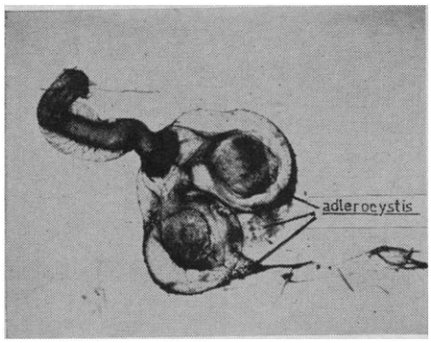

Fig. 2. The endo-spermatophore of *O. savignyi* of an interrupted copulation (× 57). The endo-spermatophore was extruded by the male after removal of the ecto-spermatophore. Preparation vitally stained with Janus green. The *Adlerocystis* were spread in the preparation.

matophore collapses, hardens, and becomes yellowish brown. It may remain attached to the female genital opening for a few hours or days.

The various stages of the formation of the spermatophore were closely followed under a stereoscopic microscope. I ascertained their temporal sequence by interrupting copulation at various stages. Although the whole process of extruding the ecto-spermatophore, filling it with sperm, and extruding the endo-spermatophore takes only 20 to 30 seconds, by close observation and quick manipulation it was possible to interrupt spermatophore formation when the ecto-spermatophore was either empty or filled only with sperm. In either case, once the male had started to copulate, it continued the chain of instinctive actions, even if they were in vain.

If, at the right moment, the still-empty ecto-spermatophore is removed from the male genital aperture, the male will eject sperm, large proteinic granules, and adlerocysts, and will extrude the endo-spermatophore on the surface of its own body (Fig. 2).

If the ecto-spermatophore is removed when it is already full of sperm, the male will eject adlerocysts and extrude the endo-spermatophore, the adlerocysts adhering closely to the exterior surface of the latter. When the endo-spermatophore is extruded from the male genital aperture to the outside (after the ecto-spermatophore has been removed), its shape is also that of a bilobate bottle, but the two lobes are considerably larger than they are inside the ecto-spermatophore (Fig. 2), although they are not as large as when they are filled with sperm in the uterus. If the endo-spermatophore is dropped into saline on a slide, one can see the huge number of adlerocysts which cover the lobes of the endo-spermatophore.

The processes that normally occur in the female genital tract after the deposition of the spermatophore on the female genital aperture can be studied in vitro. If a completely formed spermatophore is removed from the male before it is deposited on the female and if it is put into saline or left on a dry slide, within 1 to 2 minutes the endo-spermatophore evaginates from the ecto-spermatophore. The evagination occurs whether the spermatophore was taken off the male genital opening or off the male chelicerae. The evagination starts without any intervention. At first, a stem pops out (Fig. 1d), and immediately afterwards the two lobes of the endo-spermatophore are everted and evaginated (Fig. 1e). At this stage arise most of the minor differences between *O. savignyi* and *O. tholozani*, to which this description refers, and some other species of Argasid ticks. The everted neck of the endo-spermatophore extends and elongates. During evagination, the lobes turn inside out, and adlerocysts and sperm which were in the ecto-spermatophore, but outside the endo-spermatophore, stream quickly into the two everted lobes of the endo-spermatophore (Fig. 1f) until no sperm remains in the ecto-spermatophore. Only after the lobes have entirely been filled up with sperm do they separate slowly to form two distinct capsules, but both are still connected to the ecto-spermatophore by the neck (Fig. 1g). The sperm and adlerocysts, which were entirely separated in the ecto-spermatophore, are mixed in these capsules. Thus, during the whole process of transfer of sperm from the male to the female, the semen remains in a closed system.

