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Spirochaete-mediated abnormal sex-ratio (SR) condition in *Drosophila*: A second virus associated with spirochaetes and its use in the study of the SR condition

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SUMMARY

A class of abnormal sex-ratio (SR) condition in *Drosophila* characterized by the elimination of male zygotes is caused by infection with maternally transmitted SR-spirochaetes. A given spirochaete strain, WSR, was shown to have an associated DNA virus, spv-2, in a latent condition. This virus is induced to multiply when another DNA virus, previously designated spv-1, infects WSR spirochaetes; spv-1 is normally associated with another strain of SR-spirochaetes, NSR, and is in a manifest state. Spv-2 infects and lyses NSR, thus eliminating spirochaetes from fly hosts which, in these experiments, were *D. melanogaster*.

The elimination of WSR through lysis by spv-1 results in the 'curing' of the SR-condition in the host flies. However, the elimination of NSR by spv-2 does not lead to the immediate elimination of the SR-condition in the host. A hypothesis is presented in favour of the view that a substance, *androcidin*, is produced by SR-spirochaetes and that this substance is actually responsible for the death of male zygotes. NSR may produce a more potent *androcidin* than WSR.

1. INTRODUCTION

The maternally inherited SR-condition of *Drosophila*, characterized by an absence of male progeny, has been described in several species of this genus. The latter falls into two groups: those in which the condition is caused by an SR-spirochaete infection, and those in which there is no indication of a spirochaete infection (Poulson, 1963, 1968). The SR-condition referred to in this report falls into the first category. Since infection with SR-spirochaetes (Poulson & Sakaguchi, 1961) results in the selective death of male zygotes at various developmental stages (Counce & Poulson, 1962, 1966), it has been hoped that this system would provide a means of attack on the problem of sex-differentiation in *Drosophila*.

Using two strains of SR-spirochaetes derived from different host species and from different localities, Sakaguchi, Oishi & Kobayashi (1965) showed that, upon mixing *in vivo* or *in vitro*, they form clumps and gradually disappear. One strain of spirochaetes, NSR (originally called NebSR), produces a substance that incapacitates another strain, WSR (originally referred to as SRB_3). This clump

formation has since been demonstrated between other SR-spirochaete strains; that is, any two strains form clumps upon mixing (Poulson, Hyde & Oishi, 1967; Poulson & Oishi, in preparation).

The substance produced by NSR which incapacitates WSR (Sakaguchi & Oishi, 1964) is a virus, spv-1, and it is associated with NSR in a manifest condition (Oishi & Poulson, 1970). Spv-1 infects and lyses WSR, thus 'curing' the host flies of the SR-condition. In the present report it is shown that another virus, spv-2, is present in association with WSR, but in a latent state; it infects and lyses NSR. It is also shown that, while lysis of WSR by spv-1 results in curing of the SR-condition here, lysis of NSR by spv-2 does not lead to the same results. A working hypothesis to explain these results is presented and discussed.

2. MATERIALS AND METHODS

(i) *SR-spirochaete strains*

Two SR-spirochaete strains, WSR and NSR, were used (Table 1). They were maintained in Oregon-R (Ore-R) strains of *D. melanogaster*, ORWSR and ORNSR, which were established by injection of Ore-R host females with spirochaete-laden

Table 1. *Nomenclature of strains of SR-spirochaetes and their associated viruses, and of SR-flies used in the present study*

SR-spirochaete strains	Origin*	SR-flies: maintained for the present study in Oregon-R strains of *D. melanogaster*	Viruses associated with SR-spirochaetes
WSR	From a natural population of *D. willistoni* in Jamaica in 1956	ORWSR	spv-2
NSR	From a natural population of *D. nebulosa* in Haiti in 1959	ORNSR	spv-1

* Original host fly strains derived from a single mated female in each case.

hemolymph from *D. willistoni* and *D. nebulosa*, respectively. Routine maintenance was as follows: About 25 SR females were placed in a culture bottle containing standard cornmeal–molasses–agar medium with about 30 males from the Ore-R normal strain and reared at room temperature, generally $24 \pm 1{\cdot}5$ °C. To maintain stable SR-fly strains, parents were transferred into a new culture bottle every 4 days so that progeny developed in several broods. Female parents for the next generation were then taken from the fourth brood in the case of ORWSR and from the third brood for ORNSR. Flies from comparable broods were the primary experimental material for the present studies.

