References and Notes

1. G. Ungar, *Federation Proc.* **24**, 1293 (1965).
2. C. F. Code, M. M. Hurn, R. G. Mitchell, *Mayo Clin. Proc.* **39**, 715 (1964).
3. R. J. Levine, T. L. Sato, A. Sjoerdsma, *Biochem. Pharmacol.* **14**, 139 (1965).
4. J. D. Reid and D. M. Shepherd, *Life Sci.* **1**, 5 (1963).
5. R. J. Levine, *Federation Proc.* **24**, 1331 (1965).
6. Studies were done in either the metabolic ward of the Veterans Administration Hospital, West Haven, Conn., or the clinical pharmacology ward of the Yale-New Haven Medical Center.
7. J. A. Oates, E. Marsh, A. Sjoerdsma, *Clin. Chim. Acta* **7**, 488 (1962).

8. Supported by grants from NIH (GM-13016 and CA-08341) and Lederle Research Laboratories. I thank Heidi Humpert for technical assistance. NSD-1055 (CL-54998) and placebo capsules were provided by H. G. Lockard, Jr., of Lederle Research Labs., Pearl River, N.Y.

8 August 1966

Phytohemagglutinin: Inhibition of the Agglutinating Activity by N-Acetyl-D-Galactosamine

Abstract. *The effect of simple sugars on the agglutinating activity of phytohemagglutinin was studied.* N-*Acetyl*-D-*galactosamine selectively inhibits the agglutination of leukocytes and erythrocytes by phytohemagglutinin.*

Phytohemagglutinin (PHA), an extract of the red kidney bean *Phaseolus vulgaris,* agglutinates leukocytes and erythrocytes (*1*); it also stimulates lymphocytes in vitro to differentiate into large cells capable of undergoing mitosis (*2*).

It has been reported that agglutination caused by fava-bean extract, another plant hemagglutinin, could be inhibited by D-glucose, D-fructose, and maltose (*3*). Agglutination of lymphocytes by PHA is inhibited by coating the cells with Vi antigen from *Salmonella* (*4*), a substance containing *N*-acetyl-D-galactosaminuronic acid (*5*). These studies have raised the possibility that sugars may play a role in the interaction between PHA and mammalian cells. We have investigated the effect of certain sugars which occur as constituents of mammalian cell surfaces on the agglutination of cells by PHA.

The contents of each vial of PHA (*6*) were dissolved in 5 ml MEM (*7*), the diluent routinely used in our experiments, and this was used as a stock solution from which serial dilutions were made. Sugars were obtained commercially and made up in stock solutions of 100 mg per milliliter of MEM. Erythrocytes were obtained from man, rabbits, and rats by drawing blood into a syringe containing heparin. Cells from rat thymus, rabbit thymus, and human tonsil were obtained by gently teasing these tissues in MEM and filtering the suspension through a thin layer of glass wool. Rat thoracic duct cells were obtained by conventional techniques (*8*). For agglutination, cells were washed three times in MEM, and 0.2 ml of a suspension (10^7 cells/ml) was incubated with 0.1 ml of PHA and 0.25 ml of sugar solution. After 3, 10, and 30 minutes at 37°C, samples of the reaction mixtures were streaked on a slide and examined microscopically (100×). The degree of agglutination was graded on a 0 to 4 scale. Test samples from which PHA or sugar, or both, had been omitted were included as controls in each experiment.

N-Acetyl-D-galactosamine (5 mg/ml) selectively inhibits the agglutination of rat thoracic-duct lymphocytes by PHA (Table 1). A lesser degree of inhibition was observed with 10 mg of the *N*-acetyl-D-galactosamine per milliliter. The other sugars, in concentrations of 10 to 100 mg/ml, showed no consistent inhibition at 30 minutes. L-Fucose, however, at a final concentration of 50 mg/ml, showed slight and variable inhibition after 10 minutes of incubation. Cells freshly agglutinated by PHA could be disassociated by the addition of *N*-acetyl-D-galactosamine (50 mg/ml). *N*-Acetyl-D-galactosamine selectively inhibited agglutination by PHA of rat thymus, rabbit thymus, and human tonsil cells, and also of human (Table 2), rat, and rabbit erythrocytes.

Table 1. Inhibition of agglutination of rat thoracic duct cells by PHA with *N*-acetyl-D-galactosamine. In each reaction mixture the final concentration of the sugar was 50 mg/ml. The readings were taken 10 minutes after incubation at 37°C was begun.

