

LOCALIZATION OF RIBOSOMAL DNA WITHIN  
OOCYTES OF THE HOUSE CRICKET,  
*ACHETA DOMESTICUS* (ORTHOPTERA: GRYLLIDAE)

MAC DONALD CAVE

From the Department of Anatomy and Cell Biology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15213

ABSTRACT

A large DNA-containing body is present in addition to the chromosomes in oocytes of the house cricket *Acheta domesticus*. Large masses of nucleolar material accumulate at the periphery of the DNA body during the diplotene stage of meiotic prophase I. RNA-DNA hybridization analysis demonstrates that the genes which code for 18S and 28S ribosomal RNA are amplified in the ovary. *In situ* hybridization indicates that the amplified genes are localized within the DNA body of early prophase cells. As the cells proceed through diplotene the DNA which hybridizes with ribosomal RNA is gradually incorporated into the developing nucleolar mass.

INTRODUCTION

Specific amplification of the genes which code for 18S and 28S ribosomal RNA (rRNA) was first described in amphibian oocytes (Brown and Dawid, 1968; Gall, 1968). Conceptually, the process involves extensive replication of a portion of the genome containing the genes which code for rRNA in the absence of replication of the remainder of the genome. In *Xenopus laevis* the amplification process continues into the pachytene stage of meiotic prophase I, when a large cap of Feulgen-positive material is visualized at one end of the nucleus. The pachytene nucleus contains in addition to 12 pg of chromosomal DNA approximately 30 pg of DNA in the nuclear cap (MacGregor, 1968). That the nuclear cap contains the amplified ribosomal genes has been shown by the technique of RNA-DNA hybridization *in situ* (Gall and Pardue, 1969; John et al., 1969).

Large masses of Feulgen-positive material are present in addition to the chromosomes within the oocyte nuclei of various species of insects (Bauer,

1933; Bayreuther, 1956; Urbani, 1969). Such a structure, referred to as the DNA body, is present in oocytes of the house cricket, *Acheta domesticus* (Nilsson, 1966). The DNA body is limited to the oocytes and is not present in the nuclei of somatic cells. Microphotometric determinations made on the nuclei of Feulgen-stained pachytene stage cells show that the DNA body is present in addition to chromosomal DNA and accounts for approximately 25% of the DNA in the pachytene stage oocyte (Cave and Allen, 1969 *a*). During diplotene large masses of RNA-containing material accumulate at the periphery of the DNA body (Heinonen and Halkka, 1967; Lima-de-Faria et al., 1968). Electron microscope examination indicates that the RNA-containing material associated with the body is characteristically nucleolar in appearance, the granular elements measuring 200–250 Å in diameter (Allen and Cave, 1968; Cave and Allen, 1969 *b*; Kunz, 1969). Treatment with actinomycin D causes a typical segregation of components into

granular and fibrillar elements (M. D. Cave and E. R. Allen, unpublished results).

Previous studies carried out by Lima-de-Faria et al. (1969) have demonstrated the amplification of a heavy satellite DNA in ovaries of *Acheta*. The satellite DNA, a portion of which hybridizes with rRNA, accounts for approximately 14% of the DNA in ovaries of young animals, and approximately 0.8% of the DNA in testes. Like the satellite DNA the genes which code for rRNA are amplified during oogenesis. The close association of the DNA body with the developing nucleoli suggests that the body may contain the amplified genes coding for rRNA. *In situ* hybridization with labeled 18S and 28S rRNA shows that this is the case.

## MATERIALS AND METHODS

### Preparation of DNA

DNA was extracted from various tissues of male and female nymphs of the house cricket, *A. domestica*. DNA was prepared from the testes of 4–6-wk old (2–3 instar nymphs, 75–100 mg body weight) and 8–10-wk old (3–4 instar nymphs, 125–175 mg body weight) male animals according to the method of Klett and Smith (1968). DNA was prepared from the legs and from leg muscle of both males and females at various stages of development, and from the ovaries of 2–3-wk old (1–2 instar nymphs, 20–30 mg body weight) and 6–10-wk old (3–4 instar nymphs, 125–175 mg body weight) females. Organs of approximately 200 animals were dissected and immediately frozen on dry ice. They were homogenized for 5 min with a motor-driven glass-Teflon homogenizer in 5 ml 0.1 M Tris buffer (pH 7.4), 0.05 M ethylenediaminetetraacetate (EDTA), and 0.5% sodium dodecyl sulfate (SDS). The homogenate was made to a concentration of 1% in pronase and digested for 3 hr at 37°C while stirring. The tissue was then extracted with an equal volume of water-saturated phenol for 30 min with stirring. The aqueous layer was made to a concentration of 0.2 M in sodium acetate and the nucleate precipitated with ethanol. After several washes, the pellet was suspended in 5 ml 0.1 × 0.15 M NaCl and 0.015 M trisodium citrate (SSC) and digested with pancreatic ribonuclease (RNase P) (100 µg/ml) for 3 hr at 37°C, and then subsequently with pronase (1 mg/ml) for an additional 3 hr. DNA was precipitated with ethanol and wound onto a glass rod.

DNA was routinely banded by preparative ultracentrifugation in CsCl. Approximately 0.2–1 mg of DNA ( $A_{260} = 35$  µg DNA) was diluted to 5 ml in CsCl solution which was brought to density of 1.70 g/cm<sup>3</sup> and centrifuged for 60 hr, 20°C, 35,000 rpm, in the Spinco Ti 50 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). In some cases

0.05–0.25 mg of *Micrococcus lysodeikticus* DNA (density = 1.731 g/cm<sup>3</sup>) served as marker. Individual fractions were collected and brought to 1 ml with  $0.1 \times$  SSC.

### Preparation of RNA

Ribosomes were extracted from whole crickets according to the method of Kaulenas (1969). The ribosomes were suspended in 0.01 M Tris buffer, pH 7.4, containing 0.01 M KCl, 0.01 M MgCl<sub>2</sub>, and 1% SDS, and extracted with an equal volume of water-saturated phenol for 30 min (40°C) on a magnetic stirrer. The aqueous layer was made to a concentration of 0.2 M in sodium acetate, and the nucleate precipitated with ethanol. The precipitate was layered on 15–30% sucrose gradients and centrifuged (20°C) for 18 hr at 112,500 g in the Spinco SW-27 rotor. The 18S and 28S fractions of the gradients were collected separately and precipitated with ethanol. Subsequently, the RNA was purified with cetyltrimethylammonium bromide according to the procedure of Bellamy and Ralph (1968). Then 1 part 18S RNA was combined with 2 parts 28S RNA (w/w), and the reconstituted RNA was digested for 20 min at room temperature with 50 µg/ml electrophoretically purified DNase in 10<sup>-3</sup> M MgSO<sub>4</sub> brought to pH 7.0 with Na<sub>2</sub>HPO<sub>4</sub>. Subsequently, DNase was removed from the solution by means of the phenol procedure described previously, and the RNA precipitated with cetyltrimethylammonium bromide (Bellamy and Ralph, 1968). The purified RNA was made 0.2 M in sodium acetate, and passed through Sephadex G-25.

