FURTHER STUDIES ON THE RIBOSOMAL RNA CISTRONS OF *SCIARA COPROPHILA* (DIPTERA)[1]

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ABSTRACT

Additional experiments with homologous as well as heterologous hybridization confirmed our previous finding in *Sciara coprophila* that *XX* females have nearly twice the number of ribosomal RNA cistrons as *X0* males. A comparison between two different *X'* chromosomes revealed that only the one carrying the irradiation-induced Wavy mutation has a deletion of 70% of its ribosomal RNA cistrons as compared to the standard X. The deletion is relatively stable, and the remaining ribosomal RNA cistrons do not appear to undergo disproportionate replication or magnification as in Drosophila. Homologous hybridization experiments revealed an unusually low reiteration of ribosomal RNA cistrons in this fly, 45 gene copies per *X* chromosome. The question is raised as to whether such a low number of cistrons may be related to the unusual nucleolar condition encountered in the Sciaridae.

THE polytene nuclei of most Diptera contain a prominent nucleolus. This is not the case in the Sciaridae (fungus gnats) where a well-formed nucleolus is lacking (POULSON and METZ 1938; KATO and SIRLIN 1963; SAUAIA, LAICINE and ALVES 1971). In those cells where nucleolar material can be identified, some of it—in both Rhynchosciara (BREUER and PAVAN 1955; MATTINGLY and PARKER 1968) and *Sciara coprophila* (GABRUSEWYCZ-GARCIA and KLEINFELD 1966)—is associated with an organizer located on one end of the *X* chromosome, and some of it is in the form of "micronucleoli". In their exacting study on *S. coprophila*, GABRUSEWYCZ-GARCIA and KLEINFELD (1966) found the micronucleoli to be associated with a group of bands scattered over all four chromosomes and constituting approximately 18% of the total band complement. To imagine that 18% of the genome thus functioned as nucleolus organizer seemed unlikely. With great interest, therefore, molecular hybridization was undertaken in order to

* This paper is dedicated to the memory of PROFESSOR C. W. METZ who died on June 5, 1975 in his 86th year. It was he who discovered what a remarkable cytological entity Sciara is.

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ABBREVIATIONS USED: rRNA = ribosomal RNA; rDNA = DNA containing rRNA cistrons; SSC = standard saline citrate: 0.15M NaCl, 0.015M sodium citrate, pH 7.0; EDTA = ethylene diaminetetraacetic acid.

identify the rDNA in this species: (1) by heterologous hybridization on filters the majority of rRNA cistrons was found to be on the *X* chromosome (GERBI 1971), and (2) by *in situ* hybridization these cistrons were localized in the proximal end of the *X*, and also in the micronucleoli, but *not* in the chromosomal bands with which the micronucleoli were associated (PARDUE, *et al.* 1970). In view of these results, one could imagine that the micronucleoli are part of a highly ramified nucleolus.

The present paper is a continuation of our studies on the rRNA cistrons of *S. coprohila* carried out in order to determine:

1) whether the unusual nucleolus encountered in these flies may be related to the degree of cistron reiteration. In order to determine the degree of reiteration, we have prepared Sciara rRNA of sufficient radioactivity for use in homologous rRNA saturation experiments.

2) whether the *X′* chromosome, which governs sex of progeny, is actually carrying a deleted number of rRNA cistrons, as our earlier data suggested (GERBI 1971). Previously we used an *X′* chromosome carrying the Wavy marker, and we wished to determine if the deletion was correlated with the Wavy marker or with the prime differential.

MATERIALS AND METHODS

A. PERSPECTIVE

1). *The* X′ *Chromosome*

In *S. coprophila* there are two genetic kinds of sex chromosomes known as *X* and *X′* (X-prime). Female-producers are *X′X* and male-producers are *XX*. For the present study two different *X′* chromosomes were employed: the one, designated X'_W, carries the sex-linked, *X*-irradiation-induced dominant wing marker Wavy, and is the same *X′* chromosome which has been used by Sciara students since 1931 (METZ and SMITH 1931). The other, designated *X′*, carries no visible sex-linked markers, and was derived from a wild female fly collected by DR. UZI NUR for one of us (H.V.C.) in Berkeley, California in 1966. Each of these *X′* chromosomes differs from the standard *X* by a long paracentric inversion (CROUSE 1960, and also unpublished observations). As in *S. impatiens* (CARSON 1946), all crossovers between the *X/X′* homologues within the region of the inversion are selectively eliminated as dicentric chromatids during oogenesis (unpublished cytological observations by H.V.C.).

