antigens are widely dispersed throughout the cell with maximal concentration in the 10,000 *g* and 30,000 *g* sediments. 2. A low incidence of tolerance was also noted when the H_3 histocompatibility difference was crossed. This was increased either by additional pretreatment with sublethal doses of irradiation or continuation of the antigenic injection after grafting, both of which resulted in a much higher mortality rate. The use of subcellular fractions in an attempt to produce tolerance across the H_2 histocompatibility difference was not possible in this study.

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Ligation of Mouse Submandibular Glands and Its Effect on Components of Nerve Growth Factor.* (30988)

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A protein which enhances the growth of spinal and sympathetic ganglia and their processes has been found in various tissues and secretions of chicks, reptiles, and in several mammalian species(1,2). The richest source of this nerve growth factor (NGF) is the mouse submandibular gland(3). Fluorescent antibody studies have indicated that NGF is located in the serous tubule(4), a structure whose size is dependent upon androgens(5). It has been recently demonstrated that the diameter of the serous tubule (Tubular Index) and the log of the concentration of NGF in the mouse submandibular gland are directly proportional(6).

Mouse NGF has two components, both of which must be present for biological activity. As described by Schenkein and Bueker(7), these are: 1. a protein, "A," with a molecular weight of about 8,600 and 2. a dialyzable peptide, "C," with a molecular weight of about 3,500.

Junqueira reported on the effect of excretory duct ligation on the rat submandibular gland(8). Ligation caused complete atrophy of acinar cells, while the tubular cells, though diminished in size, retained their basic structure. Since this change was atrophy rather than degeneration, he called the ligated gland a "resting gland." To contribute additional observations on the mode of biosynthesis of NGF, we have investigated the concentrations of, and isotope-labeled amino acid incorporation into, "A" and "C" in mouse submandibular glands in the "resting" state which results from ligation. Of special interest was the possibility of correlating the fluorescent antibody localization of NGF in the serous tubules with the retention of only the tubules in the "resting" gland, along with the continued production of NGF in ligated glands.

Method. Adult male Swiss white mice were anaesthetized with intraperitoneal injections of pentobarbital-sodium. Their submandibular ducts were ligated with 4/0 silk sutures just at the site of leaving the glands, with individual sutures used on each side. The single midline incisions were closed with interrupted silk sutures. At 5-day intervals after the operations, 5 animals were injected intraperitoneally with ^{14}C-algal protein hydrolysate† for a total of 3.33×10^6 counts per

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† NEC-233, from New England Nuclear Corp. Boston.

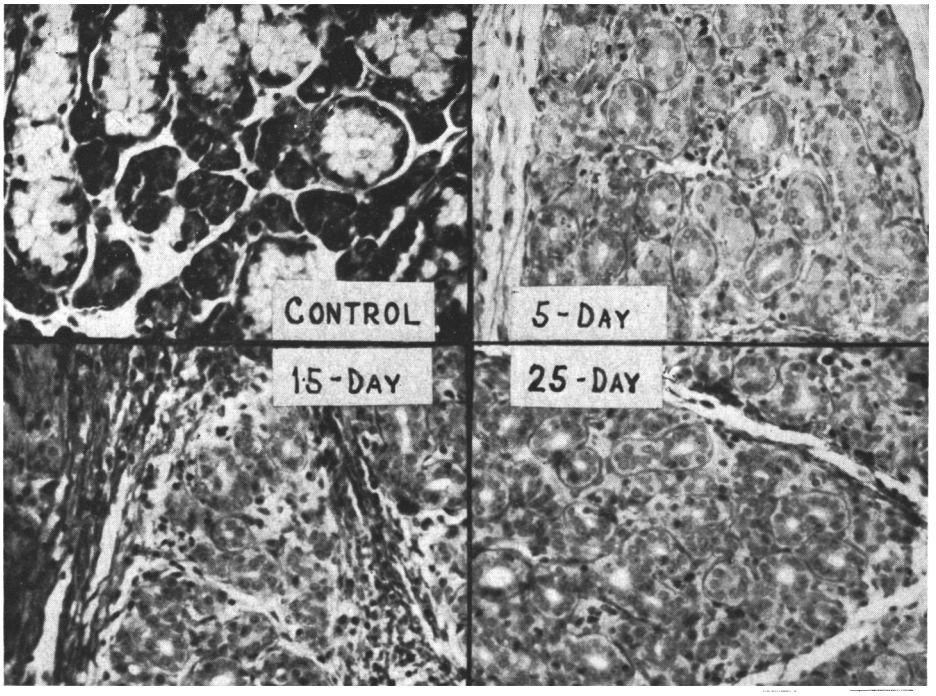

FIG. 1. Mouse submandibular glands at various times after ligation of excretory ducts; stained with H.+E. ×200.

minute (CPM) per animal. Three hours after this injection the animals were sacrificed by chloroform inhalation and their submandibular glands were removed. These glands were weighed, a sample was taken for histological study, and the remainder was frozen in buffered isotonic saline until all the groups were collected.