*Acheta* rRNA was made radioactive by means of ultraviolet photostimulated incorporation of <sup>3</sup>H from NaB<sup>3</sup>H<sub>4</sub> into RNA (Kirkegaard, 1968, as described by Vincent et al., 1969). 1 mg ( $A_{260} = 50$  µg RNA) purified 18S and 28S RNA was suspended in Tris buffer at pH 8.5, containing 100 mCi NaB<sup>3</sup>H<sub>4</sub> (15 Ci/mole), and irradiated at a distance of 1 cm for 10 min with a Mineralight UVS 12 illuminator (Ultra-Violet Products, Inc., San Gabriel, Calif.) with constant stirring. The reaction was stopped by the addition of several drops of cyclobutanone, and the radioactive cyclobutanone removed by repeated ether extraction. After precipitation with cetyltrimethylammonium bromide the RNA was passed through Sephadex G-25 and precipitated with ethanol. The specific activity of the resulting RNA was 9100 cpm/µg.

To obtain rRNA of higher specific activity than that available from *Acheta*, labeled 18S and 28S RNA was prepared by growing cell cultures in medium containing uridine-<sup>3</sup>H. A cell line of *X. laevis* kidney cells originally established by K. A. Rafferty, Jr. (line CCL 102A obtained from the American Type Culture Collection, Rockville, Md.) was grown in modified

Leibovitz L-15 medium with 15% fetal calf serum. Cells at low density were grown for 3–5 days in medium containing 25  $\mu\text{Ci/ml}$  uridine-5- $^3\text{H}$  (specific activity 29.2 Ci/mmol), and then for an additional 24 hr in two changes of fresh medium containing unlabeled uridine. Subsequently, RNA was extracted from the cells by a modification of the phenol procedure and 18S and 28S RNA isolated on sucrose gradients as described previously. rRNA was reconstituted by combining 1 part 18S RNA with 2 parts 28S RNA (w/w). The RNA was purified by means of cetyltrimethylammonium bromide precipitation (Bellamy and Ralph, 1968) and stored frozen in 0.01 M Tris, pH 7.4.

### DNA-RNA Hybridization on

#### Nitrocellulose Filters

A modification of the membrane filter technique was utilized (Gillespie, 1968). DNA in pooled or individual fractions of cesium chloride gradients was denatured by addition of NaOH to 0.2 M. Denaturation was monitored by measurements of hyperchromicity ( $A_{260}$  m $\mu$ ). After denaturation the solution was neutralized with HCl and Tris buffer (pH 7.5) and brought to 4  $\times$  SSC. Samples were allowed to pass through Millipore HAWP filters (Millipore Corp., Bedford, Mass.) by gravity filtration. Each filter was washed with 50 ml 4  $\times$  SSC with suction, blotted, and dried at room temperature for 2 hr and subsequently at 70°C for 16 hr.

Hybridization was carried out in 15-ml stoppered culture tubes. Several filters were placed in tubes containing 1–10 ml RNA-containing medium (4  $\times$  SSC, 0.4% SDS). At least 0.5 ml was used per filter with a minimum volume of 1.0 ml. The filters were hybridized for 16 hr at 68°C. Then they were extensively washed with 2  $\times$  SSC and digested for 1 hr (room temperature) with 100  $\mu\text{g/ml}$  RNase P and 5 units/ml RNase T<sub>1</sub> on a wrist action shaker. After RNase treatment the filters were washed thoroughly with 2  $\times$  SSC, blotted, and dried at 70°C for counting. The amount of RNA bound to the filters was determined by counting the filters in 10 ml of toluene 2,5-diphenyloxazole and 1,4-bis-[2-(5-phenyloxazolyl)]benzene (PPO-POPOP) scintillation fluid in a Beckman DPM 100 scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.) for 100 min, or to 1.0% SE.

The amount of DNA on the individual filters was determined subsequently by washing the filters in three changes of chloroform. The filters were dried and then hydrolyzed at 70°C for 30 min in 2 ml 5.0% perchloric acid. DNA was determined by measuring the  $A_{260}$  of the hydrolysate or by the diphenylamine procedure of Burton (1968). In both cases calf thymus DNA served as standard.

### In Situ Hybridization

A modification of the *in situ* hybridization procedure was employed (Gall and Pardue, 1969; Wimber and Steffenson, 1970). Squash preparations and paraffin sections of 3:1 alcohol-acetic acid-fixed ovaries were prepared as described previously (Cave and Allen, 1969 a). The slides were hydrated and then digested for 2 hr with 100  $\mu\text{g/ml}$  RNase P in 2  $\times$  SSC. After washing in 2  $\times$  SSC, DNA was denatured by treating the slide for 2.5 hr at 65°C in 95% formamide in 0.1  $\times$  SSC or by dipping them in 0.07 M NaOH for 2 min at room temperature. Subsequently, the slides were washed in 0.1  $\times$  SSC, passed through an ethanol series, and air-dried.

100  $\mu\text{l}$  of 6  $\times$  SSC containing 2  $\mu\text{g/ml}$  rRNA (1 part 18S to 2 parts 28S [w/w]) was placed on a slide and covered with a cover slip. The slide was placed in a moisture chamber and incubated 15 hr at 68°C. The cover slip was removed from the slide which was rinsed in several changes of 6  $\times$  SSC. The slides were digested for 1 hr at 37°C with 20  $\mu\text{g/ml}$  RNase P in 2  $\times$  SSC, washed in 2  $\times$  SSC, run through an ethanol series to 95% ethanol, and air-dried.

Slides were coated with Kodak NTB-2 emulsion (1 part emulsion to 2 parts water) (Eastman Kodak Co., Rochester, N. Y.) and exposed for 2–8 wk in light-tight boxes. The slides were developed in Kodak D-19 developer 2 min, 18°C, fixed, and stained through the emulsion with 0.5% toluidine blue at pH 9.0.

In some cases, the silver grains were removed from the radioautographs according to the method described by Bianchi et al. (1964), stained according to the Feulgen procedure, and the slides subsequently stained with 1.0% fast green dye.

### RESULTS

Fig. 1 shows a profile of a CsCl gradient of *Acheta* ovarian DNA. The main band has a buoyant density of approximately 1.70 g/cm<sup>3</sup>. The rRNA of both *Acheta* and *Xenopus* hybridizes with DNA, the buoyant density of which is approximately 1.715 g/cm<sup>3</sup>.<sup>1</sup> Similar results are obtained when *Xenopus* or *Acheta* rRNA is hybridized with DNA derived from various tissues of *Acheta* (Fig. 2). In all cases, the labeled rRNA hybridizes with DNA, the buoyant density of which is approximately 1.715 g/cm<sup>3</sup>.

In order to determine what portion of the genome of *Acheta* hybridizes with rRNA, filters containing whole *Acheta* DNA were hybridized in

<sup>1</sup>The term ribosomal RNA (rRNA) is used here and subsequently to denote RNA prepared by recombining 1 part 18S RNA with 2 parts 28S RNA (w/w).