In the normal course of copulation (but not in vitro), the two capsules are soon disconnected from the ecto-spermatophore. The separation occurs at the neck, near the capsules. The longer part of the neck, which originally belonged to the endo-spermatophore, remains attached to the ecto-spermatophore and can be seen when it falls off the female (Fig. 1h). The shorter part of the neck remains attached to the two capsules and forms a thin tube connecting them (Fig. 1i). This is the form in which the endo-spermatophore reaches the uterus. There the two capsules remain attached to each other for various lengths of time depending on external conditions, such as subsequent copulation and feeding by the female. The capsules then separate, and every two capsules found in the uterus represent one copulation. The sperm remains viable in the capsules for many months (*6*).

B. FELDMAN-MUHSAM
Department of Parasitology, Hebrew University, Jerusalem, Israel

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Submaxillary Gland of Mouse: Properties of a Purified Protein Affecting Muscle Tissue in vitro

Abstract. *A protein from the salivary gland of mice has been highly purified. It affects embryonic muscle tissue in vitro and has both esterase and peptidase activities. Addition of the pure protein to tissue culture in synthetic medium causes dissociation of muscle fibers in individual myoblasts with loss of myosin. This biological activity, as well as the esterase activity, is inhibited by low concentrations of phenylmethanesulfonyl fluoride; this suggests that the effect on the tissue is a consequence of the protein's enzymatic activities.*

The submaxillary gland of the male mouse contains a number of proteins that have specific growth-promoting properties. One of these, the nerve-growth factor, stimulates the growth and differentiation of embryonic sensory nerve cells and embryonic and mature sympathetic nerve cells (*1*). Subsequently, another protein, the epidermal-growth factor which promotes the growth of epidermal cells, was purified from the submaxillary gland (*2*). More recently, a fraction from extracts of the salivary gland was reported to stimulate in vitro the growth of mesenchymal cells and cause loss of differentiative marks in muscle and cartilage (*3*). The material responsible for the latter effects was also considered to be a pro-

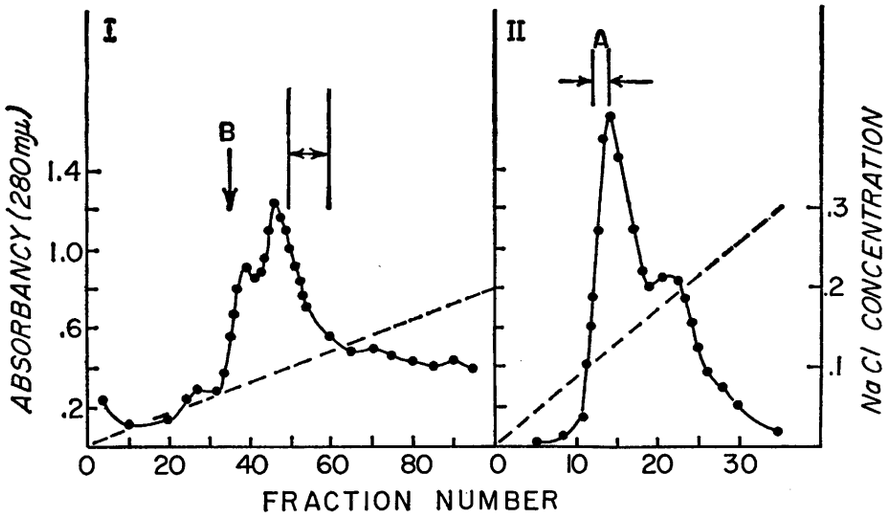

Fig. 1. Fractionation of salivary gland extract on DEAE-cellulose. Salivary gland extracts were eluted from a DEAE-cellulose column with a linear salt gradient (0 to 0.2*M* for column I and 0 to 0.3*M* for II) in 0.01*M* tris-Cl, *p*H 7.4. Tubes 50 to 60 of column I were chromatographed again for purification of A.

tein on the basis of properties noted during its isolation and partial purification. To characterize this material further and to determine its possible mode of action on embryonic muscle tissue, we have purified the factor and examined its enzymatic properties. The highly purified material is a protein containing both esterase and peptidase activities. Several properties of this enzyme have been measured and compared with another protein that was purified from the salivary gland of the mouse during the isolation of the biologically active material.

