*SATELLITE DNA'S IN THE CRABS GECARCINUS LATERALIS AND CANCER PAGURUS**

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Although Meselson, Stahl, and Vinograd[1] had noted the probable existence of heterogeneity in the base composition of calf thymus DNA, until 1961 it appeared that all DNA molecules found in a given species were sufficiently similar that the DNA isolated from a given organism could be banded in a single peak in isopycnic CsCl density gradient centrifugation. The findings that a significant fraction of the DNA's of mouse[2] and of the crab *Cancer borealis*[3] are so different from the majority of the DNA's of the respective species that they form distinct and separate bands in CsCl led to more acute observations of density gradient centrifugation patterns. Since then, similar satellite DNA's, comprising 10 to 30 per cent of the total DNA, have been found in seven other species of *Cancer;*[3–6] and satellite DNA's with quite different physical properties have been observed in other invertebrates.[6, 7] In addition, minor component DNA's, comprising less than 1 per cent of the total DNA, some of which have been localized in organelles other than the nucleus, have been described for many species.[8] The functions of the satellite DNA's are not known, although the biological role of those DNA's localized in organelles other than the nucleus may be linked to the physiology and duplication of the organelles that contain them.[8]

This paper describes our findings with the DNA of another crustacean, the land crab *Gecarcinus lateralis*. The centrifuge pattern of this DNA is composed of a main band and two satellites. One satellite is similar to, if not identical with, the adenylate- and thymidylate-rich satellite[9] which has been reported for *Cancer* species. In addition, present in both *G. lateralis* and *Cancer pagurus* is another satellite which is apparently rich in guanylate and cytidylate residues.

No studies have been reported on the biological role of satellite DNA in *Cancer*, in part because of the technical difficulties inherent in maintaining marine Crustacea in the laboratory throughout their molt cycle coupled with the problems of performing long-term labeling experiments under such conditions. The present finding of satellites in the land crab opens the possibility of such studies, since this animal is easily maintained in the laboratory and detailed knowledge of its molt cycle is available.[10, 11]

Materials and Methods.—Animals: Specimens of *Gecarcinus lateralis* were obtained and maintained in the laboratory as previously described;[10] specimens of *Maia squinado* were supplied by the staff of the Biology Station at Roscoff, France; and specimens of *Cancer pagurus* were bought at Les Viviers, Roscoff.

DNA isolation: Basically the methods of Smith[5, 6] were followed with isoamyl alcohol:chloroform (1:24, v/v) as the protein denaturant.[12] The concentration of ethylenediaminetetraacetate was increased to 0.2 *M* in the homogenizing medium because of the high concentrations of Mg^{++} and Ca^{++} in this animal.[13] Prior to the final ethanol precipitation the DNA preparations were treated with pancreatic RNase (Worthington; 50 μg/ml) for 30 min at 37°C, followed by Pronase (Calbiochem; 50 μg/ml) for 2 hr.

Analytical CsCl isopycnic density centrifugation: To 0.8 ml of a solution of CsCl (ρ = 1.848) were added 0.05 ml of 0.02 *M* Tris, pH 8, 1–2 μg *Micrococcus lysodeikticus* DNA as marker, and

0.02–0.1 ml (2–10 μg) of the DNA sample to be analyzed. The density of the final solution was adjusted to $\rho = 1.708$ by the addition of either CsCl or water. After centrifugation for at least 20 hr at 44,770 rpm and 25°C in a Spinco model E analytical ultracentrifuge, ultraviolet (UV) absorption photographs were taken. Tracings of the photographs were made on a Joyce-Loebl double beam automatic recording microdensitometer. The densities of the components of the various crab DNA's were calculated by equation (3) of Sueoka[3] where, at 44,770 rpm:

$$\rho = \rho_0 + 0.0092\ (r^2 - r_0^2), \tag{1}$$

where r and r_0 denote the peak positions of the unknown and reference DNA's, respectively. The density of the *M. lysodeikticus* marker (ρ_0) was taken as 1.727 gm/cm^3.[14]

The guanylate and cytidylate (G + C) content of the main-band DNA and the satellite rich in G + C were calculated from equation (4) of Sueoka:[3]

$$\text{Per cent (G + C)} = \frac{(\rho - 1.662) \times 100}{1.03}, \tag{2}$$

where ρ is the buoyant density of the DNA in CsCl.