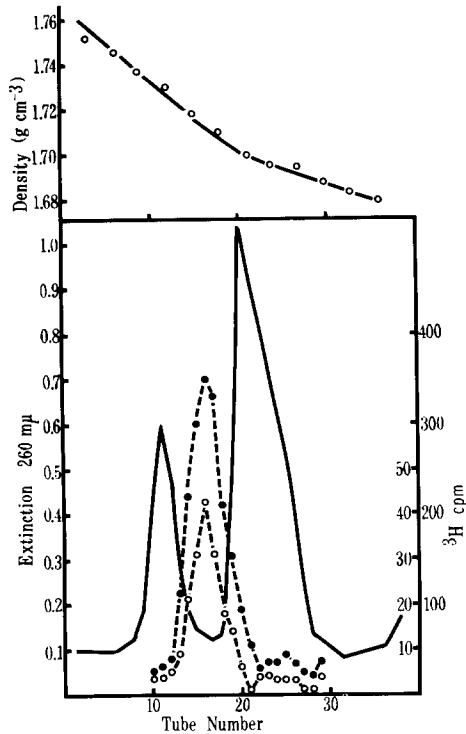


FIGURE 1 CsCl gradient of DNA from ovaries of *Acheta domestica* hybridized with *Xenopus* and *Acheta* ribosomal 18S and 28S RNA. After centrifugation to equilibrium each fraction of the gradient was diluted to 1.0 ml with  $0.1 \times$  SSC, divided into two equal parts, denatured, and bound to filters as described in Materials and Methods. One part of each fraction was hybridized with the labeled RNA of *Acheta* (sp act 9100 cpm/ $\mu$ g) (○); the other was hybridized with RNA of *Xenopus* (sp act 169,700 cpm/ $\mu$ g) (●). Densities were calculated from the refractive index of individual fractions (Ifft et al., 1961). DNA of *Micrococcus lysodeikticus* ( $1.731 \text{ g/cm}^3$ ) served as marker.

medium containing increasing concentrations of *Xenopus* rRNA (Fig. 3). Approximately 0.004% of the DNA derived from legs of male or female crickets hybridizes with rRNA. Similarly, approximately 0.004% of the DNA from testes hybridizes with rRNA. Identical values were obtained with DNA extracted from the testes of young (2–3 instar) and old (3–4 instar) animals. Approximately 0.02% of the DNA in ovaries of 1st and 2nd instar female crickets hybridizes with rRNA, and approximately 0.01% of the DNA in ovaries of 3rd and 4th instar crickets hybridizes with rRNA (Fig. 3). In somewhat older animals (5 instar), 0.007% of the DNA was found to hybridize with rRNA. The relative decrease in the per cent of the

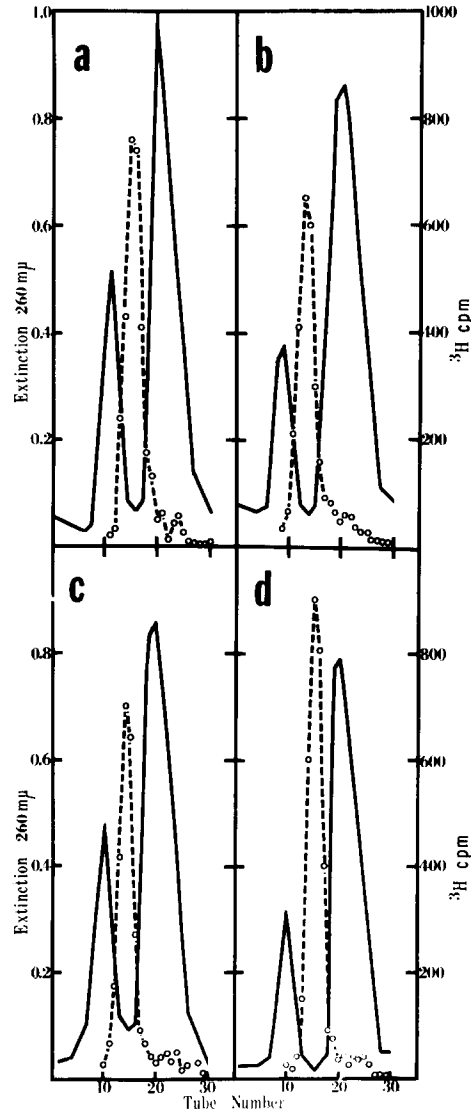


FIGURE 2 DNA from (a) male legs, (b) female legs, (c) testes, and (d) ovaries of *Acheta* centrifuged to equilibrium in CsCl and hybridized with the rRNA of *Xenopus* (sp act 169,700 cpm/ $\mu$ g). Each fraction was diluted to 1.0 ml with  $0.1 \times$  SSC, denatured, bound to filters, and hybridized as described in Materials and Methods. DNA of *Micrococcus lysodeikticus* ( $1.731 \text{ g/cm}^3$ ) served as marker.

genome hybridizing with rRNA with increasing age is best accounted for by a relative increase in the number of somatic cells in the ovary which do not appear to amplify the genes which hybridize with rRNA (see results of *in situ* hybridization experiments). Table I shows that although the num-

ber of oocytes within the ovary remains approximately the same throughout the developmental stages investigated, the amount of DNA in the ovary increases. The oocytes have completed DNA synthesis before the pachytene stage and contain a total amount of DNA equivalent to 5.5 C (Cave and

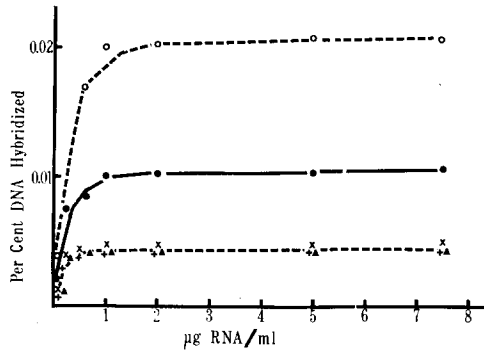


FIGURE 3 Determination of the hybridization saturation level between *Xenopus* ribosomal RNA and DNA prepared from male legs (▲), female legs (×), testes (+), and ovaries of young (20–30 mg body weight, 1–2 instar) animals (○), and old (150–175 mg body weight, 3–4 instar) animals (●). DNA was prepared and purified by centrifugation in CsCl as described in Materials and Methods. Unfractionated DNA was reconstituted by pooling all of the gradient fractions. After denaturation 25 µg of DNA was bound to each filter. Hybridization was carried out with increasing concentrations of *Xenopus* rRNA of sp act 169,700 cpm/µg. The amount of DNA on the filters was determined by the diphenylamine procedure. Retention of DNA during hybridization was about 95%.

Allen, 1969 a). The increase in DNA content of the ovary, therefore, reflects an increase in ovarian DNA present in addition to oocyte nuclear DNA, and is reflected by an increase in the number of follicle cells per ovariole. Assuming that the amplified ribosomal DNA is localized in the oocyte as is indicated by *in situ* hybridization, and that 11% of the DNA in ovaries of 1–2 instar crickets, and 4.5% of the ovary DNA in 3–4 instar crickets, is oocyte DNA as estimated in Table I, the number of ribosomal genes in oocytes is calculated to be approximately 100 times the diploid value.

