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A Virus Associated with SR-Spirochetes of *Drosophila nebulosa**

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Abstract. A virus is associated with a strain of transovarially-transmitted SR-spirochetes, NSR, of *Drosophila*. This virus, designated spv-1, is able to infect and to multiply in another strain of SR-spirochetes, WSR, thereby eliminating the SR condition from the host flies. Spv-1 contains DNA, has a buoyant density of 1.480 in CsCl, and is spherical in shape, measuring 50–60 nm in diameter.

The maternal sex-ratio (SR) condition in *Drosophila* is characterized by an absence of male progeny. One class of the SR-condition is caused by an infection with so-called SR-spirochetes.[1] The absence of male progeny is a result of selective lethality at various developmental stages.[2,3] The SR-spirochetes can be transferred into and maintained in other *Drosophila* species.[4] Interest in the analysis of the SR phenomenon has centered about determining the specific factor(s) causing male lethality in the hope that the system may provide some insight into the problem of sex-differentiation in *Drosophila*. Various workers have tried to understand the general picture of the SR-spirochete infection as a first approach to solving this problem.[4–6]

Three strains of SR-spirochetes have been maintained in the laboratory, each derived from a single female of the neo-tropical flies, *Drosophila willistoni*, *D. equinoxialis*, and *D. nebulosa*[4]; they are referred to as WSR, ESR, and NSR, respectively.[7] These SR-spirochetes appear identical under the electron microscope, approximately 5–6 μm in length and 0.15 μm in diameter.[6]

Experiments have shown that the mixing of any two strains of SR-spirochetes, either *in vivo* or *in vitro*, results in the instantaneous formation of clumps and gradual disappearance of spirochetes; *in vivo*, this generally ends in the elimination of the SR-condition of the flies.[8–10] Extracts of NSR-carrying flies, treated by heat or high-speed centrifugation to eliminate the activity of SR-spirochetes, were shown to cure WSR-carrying flies of the SR trait. This effect was thought to be parallel to the action of bacteriocins and the name *spirocin* was given to the active principle in these extracts.[8,9] Further studies indicated, however, that spirocin eliminates WSR by lysis; at the same time, the apparent activity of spirocin is increased. In this report we show that "spirocin" is a virus.

Materials and Methods. SR-spirochete strains: Two SR-spirochete strains (WSR and NSR) were used. They were maintained in Oregon-R strains of *D. melanogaster* (ORWSR and ORNSR) by mating SR females with males from the Oregon-R normal strain (Ore-R) in each fly generation. Details of maintenance and rearing are presented in full elsewhere.[11]

Observation of SR-spirochetes by dark-field microscopy: Hemolymph was routinely examined to detect the presence or absence of SR-spirochetes. Undiluted hemolymph was taken up into a small glass injection pipet (see below) and placed on a glass slide below a drop of immersion or mineral oil. A cover slip was applied and the specimen observed (×250 or 400) under a Zeiss Photomicroscope with a dark-field condenser.

Injection of flies: Flies were injected with a modified version of an apparatus described by Rizki.[12] Glass microinjection pipets were made of commercially-available capillary tubing (inner diameter, 0.5–0.9 mm). Pipets, which were used only once, were calibrated against a Hamilton microsyringe (1 μl). They were changed whenever the injection material was changed.

Extraction of lytic factor: The lytic factor associated with NSR (spirocin) was extracted as follows: ORNSR flies were collected and weighed. One g of flies was homogenized in 4 ml of 0.15 M NaCl, or in buffered sucrose solution (0.25 M sucrose–50 mM Tris·HCl buffer, pH 7.6–25 mM KCl) in an all-glass homogenizer, and centrifuged at 3000 × g for 10 min. The supernatant was heat-treated in a water bath to eliminate SR-spirochete activity (60°C for 10 min), cooled on ice, and recentrifuged at 3000 × g for 10 min. The supernatant was kept frozen at −17°C until use. The final supernatant contained a titer of the lytic factor of 10^{-4} final effective dilution (see below).

Bioassay for lytic factor: Lytic activity was measured on the basis of morphological changes in the SR-spirochetes and their disappearance from the hemolymph as ascertained with dark-field microscopy (Fig. 1).

