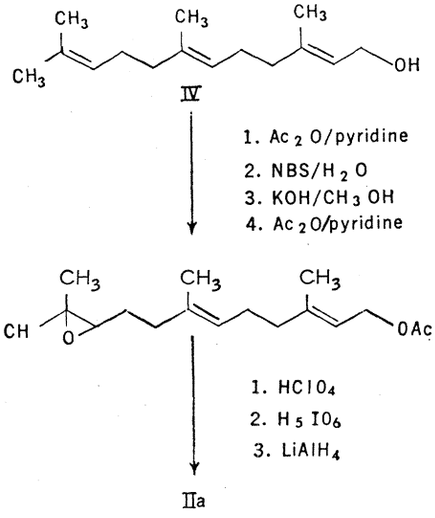
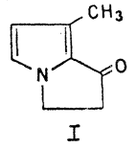


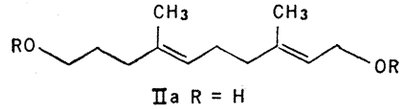
Fig. 1. Synthesis of *trans,trans*-3,7-dimethyldeca-2,6-dien-1,10-diol (IIa).

(I) previously characterized as a major component in the hairpencil secretion of the neotropical danaid *Lycorea ceres ceres* (*2*).



Additional infrared absorption in the 2.7- to 3.0-μ region indicated the presence of hydroxylic components as well. Thin-layer chromatography (TLC) on silica gel (developed with 5 percent methanol in methylene chloride) showed two major spots, along with two minor ones. The least polar component was indistinguishable from an authentic sample of I on the basis of comparisons of thin-layer gas chromatograms. This component could be isolated in crystalline form either by direct sublimation (60° to 65°C at 1 mm-Hg) from the crude extract or by preparative TLC; a mixture melting point with a synthetic sample of I was undepressed.

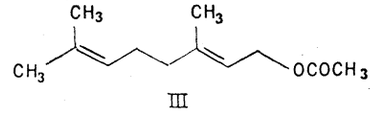
The major polar component of the queen hairpencil extract was most conveniently isolated by preparative TLC after most of the heterocyclic ketone was removed by vacuum sublimation. It was characterized as *trans,trans*-3,7-dimethyldeca-2,6-dien-1,10-diol (IIa) on the basis of the evidence summarized below.



IIa R = H
IIb R = CH_3CO
IIc R = C_6H_5CO
IId R = $Si(CH_3)_3$

The diol (IIa) was eluted from silica gel plates as a colorless, viscous oil with little characteristic infrared absorption beyond hydroxyl and carbon-hydrogen bands. It was converted into the corresponding diacetate (IIb), dibenzoate (IIc), and bis-trimethylsilyl ether (IId) by standard procedures. While neither the diol nor its diacetate could be purified by gas chromatography without decomposition, the mass spectrum (*3*) of the diacetate showed its highest mass/charge (*m/e*) peak at 222, corresponding to expectations for loss of acetic acid from a parent $C_{16}H_{26}O_4$ molecule (molecular weight, 282). The mass spectrum of IId purified by gas chromatography showed a parent peak at *m/e* 342 and an M-15 peak at *m/e* 327, thus confirming the $C_{12}H_{22}O_2$ formula for the diol itself.

The nuclear magnetic resonance (NMR) spectrum (*4*) of IIb showed a striking resemblance to that of geranyl acetate (III), with an additional low-field triplet (τ 6.0, J = 7 hz, 2H) corresponding to a second oxygen-bearing methylene group split by an adjacent methylene. The NMR spectrum of the oily dibenzoate showed all the features to be expected for structure IIc, and the formulation of the diol as an oxidatively degraded sesquiterpenoid closely related to farnesol (IV), appeared to be a most attractive hypothesis on this basis (*5*).



A synthesis of diol IIa from *trans,trans*-farnesol (IV) was accomplished as shown in Fig. 1 (*6*). The final product was identical to the natural diol in all respects. Treatment of the natural product with α-naphthyl isocyanate yielded a bis-α-naphthyl urethane which showed a melting range of 127° to 129°C after recrystallization from an ether-hexane mixture. The synthetic diol gave a bis-α-naphthyl urethane with the same melting range and a mixture melting point was undepressed.

