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Antituberculous Activity and Toxicity of Lupulon for the Mouse.*

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Wong and others have reported from the University of California on the *in vitro* activity of subtilin against *Mycobacterium tuberculosis*.[1-4] The parenteral application of this antibiotic has been interfered with by its relative insolubility in physiologic saline.[5] Antibiotics characterized by lipid solubility might overcome this difficulty. Two such agents prepared at the Western Regional Research Laboratory are lupulon and humulon derived from hops (*Humulus lupulus, L.*). Lupulon has the structure given below:[6] In humulon the side chain marked "*" is replaced by a hydroxyl group. A method for the isolation of lupulon from hops by direct crystallization was discovered by Michener *et al.*[7] and further simplified by Alderton.[8] The initial crystallization occurred in a vacuum concentrate of a direct petroleum ether extract of hops.

The antibacterial properties of hops have been recognized for many years, in connection with their use in brewing. Walker and Parker[9] reported lupulon and humulon to be 29,000 and 4,000 times, respectively, as active as phenol in restricting acid production by *Lactobacillus bulgaricus*. Shimwell[10] noted that hop extracts inhibited a considerable number of saprophytic Gram-positive bacteria, but not Gram-negative bacteria. Acid-fast mycobacteria and other pathogens were not tested. Michener *et al.*[7] reported antifungal activity for lupulon and humulon. Subsequent to the earlier observations reported in this paper, Reynolds[11] found inhibition of acid-fast organisms at 1:100,000 for lupulon and at 1:10,000 or 1:20,000 for humulon when this agent was incorporated in agar by a streak technic. Salle[12] obtained similar results against *M. tuberculosis* tested on both Long's and Dubos' media.

Hops have long been regarded as having hypnotic properties. Steidle[13] reported that hops produce paralysis and decreased excitability of the striated muscle and of motor nerve endings in frogs. Sikorski and Rusiecki[14] reported lupulon and humulon to be sedative for pigeons and small birds and somewhat less active in mice. These indications of pharmacologic action after oral administration, together with the fat-soluble nature of

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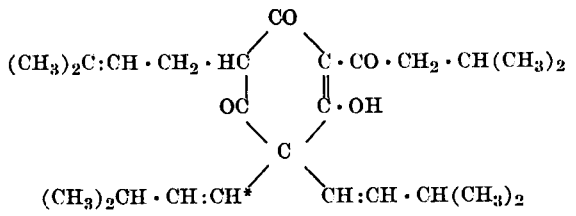

lupulon, made preliminary tests of the effect of this agent on experimental tuberculosis infections desirable.

Toxicity of lupulon. The toxicity of lupulon on single and repeated intramuscular injections was determined in inbred white mice. Crystals of lupulon were dissolved (1.5%) in cottonseed oil. The acute LD_{50} was found to be 600 mg per kilo. Deaths occurred within 1 to 12 hours. At autopsy, it was noted that incomplete absorption of the solution had occurred even after 5 days. This condition was observed in animals given only half of the LD_{50}. Daily injection of 60 mg per kilo over 4 weeks was tolerated without gross evidence of harmful effects. Histopathologic examination of tissue from these animals revealed small areas of leukocytic infiltration of the liver as well as foci of degeneration in the renal tubules.

Oral toxicity of lupulon, 5% suspended in gum acacia solution (6% in water) was determined in mice. Intragastrically, 1500 mg per kilo killed half of the animals within one hour of administration. All treated mice were depressed in contrast to those animals given the drug intramuscularly. Immediately before death, mice given doses twice the LD_{50} were excited and exhibited convulsions of a tetanic character. Congested and hemorrhagic lungs were observed at autopsy.

Antituberculous activity. Six samples of crystalline lupulon have been tested *in vitro* against tubercle bacilli (H37Rv) in Dubos' fluid medium.[15] Serial dilutions were made by pipetting appropriate amounts of lupulon solution in propylene glycol into 5 ml of the medium. Early samples were used in 1% solution in propylene glycol with the aid of heat, and later samples were dissolved first in 95% ethyl alcohol up to 10% solution and then diluted to 0.1% in propylene glycol. Growth of mycobacteria was inhibited at a dilution of 1:40,000 whether heat or ethyl alcohol was used to facilitate solution of the lupulon in the glycol. Humulon had a much lower potency and was not investigated further.

Various wetting agents,‡ including "Tween 60," "G-2144," "Nopacol," "Neutronyx," and "Triton," have been used to substitute for "Tween 80" in Dubos' medium without any effect on the tuberculostatic activity of lupulon.