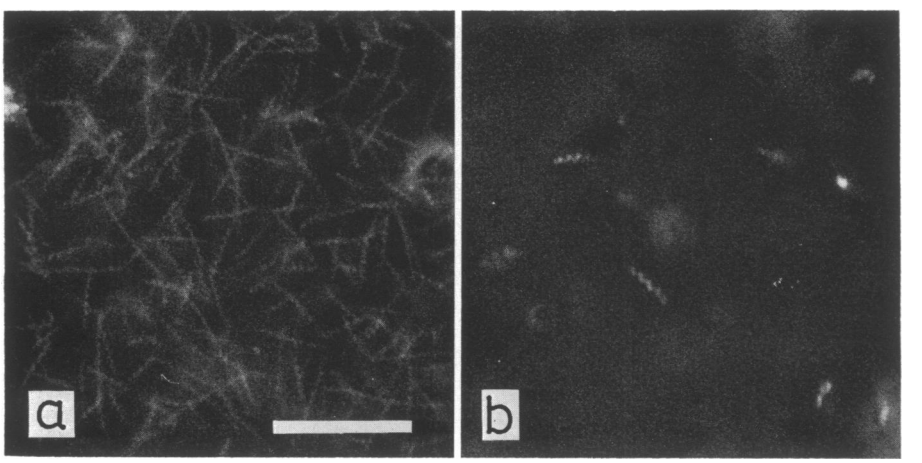

FIG. 1. WSR spirochetes in undiluted hemolymph as seen under the dark field microscope with flash illumination (Zeiss UKATRON UN 60) before and after action of the lytic factor: (*a*) Normal WSR spirochetes, (*b*) lysis completed, leaving a few survivors. Scale indicates 10 μm.

To measure lytic activity, 20 flies were homogenized with 1 ml of buffered sucrose solution, and spun at 3000 × g for 10 min. The resulting supernatant was called the first dilution. The supernatant was then serially diluted in steps of 10 with buffered sucrose solution. When lytic titer was measured in subsequent suspensions, however prepared, the initial suspension was called the first dilution. Lytic activity in each dilution was tested by injection of test flies (ORWSR) with 0.15 μl per fly. Since it was found in preliminary experiments that any lytic activity present in an extract was evident within 15 days, examination of hemolymph was routinely made after 15 days for determination of the final effective dilution. 10–15 flies were tested at each dilution.

Labeling of lytic factor with [^{3}H]thymidine: Flies carrying WSR were first injected with a suspension of the lytic factor (approximate volume, 0.2 μl/fly), and then three times with an equivalent volume of [CH_3-^{3}H]thymidine (Nuclear Chicago, 18.3 Ci/mmol). [^{3}H]thymidine was injected at days 2, 3, and 5. Flies were collected on the 10th day after the injection of the lytic factor and kept frozen until use.

Sucrose density gradient centrifugation: Linear gradients of sucrose (30 ml; 35–10%, w/w) were prepared in 0.05 M Tris buffer, pH 7.6–25 mM KCl. 0.5–1 ml of a concentrated lytic factor preparation, [^{3}H]thymidine labeled, was layered on top of the gradient and centrifuged at 19,000 rpm in a SW 25.1 rotor for 160 min in a Spinco model L ultracentrifuge.

After centrifugation, tubes were punctured from the bottom, and 45-drop fractions were collected. A portion (0.1 ml) was removed from each fraction for bioassay; the original portion was the first dilution.

After absorbance was measured, an equivalent volume of 10% cold trichloroacetic acid solution was added to each fraction, and the whole filtered through a Millipore filter (Millipore Filter Corp., pore size 0.22 μm). Filters were washed with 5% cold acid, then with anhydrous ether, dried, and placed in counting vials. 5 ml of counting liquid [1 liter toluene, 4 g 2,5-diphenyloxazole (PPO), 0.5 g 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene (POPOP)] was poured into each vial, and the radioactivity was measured in a liquid scintillation spectrometer.

CsCl equilibrium density gradient centrifugation: Lytic factor was purified as follows. About 200 flies with labeled lytic factor were homogenized, after lysis of WSR, in 10 ml of buffered sucrose solution and centrifuged. The post-mitochondrial supernatant was spun at 115,000 $\times$ g for 30 min. The pellet was suspended in 5 ml of buffered saline (0.15 M NaCl–0.01 M Tris buffer, pH 7.6). DNase (DNase I, Worthington Biochemicals) and $MgCl_2 \cdot 6\ H_2O$ solutions were added successively to make final concentrations of 15 μg/ml and 10 mM, respectively; the suspension was incubated for 10 min at 37°C. It was then spun for 10 min at 3,000 $\times$ g, and the resulting supernatant was centrifuged at 115,000 $\times$ g for 30 min. The pellet was resuspended in 2 ml of buffered saline solution. A portion (50 μl) of this suspension was applied to a filter paper and the radioactivity was measured.

