Table 1. Preservation of microtubules in vitro. Each value is the average of three grids examined as in Fig. 2. Rat brain was homogenized in hexylene glycol at *p*H 6.4. Equal parts of the crude mitochondrial fraction were distributed in three additional media, washed twice, and incubated 1½ hours at room temperature.

Exp. No.	Microtubules per grid			
	Hexylene glycol		Buffer only	
	*p*H 6.4	*p*H 7.4	*p*H 6.4	*p*H 7.4
R 54	16	5	6	0
R 55	35	14	6	1
R 56	36	12	5	1
R 57	26	9	3	0

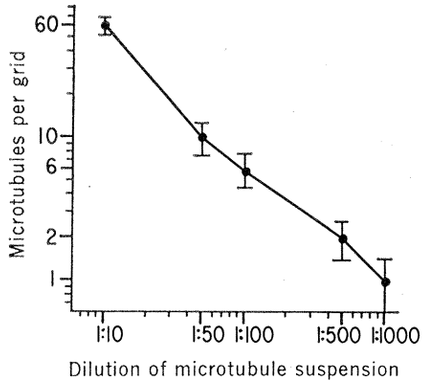

Fig. 2. Relation of microtubule counts to dilutions of homogenates. Each point represents the average of six negatively stained grids, examined in the electron microscope without knowledge of the source. Bars show ± standard error of the mean. Twenty grid squares of a No. 400 grid (about 0.017 mm²) were scanned at × 18,000, and each microtubule was counted as 1; logarithmic coordinates.

most of microtubules in brain homogenates are of neuronal origin. Rats were used in most experiments; results with mouse and rabbit brains were identical.

The negatively stained specimens showed that the brain microtubules have a filamentous substructure (Fig. 1C). With high magnification, six subfilaments could usually be identified in the side view of the microtubule (Fig. 1D). This is comparable to estimates that microtubules from plant cells (*6*) and fly sperm flagella (*7*) contain 13 subunits in cross section. The filamentous substructure of brain microtubules is virtually identical to that of microtubules of the mitotic spindle (*8*). It is also similar to the substructure of microtubules from salamander red cells (*9*), mammalian platelets (*10*), and the peripheral doublets of flagella (*11*). The helical pattern visible in microtubules of lung fluke sperm (*12*) has not been seen in brain microtubules.

The number of microtubules on a grid and the microtubule concentration of a suspension were linearly related (Fig. 2). A fivefold change in microtubule concentration could be detected. The technique is highly sensitive, microtubules being identified even in a 1 : 1000 dilution. The length of the microtubule was not taken into consideration in making the counts. Despite the apparent obstacles of minute sample size and the unknown reactions between the carbon film and suspended particles the assay has proved to be practical in daily use.

Microtubules were poorly preserved without the addition of hexylene glycol or maintenance of slightly acid *p*H (Table 1). Either hexylene glycol at *p*H 7.4 or buffer only at *p*H 6.4 exerted some protective effect, but the combination of these alterations provided the best preservation. By comparing the values in Table 1 with those of Fig. 2, it can be calculated that a 25- to 50-fold decrease in concentration occurred when microtubules were exposed to medium containing buffer only at *p*H 7.4. Hexylene glycol and acidic *p*H appear to act by decreasing the solubility of some structural subunit sufficiently to maintain the structure of the microtubule. The effect of hexylene glycol is not specific since medium containing 13 percent ethanol at *p*H 6.4 also preserved the structure of brain microtubules, as shown by Kane (*5*) in the case of mitotic spindle microtubules.

This technique of preserving microtubules in homogenates, together with the assay that has been developed, provides the essential tools for purifying brain microtubules. Analyses of purified preparations of brain microtubules may provide information that can be correlated with studies of the colchicine-binding protein (*13*), which is thought to be of microtubule origin. Further studies of the reactions of brain microtubules in vitro may help to elucidate the function of this organelle in normal and diseased states.

