In order to further evaluate our observations, the meiotic tissue was also stained with aceto-orcein, iron hematoxylin, and Sudan black B. In our preparations aceto-orcein deeply stained the meiotic chromosomes and obscured the centromeres, while preliminary results with iron hematoxylin and Sudan black B indicated that these dark staining bodies were present in each bivalent.

If the heterochromatic bodies observed in our preparations are indeed centromeres, then cytological evidence is available with regard to the XY association of man in the late diplotene stage. The XY bivalents (Figs. 1 and 2) reveal that the short arm of the Y chromosome is in association with the short arm of the X chromosome. This is a consistent finding in all of our material.

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Sex Pheromones Produced by Male Boll Weevil: Isolation, Identification, and Synthesis

Abstract. *The response of female boll weevils to males,* Anthonomus grandis *Boheman, in laboratory bioassays can be reproduced by exposure to a mixture of compounds I, II, and either III or IV, all isolated from male weevils and their fecal material. The same response was elicited by mixtures of the synthesized compounds. Compound I is* (+)-cis-*2-isopropenyl-1-methylcyclobutaneethanol; II,* cis-*3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol; III,* cis-*3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde; and IV,* trans-*3,3-dimethyl-$\Delta^{1,\alpha}$cyclohexaneacetaldehyde.*

We have isolated and identified two terpene alcohols and two terpene aldehydes from male boll weevils, *Anthonomus grandis* Boheman, and their fecal material. These compounds are the components of the pheromone to which only female boll weevils respond in laboratory tests. They may also be aggregating pheromones, because live males attract only females in the laboratory, but attract both sexes in the field in the spring and fall (*1*). The active compounds, each of which has been synthesized, are shown in Fig. 1. The success in isolating, identifying, and synthesizing the pheromone may point the way to new methods of surveying and controlling the boll weevil (*1*).

In laboratory bioassays (*1*), mixtures containing all four compounds elicited a response by females equivalent to or better than that elicited by live males. Absence of either alcohol (I, II) or both aldehydes (III, IV) from the mixture resulted in almost complete lack of response (Table 1). The response to mixtures of synthetic compounds was identical to that obtained from corresponding mixtures of the natural compounds. The synergistic effect observed with these four compounds is similar to, although slightly more complex than, that observed for the sex pheromone of the male *Ips confusus* (Le Conte) (*2*).

The four compounds were isolated from insects (67,000 males and 4.5 million weevils of mixed sexes) and from fecal material (54.7 kg) in identical fashion. No masking of the activity was evident in material derived from weevils of mixed sexes after steam distillation. Neither males nor females were responsive to extracts or steam distillates of females.

In brief, the isolation procedure (*3*) involves extraction of the source material, steam distillation, and subsequent fractionation by column chromatography on Carbowax 20M-coated (*4*) silica gel eluted successively with pentane, pentane–ether (90 : 10), pentane–ether (50 : 50), ether, and methanol. None of the individual fractions from this column were active, but the combination of fractions two and three, eluted with pentane–ether (90 : 10) and (50 : 50), respectively, was as active as the original distillate. Each of these fractions was then further fractionated on a column containing Adsorbosil-CABN (25 percent $AgNO_3$ on silica gel), which was eluted with the same series of solvents.

The material eluted with pentane–ether (90 : 10) from Carbowax 20M/silica gel and pentane–ether (50 : 50) from $AgNO_3$-silica gel gave six peaks on gas-liquid chromatography (GLC) on Carbowax 4000. The sixth peak, whose Kovats index [I_k (*5*)] was 1782 on Carbowax 4000, and which appeared homogeneous on the SE-30 column ($I_k = 1248$) gave two peaks, compounds III and IV, on a 15.2-m SCOT column coated with Carbowax 20M.

The material eluted with pentane–ether (50 : 50) from both liquid columns gave eight peaks on GLC on Carbowax 4000. Peak eight ($I_k = 1820$ on Carbowax 4000) gave two peaks, compounds I and II ($I_k = 1205$ and 1228, respectively), on the SE-30 column.

Compounds I and II were collected individually for bioassay by bubbling the effluent from the SE-30 column through dichloromethane. Compounds III and IV, combined in a single peak, were similarly collected from the SE-30 column. For the spectral studies, collections were made in carbon tetrachloride.