The stability of the SR-condition under these conditions had been previously established. Thus, progeny were counted and the sex-ratio recorded according to brood for each SR strain of flies. The accumulated data for five fly generations (Fig. 1) demonstrated the stable pattern of the SR phenomenon when so handled.

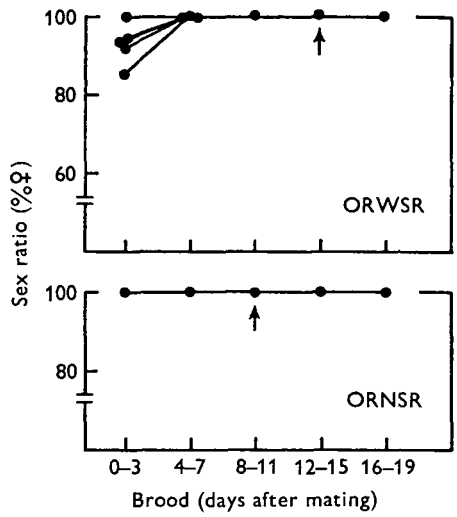

Fig. 1. Typical brood patterns of host fly strains, ORWSR and ORNSR. Arrows indicate broods supplying female parents for the next generation. Fly generations counted were F_{80-84} for ORWSR, Aug. 1969 to Dec. 1969; and F_{86-90} for ORNSR, Aug. to Nov. 1969.

(ii) *Bioassay for viruses*

Virus titre was measured on the basis of the lytic activity of the virus on SR-spirochaetes, as examined under a dark-field microscope (Oishi & Poulson, 1970). Twenty flies in which virus titre was to be measured were homogenized with 1 ml buffered sucrose solution (0·25 M sucrose in 0·05 M Tris-HCl buffer, pH 7·6, containing 0·025 M-KCl), and spun at 3000*g* for 10 min. The supernatant was heat-treated to eliminate spirochaetes at 60 °C for 10 min, cooled on ice, and spun again at 3000*g* for 10 min. The resulting supernatant was called 10^0 dilution. It was then serially diluted in tenfold steps with buffered sucrose solution.

Virus titre in the supernatant was determined by injecting test flies with 0·15 μl/fly of each dilution; examination of hemolymph after 15 days was used as an indication of whether lysis took place, and for determination of the *Final Effective Dilution* (FED).

Following sucrose-density gradient centrifugation of concentrated viral suspension, virus titre in each fraction was quantitated by calling each undiluted fraction 10^0 dilution and determining their FED's.

Other procedures, i.e. injection of flies, microscopic observation of SR-spirochaetes, extraction of spv-1, labelling of viruses with radioactive nucleic acid precursor, and sucrose-density gradient centrifugation, are as previously described (Oishi & Poulson, 1970).

3. RESULTS

(i) *Spv-2: A virus associated with WSR*

In the course of studying the manifest spv-1 associated with NSR (Oishi & Poulson, 1970) it was noticed that extracts from WSR which had been lysed

by spv-1 were able to lyse NSR, while extracts from unlysed WSR were not. As will be seen, the agent which lyses NSR now appears to be a virus, spv-2, which is associated with WSR in a latent condition (Fig. 8).

(*a*) *Development of spv-2 activity in WSR during lysis by spv-1*

A crude spv-1 suspension was injected into flies (0·15 μl each) carrying WSR and the subsequent development of lytic activity examined: bioassays were made for spv-1 activity on ORWSR, and those for spv-2 activity on ORNSR. Results are shown in Fig. 2. The FED of spv-2 reached 10^{-2} by day 6. The FED of spv-1 reached 10^{-4} in the same time. Note that no spv-2 activity could be detected in untreated WSR.