Competition experiments indicate that under the conditions of the hybridization experiments in Fig. 3 (up to 20 µg/ml RNA) *Xenopus* rRNA reacts with *Acheta* DNA only about 40% as efficiently as does *Acheta* rRNA (Fig. 4). Unlabeled *Acheta* rRNA effectively competes with *Xenopus* rRNA in hybridizing to *Acheta* DNA. Unlabeled *Xenopus* rRNA competes with the hybridization of *Acheta* rRNA to *Acheta* DNA only about 40% as efficiently as does unlabeled *Acheta* rRNA.

When *Acheta* DNA is hybridized with *Acheta* rRNA, 0.012% of the DNA from male and female legs hybridizes under saturating conditions of rRNA concentration. The data are consistent with the competition experiments indicating that *Xenopus* rRNA hybridizes with *Acheta* DNA about 40% as efficiently as does *Acheta* rRNA.

In pachytene stage cells hybridized with *Xenopus* rRNA virtually all of the label is localized over the DNA body. Little if any label is associated with the chromosomes (Fig. 5). Between 1 and 2% of the

TABLE I  
Relative Amount of Oocyte DNA in Ovaries of *Acheta* at Various Stages of Development

Stage	Total No. ovarioles/ovary	Total No. oocytes/ovariole	Total No. oocytes/ovary	Oocyte DNA/ovary*	DNA/ovary‡	Per cent ovarian DNA in oocytes	Total No. follicle cells/ovariole
				µg			
1–2 instar (30 mg)	132.3 (±1.74)	20.2 (±0.47)	2672.4	0.088	0.78 (±0.04)	11.2	162.4 (±3.5)
3–4 instar (150 mg)	126.8 (±1.48)	17.8 (±0.40)	2257.0	0.074	1.65 (±0.11)	4.48	421.0 (±17.6)
4–5 instar (250 mg)	134.1 (±1.57)	17.2 (±0.34)	2304.8	0.076	2.94 (±0.19)	2.58	715.2 (±47.5)

\* Haploid DNA value of *Acheta* equals  $6 \times 10^{-6}$  µg (Durand, 1955). The amount of DNA in an oocyte is equivalent to the amount of chromosomal DNA (four times the haploid value) and the amount in the body (1.5 times the haploid value) (Cave and Allen, 1969 b).

‡ Determined by diphenylamine procedure.

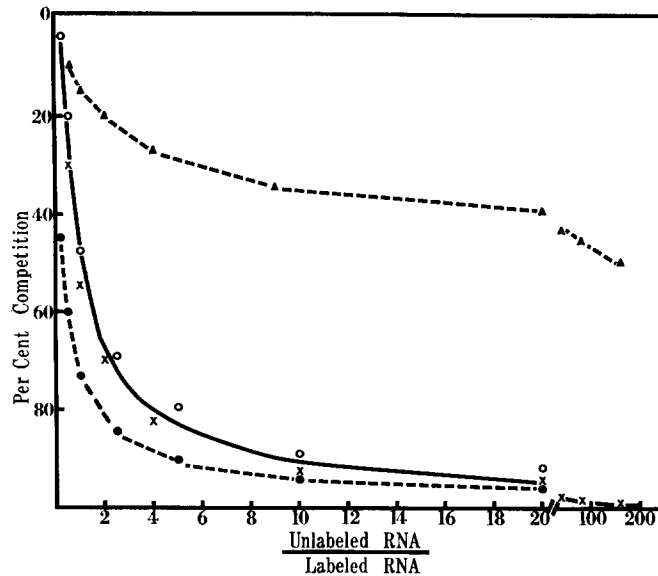


FIGURE 4 Competition hybridization of *Xenopus* and *Acheta* rRNA with *Acheta* DNA. DNA obtained from testes of *Acheta* was purified by centrifugation in CsCl as described in Materials and Methods. The portions of the gradients which hybridize with rRNA were pooled and denatured, and samples were bound to filters. Labeled *Xenopus* rRNA was competed with unlabeled *Xenopus* rRNA (○) and unlabeled *Acheta* rRNA (●). Labeled *Acheta* rRNA was competed with unlabeled *Acheta* rRNA (×), and unlabeled *Xenopus* rRNA (▲). Solid line shows the expected curve for homologous RNA competition. Hybridization was carried out under standard conditions. 1.0 μg of labeled RNA was added per milliliter of 4 × SSC. This more than saturated the DNA on the filters.

pachytene stage cells demonstrate, in addition to the DNA body, a limited area of labeling associated with the chromosome complement (Fig. 6). The label is over structures morphologically similar to, but much smaller than, the DNA body. Such structures probably represent portions of the DNA body which have been separated from the main mass as a result of the squashing procedure. Association of the DNA body with a single chromosome bivalent is demonstrated in Fig. 7.

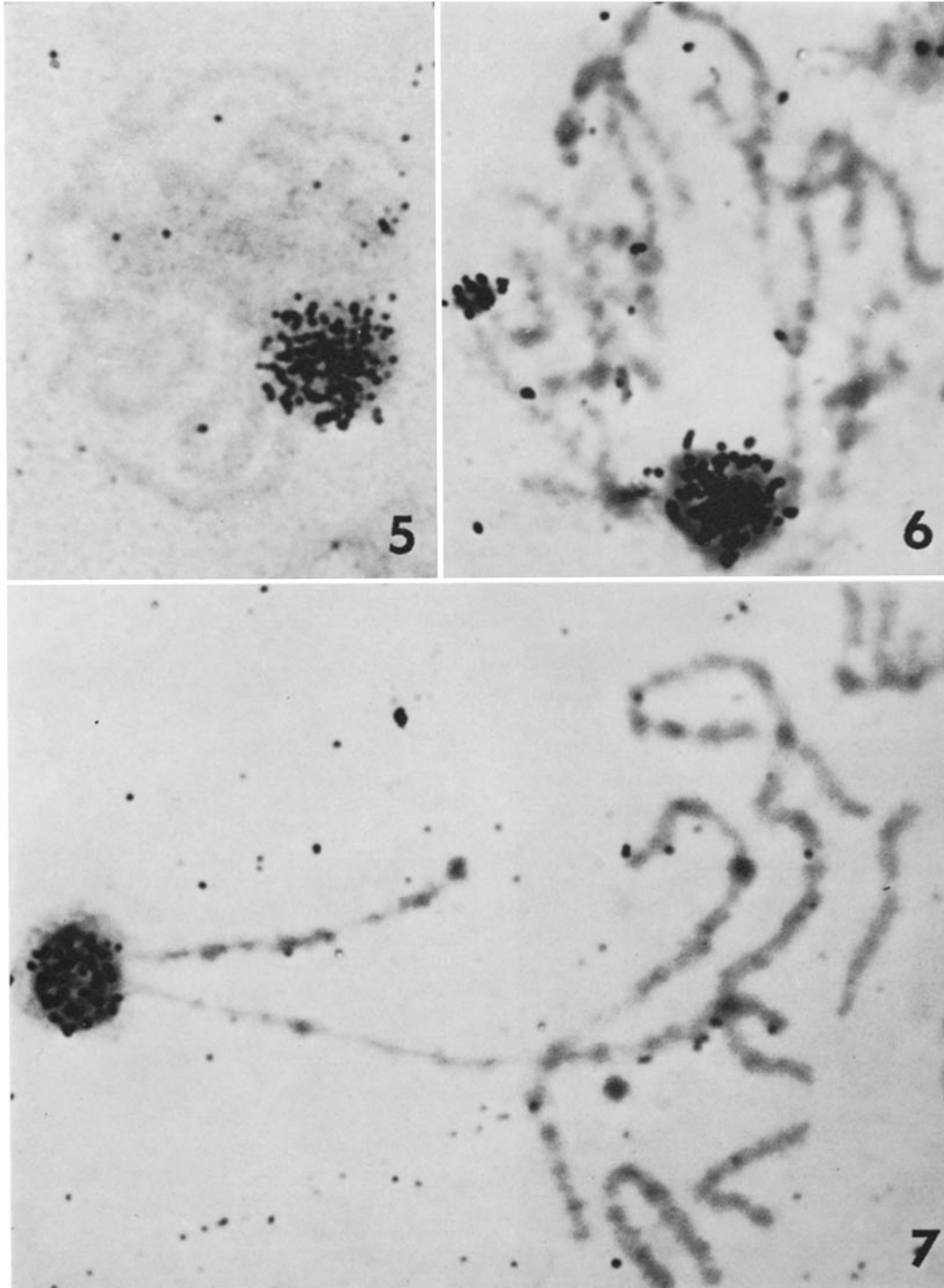
The DNA body of diplotene stage cells is localized within the developing nucleolar mass. Fig. 8 shows an early diplotene cell. A heavy concentration of label is localized over the DNA body. In addition to label localized over the DNA body, silver grains extend into the nucleolar mass indicating that the genes which code for rRNA extend from the DNA body into the surrounding nucleolar material.

