

## Concanavalin A-dorsalized Ventral Mesoderm Mimics the Neural-inducing Activity of the Organizer in *Cynops* Embryo

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**ABSTRACT**—The neural-inducing activity of ventral mesoderm aged after Con A treatment was examined in detail. To determine the time at which this function was attained, Con A-treated ventral mesoderm was incubated at different time lapses and sandwiched between competent ectoderm explants. Neural-inducing activity was found have been acquired as early as about 15 hr after Con A treatment and was maximal about 24 hr after Con A treatment. Brain, spinal cord and small neural tissues were observed.

Oligomannose-type oligosaccharide, a specific ligand of Con A, failed to inhibit dorsalization one hour after Con A treatment. This possibly hints that the ventral mesoderm might have been determined to proceed to dorsal differentiation about one hour from application of Con A. Autoradiographic observation showed accumulation of <sup>125</sup>I-Con A on or around the nucleus 24 hr after Con A treatment. This may suggest a possible role of the nucleus in the acquisition of neural-inducing activity of Con A-dorsalized ventral mesoderm.

### INTRODUCTION

In the early amphibian embryo, differentiation into a highly organized array of tissues and organs starts off as a result of a series of embryonic inductions. Two of these primary embryonic inductions, dorsalization and neural induction, are governed by the dorsal lip of the blastopore, so-called Spemann's organizer [1–4]. Dorsalization takes place in the mesodermal marginal zone and involves the formation of a dorso-ventral hierarchy of tissues under the influence of the organizer, the dorsalmost mesoderm. Neural induction involves the formation of neural tissues from the archenteric roof through the influence of the invaginating organizer.

Recently, we found evidence that treatment of the ventral mesoderm with Con A resulted in the differentiation of the dorsalmost tissue, the

notochord, with accompanying muscle and pronephros [5]. This dorsalizing effect of Con A on the ventral mesoderm appears to require internalization of the substance since immobilization of Con A on Sepharose beads did not result in dorsalization. On the other hand, Takata *et al.* [6–9] have suggested that in neural induction, Con A's target site probably resides in the asparagine-linked, high mannose-type oligosaccharide-rich receptors on the interior surface of the ectoderm. Con A has been unambiguously proven to mimic both the neural-inducing and dorsalizing functions of the organizer. Con A's modes of action in dorsalization and in neural induction appears to be different from each other.

In the foregoing paper [5], we presented preliminary evidence that Con A-treated ventral mesoderm was capable of inducing the competent ectoderm to form neural tissues. This time, the acquisition of neural-inducing activity by Con A-treated ventral mesoderm was followed through time in greater detail, and the pattern was com-



pared with that of the living organizer. Additional data on the mechanism of Con A action in dorsalization are also presented.

## MATERIALS AND METHODS

### Egg Handling

Female Japanese newts (*Cynops pyrrhogaster*) were injected with 25–50 IU's of gonadotropin (Gonadotropin, Teikoku Zoki, Tokyo, Japan) for three consecutive days. Eggs were collected and reared to early gastrula stage (St 11 according to the table of Okada and Ichikawa [10]) at 20°C. All microsurgical operations were done in Steinberg's balanced salt solution (SBSS: 58 mM NaCl, 0.67 mM KCl, 0.58 mM  $\text{CaNO}_3 \cdot 4\text{H}_2\text{O}$ , 1.3 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and 4.6 mM Tris), pH 7.8, with 20  $\mu\text{g}/\text{ml}$  of gentamycin (Gentamicin, Flow Laboratories, Irvine, Scotland) at 20–25°C.

### Sandwich culture

The outline of the procedure is shown in Figure 1. All cultures were incubated at 20°C. Ventral mesoderm was microsurgically excised and for ease of handling, the explants were first allowed to remain in SBSS for about an hour until they have rounded up. Ventral mesoderm explants were then treated with 2.83  $\mu\text{M}$  Con A (Lot No. 070207 CA, EY Labs, Inc., San Mateo, California, USA) in SBSS for 3 hr. The explants were then washed with excess SBSS, cultured for different time lengths, and sandwich between ectoderm explants. The explants were allowed to remain in sandwich for 8 hr during which the edges of the ectoderm explants come together and heal. With a glass needle, an incision was made approximately along the line where the edges meet and the ventral mesoderm was very carefully separated from the ectoderm taking care that every remaining mesoderm cell was removed. The ectoderm and ventral mesoderm were separately cultured for about 2 weeks. The explants were fixed overnight in Bouin's fluid. Embedding was done using paraffin wax. The sections were stained with Mayer's haemalum and examined for the presence of neural tissues in the ectoderm and dorsal mesodermal structures in the Con A-treated ven-