Groups of 5 mice with ligated glands were sacrificed at 5-day intervals from 5 to 30 days, and at 45 days. Frozen glands of each group were thawed, and completely macerated with a glass homogenizer. Crude homogenates were centrifuged at 6,000 × *g* for 15 minutes and supernates analyzed for protein concentration by the Lowry method(9). Each preparation was bioassayed for nerve growth activity with spinal ganglion explants from 8-day chick embryos, suspended in hanging drop cultures of rooster plasma containing aliquots of the supernate(10) at dilutions of 10^{-1} to 10^{-6}. The remainder of each group was purified according to the method recently reported by Schenkein and Bueker(7), with substitution of CM- and DEAE-cellulose chromatography by Rivanol‡ precipitation, a method especially suited for small fractions of NGF.§ This was followed by gel filtration of the Rivanol supernate on Sephadex G-25. This separated the NGF material into peaks of "A," "AC," and "C." Sephadex fractions were each analyzed for total protein by the Lowry method, and the incorporation of labeled amino acids measured with a Geiger-Müeller counter

Results. The histological changes in the mouse submandibular glands due to ligation are shown in Fig. 1. The acini rapidly underwent atrophy; by the 15th day their basophilia had disappeared and they appeared as amorphous clumps of cells. An inflammatory reaction, with infiltration of lymphocytes, occurred in the acinar areas. Proliferation of connective tissue in these areas was so extensive that homogenization of the glands was accomplished with difficulty. The serous tubules diminished in size (Fig. 2a), and loss of secretory granules and mitochondria, along with an increased diameter of the lumina,

‡ Rivanol (Ethodin) from Winthrop Laboratories, New York City.

§ I. Schenkein, unpublished data.

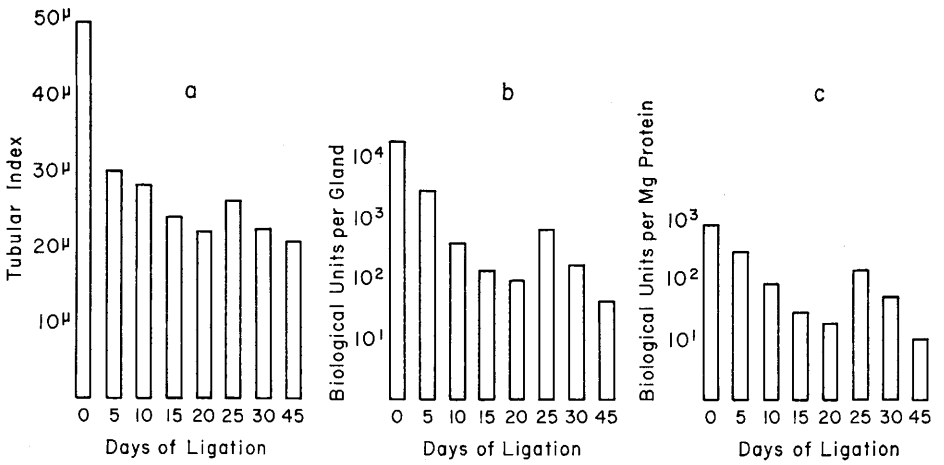

FIG. 2. Effect of excretory duct ligation on: a. Tubular Index, b. biological activity per gland, and c. relative concentration of NGF.

indicated a loss of secretory activity. However, the basic structure of the tubules was retained.

Bioassays at first showed a rapid decrease in the NGF content, with a sloping off after the 20th day (Fig. 2b & 2c). Typically, bioassays show a dense halo of nerve outgrowth in a 3+ response (Fig. 3a) when in the presence of one biological unit(10). However in the 15- and 20-day groups we found that neurites grew out as far as in a normal 3+ response but that the density was relatively sparse (Fig. 3b). This was similar to bioassays in which we assay NGF insufficient in the amount of the dialyzable polypeptide "C."