Preparative CsCl isopycnic density centrifugation: Essentially, the method of Flamm *et al.*[15] was used. Pure samples of satellite DNA's were obtained by rebanding selected fractions from a preliminary CsCl centrifugation in a Spinco no. 40 rotor for 62 hr at 20°C. The second centrifugation of the (G + C)-rich DNA plus some contaminating main-band DNA was run in CsCl of $\rho = 1.715$, while the DNA rich in A + T was purified by centrifuging in CsCl of $\rho = 1.696$. The purity of the final preparations was checked by analyzing 10 to 20 times the usual amount of DNA (i.e., 10–20 μg) in the model E ultracentrifuge. In this way, a 5% contamination could be detected.

Chromatography: Purified light satellite DNA (16 μg) was hydrolyzed for 45 min at 175°C in formic acid, dried, redissolved in water, and chromatographed on Whatman no. 1 paper with isopropanol:HCl:H_2O (4:1:12, v/v) as the solvent.[16]

Irradiation of DNA's: Purified samples of main-band and satellite DNA's of at least 24 μg/ml in 0.04 *M* potassium phosphate buffer, pH 7, were irradiated in a large Hilger monochromator.[12] The UV absorbancy of each sample at 270 mμ was followed with an unirradiated sample of the same DNA as a blank.

Results.—Buoyant densities of the DNA's of the three species: The results of the analytical ultracentrifuge analysis are summarized in the densitometer tracings of Figure 1.

Approximately 72 per cent of the testis DNA from *C. pagurus* is composed of a main band of $\rho = 1.701$ and 24 per cent of a satellite of $\rho = 1.677$, as observed previously.[18] In addition, there is a second satellite of $\rho = 1.721$ comprising approximately 4 per cent of the total DNA. Careful examination of the published melting curves, relating absorbancy at 260 mμ to temperature, reveals a small inflection at high temperatures which is probably due to this second satellite.[18] The second satellite does not appear to exist in measurable quantities in the other species of *Cancer* which have been studied.[4–6, 19]

DNA from both the testis and midgut gland (hepatopancreas) of *G. lateralis* has a buoyant density pattern similar to that found in *Cancer*, although the relative quantities of the satellites are different. From densitometer tracings of three preparations of *G. lateralis* DNA (1–3.3 μg/ml), 78.2 ± 0.2 per cent of the total DNA is in the main band, 17.8 ± 0.6 per cent is in the light satellite, and 4.6 ± 1.2 per cent is in the heavy satellite.

These results are corroborated by calculations of hyperchromicity from the melting curves (Fig. 2). In *C. pagurus* DNA, 25.4 per cent of the total hyperchromicity occurs with a T_m of 51°C and 71 per cent occurs with a T_m of 69°C; in *G. lateralis*, 20.5 per cent of the total hyperchromicity occurs with a T_m of 52°C