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15. Presented in part at the meeting of the American Society of Experimental Pathologists, Atlantic City, N.J., April 1968 [*Fed. Proc.* (abstr.) **27**, 247 (1968)].

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Hemophilia: Role of Organ Homografts

Abstract. *The concentration of factor VIII and partial thromboplastin times became normal and have remained normal for 140 days after orthotopic tranplantation of a normal liver to a hemophilic dog. Transplantation of a normal spleen into a hemophilic recipient did not result in a significant increase in factor VIII although the splenic graft was viable for at least 47 days. Transplantation of normal marrow to a lethally irradiated hemophilic dog did not result in an increase in factor VIII during 34 days of observation.*

The site of formation of factor VIII is not known. Some experiments indicate that the spleen may synthesize factor VIII (*1*, *2*). Most clotting factors have their origin in the liver, but the usually normal concentrations of factor VIII in patients with severe liver disease (*3*) suggest that the liver does not have a significant role in factor VIII production. In a recent study, perfusion of the isolated liver resulted in some increase of factor VIII in the effluent (*4*). The following experiments were undertaken to determine what role individual organs might play in the production of factor VIII.

Hemophilic beagle dogs (*5*) were used in the experiment (*6*). Three dogs with factor VIII deficiency were used as recipients; dog 1 received an orthotopic hepatic homograft, dog 2 a

splenic homograft, and dog 3 a bone marrow homograft. In each case the donor and recipient were unrelated. The donor was selected to match the hemophilic recipient by means of six canine lymphocytotoxic antiserums. Four of these typing serums were developed in this laboratory (*7*), and two were obtained from Dr. J. W. Ferrebee, Cooperstown, New York.

For coagulation assays, blood was collected in plastic tubes containing 3.8 percent sodium citrate (volume ratio 9:1). The partial thromboplastin times (PTT) were determined by the method of Proctor and Rapaport (*8*). In normal dogs, values were 18 to 22 seconds, and in untreated hemophilic dogs, the values ranged from 40 to 58 seconds. Factor VIII was assayed by a one-stage technique in which the partial thromboplastin time with kaolin of plasma from one of the untreated hemophilic dogs was determined after the addition of a 1 to 5 dilution of the fresh plasma being tested. Fresh plasma from a normal beagle was used as the reference standard for factor VIII and was assumed to contain 100 percent factor VIII. As the substrate was from one of the hemophilic dogs, the absolute value of factor VIII in the hemophiliacs in this colony could not be determined. Plasma from an unrelated colony of dogs with clinically more severe hemophilia was obtained from Dr. K. Miller, Albany, New York. With this plasma as substrate, the plasma of the Oklahoma beagles appeared to contain from 0 to 3 percent of factor VIII.

Just before transplantation in dog 1, donor and recipient were subjected to cross circulation for 15 minutes in order to provide the recipient with enough factor VIII to prevent bleeding during surgery. The recipient's liver and spleen were removed, and the normal orthotopic liver graft was carried out (*9*). Immunosuppression was maintained with daily doses of azathioprine. Methylprednisolone was given during the first 5 days after transplantation and again from the 34th to the 48th day when a rise in serum bilirubin to 4 to 6 mg per 100 ml of serum suggested a rejection reaction. All treatment for maintaining immunosuppression was discontinued on day 116 in order to determine the influence of the drug on factor VIII concentrations. The dog died on day 145 from rejection of the liver graft.

The increase in factor VIII with cross circulation, the subsequent fall over a period of 2 days, and the rise to normal values that was maintained for 141 days (the last value determined before death) is shown in Table 1. Citrated plasma from dog 1 was collected and frozen on the 16th, 17th, 23rd, and 61st days after surgery. This plasma was pooled to give a volume of 132 ml that was infused into an untreated hemophilic beagle. The calculated volume of plasma of the hemophiliac was 350 ml. The infusion expanded this to 482 ml, giving a calculated value of 27 percent factor VIII, if we assume 100 percent in the infused plasma and 0 percent in the recipient. The value determined after 4 hours was 20 percent. Normal plasma (132 ml) was later infused into the same hemophilic dog, and a value of 23 percent was found 4 hours later.