The amounts of I obtainable from the male butterflies varied. Thus, in different batches of insects caught in the wild, recoveries of crude I averaged from 0.1 to 0.02 mg per male. The amount of diol II was very difficult to estimate, since no procedure for efficient isolation and purification of this component was developed. The secretion did not appear to be subject to qualitative variation. Extracted hairpencils from males of another subspecies (*Danaus gilippus strigosus*, from Rodeo, New Mexico) also yielded compounds I and IIa.

Whether these compounds are elaborated *de novo* by the butterflies or are obtained by transformations carried out on closely related precursors which might be available in their food remains to be investigated.

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3. We are indebted to Dr. G. Dudek, Harvard University, for the mass spectral results presented in this paper. All measurements were made on an AEI (Calvert Electronics) MS-9 double-focusing instrument. A more thorough study of the mass spectral fragmentation pattern of IIa and many related molecules has been carried out by Drs. S. Shrader and A. M. Chalmers with the M-902 C1 instrument at Cornell University.
4. These NMR data were obtained through Dr. R. Pitcher, Varian Associates.
5. Subsequent to the completion of this work, a C_{13} allenic ketone and a C_{12} diacid, both oxidatively degraded terpenoids, have been characterized as components of other insect secretions [J. Meinwald, K. Erickson, M. Hartshorn, Y. C. Meinwald, T. Eisner, *Tetrahedron Lett.* **1968**, 2959 (1968); J. Meinwald, A. M. Chalmers, T. E. Pliske, T. Eisner, *ibid.*, p. 4893].
6. These results were described at the 4th International Union of Pure and Applied Chemistry Symposium on the Chemistry of Natural Products, Stockholm, June 1966 (Abstr., p. 147). A portion of this synthetic sequence had been carried out earlier by Prof. E. E. van Tamelen at Stanford University, whom we thank for providing us with the appropriate experimental procedures.
8. Supported by NIH grant AI-02908, and by a generous gift from Hoffman-La Roche, Inc. Extracts supplied by R. Alsop, T. Eisner, and T. Pliske.

22 October 1968

Polyvinylsulfate: Interaction with Complexes of Morphogenetic Factors and Their Natural Inhibitors

Abstract. *The inactivation of the mesodermalizing inducing factor by its natural inhibitor is completely abolished by low concentrations of polyvinylsulfate.*

The differentiation of the early gastrula ectoderm of *Triturus* can be channeled into different pathways. The "neuralizing" factor initiates the differentiation of neural tissue, especially forebrain; the "mesodermalizing" factor, which is protein in nature, induces the formation of muscle and notochord (*1, 2*). The formation of complex trunk and tail structures (with spinal cord,

Table 1. Inducing activity of the protein fraction and of recombinations between the protein fraction and the inhibitory aqueous phase in the presence or absence of polyvinylsulfate. For preparation of the protein fraction and the aqueous phase, supernatant obtained after centrifugation at 105,000*g* was extracted with an equal volume of 80 percent phenol for 5 minutes at 60°C. After the mixture was cooled, the phenol layer and the aqueous layer were separated by centrifugation (15 minutes, 20,000*g*, +4°C), and the phenol layer together with the middle layer was precipitated with 4 volumes of 96 percent ethanol. The precipitate was washed six times with 96 percent and twice with 66 percent ethanol and then suspended in 0.085*M* NaCl (~ 10 mg/ml). The aqueous layer was extracted again with an equal volume of 80 percent phenol, precipitated with 2 volumes of 96 percent ethanol at −20°C, and washed six times with 96 percent ethanol and twice with a mixture of 66 percent ethanol and 0.085*M* NaCl. The precipitate was dissolved in 0.085*M* NaCl (~ 800 μg of RNA per milliliter). The yield was ~ 400 mg of protein and ~ 6 mg of RNA from 50 g of trunks. In experiments 2 and 4, the aqueous phase was incubated for 60 minutes at 37°C before combination with protein. In some cases of experiment 4, PVS was added before incubation at 37°C, in other cases after incubation at 37°C. The results were identical. To the proteins or the combined fractions urea (6*M*) was added. The fractions were dialyzed for 24 hours against 5 liters of water (two times changed) at +2°C, precipitated with two volumes of ethanol at 0°C after addition of 0.05 volume of 10-percent NaCl, and centrifuged. After being washed twice with 66 percent ethanol, the pellet was dried on a suction pump (18 torr, 18°C, 2 hours) and tested on *Triturus alpestris* (*13*).

