

REGULATION OF PROTEIN PRODUCTION IN THE SILK GLANDS OF SPIDERS*

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Abstract—1. A single injected dose of acetylcholine into the spider, *Araneus sericatus*, causes secretion of performed fibroin from the ampullate silk glands. This is followed by increased protein synthesis, measured by incorporation of labelled amino acids and nucleic acid precursors.

2. Puromycin inhibits the increased synthesis of new protein but does not affect the initial secretion. Thus the processes of secretion and synthesis can be separated.

3. A scheme is proposed for the overall regulation—both central and local—of the protein production in the ampullate gland of *Araneus sericatus*.

INTRODUCTION

THE silk gland of the spider has been found to be a convenient system for the study of a mechanism which regulates protein synthesis in relation to the total economy of a simple organ. The ampullate silk gland has as its sole function the rapid production of a single protein which is used to construct the scaffolding of the daily web (Peakall, 1964a). Previous experiments have demonstrated that the rate of incorporation of amino acids can be stimulated by cholinergic materials both *in vivo* and *in vitro* (Peakall, 1964b, 1965). Stimulation of protein synthesis can also be accomplished by emptying of the gland. In this paper the effects of a single dose of acetylcholine are examined and a scheme for the overall regulation of the gland is presented.

MATERIALS AND METHODS

The spiders (*Araneus sericatus*) were emptied of thread 24 hr before the start of the experiments. This was to ensure that the ampullate glands were in as uniform a physiological state as possible. In all experiments sections were made of the abdomen and alternate sections were stained with Azure B, or hemaoxylin and eosin, or prepared for autoradiographs.

One group of spiders were injected with T³-alanine (5 mc/kg) and acetylcholine (0.1 mg/kg) at the start of the experiment and killed by severing at the pedicel after a given time period (5, 10, 20, 30 and 60 min). The control group of spiders were injected with T³-alanine only. In the second series of experiments puromycin (1 mg/kg) was injected 30 min before the start of the experiment and T³-alanine

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was injected at the start of the experiment. Half of the spiders were given acetylcholine and killed after given time intervals as before.

Two sets of experiments were performed to observe the time sequence of changes in nucleic acids following stimulation. The spiders were injected with T³-thymidine (10 mc/kg) 2 hr prior to the start of the experiment. Half of the spiders were given acetylcholine and were killed after given time intervals as before. The glands were immediately removed and fixed in buffered formaldehyde. The sections were treated with RNAase [0.2 mg/ml, 45°C, for 4 hr, Flax & Himes (1952)] and then alternate sections were made into autoradiographs or stained with Azure B. In the second set of experiments T³-orotic acid (experiments failed to show incorporation of uridine) and DNAase were used in place of thymidine and RNAase. The conditions for DNAase treatment used were 0.1 mg/ml in phosphate buffer, pH 7.5, 0.1% gelatin, 0.03 M MgSO₄, 3 hr at room temperature and Azure B, 0.1%, pH 4.0, for 2 hr at 40°C, washed briefly in water, differentiated overnight with *t*-butyl alcohol. The density of the staining with Azure B was measured cytophotometrically at 545 m μ (Ritter *et al.*, 1961).

The localization of injected acetylcholine was examined by injecting C¹⁴-acetylcholine (5 mc/kg) in the abdomen and killing the spider 5 min later. Autoradiographs were then made of the sections.

Gland tissue was excised from the animal in Krebs-Ringer and immediately dropped into a fixative consisting of 1 part 0.25 M sucrose, 1 part 7.3% P.V.P. (polyvinyl pyrrolidinone) and 2 parts osmium tetroxide (Di Stefano & Dougherty, 1964). The fixative was maintained at pH 7.4 with 0.01 N sodium hydroxide. The specimens were then double embedded in agar and maraglass (Freeman & Spurlock, 1962), sectioned on a LKB ultratome and examined in an RCA EMU-3F electron microscope.

RESULTS

Acetylcholine causes the appearance of droplets of protein throughout the epithelium within 10 min, this is shown diagrammatically in Fig. 1. Examination of the autoradiographs shows that these droplets are unlabelled and therefore consist of material which was present in the cell before the injection of acetylcholine. The presence of small droplets (3–5 μ dia.) in the epithelium of the resting gland can be seen in the low-powered electron micrograph (Fig. 2). This photograph shows the overall structure of the epithelial cell and is part of a study of the changes in the fine structure of this gland after stimulation (Bell & Peakall, 1966). The droplets move towards the epithelium-lumen boundary, and after 30 min the droplets are largely in the luminal portion of the epithelium (Fig. 1c) and all droplets are discharged into the lumen within an hour. Histological examination (both light and electron microscope) shows that the secretion causes considerable disturbance in cell structure.

