J. Insect Physiol., 1976, Vol. 22, pp. 891 to 895. Pergamon Press. Printed in Great Britain.

SPERMATOPHORE OF THE MEALWORM BEETLE: IMMUNOCHEMICAL CHARACTERISTICS SUGGEST AFFINITIES WITH MALE ACCESSORY GLAND

ELLEN FRENK[1]* and GEORGE M. HAPP[1,2]†

[1]Department of Biology, New York University, Bronx, NY 10453
[2]Department of Zoology and Entomology and Graduate Faculty of Cellular and Molecular Biology, Colorado State University, Fort Collins, CO 80523

(*Received* 23 *December* 1975)

Abstract—Acid hydrolysis of the spermatophore and of the bean-shaped accessory gland (BAG) of male *Tenebrio* yields strikingly similar amino acid components; proline, glutamic acid, aspartic acid, and alanine predominate in both. Antisera were prepared against extracts of the spermatophore and BAG. The two antisera shared common cross-reactive male-specific components as shown by both complement fixation tests and immunodiffusion procedures.

INTRODUCTION

IN MANY terrestrial arthropods, semen is passed from male to female within a spermatophore. The spermatophore may be deposited externally and subsequently retrieved by the female (SCHALLER, 1971) or it may be directly placed within the female reproductive tract (WIGGLESWORTH, 1972; DAVEY, 1965; ENGLEMANN, 1970). Once within the female bursa copulatrix, the spermatophore ruptures to liberate spermatozoa which subsequently are transported to the spermatheca.

Many spermatophores are multi-layered structures, which undergo a programmed sequence of expansions before liberation of sperm (KALIFA *et al.*, 1949; FELDMAN-MUHSAM *et al.*, 1973; GADZAMA and HAPP, 1974). It is often assumed that the spermatophore is derived from the paraseminal secretions of male accessory reproductive glands. The proteins, carbohydrates, and lipids of spermatophores have been little studied. Amino acid analyses of the proteins are available for two invertebrates: a pseudoscorpion (HUNT and LEGG, 1971) and an octopus (MANN *et al.*, 1971).

The male reproductive tract of the mealworm beetle (*Tenebrio molitor* L.) consists of the paired testes, paired vas deferentia, and paired seminal vesicles which converge upon the ejaculatory duct. At the junction of the seminal vesicles and the ejaculatory duct, two pairs of accessory reproductive glands export their products. The larger, stubbier gland type is herein referred to as the bean-shaped accessory gland (BAG) and the elongate cylindrical gland type is termed the tubular accessory gland (TAG).

Sperm are transferred to the female within a spermatophore (GADZAMA and HAPP, 1974). After rupture of the spermatophore, the sperm reach the spermatheca (HAPP and HAPP, 1975). The outer, frothy layer of this spermatophore is lipoprotein (on histochemical criteria) and the inner fibrous layer contains mainly protein (GADZAMA and HAPP, 1974). In the present paper, we report the results of an amino acid analysis of the spermatophore of *Tenebrio*. Similarities in amino acid composition and immunological characteristics indicate that, at least in part, the spermatophore is derived from BAG.

MATERIALS AND METHODS

Specimens and maintenance of culture

Tenebrio molitor were obtained from a laboratory stock culture and maintained on chicken feed (Purina Startena) and sliced potato. The insects were sexed at the pupal stage, and males and females were segregated for about 10 days after ecdysis. Mating occurred promptly when males were allowed access to females. Spermatophores were retrieved by separating male and female thirty seconds after the onset of copulation—at which time the spermatophore could be found protruding from the male intromittant organ. All dissections were performed in *Tenebrio* saline (BUTZ, 1957).

Amino acid analyses

Sperm sacs (retrieved after the rupture of the spermatophore in insect saline) and bean-shaped accessory glands were hydrolyzed in 2 ml of 6N hydrochloric acid for twenty hours at 105°C (under nitrogen) in sealed glass-stoppered tubes. The hydrolysates were taken to dryness at room temperature and redissolved in 5 ml pH 2.2 citrate buffer. Aliquots

* Present address: College of Physicians and Surgeons, Columbia University, New York, NY 10032.
† To whom reprint requests should be addressed.

of the hydrolysed material were taken for analysis on a Beckman Model 121 Amino Acid Analyser.

