界面活性剤処理したショウジョウバエ唾腺における細胞膜の透過性

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Permeabilization of *Drosophila* Salivary Glands with Mild Detergents

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Introduction

The cells of an organism are generally accepted to differ from each other, not because they contain different genes, but because they express different genes. One of the most important subject in the developmental biology today, therefore, is to understand the mechanisms that underlie the regulated expression of specific genes in specific cells of an organism.

Salivary gland polytene chromosomes of dipteran insects provide a unique system for studying regulation of eukaryotic gene expression because their large sizes allow us to "see" actively transcribing gene loci as puffs under the light microscope (reviewed by Ashburner and Berendes, 1978). On the other hand, cytoplasmic factors are suggested to be important in the regulation of gene expression (Bonner, 1981 ; Craine and Kornberg, 1981 ; Anderson and Nüsslein-Volhard, 1984 ; Deno and Satoh, 1984 ; Iwakura *et al.*, 1985 ; Nomura *et al.*, 1986 ; also see Gurdon, 1985, for review). However, little is known of the molecular basis of these postulated factors. The use of polytene nuclei in an *in vitro* assay system to elucidate the gene-regulating activity of these cytoplasmic factors should have the following advantages : (1) gene expression can be visualized as puffing and, therefore, it is easy to locate loci being regulated by a given cytoplasmic factor, and (2) since cytoplasmic factors need not be purified before application to polytene nuclei, monitoring of gene regulation activities is possible during factor purification.

Polytene chromosomes are feasible for assaying gene-regulating activities of a cytoplasmic factor only if the factor can reach the nuclei so that it can

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function. Thus, in previous studies, we treated *Drosophila* salivary glands with a mild detergent, digitonin, to permeabilize the cell membranes, and showed that digitonin-permeabilized *Drosophila* salivary glands are effective as an assay system for gene-regulating factors (Myohara and Okada, 1987, 1988 a, b, c).

In the present study, we investigated and compared the permeabilizing effects of digitonin and three other detergents, i.e., Nonidet-P 40, Triton X-100, and saponin, using two marker molecules, Trypan blue (MW 960 Da) and DNase I (MW 31 kDa). These substances were adopted as index markers because their presence is easily detectable cytochemically.

Materials and Methods

Materials and methods were as described previously (Myohara and Okada, 1987), unless stated otherwise. In brief, salivary glands at puff stage 1 (PS 1) were dissected from third (final) instar larvae of *Drosophila melanogaster* in the medium MTB 1 (15 mM K-phosphate, pH 7.0, 60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, and 1% polyethylene glycol 6000), then transferred to MTB 1 containing a detergent, and incubated for 0-60 min at room temperatures (20-25°C). Less than 20 lobes of salivary glands per 2 m*l* of detergent solution were placed on a siliconized depression slide (36-mm-diameter depression). After the detergent treatment, the glands were washed for 30 seconds each in four changes of MTB 1. The permeabilizing effects of four detergents, i.e., digitonin and Nonidet-P 40 (both from BDH Chemicals), saponin (Sigma), and Triton X-100 (Wako Pure Chemical Industries) on salivary glands were judged

Table 1 Rank of Trypan Blue Staining *

Rank	State of Trypan blue staining	Time length of treatment with 0.05% Nonidet-P 40
0	No trace of staining	0 min
1	Light blue in part of salivary gland	2 min
2	Light blue in whole salivary gland	4 min
3	Light blue in whole gland and dark blue in part	8 min
4	Dark blue in whole gland with nuclei recognizable	12 min
5	Blue black in whole gland with nuclei unrecognizable	18 min

* Salivary glands were treated with 0.05% Nonidet-P 40 for the indicated length of time, washed, and stained with 0.06% Trypan blue for 20 min. The extent of staining in these glands was used as the standard in determining the ranks of Trypan blue permeation.

on the extent of permeation of the marker molecules, DNase I and Trypan blue.

For the examination of the permeability of detergent-treated salivary glands to Trypan blue, the glands were incubated in MTB 1 containing 0.06% Trypan blue for 20 min, washed with MTB 1, and then ranked the staining on the basis from 0 to 5. The standards for ranking were the extent of Trypan blue staining in glands which had been treated with 0.05% Nonidet-P 40 (NP 40) for 0, 2, 4, 8, 12, 18 min (Table 1). NP 40 was used to make the standards because it rendered salivary glands permeable to Trypan blue in more various degrees than other detergents (Fig. 1).

