THE LOCALIZATION OF SPERMIDINE IN SALIVARY GLAND CELLS OF DROSOPHILA MELANOGASTER AND ITS EFFECT ON H²-URIDINE INCORPORATION

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Communicated by Esmond E. Snell, October 25, 1967

Although the polyamines—putrescine, spermidine, and spermine—are ubiquitous biological compounds, their specific distribution and function at the subcellular level remain uncertain. As cellular cations they probably serve an important function as part of the ionic milieu in the mediation of some cellular processes. Stabilization of cellular membranes, 1, 5 mitochondria, 2 ribosomes, 3, 4 and nuclei 5 by polyamines has been demonstrated. Stabilization of polynucleotide by the polyamines 6, 7 is reflected in the stimulation of RNA synthesis 8, 9 DNA synthesis, 10 and, in the case of ribosomes, in the enhancement of amino acid incorporation. 11

The problem of polyamine redistribution, 12, 13 inherent in cell fractionation, has discouraged and cast doubt on investigations of polyamine localization at the cellular level. The present paper describes a reliable method for the radioautographic localization of spermidine, and the effect of polyamines on RNA synthesis in situ in polytene chromosomes.

Materials and Methods.—Polyamine and RNA analyses: Stocks of Drosophila melanogaster were maintained at 23.0 ± 0.5°C on a medium described by Pearl et al., 14 modified by the addition of propionic acid (0.90 ml/liter) for the control of mold. Various stages of development were collected, washed in Drosophila saline, and frozen at –20°C. The polyamines were concentrated from TCA extracts of the developmental stages according to the method of Raina. 15 The separation and quantitation of the polyamines were effected by the thin-layer chromatographic method of Hammond and Herbst. 16 The hydrochloride salts of the polyamines (Calbiochem) were used in all experiments. RNA analyses 17 were performed on the TCA-insoluble precipitates, using yeast RNA as the standard.

Radioautography.—Localization of H²-uridine: Salivary glands of late third-instar larvae of D. melanogaster were cleanly dissected in a drop of Medium 199 (Difco Laboratories, Detroit, Michigan), washed briefly in a fresh drop of the same medium, and preincubated for 1 hr in sealed depression slides containing Medium 199 (50 μl) in the presence and absence of spermidine. At the end of the preincubation period the glands were rapidly rinsed in Medium 199, transferred to Medium 199 containing H²-uridine (30 μc/ml; 4 c/mmole), and incubated for 30 min. The preincubation and incubation procedures were carried out with shaking in a Dubnoff water bath at 27–28°C. The incubations were stopped by adding an equal volume of 10% TCA (w/v), and processed according to Sirlin et al. 18

The squashed preparations were coated with NTB-2 liquid emulsion, 19 and exposed for 1 week at 4°C in bakelite slide boxes containing Drierite. After the indicated exposure period the radioautograms were developed in Microdol-X (5 min at 18–19°C), stopped in a water bath (30 sec), and fixed (5 min). The developed slides were then washed in distilled water, air-dried, and stained lightly through the emulsion with 0.05% toluidine blue.

Radioautography—Localization of H²-spermidine: Larval salivary glands of D. melanogaster were isolated as described above and incubated in Medium 199 (50 μl) containing H²-spermidine (5 μc/ml; 30 mc/mmole), obtained from New England Nuclear Corp. (Boston, Mass.), for a period of 1.5 hr. After incubation the salivary glands were rapidly rinsed in fresh Medium 199 and placed in a small drop of the same medium on a subbed slide (slide dipped in 0.1% gelatin and 0.05% chrome alum). Excess medium was removed with bibulous paper, and the glands were frozen in isopentane at liquid nitrogen temperature. The slide containing the glands was then placed in a precooled chamber which was submerged in a dry ice-acetone mixture and lyophilized for 24–48
hr. After lyophilization the slide was transferred to a vacuum desiccator containing paraformaldehyde and Drierite. The glands were fixed in formaldehyde vapor at 60°C for 24 hr, and embedded in Epon according to Luft.26 Beem capsules filled with degassed and accelerated Epon were inverted over the dehydrated and fixed salivary glands, allowed to infiltrate for 48 hr at room temperature, and finally polymerized at 60°C. This completed, with certain modifications, the procedure outlined by Wilske and Ross41 for the localization of water-soluble compounds. Finally the polymerized Epon capsules were removed from the glass slides by immersion in liquid nitrogen.