In the absence of puromycin, injection of acetylcholine brings about a significant increase in the uptake of labelled alanine after 1 hr (see Table 1). The values for 30 min do not vary significantly from controls. There is a significant

increase in nuclei size after 30 min and by 1 hr the size has approximately doubled. In the experiments in which the spiders were pretreated with puromycin the uptake of labelled amino acids was almost completely stopped and was, in fact, much less than the amount taken up by the resting gland. Also there was no alteration in the size of the nuclei of the epithelial cells. These results are also given in Table 1. Thus pretreatment with puromycin separates the stages of secretion and synthesis.

Histological changes following stimulation of ampullate silk gland

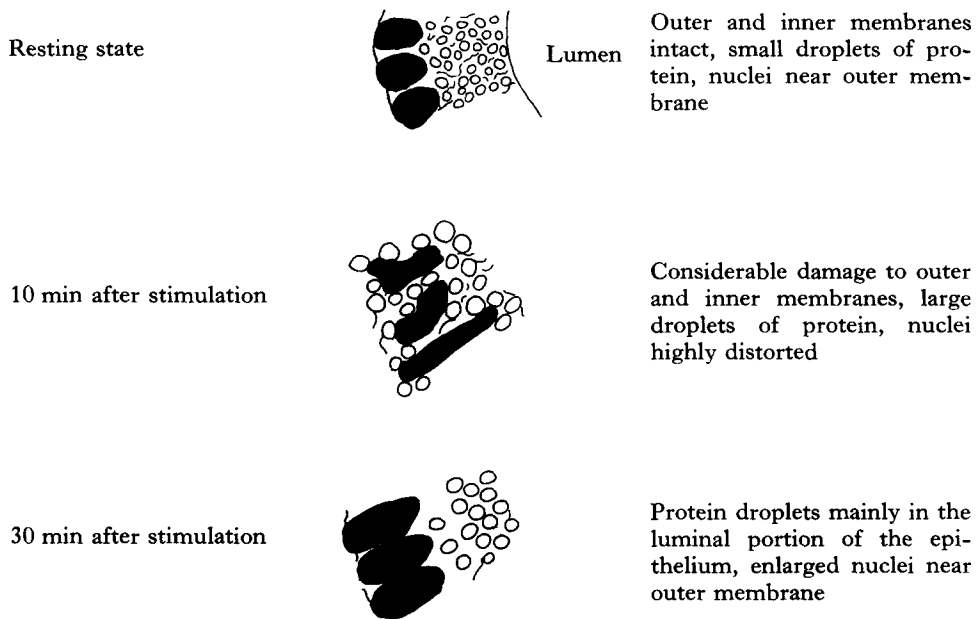


FIG. 1. Ampullate glands of *Araneus sericatus* showing histological effects of stimulation with acetylcholine (0.1 mg/kg, injected). Stain: hematoxylin and eosin.

The time sequence of changes in nucleic acids following stimulation were studied histologically and by observations on the uptake of labelled materials. In the resting gland the nuclei have a few small dense particles of DNA, 10–20 min after stimulation these particles increase in size and after 30 min the nucleus is largely, although diffusely, filled with DNA. The autoradiographs show a marked increase in labelling 20 min after stimulation and no appreciable increase thereafter (Table 2).

The results of the cytophotometric determination of RNA are given in Table 2. There is a marked rise in the concentration of RNA in the cytoplasm starting 10 min after stimulation and maximum values are reached within 30 min. Glands pretreated with puromycin showed a distinct rise in the 10 and 20 min values, almost paralleling the non-puromycin experiment, but this rise stops after 20 min

and has returned to control values within 60 min. It is considered that this rise is due to concentration of the ribosomes by the droplets of protein which are released at this time (Fig. 1). The amount of new RNA is indicated by the difference between the puromycin and non-puromycin values. This is then in agreement with the orotic acid incorporation data (Table 2). That the incorporation of both

TABLE 1—CHANGES IN SIZE OF NUCLEI AND OF C^{14} -ALANINE INCORPORATION FOLLOWING STIMULATION

Pretreatment	Treatment	Time of expt. (min)	Size of nuclei ($mm^2 \times 10^{-7}$), standard deviation and no. of determinations	Autoradiographic counts in epithelium per mm^2 at $860 \times$. Average of 100 counts with standard deviation
None	None	30	20.9 ± 4.3 (51)	9.8 ± 2.1
None	None	60	19.7 ± 3.6 (59)	29.7 ± 5.6
None	Acetylcholine	30	31.3 ± 6.2 (40)*	10.9 ± 2.6
None	Acetylcholine	60	43.3 ± 7.3 (48)*	$56.3 \pm 6.7^*$
None	Emptying gland	60	33.0 ± 8.2 (123)*	$77.3 \pm 8.8^*$
Puromycin	None	60	18.1 ± 5.9 (56)	Background
Puromycin	Acetylcholine	60	21.8 ± 4.9 (68)	Background
Puromycin	Emptying gland	60	21.8 ± 5.5 (84)	Background

* Significantly different from control at the 1 per cent level.