Our previous experiments using heterologous hybridization had suggested that the X'_W chromosome was deficient in rRNA cistrons: whereas *XX* adult females contained approximately twice the number of rRNA cistrons as *X0* males, the number found in X'_WX females was only slightly higher than the *X0* male value (GERBI 1971). It was important, therefore, in our new studies, to be able to examine the number of rRNA cistrons in an *X′* chromosome which did not carry Wavy, and had not been exposed to high dosages of irradiation.

2). *The* X *chromosome*

The new experiments reported here also offer advantages in the sex-linked markers used for identifying the *X* chromosomes. Previously the separation of X'_WX *versus XX* female flies was made on the basis of Wavy wings *versus* wild-type straight wings. There is known to be overlap between Wavy and wild type, but the error in classification was kept at a minimum by using only those individuals whose wings were markedly Wavy. In our new experiments two different recessive *X*-chromosome markers were used, either swollen (sw) (METZ and ULLIAN 1929) of petite (p) (CROUSE 1961). Petite wings are vestigial-like and are 100% classifiable, and swollen wing venation is nearly so. DNA was extracted from flies of the following genotypes:

X'X females: X'_WX, X'_WX_p, $X'X_p$ $X'X_{sw}$; *XX* females: *XX*, $X_{sw}X_{sw}$, X_pX_p; and *X0* males: *X0* and X_p0.

By comparison of the rRNA saturation values we could determine whether the wild-type *X'* chromosome as well as the X'_W carried the rDNA deficiency.

B. NUCLEIC ACID PURIFICATION

1). *DNA purification*

Radioactive ^{14}C-thymidine *S. coprohila* were grown as previously described (GERBI 1971), and these radioactive flies were pooled with non-radioactive flies of the same phenotype, and stored at —20°. The frozen flies were ground with a chilled mortar and pestle, and then homogenized in 0.1 M EDTA, 0.15 M NaCl, pH 8.0 (MARMUR 1961). DNA was extracted by the method of MARMUR (1961) and enzymatically purified as described earlier (GERBI 1971). After enzyme treatment, the solution was re-extracted with 24:1 chloroform-isoamyl alcohol (v/v), and the aqueous layer centrifuged in 0.1 × SSC at 35,000 rpm and 15° in a Beckman Ti-50 or Ti-60 angle rotor overnight. The resulting pellet was gently dissolved in 0.1 × SSC and stored with a drop of chloroform at 4° until further use. The specific activity of the DNA was 20 cpm/μg.

2). *RNA Purification*

^{3}H-uridine-labelled RNA from a *Xenopus laevis* kidney tissue culture line was extracted with phenol as previously described (GERBI 1971), and the 28S and 18S rRNA fractions from a 5–20% sucrose gradient were pooled. The specific activity was 250,000 cpm/μg.

Radioactive *S. coprophila* RNA was prepared by feeding radioactive yeast to the larvae, as originally suggested by DR. K. C. ATWOOD. A yeast haploid spore culture, XV104–2D, requiring both uracil and adenine was generously provided by DR. R. C. VON BORSTEL. Radioactive yeast were grown at 22° for 85 hours in a medium containing 0.67% yeast nitrogen base without amino acids (Difco), 2% dextrose, 0.002% adenine and 10 μCi/ml ^{3}H-adenine (Schwarz, 22.6 Ci/mM), 0.002% uracil and 40 μCi/ml ^{3}H-uridine (Schwarz, 20 Ci/mM). The cells were then centrifuged for 20 minutes at 12,100 g, resuspended in water, and lyophilized. *S. coprophila* larvae were fed this yeast from the time of hatching until mid-fourth instar, when their RNA was extracted as described earlier (GERBI 1971). The specific activity of this rRNA was 30,000 cpm/μg.

C. MOLECULAR HYBRIDIZATION ON FILTERS

Radioactive homologous or heterologous rRNA was hybridized with about 50 μg *S. coprophila* DNA baked on a 0.45 μ nitrocellulose filter (Sartorius from Science Essentials, Anaheim, California; n.b.: other brands of filters do not have quite as good DNA retention, nor do they wet as well, and they are rather brittle), as described earlier (GERBI 1971). Over 90% of the DNA remained on the filter at the end of the hybridization, as judged by ^{14}C-thymidine counts. The hybridization data represents an accumulation of points obtained from separate experiments done in New Haven, Connecticut; Tübingen, West Germany; and Providence, Rhode Island. Replicate points fell along the same curve despite geographical and temporal distance between experiments. Saturation values were corrected by 10% for male-derived DNA since male diploid somatic cells contain only seven chromosomes and not eight as in female flies; the sex chromosome complement of male germ cells is *XX* but is insignificant in amount to warrant additional correction (for further discussion of this point see GERBI 1971).

