut on an LKB-Ultrotome III with diamond knives, collected on naked grids or grids covered with collodion and carbon films, and examined unstained or stained with 2% aqueous uranyl acetate and lead citrate (18) in a Siemens Elmiskop I at initial magnifications of 2,000–40,000. For light microscopy, 0.5 μ thick sections were cut with glass knives and photographed unstained in a Zeiss Ultraphot III.

Enzyme Assays in Tissue Fractions

100 adult rats, in groups of five animals, were perfused through the heart with 150–200 ml of ice-cold 0.9% saline. Both external orbital glands were freed of connective tissue, cut into small pieces, and homogenized with 0.25 M sucrose containing 1 mM ethylenediaminetetraacetate (EDTA) (1:10 w/vol) in a Potter-Elvehjehm glass homogenizer with a Teflon pestle for 2 min at a speed of 900 rpm in an ice-bath, with a stop of 30 sec after the first min. 5 ml of 0.35 M sucrose containing 1 mM EDTA were overlayed with 5 ml of homogenate and centrifuged

[1] Glutaraldehyde 50%, Biological Grade, was obtained from Fisher Scientific Co., Fair Lawn, N. J., and was purified by shaking with charcoal (13); freshly distilled 25% glutaraldehyde (Schuchardt, GmbH, Munchen) was also used; its extinction quotient: (E 235 nm)/(E 280 nm) was 0.32.

[2] Osmolality was measured with an electronic osmometer, type M, Knauer, Berlin.

for 20 min at 700 *g* and 4°C. The supernatant of the crude nuclear fraction was centrifuged for 10 min at 5000 *g* and 4°C to sediment mitochondria. The supernatant of this crude mitochondrial fraction was then centrifuged for 60 min at 54,000 *g* in a Beckman Spinco L 50 centrifuge (rotor 40) to give the microsomal pellet and the postmicrosomal supernatant. A part of these pellets was fixed and embedded in Epon, either untreated or after incubation in the peroxidase medium as described above.

The microsomal pellets from two groups of five rats were pooled for each experiment, resuspended in 1 ml potassium phosphate buffer, pH 7.0, and brought to a volume of 15 ml with the same buffer. The postmicrosomal supernatants of two groups of five rats were also pooled and had a volume of ~30 ml. Ammonium sulfate was added in solid form to the resuspended microsomal pellets and the postmicrosomal supernatants and stirred for 20 min. The concentration was raised to 40, 50, 60, 70, 80, and 90% saturation, and the pH was kept at 7.0 with 0.1% $(NH_4)OH$. After centrifugation for 15 min at 5000 rpm the precipitates were washed twice with ammonium sulfate of the respective saturation, dissolved in 0.1 M potassium phosphate buffer, pH 7.0, containing 1 mM EDTA, and dialyzed for 12 hr at 4°C against 0.025 M potassium phosphate buffer, pH 7.0, containing 1 mM EDTA. Protein was determined in the various ammonium sulfate fractions according to Lowry et al. (19) with bovine serum albumin as standard. Catalase activity was determined after Bergmeyer (20) by measuring the rate of H_2O_2 decomposition at 240 nm and 25°C in a Zeiss PMQ II spectrophotometer. Peroxidase activity was measured with the guaiacol test (21) in an Eppendorf photometer (Eppendorf Gerätebau, Hamburg) at 436 nm and 25°C.

Fractions obtained at 70–90% ammonium sulfate saturation contained peroxidase, but no catalase; they were used to determine substrate (H_2O_2) dependence, pH optimum, temperature dependence, and inhibition by aminotriazole of the orbital gland peroxidase.

In addition, endogenous peroxidase was measured with the pyrogallol test (22) in the total homogenate (obtained from the pooled glands of four to seven rats in two different experiments), the crude nuclear, mitochondrial, and microsomal fractions, and in the postmicrosomal supernatant. This test does not require a separation of peroxidase from catalase, but is less exact and does not permit the determination of enzyme activity in international units.

Enzyme Assays in Lacrimal Fluid

Secretion of lacrimal fluid was stimulated by intraperitoneal injection of 2 mg pilocarpine. 4–5 min after injection the lacrimal sacs of groups of three to five rats were rinsed with 0.1 M phosphate buffer, pH 7.6, and the fluid was collected with a Pasteur pipette. The pooled fluids were filtered through a Millipore filter (Millipore Corporation, Bedford, Mass.) (pore size 0.2 μ) after centrifugation and monitored for the absence of cells. Catalase and peroxidase activities were then determined photometrically as described above, and the protein content of the lacrimal fluid was measured for each experiment.

Substrate (H_2O_2) dependence, pH optimum, temperature dependence, and inhibition by aminotriazole of the peroxidase in the lacrimal fluid were determined and compared with the same parameters obtained from the microsomal fractions and the postmicrosomal supernatants.

RESULTS

General

The secretory acini of the adult external orbital gland are composed of a single layer of six to eight pyramid-shaped cells (Fig. 1). The cells are 10–12

FIGURES 1 and 4–10 are electron micrographs of secretory acinar cells of the glandula orbitalis externa of the rat. All tissues were stained in block in buffered uranyl acetate. Figs. 4, 6, 8, 10, and 13 were taken from unstained sections, Fig. 11 from a section doubly stained with aqueous uranyl acetate and lead, all others from sections stained with lead alone. Figs. 2 and 3 are light micrographs taken from unstained, 0.5 μ thick sections of tissue embedded in Epon.

FIGURE 1 Part of a secretory acinus, 10 days after birth. Tissue fixed in paraformaldehyde-glutaraldehyde and osmium tetroxide. The nucleus (*n*) is located in the basal part of the pyramid-shaped cell and is surrounded by cisternae of the rough endoplasmic reticulum (*er*) which are scarce at this stage. The Golgi apparatus (*G*) lies above the nucleus. Secretory granules (sg_1) fill the apical part of the cell. Microvilli (*mv*) project into the lumen (*L*). The cell to the right contains larger granules (sg_2) with a content of lower opacity. A myoepithelial cell (*mc*) is interposed between the basement membrane (*bm*) and two acinar cells. × 12,000.

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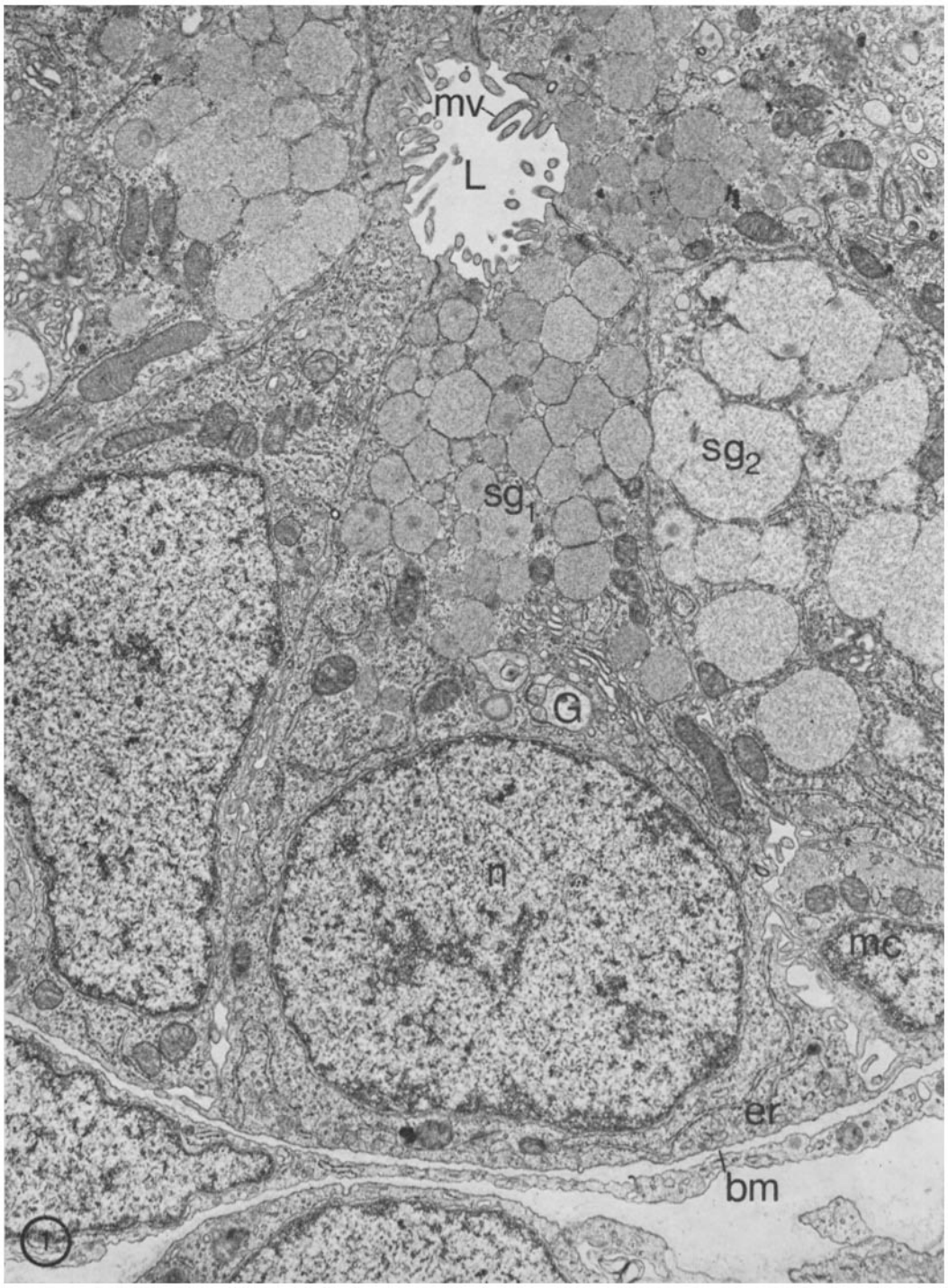

