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Staining of substances adjacent to the sex bivalent in certain weevils of Puerto Rico^{1,2}

Niilo Virkki,³ Cristina Mazzella⁴ and Aida Denton⁵

ABSTRACT

A nucleolar substance adjacent to early prophasic sex bivalents is observed and studied by cytochemical means in certain broad-nosed male weevils. The substance soon disintegrates, possibly because of an active nucleolus organizer. By early diplotene, the sex bivalent assumes a parachute shape (Xy_p). An argyrophilous, fibrous substance appears first at the inner borders of X and y, then invades the whole space between them. Most commonly used nucleolus and protein stains leave it unmarked. RNA and histones seem to be excluded. Only AgNOR staining marks the substance clearly, especially if a formalin-containing fixative has been used.

RESUMEN

Tinción de substancias adyacentes al bivalente del sexo Xy_p en algunos gorgojos de Puerto Rico

Una substancia del nucléolo adyacente a los bivalentes sexuales profásicos tempranos se ha observado y estudiado mediante métodos citoquímicos en los machos de unos picudos de proboscis corta. La substancia se desintegra rápidamente probablemente por no tener un organizador activo. En el diploteno temprano el bivalente sexual adquiere la forma de paracaídas (Xy_p). Una substancia argirofílica aparece en los bordes interiores de X y y, para después invadir todo el espacio entre ellos. La substancia queda sin marcar por la mayoría de los colores comunes del nucléolo o de las proteínas. ARN y los histones parecen excluidos. Solo la tinción AgNOR la marca claramente, especialmente si se ha usado un fijador que contenga formalina.

INTRODUCTION

The parachute-formed sex bivalent (Xy_p) of male meiosis occurs widely in Coleoptera Polyphaga (15). In profile views it appears as an airborne parachute, where the X chromosome represents the canopy, and the much smaller y chromosome, the load. This X-to-y association is

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apparently ancient in origin (15). Although the size and shape of the parachute sex chromosomes may vary during evolution, the mode of association is very persistent. Thus, it still prevails in Curculionidae, a large beetle family of advanced phylogenetic status.

Several hypotheses have been forwarded to explain Xy_p association. Nucleolar association, as conceived by John and Lewis (6), and by others, was widely accepted until Uruguayan school of cytologists challenged it on the grounds that no nucleolus organizing region (NOR) was found in the sex chromosomes of the beetles they studied (3,10,11,24). They attributed the Xy_p association to chromosomal end contacts of heterochromatic nature, but did not discuss the possible role of a substance seen in the Xy_p lumen. Observations of both "prenucleolar" end contacts (15) and autosomal NORs have been reported also in other beetles (18-20,23,25).

The nucleolus hypothesis was based on a non-chromosomal substance seen in the Xy_p lumen. Such a substance might participate in the maintenance of the Xy_p association. Accordingly, its identification and compositional information might contribute to understanding the evolution of the Xy_p bivalent. The work herein presented examines the presence of such substances adjacent to the sex bivalent in four Puerto Rican weevil. We used light microscopy and methods of classical cytochemistry.

MATERIALS AND METHODS

During the period 1981-89, living weevils were collected in several localities on Puerto Rico's north coast (table 1). The live beetles were brought to the laboratory, where testes were processed for squash and air dried preparations.

1. *Sample fixation*: Kahle-Smith (KS) fixative (1 part glacial acetic acid: 3 parts formalin: 7.5 parts 95% ethanol) was applied to the opened abdomen, from where the testes were excised to a vial containing the same fixative. Total fixation time did not exceed 2 min. The testes are

TABLE 1.—Systematics, host-plant association, and insular Puerto Rican collection sites of the examined weevil materials

Weevil identification	Host-plant affiliations	Insular PR sample location
1. Otiorrhynchinae: Phyllobiini		
<i>Diaprepes abbreviatus</i> (L.)	<i>Swietenia macrophylla</i> G. King	El Yunque, Catalina
<i>Exophthalmus roseipes</i> (Chevrolat)	<i>Dalbergia ecastaphyllum</i> (L.) Taub.	Dorado, Road 165
<i>E. quindecimpunctatus</i> (Olivier)	<i>Guapira fragrans</i> (Dum.-Cours) Little	Vega Alta, Road 675
2. Brachyderinae: Barynotini		
<i>Lachnopus curvipes</i> (F.)	<i>Dalbergia ecastaphyllum</i> (L.) Taub.	Vega Baja Beach, end of Road 692

composed of two follicles each. One fixed follicle was placed on an albuminized slide in a drop of 45% acetic acid, where it was teased with pins and watchmaker's forceps to pieces as small as possible. The teased material was spread on an area approximately 15 x 15 mm, covered with a cover slip of 22 x 22 mm, and squashed by thumb pressure. The preparation was examined by light microscopy. Selected cells were photographed under phase contrast. The cover slip was then removed in 50% ethanol and the preparation was air dried. It was cleaned with two changes of xylene to remove residual traces of immersion oil and again air dried. These preparations were stored for intervals ranging from overnight to six months before further processing. This presumably will have eliminated volatile compounds, such as formalin, that can affect the staining process.