In late diplotene oocytes, silver grains are visualized throughout the area occupied by the nucleoli. In Fig. 9 some small masses of Feulgen-positive material are detectable within the nucleolar mass. The silver grains, however, are distributed throughout the nucleolus. The results are consistent with the interpretation that DNA

originally localized within the DNA body is distributed to the individual nucleoli of the late diplotene stage oocyte.

In control preparations in which the DNA was not denatured with formamide, the DNA bodies were not labeled, nor were the DNA bodies labeled when the cells were treated with DNase after the initial RNase digestion. No detectable Feulgen-staining material could be visualized in the DNase-treated cells. Labeling of the DNA bodies was markedly reduced when unlabeled *Xenopus* or *Acheta* rRNA was added to a concentration of 7–15 times the concentration of labeled rRNA. Addition of unlabeled 4–7S RNA to similar concentrations had little if any effect on labeling. The results indicate that labeling of the DNA body is accounted for by specific hybridization of labeled rRNA with single-stranded DNA complementary to 18S and 28S ribosomal RNA.

Few if any silver grains can be detected over the nuclei of interphase somatic cells in radioautographs exposed for 2–4 wk. Failure to detect genes coding for rRNA in the nuclei of somatic cells, in spite of the fact that 0.004% of the DNA in somatic tissues hybridizes with rRNA, indicates that the number of ribosomal genes in somatic



**FIGURE 5** Radioautograph of pachytene stage cell hybridized with *Xenopus* rRNA of sp act 946,500 cpm/ $\mu$ g as described in Materials and Methods. Virtually all of the silver grains are localized over the DNA body. Squash preparation. 22 days' exposure.  $\times$  2440.

**FIGURE 6** Radioautograph of pachytene stage cell hybridized with *Xenopus* rRNA of sp act 946,500 cpm/ $\mu$ g as described in Materials and Methods. Note, in addition to label over DNA body, labeled area associated with one of bivalents. Squash preparation. 22 days' exposure.  $\times$  2440.

**FIGURE 7** Radioautograph of pachytene stage cell hybridized with *Xenopus* rRNA of sp act 946,500 cpm/ $\mu$ g as described in Materials and Methods. Note association of DNA body with single chromosomal bivalent. Squash preparation. 22 days' exposure.  $\times$  2440.

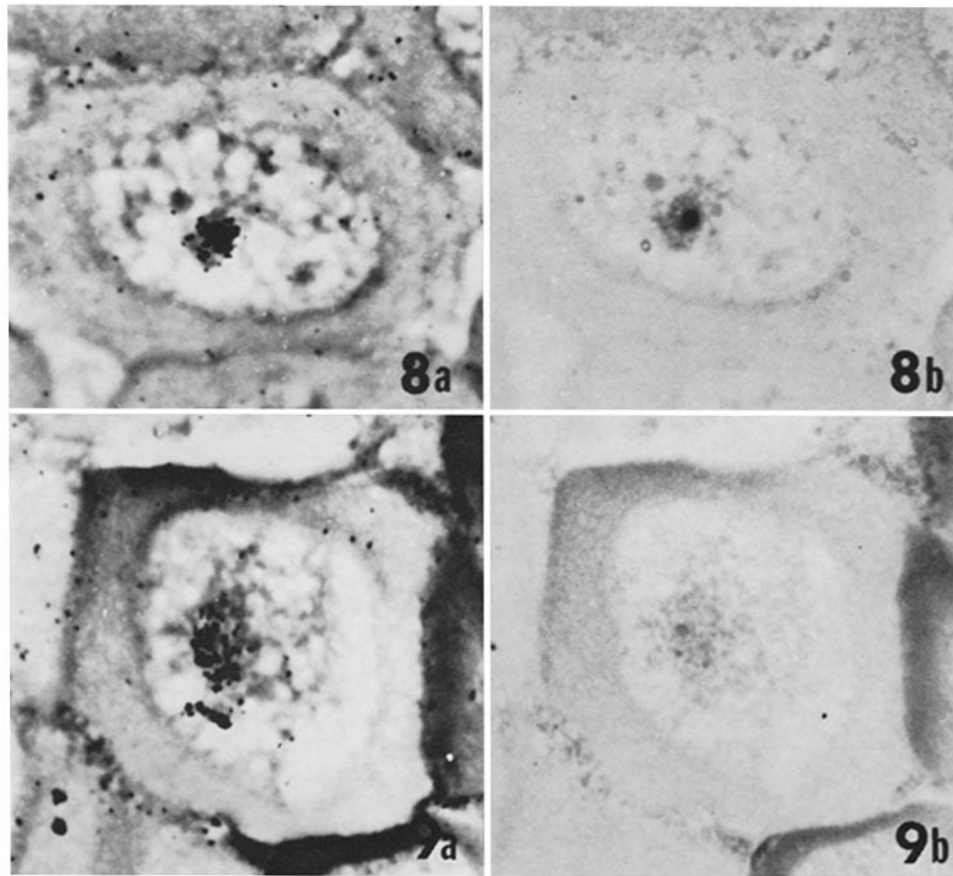


FIGURE 8 *a* Radioautograph of early diplotene stage cell hybridized with *Xenopus* rRNA of sp act 946,500 cpm/ $\mu$ g as described in Materials and Methods. Heavy labeling of the DNA body is apparent. Silver grains extend out into the nucleolar mass. Sectioned ovary stained with toluidine blue. 40 days' exposure.  $\times$  1100.