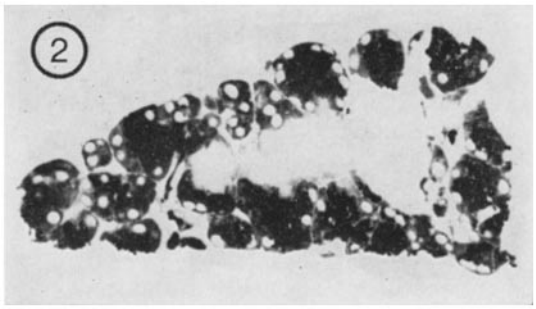

FIGURE 2 Unstained section from peroxidase-incubated tissue of an adult rat, demonstrating the depth of penetration of the incubation medium into the tissue block. Dark-brown reaction product is found in the outer zone (~20 μ thick) of the block, but not in the nuclei and the interacinar spaces. No reaction product is visible in the central part of the block. × 300.

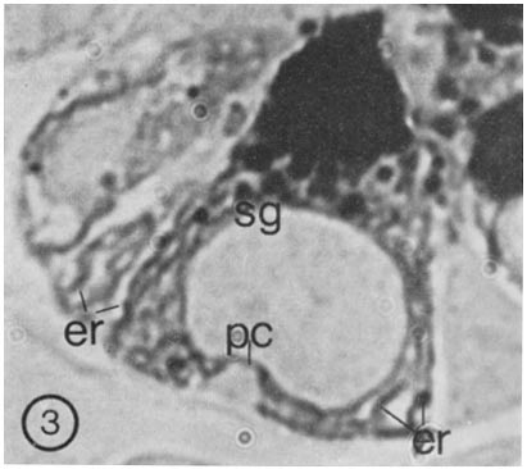

FIGURE 3 Unstained section of an acinar cell from peroxidase-incubated tissue, 4 days after birth. Brown reaction product is visible in the strands of the *er*, including the perinuclear cisterna (*pc*) and in the secretory granules (*sg*) filling the apical pole × 3300.

μ high, measure 8–10 μ at their bases and taper towards the lumen to a width of 2–4 μ. Microvilli project into the lumen. The membranes of adjacent cells form junctional complexes (23) near the lumen, usually run parallel in the apical part and interdigitate in the basal half of the cell. A loosely textured basement membrane, ~60 nm thick, surrounds the acinus, myoepithelial cells are interspersed between basement membrane and secretory cells. The round nucleus is located in the basal part of the cell. The apical portion of the cell is filled with two types of secretory granules: a larger one (diameter 1.2–1.5 μ) with partially disrupted membranes and granular contents of little density, and a smaller one (diameter ~ 0.8 μ) with denser, homogenous content. About 50% of the cells of an acinus contain only large granules, 50% only small granules. Rarely, a cell will contain both types together. The rough er surrounds the nucleus and extends upwards into the close vicinity of the Golgi apparatus which is built up of four to six membrane stacks. The cisternae of the er and of the Golgi stacks contain loosely textured material Before birth and until 2 days after birth, the secretory cells contain few profiles of the er and abundant unattached ribosomes. The Golgi apparatus is fully developed, but secretory granules are scarce or absent.

Cytochemistry

After 2 hr of incubation the peroxidase reaction is positive in a zone extending ~20 μ into the tissue from the surface in adult rats (Fig. 2), while regions deeper in the tissue do not react In fetal or young rats the reaction zone is broader because of the looser texture of the interstitial tissue. Care was, nevertheless, taken to incubate slices not thicker than 40 μ

In the light microscope the er is visible in peroxidase-positive cells as faint, light-brown strands up to 15th day of life (Fig. 3); later the reaction in the cytoplasm appears diffuse, because the cisternae of the er are packed more tightly.

FIGURES 4–6 Cross-sections through developing acinar cells at the level of the nuclei. Tissues incubated for peroxidase activity Fig 4 illustrates that 2 days after birth the majority of the acinar cells are peroxidase-negative. In the two peroxidase-positive cells reaction product lies in the scant cisternae of the er and in the perinuclear cisternae. Fig. 5 shows that 4 days after birth the situation is reversed; cells c_1 and c_2 are peroxidase-negative while all others are reactive. Fig. 6 demonstrates that 10 days after birth all the cells are reactive. The elements of the er are packed more tightly. Fig. 4, × 5700; Fig. 5, × 5500; Fig. 6, × 5500.

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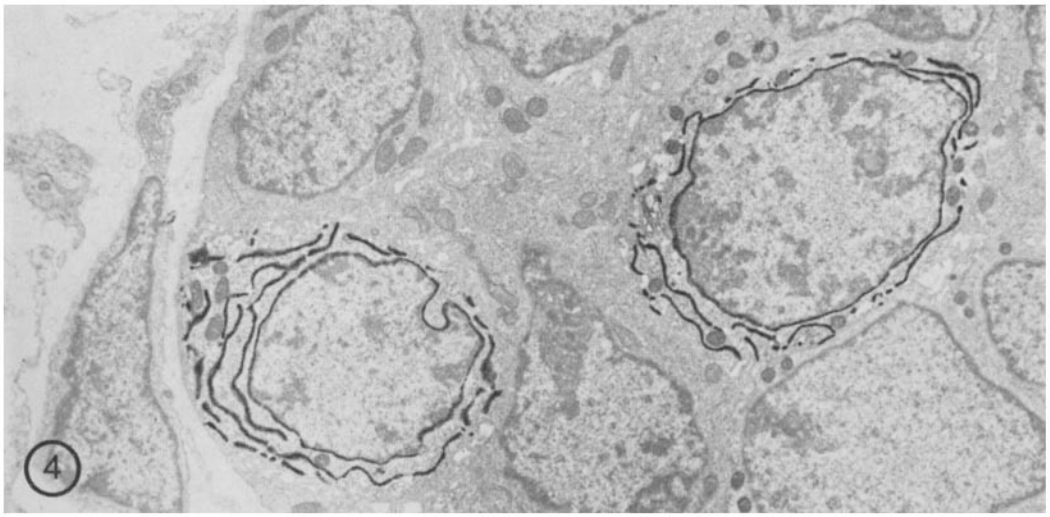