The concentrations of compounds I and II in fecal material were determined by combining a weighed amount of *p*-menth-1-en-4-ol (terpinen-4-ol) with the fraction eluted from both liquid columns with pentane–ether (50:50), by gas chromatography of the mixture, and by determination of the relative peak areas of the standard and the two natural products. Compounds I and II were thus calculated to be present in fecal material in concentrations of 0.76 and 0.57 ppm, respectively. Similarly, with 3,7-dimethyl-6-octenal (citronellal) as an internal standard, compounds III and IV were each found to constitute 0.06 ppm of fecal material. Concentrations in mixed weevils were about tenfold less.

The identity of compound I was determined on the basis of its mass, nuclear magnetic resonance (NMR), and

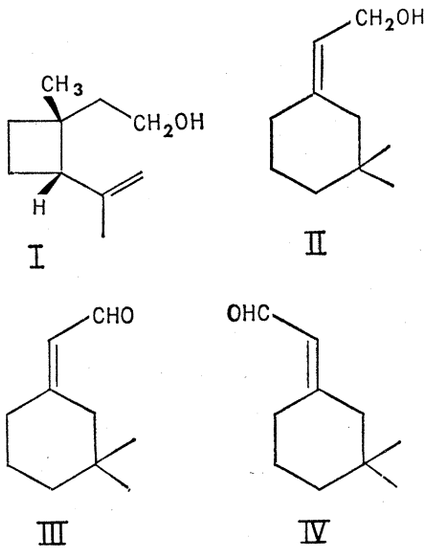

Fig. 1. Compounds I, (+)-*cis*-2-isopropenyl-1-methylcyclobutaneethanol; II, *cis*-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol; III, *cis*-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde; IV, *trans*-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde.

infrared spectra. The mass spectrum showed the following diagnostic peaks (mass to charge, *m/e*; parent peak, *P*): 154 (*P*), 139 (*P*-CH_3), 136 (*P*-H_2O), 121 (*P*-33), and 109 (*P*-CH_2CH_2OH); the base peak was 68. The infrared spectrum (CCl_4 solvent) showed the following diagnostic peaks (cm^{-1}): 3630 (free OH), 3250-3550 (H-bonded OH), and 885 and 1642 (terminal methylene). The NMR spectrum [CCl_4, external tetramethylsilane (TMS) standard, δ, ppm] was: 4.88 and 4.71 (broadened singlets, R,R′C=CH_2), 3.63 (triplet, -CH_2CH_2OH), 2.60 (broadened triplet, methinyl), 2.59 (singlet, OH), 1.72 (singlet, vinyl methyl), 1.22 (singlet, R,R′R″C-CH_3), and 1.3 to 2.2 (multiplet, broad, six protons). The optical rotation was measured on 11 mg of the pure natural compound. The specific rotation was estimated to be about +50° (±10°).

The mass spectrum of compound II showed the following diagnostic peaks: 154 (*P*), 139 (*P*-CH_3), 136 (P-H_2O), 121 (*P*-33), and 107 (*P*-CH_3OH and CH_3); the base peak was 69. The infrared spectrum showed a band at 3610 cm^{-1} (free OH) with less hydrogen bonding than in compound I. The NMR spectrum was: 5.53 (triplet, =CH—) coupled to 4.05 (doublet, RR′C=CHCH_2OH); 2.09 (multiplet, two protons) overlapping 2.00 (singlet, two protons) suggesting two methylenes adjacent to a double bond, one unsplit; 1.83 to 1.13 (multiplet, broad, four protons); and 0.95 (singlet, geminal dimethyls). The *cis* configuration about the double bond was assigned by comparison of the NMR spectra of the *cis* and *trans* ester precursors in the synthesis of compound II. Compound II, subjected to microozonolysis (*6*), yielded 3,3-dimethylcyclohexanone, which was determined by GLC and spectral comparison with the known compound.

Compounds III and IV, when eluted as one peak from SE-30 into a drop of 2,4-dinitrophenylhydrazine reagent on a thin-layer plate (*7*), produced a derivative that chromatographed on thin-layer very similarly to standard terpene carbonyl compounds. The mass spectra of these two compounds were very nearly identical with a parent peak at 152. They were also very similar to the mass spectrum of compound II. Active MnO_2 oxidation (*8*) of compound II (both natural and synthetic) produced a compound identical in GLC behavior, mass spectrum, and biological activity to compound III. Similarly, oxidation of the synthetic *trans* isomer of alcohol II produced aldehyde IV.