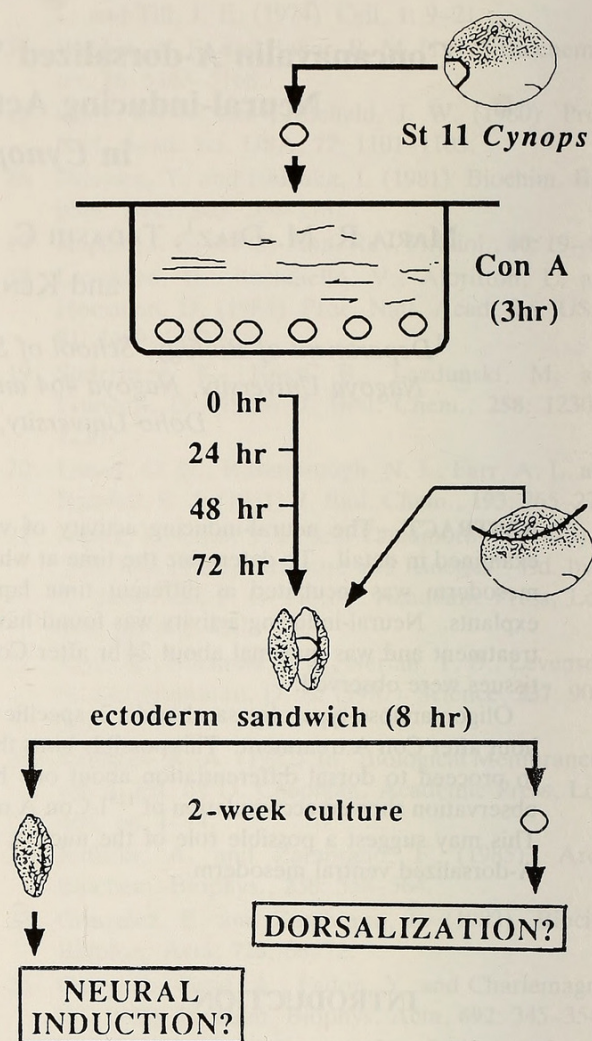


FIG. 1. Scheme for the sandwich experiment.

tral mesoderm. Ectoderm explants contaminated with mesoderm tissues were excluded from the scores.

A series where the sandwich was cultured as is for two weeks, without separating the ectoderm and Con A-treated ventral mesoderm components, was also done.

In the case where the organizer was used as inducer, the organizer explants used were obtained from early gastrula embryos. The sandwich experiment was done as described above where Con A-treated ventral mesoderm was used as inducer, except that the sandwich time was 4 hr.

### Inhibition experiment using oligomannose-type oligosaccharide

Ventral mesoderm was treated with 2.83  $\mu\text{M}$



Con A in SBSS for certain time lapses, immediately removed from the solution and washed in 30  $\mu$ M oligomannose-type oligosaccharide (a high-mannose type oligosaccharide isolated from ovalbumin [11]) in SBSS for 30 min. The explants were then washed in excess SBSS, cultured for about 2 weeks and processed for histological observation as in the above.

#### Microautoradiography

$^{125}$ I-Con A was prepared using Iodo-Gen (Pierce Chem. Co., Rockford, Ill., USA). The labelled sample was run in Bio-Gel P6DG filtration column (Bio-Rad, California, USA) and the fractions with the highest activities were collected. 2.83  $\mu$ M of  $^{125}$ I-Con A (655 kBq/nmol) was used in treating ventral mesoderm explants for 5 or 30 min at 20°C. Ventral mesoderm explants were thoroughly washed with SBSS, cultured for various time lengths and then fixed in 2.5% glutaraldehyde at 4°C overnight. In the case of the 5 min-labelling series, the explants were immediately fixed. The explants were then embedded in paraffin and sectioned at 6  $\mu$ m thickness. The sections were immersed in 10% H<sub>2</sub>O<sub>2</sub> overnight for depigmentation, air-dried and coated with NR-M2 autoradiographic emulsion (Konica Co., Japan). After 20 days exposure, the sections were developed in Rendol X (Fuji Film Co., Japan) and stained with Mayer's haemalum.