Experiment	Aqueous phase (ml)	Protein (mg)	PVS (μg)	Total cases (No.)	Positive (%)	Size of induction (%)			Induced region (%)			
						Large	Medium	Small	Forehead	Hindhead	Trunk-tail	Other
1	0	2.5	0	86	78	37	30	10	0	16	62	12
2	0.5	2.5	0	53	38	8	6	25	0	4	6	28
3	0.52	3.0	0	29	52	3	10	38	0	3	3	45
4	0.5	2.5	5	88	72	23	14	35	0	5	53	17
5	0.5	2.5	25	28	93	64	18	11	4	61	18	25

muscle, and notochord) and of deuterencephalic structures (with hindbrain, ear vesicles, and head muscle) depends on the interaction between the induced tissues (*3*). Earlier data support the hypothesis that both inducing factors are present in ectoderm of early newt gastrulas in inactive form (*4*). Neural or mesodermal differentiation proceeds in the ectoderm when one of them is activated or when one of the active factors is added in excess. Our results contribute to the understanding of the activation and inactivation of the mesodermalizing factor.

The mesodermalizing factor has been extracted from chicken embryos (and amphibian embryos) in an inactive form. Frozen and thawed trunks from 9- to 11-day-old chicken embryos were homogenized with two volumes of 0.085*M* NaCl and centrifuged for 2 hours at 105,000*g*. The supernatant shows only a very weak inducing capacity. Mesodermalizing factor present in the supernatant can be activated by extraction with phenol (*2*). The protein fraction, which is precipitated from the phenol phase, induces the formation of trunks and tails in the belly ectoderm (Table 1, experiment 1). The factors are tested by implanting pieces of equal size of ethanol-precipitated pellets into the blastocoele of early *Triturus* gastrulas (*5*). The mesodermalizing factor is also activated if the supernatant is electrophoretically separated into a protein and a ribonucleoprotein fraction (which also contains glycoproteins). The protein induces trunk and tail structures (*6*).

The inducing activity can be inhibited again if the phenol-activated protein fraction is recombined with the aqueous phase in 6*M* urea, dialyzed for 24 hours against H_2O, and prepared for testing (Table 1, experiments 2 and 3). The aqueous phase contains RNA of low molecular weight, proteins, and polysaccharides. The inhibitory activity of the aqueous phase is partially reduced by treatment with trypsin and by treatment with pancreatic ribonuclease. Whether polysaccharides are involved in the inhibition is not known. Ribosomal RNA from chicken embryos, as well as supernatant RNA from baker's yeast and from *Salmonella typhimurium*, exert no inhibitory activity (*7*).

The inhibition is reversed if the polyanion potassium polyvinylsulfate (PVS) (*8*) is added to the mixture of the inducing protein fraction and the aqueous phase in 6*M* urea before dialysis and precipitation with ethanol. A concentration as low as 5 μg of PVS per 1.2 ml abolishes the inhibition almost completely (Table 1, experiment 4). If the concentration of PVS is increased to 25 μg/1.2 ml, the percentage of spinocaudal inductions is reduced but the percentage of deuterencephalic inductions is increased (Table 1, experiment 5). Although PVS cannot be precipitated with two volumes of ethanol at concentrations mentioned above, it is however coprecipitated with protein and ribonucleic acid under the conditions of the recombination experiments (*9*).

Gastrula ectoderm contains a small quantity of mesodermalizing factor besides a larger amount of neuralizing factor in inactive form. Therefore one has to exclude the possibility that PVS can activate the mesodermalizing factor by interacting with control circuits in the tissue. Polyvinylsulfate has been coprecipitated with an inert protein, like γ-globulin, which has almost no inducing capacity. γ-Globulin itself induces neural structures only to a very small extent (Table 2). If a coprecipitate of 5 μg of PVS and 2.5 mg of γ-globulin is tested, neural structures (forebrain and eyes) differentiate in 23 percent of all cases; small tissue complexes which consist of neuroid cells, mesenchyme, and melanophores are also formed (Table 2, experiment 1) (*10*). If PVS is added in larger amounts, neural differentiations—especially forebrain and eyes—are formed in a higher percentage of cases (Table 2, experiment 2). Obviously the neuralizing factor in the ectoderm is activated under these conditions.