Compound I, its *trans* isomer, and the 1,3-substituted cyclobutane isomers were synthesized by the sequence in Fig. 2. The photocycloaddition produced several other products besides those in Fig. 2. The GLC behavior and spectral data for synthetic *cis*-2-isopropenyl-1-methylcyclobutaneethanol were identical to those of natural compound I. Synthetic *trans*-compound I had almost identical GLC behavior to the *cis* isomer. Its NMR spectrum was also very similar except for the upfield shift of the methyl singlet from 1.22 ppm in the *cis* to about 0.95 ppm in the *trans*. The *trans* isomer was active in laboratory assays at concentrations 100- to 200-fold greater than the *cis*.

Table 1. Response of female boll weevils in laboratory bioassay to optimum concentrations of compounds I, II, III, and IV. The T/S equals net response to test sample divided by net response to a standard. The sample in each case consisted of the compound or the indicated mixture of compounds in 0.2 ml of dichloromethane. The standards used were live male boll weevils or steam distillates that were equivalent in attractancy to live males.

Sample	Respective amounts (μg)	Average T/S
I	0.09	0.11
II	0.07	0.06
III	0.12	0.00
IV	0.12	0.11
I, II	0.09, 0.07	0.15
I, III	0.09, 0.12	0.06
I, IV	0.09, 0.12	0.12
II, III	0.07, 0.12	0.24
II, IV	0.07, 0.12	0.12
III, IV	0.12, 0.12	0.16
I, II, III	0.09, 0.07, 0.12	1.00
I, II, IV	0.09, 0.07, 0.12	0.84
I, III, IV	0.09, 0.12, 0.12	0.07
II, III, IV	0.07, 0.12, 0.12	0.18
I, II, III, IV	0.09, 0.07, 0.12, 0.12	1.26

Compounds II, III, and IV were synthesized (*3*, *9*) by the addition of ethyl bromoacetate (Reformatsky) to 3,3-dimethylcyclohexanone, followed by dehydration to yield the *cis* and *trans* unsaturated ester precursors. These unsaturated esters were separated by GLC, and the *cis* and *trans* configurations were assigned on the basis of NMR spectra. The ethyl ester of *cis*-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexylacetic acid was reduced to compound II with $LiAlH_4$. Compound II was then oxidized to compound III with active MnO_2. Similarly, compound IV was obtained from

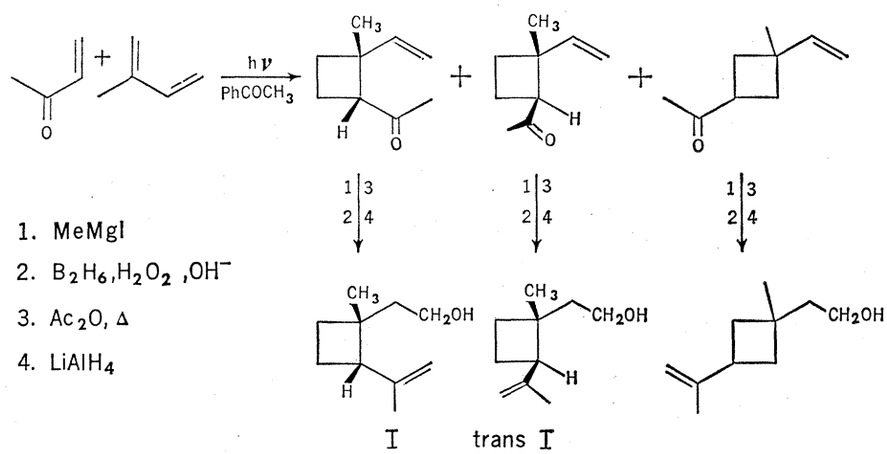

Fig. 2. Synthesis of compound I and isomers. The photocycloaddition of 3-buten-2-one and isoprene produced several other products, some of them in greater yields than the products shown.

the *trans* ester. The mass, infrared, and NMR spectra and the biological activity of the synthetic compounds were identical to those of the corresponding natural products.

