# Depressant Agent from Walnut Hulls

Abstract. Crushed unripe walnut hulls (Juglans nigra), when extracted with ether, yield an extract which sedates or at least depresses the movements of Daphnia magna, leopard frogs, perch, catfish, goldfish, mice, rats, and rabbits. One purified depressant compound, 5-hydroxy-1,4-naphthoquinone (juglone), has been isolated and tested on most of these species.

Crushed unripe walnut hulls (Juglans nigra) have been used for generations in various types of folk medicine, including treatment of fungus infections (1). In certain regions of the United States fresh walnut hulls are employed illegally to immobilize fish, and consequently take them from small streams and impoundments. The fish gradually lose equilibrium and awareness to changes in environment, and finally can be caught by hand. When placed in fresh water, many of these animals seem to recover fully. The use of walnut hulls in folk medicine and as a depressant of fish initiated our interest in extracting the active principle from green walnut hulls, testing it on different species of fish, and assessing its effects on several other species of animals.

After using several extractive solvents-including water, acetone, ethyl alcohol, chloroform, diethyl oxide, and petroleum ether-diethyl oxide (ether, U.S.P.) and petroleum ether were judged the two best solvents for extracting the active principles from fresh, finely ground walnut hulls. After the

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hulls had been macerated in ether, the solvent was allowed to evaporate at room temperature, leaving a dark, scaly residue. One kilogram of the fresh hulls extracted with 1.4 kg of ether yielded 2.13 g of residue. This residue produced the typical depression in young yellow perch (Perca flavescens), channel cat fish (Ictalurus punctatus), and goldfish (Carassius auratus). All of these fishes could be depressed markedly by the water-soluble fraction from 7 to 15 mg of the dried ether extract per liter of tap water. Perch were the most sensitive and goldfish the most resistant of the fishes tested. The small amount of extract used in these tests produced no measurable effect on the pH or oxygen content of the water.

A study was made also to determine whether this material would be specific for fishes or would have a similar effect on more primitive organisms (Daphnia magna) and more highly developed animals (frogs and mammals). Daphnia were immobilized and, if not placed in fresh water, would die when exposed to approximately the same concentration that was lethal to perch. The watersoluble fraction was dissolved in saline (0.7 percent) and injected into frogs. The frogs became quiet and recovered in a few hours, providing the dose did not exceed 1 mg/g of body weight. Albino mice and rats also were sedated after intraperitoneal injection of this substance dissolved in corn oil.

Attempts are being made to separate the sedative principles from this ether extract. One purified compound, 5hydroxy-1,4-naphthoquinone (juglone), has been isolated to date. This juglone was separated from the crude ether extract by sublimation and then identified by melting point, infrared spectrophotometry, and crystalline studies by comparing it with known samples of synthesized 5-hydroxy-1,4-naphthoquinone. One gram of the crude ether extract yielded 0.156 g of the 5-hydroxy-1,4-naphthoquinone. Although it has been known for years that juglone

occurs in various species of the walnut tree (2), published work on the pharmacological actions of this naphthoquinone does not include a sedative effect.

Our purified 5-hydroxy-1,4-naphthoquinone was tested on goldfish (LD<sub>50</sub>  $\approx$ 0.2 mg/liter of water in the aquarium),mice  $(LD_{50} = 0.25 \text{ mg}/100 \text{ g of body})$ weight), and rats, and was found to have a calming or sedative effect in all of these species. Rabbits were injected intravenously with 0.07 mg of juglone (in 5-percent glucose solution) per kilogram of body weight. This resulted in tranquilization and general quietude of the animals for a period of 2 to 3 hours. Although sedation is produced by quite small amounts, the toxicity is such that profound sleep cannot be produced with less than lethal doses in these mammals.

Although the purified juglone produces sedation, there seems to be a difference between the quality of depression produced by the crude material and that produced by juglone. This suggests the presence of compounds other than juglone which may have synergistic action (3).

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## **Proof of an Adaptive**

#### Linkage Association

Abstract. Linked X-chromosome inversions of an eastern woods fly. Drosophila robusta, occur in two different nonrandom arrays. Since only one can be the nonrandom condition expected on the approach toward random equilibrium, at least one of the observed arrays must have an adaptive basis.

It is fashionable in modern evolutionary genetics to state: "The raw materials of evolution arise by mutation and recombination" and then to proceed to discuss how alleles may change frequencies under directional or nondirectional forces. Very little attention has

Instructions for preparing reports. Begin the report with an abstract of from 45 to 55 words. The abstract should not repeat phrases employed in the title. It should work with the title to give the reader a summary of the results presented in the report proper.