Acetic ethanol and acetic methanol (both 1:3) were also used as media for fixation. The procedure was normally extended to 2 h, with one change of fixative. Shorter times (down to 2 min.) were used for comparison with Kahle-Smith fixative. Preparation procedure was otherwise as described above. For non-squashed, air dried samples, acetic ethanol or acetic acid (45%) were used as medium for fixation, teasing, and evaporation procedures.

2. *Extraction of RNA.* The Kunitz' method of RNA extraction, employing RNase (Sigma Co.),⁶ was used as recommended by Darlington and LaCour (2). Extraction by perchloric acid was performed in accordance with Erichson et al. (4).

3. *Staining of nucleolus and NOR.* A series of staining reagents and preparations was evaluated. Acetocarmine was used in the conventional way, or following acid hydrolysis in accordance with Rattenbury (12). Because Rottenbury's fixative is reliably similar to the Kahle-Smith fixative, the latter was used. The Unna-Pappenheim stain, employing Pyronin Y (Eastman) and methyl green (Harleco) was used as recommended by Darlington and LaCour (2). Toluidine Blue O (Eastman, 0.1%), preferred by Kurnick (7) to pyronine as an indicator of RNA, was used in 1% aqueous sodium borate solution. Staining lasted at least 2 h. After rinsing in water, the preparations were differentiated in 70% ethanol. Alkaline Azure A (Chroma-Gesellschaft), closely related to Toluidine Blue, was evaluated in an attempt to stain nucleoli reddish violet. Eighteen parts of 0.1% solution prepared in 2-methoxyethanol were mixed with two parts of 0.2M disodium phosphate (v/v) just prior to use. Luxol Brilliant Green (Du Pont de Nemours; discontinued) which stains nucleoli green, was also tested as a 1.0% solution in 2-methoxyethanol.

⁶Trade names in this publication are used only to provide specific information. Mention of a trade name does not constitute a warranty of equipment or materials by the Agricultural Experiment Station of the University of Puerto Rico, nor is this mention a statement of preference over other equipment or materials.

The AgNOR staining was ordinarily performed according to Pathak and Elder (8). In fewer instances the method of Rufas et al. (13) was utilized. In the former method, one drop of 3.5% formalin per 1 ml of 50% AgNOR₃ solution was used. The slides were incubated in a moist chamber at 65° C, from 75 to 150 min. In some trials, AgNOR staining was performed after Rattenbury's acetocarmine or Feulgen staining. Because HCl hydrolysis eliminates histones and RNA, the results of such double staining should be evaluated with this in mind. We have obtained very clear and intense staining after Feulgen. Possibly a reducing agent, simple triose or hexose sugars, for example, are released by the HCl hydrolysis.

Elimination of silver after AgNOR staining was obtained by overnight treatment of the slides in 1.5% ferricyanide at 25° C. After three 5-min. changes in distilled water, the destained slides were suitable for other treatments and stains.

4. *Staining of proteins.* At metaphase, the AgNOR methods are thought to mark NOR-associated proteins rather than RNA because the nucleolus normally disappears during the preceding prophase. Formalinic fixation enhances argyrophily of acid proteins through induction of carboxyl and aldehyde terminals that are argyrophilous (5). In addition, Remazol Brilliant Blue (Sigma Co.), a reactive protein stain which most probably marks amino groups (Horobin, personal communication), was tested in buffered aqueous solutions at pH 3.0, 5.0, 7.0, 8.0, and 10.0. Routine Feulgen and Giemsa staining was sometimes performed in addition to the above-mentioned staining methods, as well as C-banding in accordance with Vidal and Giacomozzi (16).

A Zeiss Photomicroscope II was used for the study. Photomicrographs were prepared with the films Kodak Plus-X-Pan and Ektachrome 200 (daylight).

RESULTS AND DISCUSSION

The early prophasic sex bivalent

Prior studies at this laboratory with *Diaprepes* and other Puerto Rican Curculionidae have indicated that the initial pairing of X and y is by one terminal contact (21,22, unpublished). The sex chromosomes and procentric blocks of all autosomal bivalents are strongly marked by AgNOR staining. This apparently is due to a precocious condensation rather than to nucleolar or NOR substances. However, after the X-y chain has bent to variable configurations, its stainable substance seems to grow unexpectedly large. Most of this substance is lost during a short diffuse phase in the earliest diplotene (*cf* fig. 3A to C in reference 22).

