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Yin-Ch'Ang Chin, … , Nai-Ch'U Chang, Hamilton H. Anderson

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Research Article

FACTORS INFLUENCING THE ANTIBIOTIC ACTIVITY OF LUPULON [1, 2, 3]

By YIN-CH'ANG CHIN, NAI-CH'U CHANG,[4] AND HAMILTON H. ANDERSON

(Division of Pharmacology and Experimental Therapeutics, University of California Medical School, San Francisco, California)

Lupulon is one of the two lipid-soluble antibiotics prepared from hops (*Humulus lupulus* L.) (1, 2). Michener *et al.* reported antifungal activity for both lupulon and humulon. Salle *et al.* (3) confirmed Shimwell (4), that Gram-positive organisms are more susceptible to lupulon than are Gram-negative bacteria. Chin *et al.* (5) reported its antituberculous activity both *in vitro* and *in vivo*. These findings and its relatively low toxicity (6) indicated that lupulon may be an effective chemotherapeutic agent. It becomes, therefore, desirable to investigate some important factors which may influence its activity either *in vitro* or *in vivo*.

MATERIALS AND METHODS

Crystalline lupulon used throughout this experiment was prepared at the Western Regional Research Laboratory, Albany, California. One % solution can be made in alkaline water at pH around 11.5 or by dissolving the crystals first as 10% solution in ethyl alcohol and then diluting up to volume with propylene glycol. The antibiotic activity has been found to be the same whether the solution was first made in alkaline water or in propylene glycol. The latter solution has a pH at 4.8 and has been used, freshly prepared with aseptic precautions, as the stock for further dilutions in alkaline water, serum, or culture media.

Staphylococcus aureus (FDA No. 209), *Mycobacterium phlei*, and *Mycobacterium tuberculosis* H37Rv were used in the experiments. Unless otherwise mentioned, veal-glucose broth (7) was used for growth of and tests on *Staph. aureus*, and Dubos and Davis fluid medium (8) for the mycobacteria. The Coleman junior spectrophotometer was used for turbidimetric determinations of the growth of organisms.

RESULTS

Effect of size of inoculum. The effect of the size of inoculum of *Staph. aureus* on the antibiotic activity of lupulon was studied with the serial dilution method. Two-fold dilutions of the antibiotic were made in 0.5 ml. of the medium in nine 10-cm. test tubes, starting from a concentration of 200 μg./ml. A tenth tube containing the same amount of the medium served as the control in a series. Each tube was then inoculated with 1.5 ml. of a diluted 24-hour culture. Each of four different dilutions, namely, 1:100, 1:1000, 1:10,000, and 1:1,000,000, was inoculated to one series of tubes. The tubes were incubated at 37° C. and observations were made four, 24, and 48 hours later. The results are shown in Table I where even a faint growth was recorded positive.

Apparently the end point of no growth varied with the concentrations of the inoculum and also the time when the observation was made. Repeated experiments showed that observations at four hours after inoculation gave consistent end point at 1:640,000 or 1.56 μg./ml. in the series where the concentration of the inoculum was 1:1,000 of a 24-hour culture. This size of inoculum and time of incubation have been adopted in later experiments where serial dilution method was used.

Effect of constitution of medium. The serial dilution method was used to determine the bacteriostatic level of lupulon against *Staph. aureus* in three different media. These media,[5] namely,

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[2] Part of a cooperative study with Drs. J. Lewis and G. Alderton, Western Regional Research Laboratory, U. S. Department of Agriculture, Albany, California.

[3] Supported, in part, by Eli Lilly and Company, Indianapolis 6, Indiana.

[4] Peiping Union Medical College Fellow in Pharmacology.

[5] The compositions of these media are given below:

Modified Dubos and Davis medium:	
Casein hydrolysate	1.0 gm.
$Na_2HPO_4 \cdot 12H_2O$	6.3 gm.
KH_2PO_4	1.0 gm.
Na_3citrate$\cdot 2H_2O$	1.5 gm.
$MgSO_4 \cdot 7H_2O$	0.6 gm.
Distilled water to	1000.0 ml.
Beef heart infusion broth:	
Beef heart	500 gm.
Difco neopeptone	10 gm.

