equatorial side predominantly. The similar explanation was made in the reaction of benzomorphan system by H. Kugita, *et al.*[6]

Experimental*3

(+)-Lupinine Methiodide——To the 100 ml. flask was added 0.7 g. (0.0046 mole) of S(+)-1-methylenequinolizidine, α_D^{26} +2.787°(l=1 cm., neat)[2] and 0.3 g. (0.0075 mole) of $NaBH_4$ in 20 ml. of diglyme. A solution of 1.4 g. (0.01 mole) of borontrifluoride etherate in 20 ml. of diglyme was added dropwise to the stirred reaction mixture over a period of 40 min. under N_2 atmosphere, while the temperature was maintained at 5~7°. The mixture was kept for 1 hr. at this temperature and then 1 hr. at room temperature. An excess of hydride was then decomposed by careful dropwise addition of 1 ml. of H_2O. The organoborane is oxidized by the addition of 4 ml. of 3*N* NaOH, followed by 4 ml. of 30% H_2O_2. The inorganic compound precipitated was filtered off, the filtrate acidified with dil. HCl and evaporated to dryness *in vacuo*. The residue was basified with K_2CO_3 and extracted with benzene. The extract was dried and evaporated *in vacuo* to leave 0.6 g. of colorless viscous liquid, a part (0.3 g.) of which was chromatographed on neutral alumina (Woelm activity III) to give 110 mg. of colorless oil. This was proved to be a mixture (10:1) of lupinine (III) and epilupinine (IV) by gas chromatographic analysis using a 3% carbowax 20*M* column at 174°. The methiodide was formed in benzene and recrystallized several times from EtOH to afford colorless needles, m.p. 288~290°, $[\alpha]_D^{15}$ −6.3°(c=0.72, MeOH). $[\alpha]_{260}^{15}$ −45°. *Anal.* Calcd. for $C_{11}H_{22}ONI$: C, 42.58; H, 6.78; N, 4.52. Found : C, 42.61; H, 6.79; N, 4.66. The IR spectrum (KBr) was identical with that of the authentic methiodide, m.p. 294°, $[\alpha]_D^{15}$ +11.2°(c=1, MeOH), prepared from natural(−)-lupinine. The epilupinine methiodide could not be isolated.

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*3 Melting points are uncorrected.

6) H. Kugita, M. Takeda : This Bulletin, **12**, 1166, 1172 (1964).

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Tetsuro Ikekawa,*1 **E. Lin Wang, Masa Hamada, Tomio Takeuchi, and Hamao Umezawa :** Isolation and Identification of the Antifungal Active Substance in Walnuts.

(*Institute of Microbial Chemistry**2)

The substance in walnuts of *Juglans regia* Linn. and *J. Sieboldiana* Maxim. exhibiting growth inhibition of *Trichophyton mentagrophytes* has been isolated, and it is confirmed that the active substance is identical with juglone (5-hydroxy-1,4-naphthoquinone).

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Pericarps of walnuts have been known since earlier days as a crude drug for treatment of Trichophytiasis,[1] but no report has been published about the active agent inhibiting *Trichophyton*.

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The present paper concerns the isolation of the antifungal active substance in walnuts. As methanol extract of green pericarps of walnuts inhibits growth of *Trichophyton*, extraction of the active substance was carried out by application of a cylinder plate method using *Trichophyton mentagrophytes* as a test organism, so that the active agent could be determined. Antifungal test showed that the active substance was quantitatively extracted with hexane, ether, benzene, ethyl acetate, or chloroform and was slightly unstable in alkaline condition but stable in acidic and neutral.

Walnuts of *Juglans regia* Linn. or *Juglans Sieboldiana* Maxim. were peeled in the green stage, and the pericarps were ground and the solid part was separated from the liquid part. The solid part was extracted with hexane or petroleum ether using the Soxhlet and the liquid part was also extracted with hexane or benzene. It was shown by bioassay that the active substance was nearly all extracted when the yellow color disappeared in the residues.

After careful evaporation of the organic solvent from the extract under vacuum, the crude active substance was obtained by extraction with a small amount of ethyl ether. Further purification was made by a column chromatography of calcium diphosphate using hexane for the elution or by sublimation under vacuum.