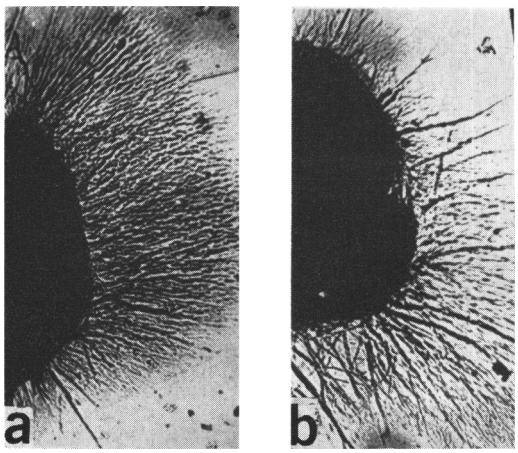

FIG. 3. Bioassays, using eight-day chick embryo spinal ganglia in hanging drop cultures: a. 3+ response, indicating presence of one biological unit (B.U.) per ml; b. best response possible from glands ligated 15 to 20 days.

An indication of the cause for this result is seen in Fig. 4 which shows Sephadex profiles of the various ligation-time groups. In the control there were 3 peaks: peak I is "A"; peak II, "A-C"; peak III, "C." Peak I ("A") was present in all the groups, but in decreasing amounts. Peaks with "C," on the other hand, decreased rapidly, and by the 25th day were practically undetectable.

For verification of this loss of "C," an agar double diffusion test was employed. Using bovine anti-NGF-serum,‖ "A" and "A-C" routinely give precipitin bands(11). With material from ligated glands, the precipitin band associated with "AC" was undiscernible after 20 days (Fig. 5). The precipitin band associated with "A" remained.

A summary of all data from ligation experiments is shown in Table I. In the Sephadex peak I line of the Table we see that the amount of component "A," although steadily decreasing in total amount, was still synthesized, as measured by the uptake of ^{14}C-amino acids. The peaks containing "C," on the other hand, rapidly decreased in concentration, and by day 20, measurement of labeled amino acid incorporation into "C" was barely above background.

‖ Courtesy of Abbott Laboratories, North Chicago, Ill.

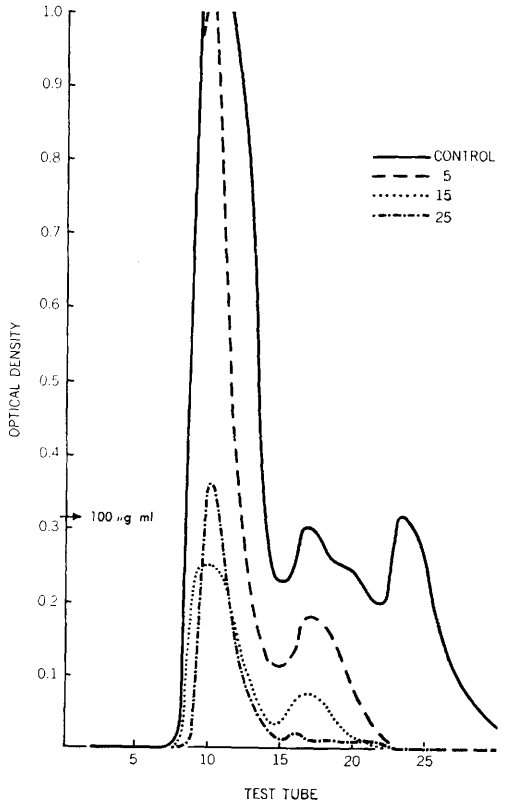

FIG. 4. Gel filtration of Rivanol-soluble protein from 0-, 5-, 15-, and 25-day ligated glands: Lowry protein determinations(9) of materials eluted from 21 × 350 mm column of Sephadex G-25.

Discussion. Ligated glands maintained their production of component "A," but "C" was lost quickly. The nerve growth obtained was due largely to "C" which apparently was stored rather than produced *de novo*. Preliminary studies on the *in vitro* biosynthesis of NGF have indicated that only component "C" is androgen-dependent, and that submandibular duct ligation in some way interferes with normal gonadal function. Suzuki reported gonadal atrophy resulting from submandibular gland ablation in guinea pigs(12), but Junqueira found no testicular changes after submandibular gland ligation in rats (although he was able to bring about a reappearance of mitochondria in the tubular cells by means of testosterone injections(8). In our experiments, mice showed testicular atrophy (Fig. 6) which was seen at 20 days of ligation as a slight disorganization of the germinal epithelium in a few spermatic tubules, and at 120 days of ligation as complete degeneration of the germinal epithelium in 50-90% of the spermatic tubules. This was accompanied by a slight but constant testicular weight loss and deposition of fat around the epididymis. It was thought that this interference with gonadal structure might be related to the diminution of "C," and a parallel experiment was performed in which testosterone propionate pellets were implanted in mice with ligated glands. As a result of the presence of the exogenous testosterone the tubular cells were slightly larger than in the comparable untreated ligated glands, and the amount of "A" which was synthesized was proportionately increased; there were still