RESULTS

We have previously shown that the DNA sequences of *Sciara coprophila* which bind *Xenopus laevis* rRNA band at a buoyant density in neutral CsCl corresponding to a G+C value of 44%. The data presented here extend this observation to homologous hybrids (Figure 1). *S. coprophila* DNA was centrifuged to equilibrium in a CsCl gradient, and half of each fraction was hybridized with

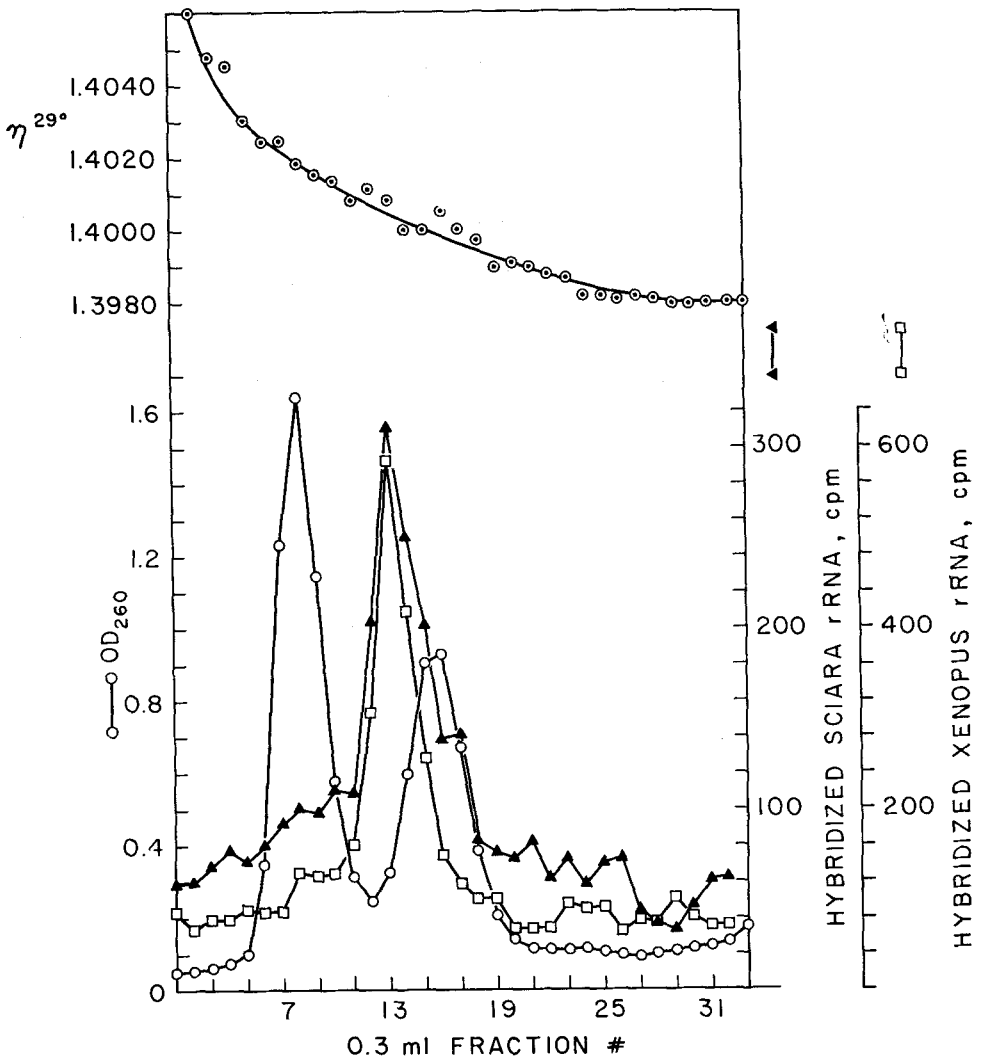

FIGURE 1.—rRNA hybridization of *S. coprophila* DNA fractions from a CsCl gradient. *S. coprophila* DNA was centrifuged in CsCl-0.01M tris, 0.01M EDTA, pH 7.7 at a mean buoyant density of 1.70 gm/cc for 60 hours at 20° and 35,000 rpm in a Beckman Ti-60 rotor. Fractions were collected and the refractive index (⊙) and absorbance at 260 nm (○) determined. *S. coprophila* DNA is located in fractions # 13–18 ($\rho = 1.697$ gm/cc) and marker bacterial DNA is in fractions # 6–10 ($\rho = 1.720$ gm/cc). Each fraction from the gradient was divided in half, denatured by 0.1N NaOH, neutralized, and loaded on nitrocellulose filters. One set of filters was hybridized with ^{3}H-*S. coprophila* rRNA (▲), and the second set of filters was hybridized with ^{3}H-*X. laevis* rRNA (□).