Special attention was directed to this relatively short phase to determine whether a substance is leaving the sex bivalent. In AgNOR-stained slides we have indeed encountered a racemose substance, in various

stages of coalescence, adjacent to the sex bivalent. The density of this substance is thought to be low because of difficulty in detection in unstained preparations under phase contrast. Its staining by silver suggests nucleolar material. It is yellow to brown, when the adjacent compact sex chromatin is brown to black (fig. 1). Unna-Pappenheim stain applied to the same cells after removal of silver also suggests the presence of nucleolar substance (fig. 2). When ferricyanide-washed cells are treated with RNase (fig. 3), or with perchloric acid (fig. 4), and then again submitted to AgNOR staining, the substance has faded or disappeared.

The association of this substance with sex chromosomes appears so intimate that its formation there is strongly suspected. This is opposite to findings in a chrysolimid, where the early nucleolus marked with a fluorochrome is always associated with an autosomal NOR (11). Our staining methods have not revealed any functioning NOR in these phases, although the substance usually attached to the sex bivalent is sometimes seen detached and associated with an autosomal bivalent (fig. 1). It is likely that we are discussing two different kinds of nucleoli: the classical, autosome-associated one destined to disappear during prophase, and another, usually Xy_p -associated substance which has to do with the diffuse phase and the growth of the spermatocyte I during this phase. Thus this ephemeral droplet substance attached to early sex bivalent of the weevils might be akin to the huge nucleolus-like masses found in the very large spermatocytes of the oedionychine fleabeetles (19).

We conclude from these findings that a nucleolar substance forms at the sex bivalent of the latest bouquet stage, but there is lack of active NOR in these nuclei. The nucleolar substance appears to form as coalescent condensation droplets. The latter are neither stable nor permanent, soon becoming detached from the sex bivalent and disappearing from view.

The Xy_p

In the early diplotene, a double contact between X and y chromosomes becomes clearly visible and the parachute shape of the bivalent emerges. Because the parachute association has been attributed to the nucleolus, application of the AgNOR staining is of special interest here. We have applied it routinely to all species of the present study, and to many other curculionids and chrysolimids, with consistent results. Kahle-Smith fixative works well, but acetic alcohols must be applied at least for 2 h, to avoid notable variations in the results.

Late diplotenic and diakinetiic parachutes reveal an empty lumen under phase contrast and when Feulgen-stained (fig. 5A and B). If AgNOR staining is added, a visibly compact deposit of silver appears at the inner borders of both sex chromosomes (fig. 5C). In addition, a fi-