The mesodermalizing factor on the other hand is reactivated because PVS interferes directly with the recombination of the protein fraction (which contains the mesodermalizing factor) with

Table 2. Neuralization of ectoderm after implantation of polyvinylsulfate-γ-globulin. γ-Globulin and γ-globulin–PVS were dissolved in 1.2 ml of 6*M* urea and prepared for testing as described in the legend of Table 1.

Experiment	PVS (μg)	Cases (No.)	Positive (%)	Size of induction (%)			Induced region (%)			
				Large	Medium	Small	Forehead	Hindhead	Trunk-tail	Other
1	5	30	57	10	13	33	23	0	0	33
2	25	18	83	56	17	11	61	0	0	22
3	0	72	17	0	3	14	7	0	0	10

the inhibitor (Table 1, experiment 4). The shift of the inducing activity from spinocaudal to deuterencephalic structures at 25 μg PVS (Table 1, experiment 5) can be explained by the activation in vitro of the mesodermalizing factor and the activation in vivo of the capacity of the ectoderm to differentiate into neural structures.

It is not unlikely that PVS competes with binding sites for a ribonucleoprotein or acidic glycoprotein in the complex of mesodermalizing factor and inhibitor complex (*11*). The action of PVS in ectoderm in vivo is more difficult to explain. The neuralizing action could be caused by interaction of PVS with a complex between the neuralizing factor and its inhibitor. The neuralizing factor is more easily activated than the mesodermalizing one. But the effect of PVS on ectoderm could also be caused by the interaction of PVS with DNA-histone bonds in chromatin or by interaction with cytoplasmic membranes. Polyethylene sulfate activates the aggregate RNA polymerase from rat liver nuclei (*12*).

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8. The polyvinylsulfate (Eastman Kodak) migrates in the analytical ultracentrifuge as a single peak with $s_{25°,w} \sim 7.4$ ($c \to 0$).
9. We thank the analytical laboratory of the Schering AG., Berlin, for performing the sulfur analyses.
10. Under these conditions the PVS which is implanted into a gastrula amounts to about 5×10^{-2} μg.
11. PVS has no effect on the inhibition of trypsin by soybean trypsin inhibitor.
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13. Other species are less suitable for these experiments. Gastrula ectoderm of *Ambystoma punctatum* and of *Triturus taeniatus* show a stronger neural reaction when mixed inducers like the phenol-extracted protein, which contain besides the mesodermalizing factor also some neuralizing factor, are tested.
14. Supported by the Deutsche Forschungsgemeinschaft.

3 January 1969; revised 14 March 1969

Colchicine-Inhibited Cilia Regeneration: Explanation for Lack of Effect in Tris Buffer Medium

Abstract. *In* Stentor coeruleus *growth of new daughter ciliates and experimentally induced regeneration of oral membranellar cilia are reversibly inhibited by low, nontoxic concentrations of colchicine. However, if the culture medium containing colchicine (or Colcemid) is made up in tris(hydroxymethyl)aminomethane buffer, growth of ciliated daughters and regeneration of oral cilia proceed normally. The evidence suggests that the mechanism of this reversal of the effects of colchicine (or Colcemid) is due to a chemical reaction between tris(hydroxymethyl)-aminomethane (or its hydrochloride, or both) and colchicine (or Colcemid), which reduces the effective concentration of these mitotic spindle inhibitors reaching the stentors.*

Stentor coeruleus can be experimentally induced to shed its oral membranellar cilia in response to toxic substances in the medium (*1*). If replaced into normal medium all shed cells regenerate their oral cilia within hours (*1*). Cilia and flagellar regeneration is sensitive to colchicine (*2*, *3*). Colchicine, approximately 10^{-6} to $10^{-8}M$, reversibly inhibits regrowth of the membranellar band in *Stentor* for up to 8 hours—as long as the cells remain in colchicine (*3*). The effect of colchicine and Colcemid in blocking cilia regeneration is entirely obliterated when Trizma® buffer (*4*) is used in the culture medium (Table 1). We now present evidence that colchicine and Colcemid react with tris(hydroxymethyl)aminomethane(tris) or with tris hydrochloride or with a combination (Trizma) to form new compounds.