The activity of lupulon has also been tested in serial dilution in the presence of a number of other antituberculous substances such as promin, streptomycin, subtilin, and 2,3-dimercaptopropanol (BAL). Sub-bacteriostatic concentrations of the latter agents (*e.g.*, 1:800, 1:3,200,000, 1:400,000 and 1:100,000, respectively) were used in the Dubos medium. Lupulon checked the growth of tubercle bacilli at 1:40,000 despite the presence of any of these agents. No synergism or antagonism occurred. The lack of synergism between lupulon and BAL is in contrast with the potentiation of action of subtilin and streptomycin by BAL, previously reported.[3]

Groups of 20 mice were infected intravenously with 0.02 mg of the H37Rv strain of *M. tuberculosis* grown in Dubos' medium. Lupulon was administered by two different routes. In one group of infected animals, it was given intramuscularly as a 1.5% solution in cottonseed oil at a daily single dose of 60 mg per kilo. In another group, it was administered intragastrically as a 3% suspension in 6% gum acacia solution (aqueous)

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‡ Kindly supplied by Dr. R. J. Dubos, Rockefeller Institute for Medical Research, New York. Mention of this and other products does not imply that they are endorsed or recommended over others of a similar nature not mentioned.

TABLE I.
Tuberculostatic Activity of Lupulon in Infected Mice.

Antibiotic	Experiment	Route	Approx. LD_{50} mg/kg	Daily dose mg/kg over 30 days	Local effects noted grossly	Avg gross lung lesions*	Tissue reaction in all organs examined	Relative abundance of T.B. in all organs†	No. animals in each group
Controls	I	—	—	—	—	3.7	18	713	20
"	II	—	—	—	—	5.0	24	1,514	20
Lupulon	II	Oral	1500	150 (twice daily)	None	2.3	18	382	20
"	I	I.M.	600	60 (daily)	"	1.9	12	231	20
"	II	I.M.	600	60 (daily)	"	2.8	16	368	20

* *Tissue Reaction:* Number indicates total (average) degree of lesions (larger means greater involvement); *e.g.*, Lung: 8 = 50-100% of tuberculous tissue in the whole organ as observed grossly as well as microscopically; 4 = 25-50%; 2 = 10-25%; 1 = 1-10%; 0 = no gross lesions. Spleen and liver: 8 = 10 lesions per low power field; 4 = 5-10 lesions; 2 = 2-5; 1 = lesions in any portion; 0 = no apparent lesions. Kidney and heart: Number = No. of lesions in the section; exception 4 = 4 or more.

† *Abundance of Mycobacteria:* Number indicates total (average) relative number of mycobacteria in all lesions; *e.g.*, 1,000 = more than 10 bacilli to each cell; 100 = 1-10 to each cell; 10 = 1 bacillus to 1-10 cells; 1 = 1 bacillus to 1-5 lesions; 0 = no organisms.

at a dose of 150 mg per kilo at 12-hour intervals. Other infected animals served as untreated controls. The results are shown in Table I. The evaluation of the gross tuberculous lesions in the organs was based on histopathologic sections stained with hematoxylin and eosin for lesions and by acid-fast methods for determining abundance of tubercle bacilli present.

Despite its relatively low *in vitro* activity, lupulon exerted considerable suppressive effect on the development of tuberculosis in mice, whether it was given intramuscularly or orally. It appeared to check the multiplication of tubercle bacilli to a similar extent when given orally. The latter method gave greater suppression in the development of tuberculosis in the second series of experiments where a difference in multiplication of mycobacteria was apparent.

Table II gives further details regarding the relative abundance of mycobacteria in lesions of treated and untreated mice. In comparing the prevalence of microorganisms in the lungs, spleen, liver, kidneys, and heart of the control animals, notable differences were apparent at the end of the 30-day period with their relative abundance in lupulon-treated animals. The ratios (in Experiment I) approximated from 34 to 1 (comparison of controls with lupulon-injected mice) for liver, 8 to 1 for heart, 4 to 1 for spleen, and 4 to 1 for lungs. After oral use (in Experiment II) the ratios were: Liver 10 to 1, heart 3 to 1, spleen 10 to 1, lungs 5 to 1, kidneys 2 to 1: an over-all 4 to 1 difference. Only the renal lesions had a slight and perhaps insignificantly greater number of mycobacteria in lupulon-injected animals, in the first but not in the second series. Renal damage might be related to tubular changes which followed continual administration of this antibiotic, and these may have been accentuated by the extent of renal infection, with concurrent drug damage to this tissue. Another possibility is that the foci of degeneration noted in the renal tubules (in infected intramuscularly treated animals) may have permitted more extensive tuberculous involvement.

The lupulon-treated mice had lower indices

TABLE II.
Relative Abundance of Mycobacteria* in Lesions of Treated and Untreated Mice.