## RESULTS

### *Neural induction by Con A-treated ventral mesoderm*

To examine whether Con A-treated ventral mesoderm, which differentiated into dorsal tissues such as notochord and muscle, is capable of inducing the competent ectoderm to form neural tissues, it was sandwiched between ectoderm explants and cultured intact for about two weeks. High incidence of neural differentiation took place (Fig. 2a). When untreated ventral mesoderm was used as inducer, no neural differentiation took place at all (Fig. 2b).

To determine the time at which neural-inducing activity was attained by the Con A-treated ventral mesoderm, the ventral mesoderm was incubated at different time lapses (aging) and sandwiched between ectoderm explants. The sandwich components had to be separated after a minimum time lapse of sandwich culture, since the specific time at which neural-inducing activity was attained could not be determined with continuous culture of the ventral mesoderm together with the ectoderm in a sandwich.

In routine sandwich methods where the organizer was used as inducer [12, 13], 0.5–4 hr sandwich time was sufficient to bring about neural differentiation. However, in the case where Con A-treated ventral mesoderm was used as inducer, 4 hr sandwich time only resulted in the differentiation of small neural tissues; hence, the sandwich

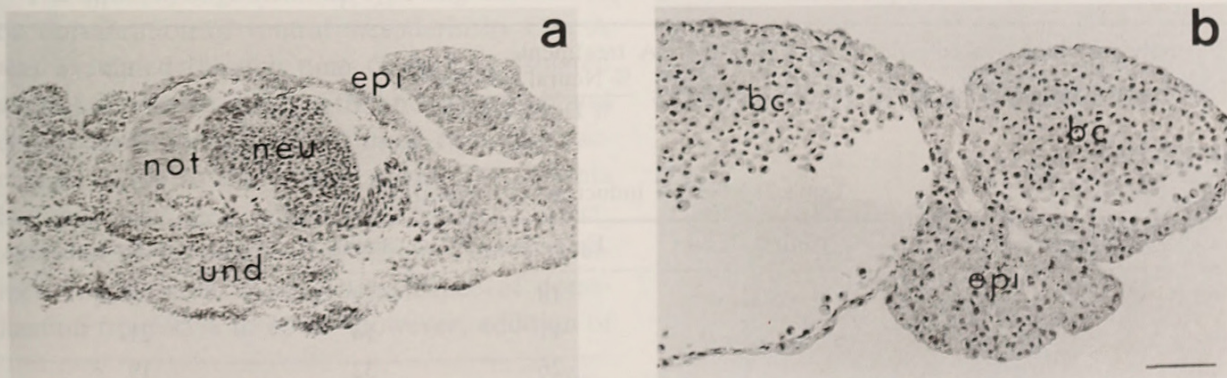


FIG. 2. a) Neural structures induced by Con A-treated ventral mesoderm continuously sandwiched in ectoderm for 2 weeks. Notochord (not), neural tissue (neu), epidermis (epi) and undifferentiated mesoderm cells (und). b) Absence of neural structures in ectoderm explants used in a sandwich with an untreated ventral mesoderm. Blood cells (bc). Scale bar is 100  $\mu$ m.



time was increased to 8 hr. With 8 hr sandwich time, larger neural structures could be obtained.

Another problem that had to be overcome is the fact that Con A is a potent neuralizing agent [6-9, 14, 15]. Therefore, the possibility exists that Con A remaining on the ventral mesoderm might contribute to neural induction. To distinguish between the neuralizing effect of remnant Con A and that of the dorsalized ventral mesoderm, the 0-hr control series where the ventral mesoderm was not allowed to age or develop further in culture, but sandwiched immediately after Con A treatment, was done. Incidence of neural induction in this series could then be attributed to the effect of remnant Con A and minimally, if any, to the dorsalized ventral mesoderm. Only one very small neural structure of which regionality could not be identified was detected out of 30 explants (3%). This indicates that the effect of remnant Con A is negligible, and it is expected that this residual

effect will diminish as the aging period gets longer.

The neural-inducing activity of ventral mesoderm aged after Con A treatment is expressed relative to the extent of dorsalization. Thus, "relative neural induction" or RNI was adopted as an index of neural differentiation caused by the dorsalized ventral mesoderm (Table 1).