To 1.5 ml of suspension, prepared as above, was added 0.1 ml ^{32}P-labeled bacteriophage lambda suspension and buffered saline to make a total weight of 2.50 g; 2.04 g solid CsCl was then added. For density measurement, 2.50 g of buffered saline, to which 2.04 g of CsCl was added, was prepared.

Centrifugation was in a Spinco L-2 ultracentrifuge with a SW 50 rotor for 24 hr at 40,000 rpm and 25°C. 3-drop fractions were collected from the tube containing labeled lytic factor and lambda phage, and the radioactivity in each fraction was measured by scintillation counting. 15-drop fractions were collected from tubes containing buffered saline only. The density of each fraction was determined by measuring its refractive index.

Electron microscopy: A drop of diluted hemolymph was placed on a copper grid (300 mesh, coated with 0.2% Formvar and then covered with carbon) and fixed with the vapor from 25% glutaraldehyde for 10 min. Hemolymph was removed from the grid by blotting the sample with filter paper, and a drop of 1% potassium phosphotungstate solution (pH 7.6) was applied for 30 to 60 sec. The solution was removed and the grid was air-dried. Observations were made with a Phillips 200 electron microscope.

Results. Increase in lytic activity: That "spirocin" is a virus was first suspected when it was found that spirocin did not simply incapacitate WSR spirochetes, but actually lysed them. Lysis was detected by dark-field examination of fly hemolymph after the injection of spirocin.

Spirocin preparation was injected into flies which were harvested, 20 each, on the days shown (after the initial injection) and kept frozen until use. It can be

TABLE 1. *Increase in activity of the lytic factor in spirochete-infected flies.*

	Lytic factor activity on WSR — Dilutions tested:						
Days in host flies	First	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
In ORWSR strain							
0	+(5/5)	±(3/6)	−(0/10)	−(0/8)			
2	+(9/9)	+(9/9)	±(4/10)	−(0/9)	−(0/10)		
6		+(8/8)	+(4/4)	+(8/8)	±(7/8)	−(0/5)	−(0/9)
12				+(8/8)	+(8/8)	±(5/6)	−(1/8)
In Ore-R (control)							
0	+(8/8)	+(9/9)	±(1/6)	−(0/9)	−(0/10)		
12	+(6/6)	±(1/7)	−(0/7)	−(0/8)			

Crude spirocin preparations were injected into ORWSR and control flies, and collected at specified intervals. The lytic factor was extracted and dilutions were injected into ORWSR flies whose hemolymph was examined for the presence of lysis: (+) lytic factor active at the dilution, (−) lytic factor inactive at the dilution, (±) final effective dilution (some injected flies carried lysed WSR and some did not). The ratio of flies carrying lysed WSR to the total number of flies injected and examined is shown in parentheses.

seen in Table 1 that the final effective dilution increased 10,000 times in the spirocin-treated flies, while it decreased 10 times in the controls. Spirocin activity in the treated flies on day 0 is 10-times less than that in the control flies. The same result was obtained in another experiment using independently prepared material. This low recovery suggests that the lytic factor enters into WSR and is temporarily not extractable as infectious particles.

Step-wise sedimentation of the lytic factor: Flies were injected with crude spirocin suspension and collected after 10 days. 100 flies were homogenized in 5 ml of buffered sucrose solution. The homogenate was sedimented step-wise: (*a*) 3000 × *g* for 10 min (supernatant, sample 1), (*b*) 15,000 × *g* for 20 min (supernatant, sample 2), and (*c*) 115,000 × *g* for 90 min (supernatant, sample 3; and pellet to which the original volume of buffered sucrose was added, sample 4). As shown in Table 2 the final effective dilution in each sample was 10^{-4}, 10^{-4}, 10^{-1}, and 10^{-4}, respectively.

This method of partially purifying and concentrating the lytic factor (by resuspending the 115,000 × *g* pellet fraction in a smaller volume) was used for the sucrose density gradient analysis. Later experiments, however, showed that it was sufficient to centrifuge the post-mitochondrial supernatant for 30 min at 115,000 × *g* to recover essentially all the lytic activity. This preparation was used for CsCl density gradient centrifugation.

TABLE 2. *Step-wise sedimentation of the lytic factor activity.*

	Lytic factor activity on WSR* — Dilutions tested:					
Fraction	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
3,000 × *g*, 10 min supernatant				±(6/9)	−(0/10)	−(0/13)
18,000 × *g*, 20 min supernatant				±(8/11)	−(0/10)	−(0/11)
115,000 × *g*, 90 min supernatant	±(3/10)	−(1/9)	−(0/9)			
Pellets (resuspended in the original volume)			+(10/10)	±(7/9)	−(0/10)	

* See Table 1.