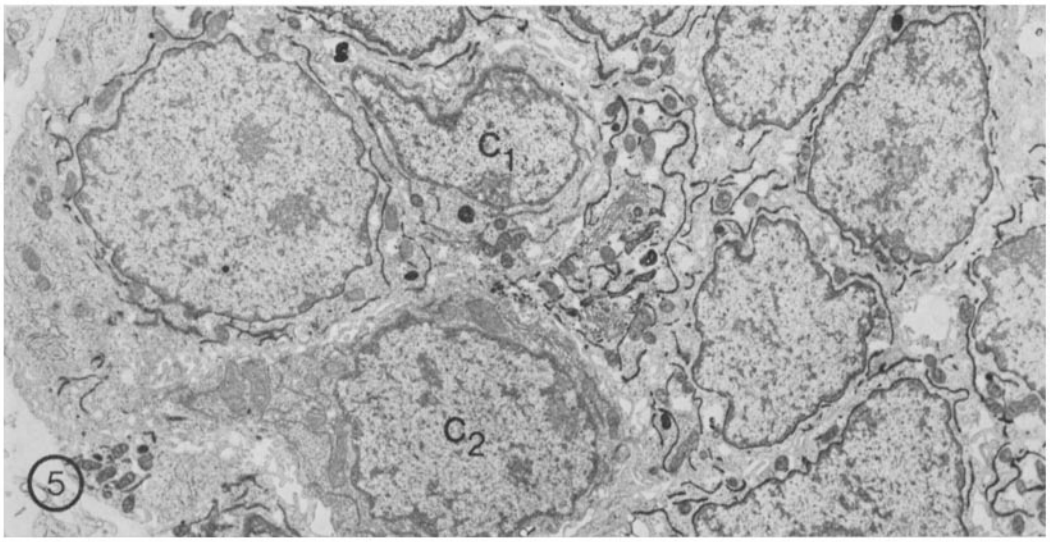

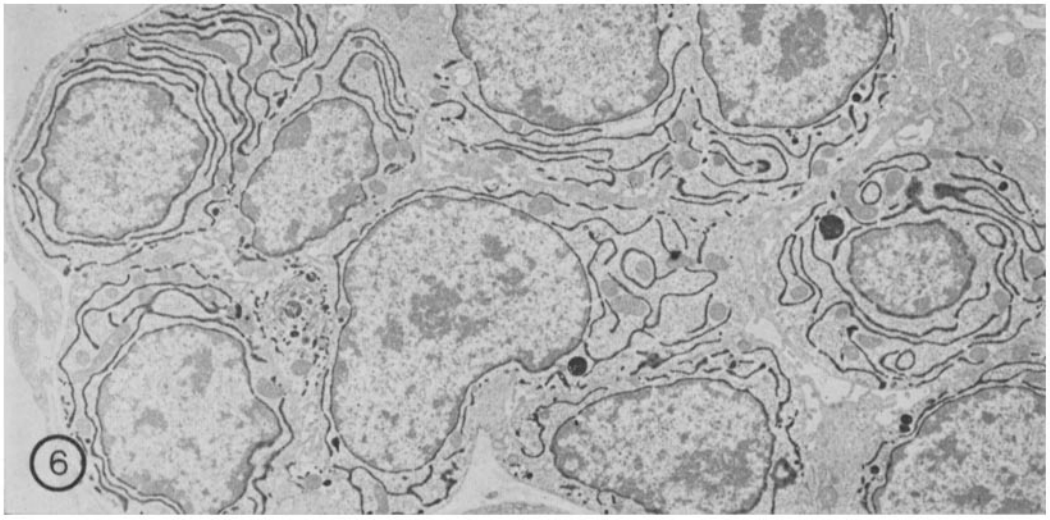

Before birth the peroxidase reaction is negative; it becomes first positive 6 hr after birth in a few scattered cells in different acini 2 days after birth, the majority of the cells (75–80%) is still negative (Fig. 4), whereas at 4 days the situation is reversed; ~80% of the cells are positive (Fig. 5); the number of peroxidase-positive cells increases rapidly after this time point (Fig. 6); at 15 days, 1 day after the lids have opened, all secretory cells are reactive. Peroxidase reaction product is then found

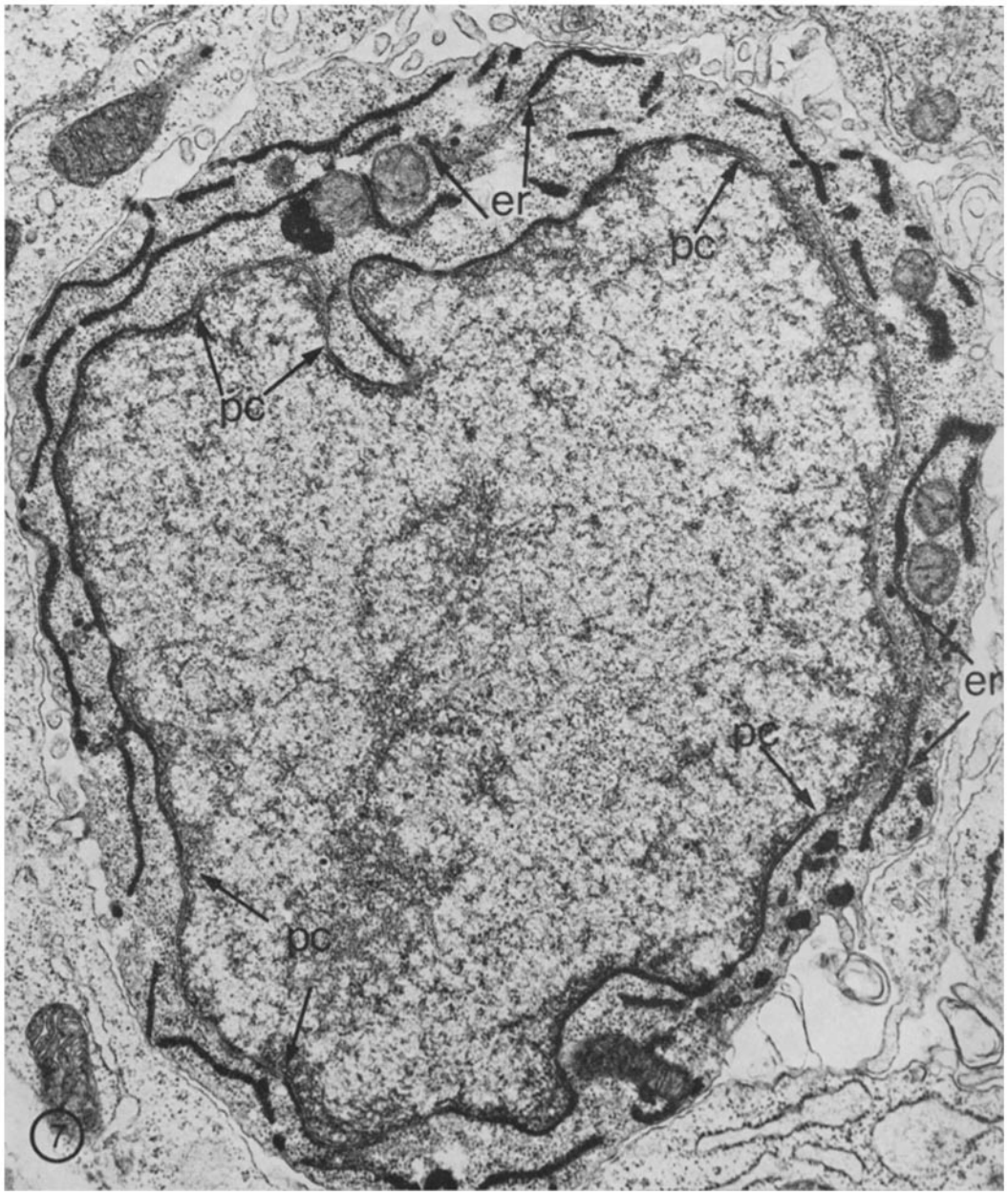

FIGURE 7 Cross-section through an acinar cell 6 hr after birth. Tissue reacted for peroxidase activity. Reaction product fills most of the scant er cisternae and of the perinuclear cisterna, but segments of both structures between arrows marked *er* and *pc*, are devoid of reaction product. × 22,000.