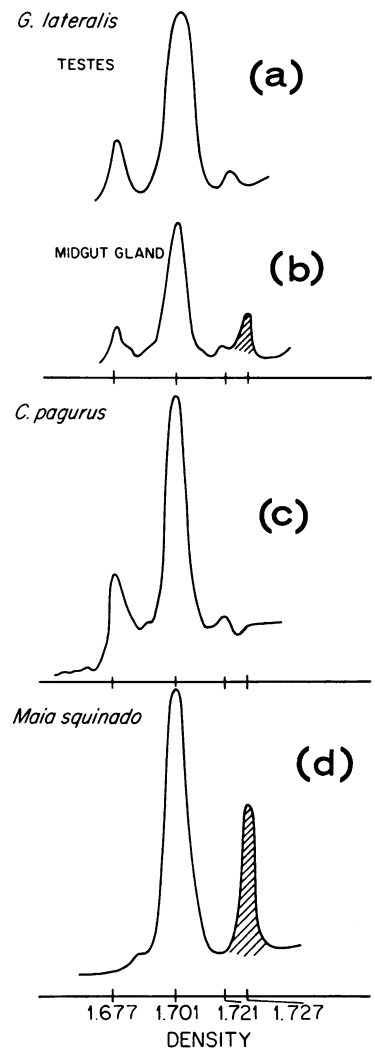

FIG. 1.—Microdensitometer tracings of photographs of crab DNA banded in CsCl at 25°C in the Spinco model E analytical ultracentrifuge. The DNA's were isolated from (*a*) testis and (*b*) midgut gland of *Gecarcinus lateralis*, (*c*) testis of *Cancer pagurus*, and (*d*) testis of *Maia squinado*. The peak on the right of traces (*b*) and (*d*) is marker DNA from *Micrococcus lysodeikticus* (ρ = 1.727 gm/cm^3). To visualize more clearly the heavy satellite (ρ = 1.721 gm/cm^3) present in *G. lateralis* and *C. pagurus*, traces (*a*) and (*c*) are of samples which contained no marker.

and 77 per cent occurs with a T_m of 71°C. The material with the lower T_m is the light (A + T)-rich satellite (see below), while the material with the higher T_m is the main-band DNA. At a very high temperature an additional small inflection occurs in both melting curves. These are due to the (G + C)-rich satellites. The fractional amounts of the A + T satellites as obtained from melting curves are slightly greater than those obtained from densitometer tracings because of the greater hyperchromicity of (A + T)-rich DNA's.[20]

The DNA from the third species of crab, *M. squinado*, (Fig. 1) appears to occur as a single peak of ρ = 1.701, although there is a suggestion of a small shoulder on the light side of this peak. There is no satellite present in any significant quantity (i.e., more than 1% of the main band).

The main band of all three species, then, has a density of 1.701, which corresponds to a G + C content of 38 per cent as calculated from equation (2). Since we have

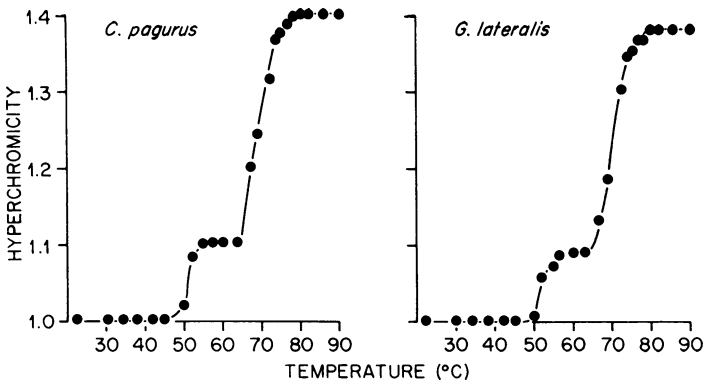

FIG. 2.—Melting curves of DNA preparations from *C. pagurus* and *G. lateralis*. The DNA's were dissolved in 0.015 *N* NaCl, 0.0015 *N* sodium citrate, pH 7 (SSC/10) and the optical density was read at 260 mμ. Ten min was allowed for equilibration at each temperature.

now demonstrated that the DNA of *M. squinado* is unimodally distributed, this calculated base composition may be compared with the results of chemical analysis of total DNA from *M. squinado*, which Lee and Barbu[21] found to be 42 per cent.

The heavy satellite of $\rho = 1.721$ has a calculated G + C content of 57 per cent. The light satellite of *G. lateralis* has a density identical to that of the "poly d(A − T)" of the *Cancer* species. As has been pointed out,[3] the base composition of such DNA cannot be calculated from equation (2). We have therefore used other methods to investigate the nature of this satellite.

Nature of the satellite, $\rho = 1.677$: The light satellites of both *C. pagurus* and *G. lateralis* were purified by two consecutive runs of preparative isopycnic CsCl centrifugation (see *Materials and Methods*). A sample of the material finally collected was rerun in the analytical ultracentrifuge; there was no detectable (i.e., less than 5%) contamination of the light satellite with main-band DNA.