TABLE I

The effect of size of inoculum of Staph. aureus *on its growth in various concentrations of lupulon at 37° C.*

Concentration of the inoculum	Hours of incubation	Concentration of lupulon, μg./ml.									
		50.0	25.0	12.5	6.25	3.12	1.56	0.78	0.39	0.20	0
1:1,000,000	4	−	−	−	−	−	−	−	−	−	−
	24	−	−	−	−	−	+	++	+++	+++	+++
	48	−	−	−	−	+	++	+++	+++	+++	+++
1:10,000	4	−	−	−	−	−	−	+	+	+	+
	24	−	−	−	−	−	++	+++	+++	+++	+++
	48	−	−	−	−	+	+++	+++	+++	+++	+++
1:1,000	4	−	−	−	−	−	−	+	++	++	+++
	24	−	−	−	−	−	+	++	+++	+++	+++
	48	−	−	−	−	++	+++	+++	+++	+++	+++
1:100	4	−	−	−	−	+	+	++	+++	+++	+++
	24	−	−	−	−	++	+++	+++	+++	+++	+++
	48	−	−	−	−	++	+++	+++	+++	+++	+++

modified Dubos and Davis medium, a beef heart infusion, and veal-glucose broth, had been shown by turbidimetry to support the growth of *Staph. aureus* to different extents. It was found, however, that the growth was completely inhibited by lupulon at the same concentration, 1.56 μg./ml., no matter in which medium this antibiotic was incorporated.

Effect of pH

Studied with Staph. aureus. Veal-glucose medium was titrated to pH's 7.1, 7.6 and 8.1. For each pH duplicate series of six tubes were used. Into each of the six tubes in a series, 3 ml. of the medium was dispensed, containing an appropriate amount of lupulon so that after the inoculum was added to it the final concentrations of lupulon were 0, 0.2, 0.4, 0.6, 0.8, and 1.0 μg./ml. An inoculum consisted of 3 ml. of 1 : 50 dilution of a 24-hour culture so that the final concentration of the bacteria in each tube was 1 : 100 of the culture. The growth in each tube was measured turbidimetrically after four hours incubation at 37° C. The results, as shown in Figure 1, indicated greater inhibitory power of lupulon at lower pH.

Plate-well assay was performed by following Pratt and Dufrenoy's method (9), with some modifications. The inoculum of *Staph. aureus* of the top agar was 2%. The inhibition zones were measured after overnight incubation at 37° C., and the staining processes with 1% potassium ferricyanide and 1% ferric sulfate were applied only when necessary. Dilutions of lupulon were made in alkaline water adjusted to pH's 7, 8, 10, and 11.5. The inhibition zones produced by 50, 20, 10, and 5 μg./ml. were fairly consistently 28, 24, 21, and 18 mm. for respective concentrations at all pH's.

Studied with M. phlei. Dubos medium used in this experiment was titrated to pH's 5, 6, 7, and 8. The concentrations of lupulon in duplicate series of tubes were 0, 30, 35, 40, 45, 50, 55, 60, 70, and 80 μg./ml. The inoculum was made so that the final concentration of bacteria in a tube was 1 : 100 of a 48-hour culture. The end points, taken as the concentration in which no growth occurred after 24 hours incubation at 37° C., were 50 μg./ml. for pH 7 and 8, and 40 μg./ml. for pH 5 and 6.

Studied with M. tuberculosis (*H37Rv*). The experiment was performed in a similar manner to that with *M. phlei.* The concentrations of lupulon in a series of tubes were 0, 5, 10, 15, 20, 25, 30, and 40 μg./ml., and the inoculum was 0.05 ml. of 14-day culture to a volume of 5 ml. of the medium. End points were taken at the end of 72 hours: At pH 7 and 8 it was 25 μg./ml.; at pH 6, 15 μg./ml. The organisms did not grow well at pH 5.

Effect of NaCl

Studied with Staph. aureus. The veal-glucose medium contains 0.5% NaCl. The salt concen-

NaCl	5 gm.
Distilled water	1000.0 ml.
Veal-glucose broth:	
Veal infusion	500.0 gm.
Bacteriological peptone	10.0 gm.
NaCl	5.0 gm.
Dextrose	1.0 gm.
Distilled water	1000.0 ml.

tration was also increased to 1 and 2%. Since NaCl itself at concentrations higher than 2% inhibited partly the growth of the bacteria, they were not included in this experiment. The results of the turbidimetric measurements indicated that 2% sodium chloride increased slightly the activity of lupulon (Figure 1).

Plate-well assay showed no influence of 0.5, 1, 2, and 5% NaCl on the activity of lupulon when the salt was incorporated in the lupulon solutions. However, as illustrated in Table II slightly larger inhibition zones were produced by the same concentrations of lupulon when the salt was incorporated in the agar plates.