Antibiotic	Experiment	Route	Lungs	Spleen	Liver	Kidneys	Heart	Sum total
Controls	I	—	471	19.3	68.3	70.7	83.8	713
"	II	—	353	277	278	170	436	1514
Lupulon	II	Oral	78	27	27	80	170	382
"	I	I.M.	121	4.5	2.0	93.4	10.1	231
"	II	I.M.	14.6	3.8	12.9	2.7	334	368

* See Table I for key.

of tissue reaction, as measured by the development of lesions, in all but the renal and cardiac tissues. The latter organs in all groups did not differ appreciably in occurrence of lesions. In the lungs, not only was there a lower percentage of tuberculous tissue in lupulon-treated mice than in the controls but the type of tissue reaction was also different. The lesions in the lungs in the lupulon-treated mice were predominantly proliferative, while those in the controls were predominantly necrotic and exudative.

Discussion. An over-all examination of these data showed a significantly lower number of mycobacteria in lesions of lupulon-treated mice. Thus far, in chemotherapeutic trials in animals, only indefinite numbers of mycobacteria in lesions have been reported.[16-18] In our opinion, the numbers of mycobacteria in the lesions is of great importance to the solution of the problem of ultimate control. The use of a drug should be for a relatively short period in terms of the life span of the individual. On cessation of therapy, there should be left in lesions a small enough number of bacilli for the natural defense mechanism of the host to combat successfully.

Since the lipid fraction of tubercle bacilli is a major part of the organism, it would appear reasonable to expect a fat-soluble antibiotic to have a marked affinity for these bacteria, and that this affinity might be reflected in the animal. Fat-soluble usnic acid, which has a relatively low tuberculostatic effect *in vitro*, has a definite effect in guinea pigs.[19] This hypothesis is also suggested by our observations; *e.g.*, the lipid soluble lupulon is active *in vivo*.

Lupulon, like the aerosporins and polymyxins, appears to have an affinity for the renal tubules when it is given intramuscularly. Whether these changes in the tubules are reversible or not remains for further experiment. The relatively mild leukocytic infiltration of the liver may not be significantly harmful. This may indicate a mobilization of the antibiotic from the site of injection and its storage in the liver. Storage in the liver would be compatible with the finding that the greatest reduction in mycobacteria occurred, among lupulon-treated animals, in this organ.

Summary. Lupulon, a fat-soluble antibiotic derived from hops, has relatively low *in vitro* activity (1:40,000) as compared with other antituberculous agents. Despite this observation, lupulon (given orally or intramuscularly) *was active* against experimental mouse infections of *M. tuberculosis*. Following intramuscular administration, significantly lower numbers of acid-fast organisms occurred in lesions of treated animals. The approximate numbers relative to the control were: In the liver, 34 to 1; heart, 8 to 1; spleen, 4 to 1; and lungs, 4 to 1; but not in the kidneys. In orally treated animals the ratios were: Liver, 10 to 1; heart, 3 to 1; spleen, 10 to 1; lungs, 5 to 1, and kidneys, 2 to 1. The over-all difference was a reduction of approximately 4 to 1 by either route of administration.

Lupulon given intramuscularly within its effective range produced some foci of degen-

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eration in renal tubules. Such pathologic changes were not observed in infected animals given this antibiotic orally in effective doses. The single LD_{50} on intramuscular administration was 600 mg per kilo in mice; on oral application, 1,500 mg per kilo.

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Tocopherol vs. Tocopherol Acetate as a "Sparer" of Vitamin A.

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The early observation[1] that materials containing vitamin E delayed the auto-oxidative destruction of carotene and vitamin A in fatty food mixtures was explained when the tocopherols were shown to be antioxidants.[2] That such protection may augment and conserve the vitamin A stores of an animal was first demonstrated by Moore[3] and has been confirmed repeatedly, as detailed in several reviews and some recent reports.[4]

The evidence suggests that inhibited oxidation in or near the alimentary tract[5] accounts for the survival of carotene and vitamin A. Recently,[6] this protective action appeared to be confined to preserving the store of vitamin A already deposited in the liver. The various forms of vitamin A behave differently. The results are modified by the form and method of administering the preparations of vitamin A[7] and vitamin E[8]. Other compounds having possible stabilizing effects[9] may be present and effective.

Several reports have stated or implied that the stabilizing action of the tocopherols is not limited to the alimentary tract but extends to the tissues of rats.[8,10] This was not demonstrable in rabbits.[11] Some information on this question might emerge from experiments with the esters or α-tocopherol, among them the acetate, which is also an oil, and is a dependable source of vitamin E, perhaps because it is not auto-oxidizable and has no antioxygenic action. Presumably, α-tocopherol acetate is hydrolyzed in the intestinal tract, and the free alcohol should therefore be available in the tissues in quantities undiminished by having provided prior stabilization in the food.

The only pertinent observation on the acetate appears to be that of Bacharach,[12] to the effect that only at high levels of feeding and for an extended period (60 days) was any conservation of vitamin A demonstrable. A comparison of α-tocopherol and its acetate was therefore undertaken, to assess the relative protective value of each by simple experiments patterned after the U.S.P. bio-

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