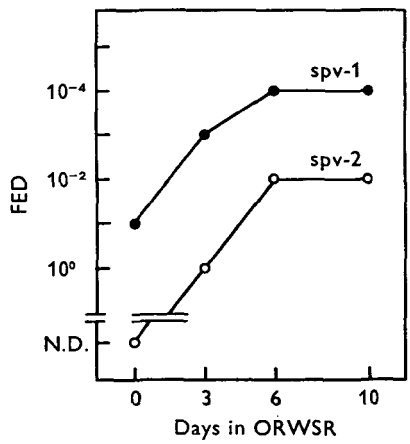

Fig. 2

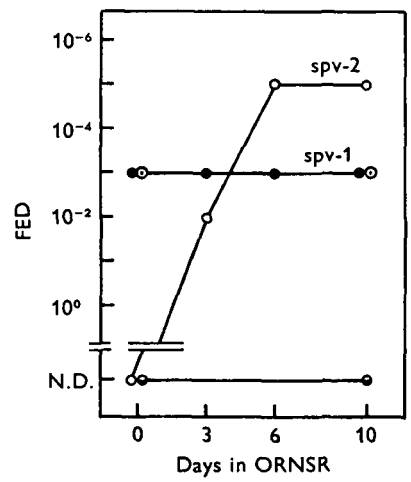

Fig. 3

Fig. 2. Development of lytic activities during the lysis of WSR, following injection of spv-1 into ORWSR flies. The FED's of spv-1 (closed circles) and spv-2 (open circles) were determined by bioassays on ORWSR and ORNSR, respectively, for samples collected on days 0, 3, 6 and 10 after the initial injection. N.D.: not detectable.

Fig. 3. Development of lytic activities during lysis of NSR. Lytic factors extracted from ORWSR after lysis by spv-1 were injected into ORNSR. The FED's of spv-1 (closed circles) and spv-2 (open circles) were determined at specified intervals. Control injections with buffered sucrose were made into ORNSR, and the FED's of spv-1 (double circles) and spv-2 (open double circles) were determined. N.D.: not detectable.

These results do not resolve questions such as whether the NSR-lysing activity is the result of a high multiplicity effect or host modification of spv-1, to mention two possibilities. The following experiment demonstrated the reality of a virus being associated with WSR and its ability to lyse NSR.

(*b*) *Development of spv 2 activity in NSR*

Twenty ORWSR flies were injected (0·15 μl each) with a crude spv-1 suspension, and an extract was prepared from them 10 days later, in the usual way. This extract was then injected into a number of ORNSR flies (0·15 μl each), and extracts

from these flies in groups of 20 were made 0, 3, 6 and 10 days after the injection. These extracts were then assayed for spv-1 on ORWSR and for spv-2 on ORNSR.

The FED of spv-1, which exists in a manifest condition (i.e. the lytic activity is detectable in homogenates prepared according to procedures described in *Methods*), was 10^{-3} for all samples tested as shown in Fig. 3. On the other hand, no lytic activity was detectable when the 0 day homogenate was tested on ORNSR.

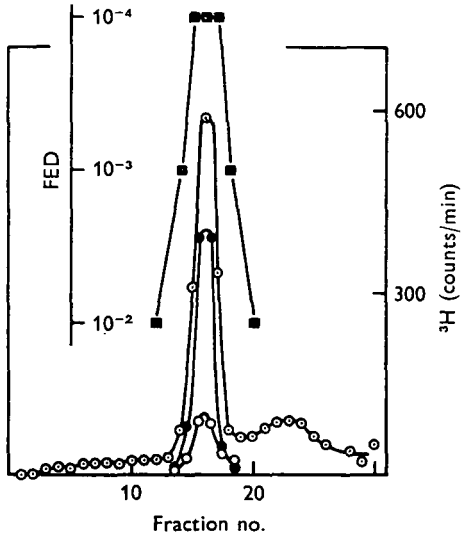

Fig. 4

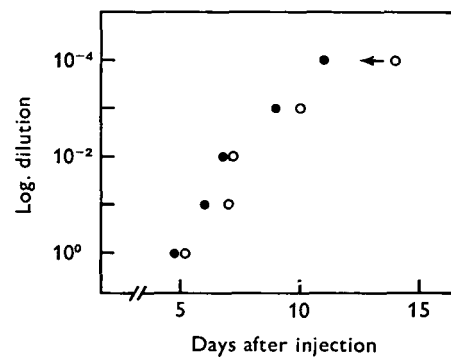

Fig. 5

Fig. 4. Sucrose-density gradient (35–10%, w/w) analysis of [^{3}H]thymidine labelled spv-1 (open circles), spv-2 (closed circles), and a mixture of spv-1 and spv-2 (double circles). Bioassays were made for selected fractions (12, 14, 15, 16, 17, 18 and 20) from the mixture of spv-1 and spv-2 to determine the FED (closed square, insert).