The active compound thus obtained was recrystallized from ethyl ether, hexane or petroleum ether to yellow needle crystals.

The crystals showed a single spot by thin-layer chromatography using several solvent systems, and a single peak by a gas chromatography. Ultraviolet, infrared and nuclear magnetic resonance spectra showed that it had an aromatic quinoid structure.

The quinoid structure was also suggested by the following color reactions : red by magnesium acetate,[2] reddish purple by alkaline, reddish orange by ferric chloride, positive by potassium permanganate and bromophenol blue,[3] negative by Brady, Molisch, Tollens, Benedict, maltol, anthrone, Fehling and diazo reactions. It contained no nitrogen, sulfur or halogen and was optically inactive by optical rotatory dispersion curve. Nuclear magnetic resonance spectrum showed that it had no aliphatic side chain.

From the above results and analytical data showing the formula of $C_{10}H_6O_3$, the crystalline active compound is suggested to be juglone (5-hydroxy-1,4-naphthoquinone).[4] The identity with juglone is proved by the mixed melting points, infrared spectra, thin-layer and gas chromatography.

Thus, it is confirmed that the substance in walnuts exhibiting inhibition of *Trichophyton* is 5-hydroxyl-1,4-naphthoquinone.

The antifungal and antibacterial spectra of juglone are shown in Table I and II, and green pericarps of walnuts contains the active substance, but the nut fruits show no activity against *Trichophyton*..

Experimental*3

Isolation of an Antifungal Agent Inhibiting *Trichophyton* from Walnuts——a) 16 kg. of green pericarps of *Juglans Sieboldiana* Maxim. were added to 16 L. of water and ground into pieces and the solid part was separated by filtration.

The solid part was extracted with hexane (11 L.) three times, and the filtrate was extracted with the same amount of hexane twice.

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*3 All melting points are uncorrected.

2) S. Shibata : Yakugaku Zasshi, **61**, 320 (1941); S. Shibata, M. Takido, O. Tanaka : J. Am. Chem. Soc., **72**, 2789 (1950).
3) E. Akita, T. Ikekawa : J. Chromatog., **12**, 250 (1963).
4) T. Shoji : Yakugaku Zasshi, **79**, 1034, 1041 (1959).

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After concentration about 6.8 g. of orange crystalline powder was obtained. It was dissolved in 300 ml. of ethyl ether and filtered. The residue was washed with 100 ml. of ethyl ether.

The extrated and washed solvents were evaporated under vacuum.

About 4.3 g. orange needle crystals were obtained. The yield from green pericarps was about 0.027% (g./g.). 20 mg. of the orange needles were sublimed under 1～3 mm. Hg vacuum at 75° for 2 hr. and orange crystals of 18.8 mg. were obtained. The yield shown by the bioassay was about 94% from the original needles.

b) Green pericarps of 30 nuts of *Juglans regia* Linn. were peeled and 1230 g. were obtained. They were ground and filtered to remove the liquid part and extracted with hexane using Sohxlet.

The extraction continued until the yellow color had vanished, and all of the active substance was extracted with hexane and the residue showed no activity. The extracted hexane was evaporated under vacuum and the extract was dissolved in 120 ml. of ether.

After evaporated the solvent and drying, the greenish yellow powder thus obtained (366.4 mg.) was subjected to a column chromatography of calcium diphosphate using hexane or petroleum ether for the elution. After concentration of the solvent, yellow crystals (308.6 mg.) were obtained and recrystallized from hexane or ethyl ether. The liquid part (800 ml.) was extracted with hexane, and after evaporated the active substance was extracted with ether and subjected to the calcium diphosphate column chromatography and recrystallized, yielding 48 mg. of the orange crystals. Thus totally 356.6 mg. of the crystalline material was obtained from 30 walnuts.

c) 353 g. of green pericarps were obtained from 30 nuts of *Juglans Sieboldiana* Maxim. and 170 g. of a wet solid part and 175 ml. of a liquid part were obtained. The active substance of the solid part was extracted with hexane and the extraction continued for about 1.5 days until the yellow color in the residue had vanished.

After evaporation, the extract was dissolved in 190 ml. of ethyl ether and 172 mg. of crude crystals were obtained. They were purified by sublimation under vacuum and recrystallized from hexane to obtain 144.1 mg. of yellow crystals. The liquid part was extracted with the same amount of hexane three times, yielding 18.7 mg. of yellow crystals by the same purification procedure.