TABLE 2—CHANGES IN NUCLEIC ACIDS IN THE EPITHELIUM OF THE AMPULLATE GLAND FOLLOWING THE ADMINISTRATION OF 0.1 mg/kg ACETYLCHOLINE

Time (min)	Autoradiographic counts of		Extinction coefficient of Azure B in the cytoplasm†	
	Thymidine-5- T^3 in nucleus*	Orotic acid-5- T^3 in cytoplasm*	Absence of puromycin	Pretreated with puromycin
0	Background	Background	0.45 ± 0.05	0.48 ± 0.04
5	Background	Background	0.43 ± 0.04	0.45 ± 0.04
10	4.7 ± 1.3	Background	0.59 ± 0.04	0.56 ± 0.07
20	19.1 ± 4.0	Background	0.68 ± 0.04	0.71 ± 0.06
30	21.6 ± 5.2	25.0 ± 5.7	0.97 ± 0.06	0.53 ± 0.05
60	24.1 ± 5.4	33.4 ± 6.6	0.86 ± 0.07	0.46 ± 0.04

* Counts per mm^2 at $\times 1940$. Each figure represents the average, with standard deviation of 50 counts. Background was 0.4 counts/ mm^2 .

† Sections pretreated with DNAase (0.1 mg/ml in phosphate buffer, pH 7.5, 0.1% gelatin, 0.03 M $MgSO_4$, 3 hr at room temperature). Stained with Azure B (0.1%, pH 4.0, for 2 hr at 40°C, washed briefly in water, differentiated overnight with *t*-butyl alcohol) and measured at 545 $m\mu$ (5).