Fig. 5. 'Curing' the SR-condition in ORWSR host flies injected with spv-1: first date of WSR lysis detection (open circles) and male appearance in fly progeny (closed circles). Arrow: see text for explanation.

However, rising lytic activity on the following days (Fig. 3), which reached an FED of 10^{-5} by day 6 and continued at that level to the conclusion of the experiment, demonstrated the reality of the virus, spv-2.

It is concluded that (1) spv-2 is associated with WSR spirochaetes maintained in Ore-R host flies in a latent condition, (2) spv-2 is 'induced' to multiply when spv-1 replicates in the same SR-spirochete, and (3) spv-2 is lytic to NSR.

(*c*) *Sucrose density-gradient centrifugation analysis*

Since spv-2 increased its activity in NSR, while spv-1 remained at the same titre, selective labelling of spv-2 with radioactive nucleic acid precursors should be possible. This was done using [^{3}H]thymidine (methyl-T) according to the procedures used for spv-1 labelling (Oishi & Poulson, 1970). Thus, both viruses contain DNA.

Fig. 4 shows the results of sucrose density-gradient centrifugation analysis of

the viruses. In order to compare spv-2 with spv-1, samples were prepared for both of these viruses separately. Spv-1 was labelled while it was replicating in WSR, and spv-2 while replicating in NSR. Three samples were analysed; the first tube contained only [^{3}H]thymidine-labelled spv-1, the second only labelled spv-2, and the third received a 1:1 mixture of spv-1 and spv-2, both labelled. Since the first few drops were lost in the course of fractionation in samples 1 and 2, fractions were counted from the last fraction and the curves in Fig. 4 were shifted accordingly.

It is clear that all the curves have the radioactive peak at the same point. The curve for the mixture of two factors (sample 3) has no noticeable shoulder, indicating that both viruses sediment at the same rate. Bioassays were made for seven selected fractions (12, 14, 15, 16, 17, 18 and 20) from sample 3 (Fig. 4): three fractions at the centre of the radioactive peak had a FED of 10^{-4}; fractions immediately adjacent to the peak on both sides showed an FED of 10^{-3}; and fractions further away from the peak showed a still smaller FED of 10^{-2}.

(ii) *SR-spirochaetes and SR-condition in* Drosophila

Studies of spv-2 revealed one important aspect of the problem of the specific male-killing effect of SR-spirochaetes on the host organism, namely that the

Table 2. *Effects of injecting a mixed population of spv-1 and spv-2 into flies, as determined by their progeny's sex-ratio (Fig. 6) and spirochaete transmission (Fig. 7)*

Flies injected with a mixed population of spv-1 and spv-2	Days after injection					
	0–2	3–6	7–9	10–12	13–15	16–18
ORNSR (19 flies)						
(1) No. progeny, female: male	195:0	941:0	521:0	391:2	131:1	60:0
(2) Sex-ratio (% female)	100·0	100·0	100·0	99·5	99·2	100·0
(3) No. progeny examined for SR	56	55	56	56	51	39
(4) No. progeny SR	56	33	22	3	1	0
(5) No. progeny haemolymph examined	19	51	44	45	47	36
(6) No. progeny carrying spirochetes	19	28	13	3	0	0
ORWSR (control, 13 flies)						
(1)	150:30	594:478	366:442	356:295	155:133	80:66
(2)	83·3	55·4	45·3	54·7	53·8	54·8
(3)	40	39	34	37	37	28
(4)	30	20	0	0	0	0
(5)	29	29	34	31	32	27
(6)	20	11	0	0	0	0
Ore-R (control, 15 flies)						
(1)	194:206	700:685	460:503	348:385	152:156	82:72
(2)	48·5	50·5	47·8	47·5	49·4	53·2

The mixed population of spv-1 and spv-2 was prepared by extracting lytic factors from ORWSR after lysis by spv-1. FED's of spv-1 and spv-2 in the extracts were 10^{-5} and 10^{-2} respectively.

effect may depend upon the lytic activity of the spirochaetal virus. Although spv-2 infects, multiplies, and lyses NSR, the elimination of NSR by lysis does not cure the host flies of the SR-condition. However, the situation is entirely different in ORWSR, where the lysis of WSR spirochaetes by spv-1 immediately leads to the production of male progeny.