Epon sections of 1–2 μ were cut with glass knives on a Porter-Blum MT-2 Ultramicrotome (Ivan Sorvall, Inc., Norwalk, Conn.). The sections were transferred to subbed slides, coated with NTB-2 emulsion, and processed as described above.

Results.—Polyamine and RNA levels: Figure 1A represents RNA levels of the indicated developmental stages of D. melanogaster. The initial decrease in RNA during the prepupal stage is followed by a significant increase in RNA on pupation (on a wet weight basis). A second increase in RNA coincides with the maturation of the adult organs of the imago.22 Although differing in amounts, the proportionate increases in RNA during pupation and at the time of emergence of the adult are in good agreement with those obtained by Church22 for Pacific stocks of D. melanogaster.

Polyamine levels during development are given in Figure 1B. The increase in polyamine content, most notable for putrescine, is followed by a decrease upon pupation. At approximately the midpupal period, i.e., when the imaginal eyes are pigmented, the prepupal polyamine levels are re-established. Upon eclosion the level of putrescine drops markedly, and concurrently small increases in the levels of spermidine and spermine are observed.

Effect of spermidine concentration of H3-uridine incorporation: Table 1 illustrates the distribution of labeled nuclei in salivary gland cells of D. melanogaster as a function of spermidine concentration. Spermidine at concentrations of $5.0 \times 10^{-5}$ and $10^{-4} M$ substantially inhibited H3-uridine incorporation; higher concentrations had little or no effect ($2.0 \times 10^{-4}$) or stimulated H3-uridine incorporation ($4.0$ and $6.4 \times 10^{-4} M$). Spermidine at a concentration of $6.4 \times 10^{-4} M$ produced concomitant changes in nuclear structures. As is evident in Figure 2A and B, both chromosoma! and nucleolar material appear enlarged.

All spermine concentrations so far investigated ($0.29–4.6 \times 10^{-4} M$) strongly inhibit the incorporation of H3-uridine. Only the lowest concentration, viz. $0.29 \times 10^{-4} M$, did not lead to extensive nuclear destruction. Nuclear destruction by higher concentrations of spermine was assumed from low numbers of scorable nuclei.

The localization of H3-spermidine: The localization of tritiated spermidine in distal cells34 of the salivary gland of D. melanogaster is demonstrated in Figure 3A and B. Although the nuclear-to-cytoplasmic labeling ratio, computed on the basis of grain counts per unit area, is approximately 2 in distal cells, the label appears uniformly distributed in proximal cells.
TABLE 1

**Distribution (Per Cent Frequency) of Labeled Salivary Gland Nuclei of**

*Anopheles* **melanogaster**

<table>
<thead>
<tr>
<th>Spermidine concentration: $10^{-4} M$</th>
<th>25</th>
<th>50</th>
<th>75</th>
<th>100</th>
<th>125</th>
<th>150</th>
<th>175</th>
<th>200</th>
<th>&gt;200</th>
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<tr>
<td>0</td>
<td>25.6</td>
<td>11.2</td>
<td>16.8</td>
<td>11.4</td>
<td>8.72</td>
<td>7.2</td>
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<td>6.73</td>
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<tr>
<td>0.5</td>
<td>32.0</td>
<td>20.4</td>
<td>23.4</td>
<td>4.7</td>
<td>3.9</td>
<td>5.4</td>
<td>4.7</td>
<td>3.9</td>
<td>2.3</td>
</tr>
<tr>
<td>1.0</td>
<td>61.0</td>
<td>8.2</td>
<td>4.9</td>
<td>9.9</td>
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<tr>
<td>2.0</td>
<td>37.4</td>
<td>13.1</td>
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<tr>
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<td>25.0</td>
<td>3.1</td>
<td>3.9</td>
<td>18.9</td>
<td>15.6</td>
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<td>2.5</td>
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<td>3.7</td>
<td>4.9</td>
<td>6.2</td>
<td>55.5</td>
</tr>
</tbody>
</table>

*Preincubated in Medium 199 with the indicated concentration of spermidine. At least 200 nuclei were scored for each indicated concentration of spermidine.