TABLE II

The effect of NaCl incorporated in agar plates on the activity of lupulon against Staph. aureus

Concentration of NaCl in agar %	Concentration of lupulon, μg./ml.			
	50	20	10	5
	Average diameter of inhibition zones, mm.			
0.5	28	24	21	17.5
1.5	27.5	24	21.2	19
2.5	27.5	24.5	21.5	19.5
5.5	27.5	24.5	22	20.2

Studied with M. tuberculosis (*H37Rv*). It has also been shown by experiment, as described previously, that 2% sodium chloride increased the

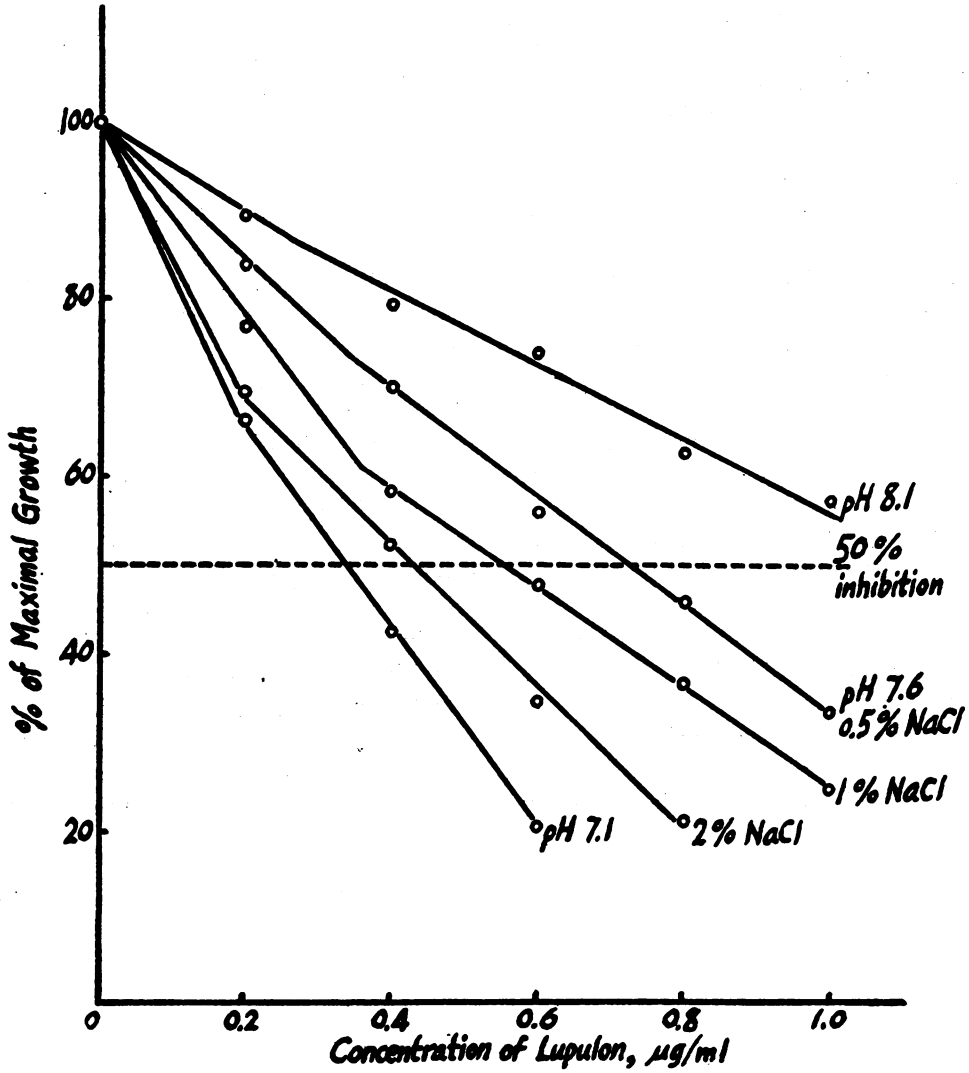

FIG. 1. EFFECT OF pH AND NaCl ON GROWTH OF *Staph. aureus* IN VARIOUS CONCENTRATIONS OF LUPULON

TABLE III

The effect of NaCl in Dubos fluid medium on the growth of M. tuberculosis *(H37Rv) in various concentrations of lupulon*

Concentration of NaCl %	Concentrations of lupulon, μg./ml.							
	40	30	25	20	15	10	5	0
0	−	−	−	++	+++	+++	+++	+++
0.5	−	−	−	+	+++	+++	+++	+++
1.0	−	−	−	+	++	+++	+++	+++
2.0	−	−	−	−	−	++	+++	+++

antibiotic activity of lupulon against *M. tuberculosis* (H37Rv) (Table III).