In dog 2, a normal splenic homograft was inserted heterotopically in the left iliac fossa (*10*). Venous blood from the spleen entered the systemic rather than the portal venous circulation. The recipient's spleen was removed. Immunosuppression was similar to that described for dog 1. Spleen scans with ^{99m}Tc-labeled sulfur colloid obtained on the 3rd, 6th, and 45th days after surgery, and open biopsies of the spleen on the 10th and 47th days indicated the presence of a viable spleen at these times. Although the mean values appear higher than those of the untreated hemophilic dogs, the significance of these low concentrations is uncertain. After the 4th day, the PTT's ranged from 35 to 43 seconds and tended to be shorter than those of the untreated hemophilic dog.

Dog 3, a hemophilic male beagle, was given a supralethal dose of 1200 r total body irradiation from dual opposing Co^{60} sources; the radiation was followed by infusion of homologous bone marrow cells (14.4×10^9) obtained from a normal female beagle. Methotrexate was given as an added immunosuppressive measure (*11*). Marrow engraftment occurred promptly, as shown by return to normal white blood cell and platelet counts by the 12th day after irradiation. The dog appeared healthy until the onset of secondary disease about the 25th day, and it died on day 34. At this time the concentration of factor VIII did not exceed 5 percent, and the PTT values ranged from 32 seconds to 55 seconds.

With the two-stage assay (*12*) (0.2-percent dilutions) with human plasma as the standard reference, values of 451 percent on the normal canine plasma, less than 1 percent on canine hemophilic plasma, 550 percent on the liver homograft plasma, and 22 percent on the splenic homograft plasma were obtained by an independent investigator.

Our experiments show that the liver

Table 1. Representative values of factor VIII and partial thromboplastin time (PTT).

Time of sampling	Liver transplant		Spleen transplant	
	Activated PTT (sec)	One-stage factor VIII (%)	Activated PTT (sec)	One-stage factor VIII (%)
Before transplant	40	0	41	0
After cross circulation	19.5	42	30	30
After transplant	33	17	29	25
After surgery				
1 day			28	25
2 days	30.5	20		12
3 days	28	78	13*	>1
4 days	15	180	43	>1
6 days	14	360		
10 days			35	>1
13 days			39	0
15 days	19	80		
20 days			41	0
24 days			42	11†
27 days	21	70		
29 days			40	3
35 days			42.5	6
41 days	18	55	42	4.5
55 days	20	100		
72 days	22.5	90		
91 days	25	130		
107 days	17.5	125		
118 days	29	72		
126 days	27	120		
141 days	24	66		
Normal dog	18–21	100		

* Read at end point of kaolin clumping. † Specimen obtained after 4 ml blood already withdrawn.

takes part in the maintenance of normal factor VIII concentrations in the hemophilic dog. However, they do not exclude a minor contribution by other organs, including the spleen and bone marrow. Norman, Covelli, and Sise (*2*) have reported an "experimental cure" of hemophilia by transplantation of normal spleen into hemophilic Oklahoma beagles. Their higher factor VIII concentrations may be due to the use of human substrate in the assay. Our studies were performed with canine substrate. The very low amounts of factor VIII observed in dog 2 are not subject to critical interpretation. In the case of dog 3, the bone marrow graft did not appear to influence factor VIII concentration, although long-term chimerism without secondary disease will be necessary for a definitive answer to this question.