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4. Mention of a proprietary product does not necessarily imply its endorsement by the U.S. Department of Agriculture.
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Changing Sensitivity of the Pubertal Gonadal Hypothalamic Feedback Mechanism in Man

Abstract. *Clomiphene citrate in doses which stimulate gonadotropin production in the adult suppresses urinary follicle stimulating hormone (FSH) excretion and plasma testosterone concentration in prepubertal children. Such results indicate that feedback between gonad and hypothalamus is operative and highly sensitive in prepubertal humans. Puberty in man, as in the rat, is accompanied by a decrease in the sensitivity of the feedback mechanism.*

Gonadotropins (*1*) and sex steroids (*2*, *3*) have been quantitatively determined in urine and blood of prepubertal children. The prepubertal gonad can be stimulated by gonadotropins (*4*), and the immature pituitary gland is responsive to hypothalamic gonadotropin-releasing factors (*5*). We now offer evidence for the existence of an operative feedback relation between the gonad and hypothalamus of the prepubertal child, the sensitivity of which changes during sexual maturation.

Clomiphene citrate, an antiestrogen, stimulates gonadotropin release in the adult, presumably by competing with steroids at hypothalamic receptor sites (*6*). The effect of this agent on gonadotropin secretion in the child is not known. Accordingly, doses of clomiphene which augment gonadotropins in the adult, approximately 1 to 100 mg/m^2 per day (*7*), were administered for 1 week to 16 children in various stages of sexual maturity. Clomiphene was administered to patients admitted for evaluation of short stature, delayed adolescence, or precocious puberty. Informed consent was obtained from the parents. Careful monitoring of hepatic and renal function was carried out before and after drug administration, as was close clinical follow-up. No untoward effects of clomiphene have been observed in any of these patients.

Urinary concentrates processed by the kaolin-acetone technique (*8*) were made from 30- to 60-ml portions of 24-hour urine specimens collected under hospital supervision. The concentrates were suspended in 2 ml of water, and samples of 50 to 200 μl were used for the radioimmunoassay of follicle-stimulating hormone (FSH) (*9*). Samples of concentrates from one or more urine specimens from each patient were parallel to samples of standard material, the 2nd International Reference Preparation of Human Menopausal Gonadotropin (2nd IRP). The three serial dilutions used to show this parallelism were also appropriate for testing assay sensitivity with reference to the unknown material. Measurements of urinary luteinizing hormone (LH), and of plasma FSH and LH as well, were made. Serial dilutions could not be carried out since these unknowns fell at the lower limits of the standard curve; consequently, for technical reasons, suppression of urinary LH and plasma gonadotropins could not be demonstrated. Testosterone in plasma was measured by a competitive protein-binding method (*3*) on the first and last day of clomiphene treatment in a number of the male patients.

Figure 1a shows the effects of daily administration ration of 100 mg of clomiphene per square meter of body surface for 7 days to two normal adult males and three prepubertal children. After the drug course, both FSH and plasma testosterone increased in the adult males, but these hormones appeared to decrease in the children. Indirect evidence for the fact that suppression of FSH in the children was caused by the known intrinsic estrogen action (*10*) of clomiphene (despite its antiestrogen activity) was the appearance of a small nubbin of breast tissue after the 1-week clomiphene course in both boys whose data appear in Fig. 1a.

Figure 1b shows a second series of patients given clomiphene, seven children between the ages of 5 and 16 years, who received 10 mg/m^2 per day for 1 week. All were prepubertal except for one boy, deficient in growth hormone and ACTH, who was in the very early stages of sexual maturation. In six of the seven individuals receiving this lower dose, clomiphene suppressed either the FSH in the urine or testosterone in the plasma.

Figure 1c shows the effects of still further decreases in clomiphene dosage, namely to 1.0 mg/m^2 per day. Urinary FSH was suppressed in three boys on this very small amount of the drug. One of these three patients received 10 mg/m^2 per day for seven additional days immediately after the first week at the 1.0 mg dose; little further decrease in FSH excretion took place during the second week of drug administration.

Since clomiphene appeared to elicit contrary effects in the pre- versus the postpubertal patient, the transitional period of puberty was next examined. Three girls in the early stages of sexual maturation were given 100 mg/m^2 per day for 1 week. The results (Fig. 1d) reveal FSH stimulation in one girl, suppression in another, and no change in the third. Such variable responses might be expected during the period of change from child to adult.

The interpretation of our results may be based on data available from lower animals, particularly the rat. For many years it has been known that the amount of estrogen necessary to suppress gonadotropin production in the prepubertal rat is far less than that needed for stimulation of the acces-