Immunological studies of proteins

Preparation of antisera. Bean-shaped accessory glands (BAG) and spermatophores for immunization were prepared by the method of DORSETT and IOACHIM (1973). After homogenization in a ground glass homogenizer, specimens were mixed with an equal volume of complete Freund's adjuvant and emulsified by a brief sonification in the cold. Female Sprague–Dawley rats were used for the preparation of antisera. Immunization was effected by injecting the specimen–adjuvant emulsion subcutaneously at four sites with 0.25 ml at each site. Rats were boosted every ten days, three times, followed by the collection of sera, 10 days later.

Preparation of extracts. Soluble antigen extracts to be used in immunoprecipitation tests and for the absorption of antisera were prepared by sonifying homogenates for 60 sec at 4°C in a MSE 100 W Ultrasonic disintegrator. The material was centrifuged at 20,000 rev/min for 1 hr at 4°C and the supernatant was then concentrated by lyophilization.

Absorption of sera. Absorption was performed by suspending 50 mg of material in antisera and storing for 15 hr at 4°C. Sera was then clarified by centrifugation at 4°C at 700 *g* for 10 min.

Complement fixation. Dilutions of sera were made in modified barbital buffer to be used in complement fixation against a standard preparation of 1 mg antigen/ml modified barbital buffer. A constant amount of complement was added and the mixture was incubated for 15 hr at 4°C. After incubation, 25 λ of a mixture of 3 ml of a dilution of sensitized sheep red blood cells and 3 ml of a 1:200 dilution of antibody against sheep red blood cells, was added to each antigen–antibody–complement mixture. The mixture was heated at 37°C for 1 hr.

Immunodiffusion tests. Immunodiffusion tests were performed by the method of Ouchterlony in Kallsted immunodiffusion plates. Precipitation patterns were read after 24 hr, 2 days, and then 5 days.

Immunofluorescence tests. Frozen-sectioned specimens for examination by immunofluorescence studies were directly embedded in O.C.T. medium (product of Lab-Tek) 2nd cut 6μ thick on an International Harris Cryostat. Immunofluorescence tests were performed by the incubation of the prepared slides with the appropriate dilution of antisera for one hour at 37°C in a moist chamber, followed by 2 × 5-min washings in insect saline and 2 × 5 min washings in distilled water. The slides were counterstained with 0.6% Evans Blue solution for 5 min and then air dried. Fluorescein conjugated goat anti-rat immunoglobulin antisera (Hyland) was applied and the slides were incubated again for 1 hr at 37°C and washed as before. Slides were examined under a Leitz fluorescence microscope using an HBO 200 mercury light source.

RESULTS AND DISCUSSION

Amino acid analyses of bean-shaped accessory gland and spermatophore

The amino acid composition of the spermatophore and BAG is given in Table 1. Sixteen amino acids were detected and there appeared to be no unidentifiable amino acid peaks present on the analyser trace. There was, however, some evidence of amino sugars. Quantitatively, the most important amino acids present are proline, glutamic acid, aspartic acid, alanine, and glycine. The amino acid analysis of BAG is also shown in Table 1. Sixteen amino acids were detected, with no unidentifiable peaks on the analyser trace. Quantitatively, the most important amino acids present in the BAG are glutamic acid, aspartic acid, alanine, glycine, proline, and serine. With the exception of proline, the major amino acids of the spermatophore and BAG are strikingly similar.

It is difficult to compare proteins of diverse function in diverse organisms. To facilitate comparison of amino acid compositions, it is useful to group the amino acids according to their various classes of side chains. Such a comparison is provided in Table 2. In addition to the BAG and spermatophore of *Tenebrio*, we have included data on other proteins which might have functional or phylogenetic affinities to structural proteins of the spermatophore.

