THE RADIOAUTOGRAPHIC DETECTION OF DNA WITH TRITIATED ACTINOMYCIN D

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The specificity with which actinomycin D is bound to DNA both in vivo and in test tube experiments (for a review, see Reich and Goldberg, 1964) and the recent availability of the drug in tritiated form with high specific activity have suggested its possible usefulness as a means to detect DNA radioautographically in fixed cytological preparations. Brachet and Ficq (1965) had previously employed ^{14}C-labeled actinomycin D in this fashion.

This communication presents a technique which has led to the successful labeling of DNA in chromosomes and nucleoli of dipteran salivary gland preparations and in both nuclei and kinetoplasts of Leishmania. We are also reporting the results of extraction and fixation experiments which lead to the conclusion that actinomycin-D binding under these conditions is specific and suggest that the mode of binding in fixed preparations is similar to that previously reported on the basis of biochemical experiments (Reich and Goldberg, 1964).

MATERIALS AND METHODS

Salivary glands were derived from third instar larvae of *Drosphila melanogaster* and *D. hydei*. Leptomonad forms of *Leishmania tarentolae* and *L. donovani* were obtained through the courtesy of Dr. L. Simpson, The Rockefeller University.

The salivary glands were squashed on gelatinized slides in 45% acetic acid and, after coverslip removal with liquid nitrogen, postfixed in 3:1 ethanol-acetic acid and passed through a decreasing ethanol series. Alternatively, 4% neutral formaldehyde was substituted for 45% acetic acid for squashing or, in one

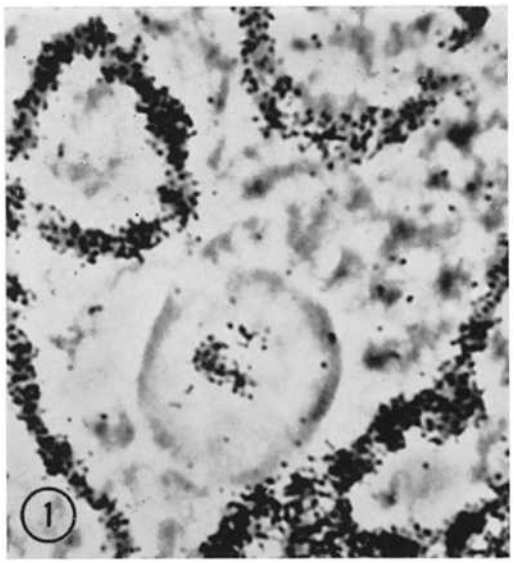


FIGURE 1 Radioautograph of acid-fixed salivary gland squash preparation of *Drosophila hydei* incubated, after fixation, with actinomycin D-^{3}H for 5 min. Radioautographic exposure was 4 days. Note labeled DNA in nucleolus. Magnification about 2500.

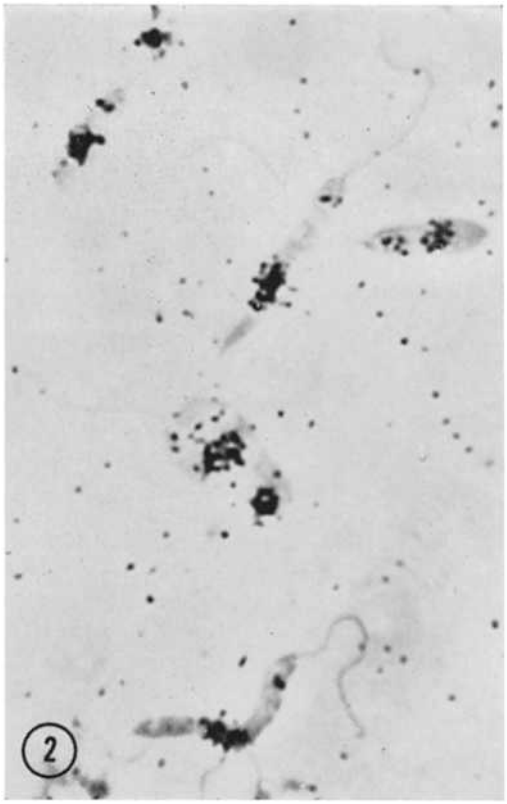


FIGURE 2 Radioautograph of leptomonad forms of *Leishmania sp.* incubated for 30 min with actinomycin-D-^{3}H after air drying and methanol fixation. Radioautographic exposure was 5 days. Note labeled DNA in nuclei and kinetoplasts. (One apparent division figure is present in the field.) Magnification about 3500.

series, 5% acetic acid in 50% ethanol; in the latter case, postfixation in 3:1 was omitted.