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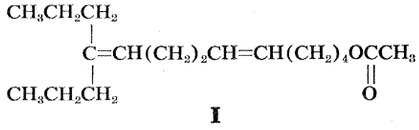
Sex Pheromone of the Pink Bollworm Moth: Biological Masking by Its Geometrical Isomer

Abstract. *A mixture of the* cis *and* trans *forms of propylure (10-propyl-trans-5,9-tridecadienyl acetate), the sex pheromone of the female pink bollworm moth, has been separated into its pure isomers by thin-layer chromatography. The* cis *isomer inhibits or masks the activity of the* trans *isomer, as little as 15 percent of the* cis *isomer being sufficient to completely nullify the activity of the* trans *isomer.*

In 1966, the sex pheromone produced by the female pink bollworm moth, *Pectinophora gossypiella* (Saunders), was isolated, identified, and synthesized (*1*). The compound, which was shown to be 10-propyl-*trans*-5,9-tridecadienyl acetate (structure I) and was designated "propylure," elicits a high degree of sexual excitement, including copulatory attempts, among caged male moths exposed to it in the laboratory.



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Eiter *et al.* (*2*) prepared propylure by a different method and reported that it showed no activity, although the details of their biological tests were not given (*3*). They concluded that propylure is not the pink bollworm sex pheromone, despite the fact that their preparation was admittedly geometrically impure. Active propylure prepared in our laboratories was a pure single isomer (*trans*), and this prompted me to investigate their prepartion in an effort to explain the contradiction.

An explanation for the lack of activity appeared to be a possible masking effect caused by isomers admixed with propylure in the preparation of Eiter *et al.* Although these investigators discount a masking phenomenon as a factor in pheromone inhibition or inactivation (*2*), authentic cases of pheromone masking by admixed contaminants have already been reported for the sex pheromones of the gypsy moth, *Porthetria dispar* (L.) (*4*), introduced pine sawfly, *Diprion similis* (Hartig) (*5*), corn earworm, *Heliothis zea* (Boddie) (*6*), tobacco budworm, *H. virescens* (F.) (*6, 7*), American cockroach, *Periplaneta americana* (L.) (*8*), cynthia moth, *Samia cynthia* (Drury) (*9*), cabbage looper, *Trichoplusia ni* (Hübner) (*10*), and omnivorous leaf roller, *Platynota stultana* (Walsingham) (*11*). In the case of *Trichoplusia ni,* whose sex pheromone is *cis*-7-dodecenyl acetate (*12*), the agent responsible for masking is the corresponding *trans* isomer. This is likewise true for gyplure (12-acetoxy-*cis*-9-octadecen-1-ol), a synthetic sex attractant for the gypsy moth, which is completely inactivated by admixture with 20 percent of its *trans* isomer (*13*) or even smaller amounts of ricinoleyl (*cis*) alcohol (*14*).

Eiter *et al.* (*2*) reported that their preparation of propylure was a 1:1 mixture of the *cis* and *trans* isomers, and it therefore seemed highly likely that the activity of the latter was being completely inhibited by the former isomer. That this was indeed the case has now been shown by preparing propylure according to the exact procedure described by Eiter and co-workers (*2*), separating the resulting mixture into its geometrical isomers, and testing them alone and in combination for activity on male pink bollworm moths.

The propylure obtained by Eiter's procedure was a colorless liquid [b.p., 130° to 135°C at 0.1 mm-Hg; n_D^{25} 1.4610; Eiter *et al.* (*2*) reported b.p., 90° to 100°C at 0.05 mm; n_D^{20} 1.4612] whose gas-liquid chromatogram (*15*) showed one major and one minor component with retention times of 13.5 and 12.5 minutes, respectively. The minor component, comprising approximately 10 percent of the mixture, was identified as the isomeric 9-propyl-5,9-tridecadienyl acetate (*cis* and *trans*) as reported by Eiter *et al.* This contaminant was efficiently separated by slow distillation, at 0.1 mm-Hg pressure, through a spinning band column; it boiled at 129° to 131°C (n_D^{25} 1.4600), whereas the major component boiled at 134°C (n_D^{25} 1.4630).

The major component was readily separated into *cis* and *trans* forms of propylure by preparative thin-layer chromatography on silica gel impregnated with silver nitrate (*16*). With benzene-hexane (80:20) as the developing solvent, the *cis* and *trans* isomers showed R_F values of 0.31 and 0.50, respectively; propylure prepared by the method of Jones *et al.* (*1*) showed R_F 0.51 under these conditions. The