FIGURE 8 *b* Same cell as in Fig. 8 *a* after removal of silver grains and staining with the Feulgen procedure followed by fast green.  $\times$  1100.

FIGURE 9 *a* Radioautograph of late diplotene stage cell hybridized with *Xenopus* rRNA of sp act 946,500 cpm/ $\mu$ g as described in Materials and Methods. Label is dispersed throughout the nucleolar mass. Sectioned ovary stained with toluidine blue. 40 days' exposure.  $\times$  1100.

FIGURE 9 *b* Same cell as in Fig. 9 *a* after removal of silver grains and staining with the Feulgen procedure followed by fast green. Note that some small masses of Feulgen-positive material are still detectable.  $\times$  1100.

tissues is under the limits of sensitivity. Longer exposure of the radioautographs (5–6 months) produces some silver grains in the vicinity of the nucleolus of interphase nuclei; the extreme density of silver grains over the DNA body at this time does not allow direct comparison of labeling.

#### DISCUSSION

Large DNA-containing bodies which are present in addition to chromosomal DNA have been observed in the oocyte nuclei of various species of insects representing the orders Siphonaptera (Bayreuther, 1957), Diptera (Bauer, 1933; Bayreuther,



1956; Lima-de-Faria and Moses, 1966), and Coleoptera (Urbani, 1969; Urbani and Russo-Caia, 1964; Kato, 1969; Bier et al., 1967; Gall et al., 1969). In oocytes of Orthoptera, DNA which is present in addition to the chromosomes is closely associated with the oocyte nucleoli of several species of grasshoppers belonging to the family Acrididae (Kunz, 1967), and of crickets (Gryllidae) belonging to the subfamilies Gryllinae and Nemobiinae (Johnson, 1938; Favard-Sereno, 1968; Heinonen and Halkka, 1967; Cave and Allen, 1969 *b*; Kunz, 1969; Allen and Cave, 1972).

In oocytes of *A. domesticus* a heavy satellite DNA accounts for approximately 14% of the DNA in the ovaries of young animals (36–40 days old). Satellite DNA hybridizes with rRNA, but the efficiency of hybridization is low. That the increased amount of satellite DNA in ovaries is not accounted for by mitochondrial DNA is indicated by the fact that the mitochondrial DNA of *Acheta* is of the same density as main band DNA (1.699 g/cm<sup>3</sup>) (Hansen-Delkeskamp, 1969 *b*) and mitochondrial DNA does not hybridize with rRNA (Dawid and Brown, 1970). Like the DNA hybridizing with rRNA, the per cent of total DNA in the satellite is lower in the ovaries of more mature animals. Thus, there is a parallel decrease with development in the amount of satellite DNA and the per cent of the genome coding for rRNA. This is the result of a relative increase in the amount of somatic cell DNA within the ovary (Table I). Because of possible differences in the developmental stage of the ovaries used in the two studies (those reported by Lima-de-Faria et al., 1969, were in the “interphase of meiosis”), direct comparison of the degree of amplification for the satellite and for DNA complementary to rRNA is not possible. However, the data from the two studies suggest that in addition to DNA complementary to rRNA a large amount of DNA similar in density and GC content to rRNA is amplified in the ovary. A similar situation has been described in the oocytes of various members of the family *Dytiscidae* where amplification of a satellite DNA of high GC content is associated with the presence of a large extrachromosomal DNA body (Giardina's mass). All of the DNA complementary to rRNA is localized within the satellite, but the genes hybridizing with rRNA make up only a small portion of the satellite (6.5% in *Colymbetes fuscus*), all of which is amplified in the oocyte.

A round Feulgen-positive body is seen within

the nucleus of spermatogenic cells of *A. domesticus* (Wolstenholme and Meyer, 1966; Sotelo and Wettstein, 1964; Kunz, 1969). The heterochromatic body, seen in the spermatogonium through the spermatid stages, is morphologically similar to but smaller than the DNA body of oocytes. It lies inside of the nucleolus (Kunz, 1969). Whether or not the round body is present in addition to the chromosomal DNA or simply represents a chromocenter is not established. The association of the round body with the nucleolus raises the question of whether or not it may represent, like the DNA body, an amplification of the genes which code for rRNA. The fact that the per cent of the genome coding for rRNA is the same in testis as it is in leg muscle indicates that amplification of genes coding for rRNA does not occur during male gametogenesis. Moreover, *in situ* hybridization of *Xenopus* rRNA with squash preparations of testes has failed to demonstrate amplification of DNA complementary to rRNA in any of the stages of male gametogenesis.

Studies on spermatogenesis have further indicated that the nucleolus forms in contact with the X chromosome (Sotelo and Wettstein, 1964; see discussion of Kunz, 1969). Since the sex-determining mechanism in this organism is of the XX-XO type (Nilsson, 1968), one would expect that if the nucleolar organizers were localized on the X chromosomes, nuclei of female cells which have two X chromosomes should have twice the amount of nucleolar organizer DNA as male cells with a single X chromosome. The per cent of the genome coding for rRNA is proportional to the number of nucleolar organizers present in the cell (Ritossa et al., 1966). The fact that the per cent of the genome coding for rRNA is the same in male as in female somatic tissues indicates that the nucleolar organizer regions are localized on the autosomes rather than the sex chromosomes. A similar conclusion based on measurements of nucleolar number in somatic tissues (nerve ganglia) was reached by Kunz (1969).

Alternatively, the data could be explained if there were a large number of nucleolar organizers distributed throughout the chromosome complement as has been described in some vertebrates. Under these conditions the absence of a single organizer localized on one of the sex chromosomes might go undetected. The intimate association of the DNA body with a single chromosome bi-

valent during early prophase of meiosis suggests that the nucleolar organizer is singular rather than multiple (Cave and Allen, 1969 *a*).

*In situ* hybridization experiments demonstrate that the amplified genes hybridizing with rRNA are localized within the DNA body of the pachytene stage oocyte. Synthesis of extrachromosomal DNA is completed at this time and DNA synthesis cannot be detected in the nuclei of postpachytene stage cells (Cave and Allen, 1969 *a*). As the cells enter diplotene, large masses of nucleoli begin to accumulate at the periphery of the body. Previous studies have shown that as the cells proceed through diplotene the amount of RNA-containing material surrounding the DNA body gradually increases. Simultaneously, the amount of detectable Feulgen-positive material within the body gradually decreases (Cave and Allen, 1969 *a*). The fact that DNA-RNA hybridization is detected in the nucleolar mass suggests that genes hybridizing with rRNA localized in the DNA body protrude into the surrounding nucleolar mass. The DNA body is no longer visible in nuclei of late diplotene stage cells. Hybridization with rRNA, however, is visualized throughout the area occupied by the nucleoli. Since no detectable DNA synthesis occurs in the oocyte after the pachytene stage, the data indicate that DNA complementary to rRNA originally localized in the body is incorporated into the individual nucleoli of the late diplotene stage oocyte. Previous studies have described the nucleoli of late diplotene stage oocytes as small ring-shaped structures which are disrupted by treatment with DNase (Kunz, 1967). That the DNA within the nucleoli acts as a template for rRNA synthesis is shown by the fact that the nucleolar mass incorporates uridine-<sup>3</sup>H to a greater extent than does the remainder of the nucleus, and that such incorporation is sensitive to concentrations of actinomycin D which selectively inhibit 18S and 28S rRNA synthesis (Cave and Allen, 1969 *a*; Cave, unpublished results). Similar cores of DNA have been described in the nucleoli of germinal vesicles of *X. laevis* (Miller and Beatty, 1969), and are probably derived from the "nuclear cap" when this disperses in the nucleus.