In previous fractionation of salivary gland extracts, the biologically active factor was eluted from a DEAE-Sephadex column with 0.1*M* NaCl. Accordingly, partially purified material which had been first fractionated with ammonium sulfate and passed over a Sephadex G-100 column (*3*) was dialyzed against 0.01*M* tris-Cl, *p*H 7.4, and pumped onto a DEAE-cellulose column (Fig. 1). Those fractions with high biological activity were pooled, dialyzed, and pumped again onto a DEAE-cellulose column. Fraction A (Fig. 1, II) had high biological activity and gave essentially a single protein band when examined by acrylamide-gel electrophoresis (Fig. 2). A similar electrophoretic analysis of other eluates from these column separations showed that tube 35 of column I contained primarily a single protein band (B). This protein was subsequently found to have active esterase activity and some effect on the embryonic tissue.

The highly purified proteins, A and B, have been tested for a number of enzymatic activities. The results of these experiments (Table 1) show that both proteins have strong esterase activities when assayed with benzoylarginine ethyl ester. Both also possess peptidase activity, and B has demonstrable protease activity. The latter conclusion is based on the observation that incubation of alkaline phosphatase polypeptide chains of *Escherichia coli* with fraction B inhibits by 46 percent the ability of these chains to dimerize and form an active enzyme. The subunits of this enzyme are susceptible to proteolysis, but in the absence of proteases they can reassociate to form native material unaffected by proteolytic enzymes (*4*). Despite the apparent proteolytic activity of B, it has only one-tenth the peptidase activity of A. Even more striking is the effect of the protease inhibitor, phenylmethanesulfonyl fluoride (*5*). Under conditions in which trypsin is inhibited 90 percent by this chemical, A is inhibited to 80 percent, whereas B is unaffected. Thus, these two proteins must have distinctive catalytic centers despite their relative similarity in substrate specificities.

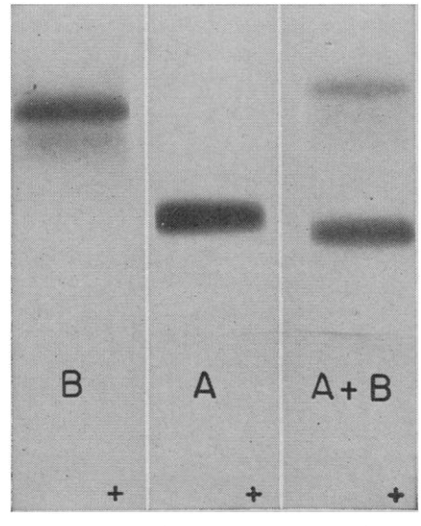

Fig. 2. Acrylamide-gel electrophoresis patterns of salivary gland proteins. Fractions A and B (from column eluates of Fig. 1) and a combination of the two were subjected to electrophoresis at *p*H 8.5 in the standard acrylamide gel; 25 μg of protein were applied.

Protein A has been examined in the analytical Spinco ultracentrifuge (Model E) and found to have a sedimentation coefficient ($s_{20,w}$) value of 3.3*S*. If we assume this to be a globular molecule, the molecular weight is estimated to be between 30,000 and 40,000.

The mechanism of action of protein A on embryonic muscle tissue has not been ascertained. After 24 hours in the presence of protein A, fragments of embryonic skeletal muscle tissue are radically modified in their structure when compared with a control culture. The dissociation of the muscle fibers into individual myoblasts with a loss of myosin is a striking feature of the treated tissue, readily observable in histological sections (Fig. 3). Tissue exposed to chemically inhibited protein A, however, shows no substantial difference from the control culture (Fig. 3c). Thus, inhibition of enzymatic activity of protein A also leads to a loss of biological activity. Possibly, protein A cleaves specific peptide bonds essential to maintenance of the differentiative state of the tissue and thus leads to a series of events that result in the loss of cell specificity and disappearance of myosin. Unlike trypsin which causes eventual tissue disaggregation, the presence of protein A results in the formation of a very compact mass of undifferentiated cells, even when it is added in large amounts.