The purified satellite of *Gecarcinus* was hydrolyzed with formic acid and chromatographed. It was apparent from the appearance of the chromatogram that the base composition of the light satellite was in excess of 90 per cent thymine + adenine, with traces of cytosine and guanine.

The light satellite of the genus *Cancer* has been shown previously to consist largely of *alternating* adenylate and thymidylate residues. The evidence on which this conclusion was based is twofold: (*a*) If the light satellite from *C. borealis* is used as a primer with *E. coli* DNA-polymerase and the product is subjected to nearest-neighbor analysis, only 1.3 per cent of the thymines are found adjacent to another thymine.[9] (*b*) If the satellite of *C. pagurus* is depurinated and then hydrolyzed in acid and chromatographed, the principal compound recovered is thymidine 3′,5′diphosphate. If there had been adjacent thymines in the original DNA, the hydrolysis products would have included oligothymidylic acids; however, none were detected.[18]

We have studied the primary structure of the light satellites from *C. pagurus* and *G. lateralis* by following (*a*) the reversibility of the melting curve and (*b*) spectral changes with ultraviolet irradiation.

(*a*) In Figure 3*a* and *b* are depicted the melting curves of the purified light satellites from *C. pagurus* and *G. lateralis*. During heating, readings were taken only after the absorbancy changes had stabilized (i.e., after 10 min at each temperature). Once the peak of the melting curve had been attained, the sample was allowed to

cool at a rate of less than 0.5°/min. Initially the hyperchromicity fell rapidly, lagging behind the melting curve by only 1 or 2 degrees. Such a result would be expected of a DNA composed primarily of alternating adenylate and thymidylate residues. This result does not imply that the reannealing returns the DNA to its native form[18, 22] but only that extensive regions of complementarity exist between the denatured strands. However, despite the relatively slow rate of cooling used in this experiment, the descending curve soon departs from the melting curve. This departure indicates that after the initial random pairing of alternating A — T sequences, further reannealing is inhibited by the apposition of noncomplementary sequences on opposite strands. In a purely alternating DNA copolymer composed of two bases, the strands are everywhere complementary, and such a departure is not observed.[20]

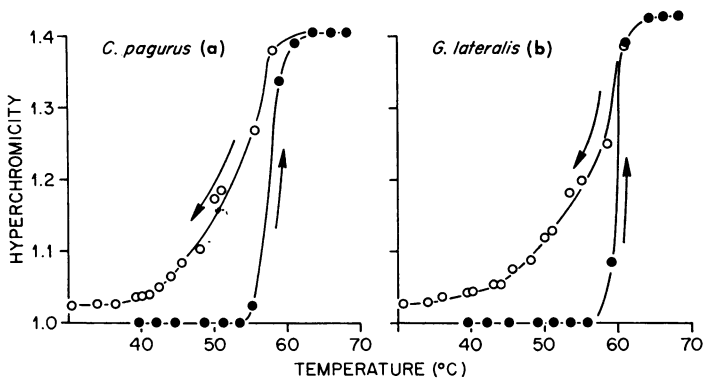

FIG. 3.—Melting curves of light satellite DNA preparations from *C. pagurus* and *G. lateralis* in 0.04 *M* potassium phosphate, pH 7 and SSC/10. *Closed circles* = heating (10 min allowed for equilibration at each temperature); *open circles* = cooling (at a rate of less than 0.5°/min).