Smear preparations of *Leishmania* were air dried and subsequently fixed in methanol, 4% formaldehyde, or 3:1.

Staining for fluorescence microscopy was carried out with coriphosphine O, using previously described methodology (Keeble and Jay, 1962). The preparations were examined with BG-12 filtered mercury light and a Zeiss No. 50 barrier filter. Photographs were taken with Kodak High Speed Ektachrome film (daylight type) and directly printed in black and white on Kodak Panalure photographic paper.

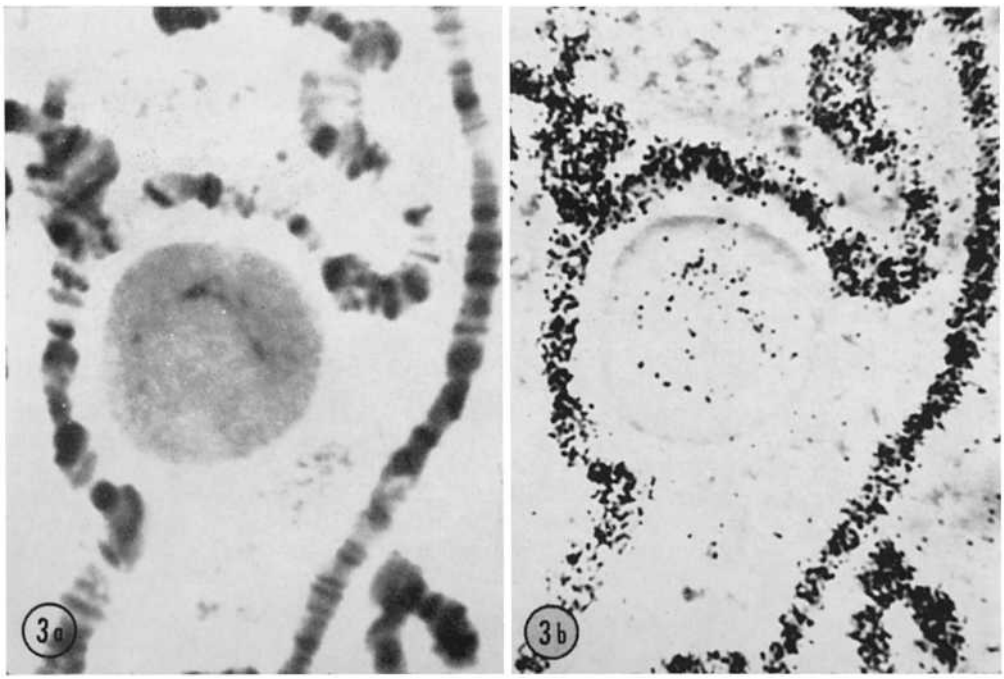
Tritiated actinomycin D was obtained from Schwarz BioResearch Inc. (Orangeburg, N. Y.), at a specific activity of 3.8 c/mmole and a concentration of 0.5 mc/ml. In a typical experiment, 0.3 ml of 10 μc of actinomycin D-^{3}H per ml of water was deposited over the squash or smear and permitted to remain for periods ranging from 1 min to 1 hr. After this incubation the slides were rinsed in water, passed through 75%, 95%, and absolute ethanol, dried, and covered with radioautographic emulsion.

The radioautographic preparations were made with Ilford K-5 liquid emulsion, diluted 1:1. Exposure periods varied from 24 hr to 5 days. The slides were developed in Kodak D-19b developer for 3 min at 18°C, washed, and fixed.