In the examination of the permeability of detergent-treated salivary glands to DNase I, four lobes of glands were incubated in 1 m*l* of MTB 1 containing 0.05 mM $CaCl_2$ and 100 U/m*l* of DNase I (MW 31 kDa, Sigma) for 20 min at room temperatures (20-25°C). The digestion was stopped by the addition of 0.1 m*l* of 220 mM EDTA to the enzyme solution. The salivary glands were then washed with MTB 1, fixed, stained with lactic-acetic-orcein (10 : 45 : 2), and then squashed for the observation of the chromosomes. The permeabilities of glands to DNase I were judged according to the percentage of nuclei in which chromosomes were digested by the enzyme.

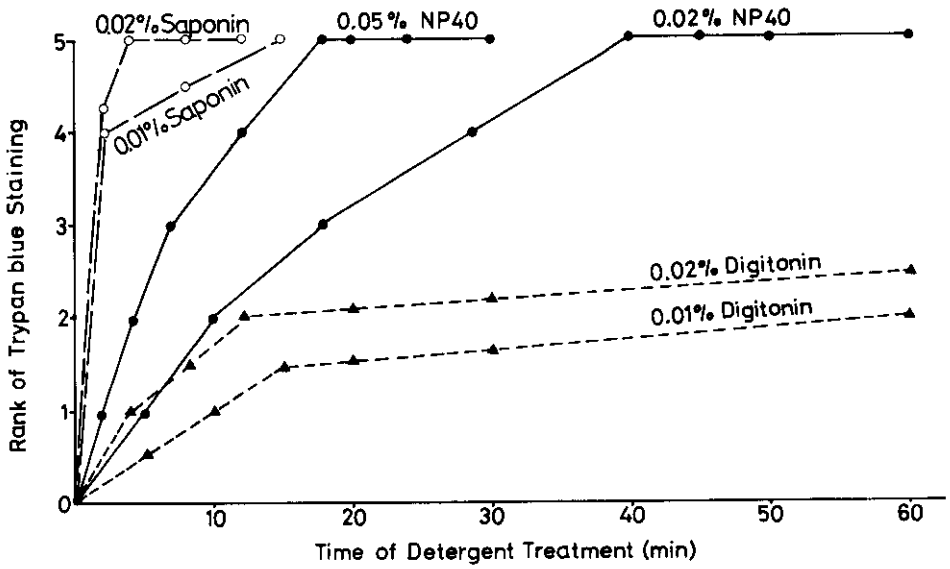

Fig. 1. Permeability of detergent-treated salivary glands to Trypan Blue.
Salivary glands were treated with a detergent at the indicated concentrations for 0-60 min, washed with MTB 1, and then incubated in MTB 1 containing 0.06% Trypan blue for 20 min. Permeations of Trypan blue into these detergent-treated glands are shown as ranks of Trypan blue staining ; the ranks were determined on the basis of the criteria listed in Table 1.

Results

1. Permeability to Trypan Blue

As shown in Fig. 1, all of the detergents examined here rendered salivary glands permeable to Trypan blue, but their effectiveness was different. Saponin was most effective and rendered salivary glands permeable to Trypan blue not only at a lower concentration but also at a shorter time period than the other detergents. Nonidet-P 40 (NP 40) permeabilized salivary glands as well as saponin but at a longer time of treatment, and the permeability of the NP 40-treated glands was reduced with time after the treatment (Fig. 2). In contrast, digitonin did not render the salivary glands permeable to the same extent as the other detergents even if the time of treatment was prolonged to 60 min (Fig. 1). Moreover, the permeability of the digitonin-treated glands,

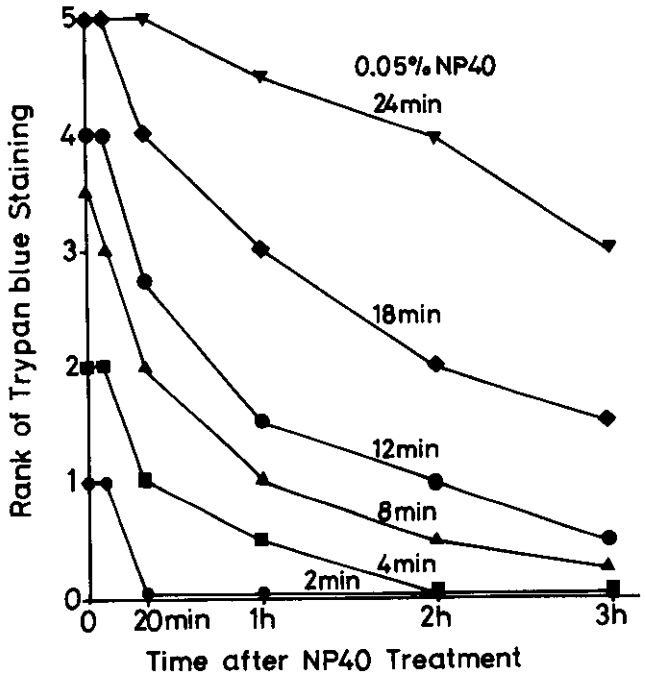

Fig. 2. Reduction of the permeability to Trypan blue in NP 40-treated salivary glands with the time after treatment.