radioactive *X. laevis* rRNA while the other half of each fraction was hybridized with radioactive *S. coprophila* rRNA. It can be seen that the same fractions of DNA, located at the denser side of the main band, hybridize with both heterologous and homologous rRNA. Earlier competition hybridization experiments

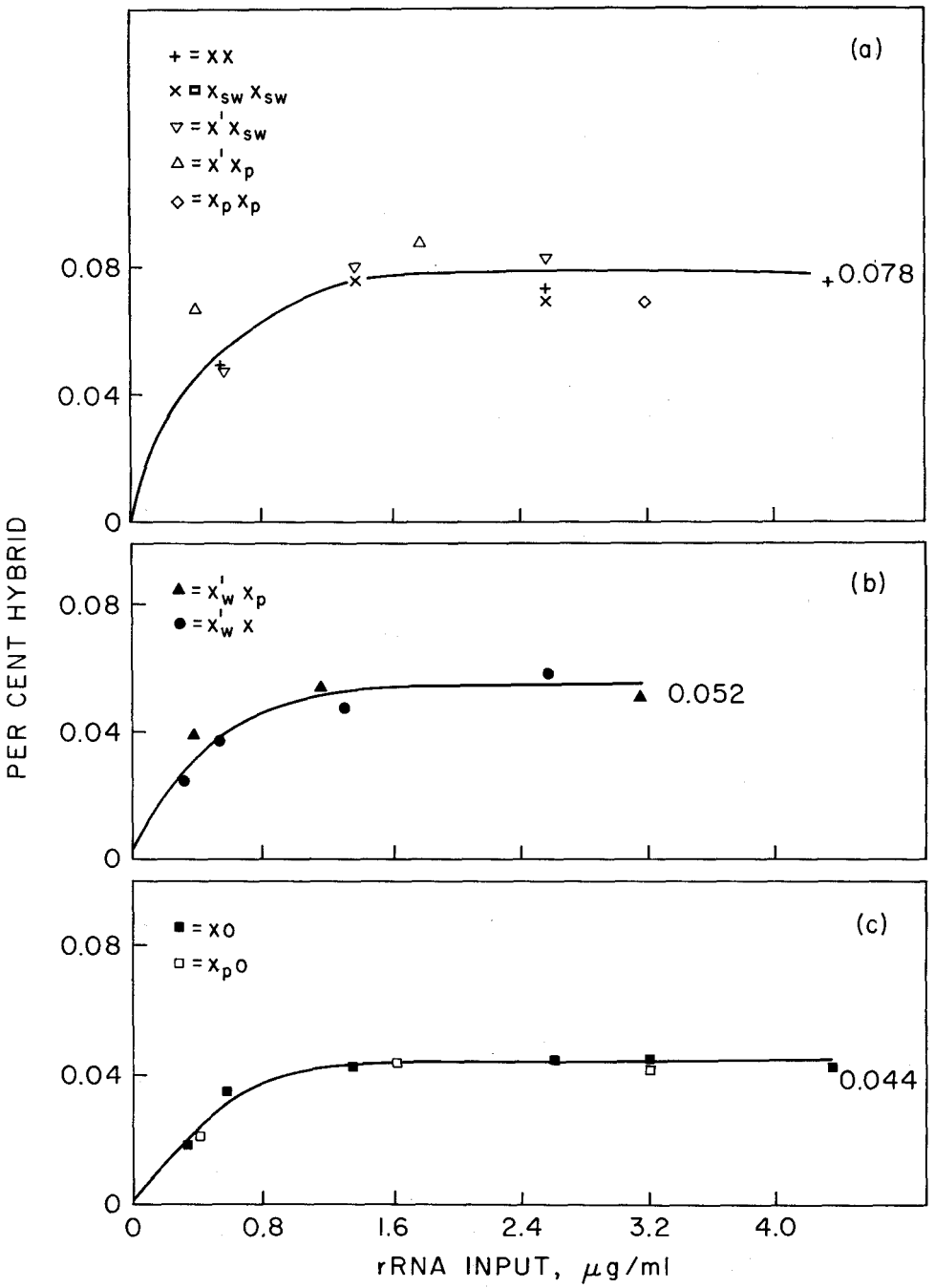

FIGURE 2.—Homologous hybridization of different *S. coprophila* DNAs. *S. coprophila* DNA from a variety of stocks of *XX* females, *X'X* females, and *XO* males (see KEY on Figure) was loaded in nitrocellulose filters and hybridized to saturation with ^{3}H–*S. coprophila* rRNA as described in METHODS.

had shown that the DNA sequences which bind heterologous rRNA are the same as those which bind homologous rRNA (GERBI 1971).

Our previous saturation experiments using *X. laevis* rRNA demonstrated that the X'_W chromosome carries a 60% deletion in rDNA (GERBI 1971). Since, on rare occasions, conclusions from heterologous hybridization are not validated when repeated with homologous rRNA (GAMBARINI and LARA 1974), we extended our previous experiments to the homologous rRNA system. Figure 2 shows that DNA from X'_WX female flies hybridizes with homologous rRNA 1.30 times as well as DNA from *XO* males; whereas DNA from *XX* females binds 1.94 times as much homologous rRNA as the DNA from *X0* males. Table 1 summarizes the ratios of homologous saturation values which are in striking agreement with the ratios of the heterologous saturation values. This confirms our deduction from heterologous rRNA hybridizations that the X'_W chromosome must have a substantial deletion of rRNA cistrons. This is also in accord with our previous observation from *in situ* hybridization with rRNA which showed asymmetry of silver grains over the X'_WX homologues (GERBI 1971).

We wished to know whether the deletion of rRNA cistrons on the X'_W chromosome was associated with the Wavy mutant. As stated above, we were fortunate to have available for study a wild-type X' chromosome which lacked Wavy. The results of numerous hybridization experiments using DNA from the various stocks of flies and heterologous rRNA from *X, laevis* or homologous rRNA from *S. coprophila* (Figure 2) show that the X' chromosome lacking Wavy has the same rRNA hybridization ability as the *X* chromosome. Table 1 summarizes these findings which (1) confirm that the X'_W chromosome has about one-third of the repeated rRNA cistrons found in the *X* chromosome and (2) demonstrate that the wild-type X' chromosome is not deficient at all.