Type manuscripts double-spaced and submit one

ribbon copy and one carbon copy. Limit the report proper to the equivalent of 1200 words. This space includes that occupied by illustrative material as well as by the references and notes.

Limit illustrative material to one 2-column figure (that is, a figure whose width equals two col-umns of text) or to one 2-column table or to two 1-column illustrations, which may consist of two figures or two tables or one of each. For further details see "Suggestions to contrib-utors" [Science 125, 16 (1957)].



Fig. 1. Three possible pathways of linkage phylogeny (see text).

been paid to the question of how combinations of alleles produced by recombination-and surely few if any major adaptions have been produced by single loci-are incorporated into the genome. Especially lacking have been empirical studies of linkage situations. Undoubtedly the mathematicians of genetics early discouraged such studies by demonstrating that linked combinations would disintegrate to random frequencies unless selection could overcome the crossover rate. Many a geneticist has concluded that crossover rates are usually so large that only the accident of close proximity of loci (by translocation or other shifts of arrangement) or abandonment of sexual reproduction could achieve the conditions for effective selection for linkage combinations. Were linked loci encountered in a nonrandom condition, other geneticists probably realized, one could not readily distinguish whether the observed data represented an adaptive development or merely a stage in the mathematically predicted breakdown of the linkages to randomness.

The latter was my predicament when I wandered innocently into this problem. Studying the linked gene arrangements (chromosomes varving by paracentric inversions) in Blacksburg, Va., populations of Drosophila robusta Sturtevant, I found three different nonrandom associations in the same population (1). In spite of circumstantial evidence pointing to an adaptive basis for the associations, the doubt remained-that is, the doubt that they were merely historical relics on their journey toward random equilibrium. Data from a D. robusta population in the Great Smoky Mountain National Park near Gatlinburg, Tenn., prove, however, that at least one of the associations is adaptive.

The Blacksburg and Gatlinburg populations contain appreciable, fairly similar, quantities of two widespread gene arrangements of the left arm of the X-chromosome, XL and XL-1, of the two right-arm arrangements, XR and XR-2 (2), and of all of the four possible combinations between them. In Table 1 the numbers of the combinations in two woods near Blacksburg and in a woods at a 2000-foot elevation near Gatlinburg are compared with the number expected in each sample on the assumption of randomness. Whereas both of the Blacksburg populations contain a highly significant excess of the so-called "coupling" combinations,  $XL \cdot XR$  and  $XL \cdot I \cdot XR \cdot 2$ , and corresponding deficiency of the "repulsion" types,  $XL \cdot XR - 2$  and  $XL - 1 \cdot XR$ , the Gatlinburg flies exhibit the reverse situation: excess of the "repulsion" types and deficiency of the "coupling" ones. The differences from expected are highly significant. An association of the Blacksburg type apparently exists also in a Lexington, Ky., population (1)and, in an extreme form, in a population from the Piedmont region of Virginia (3). In 1959 the Gatlinburg result was repeated at the 2000-foot woods, and this association pattern was found in collections from other locations near Gatlinburg and from the Blue Ridge Mountains of Georgia (3).

The exact phylogeny of the gene arrangements of D. robusta is not known, but XL and XR are probably ancestral to XL-1 and XR-2 (4). Hence the linkage phylogeny could have followed three pathways. In the first two (Fig. 1, A and B) both coupling types appeared, followed by (bracket) the formation of the missing repulsion type by crossing over between them. In the third (Fig. 1C), both repulsion types appeared independently, followed by formation of the missing coupling type by crossing over.

Schemes A and B may be termed "couplings first" and scheme C "repulsions first." Under any scheme the fourth linkage would be produced by crossing over in the available double-

Table 1. Actual (a) and expected\* (e) numbers of X-chromosomes in adult D. robusta from two woods near Blacksburg, Va. (1950-55), and from a woods at a 2000-foot elevation in Great Smoky Mountains National Park near Gatlinburg, Tenn. (1958).

Chromosome	Blacksburg				Gatlinburg			
	Crumpacker Woods		Heth Woods		April 1958		August 1958	
	a	е	a	е	a	е	а	е
XL•XR	247	212.4	197	162.2	7	15.0	47	87.5
XL•XR-2	553	587.6	403	437.8	30	22.0	160	119.5
XL-1•XR	214	248.6	221	255.8	38	30.0	186	145.5
XL-1•XR-2	724	689.4	726	691.2	36	44.0	158	198.5
Total	1738	1738.0	1547	1547.0	111	111.0	551	551.0
Chi-square <sup>†</sup>	14.2‡		16.7‡		10.8‡		52.0‡	

\* Expected on the assumption that left- and right-arm arrangements are independent.  $\dagger$  Chi-square testing goodness-of-fit of *a* to *e* in each sample; each has 1 degree of freedom.  $\ddagger$  Highly significant ( $P \le .01$ ).