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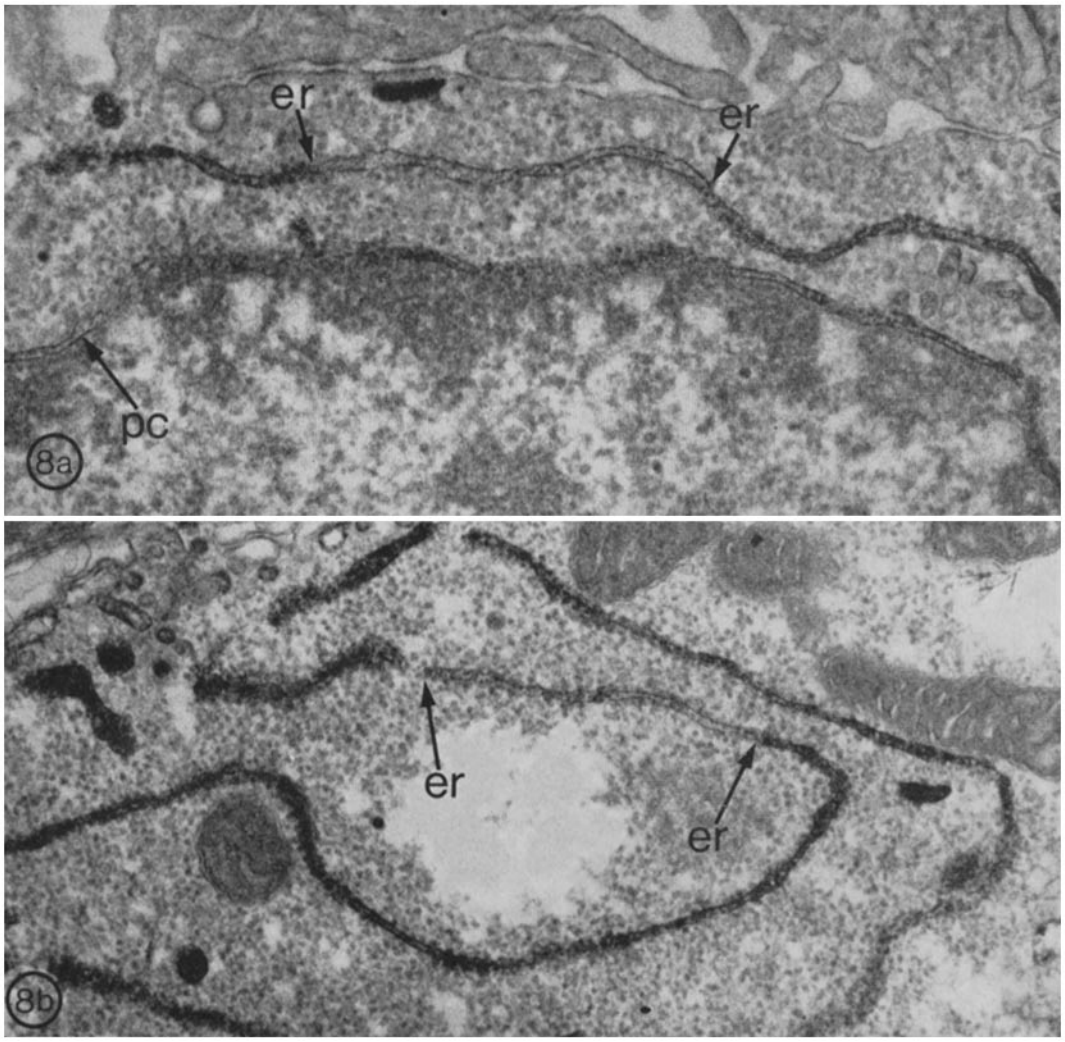
in the cisternae of the er including the perinuclear cisterna, in small smooth vesicles between the er and the Golgi stacks, in condensing vacuoles, and in all secretory granules of both types. The smaller granules appear denser than the larger ones after the reaction, but this does not imply that they contain more reaction product because their density is already greater in unreacted tissue. The Golgi cisternae contain less reaction product than the other structures, or none at all.

In acinar cells 6 hr after birth, reaction product is present in the cisternae of the scant er and in the perinuclear cisterna but does not fill these spaces completely: segments of the perinuclear cisterna up to 5 μ long and of the er cisternae are devoid of reaction product, yet are continuous with peroxidase-positive parts of the same structures (Figs. 7, 8 *a*, 8 *b*). The cisternae of the Golgi stacks rarely contain reaction product at this time point. Smooth-surfaced vesicles with a diameter of 50–70 nm, located between smooth transitional elements of the er and the Golgi stacks (Fig. 9), partly con-



FIGURES 8 *a* and *b* Small fields from two acinar cells 6 hr after birth. Tissue reacted for peroxidase activity. Segments of the cisternae of the rough er (between arrows marked *er*) and of the perinuclear cisterna (arrow *pc*) are devoid of reaction product. Fig. 8 *a*, × 45,000; Fig. 8 *b*, × 38,000.

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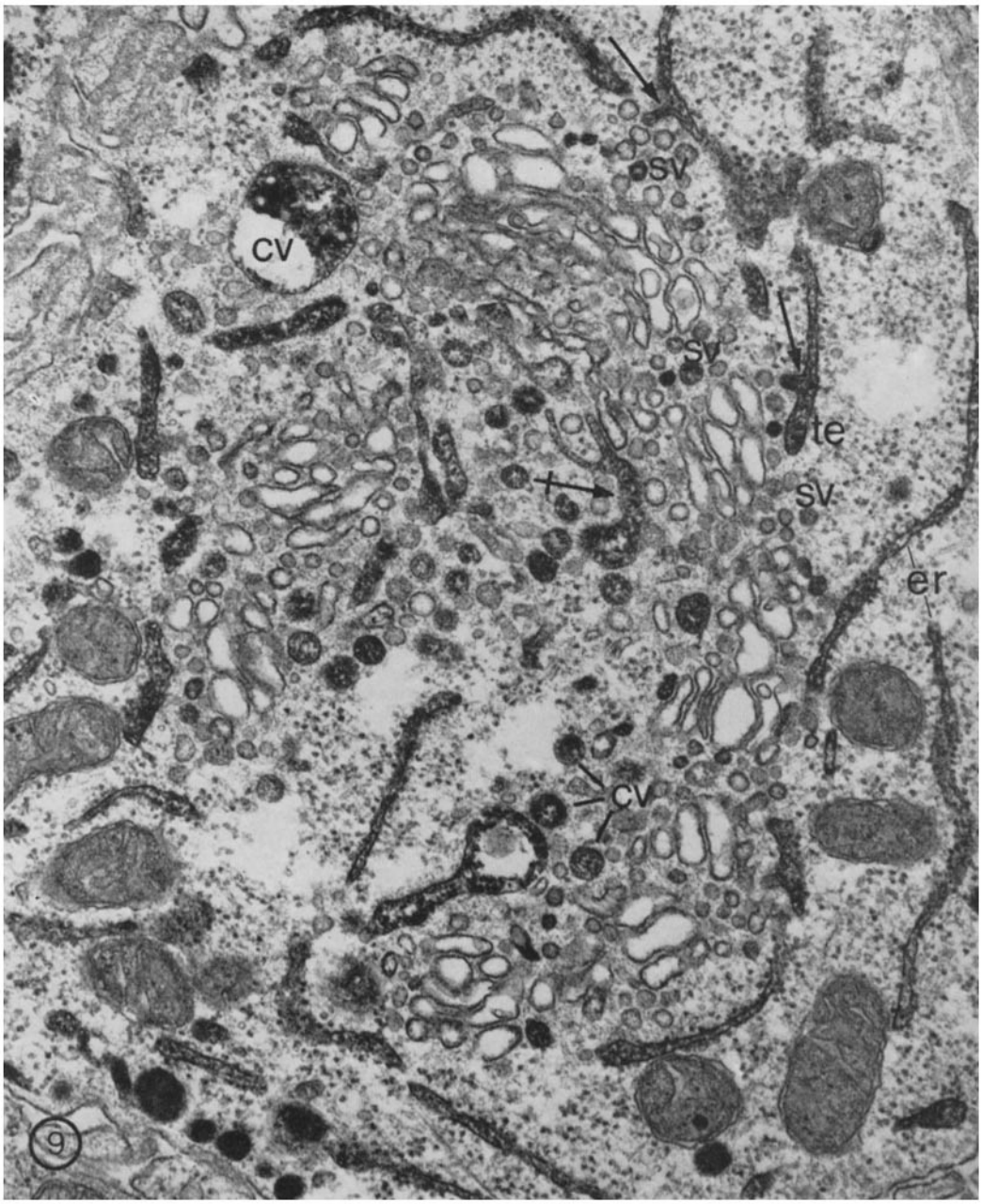

FIGURE 9 Golgi apparatus of an acinar cell 6 hr after birth. Tissue reacted for peroxidase activity. Elements of the rough *er* containing reaction product surround or are interposed between the stacks of the Golgi cisternae. Smooth-walled vesicles (*sv*) lie between smooth, transitional er elements (*te*) and the Golgi stacks. A part of these vesicles which seem to bud off from the transitional elements (arrows) is peroxidase-positive. At the concave face of the Golgi apparatus some condensing vacuoles (*cv*) of varying diameter and a Golgi cisterna (crossed arrow) contain reaction product, but the majority of the Golgi cisternae are peroxidase-negative. × 43,000.

tain reaction product, but some appear peroxidase-negative. Few condensing vacuoles containing reaction product are present.