The specific intrachromosomal sites of labeling are presently being investigated.

**Discussion and Conclusions.**—The hormonal control of insect development permits a critical evaluation of hormone levels, RNA, and polyamine contents. Burdette and Shaaya and Karlson determined the ecdysone titer in *Bombyx mori* and *Calliphora erythrocephala*, respectively. The titer in these insects increased gradually during the last larval instar, rising precipitously during the prepupal period. A commensurate decrease in the ecdysone titer was characteristic of the early pupal stage. A second broad peak was observed during histogenesis. As pupation proceeded, the level of ecdysone gradually decreased, and then increased, presumably in preparation for the emergence of the adult. Assuming that such a pattern of ecdysone levels is common to most holometabolous insects, e.g., *D.*

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**Fig. 2.**—The effect of spermidine on the incorporation of H3-uridine and on the nuclear structure of salivary gland nuclei of *D. melanogaster*. (A) Nucleus from salivary gland preincubated in Medium 199 containing no additions, followed by incubation in the same medium containing H3-uridine (30 μc/ml). (B) Nucleus from sister gland preincubated in Medium 199 containing spermidine ($6.4 \times 10^{-4} M$), and then incubated in Medium 199 containing H3-uridine.
Fig. 3.—The localization of H^4-spermidine in a distal salivary gland cell of *D. melanogaster*. Salivary glands were incubated in Medium 199 containing 5 μc/ml H^4-spermidine for 1 hr. Two-micron sections were coated with NTB-2 emulsion, exposed for 2 weeks, and stained through the emulsion with Azur II-Methylene Blue. *(A)* and *(B)* focused on tissue and emulsion, respectively.

*melanogaster*, a similarity between ecdysone and polyamine levels is evident. It is noteworthy that Ackermann\(^7\) also described a reduction in polyamine, viz. spermidine, on pupation for *Bombyx mori*. Thus, the increased RNA concentrations observed during pupation could be interpreted as the derepression of stage-specific chromosomal loci by the combined action of ecdysone and polyamines. Evidence for the possible hormonal control of polyamine levels in the liver of hypophysectomized rats has been communicated by Kostyo.\(^9\)

The inhibition and stimulation of RNA synthesis by low and high concentrations of polyamine, respectively, have been reported by Chung *et al.*\(^8\) for crude chick embryo polyribonucleotide polymerase. The *in vitro* stimulation of the RNA polymerase reaction by polyamines has been explained in essentially two ways: (1) polyamine reversal of product inhibition by the dissociation of the enzyme-bound RNA,\(^4\) and (2) polyamine modification of the RNA product so as to render it unable to bind to the template site.\(^9\) The inhibition and stimulation of RNA synthesis, dependent on polyamine concentration, necessitates a different explanation based on spermidine-DNA interactions.
X-ray diffraction evidence for the binding of polyamines to DNA has been presented.\textsuperscript{21} In addition, Mahler et al.\textsuperscript{32} have described, on theoretical grounds, possible changes in DNA conformation as a consequence of steroidal diamine concentration, i.e., low concentrations effect a condensation of DNA through charge neutralization, while high concentrations extend DNA conformation via charge reversal. The possibility that similar interactions control puff size, and hence RNA synthesis,\textsuperscript{33} appears plausible. That spermidine interacts directly with salivary gland nuclei has been demonstrated by radioautography. The differences in the subcellular distribution of spermidine in distal and proximal cells have also been noted, and may be under hormonal control.\textsuperscript{34} Further investigations concerning a possible ecdysone-polyamine synergism are presently in progress.

* Published with the approval of the Director of the New Hampshire Agricultural Experiment Station as Scientific Contribution no. 425. Supported by U.S. Atomic Energy Commission Contract AT(30-1)-3721.

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