Salivary glands were treated with 0.05% Nonidet-P 40 (NP 40) for the indicated length of time (2-24 min), incubated in MTB 1 for 0-3 hr, and then stained with 0.06% Trypan blue for 20 min. Permeations of Trypan blue into the NP 40-treated glands are shown as ranks of Trypan blue staining ; the ranks were based on the criteria listed in Table 1.

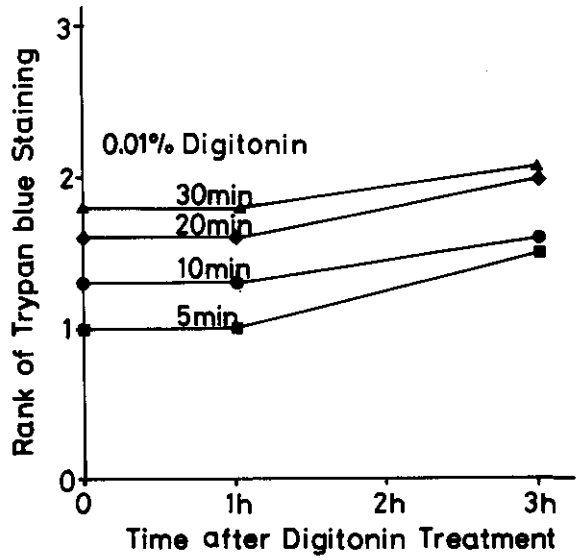

Fig. 3. Constancy of the permeability to Trypan blue in digitonin-treated salivary glands.

Salivary glands were treated with 0.01% digitonin for the indicated length of time (5–30 min), incubated in MTB 1 for 0–3 hr, and then stained with 0.06% Trypan blue for 20 min. Permeations of Trypan blue into the digitonin-treated glands are shown as ranks of Trypan blue staining ; the ranks were based on the criteria listed in Table 1.

unlike that of the NP 40-treated glands, was not reduced for at least 3 hr after treatment (Fig. 3).

2. Permeability to DNase I

The permeability of detergent-treated salivary glands was also examined using another index marker, DNase I, whose molecular weight is more than 30 times larger than that of Trypan blue. When detergent-treated salivary glands were incubated in a medium containing DNase I, the enzyme digested salivary gland nuclei and caused either fragmentation (Fig. 4, b) or disappearance (Fig. 4, c) of the chromosomes, but the digestion did not occur when untreated salivary glands were incubated with DNase I (Fig. 4, a). These results indicated that the enzyme permeated detergent-treated salivary glands but not untreated glands. Judging from the extent of nuclear digestion by DNase I (Table 2), saponin again was most effective among the four detergents. In contrast to the results of the experiments using Trypan blue as an index marker, digitonin was superior to NP 40 and Triton X-100 (TX 100) in the permeabilization of salivary glands to DNase I.