Effect of serum

Studied with M. tuberculosis *(H37Rv)*. Pooled human serum, Seitz-filtered and inactivated at 56° C. for 30 minutes, was added to Dubos medium at three different concentrations, namely, 5, 7, and 10%. Similarly treated horse serum was used at 10%. Since Dubos medium containing 20% of either human or horse serum became turbid and then produced sediments after 24 hours incubation at 37° C. which interfered with reading the end points, this concentration was not included in the present study. The results of repeated experiments by serial dilution method showed consistently that the growth of tubercle bacilli was completely inhibited by lupulon at a concentration of 1:40,000 or 25 μg./ml. regardless of the presence of either serum up to 10%.

Studied with M. phlei. Table IV shows the effect of various concentrations of inactivated horse serum on the bacteriostatic level of lupulon against *M. phlei.* In the presence of 10% serum the growth was completely inhibited by 70 μg./ml. instead of 50 μg./ml. in its absence. There was, therefore, an apparent 30% reduction of the activity.

Studied with Staph. aureus. By the serial dilution method, using 1:1,000 dilution of 24-hour culture for inoculation, the concentration of lupulon which caused complete inhibition of growth of this bacteria in veal-glucose medium containing 10% inactivated horse serum was 12.5 μg./ml. in contrast to 1:640,000 or 1.56 μg./ml. in the absence of the serum. The activity of lupulon was apparently reduced to about one-tenth of its original level.

Turbidimetric and plate-well assay were then performed to give this effect a further analysis. In turbidimetry, six duplicate series of six tubes containing various amounts of lupulon were prepared as described previously in this paper. In five of such series the medium contained five different concentrations of inactivated horse serum, namely, 50, 20, 10, 5, and 2%. The sixth series served as the control without serum. The results, as shown in Figure 2, indicated that there was a reduction of the activity of lupulon to one-tenth. The growth of *Staph. aureus* was practically not inhibited by lupulon in the presence of 50% serum.

For plate assay, 0.9% NaCl was adjusted to pH 11.5. Inactivated horse serum was added to it at 2, 10, and 50%. Lupulon was diluted in these solutions at 50, 20, 10, and 5 μg./ml. It was also diluted in 100% serum as well as the saline without serum. The inhibition zone was plotted against the logarithm of the concentrations (Figure 3). That produced by 50 μg./ml. in 100% serum was of approximately the same size as one produced by 5 μg./ml. in saline. The inhibitory power of lupulon in 10% serum was about 30% of that in its absence; in contrast, only one-tenth was found in the fluid medium as determined by serial dilution or turbidimetric method.

The apparently dissimilar effect of serum on the antibiotic activity of lupulon against different bacteria as well as against the same bacteria in dif-

TABLE IV

The effect of horse serum in Dubos medium on the growth of M. phlei *in various concentrations of lupulon*

Concentration of serum %	Concentration of lupulon, μg./ml.									
	80	70	60	55	50	45	40	35	30	0
0	−	−	−	−	−	+	+	++	+++	+++
2	−	−	−	−	−	+	+	+++	+++	+++
5	−	−	−	+	+	+++	+++	+++	+++	+++
10	−	−	++	++	+++	+++	+++	+++	+++	+++