*Xenopus* rRNA hybridizes with the DNA of a wide variety of organisms (Brown et al., 1967). In spite of variability in the density of the DNA coding for rRNA among different organisms, hybridization occurs in the gradient fractions containing the DNA complementary to rRNA (as determined with homologous rRNA) regardless of the base

composition of the rRNA, or the density of the ribosomal DNA in the species providing the RNA. The data suggest that the ribosomal DNA of various eucaryotes may contain similar sequences responsible for cross-reactions and dissimilar sequences responsible for differences in density (Pardue et al., 1970). Approximately 60% of the rRNA of *Urechis caupo* competes with *Xenopus* rRNA in hybridizing with *Xenopus* DNA (Dawid and Brown, 1970). A comparison of the hybridization of *Acheta* DNA with *Acheta* rRNA and with labeled *Xenopus* rRNA indicates that *Xenopus* rRNA binds with *Acheta* DNA approximately 40% as efficiently as does *Acheta* rRNA. It cannot be excluded that *Acheta* DNA which hybridizes with *Acheta* rRNA but not *Xenopus* rRNA under the conditions of these experiments is distributed differently in the nucleus than is that which hybridizes with *Xenopus* rRNA. That this is unlikely is indicated by the similar distribution of *Rhynchosciara* DNA complementary to *Xenopus* rRNA and DNA complementary to satellite DNA prepared by *in vitro* transcription of heavy satellite DNA using *Escherichia coli* RNA polymerase (Pardue et al., 1970).

That amplification of ribosomal DNA is advantageous to the organism has been demonstrated in oocytes of *X. laevis* where it is calculated that in the absence of amplification of the ribosomal cistrons it would take approximately 465 yr for the oocyte to synthesize the 4  $\mu$ g of rRNA normally found within it at maturity (Perkowska et al., 1969). Amplification of the ribosomal genes to approximately the level known to exist enables this to occur in 65 days. Ribosomes synthesized during oogenesis provide the protein synthetic machinery for early embryogenesis (Brown and Littna, 1964; Brown and Gurdon, 1964, 1966).

In *Acheta*, as in *Xenopus*, synthesis of rRNA cannot be detected during early embryogenesis until blastoderm formation (Hansen-Delkeskamp et al., 1967). Active incorporation of amino acids into protein is readily detected during all of this time (Hansen-Delkeskamp, 1969 *a*). During oocyte development little transfer RNA and much rRNA is synthesized. This RNA is probably stable up to the stage of yolk cleavage, providing ribosomes for the synthesis of proteins during early embryogenesis (Hansen-Delkeskamp, 1969 *a*).

In contrast to *Xenopus*, the amplified ribosomal genes of *Acheta* remain organized in a dense Feulgen-positive mass, the DNA body, during the early diplotene stage of oogenesis. This enables

visualization of the amplified DNA during its synthesis and transcription. The factors influencing such differences in the organization of amplified DNA are presently under investigation.

This work is dedicated with admiration and affection to Professor S. R. M. Reynolds, on the occasion of his 68th birthday. I thank Dr. M. Vaughan for his advice and help in the preparation of RNA and I gratefully acknowledge the technical assistance of Mrs. Marcia Andrews.

Supported by Public Health Service Grant No. GM 16440 from the Institute of General Medical Sciences, and by Grant No. L-16 from the Health Research and Services Foundation of Pittsburgh.

Received for publication 4 April 1972, and in revised form 19 June 1972.

## REFERENCES

- ALLEN, E. R., and M. D. CAVE. 1968. Formation, transport, and storage of ribonucleic acid containing structures in oocytes of *Acheta domesticus* (Orthoptera). *Z. Zellforsch. Mikrosk. Anat.* **92**:477.
- ALLEN, E. R., and M. D. CAVE. 1972. Nucleolar organization in oocytes of Gryllid crickets: subfamilies *Gryllinae* and *Nemobiinae*. *J. Morphol.* **137**:433.
- BAUER, H. 1933. Die wachsenden Oocytenkerne einiger Insekten in ihrem Verhalten zur Nuklealfärbung. *Z. Zellforsch. Mikrosk. Anat.* **18**:254.
- BAYREUTHER, K. 1956. Die Oogenese der Tipuliden. *Chromosoma.* **7**:507.
- BAYREUTHER, K. 1957. Extrachromosomal DNS-haltiges Material in der Oogenese der Flohe. *Z. Naturforsch. Teil B.* **12**:458.
- BELLAMY, A. B., and R. K. RALPH. 1968. Recovery and purification of nucleic acid by means of cetyltrimethylammonium bromide. *Methods Enzymol.* **12B**:165.
- BIANCHI, N. O., A. LIMA-DE-FARIA, and H. JAWORSKA. 1964. A technique for removing silver grains and gelatin from tritium autoradiographs of human chromosomes. *Hereditas.* **51**:207.
- BIER, K., W. KUNZ, and D. RIBBERT. 1967. Struktur und Funktion der oocytenchromosomen und nukleolen sowie der extra DNS während der oogenese panoistischer und meroistischer Insekten. *Chromosoma.* **26**:169.
- BROWN, D. D., and I. B. DAWID. 1968. Specific gene amplification in oocytes. *Science (Wash. D. C.)*. **160**:272.
- BROWN, D. D., and J. GURDON. 1964. Absence of ribosomal RNA synthesis in an anucleolate mutant of *Xenopus laevis*. *Proc. Natl. Acad. Sci. U. S. A.* **51**:139.
- BROWN, D. D., and J. GURDON. 1966. Size distribution and stability of DNA-like RNA synthesized during development of anucleolate embryos of *Xenopus laevis*. *J. Mol. Biol.* **19**:399.
- BROWN, D. D., and E. LITINA. 1964. RNA synthesis during the development of *Xenopus laevis*, the South African clawed toad. *J. Mol. Biol.* **8**:669.
- BROWN, D., G. C. WEBER, and J. H. SINCLAIR. 1967. Ribosomal RNA and its genes during oogenesis and development. *Carnegie Inst. Wash. Year Book.* **66**:580.
- BURTON, K. 1968. Determination of DNA concentration with diphenylamine. *Methods Enzymol.* **12B**:163.
- CAVE, M. D., and E. R. ALLEN. 1969 a. Synthesis of nucleic acids associated with a DNA containing body of *Acheta*. *Exp. Cell Res.* **58**:201.
- CAVE, M. D., and E. R. ALLEN. 1969 b. Extrachromosomal DNA in early stages of oogenesis in *Acheta domesticus*. *J. Cell Sci.* **4**:593.
- DAWID, I., and D. BROWN. 1970. The mitochondrial and ribosomal DNA components of oocytes of *Urechis caupo*. *Dev. Biol.* **22**:1.
- DURAND, M. C. 1955. L'acide désoxyribonucléique des gamètes des *Gryllus domesticus*. *C. R. Hebd. Seances Acad. Sci. Ser. D Sci. Nat. (Paris)*. **241**:1340.
- FAVARD-SERENO, P. 1968. Evolution des structures nucléolaires au cours de la phase d'accroissement cytoplasmique chez le Grillon (Insecte, Orthoptère). *J. Microsc. (Paris)*. **7**:205.
- GALL, J. G. 1968. Differential synthesis of the genes for ribosomal RNA during amphibian oogenesis. *Proc. Natl. Acad. Sci. U. S. A.* **60**:553.
- GALL, J. G., H. C. MACGREGOR, and M. E. KIDSTON. 1969. Gene amplification in the oocytes of Dytiscid water beetles. *Chromosoma.* **26**:169.
- GALL, J. G., and M. L. PARDUE. 1969. Formation and detection of RNA-DNA hybrid molecules in cytological preparations. *Proc. Natl. Acad. Sci. U. S. A.* **63**:378.
- GILLESPIE, D. 1968. The formation and detection of DNA-RNA hybrids. *Methods Enzymol.* **12B**:112.
- HANSEN-DELKESKAMP, E. 1969 a. Synthese von RNS und Protein während der Oogenese und Frühen embryogenese von *Acheta domestica*. *Wilhelm Roux' Arch. Entwicklunsmech. Org.* **162**:114.
- HANSEN-DELKESKAMP, E. 1969 b. Satelliten-desoxyribonuclein säure in Gonaden und somatischen Gewebe von *Acheta domestica* L. *Z. Naturforsch. Teil B.* **246**:1331.
- HANSEN-DELKESKAMP, E., W. SAUER, and F. DUSPIVA. 1967. Ribonuclein-säuren in der embryogenese von *Acheta domestica* L. *Z. Naturforsch. Teil B.* **226**:540.
- HEINONEN, L., and O. HALKKA. 1967. Early stages of oogenesis and metabolic DNA in oocytes of the house cricket, *Acheta domestica* (L.). *Ann. Med. Exp. Biol. Fenn.* **45**:101.
- IFFT, J. B., D. H. VOET, and J. VINOGRAD. 1961. Determination of density distributions and density