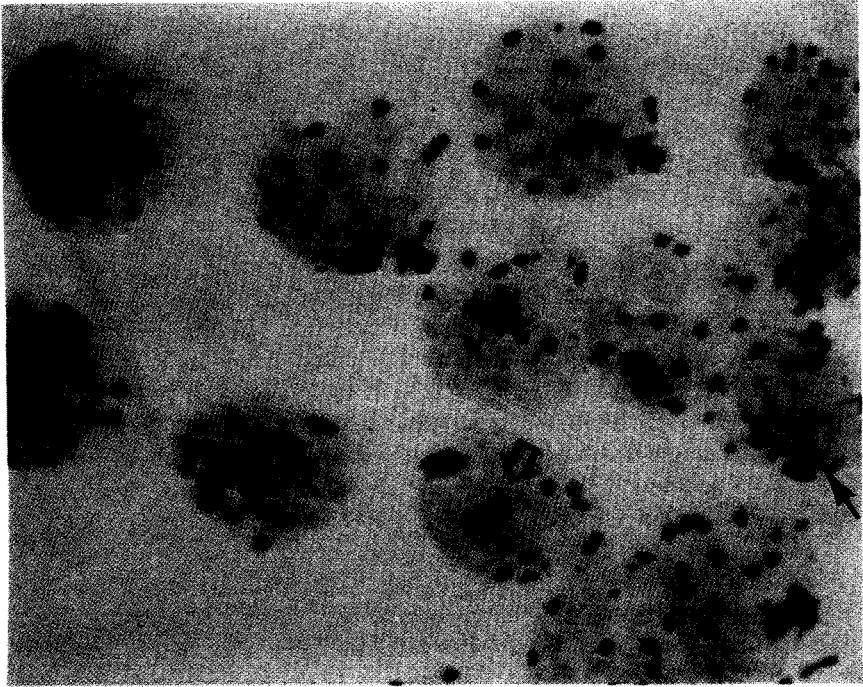


FIG. 1.—*Diaprepes abbreviatus*. AgNOR stained bouquet stages. Silver has stained condensed chromatin (sex bivalent, and procentric regions of all ten autosomal bivalents) dark brown. Paler brown color suggesting nucleolar substance marks the more or less racemose body (thick arrows) adjacent to the sex bivalent. In one nucleus, the substance is detached from the sex bivalent (hollow arrow). Three smaller nuclei to the extreme right are zygotes showing a higher number of procentric knobs. They may already show the pale brown substance (thin arrow).—1780 \times .

brous or flocculate structure can become recognizable in the lumen. Phase contrast enhances the visibility of this structure, and the most compact silver deposit at the inner border of X seems white, which is presumably due to a mirror-like glare of the dense silver surface (21) (fig. 5D).

Instead of a fibrous or flocculate content and a denser silver deposit at both ends of the lumen, the AgNOR stained Xy_p of MI indicates a dense deposit filling the entire lumen (fig. 6C). Under phase contrast, this latter deposit appears white, whereas areas of lesser density bordering the X and y chromosomes are visibly darker or black altogether (fig. 6D). Feulgen stained, or under phase contrast without AgNOR staining, the lumen seems empty (fig. 6A and B).

Other stains applied to the parachutes of the four weevils do not reveal much material in the lumen. Feulgen-staining reveals a visibly-

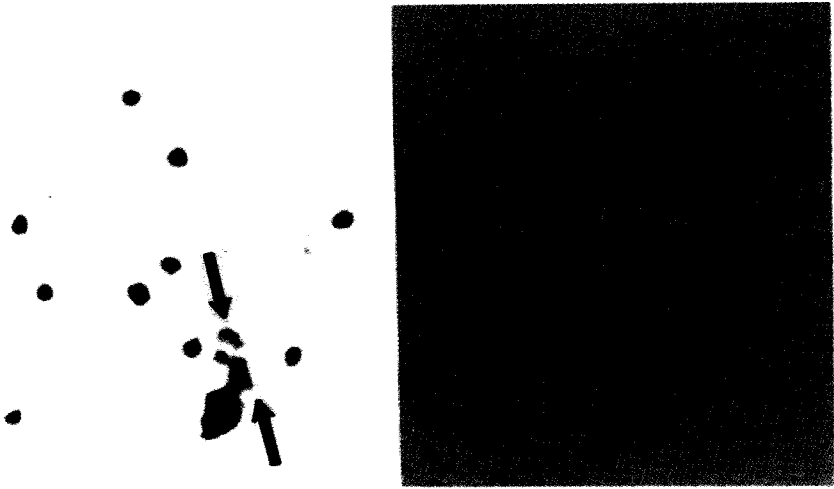


FIG. 2.—*Diaprepes abbreviatus*. AgNOR-stained diffuse bouquet (left). The three granules (between arrows) adjacent to sex bivalent, when washed in $K_3Fe(CN)_6$ and restained in Unna-Pappenheim (right), acquire a reddish tint suggestive of nucleolar substance.—1780 \times .

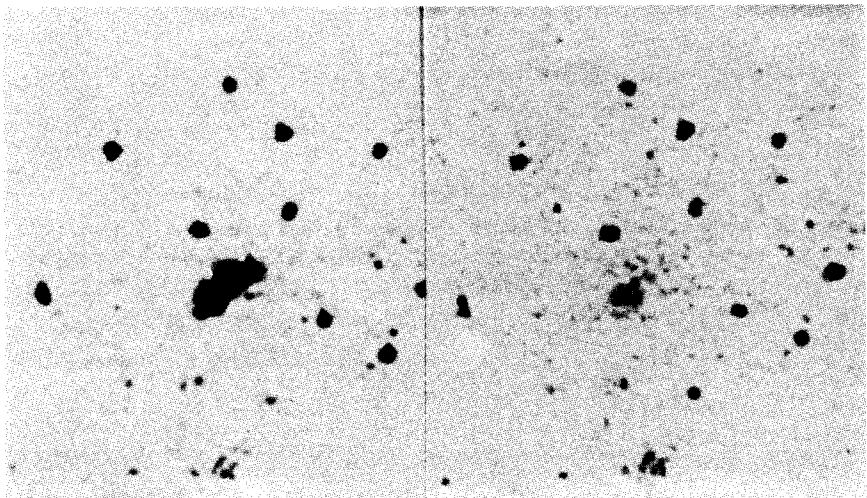


FIG. 3.—*Diaprepes abbreviatus*. AgNOR-stained diffuse bouquet (left) showing ten procentric autosomal knobs and the bulky sex bivalent.—The same nucleus (right) after washing in $K_3Fe(CN)_6$, RNAse treatment, and a new AgNOR-Staining. Most of the substance surrounding the sex chromosomes has disappeared.—1780 \times .