(*b*) In the studies of the spectral changes with ultraviolet irradiation, a solution containing roughly 24 μg/ml of DNA in 0.04 *M* potassium phosphate buffer, pH 7.0, was placed in a quartz cuvette, and its absorbancy was measured at 270 mμ. The solution was then irradiated at 280 mμ, which leads to the formation of cyclobutyl dimers between adjacent pyrimidines if any are present in the DNA. Since the absorption of the dimers is negligible at 270 mμ, their formation is detectable by a drop in absorbancy at this wavelength. The reaction is reversible; reirradiation at short wavelength (239 mμ) splits 75 per cent of the dimers, resulting in partial recovery of the absorbancy at 270 mμ.[17] Moreover, from the rate of the absorbancy increase, an estimate may be made as to whether the dimers being split are thymine-thymine, cytosine-thymine, or cytosine-cytosine.[23–25] Enzymatically synthesized alternating poly d(A — T) shows no spectral changes when irradiated with the doses used here.[26]

Figure 4 shows the results of such irradiation experiments with the light satellite from *C. pagurus* and with the light satellite of *G. lateralis* and, as a control, the main band of *G. lateralis*. The reversible absorbancy changes that are characteristic of adjacent pyrimidines are evident. The extent of the decrease is 2.4 and 2.9 per cent for the light satellites of *C. pagurus* and *G. lateralis*, respectively, and 6.3 per cent for the main band of *G. lateralis* DNA. For the results with the satellite DNA to be due to the presence of contaminating main-band DNA, the contamination would have to be 30 per cent. As mentioned above, we were unable to detect

such a contamination at the 5 per cent level. Therefore, it is certain that at least 85 per cent of the spectral changes are due to the light satellite DNA.

As adjacent pyrimidine residues are dimerized by irradiation at 280 mμ, there is local melting of the DNA and a concomitant hyperchromic effect arising from the interaction of the neighboring bases. Setlow and Carrier[17] have derived an equation relating the base composition of a particular DNA to the extent of the absorption change of that DNA following 239 mμ irradiation (due to dimer breakage) after 280 mμ irradiation (due principally to dimer formation). From this relationship, the *minimum* number of adjacent thymidylate residues in the *C. pagurus* and the *G. lateralis* light satellites and the *G. lateralis* main-band DNA may be calculated

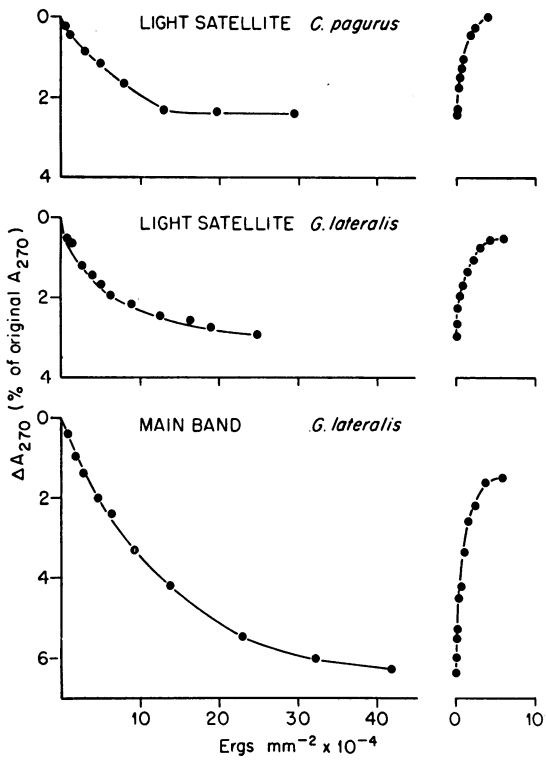

FIG. 4.—Absorbancy at 270 mμ of various crab DNA's as a function of ultraviolet radiation dose. Curves on the left show the decrease in absorbancy with irradiation at 280 mμ; curves on the right show the reversal of this decrease with subsequent irradiation at 239 mμ.

to be 2.7, 3.0, and 5.8 per cent of the total number of nucleotide pairs, respectively. Setlow and Carrier found that in fact their equation underestimated the number of adjacent thymines by roughly 50 per cent when their UV results were compared with nearest-neighbor analyses on eight different DNA's.[17] Therefore, it is likely that the numbers of adjacent thymidylate residues in the *G. lateralis* main-band DNA and the light satellites of both species are more nearly 12, 5, and 6 per cent, respectively. Although the majority of the adenylate and thymidylate residues in the light satellites must be alternating, a significant fraction are not. This interpretation of the light-satellite structure from the irradiation effects is consistent with the interpretation drawn from the hysteresis observed in the melting curves (Fig. 3).