Sugar added	Agglutination at dilution of PHA					
	1:25	1:50	1:100	1:250	1:500	1:750
None	+++	+++	++	++	++	+
N-Acetyl-D-galactosamine	++	+	+	0	0	0
N-Acetyl-D-glucosamine	++++	+++	+++	++	++	+
D-Glucose	++++	++++	++++	++	++	0
D-Galactose	++++	+++	+++	++	++	+
D-Mannose	++++	++++	+++	++	++	+
DL-Fucose	++++	++++	++	+	+	±

Table 2. Inhibition of agglutination of human erythrocytes by PHA with *N*-acetyl-D-galactosamine. In each reaction mixture final concentration of sugar was 50 mg/ml. The readings were made 10 minutes after incubation at 37°C.

Sugar added	Agglutination at dilutions of PHA			
	1:250	1:500	1:750	1:1000
None	+++	+++	++	+
N-Acetyl-D-galactosamine	+	+	±	0
N-Acetyl-D-glucosamine	+++	+++	+	+
D-Galactose	+++	++	+++	++
D-Glucose	++++	++	+	±
L-Fucose	+++	++	+	+

Incubation of rat lymphocytes or erythrocytes with *N*-acetyl-D-galactosamine (50 mg/ml) for 10 minutes followed by two washings with MEM did not result in a decrease in their agglutinability by PHA. Moreover, PHA incubated with *N*-acetyl-D-galactosamine and then dialyzed against 1000 volumes of MEM for 16 hours agglutinated rat lymphocytes to the same extent as dialyzed samples of PHA which had not been exposed to the sugar. These experiments indicate that the inhibitory effect of *N*-acetyl-D-galactosamine is due neither to the destruction of PHA nor to an irreversible alteration of the cell. Magnesium ion which overcomes the inhibitory action of ethylenediaminetetraacetic acid on PHA hemagglutinating activity (*9*) had no effect on the inhibition by *N*-acetyl-D-galactosamine.

The mechanisms by which PHA's cause agglutination of leukocytes and erythrocytes are unknown. In our study evidence has been obtained that *N*-acetyl-D-galactosamine specifically inhibits hemagglutination and leukoagglutination by PHA without destroying either the cells or the PHA. One explanation for the selective inhibition by *N*-acetyl-D-galactosamine of the interaction between the cell and PHA is that this sugar acts in a manner analogous to a hapten and it might participate in this interaction either as a constituent of the cell surface or of the PHA. Since it is known that *N*-acetyl-D-galactosamine occurs as an externally accessi-

ble constituent of mammalian cell surfaces (*10*), it would seem more likely that *N*-acetyl-D-galactosamine participating in this interaction is provided by the cell. This possibiilty is consistent with the report that in purified PHA *N*-acetyl-D-galactosamine was not found though other sugars such as glucosamine were (*11*).

H. BORBERG, J. WOODRUFF
R. HIRSCHHORN, B. GESNER
P. MIESCHER, R. SILBER
Department of Medicine, New York University School of Medicine, New York

References and Notes

1. D. A. Rigas and E. E. Osgood, *J. Biol. Chem.* **212**, 607 (1955).
2. P. C. Nowel, *Cancer Res.* **20**, 462 (1960).
3. C. B. Perera and A. M. Frumin, *Science* **151**, 821 (1966).
4. J. Borjeson, L. N. Chessin, P. Welsh, *Fed. Proc.* **25**, 306 (1966).
5. K. Heyns, G. Kiessling, W. Lindenberg, H. Paulsen, M. E. Webster, *Chem. Ber.* **92**, 2435 (1952).
6. Phytohemagglutinin was purchased as PHA-P from Difco; lot Nos. 47-97-72 and 47-13-73.
7. Minimal essential medium, H. Eagle, *Science* **130**, 432 (1959).
8. J. L. Gowans, *Brit. J. Exp. Pathol.* **38**, 67 (1957).
9. M. Tunis, *J. Immunol.* **95**, 878 (1965).
10. E. Kabat, *Blood Group Substances, Their Chemistry and Immunochemistry* (Academic Press, New York, 1956), pp. 162–183.
11. D. A. Rigas, E. A. Johnson, R. T. Jones, J. M. McDermed, V. V. Tisdale, *Fed. Proc.* **25**, 795 (1966).
12. P.M. and R.S. are career scientists, Health Research Council of the City of New York. Supported by USPHS grant CA 06657 and the American Cancer Society grant P-360.