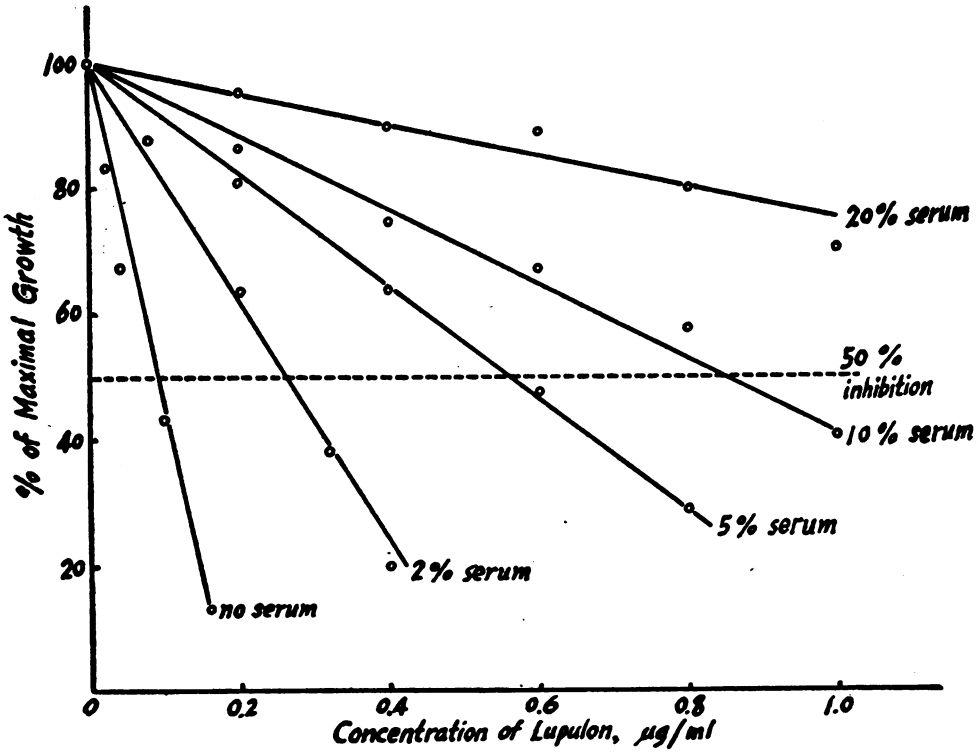

FIG. 2. EFFECT OF HORSE SERUM ON GROWTH OF *Staph. aureus* IN VARIOUS CONCENTRATIONS OF LUPULON

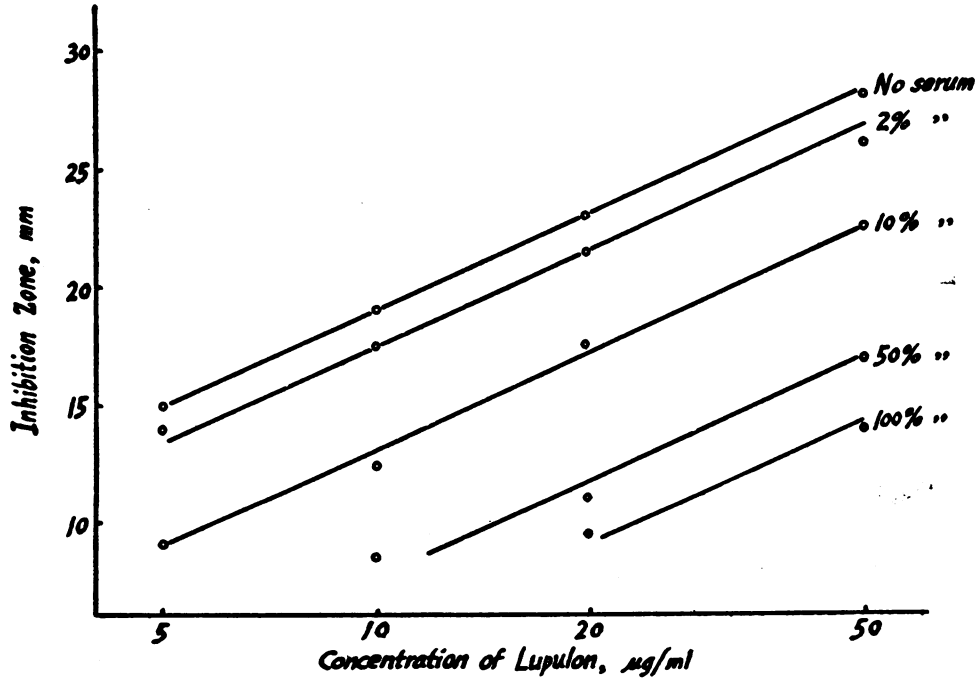

FIG. 3. EFFECT OF HORSE SERUM ON ACTIVITY OF LUPULON BY PLATE-WELL ASSAY AGAINST *Staph. aureus*

ferent media indicated that the effect was unlikely exerted on the antibiotic itself. This point was substantiated by the following experiment.