When ventral mesoderm cultured 15 hr after Con A treatment was used as inducer RNI was 11%. When ventral mesoderm cultured 20 hr after Con A treatment was used, RNI increased to 20%. RNI consistently increased to 42% in the 24 hr-series. This is the highest value obtained in all the series. Thirty hours after Con A treatment, average RNI decreased to 32%. In the Con A-treated ventral mesoderm cultured for 36 and 42 hr, neural induction was not observed. Forty-eight and 72 hr after Con A treatment, the ventral mesoderm exhibited a rather weak neural-inducing activity.

Brain-like structures and spinal cord were

TABLE 1. Neural induction by Con A-treated ventral mesoderm

Ventral Mesoderm Culture Time <sup>a</sup> (hr)	Total no. of sandwiches (no. of experiments)	Neural Differentiation in the ectoderm (%)	Dorsalized Ventral Mesoderm (%)	Relative Neural Induction (%) <sup>b</sup>
0	30 (2)	3	48	6
15	34 (3)	9	82	11
20	31 (2)	16	81	20
24	63 (3)	35	84	42
30	29 (2)	21	66	32
36	19 (2)	0	63	0
42	31 (3)	0	58	0
48	30 (2)	13	83	16
72	28 (2)	7	71	10

<sup>a</sup> Number of hours in culture elapsed after Con A treatment.

<sup>b</sup> Relative neural induction (RNI) is calculated as  $\frac{\% \text{ Neural Induction}}{\% \text{ Dorsalization}} \times 100$

TABLE 2. Neural induction by organizer

Organizer culture time (hr)	Neural Tissue	Epidermis	Total	Neural Induction (%)
0	11	18	29	38
3	3	11	14	21
6	6	26	32	19
10	4	20	24	17
15	1	25	26	4
20	4	28	32	12



TABLE 3. Inhibition of Con A-induced dorsalization by oligomannose-type oligosaccharide

Treatment	Not	Mus	Pron	Blood	Meso	Undiff	Total	Dorsal	Ventral	Undiff	Dorsal (%)
ventral mesoderm alone	0	0	0	15	0	2	17	0	15	2	0
vm + oligo <sup>a</sup>	0	0	0	5	0	10	15	0	5	10	0
Con A (0.5 hr)	12	15	2	3	0	0	19	18	1	0	95
vm + Con A + oligo <sup>b</sup>											
0.5 hr	5	13	4	13	8	4	33	14	15	4	42
1 hr	20	20	4	5	1	3	28	21	4	3	75
2 hr	12	11	5	3	3	0	15	13	2	0	87

<sup>a</sup> vm (ventral mesoderm) treated with oligo (oligomannose-type oligosaccharide), 30  $\mu$ M, for 30 min.

<sup>b</sup> Ventral mesoderm was treated with Con A (2.83  $\mu$ M) for different time lapses before washing with oligomannose-type oligosaccharide for 30 min.

observed in 83% of neural tissues induced by Con A-treated ventral mesoderm in the early phase of aging (data not shown). Only small, unidentifiable neural structures surrounded by epidermal cells were observed when Con A-treated ventral mesoderm incubated for more than 24 hr was used.

#### Neural induction by the living organizer

Thirty-eight per cent neural induction was observed when isolated St 11 organizer (0 hr organizer) was used as inducer (Table 2). Incidence of neural differentiation gradually went down to 4% as the organizer was aged for 15 hr, but it went slightly higher (12%) when the organizer used was aged for 20 hr.

#### Inhibitory effect of oligomannose-type oligosaccharide on Con A-induced dorsalization

The effect of oligomannose-type oligosaccharide on dorsalization of ventral mesoderm by Con A was examined through time (Table 3). Isolated ventral mesoderm simply differentiated into blood cells. Addition of oligomannose-type oligosaccharide alone to the ventral mesoderm explants had no toxic, deleterious or dorsalizing effect. Addition of the sugar after 30 min Con A treatment significantly decreased incidence of dorsalization from 95% to 42%. However, addition of