All of these structural proteins show some similarities in their amino acid constituents. Non-polar amino acids are 40 to 65% of the total. Glycine residues may be 8 to 10% of the total in some, namely the spermatophore of *Tenebrio* and octopus, BAG,

Table 1. Amino acid composition of spermatophore and BAG in residues/1000 total residues

Amino Acid	Spermatophore	BAG
Lysine	47.6	56.2
Histidine	19.8	21.1
Arginine	39.2	46.0
Aspartic acid	103.6	114.4
Threonine	42.7	41.8
Serine	74.5	71.3
Glutamic acid	119.4	122.6
Proline	134.2	70.5
Glycine	90.5	88.6
Alanine	98.9	113.9
Valine	46.9	55.4
Methionine	13.8	15.7
Isoleucine	57.6	42.9
Leucine	77.3	69.9
Tyrosine	34.7	38.2
Phenylalanine	25.5	31.6

Table 2. Comparisons of amino acid composition by classes of side-chains. Amino acids in residues/1000 total residues

Class of Amino Acid	T. molitor			Octopus Spermatophore	Pseudoscorpion Spermatophore	Nematode Collagen	Locust Soft Cuticle	Dragonfly Resilin	Wasp & Bee Silk (4 species)
	Spermatophore	BAG	Eggshell (CMI)						
Non-polar	531	473	460	405	552	699	646	639	517
Small	264	274	221	187	474	378	395	620	476
Glycine	91	89	68	70	240	286	198	422	55
Amino	134	71	62	50	5	304	115	75	44
Basic	107	123	115	196	74	88	84	41	67
Acidic	223	237	174	188	198	177	159	136	229
Charged	330	360	289	384	272	265	243	177	296
Tyrosine	35	38	35	20	47	1	17	12	13
Hydroxlated	151	151	124	173	158	66	113	160	---

Classes of amino acids:

Non-polar: Glycine, alanine, valine, leucine, isoleucine, phenylalanine, proline.
Small (i.e. small side-chain): glycine, alanine, serine.
Basic: lysine histidine, arginine.
Acidic: aspartic and glutamic acid.
Charged: both acidic and basic.
Hydroxylated: Serine, threonine, tyrosine, hydroxyproline.

Refs: *Tenebrio* eggshell CMI (KAWASAKI *et al.*, 1975); Octopus spermatophore (MANN *et al.*, 1973); pseudoscorpion spermatophore (HUNT and LEGG, 1971); Nematode collagen (BAILEY, 1968); Locust soft cuticle (neck membrane) (ANDERSON, 1971); Dragonfly tendon (ANDERSON, 1971); Wasp and Bee silk (as summarized by HUNT and LEGG (1971) from LUCAS and RUDALL (1968)).

the eggshell of *Tenebrio* or wasp silk. In other proteins, glycine may be 20 to 45% of the total, as in collagens, cuticular proteins, resilin, and the spermatophore of the pseudoscorpion. Total charged amino acids are quite consistent, comprising usually one-fifth to one-third of the total. Relative acidic and basic contributions to this total vary widely. Proline residues are a significant proportion of the amino acid in spermatophores and cuticles, yet purified collagens are much higher in this imino acid. At the present we can conclude that the spermatophore of *Tenebrio* is rather like that of the octopus and somewhat like the CMI fraction of the eggshell, but that neither collagen, nor silk, nor resilin predominate in any of these. Similarities between BAG and the spermatophore are quite marked.

Immunological investigations of proteins

Rats were immunized with either spermatophores or BAG's and sera from these rats were then screened by complement fixation procedures to test for the presence of antibodies to the respective antigens. The results of the complement fixation tests are shown in Tables 3 and 4. As shown in Table 3, antibodies to both BAG and the spermatophore were produced, and they prevent red cell lysis up to an endpoint of 128. Cross-reactivity between antibodies to BAG and to spermatophore is also seen in Table 3, but more clearly demonstrated in Table 4, which reports the complement test following absorption with extracts of whole female beetles as well as the individual antigens.

Table 3. Complement fixation tests, series I. Antibodies to BAG and spermatophores (SPTH) were prepared as described in Materials and Methods. "0" signifies complete lysis and "4" signifies no lysis. Lysis continued at dilutions to the right of the "0"'s. The complement fixing antibody titer of the serum is expressed numerically as the reciprocal of the dilution (endpoint).