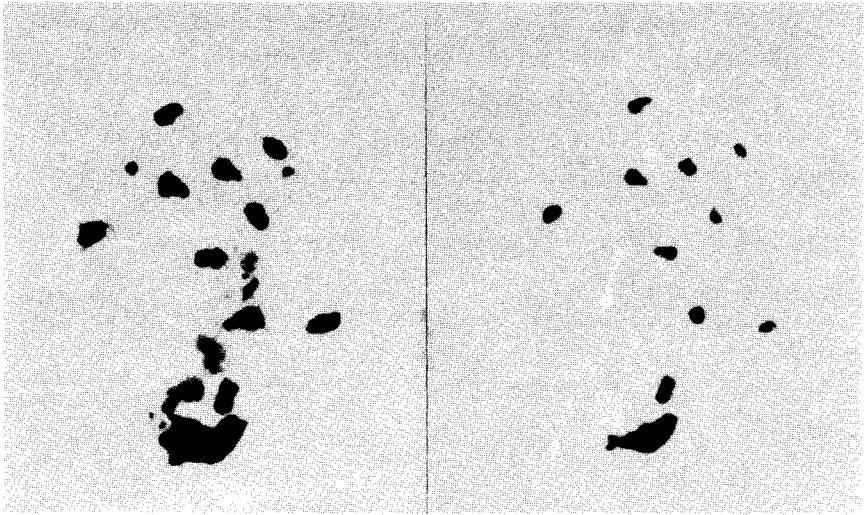


FIG. 4.—*Diaprepes abbreviatus*. AgNOR-stained diffuse bouquet (left) showing numerous granules in addition to the sex bivalent and procentric knobs of the autosomal bivalents. After washing in $K_3Fe(CN)_6$, treatment in hot + cold perchloric acid, and a new AgNOR-staining, only the sex bivalent and the ten procentric knobs remain (right).—1780 \times .

empty lumen in *Lachnopus* (fig. 7A). Giemsa can produce results similar to AgNOR staining in diplotene (fig. 7B). Both are catalytic stains and more sensitive than acid or basic dyes (Horobin, personal communication). After C-banding, Giemsa marks only the procentric heterochromatin (fig. 7C). Acetocarmine leaves the lumen visually empty (fig. 7F) or slightly shaded (fig. 7E), depending on the duration of hydrolysis. The hydrolysis does not eliminate argyrophily in the lumen of a MI parachute (fig. 7G), nor in the extremes of the lumen of a diakinetive parachute (fig. 7H). The protein stain Remazol (fig. 7I), and the nucleolus stains Toluidine Blue O, Azure A and Luxol Brilliant Green (fig. 7J to L) do not reveal a substance in the lumen.

We conclude from these findings that the lumen of Xy_p becomes filled by an argyrophilous structure which is neither nucleolar nor of basic-proteinous nature. We agree with A. Postiglioni (personal communication) that the structure could be composed of acid proteins. The structure first appears at the inner borders of both sex chromosomes. It is sufficiently dense for occasional detection with Giemsa stain. When it fills the lumen, its presence becomes clearly demonstrable via the relatively severe reaction with Ag.

On the morphology of Xy_p

Figure 7 depicts certain morphological features that are relevant to discussions of Xy_p structure.