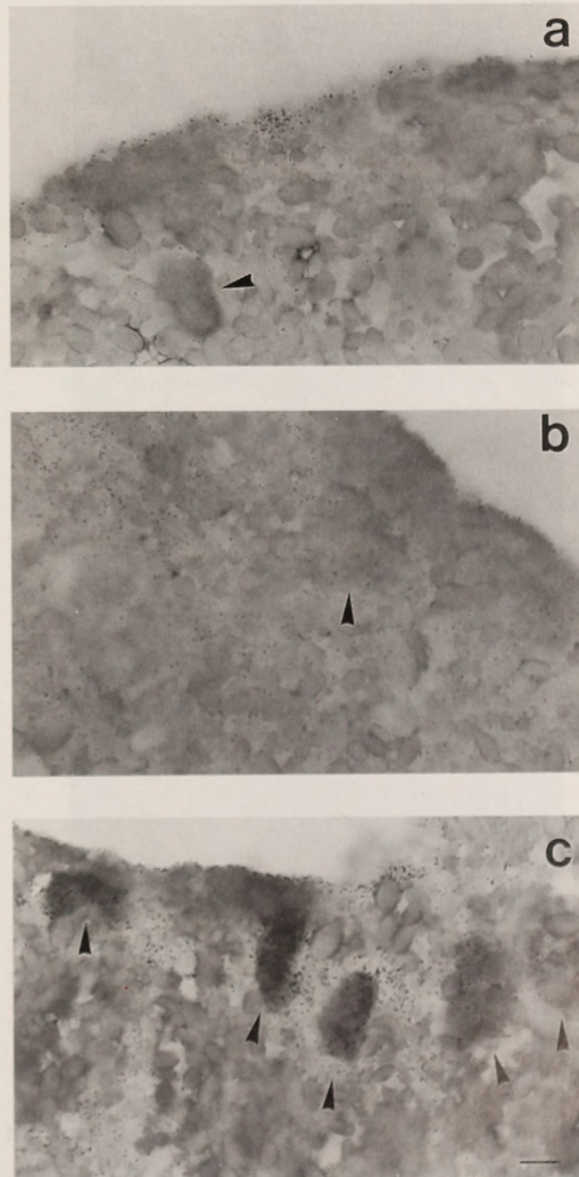


FIG. 3. a) Ventral mesoderm explant treated with <sup>125</sup>I-Con A for 5 min. b) Ventral mesoderm explant treated with <sup>125</sup>I-Con A for 30 min and further cultured for 30 min or c) further cultured for 24 hr. Arrowhead indicates nucleus. Scale bar is 10  $\mu$ m.



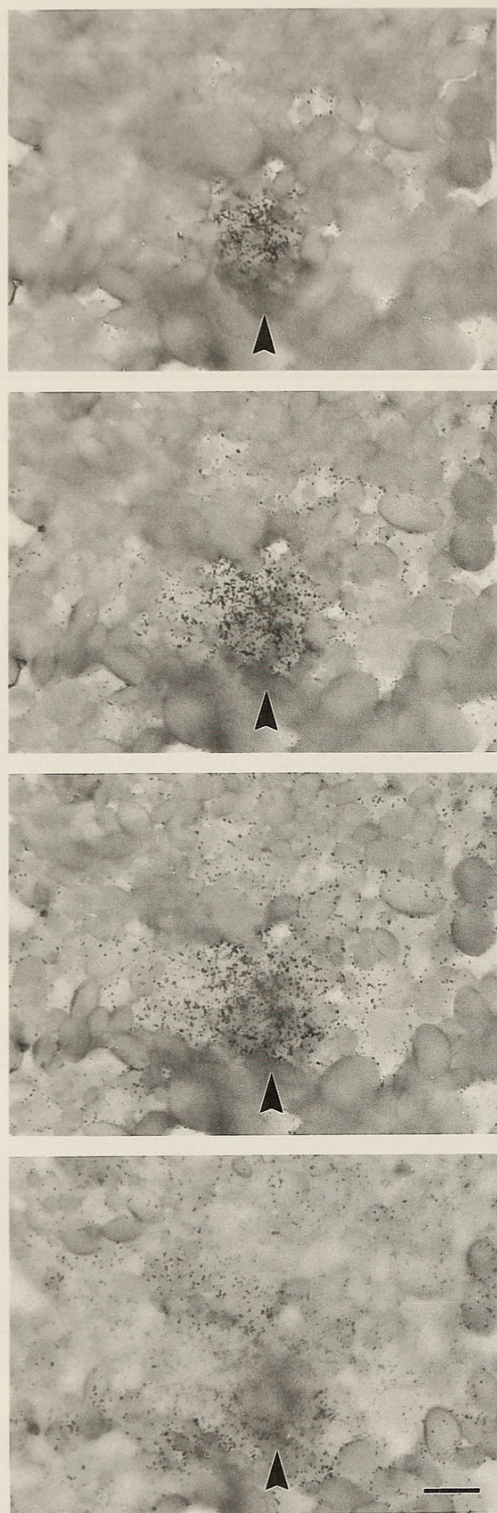


FIG. 4. Microautoradiograms taken at different focus levels of ventral mesoderm cultured 24 hr after  $^{125}\text{I}$ -Con A treatment. Arrowhead indicates nucleus. Scale bar is 10  $\mu\text{m}$ .

the sugar after 1 or 2 hr Con A treatment did not decrease incidence of dorsalization by Con A.