2–4 days after birth the profiles of the rough er of a given cell are still scant, but the reaction product now fills all cisternae uniformly, including the perinuclear cisterna. Condensing vacuoles are filled with loosely textured reaction product, but the Golgi cisternae are either devoid of it or bear only a narrow rim of it along the inner face of their membranes. 6–10 days after birth the profiles of the er within a cell have increased considerably; all cisternae contain reaction product

After the lids have opened, usually 15 days after birth (24), the profiles of the er are arranged in parallel stacks and are densely packed (Fig 10) with an average distance of 0 15–0 2 μ, all contain reaction product (Fig 11). At later time points,

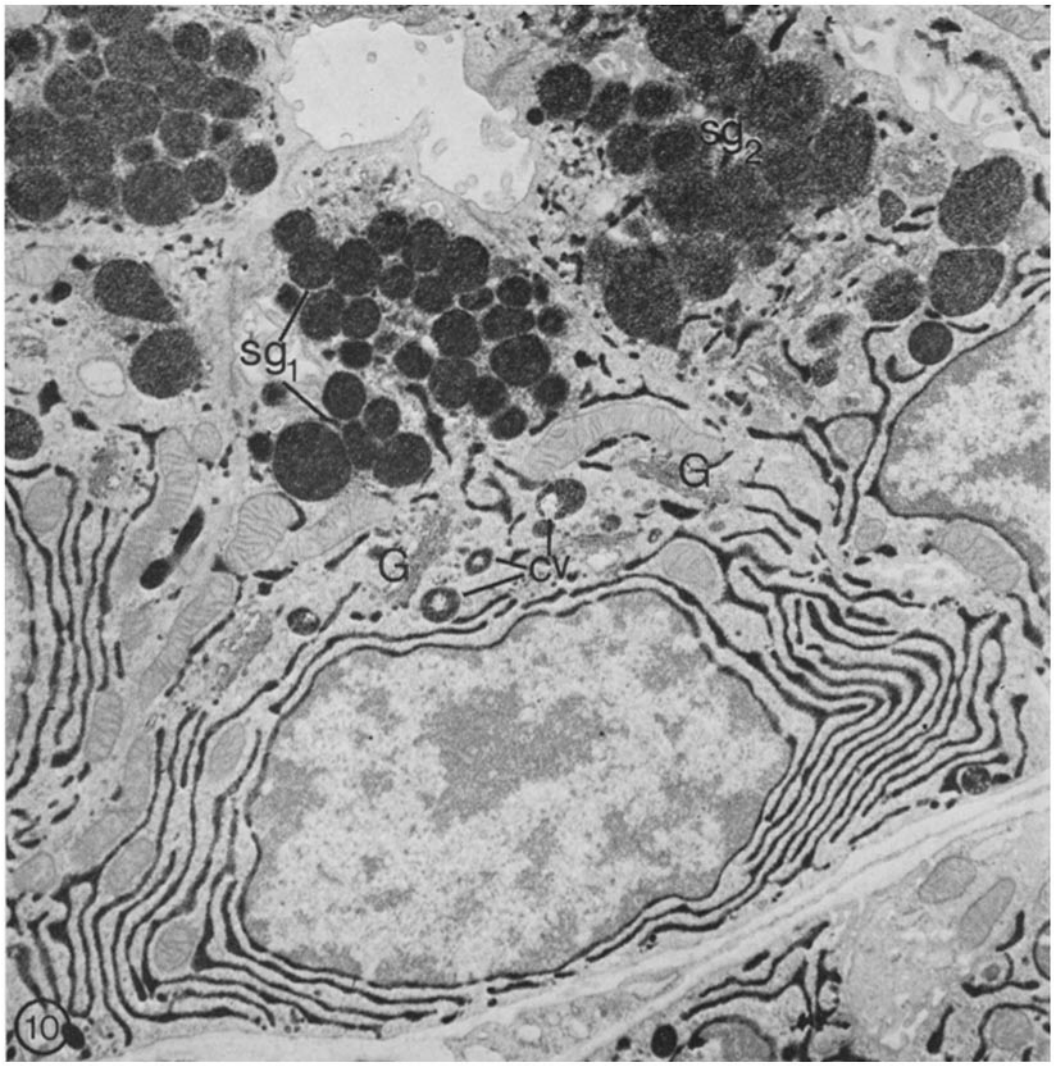

FIGURE 10 Acinar cells 15 days after birth. Tissue reacted for peroxidase activity. All elements of the rough er, mostly in parallel arrangement, contain reaction product which is also present in small vesicles and in condensing vacuoles (*cv*) near the Golgi apparatus (*G*) Two types of secretory granules are present: smaller granules (sg_1) in the apical part of the cell in the middle, and larger granules (sg_2) in the two other cells. Both granule types contain peroxidase reaction product. × 12,500.

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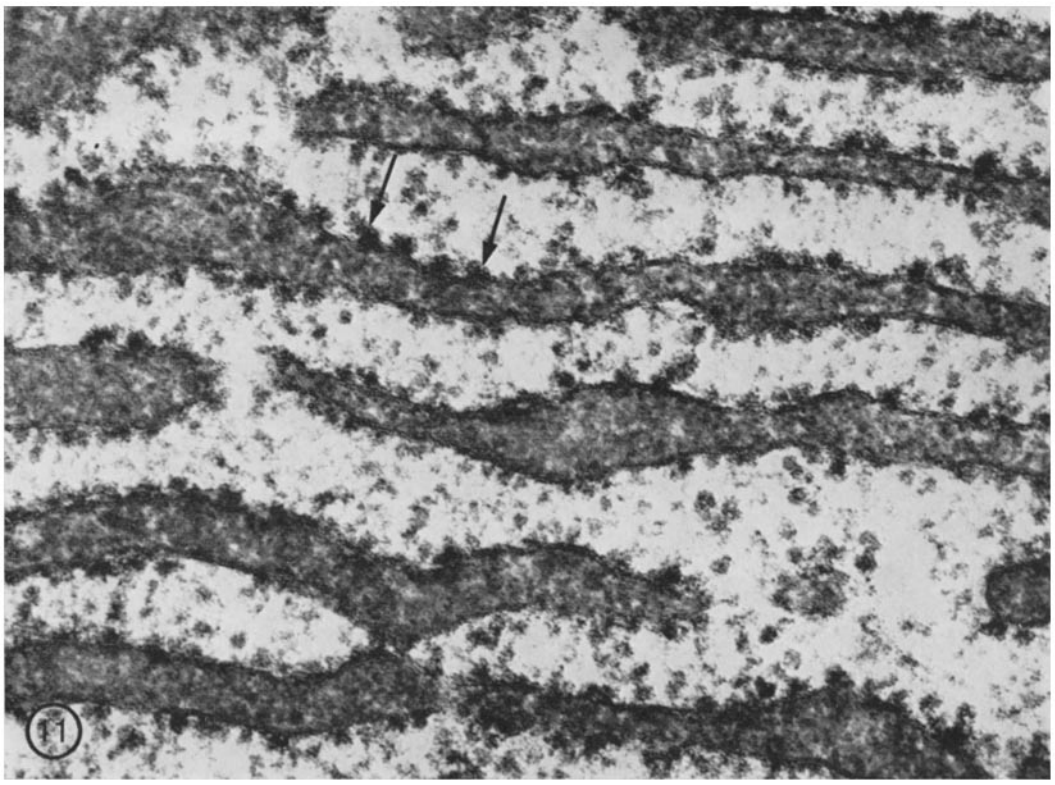


FIGURE 11 Small field of the rough er 15 days after birth. Tissue reacted for peroxidase activity. Reaction product within the cisternae is not homogeneous but appears to consist of small globules. Some of the membrane-bound ribosomes (between arrows) appear to be more electron-opaque than other (unattached?) ribosomes. × 135,000.

TABLE I

Average Number of Profiles of er between Cell Membrane and Nucleus in Cross-Sections of Cells

For each time point 20 cells were counted.

Age	Average number of profiles of er	Average distance between profiles of er
		μ
1 day before birth	1	—
1 " after "	1	—
2 days " "	2	0.6–0.7
4 " " "	2	0.6–0.7
6 " " "	4	0.4
10 " " "	6–7	0.18
15 " " "	9	0.16
28 " " "	15–20	0.15
Adult rat	20–30	0.12

21 and 28 days after birth, and in the adult rat, the er profiles increase further. Table I shows the increase in the er profiles and the decrease in the distance between them, with increasing age. In the adult rat only, a few large (~1.5 μ) pleomorphic granules of varying density and sometimes containing small vacuoles are found near the cell base or in the vicinity of the Golgi apparatus (Fig. 12). Their density does not increase after incubation for peroxidase activity. In unstained light microscope preparations, they have a yellow-brownish color.