The addition of protein B to embryonic muscle brings about a modification which is clearly distinct from that of A in the first 24 hours of incubation (Fig. 3d) but is somewhat similar to the effect with very low doses of trypsin. Protein B and trypsin cause a disaggregation of muscle fibers with sloughing of cells from the periphery of the tissue fragment.

In previous studies with the partially purified fraction of the mouse gland, a variety of tissues of mesodermal origin were found to be affected to various

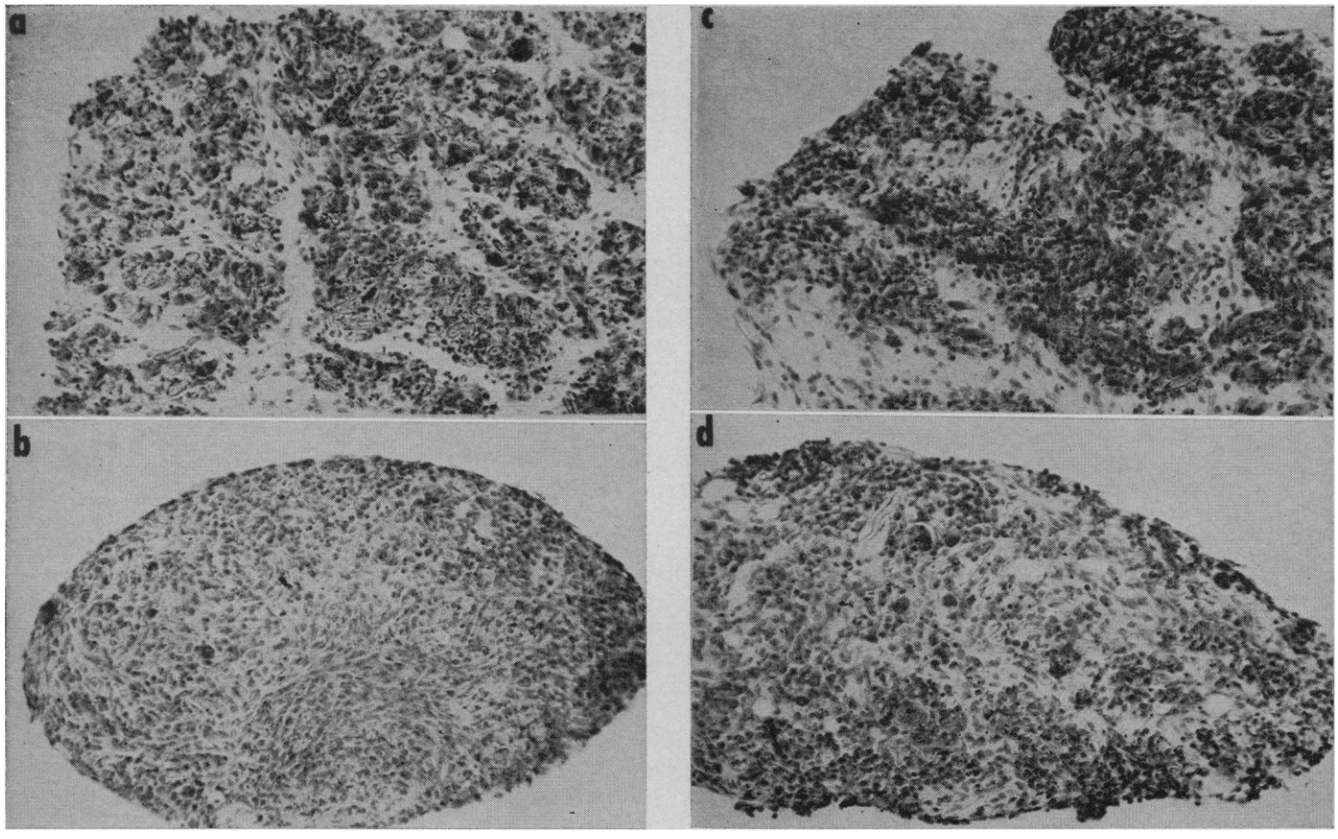

Fig. 3. Histological preparations of skeletal muscle from an 11-day-old chick embryo cultivated in vitro with salivary gland proteins. Fragments of embryonic muscle tissue were incubated in 1 ml of Eagle's medium, and sections were prepared according to conditions previously described (*3*). (a) Control, no addition; (b) 100 μg of protein A; (c) 100 μg of protein A previously incubated with a 10:1 molar ratio of phenylmethanesulfonyl fluoride; (d) 100 μg of protein B. All samples were fixed after 24 hours of incubation at 38°C. Magnification × 200.

extents (*3*). The highly purified material, however, has been tested thus far only on skeletal embryonic muscle.

Our results show that one of the enzymes present in mouse salivary gland can bring about dedifferentiative changes in the muscle tissue. This purified enzyme has both esterase and peptidase activities inhibited by phenylmethanesulfonyl fluoride, a chemical reported to inhibit proteolytic activity strongly. The observation that the inhibited protein also loses its biological activity supports the conclusion that the action of this protein on muscle tissue is a result of its enzymatic activity. Thus, our results are consistent with the hypothesis that changes in the muscle may arise from the release of biologically active peptides liberated by this enzyme (*3*).