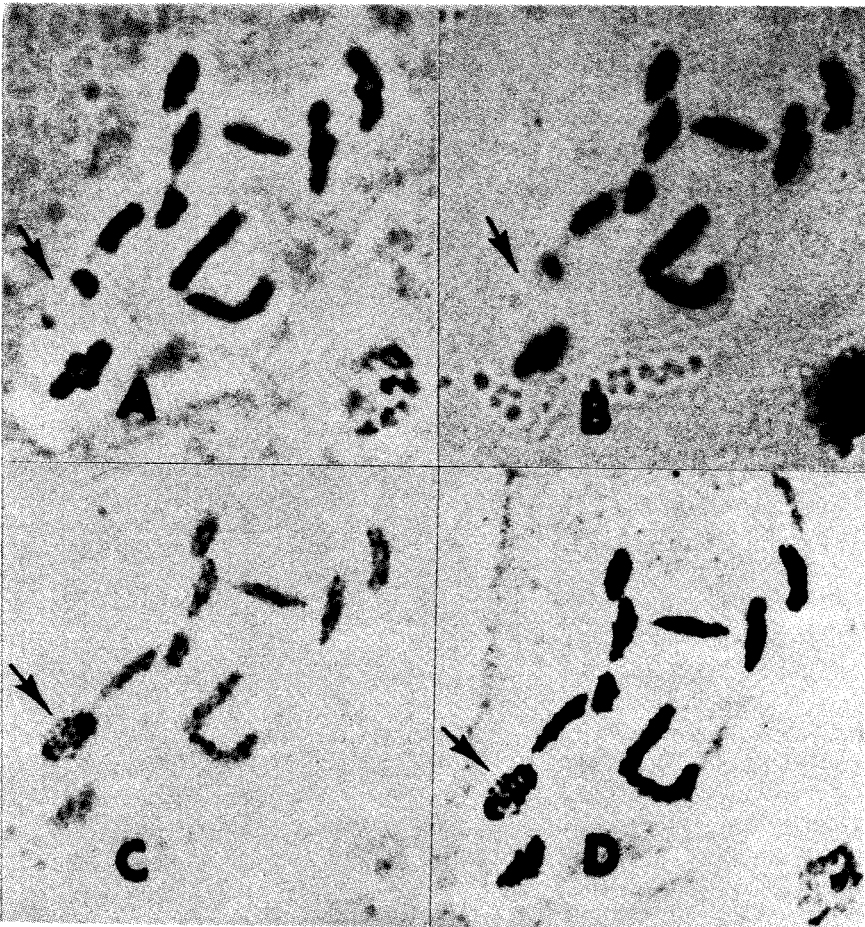


FIG. 5.—*Diaprepes abbreviatus*. KS-fixed early diakinetic bivalents under different treatments and optical conditions. Arrow shows the Xy_p sex bivalent. —A.—Unstained, under phase contrast. —B.—Feulgen stained, normal optics. —C.—AgNOR after Feulgen stain, normal optics. —D.—The same under phase contrast.—2240 ×.

Figure 7J shows a parachute which seems to have two y chromosomes. However, we interpret this in accordance with Drets et al. (3), who say the extra piece is mainly composed of the extreme portion of X, opposite to the first contact with y, and is now withdrawing towards the main body of X. In J it is half-way, in B it touches the main body of X, and in K there is integration with it. Since Shaw (14) published his experiments on accumulation of y chromosomes in *Dermestes* Xy_p , parachutes such as the one depicted in figure 7J have been interpreted as Xyy_p s. From present findings it appears that such an interpretation should be sustained by observing the segregation at A I to M II. In *Lachnopus*