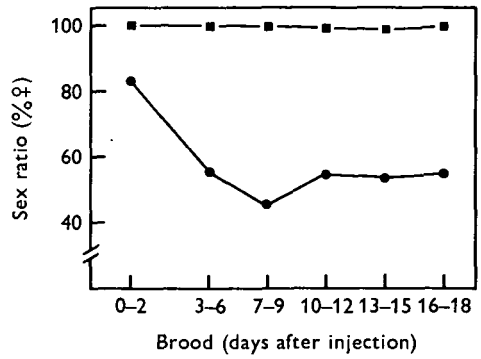

Fig. 6. Sex-ratios in progenies of ORNSR (squares) and ORWSR (circles) after lysis of their spirochaetes by a mixed population of spv-1 and spv-2 (see Table 2).

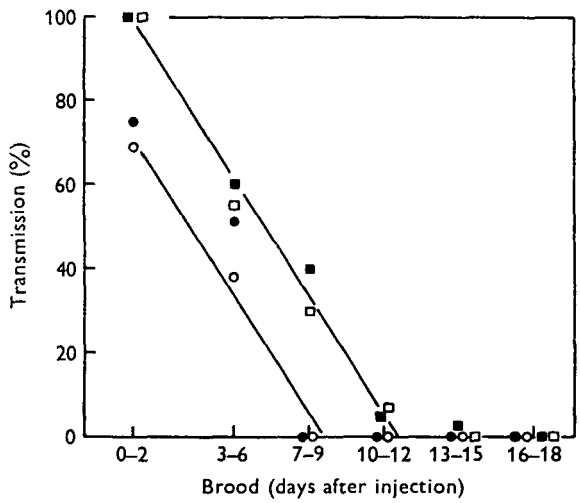

Fig. 7. Percentage transmission of SR-spirochaetes and of SR-condition after lysis of WSR (filled circles, SR-phenotypes; open circles, presence of SR-spirochaetes), and NSR (filled squares, SR-phenotype; open squares, SR-spirochaetes) (see Table 2).

(*a*) *Lysis of WSR and the SR-condition*

Fig. 5 shows the relationship between the elimination of WSR by lysis and the SR-condition in the fly host. Crude spv-1 suspension was serially diluted 10 times each up to 10^{-5} dilution. ORWSR flies were injected with each of these dilutions (0·15 μl each). Five flies for each dilution were mated and placed individually in a small culture bottle and transferred into new culture bottles every day. Sex-ratios of progeny flies were determined for single-day broods.

Other injected flies were kept in mass culture. Three or more flies were taken out every day and examined to see if they carried lysed SR-spirochaetes.

The first day when lysis was detected and the first day on which male progeny appeared were plotted against the log dilution (Fig. 5). The FED of the spv-1 suspension used was 10^{-4}. At this dilution, not all flies injected carried lysed SR-spirochaetes. Since only a small number of flies were examined daily for SR-spirochaetes in their haemolymph, it took longer to detect lysis in the 10^{-4} dilution (arrow in figure). Except for this point, lysis and the appearance of males after injection of various dilutions of spv-1 almost coincide, i.e. lysis leads immediately to the appearance of male progeny.