- gradients in binary solutions at equilibrium in the ultracentrifuge. *J. Phys. Chem.* **65**:1138.
- JOHN, H. A., M. L. BIRNSTIEL, and K. W. JONES. 1969. RNA-DNA hybrids at the cytological level. *Nature (Lond.)*. **223**:582.
- JOHNSON, M. 1938. A study of the nucleoli of certain insects and crayfish. *J. Morphol.* **62**:113.
- KATO, K. 1969. Cytochemistry and fine structure of elimination chromatin in *Dytiscidae*. *Exp. Cell Res.* **52**:507.
- KAULENAS, M. S. 1969. Factors affecting ribosome isolation from adult crickets, *Acheta domesticus*. *J. Insect Physiol.* **16**:813.
- KLETT, R. P., and M. SMITH. 1968. Isolation of DNA from invertebrates. *Methods Enzymol.* **12B**:112.
- KUNZ, W. 1967. Lampbürsten chromosomen und multiple nukleolen bei orthopteren. *Chromosoma*. **21**:446.
- KUNZ, W. 1969. Die Entstehung multipler Oocyte-nukleolen aus akzessorischen DNA Körpern bei *Gryllus domesticus*. *Chromosoma*. **26**:41.
- LIMA-DE-FARIA, A., M. L. BIRNSTIEL, and H. JAWORSKA. 1969. Amplification of ribosomal cistrons in heterochromatin of *Acheta*. *Genetics*. **61**(Suppl.):145.
- LIMA-DE-FARIA, A., and M. MOSES. 1966. Ultrastructure and cytochemistry of metabolic DNA in *Tipula*. *J. Cell Biol.* **30**:177.
- LIMA-DE-FARIA, A., B. NILSSON, D. CAVE, A. PUGA, and H. JAWORSKA. 1968. Tritium labeling and cytochemistry of extra DNA in *Acheta*. *Chromosoma*. **25**:1.
- MACGREGOR, H. C. 1968. Nucleolar DNA in oocytes of *Xenopus laevis*. *J. Cell Sci.* **3**:437.
- MILLER, O., and B. BEATTY. 1969. Visualization of nucleolar genes. *Science (Wash. D. C.)*. **164**:995.
- NILSSON, B. 1966. DNA bodies in the germline of *Acheta domesticus* (Orthoptera). *Hereditas*. **56**:396.
- NILSSON, B. 1968. *Acheta domesticus* (Orthoptera) some cytological observations. *Lantbrukshogsk. Ann.* **34**:437.
- PARDUE, M. L., S. GERBI, R. ECKHARDT, and J. GALL. 1970. Cytological localization of DNA complementary to ribosomal RNA in polytene chromosomes of Diptera. *Chromosoma*. **29**:268.
- PERKOWSKA, E., H. C. MACGREGOR, and M. L. BIRNSTIEL. 1968. Gene amplification in the oocyte nucleus of mutant and wild-type *Xenopus laevis*. *Nature (Lond.)*. **217**:649.
- RITOSSA, F. M., K. C. ATWOOD, D. L. LINDSLEY, and S. SPIEGELMAN. 1966. On the chromosomal distribution of DNA complementary to ribosomal and soluble RNA. *Natl. Cancer Inst. Monogr.* **23**:449.
- SOTELO, J. R., and R. WETTSTEIN. 1964. Electron microscope study on meiosis. The sex chromosome in spermatocytes, spermatids, and oocytes of *Gryllus argentinus*. *Chromosoma*. **15**:389.
- URBANI, E. 1969. Cytochemical and ultrastructural studies of oogenesis in the *Dytiscidae*. *Monit. Zool. Ital. (N.S.)* **3**:55.
- URBANI, E., and S. RUSSO-CAIA. 1964. Osservazioni citochimiche e autoradiografiche sul metabolismo degli acidi nucleici nella oogenesi di *Dytiscus marginalis* L. *Rend. Ist. Sci. Univ. Camerino*. **5**:19.
- VINCENT, W. S., H. O. HALVORSON, H. R. CHEN, and D. SCHIN. 1969. A comparison of ribosomal gene amplification in uni- and multinucleolate oocytes. *Exp. Cell Res.* **57**:240.
- WIMBER, D. E., and D. M. STEFFENSEN. 1970. Localization of 5S RNA genes on *Drosophila* chromosomes by RNA-DNA hybridization. *Science (Wash. D. C.)*. **170**:639.
- WOLSTENHOLME, D., and G. MEYER. 1966. Some facts concerning the nature and formation of axial core structures in spermatids of *Gryllus domesticus*. *Chromosoma*. **18**:272.