It is unlikely that many of the dimers formed contain cytosine, since at 280 mμ the

formation of C-C dimers saturates at approximately 1–2 $\times$ 10^4 ergs mm^{-2}, whereas the dimers formed in the light satellite saturate above 10 $\times$ 10^4 ergs mm^{-2}. Furthermore, the data obtained for splitting the dimers are compatible with the fact that they are mostly thymine, for the 1/erg dose for splitting the dimers in the satellites is about 1.2 $\times$ 10^4 ergs mm^{-2} at 239 mμ. The 1/erg dose at this wavelength for splitting C-C dimers is 0.3 $\times$ 10^4 ergs mm^{-2},[24] and for splitting C-T dimers is 0.5–1.0 $\times$ 10^4 ergs mm^{-2}, while the 1/erg dose for splitting T-T dimers is roughly 1.5 $\times$ 10^4 ergs mm^{-2}.[23] Therefore, the irradiated satellites contain a significant fraction of T-T dimers and hence of adjacent thymine residues.

The estimate of 5 to 6 per cent thymine residues adjacent to other thymines is nearly three times higher than that of Swartz *et al.*,[9] based on the composition of the product made by *E. coli* DNA polymerase primed with the light satellite from *C. borealis*. That enzyme, however, shows a marked preference for alternating poly-d(A-T) as a primer,[27] and the presence of long runs of these alternating sequences in crab DNA could easily account for a gross underestimation of the remaining sequences.

Discussion.—The Eubrachyurans, or true crabs, have been taxonomically subdivided into two major groups:[28, 29] the cancroids, to which *Cancer* and *Carcinus* belong, and the grapsoids, to which *Gecarcinus* and *Maia* are assigned. The former group is considered more primitive than the latter, which are more highly specialized. The light DNA satellite has been found in eight species of the genus *Cancer* but not in *Carcinus*. In the present study, the light satellite has been demonstrated in *Gecarcinus* but not in *Maia*. It is evident that the distribution of this particular satellite among the Eubrachyurans does not coincide with the classification of these animals on a taxonomic basis as was once suggested.[6] In fact, satellite DNA's of this buoyant density have been found in much more divergent species than in the various crab genera, having been described in *Euglena* chloroplasts[30] and in human lymph node and bone marrow tissue,[31] although the base composition and structure of these light DNA's have not been determined.

The heavy satellite found in *Cancer* and *Gecarcinus* is unusual among the metazoa in that its G + C content, as calculated from equation (1), is about 57 per cent. The G + C content of metazoan DNA is almost invariably less than 50 per cent,[3] resulting in a density of less than 1.710 gm/cm^3. The only previously described exceptions of which we are aware are a satellite in the barnacle *B. nubilis*[6] (ρ = 1.714), a satellite in the bivalve *A. inflata*[7] (ρ = 1.710), and a mitochondrial DNA from sheep heart[32] (ρ = 1.714).

If one examines directly the photographic plates from the analytical ultracentrifuge experiments, one can see distinctly the main band of *C. pagurus* DNA together with the light and heavy satellites, even though the latter comprise only some 3 per cent of the total DNA. In addition, however, one can also see at least two more faint bands, too faint to be detected by the microdensitometer and representing less than 1 per cent of the DNA. These have been seen in photographic plates from eight different centrifugation runs with such DNA in concentrations from 4 to 12 μg/ml. The total number of satellites in this species is therefore not yet certain. In eukaryotic animals, there is no *a priori* reason why the DNA from every chromosome should have the same buoyant density or base composition, and the main band itself will be composed of DNA from a number of different chromosomes.

Since the DNA molecules making up the main band are heterogeneous, satellite DNA's from the nucleus become, by definition, those DNA's whose physical properties do not overlap the physical properties of *most* of the other DNA molecules. This distinction need not imply any unique biological function for such "satellites."