[^{3}H]thymidine labeling and sucrose density gradient centrifugation: Since the available amount of lytic factor could not be correlated with absorbance on sucrose density gradients, labeling of the lytic factor (suspected virus) with a radioactive nucleic acid precursor was attempted.

The sucrose density gradient profile of the lytic factor labeled with [^{3}H]-thymidine is presented in Fig. 2. A single sharp peak of radioactivity was centered about fraction 16. Five fractions (6, 13, 16, 19, and 27) were bioassayed. The radioactive fraction contained 100 times more lytic activity than the other fractions.

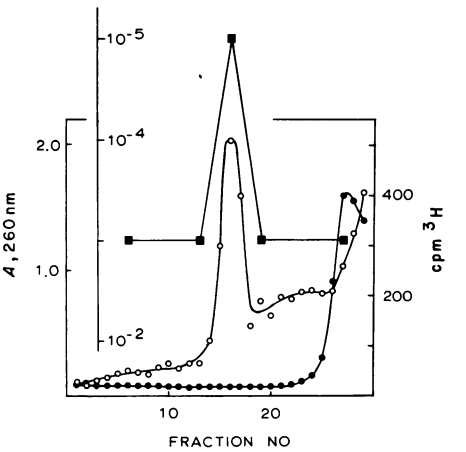

FIG. 2. Sucrose-density gradient profile of [^{3}H] thymidine-labeled lytic factor. For selected fractions bioassays were made to determine the final effective dilution. *Open circles*, cpm. *Closed circles*, *A*. *Closed squares*, activity (inserted scale) of lytic factor.

CsCl equilibrium density gradient centrifugation: Results of CsCl equilibrium density gradient centrifugation are shown in Fig. 3. One sharp peak of radioactivity for ^{3}H was obtained at about fraction 59. The peak for ^{32}P (reference) was at fractions 49 and 50. Density measurement showed that the

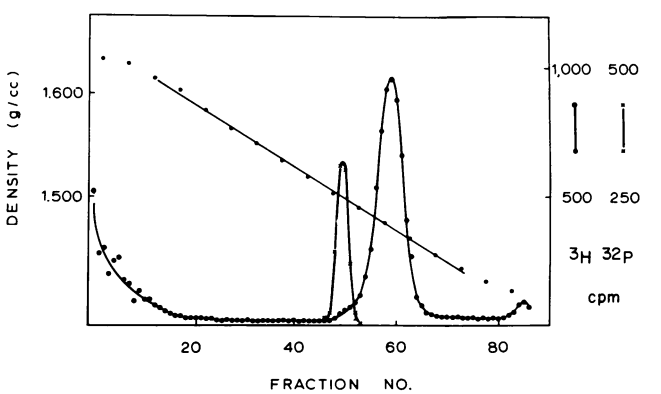

FIG. 3. CsCl equilibrium density gradient profile of the lytic factor. [^{3}H]thymidine-labeled lytic factor was mixed with ^{32}P-labeled coliphage lambda (reference) and subjected to CsCl (initial density, 1.5 g/cc) density gradient centrifugation.