DISCUSSION

A. HOMOLOGOUS RRNA SATURATION VALUE

The absolute number of rRNA cistrons in *S. coprophila* can be calculated from

TABLE 1

rRNA saturation values

Genotype	*X. laevis* rRNA	*S. coprophila* rRNA
XX	.023 ± .004	.072 ± .001
X'X	.023 ± .001	.083 ± .002
X'_WX	.014 ± .0005	.052 ± .002
XO	.011 ± .0004	.044 ± .0005
XX/XO	2.29	1.80
X'X/XO	2.29	2.08
X'_WX/XO	1.40	1.30

The mean values for percent saturation of *S. coprophila* DNA ± the standard error are reported above. The *X* chromosome may either be wild type or carry the markers swollen or petite. The ratios of saturation values have been corrected by 10% for males (see METHODS).

the homologous rRNA saturation value of 0.078% for DNA from *XX* female flies. The haploid DNA content of *S. coprophila* is 2.0×10^{-13} gm DNA or 1.2×10^{11} daltons (RASCH 1970b). Thus, there are 9.35×10^{7} daltons of haploid DNA complementary to rRNA. We estimate *S. coprophila* 18S + 28S rRNA to be about 2.1×10^{6} daltons; this is a value determined for other Diptera (LOENING 1968; RUBENSTEIN and CLEVER 1971; SERFLING, PANITZ, and WOBUS 1972). Thus, there are 45 rRNA cistrons in one *X* chromosome of *S. coprophila*. This is five-fold less than the 250 rRNA gene copies found in a single nucleolus organizer of *Drosophila melanogaster* (RITOSSA, *et al.* 1966; TARTOF 1971; SPEAR and GALL 1973). A value of 45 rRNA cistrons in *X0* male *S. coprophila* is one of the lowest rDNA contents known for a eukaryote. A case of even lower rRNA cistron redundancy is the micronucleus of the protozoan *Tetrahymena pyriformis* with fewer than 20 gene copies (YAO, KIMMEL and GOROVSKY 1974). In other Diptera, however, such a small number of rRNA cistrons probably would be lethal. For example, viable bobbed mutants of *D. melanogaster* with less than 80 rRNA gene copies have never been found (K. TARTOF, personal communication). It is difficult to imagine technical errors in our experiments which would give such a low homologous saturation value. One remote possibility might be that the multiple tandem rRNA cistrons are linearly arranged as reverse repeats as in Tetrahymena (K. KARRER and J. GALL, personal communication); upon denaturation these could snap back into double-stranded hairpin loops which would not bind to nitrocellulose filters nor be available for rRNA hybridization.