TABLE I. Summary of Data from Ligation Experiments.*

Duration of ligation	Control	5 days	10 days	15 days	20 days	25 days	30 days	45 days
No. of animals	5	5	5	5	5	5	3	4
Wet wt of glands (mg)	247	233	126	81	94	66	62	84
Soluble protein (mg)†	28.1	9.40	5.90	4.64	5.38	4.30	3.31	4.25
Nerve growth activity (μg/B.U.)	1.0	3.0	10	30	50	6.0	17	100
B.U./gland	28,100	3130	590	153	106	717	183	42
B.U./mg protein	1000	333	100	33.3	20.0	167	59.0	10
T.I. (μ)	50	30.3	28.5	24.3	22.5	26.2	22.7	20.8
Sephadex fractions								
I. ("A") protein (mg)†	1.85	1.16	.31	.31	.39	.34	.20	.13
CPM/mg	777	683	770	373	688	657	220	440
II. ("AC") protein (mg)†	.51	.28	.06	.09	.08	Trace	Trace	Trace
CPM/mg	692	332	179	118	37			
III. ("C") protein (mg)†	.44	—	—	—	—			
CPM/mg	598	—	—	—	—			

* All data expressed as value per mouse.
† Protein determinations by method of Lowry *et al*(9).

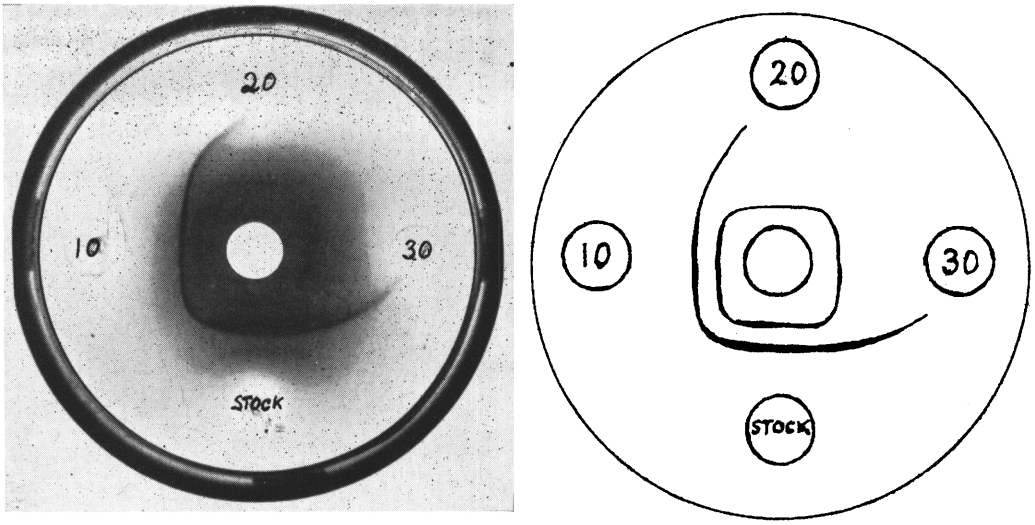

FIG. 5. Agar immunodiffusion test of NGF from ligated glands *vs.* bovine anti-NGF-serum: inner precipitin band is associated with "A" and outer band with "AC." Stock material is added for comparison. Numbers indicate time of ligation in days. Stained with naphthol blue-black.