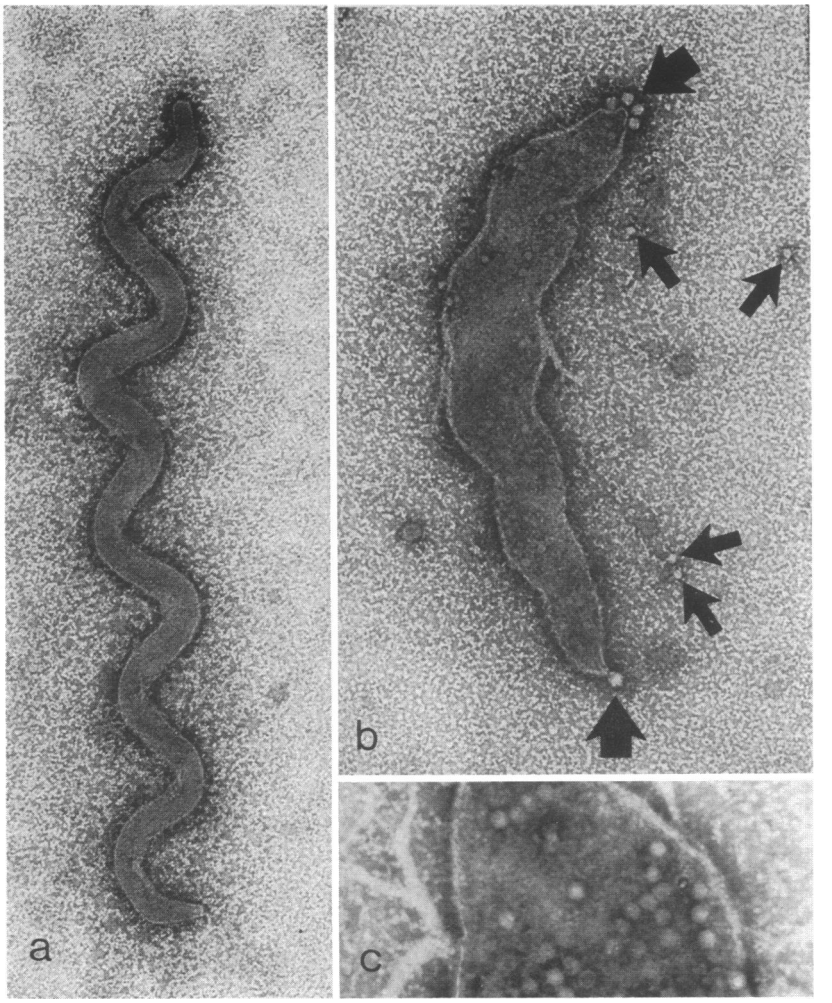

FIG. 4. Electron micrographs of normal (*a*) and lysing (*b*) WSR spirochetes at a magnification of ×32,500. Particles in (*b*) are associated with WSR, as well as free (*arrows*). Heavy arrows indicate particles presumably just extruded from, and still coated with, surface material of the SR-spirochete. Particles within lysing WSR (*c*) at higher magnification, ×58,500.

gradient was linear in the area which included both ^{3}H (lytic factor) and ^{32}P (lambda) peaks. The density of the lytic factor, calculated from the slope of the density gradient and the known density of lambda (1.508), was 1.480.

Electron microscopy: Fig. 4 shows typical electron microscopic observations made on normal and lysing WSR. Numerous particles of uniform size, 50–60 nm in diameter, were observed inside the lysing WSR spirochetes; similar particles occurred outside the cellular structure. No such particles were seen in preparations of normal WSR spirochetes.

Discussion. It is evident from these results that the entity previously called spirocin[8,9] is a virus. This virus is spherical in shape, measures 50–60 nm in

diameter, and has a buoyant density of 1.480 in CsCl. This virus, spv-1, multiplies in and lyses spirochetes of the WSR strain, and eliminates the SR condition from host flies into which it has been injected. When first released in the course of lysis, spv-1 appears encased in surface material of the WSR spirochetes from which it is subsequently freed (Fig. 4). The properties of the very small number of spirochetes that resist lysis and survive remain to be investigated. Just how spv-1 is associated with, and maintained in, NSR spirochete populations is not known at present. However, active virus can be extracted from populations of NSR spirochetes carried in *D. melanogaster* (as well as in the original host species) by our procedures.

Although the precise taxonomic position of SR-spirochetes remains to be established, they clearly belong in the order Spirochetales on the basis of overall morphology (manuscript in preparation). Since the genus *Saprospira*, in which a DNA-containing virus is known[13,14], has been removed from the order Spirochetales,[15] it appears that no other virus has been reported in association with species belonging to this order. Thus, spv-1 represents the first definitely demonstrated spirochete virus (phage). Furthermore, this virus has been successfully enployed to eliminate infections of microorganisms (susceptible SR-spirochetes) and their pathological effects (male killing) from the host organism (*Drosophila* species). The reappearance of males in the progeny of the treated flies is closely correlated with the course of spirochete lysis.[11]

Indications of the separability of male killing and spirochete transmission, particularly from the work of Williamson[6,16] and the observations presented here, suggest that SR-spirochetes produce an agent lethal to male zygotes and that virus infection, followed by lysis of spirochetes, interrupts production of this putative lethal agent, thus eliminating it from the host fly. The nature of this androcidal agent remains to be established.

There is some evidence for virus associations with SR-spirochetes from *Drosophila* species other than *D. nebulosa*.[11] It is most interesting that to date, each of the strains of SR-spirochetes examined in any detail have an associated virus (unpublished results). Should spv-1, or any of these, prove to be transducing, a powerful tool will become available for the study of SR-spirochetes and their relationships to their fly hosts. The analysis of such three-level systems—virus, spirochete, fly—is of considerable interest not only from the point of view of genetics, but also in terms of their ecological and evolutionary implications.

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