Acid and enzyme extractions were performed prior to exposing the preparations to labeled actinomycin. Depurination was carried out by hydrolysis in 1 N HCl for 10 min at 60°C. DNase treatment was done at 37°C for 3 hr with 0.3 mg DNase (Worthington) per ml, pH 6.0, with 10^{-3} M Mg SO_4. RNase (Worthington) was used at a concentration of 0.3 mg/ml in 0.01 M phosphate buffer at pH 7.6, 37°C, for 3 hr. Control slides were incubated in suitable buffer solutions.

RESULTS AND DISCUSSION

The specificity of labeled actinomycin-D binding for DNA is evident not only from the examination of the preparations, in which label is detectable only at sites at which DNA is known to exist, but also from the total absence of label in preparations previously digested with DNase. Incubation of the preparations with RNase has no measurable effect on subsequent binding of actinomycin D. The



FIGURES 3 *a* and *b* Converted fluorescent image (*a*) and radioautograph (*b*) of acid-fixed salivary gland squash preparation of *Drosophila hydei* incubated, after fixation, with actinomycin-D-^{3}H for 5 min. Radioautographic exposure was 3 days. The darker areas within the nucleolus in (*a*) correspond to regions in which DNA is visible by virtue of greenish secondary fluorescence. Note the presence of label in (*b*) at sites at which DNA fluorescence is not readily noticeable. Magnification about 2500.

sensitivity of the method is high: even with relatively short radioautographic exposure periods, we can detect low concentrations of DNA in nucleoli of Drosophila (Figs. 1 and 3) and kinetoplasts of Leishmania (Fig. 2) which are just visible in fluorescent preparations after acridine orange or coriphosphine O staining. We have some evidence that prolonged exposure shows the presence of DNase-sensitive radioactivity at locations at which nothing is visible with conventional fluorescence microscopy.

The amount of actinomycin D-^{3}H bound is affected by the method of fixation: the use of formalin alone or formalin before acid fixation reduces the amount of actinomycin bound to chromosomes subsequently. This reduction of binding is not noticed when formalin is present in the incubation mixture applied to normal acid-fixed squashes. It seems reasonable to assume that formalin as a fixative maintains some chromosomal proteins in close association with DNA and thus lowers the accessibility of the latter to the antibiotic. (We would like to point out in this context that we have gained the impression in several preparations that puffed regions of polytene chromosomes bind less actinomycin D-^{3}H than do normal regions.)

Hydrolysis of salivary gland squash preparations with HCl (1 N, 60°C, 10 min) results in the complete suppression of subsequent actinomycin-D binding. This finding is consistent with the view that actinomyin binds to DNA via the purines, specifically guanine (Reich and Goldberg, 1964), even under our conditions. Further evidence for normal binding derives from the effect of acid treatment of our preparations after exposure to actinomycin-^{3}H. Such treatment, but not treatment with formalin or alcohol, removes the antibiotic from the DNA, as would be expected in the case of a hydrogen-bonded molecule.

Several attempts to demonstrate in vivo binding of actinomycin D-^{3}H failed to yield positive results, in our hands. The realization that bound actinomycin could be removed by acid treatment suggested the possibility that our routine fixation procedure (45% acetic acid followed by 3:1,

ethanol-acetic acid) could be responsible for these failures. Indeed, the substitution of 5% acetic acid in 50% ethanol as fixative, while yielding cytologically inferior preparations in that less chromosomal detail was discernible, did result in detectable chromosomal labeling after in vivo incubation of excised salivary glands in actinomycin D-^{3}H. However, in view of the fact that chromosomal labeling can be obtained *after squashing* in the presence of the modified fixative and the labeled antibiotic, the aforementioned finding does not constitute fully convincing evidence for the radioautographic detection of actinomycin D bound in vivo in our system.

We would like to thank Mrs. B. S. Ebstein for permitting us to see her manuscript (Tritiated Actinomycin D as a Cytochemical Label for Small Amounts of DNA) while our paper was being written and for her graciousness in agreeing to delay publication of her work so that the two papers could appear together.

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