In the course of testing the possible toxic effect of these drugs on normal stentors, we found that, although the cells survived for over 3 weeks, they seemed unable to produce normal daughters, presumably because formation of new cilia is inhibited and macronuclear abnormalities are produced (Fig. 1). Thus we studied the effect of colchicine and Colcemid (*5*) on the growth rate of stentors. Cell increase did not occur in the presence of either colchicine or Colcemid. This effect was also obliterated when the colchicine or Colcemid was made up in tris buffer (Fig. 2). The toxicity of Trizma alone was also investigated. Cells grew well in 10^{-5} to $10^{-2}M$ Trizma, but died in higher concentrations of this buffer. Unlike the usual dead stentors (*6*) car-

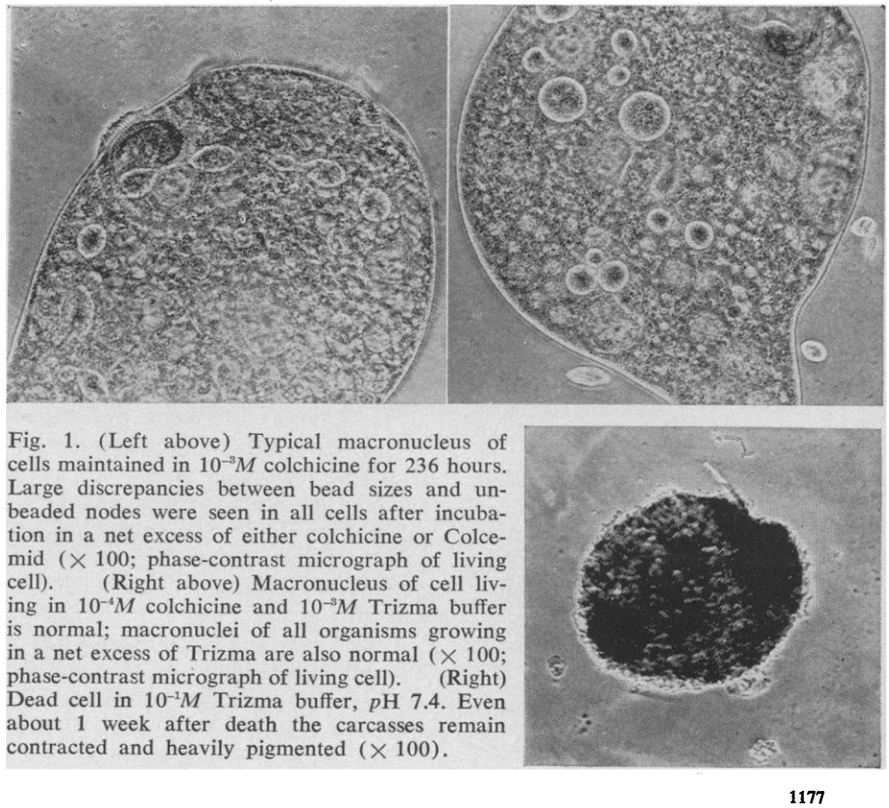

Fig. 1. (Left above) Typical macronucleus of cells maintained in $10^{-3}M$ colchicine for 236 hours. Large discrepancies between bead sizes and unbeaded nodes were seen in all cells after incubation in a net excess of either colchicine or Colcemid (× 100; phase-contrast micrograph of living cell). (Right above) Macronucleus of cell living in $10^{-4}M$ colchicine and $10^{-3}M$ Trizma buffer is normal; macronuclei of all organisms growing in a net excess of Trizma are also normal (× 100; phase-contrast micrograph of living cell). (Right) Dead cell in $10^{-1}M$ Trizma buffer, *p*H 7.4. Even about 1 week after death the carcasses remain contracted and heavily pigmented (× 100).