Morphology of Tissue Fractions

Both the nuclear and mitochondrial fractions were rather crude. The nuclear fraction contained collagen fibers, basement membrane material, large mitochondria, and secretory granules. The mitochondrial fraction was contaminated by large microsomes and contained many secretory granules. The microsomal fraction (Fig. 13 *a*) was free of mitochondria and secretory granules but contained some smooth-walled vesicles in addition to rough microsomes. After incubation for peroxidase activity, reaction product was found within many

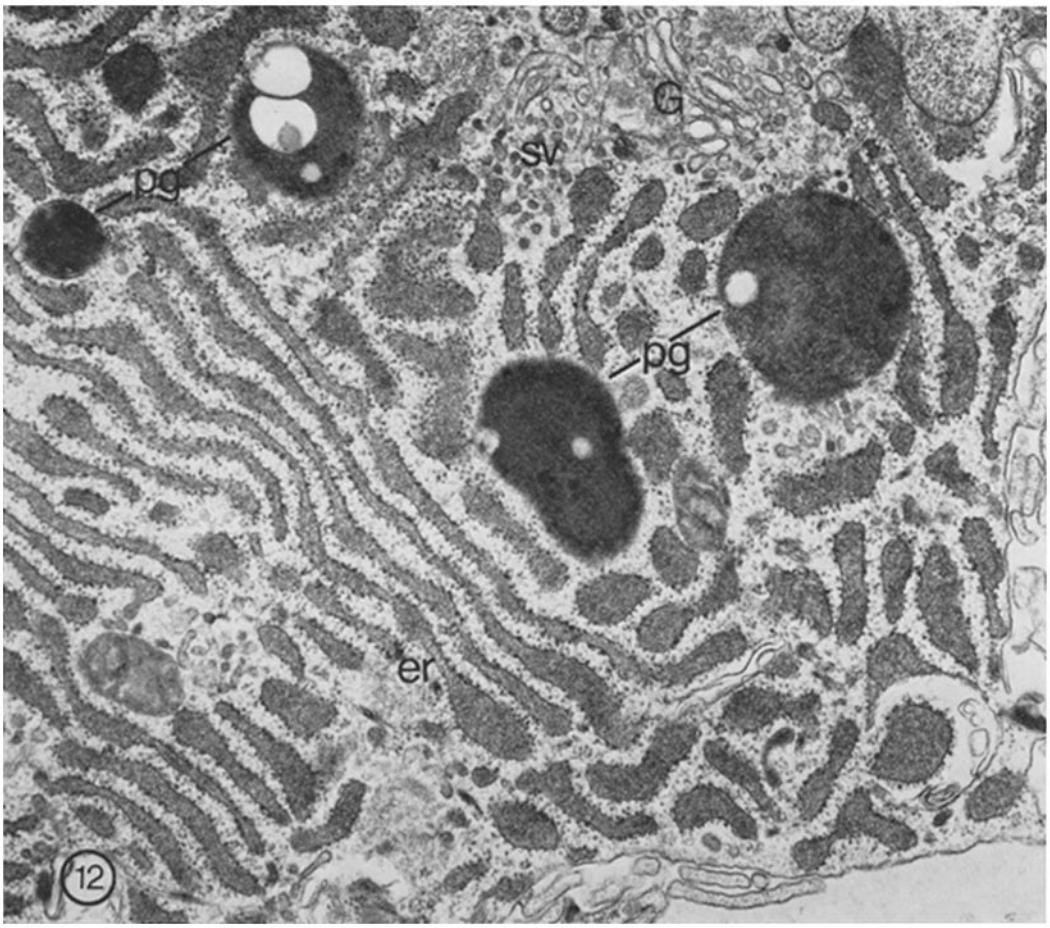


FIGURE 12 Basal part of an acinar cell from an adult rat. Tissue reacted for peroxidase activity. Reaction product is present in the parallel cisternae of the er and in some smooth vesicles (*sv*) near the Golgi cisternae (*G*) which contain no reaction product. Four pleomorphic dense granules (*pg*) resemble wear and tear pigment granules. × 20,000.

rough microsomes (Fig. 13 *b*), but some were devoid of it.

Peroxidase Assay in Tissue Fractions with the Pyrogallol Test

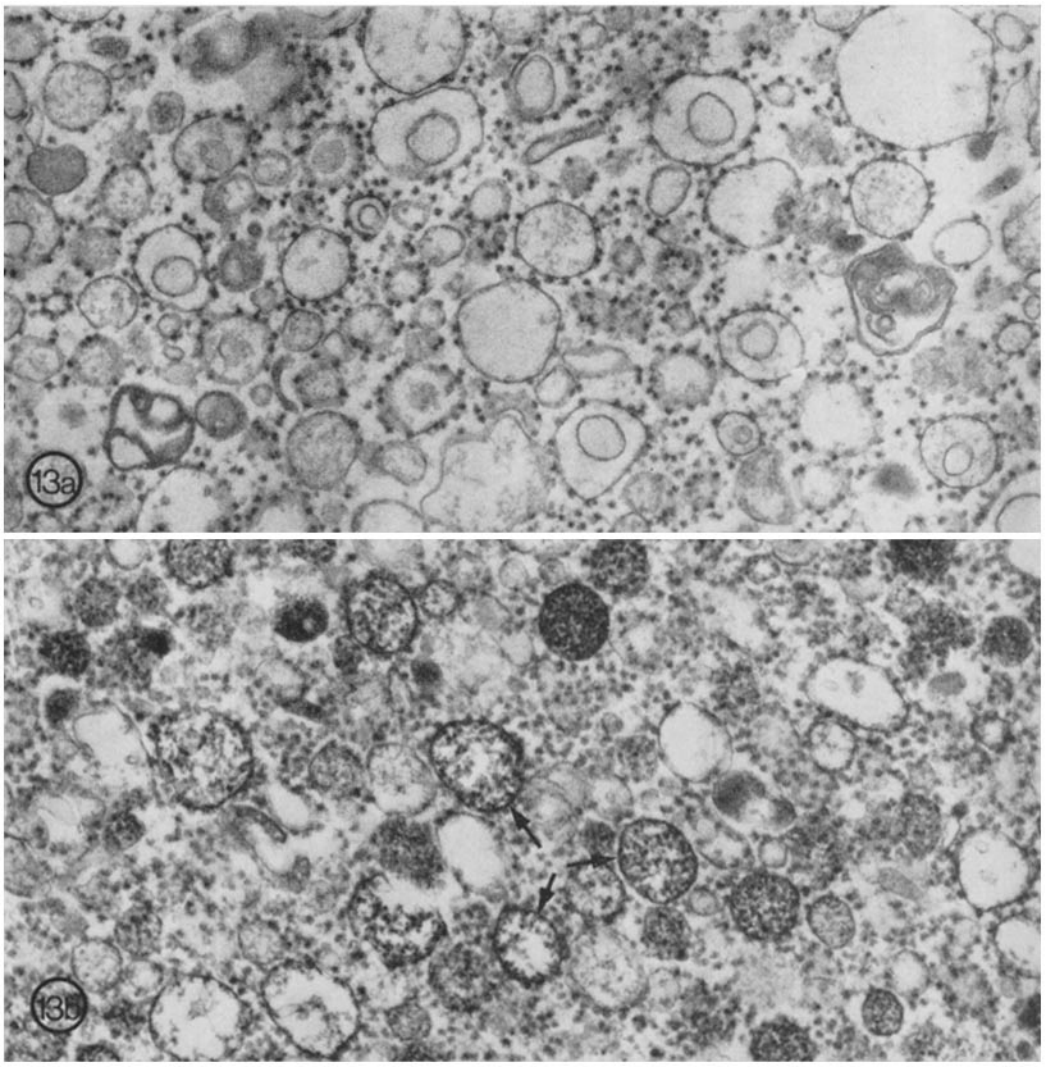
The crude fractions were used to compare their protein and peroxidase content (measured in purpurogallin units) with those of the total homogenate. Table II gives the values of two experiments and shows that the highest values are found in the postmicrosomal supernatant.

Separation of Peroxidase from Catalase by Precipitation with Ammonium Sulfate

The peroxidase assay (21) suffers from interference by catalase. Therefore, peroxidase was salted out by ammonium sulfate. Catalase precipitates at 40–60% ammonium sulfate saturation, while peroxidase precipitates at 70–90% saturation.[3] In a typical experiment on a pooled microsomal fraction the maximal specific activity of catalase was 3 units/mg protein at 40% saturation, and that of peroxidase 0.6 units/mg protein at 70% saturation (Fig. 14).

No catalatic activity was found in lacrimal fluid which contained 1.5–1.6 mg protein/ml of fluid in a typical experiment. Therefore, ammonium sul-

[3] Both, the microsomal fraction and the postmicrosomal supernatant have about equal specific activities (0.6–1.0 unit/mg protein) of peroxidase. In later experiments, both fractions were pooled, or the total postmitochondrial supernatants were used, for the separation of peroxidase.