Antibody to	Absorbed with	Antigen	Dilution								End-point
			1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	
BAG	♂	Spth	4	4	3	1	0	-	-	-	16
BAG	♀	BAG	4	4	4	3	3	3	2	0	128
Spth	♂	BAG	4	2	1	0	-	-	-	-	8
Spth	♀	BAG	4	3	3	3	1	1	0	-	64
BAG	♂	Spth	4	2	0	-	-	-	-	-	4
BAG	♀	Spth	4	4	3	3	2	1	0	-	64
Spth	♂	Spth	4	3	2	1	0	-	-	-	16
Spth	♀	Spth	4	4	4	3	3	2	1	0	128

Table 4. Complement fixation tests, series II. All antisera were also absorbed with female beetle procedures as in Table 3

Antibody to	Absorbed with	Antigen	Dilution								End-point
			1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	
BAG	BAG	Spth	1	0	-	-	-	-	-	-	2
BAG	BAG	BAG	0	-	-	-	-	-	-	-	0
Spth	BAG	Spth	1	0	-	-	-	-	-	-	2
Spth	BAG	BAG	0	-	-	-	-	-	-	-	0
BAG	Spth	Spth	2	1	-	0	-	-	-	-	4
BAG	Spth	BAG	3	2	2	2	1	1	0	0	64
Spth	Spth	Spth	1	0	-	-	-	-	-	-	2
Spth	Spth	BAG	0	-	-	-	-	-	-	-	0

Soluble extracts of spermatophores and BAG's were tested by immunodiffusion against anti-spermatophore and anti-BAG. As shown in Fig. 1, BAG and the spermatophore share a common cross-reactive component.

The cross-reactive component was shown to be male-specific by both complement fixation and immunodiffusion procedures. In the complement fixation tests, prior absorption of either antiserum with male extract decreased its titer relative to antisera absorbed with female extract. Furthermore, the specific precipitant bands in the immunodiffusion plates persisted after absorption with extract of female beetle, and the anit-BAG line disappeared after absorption with male beetle (Fig. 1, Plate 2). We cannot fully explain the weak reaction which persisted after absorption of anti-spermatophore with male extract (Fig. 1, Plate 4). However, we believe this is due simply to inadequate absorption: the amount of spermatophore antigens relative to other antigens in an extract of whole beetle is probably quite small.

Additional precipitant lines were obtained in the immunodiffusion tests. These additional lines show non-identity between BAG and spermatophore in some antigenic components. The lines of non-identity probably indicate organ-specific antigenic moieties in BAG, i.e. merely reflect the presence of proteins other than the secretory products. The persistence of the activity of anti-BAG in the complement fixation test, even after absorption with the spermatophore (Table 4; Endpoint 64), shows that BAG contains antigenic materials which are not present in the spermatophore.

Immunofluorescence tests yielded a reaction between anti-spermatophore sera from rats previously absorbed with extracts of female beetle and frozen-sections of BAG. The sites of reaction were detected in the frozen sections of the BAG by the application of fluorescence. The intensity of the reaction tended to be greatest in the mid-region of the gland.

On grounds of histochemical similarity, GADZAMA (1972) suggested that the spermatophore of *Tenebrio* was derived partially from BAG. Our data on amino acid contents, as well as the immunochemical evidence, strongly support his suggestion. The precise rôle of the tubular accessory glands remains yet undefined; it will be considered in a subsequent study.