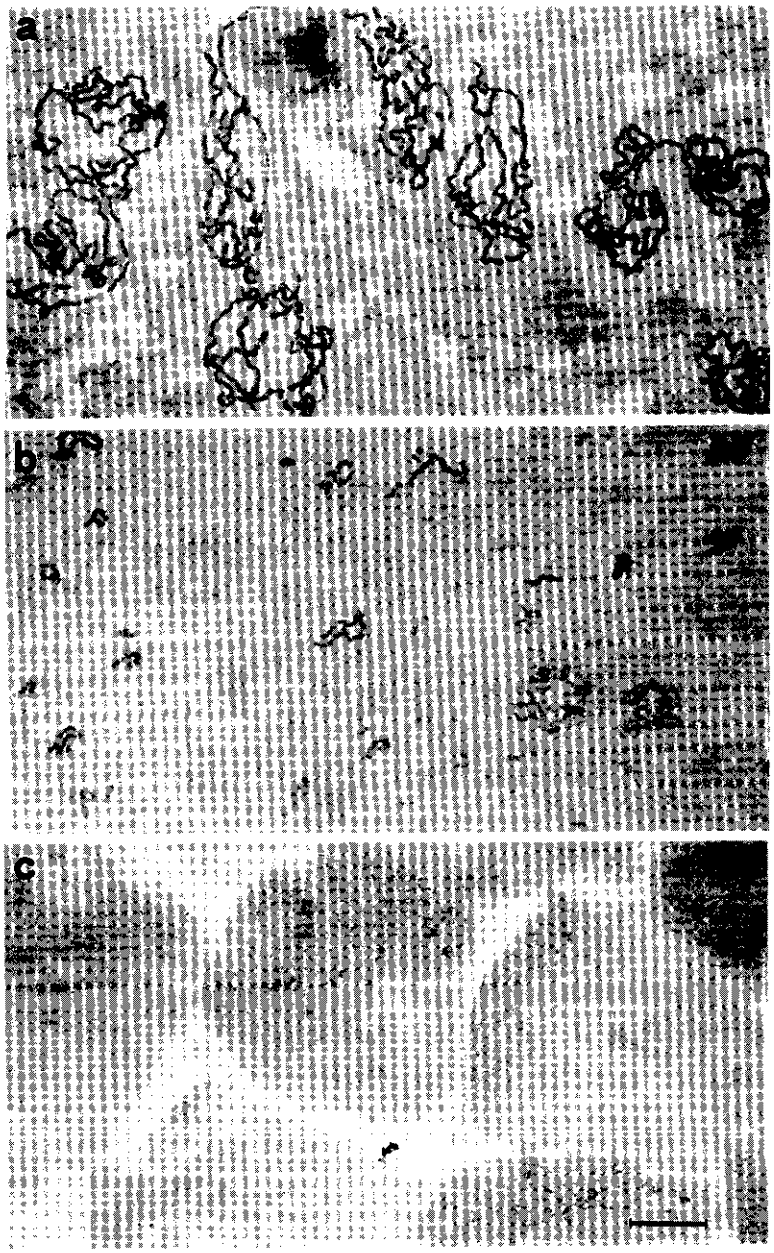

Fig. 4. Digestion of salivary gland nuclei by DNase I.

Untreated salivary glands (a), salivary glands treated with 0.005% saponin for 10 min (b), and salivary glands treated with 0.02 % saponin for 20 min (c). Glands were incubated in a medium containing 100 U/ml of DNase I for 20 min, fixed and stained with lactic-acetic-orcein, and then squashed for the observation of chromosomes. In detergent-treated salivary glands, but not in untreated glands, the fragmentation (b) and disappearance (c) of chromosomes were caused by DNase I digestion. Bar represents 50 μm.

Table 2 Permeability of Detergent-Treated Salivary Glands to DNase I*

Detergent treatment	Number of glands examined	Average number of intact nuclei per salivary gland (=A)	Percentage of nuclei digested by DNase I (=B)
None	26	134	0
Saponin			
0.005% 5 min	10	69	49
0.005% 10 min	16	42	69
0.005% 20 min	15	8	94
0.01% 20 min	11	0	100
0.02% 20 min	11	0	100
0.1% 20 min	4	0	100
0.5% 20 min	4	0	100
Digitonin			
0.01% 20 min	27	97	28
0.02% 20 min	17	40	70
0.1% 20 min	5	0	100
0.5% 20 min	5	0	100
Nonidet-P 40			
0.02% 20 min	9	109	19
0.1% 20 min	9	14	90
0.5% 20 min	4	0	100
Triton X-100			
0.02% 20 min	9	99	26
0.1% 20 min	9	3	98
0.5% 20 min	4	0	100

* The permeation of DNase I is shown as the percentage of nuclei whose chromosomes were fragmented or disappeared by enzyme digestion. Intact and detergent-treated salivary glands were incubated in a medium containing 100 U/ml of DNase I for 20 min. The percentage of digested nuclei (=B) was calculated on the presumption that the number of nuclei in a normal gland is 134 ; $B = (134-A) \div 134 \times 100$ (%).

Discussion

In the previous study (Myohara and Okada, 1987), we investigated the permeabilizing effects of the four detergents using β-galactosidase as an index marker, and found that digitonin rendered *Drosophila* salivary glands permeable to β-galactosidase at a lower concentration than other detergents, i.e., NP 40, TX 100, and saponin. Therefore, we expected that digitonin would be also more effective than other detergents in permeabilizing salivary glands to Trypan blue (MW 960 Da) and DNase I (MW 31 kDa), since the molecular weights of these substances are much lower than that of β-galactosidase (MW

465 kDa). Contrary to our expectation, however, the present study showed that digitonin was less effective than the other detergents for the permeabilization of salivary glands to Trypan blue. In the permeabilization of glands to DNase I, digitonin was superior to both NP 40 and TX 100, but was inferior to saponin. The unexpected result may be ascribed to differences in the properties of the detergents (see HELENIUS and SIMONS, 1975), i.e., NP 40 and TX 100 made a large number of small holes in the cell membrane, whereas digitonin made a small number of large holes, and saponin made a large number of intermediate sized holes.