FIGURES 13 *a* and *b* Microsomal fractions of glandular tissue not reacted (Fig. 13 *a*) or reacted (Fig. 13 *b*) for peroxidase activity. In Fig. 13 *b* most of the rough microsomes contain reaction product (arrows). Figs. 13 *a* and *b*, × 47,000.

fate precipitation was unnecessary The protein content of the pooled rinsing fluids was determined for each spectrophotometric measurement of enzyme activities. The specific activity of peroxidase was 3.5–6.4 units/mg protein. This value was 6–11–fold higher than that found in the microsomal fraction

Enzyme Assays in Microsomal Fractions, in Postmicrosomal Supernatants, and in Lacrimal Fluid

DETERMINATION OF THE SUBSTRATE (H_2O_2) OPTIMUM: The rate of the peroxidase reaction, measured with the guaiacoltest, rises with increasing concentrations of H_2O_2 and reaches a maximum at a H_2O_2-concentration of 1.5 to 2 3 × 10^{-4} M. Further increase in the H_2O_2-concentration reduces the rate of the reaction (Fig 15)

DETERMINATION OF THE PH OPTIMUM: The rate of the reaction measured with optimal H_2O_2-concentration (2 3 × 10^{-4} M) at different pH values reaches a maximum at pH 6.5[4] (Fig. 16).

[4] The cytochemical peroxidase reaction was carried out at pH 7.6, because at pH 6.5 a reaction is observed in mitochondria which is not due to endogenous peroxidase (25).

TABLE II

Protein Content and Peroxidase Activity in Tissue Fractions

	Protein		Peroxidase		
	mg/g gland tissue	%	Specific activity (u*/mg protein)	Total activity (u*/g gland tissue)	%
Total homogenate	103	100	0.935	96.4	100
	104	100	0.822	85 5	100
Crude nuclear fraction	23	22.3	0.75	17.3	17.9
	11	10.0	0.53	5.9	6.9
Crude mitochondrial fraction	20	19.4	1.07	21.4	22.2
	23	22.1	0.87	20.0	23.4
Microsomal fraction	10	9.7	0.42	4.2	4.3
	8	7.7	0.30	2.4	2 8
Postmicrosomal supernatant	41	39.9	0.99	40.5	42.5
	45	43.2	0.96	43 2	50 0
Recovery		91 3			86.9
		83.0			83.1

Values are given for two experiments

* 1 purpurogallin unit = 1 mg purpurogallin formed in 20 sec at 20°C

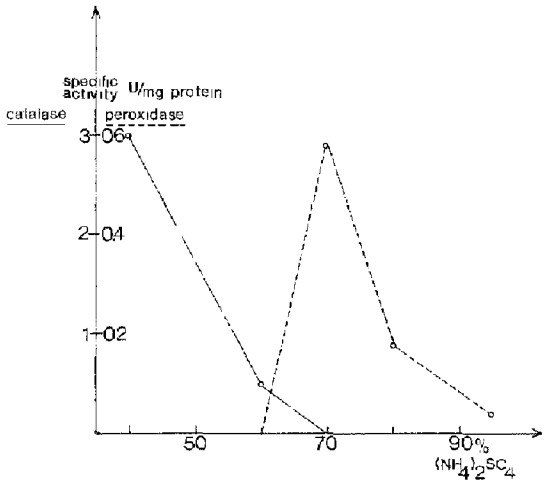


FIGURE 14 Separation of peroxidase (—) from catalase (——) by precipitation with ammonium sulfate in a pooled microsomal fraction. Catalase precipitates at 40–60%, peroxidase at 70–90 saturation with ammonium sulfate

DEPENDENCE OF ENZYME ACTIVITY ON PROTEIN CONCENTRATION At optimal H_2O_2-concentration and optimal pH, the rate of the reaction (measured in ΔE/min) is linearly proportional to the amount of protein (Fig. 17).

DETERMINATION OF THE TEMPERATURE OPTIMUM The rate of the reaction at optimal H_2O_2-concentration and optimal pH reaches a maximum at 42°C No enzyme activity was found above 60°C (Fig 18).

INHIBITION BY AMINOTRIAZOLE: Increasing concentrations of aminotriazole decrease the rate of the reaction. The inhibition begins at a concentration of 10^{-3} M and is almost complete at 10^{-2} M aminotriazole (Fig 19).

Spectropholometric Measuremenls

Peroxidase separated by ammonium sulfate precipitation from the microsomal fraction and the postmicrosomal supernatant and peroxidase in lacrimal fluid both have an absorption maximum at 415 nm which is shifted by 10 nm to 425 nm upon addition of 5 mM H_2O_2 (Fig 20).

Intracardial Injection of Horseradish Peroxidase and Beef Liver Catalase

90 sec after injection of either enzyme, reaction product is found, after incubation in the appropriate media, in the capillaries and larger blood vessels. The endogenous peroxidase in secretory cells also reacts positively in both cases. After incubation

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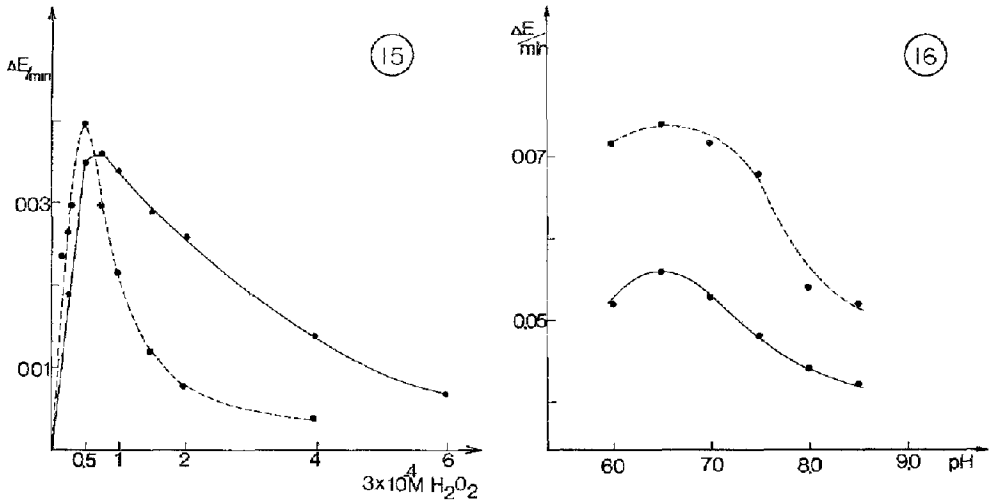

FIGURES 15–20 Some parameters of the peroxidase in microsomal and postmicrosomal fractions of the external orbital gland and in the lacrimal fluid.

FIGURE 15 Substrate optimum determined in the pooled postmicrosomal supernatant (——) after separation of peroxidase at 70% ammonium sulfate saturation and in the lacrimal fluid (---). The rate of the peroxidase reaction reaches a maximum at 1.5×10^{-4} M H_2O_2 (lacrimal fluid) or 2.3×10^{-4} M H_2O_2 (postmicrosomal supernatant). At higher H_2O_2 concentrations the reaction is inhibited.

FIGURE 16 The pH optimum of the peroxidase from lacrimal fluid (---) and from pooled microsomal fractions and postmicrosomal supernatants (——) is at pH 6.5.

in the presence of 10^{-2} M aminotriazole, the endogenous peroxidase activity of the secretory cells is completely abolished; exogenous horseradish peroxidase activity persists, while exogenous beef liver catalase activity is greatly reduced; the pseudoperoxidatic activity of erythrocytes persists in both cases.

DISCUSSION

General

The results show that a peroxidase is produced by the acinar cells of the glandula orbitalis externa of the rat, and is secreted in to the lacrimal fluid. The production begins ~6 hr after birth, but is synchronized neither within the cells composing an acinus nor within a single acinar cell. The biochemical parameters of the peroxidase found in microsomal and postmicrosomal fractions and in lacrimal fluid are qualitatively identical.