Seventy-five and 87% dorsalization, respectively, are within the range of normally expected values in Con A-treated ventral mesoderm explants (refer to Table 1).

#### *Localization of $^{125}\text{I}$ -Con A on the ventral mesoderm*

Some silver grains, predominantly on the surface of the explant, could already be observed with just 5 min treatment of the ventral mesoderm with  $^{125}\text{I}$ -Con A (Fig. 3a). A cluster of silver grains appears to be in the process of internalization. More internalized grains could be observed in the ventral mesoderm treated with Con A for 30 min and cultured for an additional 30 min (Fig. 3b). As culture time was increased, there was an apparent tendency of silver grains to converge on or around the nucleus. This tendency was most pronounced in ventral mesoderm treated with  $^{125}\text{I}$ -Con A for 30 min and cultured for an additional 24 hr (Fig. 3c). Figure 4 shows the relative distribution of silver grains on or around the nucleus and on the cytoplasm of a section whose photomicrograph was taken at different focus levels.

### DISCUSSION

Compared to the neural tissues induced by the living organizer, those by Con A-dorsalized ventral mesoderm were qualitatively inferior. Nevertheless, the incidence of neural induction by Con A-dorsalized mesoderm is almost similar to that using the organizer, at least in our experimental system. The relatively low incidence of neural differentiation and inferior architecture of induced tissues in these cultures, compared to that in continuous sandwich cultures, might have been due to the trauma caused by the separation procedure.

Using Con A-dorsalized mesoderm as inducing material, we were able to show that neural induction could take place as early as about 15 hr after Con A treatment. The peak of neural-inducing activity about 24 hr after Con A treatment may correspond to the neural-inducing activity of St 11 *Cynops* organizer. It should be noted here that acquisition of neural inducing ability by Con A-treated ventral mesoderm was rapid and after



reaching maximal activity around 24 hr, the activity gradually went down. Whether the low neural-inducing ability of the 48- and 72-hr aged Con A-treated ventral mesoderm is significant or not remains to be further confirmed since only small, unidentifiable neural structures were observed.

Since the neural-inducing potential was acquired by the Con A-treated ventral mesoderm as early as about 15 hr after Con A treatment, we could expect that determination of dorsal differentiation must have occurred during this elapsed time. The time-course inhibition experiment using oligomannose-type oligosaccharide could offer some evidence in support of this.

Addition of the oligosaccharide after 30 min Con A inhibited dorsal differentiation. However, when the oligosaccharide was added after 1 hr Con A treatment, the inhibitory effect of the sugar on dorsalization was cancelled. This might suggest that the ventral mesoderm may have been determined to proceed to dorsal differentiation as early as 1 hr after Con A treatment even in the presence of the inhibitor. However, we cannot exclude the possibility that the cause of the decrease in the frequency of dorsalization after 30 min Con A treatment was simply the reduced amount of internalized Con A and that the reason why inhibitory effect was absent after 1 or 2 hr Con A treatment was that sufficient amount of Con A had been internalized by the ventral mesoderm at these time points.

In neural induction, Con A molecules were localized on the interior surface of the ectoderm 3 hr after treatment, and cytoplasmic localization was only observed 20 hr after treatment [6]. The authors suggested that internalized Con A molecule was not responsible for neural induction. On the other hand, in dorsalization, Con A molecules were found to accumulate inside the ventral mesoderm cells rather than on the surface lending support to our hypothesis that it is internalized Con A which triggers the process [5]. Localization of Con A on or around the nucleus 24 hr after treatment suggests a possible interaction of Con A with the nucleus. It would be interesting to speculate that this interaction might play a role in the ensuing processes of dorsal differentiation, specifically that step(s) which determines the ac-

quisition of neural-inducing activity.

The model system used here, i.e., Con A-dorsalized ventral mesoderm, has made possible studies on the temporal specificity of organizer action, which might have been difficult to perform using the organizer.

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