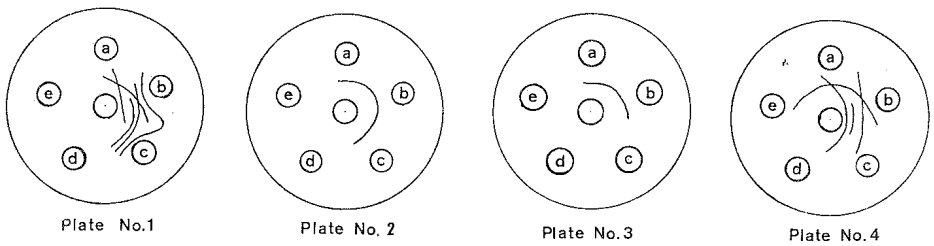

Fig. 1. Immunodiffusion tests. All antisera used were absorbed with extract of female beetle unless labelled "unabsorbed." Plate 1: Center, BAG extract; a, antispermatophore; b, anti-BAG; c, anti-BAG absorbed with spermatophore; d, anti-BAG absorbed with BAG; e, anti-spermatophore absorbed with BAG. Plate 2: Center, spermatophore extract; a, anti-BAG; b, anti-BAG unabsorbed; c, anti-spermatophore; d, anti-BAG absorbed with BAG; e, anti-BAG absorbed with extract of male beetle. Plate 3: Center, spermatophore extract; a, anti-BAG; b, anti-spermatophore; c, anti-BAG absorbed with spermatophore; d, anti-spermatophore absorbed with BAG; e, anti-spermatophore absorbed with spermatophore. Plate 4: Center, BAG extract: a, anti-spermatophore; b, anti-BAG; c, anti-spermatophore unabsorbed; d, saline; e, anti-spermatophore absorbed with extract of male beetle.

Acknowledgements—This work was partially supported by NSF Grant GB36284 and NIGMS Grant GM21711. We are grateful to ROBERT E. CANFIELD, Professor of Medicine, Columbia University, for performing the amino acid analyses, and we thank KIRBY MAXWELL and JANELLE SIMON for typing this manuscript.

REFERENCES

ANDERSON S. O. (1971) Resilin, In *Comprehensive Biochemistry*, (Ed. by FLORKIN M. and STOTZ E. H.) **26(C),** 633–657. Elsevier, Amsterdam.

BAILEY A. J. (1968) The nature of collagen, In *Comprehensive Biochemistry*, (Ed. by FLORKIN M. and STOTZ E. H.) **26(B),** 297–423, Elsevier, Amsterdam.

BUTZ A. (1957) Effects of sodium, potassium and calcium ions on the isolated heart of the mealworm, *Tenebrio molitor* L. *J. N.Y. ent. Soc* **65,** 22–31.

DAVEY K. G. (1965) *Reproduction in the Insects.* Freeman, San Francisco.

DORSETT B. H. and IOACHIM H. C. (1973) Common antigenic component in ovarian carcinomas demonstrated by diffusion immunofluorescence techniques. *Immunol. Commun.* **2,** 173–84.

ENGLEMANN F. (1970) *The Physiology of Insect Reproduction.* Pergamon Press, Oxford.

FELDMAN-MUHSAM B., BORUT S., SALITERNIK-GRANT S., and EDEN C. (1973) On the evacuation of sperm from the spermatophore of the tick, *Ornithodoras savignyi. J. Insect Physiol.* **19,** 951–962.

GADZAMA N. M. (1972) Ph.D. Thesis, New York University.

GADZAMA N. M. and HAPP G. M. (1974) The structure and evacuation of the spermatophore of *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), *Tissue & Cell* **6,** 95–108.

HAPP G. M. and HAPP C. M. (1975) Fine structure of the spermatheca of the mealworm beetle (*Tenebrio molitor* L.). *Cell & Tissue Research.* **162,** 253–269.

HUNT S. and LEGG G. (1971) Characterization of the structural protein component in the spermatophore of the pseudoscorpion *Chthonius ischnocheles* (Hermann). *Comp. Biochem Physiol.* **40B,** 475–479.

KALIFA A. (1949) The mechanism of insemination and the mode of action of the spermatophore in *Gryllus domesticus. Quart. J. micr. Sci.* **90,** 281–292.

KAWASAKI H., SATO H., and SUZUKI M. (1975) Structural proteins in the egg envelopes of the mealworm beetle, *Tenebrio molitor. Insect Biochem.* **5,** 25–34.

MANN T., MARTIN A. W. JR., and THIERSCH J. B. (1970) Male reproductive tract, spermatophores and spermatophoric reaction in the giant octopus of the north pacific, *Octopus dofleini martini. Proc. R. Soc. Lond.* (B) **175,** 31.

SCHALLER F. (1971) Indirect sperm transfer by soil arthropods. *A. Rev. Ent.* **16,** 407–446.

WIGGLESWORTH V. B. (1972) *The Principles of Insect Physiology*, 7th Ed. Chapman & Hall, London.