Localization of Peroxidase in Cell Compartments

The intracellular localization of endogenous peroxidases has been investigated in several cell systems In eosinophil promyelocytes of rat and rabbit (26, 27), and in neutrophil promyelocytes of cat (28, 29) and man (30), the entire er and the Golgi apparatus function in the segregation and condensation of endogenous peroxidase. In the parotid gland of the rat (8) few Golgi cisternae contain reaction product; peroxidase seems to be shuttled directly from the er via small smooth vesicles into condensing vacuoles, in this gland the pathways of transport of peroxidase are similar to those found for other proteins in the exocrine pancreas of the guinea pig (31, 32, 33). In the submaxillary gland of the rat, the Golgi cisternae are peroxidase negative (7). The situation as to the functional coupling between the compartments of the er, the Golgi apparatus, and the condensing vacuoles in the glandula orbitalis externa is the same as in the parotid gland: the Golgi cisternae rarely contain reaction product; if reaction product is present, it is always found in lesser amounts than it is in either the cisternae of the er or the condensing vacuoles and the secretory granules. In the external orbital gland of adult rats, Essner (34) also found reaction product only in one or two Golgi cisternae. Moreover, not all smooth-surfaced vesicles that are situated between smooth-surfaced parts of

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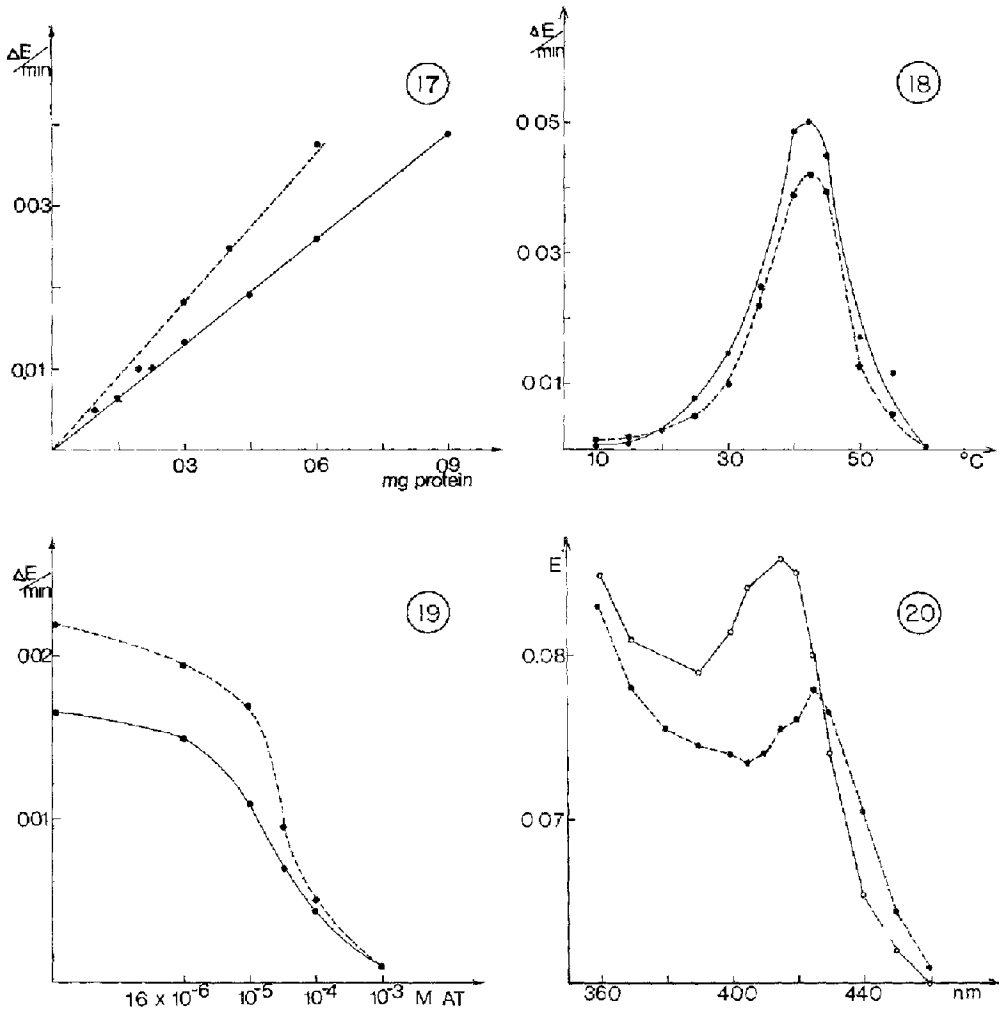

FIGURE 17 Dependence of peroxidase activity on protein concentration determined in the lacrimal fluid (---) and in the pooled postmicrosomal supernatants (——) after separation of peroxidase at 80–95% ammonium sulfate saturation. Enzyme activity is linearly proportional to protein concentration.

FIGURE 18 The temperature optimum of the peroxidase in lacrimal fluid (---) and the pooled microsomal fractions and postmicrosomal supernatants (– – –) is at 42°C.

FIGURE 19 Inhibition of peroxidase by aminotriazole in lacrimal fluid (---) and the pooled postmicrosomal supernatants (——) is almost complete at 10^{-3} M aminotriazole.

FIGURE 20 Absorption spectra of lacrimal fluid before (——) and after (---) addition of 5 mM H_2O_2.

the cr (transitional elements [31]) and the Golgi stacks and that sometimes communicate with one or the other system are peroxidase positive

The functional significance of the existence of two types of secretory granules is not clear. Scott and Pease (35) described two types of granules in the lacrimal gland a light type resembling the mucous granules of the sublingual gland and supposedly arising from the Golgi apparatus, and a dark type resembling zymogen granules and without apparent connection to the Golgi apparatus. Due to different techniques, a comparison of these early findings with the present study is not possible. Essner (34) also described two types of secretory granules, the larger one of which, contrary to our findings, was peroxidase negative, presumably due to bad preservation, and he discussed their possible significance. The pleomorphic dense granules that

occur only in the adult rat near the cell base were also seen by Essner (34) who believed them to contain partly peroxidase and partly acid phosphatase. On the basis of their morphology and color in unstained sections, they seem to belong to wear and tear pigment granules.

Localization of Peroxidase in Postnatal Development

Before birth the acinar cells are invariably peroxidase negative. Reaction product is first demonstrable 6 hr after birth in localized areas of the er cisternae and the perinuclear cisterna in a few cells. Synthesis of peroxidase and its vectorial transport into the cisternae of the er begins, therefore, in circumscript areas of a given cell. The same situation was observed in early neutrophil promyelocytes of man (3) At later time points, the entire cisternal spaces of the er contain reaction product and the number of peroxidase-positive cells increases rapidly; but it cannot be decided, at the moment, whether the initial sites of peroxidase production remain localized or spread out over wider segments of the er. In the first case, the filling of the entire er cisternae would indicate diffusion of reaction product from localized production sites, in the second case, one would have to assume that the entire rough er operates in the production of peroxidase. Staining of ribosomes by the peroxidase method has been claimed by several authors (7, 36, 37), but we were not able to distinguish on this basis between ribosomes synthesizing peroxidase and ribosomes inactive in this respect. The localized peroxidase production in a given cell after birth contrasts with the development of glucose-6-phosphatase production in rat hepatocytes; this enzyme is produced simultaneously within all of the rough er membranes of a given cell already 4 days before birth (38) and no regional differentiation within the rough er with respect to this enzyme seems to exist (39)

Differentiation between Catalatic and Peroxidatic Activity in Glandular Tissue

The cytochemical reaction with diaminobenzidine is not specific for peroxidase Reaction product, presumably due to cytochromes, is found on the cristae mitochondriales at lower pH and reduced H_2O_2 concentration of the incubation medium (25). Catalase is preferentially stained in peroxisomes when pH and concentrations of H_2O_2 and diaminobenzidine are raised (14, 15).

Both exogenous catalase and glandular peroxidase are inhibited by aminotriazole, whereas exogenous horseradish peroxidase and the pseudo peroxidatic activity of hemoglobin persist after incubation in the presence of aminotriazole. Therefore, catalase could not be distinguished from endogenous glandular peroxidase cytochemically. By fractionated precipitation with ammonium sulfate, peroxidase could be separated from catalase in glandular tissue fractions The presence of catalase in tissue homogenates remains unexplained and needs further investigation; it might be due to the presence of erythrocytes and leukocytes which contain catalase (40) Since peroxidase is also found in the postmicrosomal supernatant, we assume that the enzyme leaks out due to disruption of the er upon homogenization

Identity of Peroxidase in Glandular Tissue and Lacrimal Fluid

Catalase is not present in the lacrimal fluid. The peroxidase in tissue fractions and in the lacrimal fluid is identical with respect to substrate-, pH-, temperature-optimum, and inhibition by aminotriazole. The absorption maximum of the peroxidase in the tissue and in the lacrimal fluid is at 415 nm and is shifted to 425 nm upon addition of H_2O_2. Similar results were described in saliva. In homogenates of bovine lacrimal (5) and salivary (6) glands a peroxidase was found which had the immunological characteristics of bovine lactoperoxidase (5, 6). Lactoperoxidase has an absorption maximum at 415 nm which is shifted to 430 nm upon addition of H_2O_2 (41). Therefore, it is evident that peroxidase is secreted by the glandula orbitalis externa of the rat into the lacrimal fluid; whether the enzyme is a lactoperoxidase requires further investigations.

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