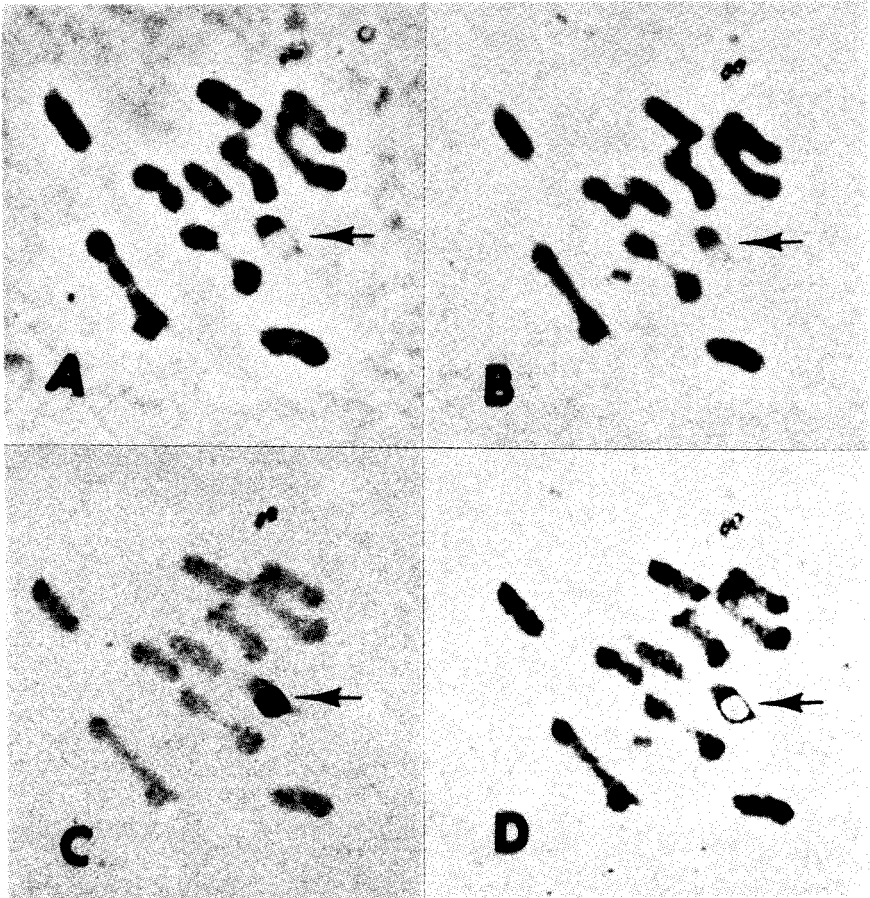
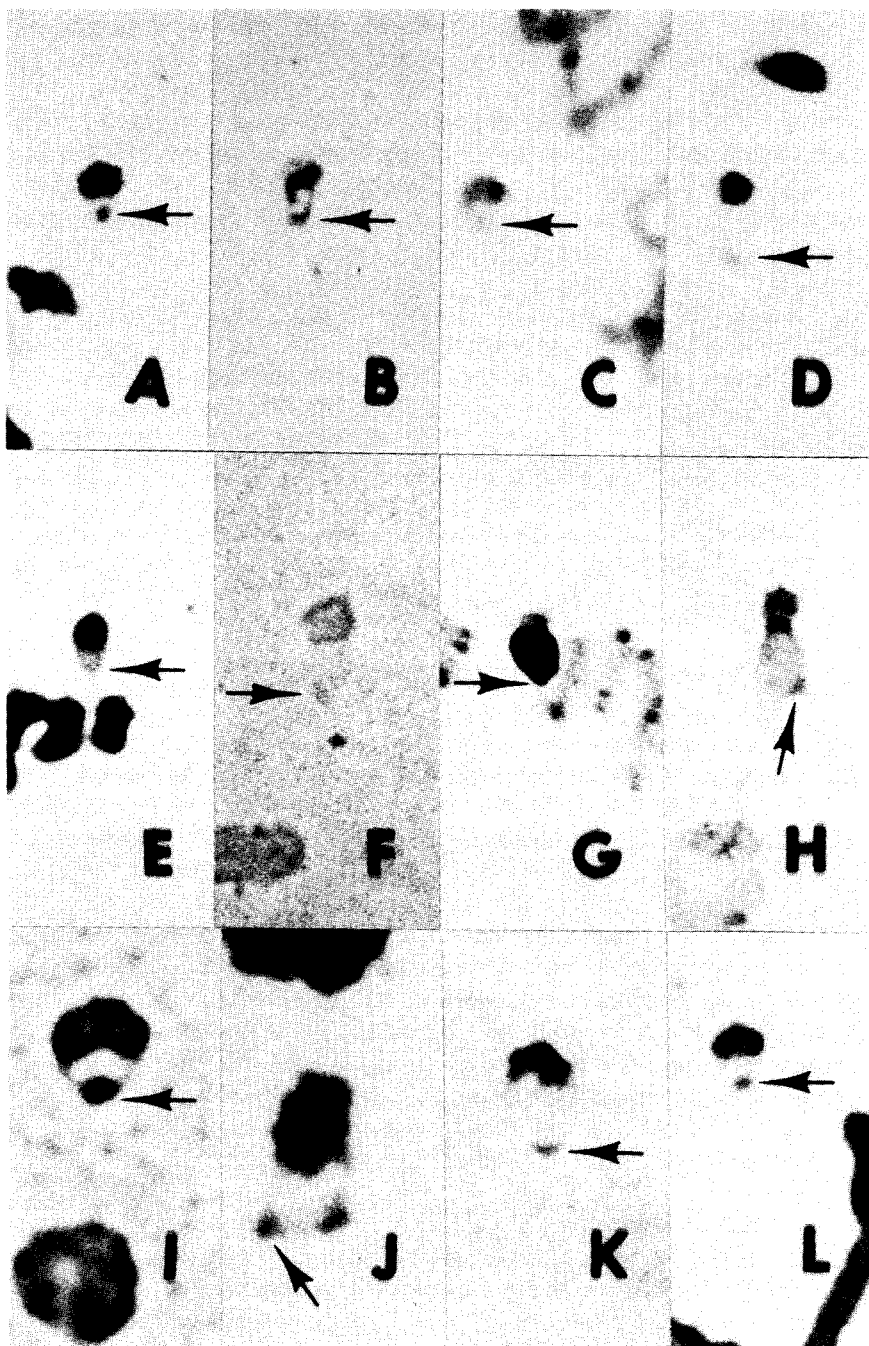


FIG. 6.—*Diaprepes abbreviatus*. KS-fixed M I bivalents under the same treatments and optical conditions as the bivalents of Fig. 5. The arrow shows the Xy_p sex bivalent. —A.—Unstained, under phase contrast. —B.—Feulgen stained, normal optics. —C.—AgNOR after Feulgen stain, normal optics. —D.—The same under phase contrast.—2240 \times .