Table 1. Comparison of enzymatic activities of purified salivary gland proteins and trypsin. Reaction 1: hydrolysis of benzoylarginine ethyl ester. Reaction 2: hydrolysis of benzoyltyrosine ethyl ester. Reaction 3: hydrolysis of benzoylarginine naphthyl amide. Reaction 4: inhibition of reaction 1 by phenylmethanesulfonyl fluoride (mole ratio inhibitor to protein, 10:1). Reaction 5: inhibition of reassociation of alkaline phosphatase subunits of *Escherichia coli*. Reaction 6: proteolysis of casein.

Test substance	Results of reactions (R)					
	R1* (unit/mg)	R2†	R3‡ (μg/min)	R4 (%)	R5§ (%)	R6‖ (%)
Fraction A	164	0	24	80	14	2
Fraction B	344	0	2.5	0	46	
Trypsin	15	0	11.2	90	99	100

* Specific activity is in units per milligram where one unit is the change in absorbancy at 253 mμ of 1.0 per minute recorded at 37°C in a cuvette of 1.0 cm in width (*6*). † The assay was performed with Dermatubes provided by Worthington Biochemical Corp. ‡ Specific activity is in micrograms of β-naphthylamine released per minute per milligram of protein at 37°C. Assay consists of coupling diazotized amine with *n*-(1-naphthyl)ethylenediamine dihydrochloride and recording absorbancy at 590 mμ (*7*). § Alkaline phosphatase subunits (50 μg) were incubated at 37°C in 1.0 ml of 0.1*M* tris-Cl, *p*H 8.0 with 5 μg of trypsin, A, or B. Activity of the dimer was recorded after 60 minutes (*4*). In the absence of added inhibitors, 80 percent of the original alkaline phosphatase activity was recovered. ‖ Denatured casein was incubated at 37°C in 0.1*M* tris-Cl, *p*H 8.0 with varying concentrations of A and trypsin. The reaction was stopped with trichloroacetic acid; solutions were filtered, and the filtrates were measured at 280 mμ. Digestion was linear from 1 to 6 μg of trypsin. The activity of A is reported relative to the activity of an equivalent quantity of trypsin.

DOMENICA G. ATTARDI
Department of Biology, Washington University, St. Louis, Missouri
MILTON J. SCHLESINGER
SONDRA SCHLESINGER
Department of Microbiology

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