A 1,000 μg./ml. solution of lupulon in inactivated horse serum (A) and another in alkaline water (B) were made from 1% propylene glycol solution. After 24 hours, a fresh solution of each (C and D) was prepared from the same propylene glycol solution. Thirty minutes later, equal volumes of 95% ethyl alcohol were added to all tubes, and those containing serum were centrifuged to separate the precipitates. The supernatant fluid and the alkaline-alcohol solution were diluted to 50, 20, 10, and 5 μg./ml. of lupulon, calculated on the basis of the amount originally added to the solutions before adding ethyl alcohol. A series of alkaline solutions (E) diluted from the same propylene glycol preparation and another series of similar dilutions of the solvents only (F) served as controls. Each series of dilutions was assayed by duplicate plates. It was definitely shown (Table V) that all the activity of lupulon was retained in the supernatant fluid. It is, therefore, believed that whatever the serum contained which produced the apparent reduction in the activity of lupulon had been removed by precipitation with 48% ethyl alcohol. When the same supernatant and alkaline-alcohol solutions were tested by serial dilution method the same end points were obtained.

TABLE V

The results of plate-well assay of the supernatant fluids after precipitation of proteins from lupulon solutions in horse serum with 48% ethyl alcohol

Solution assayed	Concentration of lupulon, based on the amount originally present in the solutions before adding ethyl alcohol, in μg./ml.			
	50	20	10	5
	Average diameter of inhibition zones in mm.			
Supernatant (A) after precipitation of proteins	27.5	22.5	19.5	16
(B) + equal volume of 95% alcohol	28	23	20.2	16.5
Supernatant (C) after precipitation of proteins	28	23.2	19.5	16.2
(D) + equal volume of 95% alcohol	28.2	22.7	19.7	16.2
Alkaline solutions (E) without alcohol	27.5	22.7	19.5	16
Solvent controls (F)	0	0	0	0

DISCUSSION

Just how far the design of *in vitro* experiments on antibacterial agents can simulate the conditions *in vivo* is not certain. However, a study of the effect of such factors as pH, sodium chloride, and serum gives information regarding the activity of these agents before or when they act on bacteria. Such a study on lupulon has indicated that at the pH, the concentration of sodium chloride, and the presence of serum, which are normal to the body, this antibiotic remained active.

Lupulon is less soluble in water but more so in lipids at low pH. The greater partition coefficient, therefore, may serve as an explanation for its availability to the bacterial cells (10) and thus its greater antibacterial activity at lower pH. Penicillin is also more active at lower, and less at higher pH, which Abraham and Duthie (11) suggested as an explanation of the action of penicillin by competition with OH^- ions for position on the cell surface in order to produce its effect. A similar theory might be suggested also for lupulon on account of the above-mentioned facts.

Addition of sodium chloride to the media increased slightly the antibiotic activity of lupulon. In no case was it decreased, although the presence of the salt in lupulon solution assayed on plates produced no effect at all. Such minor discrepancy may be explained on the basis of a difference in the rate of diffusion as in the case of streptomycin (12).

That lupulon is not inactivated by serum has been shown by the fact that it retained activity after standing in serum solution for 24 hours. The apparent effect of serum on the antibiotic activity must be explained either by a change of sensitivity of the bacteria or by the presence of some antagonizing factors in the serum. No attempt has been made to elucidate this point. However, it is known that the apparent effect could be removed by treating solutions with 48% ethyl alcohol, whatever its causal agent may be. It is unlikely that the effect was due to growth promotion by serum, because it has been found that the same

bacteriostatic level of lupulon against *Staph. aureus* was obtained from three different media which supported its growth to varying degrees.

The apparent activity of streptomycin varied inversely with the size of inoculum, which Berkman *et al.* (13) explained to be due to the presence of a greater number of resistant organisms in a larger inoculum. This explanation has been adopted here, with reservation, for a similar finding with lupulon.

SUMMARY

Lupulon inhibits the growth of *Staph. aureus* (FDA No. 209), *M. phlei* and *M. tuberculosis* (H37Rv) at a concentration of 1.56, 50, and 25 μg./ml., respectively. In the case of *Staph. aureus* a higher concentration is required for a larger inoculum. It is more active at low pH. Sodium chloride at a concentration of 2% increases slightly its activity.

Its potency against *M. tuberculosis* is not affected by 10% horse or human serum. However, a 30% decrease has been observed under the same conditions for *M. phlei.* The activity against *Staph. aureus* has been estimated by serial dilution and turbidimetric methods to be reduced to one-tenth of its original level in 10% horse serum. By plate-well assay a similar reduction has been observed in 100% serum. All the activity of lupulon has been found to be retained in the supernatant fluid after precipitation of proteins from solution in horse serum with 48% ethyl alcohol. These observations argue against any inactivation of lupulon by serum.

The results of this experiment indicate that under the conditions of the pH, the concentration of sodium chloride, and the presence of serum, which are normal to the body, lupulon should remain active.

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