FIG. 7.—A. to L.—Reaction of KS-fixed Xy_p to various DNA, nucleolus, and protein stains. Where possible, some autosomal bivalents are included to show the phase of meiosis. Arrow points to y chromosome. —A, I and J: *Lachnopus curvipes*; B: *Exophthalmus quindecimpunctatus*; C: *Exophthalmus roseipes*; D to H; K and L: *Diaprepes abbreviatus*. —A.—Diakinesis. Feulgen reaction. —B.—Diplotene. Giemsa stain. —C.—Diplotene. C-banding. —D.—Diakinesis. Acetocarmine. —E.—M I. Rattenbury's acetocarmine after hydrolysis of 1 h. —F.—Diakinesis. Rattenbury's acetocarmine after hydrolysis of 4 h. —G.—M I. AgNOR staining after Rattenbury's acetocarmine (hydrolysis of 1 h). —H.—Diakinesis. As in G. —I.—Diakinesis. Remazol stain. —J.—Diakinesis. Toluidine Blue O. —K.—Diakinesis. Alkaline Azure A. —L.—Old diplotene. Luxol Brilliant Green. —I and J: 4675 \times , the rest: 2900 \times .



curvipes, we have observed only a single y chromosome segregating from X.

Figure 7, H and K, presents views of diakinetiic Xy_p in *Diaprepes abbreviatus*. In K, the X chromosome appears much larger than in H. We interpret K to be a "frontal" view, whereas H is the "side" view; *e.g.*, the angles of observation of the bivalent differ by about 90° . In H there is a sticky contact between Xy_p and an autosomal bivalent. The contact seems to extend a string or a pellicle out of the main HXy_p body. We are publishing elsewhere more clearly-contrasted photographs of this phenomenon (21). Interestingly, they present "frontal" views of Xy_p . The likelihood is thus enhanced that the extruded structure is a pellicle rather than a string. The presence of such a pellicle in the Xy_p has been proposed (15), but is not compatible with the known structure of nucleoli.

C-banding (fig. 7C) marks constitutional heterochromatin adjacent to the centromere in autosomes and the X chromosome. Other staining methods may indicate the same (X chromosome: fig. 7B; autosomes: fig. 7C). Acrocentry of the X chromosome is common in the Puerto Rican broad-nosed weevils, as we have seen in the karyograms of *Diaprepes abbreviatus* (22) and related species (unpublished).

CONCLUDING REMARKS

Formation of a small true nucleolus just before or during the diffuse phase of spermatocyte I is probably a normal phenomenon in the beetles. Where a prolonged diffuse phase leads to unusually large spermatocytes, as in the *Oedionychina* flea beetles (19) or *Aphodius* scarab beetles (17), the production of the nucleolar substance can be materially increased. As a rule, the NOR is autosomal in the beetles. The nucleolus appears, therefore, to be separated from the sex bivalent. To the contrary, we have found nucleolar substance closely associated with the early sex bivalent in the weevils. Our methods could not exclude the possibility of association of an autosomal NOR with the sex bivalent. On the other hand, the substance is produced in droplets, being consistent with production where no NOR is present (15, pp. 31-32). This early nucleolus vanishes and apparently performs no role in maintenance of the Xy_p bivalents.

The immediate impression after Ag-NOR staining is that the Xy_p lumen becomes filled with nucleolus during the late prophase. Other nucleolus stains do not confirm this finding. Acid hydrolysis does not lessen the argyrophily. It seems that a very delicate fibrous structure invades the Xy_p lumen, the invasion starting in the late diplotene. If this structure is protein-related or protein-bearing, as in chromosomal scaffolds, it may well escape staining by methods that do not provide heavy-metal deposits (9). The fact that formalin in the fixative enhances the argyrophily is indeed consistent with an involvement of acid proteins in this staining.

Anionic carboxyls induced in the proteins by formalin might be the reason for absence of staining by Remazol (Horobin, personal communication).

The present results stress the need to apply to the studies of Xy_p structure techniques beyond the classical cytochemistry and cytomorphy. These can include application of monoclonal antibodies to "nucleosteric" (1), scaffold, synaptonemal complex, and NOR proteins, together with ultrastructural studies.

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