A possible exception to this statement is the light satellite since its structure appears to be at least 90 per cent alternating adenine and thymine. Although the function of such a DNA remains unknown, it is unlikely that it is the end product of an uncontrolled DNA polymerase reaction because the relative quantity of the light satellite remains constant from animal to animal and from one tissue to another in any given animal, at least for *Gecarcinus*. The amount of light satellite, therefore, appears to be as carefully regulated as all of the animal's DNA.

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[1] Meselson, M., F. Stahl, and J. Vinograd, these PROCEEDINGS, **43**, 581 (1957).
[2] Kit, S., *J. Mol. Biol.*, **3**, 711 (1961).
[3] Sueoka, N., *J. Mol. Biol.*, **3**, 31 (1961).
[4] Sueoka, N., and T. Y. Cheng, these PROCEEDINGS, **48**, 1851 (1962).
[5] Smith, M., *Biochem. Biophys. Res. Commun.*, **10**, 67 (1963).
[6] Smith, M., *J. Mol. Biol.*, **9**, 17 (1964).
[7] Shinomiya, T., A. Kikuchi, T. Uchida, and E. Egami, *Acta Biochim. Polon.*, **13**, 379 (1966).
[8] Granick, S., and A. Gibor, *Progr. Nucleic Acid Res. Mol. Biol.*, **7**, 143 (1967).
[9] Swartz, M. N., T. A. Trautner, and A. Kornberg, *J. Biol. Chem.*, **237**, 1961 (1962).
[10] Skinner, D. M., *Biol. Bull.*, **123**, 635 (1962).
[11] Skinner, D. M., *J. Exptl. Zool.*, **160**, 225 (1965).
[12] Sevag, M. G., D. B. Lackman, and J. Smolens, *J. Biol. Chem.*, **124**, 425 (1938).
[13] Skinner, D. M., D. J. Marsh, and J. S. Cook, *Biol. Bull.*, **129**, 355 (1965).
[14] Davidson, N., personal communication.
[15] Flamm, W. G., H. E. Bond, and H. E. Burr, *Biochim. Biophys. Acta*, **129**, 310 (1966).
[16] Wyatt, G. R., *Biochem. J.*, **48**, 584 (1951).
[17] Setlow, R. B., and W. L. Carrier, *Photochem. Photobiol.*, **2**, 49 (1963).
[18] Pochon, F., J. Massoulie, and A. M. Michelson, *Biochim. Biophys. Acta*, **119**, 249 (1966).
[19] Sueoka, N., and T. Y. Cheng, *J. Mol. Biol.*, **4**, 161 (1962).
[20] Marmur, J., and P. Doty, *Nature*, **183**, 1427 (1959).
[21] Lee, K. Y., and E. Barbu, *Compt. Rend. Soc. Biol.*, **150**, 865 (1956).
[22] Davidson, N., J. Widholm, U. S. Nandi, R. Jensen, B. Olivera, and J. C. Wang, these PROCEEDINGS, **53**, 111 (1965).
[23] Wulff, D. L., *Biophys. J.*, **3**, 355 (1963).
[24] Setlow, R. B., W. L. Carrier, and F. J. Bollum, these PROCEEDINGS, **53**, 1111 (1965).
[25] Setlow, R. B., and W. L. Carrier, *J. Mol. Biol.*, **17**, 237 (1966).
[26] Deering, R. A., and R. B. Setlow, *Biochim. Biophys. Acta*, **68**, 526 (1963).
[27] Josse, J., A. D. Kaiser, and A. Kornberg, *J. Biol. Chem.*, **236**, 864 (1961).
[28] Rathbun, M. J., *U.S. Natl. Museum Bull.*, **152**, 1 (1930).
[29] Rathbun, M. J., *U.S. Natl. Museum Bull.*, **97**, 1 (1918).
[30] Iwamura, T., *Progr. Nucleic Acid Res. Mol. Biol.*, **5**, 133 (1966).
[31] Corneo, G., E. Ginelli, and E. Polli, *J. Mol. Biol.*, **23**, 619 (1967).
[32] Corneo, G., C. Moore, D. R., Sanadi, L. Grossman, and J. Marmur, *Science*, **151**, 687 (1966).