Sciara does not have a well-formed nucleolus. Salivary gland cells sometimes have loosely organized nucleolar material at the end of the *X* chromosomes, and additionally sometimes have "micronucleoli" which contain rDNA (PARDUE *et al.* 1970). One might ask whether these "micronucleoli" are correlated with the low rRNA cistron redundancy found in this fly, since several observations suggest a correlation between partially deleted rDNA and morphologically aberrant nucleoli. For example, the blastoderm of the lethal anucleolate mutant of Chironomus does not organize a normal nucleolus, but contains instead 3–5 "pseudonucleoli" (BEERMAN 1960). Similarly, anucleolate mutants of *X. laevis* have 0–8 micronuclear "blobs" (ESPER and BARR 1964), and partial nucleolar hemizygotes in *X. laevis* carrying rDNA deletions may have 1–4 very small nucleolar-like bodies (MILLER and GURDON 1970, MILLER and KNOWLAND 1970, 1972.) Micronucleoli are found also in the microspores of *Zea mays* deficient for the nucleolus organizer, as well as those in which the nucleolus organizer has been translocated or duplicated (MCCLINTOCK 1934). Thus, anucleolate mutants fail to organize a normal nucleolus. It is tempting, but still premature, to consider that the Sciarid micronucleoli are a result of low rRNA cistron redundancy.

In *D. melanogaster* males which are *X0* undergo disproportionate replication of rRNA cistrons such that the adult males have 0.8 times the *XX* female rRNA cistron value (TARTOF 1971). It has been proposed that this disproportionate increase occurs by independent polytenization of the nucleolus organizer region (SPEAR and GALL, 1973). An independent control of rDNA replication is possible because it has previously been shown that the polyteny level of rDNA lags be-

hind the polytene class level of euchromatic regions (HENNIG and MEER 1971; SPEAR and GALL 1973). It is unclear in the Sciaridae whether the rDNA level of polytene chromosomes has kept pace with the replication class of euchromatin (GERBI 1971; GAMBARINI and LARA 1974); a tight coupling of the two could explain why *X0* males of *S. coprophila* do not show compensatory rDNA replication.

B. DELETION OF rRNA CISTRONS

The data reported here show that the *X'* chromosome carrying Wavy has a deletion of two-thirds of the rRNA cistrons. The *X'* chromosome without Wavy has the same number of rRNA cistrons as the standard *X* chromosome, indicating that the deletion of rDNA is specific for the X'_W chromosome and therefore the prime differential mechanism which governs sex of progeny in Sciara is not coupled with rDNA amounts.

D. melanogaster bobbed mutants which have reduced rDNA content develop slower than wild-type flies. However, the opposite seems to be true in *S. coprophila*, since *X0* males develop faster than X'_WX females which in turn develop faster than *XX* females (Table 2 of RIEFFEL and CROUSE 1966; Figure 1 of RASCH 1970a; and S. GERBI general observations).

The Wavy mutation in *S. coprophila* is very stable, and has been kept in laboratory stock since 1930 without reversion to wild type. This is in contrast to the bobbed mutants of *D. melanogaster* which are highly unstable, and which may undergo magnification when coupled with an rDNA-deficient homologue (RITOSSA 1968). In addition TARTOF (1973) has calculated that magnification should not be possible when less than 75 rRNA cistrons are present in an rDNA-deficient chromosome, and both RITOSSA (1973) and TARTOF (1974) have correlated magnification with crossing-over. Hence, it may be argued that magnification is absent in *S. coprophila* since the X'_W chromosome has only about 14 rRNA gene copies ($30\% \times 45 = 14$), and crossovers between the *X* and *X'* chromosomes of Sciara are eliminated by dicentric formation. Moreover, it should be noted that $X'_WX'_W$ *and* X'_W0 individuals are unknown in Sciara, and these are the genotypes which would be analogous to those in *D. melanogaster* which display magnification.

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