14 June 1966

Juvenile Hormone: Identification of an Active Compound from Balsam Fir

Abstract. *A sesquiterpenoid ester with high juvenile hormone activity for* Pyrrhocoris apterus *(L.) was isolated from balsam fir,* Abies balsamea *(L.) Miller, and identified as the methyl ester of todomatuic acid.*

Extracts of various pulp woods, in particular the balsam fir, *Abies balsamea* (L.) Miller, and paper products derived therefrom, contain a factor or factors termed "paper factor" (*1*), which show strong juvenile hormone activity in the hemipteran bug, *Pyrrhocoris apterus* (L.). Thus, when last-instar nymphs were allowed to come into contact with certain paper products or were treated with lipid extractives of these products, the insect underwent one or more supernumerary molts and eventually died without becoming sexually mature. They also noted that *P. apterus* was unique in responding to the "paper factor" since two other Hemiptera, *Oncopeltus fasciatus* (Dallas) and *Rhodnius prolixus* Stal, and several Lepidoptera were unaffected. Moreover, *P. apterus* and *O. fasciatus* were reported to be little affected by highly active extracts of the cecropia silkworm which continues to be the most active source of the "natural" insect hormone. These findings have led Slama and Williams to speculate upon the possibility of chemical evolution and diversification of the juvenile hormone itself and of its target organs.

Other investigators (*2*), making independent studies of these phenomena, differ in interpreting these effects. They maintain that the "paper factor" induced a "pathological growth pattern," which resulted in abnormal or malformed adults with apparently normal reproductive organs and external genitalia, although egg production was only 10 percent of normal with few of these developing. They also declared that any juvenile hormone effect, if present, was quite small or only superficially resembled juvenilization.

Since it is always difficult to assess the biological activities of an unknown compound, particularly when it is present in an extract containing many differing molecular species, we decided that isolation of the pure material and subsequent study of its effects on *P. apterus* might clarify these inconsistencies.

Pulverized balsam fir wood was extracted in a large glass chromatographic column by perfusion with a mixture of chloroform and methanol (3:2). The solvent was removed in a vacuum and the residue was dissolved in ether; the ether solution was filtered and dried over anhydrous sodium sulfate.

A light, mobile oil with high juvenile hormone activity for *P. apterus* was isolated from this crude extract by column and preparative thin-layer chromatography over silica gel; it was 99 percent pure (or better) by gas-liquid and thin-layer chromatographic analysis (*3*). The compound was assigned the trivial name of juvabione.

Infrared analysis of juvabione in carbon disulfide showed strong absorption at 1722 cm^{-1} for an ester carbonyl group in conjugation with a double bond, and at 1645 cm^{-1} for a double bond in conjugation with an ester carbonyl. A second band at 1712 cm^{-1} indicated the presence of an additional isolated carbonyl.

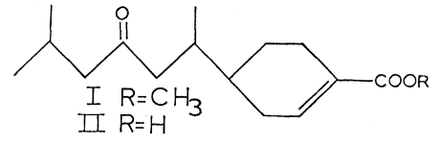
High-resolution mass spectroscopy of this ester showed a small parent ion with a molecular weight of 266.188 (*4*), a value consistent with the empirical formula $C_{16}H_{26}O_3$ (calculated, 266.187; C = 12.0000). The parent ion lost methanol which resulted in the appearance of a prominent ion at 234, a result confirming the presence of an unsaturated methyl ester (*5*).

Reduction with sodium borohydride or catalytic hydrogenation gave different products, each showing a parent ion that was two mass units higher than the original compound.

The nuclear magnetic resonance spectrum of juvabione (in deuterochloroform) showed peaks at δ (parts per million) 6.95 (1 H, olefin), δ 3.75 (3 H, methoxyl), δ 2.7 to 1.5 ($\sim$ 8 H, allylic), δ 1.5 to 1.0 ($\sim$ 5 H saturated), δ 0.88 [6 H doublet, J (spin-spin coupling constant) = 6], δ 0.86 (3 H doublet, J = 6), confirming the presence of a double bond, an isopropyl unit attached to a nonassymetric carbon atom, and a methyl group attached to an additional disubstituted carbon.

In the mass spectrum, the isopropyl fragment with mass to charge (M/e) equal to 43, a homolog M/e equal to 57 (C_4H_7) (*4*), and a related fragment at M/e 85 (C_5H_9O) were prominent and suggested the linkage $(CH_3)_2CHCH_2\dot{C}=O$.

The remainder of the molecule must contain the remaining methyl group, one ring, and the α,β-unsaturated cyclic ester. Combining these facts with the likely sesquiterpenoid nature of the material, we propose structure I.



Todomatuic acid (II), isolated from bisulfite-treated pulp wood of *Abies sachalinensis* (Schmidt) Masters, embodies all of these features (*6*). The acid, obtained on saponification of juvabione (I) crystallized from petroleum ether, gave a melting point of 57° to 59°C and a specific rotation $[\alpha]_D^{25}$ = +87° (ethanol). The physical data