The present study showed also that the permeability of NP 40-treated salivary glands was reduced with time after the treatment, whereas the permeability of digitonin-treated glands was not. This also is assumed to be a reflection of differences in properties among the detergents.

We previously showed that salivary glands treated with 0.01% digitonin for 20 min were permeable to ribonucleoside triphosphates (MW 500-600 Da), carbonic anhydrase (MW 29 kDa), egg albumin (MW 45 kDa), and bovine serum albumin (MW 66 kDa), besides β-galactosidase (Myohara and Okada, 1987, 1988 a). Considering the permeability of glands to these large molecules, it is likely that most biopolymers, including ribonuclease (MW 14 kDa), trypsin (MW 23 kDa), nuclear proteins (MW 10-100 kDa), immunoglobulin G (MW 150 kDa), and RNA polymerases (MW 500 kDa), can be introduced into the digitonin-treated salivary-gland cells. Because digitonin-permeabilized salivary glands retain their gene expression activity (MYOHARA and OKADA, 1987, 1988 a,b,c,), the method of cell permeabilization with digitonin may be applicable for investigating the effects of both purified and crude cell fractions on "living" chromosomes of not only *Drosophila* salivary glands but also of general diploid cells even of other organisms. In the search of appropriate conditions for the detergent treatment of cells or tissues other than the *Drosophila* salivary glands, the methods of Trypan blue staining and DNase I digestion, described in this study, should be useful for monitoring cell permeability. We believe that DNase I is especially suitable as an index marker because its permeation into detergent-treated cells can be evaluated simply by counting the number of digested nuclei.

Summary

Permeabilizing effects of four mild detergents (Nonidet-P 40, Triton X-100, digitonin, and Saponin) on *Drosophila* salivary glands were examined

with the following two indexes, the extent of Trypan blue staining and the percentage of digested nuclei after the incubation with DNase I. When intact (untreated) salivary glands were incubated in a medium containing either Trypan blue or DNase I, neither staining nor nuclear digestion occurred, indicating that such glands were impermeable to both Trypan blue and DNase I. In contrast, when detergent-treated salivary glands were incubated with Trypan blue or DNase I, staining or digestion occurred, indicating that the detergent rendered the salivary glands permeable to these substances. Among the four detergents examined, saponin was most effective in permeabilizing glands to both Trypan blue and DNase I. Nonidet-P 40 permeabilized glands to Trypan blue as strongly as saponin if a longer time of treatment was carried out, however, the permeability of Nonidet-treated glands was reduced with the time after treatment. Digitonin was superior to both Nonidet-P 40 and Triton X-100 in permeabilizing glands to DNase I, and the permeability of digitonin-treated cells was not reduced with the time after treatment.

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和 文 摘 要

界面活性剤処理したショウジョウバエ唾腺における細胞膜の透過性

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界面活性剤の1種ディジトニンで処理したショウジョウバエ唾腺は，細胞膜の透過性が高くなり，しかも核における遺伝子発現活性も維持されていることから，遺伝子発現調節因子の検定系として有用である。本研究では，ディジトニンおよび他の界面活性剤で処理した唾腺における細胞膜の透過性を，トリパン青による染色度とDNase I処理による染色体消化率とを指標として調べた。

界面活性剤で処理していない唾腺を，トリパン青（分子量960）を含む溶液中で培養した場合には，この染色剤が細胞膜を透過しないため細胞は全く染色されない。これに対して，界面活性剤で処理した唾腺では，トリパン青が細胞膜を透過し，処理の程度に応じた様々な強さで細胞が染色された。同様に，界面活性剤で処理をしていない唾腺をDNase I（分子量31,000）を含む溶液中で培養した場合には，細胞膜がこの酵素を通さないため，核内の染色体の消化は全く起こらないが，界面活性剤で処理した唾腺では，浸入した酵素による染色体の消化が認められた。これらのことを利用して，4種の界面活性剤（ディジトニン，サポニン，ノニデットP40，トリトンX100）の効果を比較したところ，サポニンが最も低濃度で細胞膜の透過性を高めるのに有効であった。また，ノニデットP40は濃度や処理時間の影響が著しい上に，処理後の時間経過に伴う膜透過性の低下が見られたが，ディジトニンは濃度や処理時間の影響が小さく，処理後の時間経過に伴う膜透過性の低下も認められなかった。

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