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heterozygotes, and until it achieves its random frequency the population would present a nonrandom association. Once formed, incidentally, the two "couplings first" schemes are indistinguishable from one another, because, under the 2-by-2 contingency table, the two coupling or two repulsion types show equal deviation from expected, no matter which single linkage is responsible for the deficiency or excess. Similarly, the arguments do not depend on the assumption that  $XL \cdot XR$  is the oldest combination.  $XL-1 \cdot XR-2$  may be substituted for  $XL \cdot XR$  in any scheme; having a repulsion linkage ancestral means merely that the possible phylogenies consist of two "repulsions first" alternatives and one "couplings first" instead of the reverse.

The crucial point is, however, that only one of the possible phylogenies could have occurred, for, unlike point mutations, which presumably recur again and again in the species phylogeny, inversions are probably unique events. For one thing, spontaneous chromosome aberrations are much rarer than spontaneous mutations. In addition, the chances of repeating identical breaks necessary for an exact copy of an inversion are infinitesimal. It follows that only one kind of nonrandom association attributable to the historical process of building up the fourth linkage combination by crossing over is possible, either one in which the coupling types are deficient, the phylogeny having been "repulsions first," or else one in which the repulsion types are deficient, the phylogeny having been "couplings first." This means that at least one of the associations, the Blacksburg type or the Gatlinburg, is not the historical association. It must be a newly evolved, adaptive one, since natural selection, or at least selective migration, must be postulated in order to maintain a linkage association in the face of the randomizing tendency of recombination.

This demonstration achieves the other side of the coin of the typical evolution problem. Usually the adaptive trait is noticed, and the surmise is made that it resulted from selection for interacting loci, number and location unknown. Here the interacting loci are known to be linked, but the adaptive traits produced by their interaction are unknown. Some evidence exists, however, that in Blacksburg, at least, the X-chromosome association is involved in D. robusta's seasonal adaptations to the environment (5). It is also likely that selection for

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the associations has been accompanied by selection for chiasma suppression. The region between the right- and leftarm arrangements available for crossing over includes more than 20 percent of the X-chromosome euchromatin, yet less than 1 percent crossovers (6) are obtained (7).

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## Effect of Verbalization on **Reversal Shifts in Children**

Abstract. Two questions were asked: (i) Would naming stimuli in a discrimination reversal influence the performance of 4- and 7-year-old children, and (ii) if so, would the influence be different for these two age groups? The results indicate a positive answer to the first question and an equivocal answer to the second.

There is considerable current interest among psychologists in the behavioral analysis of processes that mediate between the external stimulus and the overt response. One method developed for such analysis is the reversal-nonreversal shift technique (1), which was adapted in the following manner to the present research. Forty-eight nursery school children (mean age, 56 months) and 48 second-grade children (mean age, 91 months) were required to learn two successive discriminations. The stimuli, two-dimensional squares, varying in area (1 in.<sup>2</sup> and 3 in.<sup>3</sup>) and brightness (black and white), were presented in a portable discrimination apparatus which displayed two squares simultaneously. The child chose one of them by pressing a lever that pointed at his choice. A marble served as reinforcement. As illustrated in Fig. 1, the first discrimination presented one stimulus pair that differed simultaneously on two dimensions. The second discrimination presented this same pair with the reinforcement pattern reversed or another pair that also differed simultaneously on the same dimensions; the two pairs were presented in random alternation. Stimulus preferences were controlled by suitable counterbalancing.

The major experimental variable was the kind of verbal label the children were instructed to apply to the correct stimulus during the initial discrimination. These labels, which were always appropriate to the first discrimination, could be arranged to be descriptive of either the relevant or irrelevant dimension in the second discrimination. For example, if a child was trained to be a relevant verbalizer, he was presented with the reinforcement pattern illustrated in Fig. 1 and instructed to tell the experimenter, before making a choice, whether the large or the small one was correct. He soon learned to precede his choices with the appropriate label which, in this instance, would be "large." Since in the second discrimination "small" was the correct stimulus, the label referred to the dimension that was to become relevant. If a child was trained to be an irrelevant verbalizer. he was asked in the first discrimination to tell whether the white or the black one was correct. He would thus learn to say "black," which was descriptive of the dimension that was to become irrelevant. One-third of the children at each age level were randomly assigned to be relevant verbalizers, one-third to be irrelevant verbalizers, and the remainder to a no-verbalization group who learned with no verbal labels offered or required.

All groups learned the first discrimi-



Fig. 1. Illustration of the experimental procedure showing one of the stimulusreinforcement patterns used.