Total 162.8 mg. of the active substance was obtained from 30 nuts and the yield was 0.046% of green pericarps.

The active substance was soluble in 1*N* NaOH and 1*N* Na_2CO_3 to be reddish violet. It was insoluble in 1*N* $NaHCO_3$. It was easily soluble in ethyl acetate, chloroform, acetone, benzene and soluble in ether and sparingly soluble in methanol, ethanol, buthanol and hexane.

It was identified with juglone by mixed melting points (m.p. 151～153°) and infrared spectra. It showed the same retention time by gas chromatography using SE-30 (8.2 minutes, column temp. 148°) as juglone, and the same Rf values on thin-layer chromatograms using silica gel Rf 0.53 hexane-ethanol (20:1), Rf 0.13 hexane-ethyl acetate (20:1), Rf 0.38 hexane-ethyl acetate (4:1). *Anal.* Calcd. for $C_{10}H_6O_3$: C, 68.96; H, 3.47; O, 27.56. Found: C, 69.53; H, 3.63; O, 27.56. UV λ_{max}^{MeOH} mμ: 249.5, 340 (shoulder), 426, $\lambda_{max}^{0.01N\ NaOH\ MeOH}$ mμ: 282, 350, 533. NMR $CDCl_3$: τ 1.6 (s), 2.3～3.0 (mul.), 3.2 (s).

Table I. Antibacterial and Antifungal Spectra of Juglone

Test organisms	Minimum inhibition concentration (γ/ml.)	Test organisms	Minimum inhibition concentration (γ/ml.)
Klebsiella pneumoniae 602	100	*Penicillium chrysogenum* 49-133	50
Pseudomonas aeruginosa	>100	*P. chrysogenum* 408-701	100
Proteus vulgaris OX 19	100	*Trichophyton mentagrophytes*	25
Salmonella paratyphi A	50	*T. mentagrophytes* 598	100
S. paratyphi B	100	*T. mentagrophytes* 429	100
S. typhi 63 (T_2)	100	*Botrytis bassiana*	50
Shigella flexneri (EW 8)	100	*Aspergillus niger*	>100
Micrococcus flavus 16	3.12	*Torula utilis* 4001	100
Staphylococcus aureus 209P	50	*Saccharomyces cerevisiae*	100
Bacillus subtilis NRRL 558	25	*Candida krusei* NI-7492	100
Corynebacterium xerosis	12.5	*C. stellatoidea*	25.0
Escherichia coli NIHJ	100	*C. pseudotropicalis* NI-7494	12.5
		C. tropicalis NI-7495	100
		C. albicans yu-1200	100
		C. albicans 3147	100
		Cryptococcus neoformans NI-7496	50

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Stability Test. After the solution of the active substance was warmed at 60°, for 0.5 hr. at pH 2.0, 5.0, 7.0 and 9.0, the residual activity was 93%, 100%, 98% and 90%, respectively.

Toxicity Test. LD_{50} i.p.: 25 mg./kg. mouse; LD_{50} oral: 250 mg./kg. mouse.

Antimicrobial Spectra. Antibacterial tests were made by the usual agar dilution method, and antifungal tests and tests for bacteria and fungi of plant disease were also done by the agar dilution method, adding 1% glucose to usual agar plates.

Table II. Antimicrobial Spectrum of Juglone for Bacteria and Fungi on Plant Disease

Test organisms	Minimum inhibition concentration (γ/ml.)	Test organisms	Minimum inhibition concentration (γ/ml.)
Piricularia grisea	100	*Gloeosporium kaki*	100
P. oryzae	50	*Gibberella saubinetti*	100
Schlerotium rofsii	50	*G. fujikuroi*	100
Pseudomonas solanacearum	>100	*Fusarium roseum*	100
Glomerella cingulata	>100	*F. oxysporium*	>100
Pellicularia filamentosa	25	*F. lini*	>100
Ophiobolus miyabeanus	50	*Colletotrichum phomides*	>100
Helminthosporium sigmoideum	100	*Cladosporium sphaerosporum*	>100
H. sesanum	50		

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