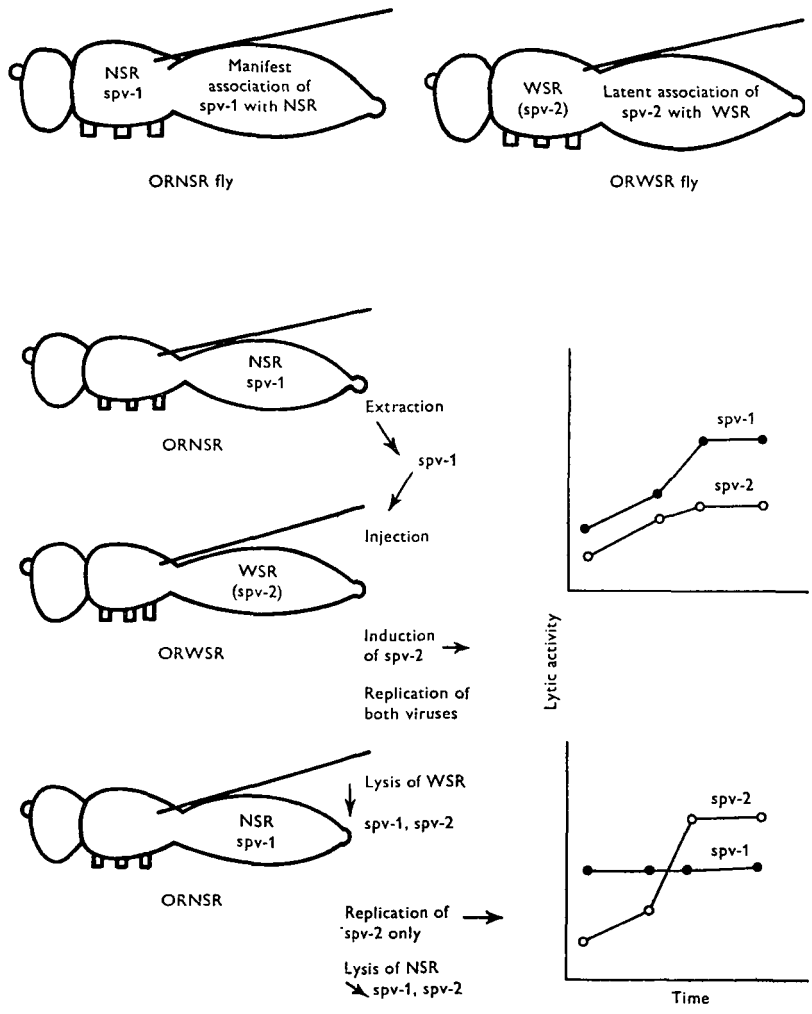

Fig. 8. A schematic representation of the spv-SR-spirochaete system.

(*b*) *Lysis of NSR and the SR-condition*

Effects of NSR-spirochaete elimination by spv-2 on the SR-condition in the fly host are shown in Table 2 and Figs. 6 and 7.

Extracts containing spv-2 were prepared by homogenizing ORWSR flies previously injected with spv-1. Such extracts contained 10^{-5} FED of spv-1 and 10^{-2} FED of spv-2. ORNSR flies were injected (0·15 μl each) with the extract thus prepared, mated, and placed individually into small culture bottles. Some of the injected flies were extracted, examined for lysed SR-spirochaetes, and returned to the cultures. Sex-ratios of progeny flies were determined according to the brood. A number of progeny flies were examined for the presence of SR-spirochaetes, and also for expression of the SR-condition when they were mated and their grandprogeny examined.

Table 3. *Summary of SR-spirochaetal virus characteristics*

Viruses	Spirochaete carrier strains	Conditions of association	Lytic to:	Inductive to:	Nucleic acid	Particle density in CsCl	Particle diameter (mμ)
spv-1	NSR	Manifest*	WSR	spv-2	DNA	1·480	50–60
spv-2	WSR	Latent*	NSR	—	DNA	?	50–60†

* Operationally defined as described in text.
† Oishi (1970).

Haemolymph examination showed that lysis of NSR was seen by the 6th day after the initial injection. Injected ORNSR flies, however, kept producing only female progeny, except for a few males which appeared in broods 4 and 5. Some progeny flies from early broods which carried SR-spirochaetes were tested for the reactions of SR-spirochaetes they carried to spv-2 and spv-1, and to NSR and WSR. All flies tested carried typical NSR, i.e. spirochaetes formed clumps with WSR but not with NSR, and were lysed by spv-2 but not by spv-1. Following lysis, however, female progeny contained no SR-spirochaetes and they all produced grandchildren in a 1:1 sex-ratio.