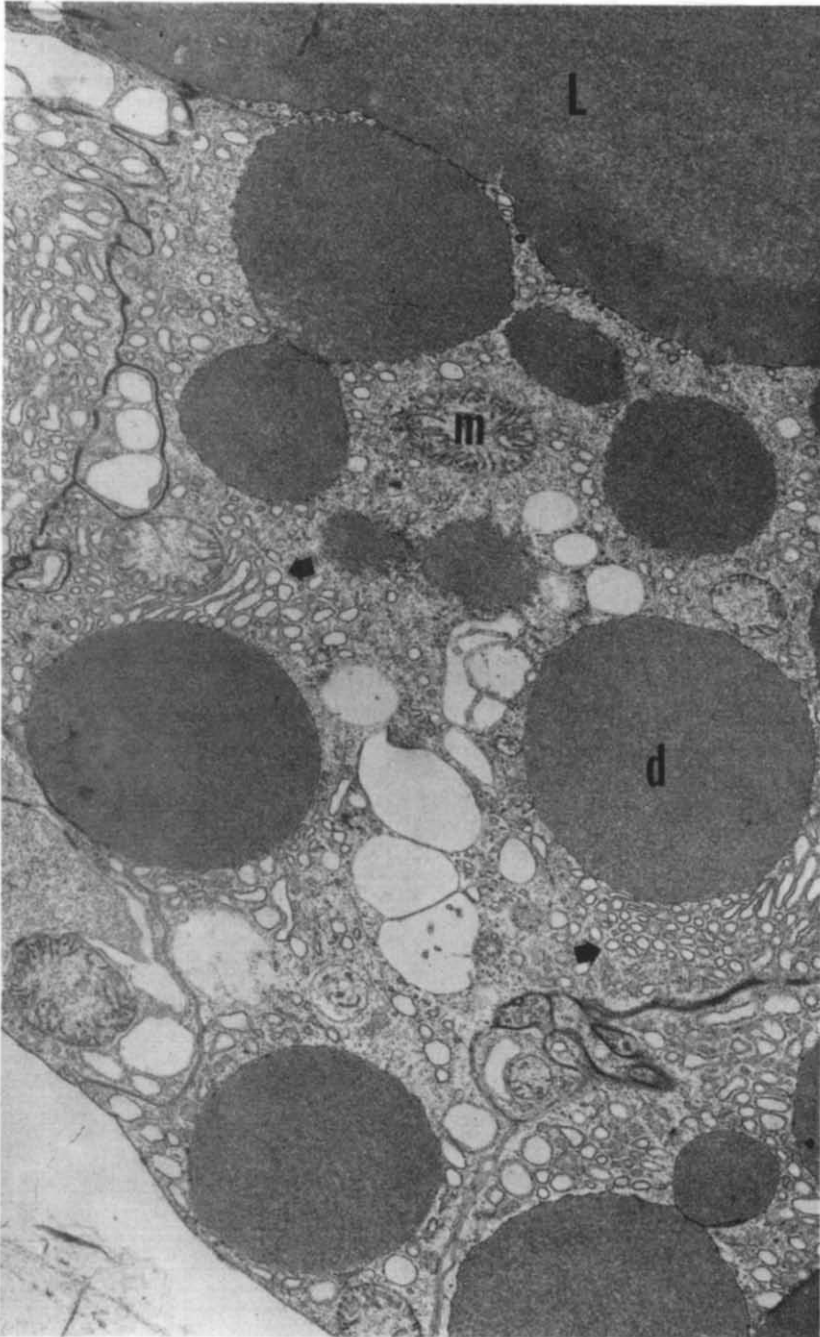


FIG. 2. Electron micrograph of control gland showing 3-5 μ protein droplets (d), mitochondria (m) and concentrations of ergastoplasm at arrows. The lumen (L) of the gland contains a substance of the same electron density as the droplets. In the center of the picture clear vesicles of unknown nature are apparent. Cell borders on adjacent gland cells may be seen at the upper left and lower right where specialized thickened areas of the plasma membrane are evident. Magnification $\times 7560$. Electron microscopy by Mr. Allen Bell.

otic acid and thymidine is blocked by puromycin suggests that some specific protein synthesizing step must be involved in the activation of the gland to increase fibroin synthesis. In this respect it is similar to the estrogen-induced protein synthesis found in the particulate fractions of rat uterus (Noteboom & Gorski, 1963).

Autoradiographs of C¹⁴-acetylcholine showed that the labelled material was highly localized on the outer membrane of the epithelium of the silk gland. The same degree of localization was found whether the label was in the acetyl or choline part of the molecule. This suggests that the labelled material is still intact when bound to the membrane.

DISCUSSION

Cholinergic regulation of the amount of thread used in web building by *Araneus diadematus* has been found by Witt (1962, 1963). Several cholinergic agents, physostigmine, carbachol and paraoxon, have been shown to increase the rate of incorporation of labelled alanine into fibroin (Peakall, 1964b, 1965). In addition, the gland can be stimulated by continuously reeling out the silk until the gland is empty. Studies on isolated glands have shown that stimulation by cholinergic drugs can be blocked by atropine, whereas stimulation caused by emptying the gland cannot be blocked by this agent. Thus, two, at least partially independent, modes of regulation exist for this simple organ. The initial stimulation may be either cholinergic (1a) or by emptying the gland (1b). These two methods of stimulation are separated by their sensitivity to atropine. This is followed in both cases by an increase in the rate of protein synthesis. The second process (2) is clearly separated from the others by pretreatment with puromycin.

(1a)	Acetylcholine binds with receptor on outer membrane of gland	Signal across <i>outer</i> epithelial membrane causes release of presynthesized fibroin	Atropine sensitive Puromycin insensitive
(1b)	Emptying of the gland	Signal across <i>inner</i> epithelial membrane causes release of presynthesized fibroin	Atropine insensitive Puromycin insensitive
(2)	Signal after release of presynthesized fibroin from epithelium	Changes in configuration of DNA; increase in nuclei size; change in RNA concentration; increased incorporation of amino acid	Puromycin sensitive

It is possible to theorize that two modes of regulation—one *via* the central nervous system which could transmit external stimuli and the other local in character dependent on the amount of fibroin present—might be useful for the quantitative control of silk production in the spider.

The nature of the various “signals” is unknown. Hokin & Hokin (1963) have found that acetylcholine stimulates the secretion of a variety of substances. This includes digestive enzymes from pigeon pancreas; mucin from rabbit and guinea-pig submaxillary glands; pepsin from the pigeon peptic mucosa and salt from the

albatross salt gland. These workers have studied the role of phosphatides and conclude that the stimulation of the secretion of a variety of water-soluble substances is associated rather specifically with an increase in the metabolism of phosphatidic acid and phosphoinositide and to a lesser extent with other phosphorus compounds. It is possible that a similar mechanism operates in this system. The signal for the second process, namely increased synthesis, can be assumed to be a type of end-product inhibition (Monod *et al.*, 1963; Atkinson, 1965). It is planned to investigate this "signal" in a cell-free system.

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