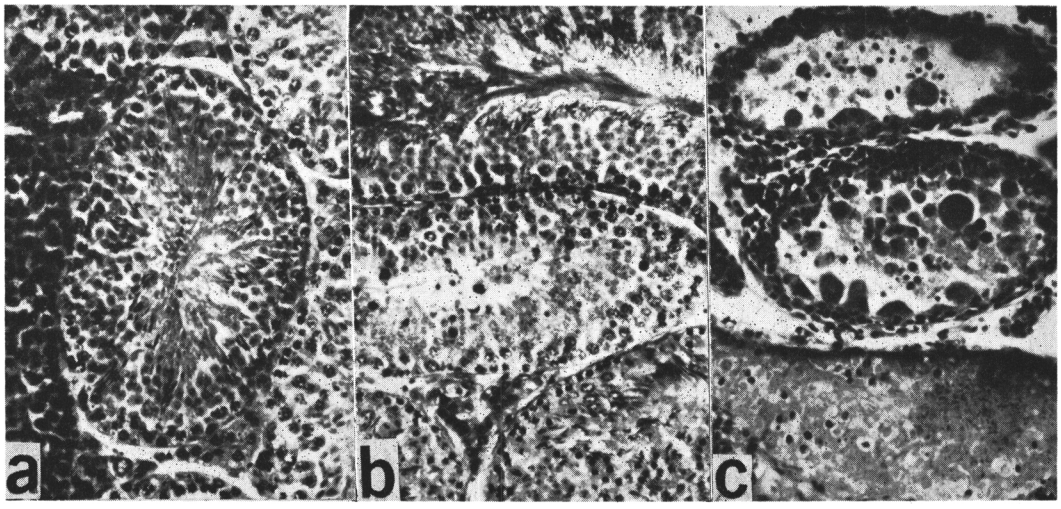

FIG. 6. Changes in spermatic tubules as a result of submandibular duct ligation: a. sham operated; b. after 20 days of ligation; and c. after 120 days of ligation. H.+E. ×150.

only traces of "C," however. There are two possible explanations for this: "C" may be synthesized elsewhere in the body, and merely stored in the gland, or it may be synthesized (or stored) in the acinar portion which is almost completely atrophied in the ligated gland.

The specificity of NGF for the sympathetic nervous system has been demonstrated directly by its injection into mice, and indirectly by injection of its antiserum which results in an immunosympathectomy(3). Thus, NGF can be said to have an endocrine-like action, and interruption of the exocrine function of the submandibular gland depresses but does not remove the endocrine-like function of its serous tubules, that is, the synthesis of the protein "A."

Summary. Ligation of submandibular salivary ducts causes a loss of production of nerve growth factor. The change is proportional to loss of the polypeptide cofactor "C" rather

than to the diminution of the protein "A," which is continually synthesized. The continued production of "A" in the ligated gland suggests an endocrine function for submandibular glands.

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Studies on Anthramycin Sensitivity in *Euglena*. (30989)

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Anthramycin(1) is a low molecular weight antitumor principle produced from the fermentation broth of *Streptomyces refuineus* var. *thermotolerans*. Even in its crude form as "Refuin"(2) there was marked antitumor activity and ability to convert fluid Erlich acites to solid tumors which, in many cases, subsequently disappeared. Preliminary clinical results with pure Anthramycin, methyl ether, confirm the early results with cruder preparations that this compound is not only a promising tumor palliative but also lacks such adverse clinical effects as bone marrow depression and renal or gastrointestinal toxicity(3).

Some of the factors which influence drug action at the cellular level are revealed in our studies reported here using the alga, *Euglena gracilis*. This organism was chosen because of a) its wide pH tolerance: it can grow in defined media ranging from pH 3-8, and b) its ability to use either photoautotrophic or heterotrophic pathways to satisfy its nutritional requirements.

Methods. Euglena gracilis, Z strain (ATCC 12716) was maintained in an undefined maintenance medium(4) which was distributed 10 ml/20 × 125 mm screw-capped tube. All maintenance cultures were incubated at 24-26°C illuminated by warm-white fluorescent tubes.

Experimental cultures were grown in Difco Bacto 0532 *Euglena*, pH 3.6 B_{12} assay medium or in a pH 5.2, B_{12} assay medium ordinarily used for *Ochromonas malhamensis*(4). Both media were supplemented with vit. B_{12} (0.1 μg%). For some experiments, the pH 3.6 medium was solidified with agar (1.5%). Anthramycin, generously supplied by Hoffmann-LaRoche Inc. as a sterile, water soluble, mannitol triturate, was added aseptically to appropriate experimental cultures.

Growth of liquid cultures was evaluated densitometrically with a Welch Densichron equipped with a red probe and calibrated so that optical density units are proportional to number of cells. Growth on solidified media was evaluated by direct colony count.

Results and discussion. Biological activity of Anthramycin is demonstrable as inhibition of growth of *E. gracilis* grown at either pH 3.6 or 5.2. The increased percent inhibition at pH 3.6 as compared with that observed at pH 5.2 (Table I) may indicate increased