4. DISCUSSION

(i) *Viruses in SR-spirochaetes*

The results presented show that a virus exists in association with WSR-spirochaetes. Some characteristics of this virus, spv-2, are compared with those of spv-1 (Oishi & Poulson, 1970) in Table 3, and the spv-SR-spirochaete system revealed is shown schematically in Fig. 8. Spv-2 exists in a latent condition in WSR, 'latent' being operationally defined here to mean that the lytic activity is not detectable under the procedures employed, but becomes evident when spv-1 infects and multiplies in NSR. Although the lysis of NSR by spv-2 results in the appearance of both viruses, for spv-1 already exists in a manifest condition

in NSR (Oishi & Poulson, 1970), the spv-1 titre does not increase (Fig. 3). No induction of spv-1 takes place in NSR infected by spv-2. Clearly no cross-immunity exists between these two viruses. Spv-2 contains DNA and sediments on a sucrose-density gradient at the same rate as spv-1 (Fig. 4). Preliminary electron-microscopic studies show that spv-2 particles are indistinguishable from spv-1 (Oishi, 1970).

The manner in which these viruses exist in association with SR-spirochaetes remains to be studied. The words 'manifest' (Oishi & Poulson, 1970) and 'latent' (present report) are used only operationally and do not imply any particular physicochemical state. Admittedly the method for measuring viral activity used in these studies has limitations. It is quite possible that spv-2 exists in a manifest condition, but at such a low level the present assay system is not sensitive enough to detect it.

The significance of these viruses to the SR-condition of the spirochaete-bearing host flies is not known. None the less, they can be a convenient tool in eliminating SR-spirochaetes from their hosts in certain cases, as shown in the second half of the present study. It is of importance to note that all SR-spirochaete strains so far studied in any detail (eight strains) carry viruses (Poulson & Oishi, in preparation). What role, then, do the viruses play in the spirochaete-dependent SR-condition of the flies? Apparently mature viruses have no male-killing effects when injected into flies. A virus-free SR-spirochaete, if recovered from a natural population or induced in the laboratory, will provide answers to this question and also to the question of the manner in which the viruses are associated with their respective SR-spirochaetes.

(ii) *SR-spirochaetes and the SR-condition*

These results suggest that the male-killing agent normally associated with SR-spirochaetes does not require the persistence of SR-spirochaetes, at least in the case of NSR, to express its effect. The indication of this 'separability' of male-killing action from SR-spirochaetes was first clearly shown in WSR maintained in *D. robusta* hosts (Williamson, 1966). In this fly host, WSR maintains its number at a very high level throughout its life, and expresses complete SR-condition. However, SR-spirochaetes are transmitted into progeny flies only for a limited period during early broods. In later broods the SR-condition is still manifested although SR-spirochaete transmission is completely blocked. The conclusion is that the male-killing agent produced by spirochaetes in the female parent can penetrate and express itself in the absence of SR-spirochaete transmission as seen in later broods.

Based on these observations, a working hypothesis may be constructed as follows. (1) An exotoxin-like substance, *androcidin*, is produced by the SR-spirochaete and is actually responsible for the death of male fly zygotes; (2) the substance produced by NSR is stable or in large quantity, while the one produced by WSR is unstable or in smaller quantity, so that flies carrying NSR continue to express the SR-condition after lysis of their spirochaetes.

The idea of an exotoxin is not new in spirochaetes. Cases of exotoxin production

are known in *Leptospira*, a true spirochaete (Alexander *et al.* 1956; Russel, 1956; Imamura, Kuribayashi & Kameta, 1957). The question of whether or not SR-spirochaetes are true spirochaetes is dealt with elsewhere (Oishi & Poulson, 1970; Poulson *et al.*, in preparation; Williamson, in preparation), but it seems reasonable to assume they are.

In the present context it is essential to develop a bioassay system to measure *androcidin* in order to study the problem further. Such a system is being developed. Thus, it may be expected that if females are injected with haemolymph of NSR-carrying flies taken after the lysis of spirochaetes, they will produce only female progenies in early broods and progenies in a 1:1 sex-ratio in later broods due to the dilution of the androcidal substance. A preliminary experiment offers some support for this expectation. Search is being continued for a strain of *Drosophila* which is both highly susceptible to the action of *androcidin* and in which the above-mentioned bioassay method will work.

Analysis of the nature of the hypothetical substance, *androcidin*, will hopefully yield information which will relate to the biochemical control of sex-differentiation in *Drosophila*.

Part of a dissertation presented to the Faculty of the Graduate School of Yale University in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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