TABLE II. Results of Pooled Blood Plasma Analyses.

Time after glycine dose, hr	B_{12} deficient		B_{12} injected	
	N.P.N., mg %	Amino N, mg %	N.P.N., mg %	Amino N, mg %
0	24	9	—	8
2	20	19	35	20
4	43	20	32	16

ately after this bleeding, capsules containing 1 g of glycine were force-fed to all remaining chicks of both groups. Two and 4 hours after the glycine feeding blood samples were obtained by heart puncture and treated as above. Segments of liver and kidney were taken for histological examination. These were fixed in Carnoy's solution and stained with hematoxylin and eosin.

Results. Two hours after the glycine administration, the birds of both the deficient and supplemented groups seemed normal with respect to appearance and behavior. Four hours after the glycine dosage, a dramatic change appeared in the vit. B_{12}-deficient chicks. Two had died and those which remained alive were extremely weak and semicomatose. Several were unable to stand and appeared near death. It was extremely difficult to obtain blood from these birds, probably because of circulatory failure. In marked contrast, the vit. B_{12}-treated chicks appeared normal and lively.

Microscopic examination of histological sections of liver and kidney tissue revealed no difference between the two groups. The results of the blood plasma analyses are shown in Table II.

In a second trial a similar procedure was followed except that food was left before the chicks at all times. Four hours after glycine feeding, the chicks of the vit. B_{12}-deficient group were noticeably listless although they were only slightly affected when compared with the starved B_{12}-deficient chicks of the first trial. The B_{12}-injected birds again seemed normal. No blood or histological examinations were performed on these birds.

Discussion. Both the plasma non-protein nitrogen and amino nitrogen levels showed that the vit. B_{12}-injected birds were able to metabolize the administered glycine better than the vit. B_{12}-deficient birds. In the former group, NPN and amino N were highest 2 hours after glycine administration and had diminished by 4 hours afterwards. In the B_{12}-deficient group, however, NPN and amino N levels did not fall after 2 hours but increased to some extent at 4 hours. These high levels were associated with the toxic effect of the glycine. It was interesting to note that vit. B_{12}-deficient chicks which were on feed during the whole period were not as affected by the glycine as those which were starved. It is possible that energy as well as vit. B_{12} is required in nitrogen metabolism.

Summary. The plasma levels for non-protein nitrogen and amino nitrogen after administration of glycine were higher in vit. B_{12}-deficient chicks than in B_{12}-injected chicks. One gram of glycine, when force fed in gelatin capsules, was toxic to starved, B_{12}-deficient chicks and less toxic to B_{12}-deficient chicks which had not been starved. Chicks which had been injected with vit. B_{12} were able to withstand the glycine whether or not they had been without food.

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Antagonistic Effect of Serum on Bacteriostatic Action of Lupulone. (18446)

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The loss of the antibiotic properties of lupulone when mixed with serum has been noted by Salle, Jann, and Ordanik(1) and by Chin, Chang, and Anderson(2). An effort has been made by this laboratory to determine the cause of this inactivation in order that the

TABLE I. Inhibition Zones of Lupulone Mixed with Serum Fractions.

Fraction added	15%* serum equivalent		5%* serum equivalent	
	B. subtilis	C7	*B. subtilis*	C7
—	21.7 mm	21.9 mm	21.7 mm	21.9 mm
I	13.7	13.2	15.7	15.2
II + III	<10	<10	10.5	<10
IV	<10	<10	<10	<10
V	<10	<10	11.2	15.5
VI	17.6	20	18.5	19
Whole plasma	<10	—	<10	<10
Whole serum	<10	<10	<10	<10

* Total solids basis.

limits of usefulness of this antibiotic(3) might be better realized.

Materials and methods. Cup plate assays were performed on Medium II* agar, with *Bacillus subtilis and Micrococcus sp* (C7) serving as test organisms. Manometric oxygen uptake experiments were carried out in 0.04 *M* phosphate buffer, pH 7, containing 0.01 *M* succinate as substrate. A suspension of *B. subtilis* grown 18 hours on Medium II at 35°C in a shaking machine served as the test organism and was added from the side-arm. The cells were washed once in 0.025 *M* phosphate, pH 7, and resuspended in buffer of the same composition. Growth experiments were carried out as follows. Tubes containing 10 ml of medium IIb† and varying amounts of lupulone were supplemented with 0.5 ml of normal or extracted plasma. All tubes were then inoculated with one drop of an 18-hour broth culture of *Streptococcus fecalis* grown on medium IIb. Turbidity was determined periodically with a Coleman Model II spectrophotometer, at 650 mμ.

Aqueous lupulone suspensions were generally prepared from concentrated alcoholic solutions of crystalline lupulone. Lecithin and cephalin preparations from eggs were obtained from E. B. Kester of this laboratory. Citrated plasma, supplied through the courtesy of Cutter Laboratories, was used rather than serum in most experiments. It had lupulone-inactivating properties equivalent to fresh serum. Serum fractions were kindly supplied by E. J. Cohn of the Physical Chemistry Department of Harvard University.

Experimental. In order to find, if possible, a particular serum component which had the capacity to antagonize lupulone antibiosis in a high degree, serum fractions were tested for this property. The fractions were dissolved in water to levels of 3.75 mg/ml and 11.25 mg/ml, which are approximately equivalent to 5% serum and 15% serum, respectively, on a total-solids basis. Five ppm of lupulone was added to the solutions, and cup assays run on the lupulone-serum fraction mixtures. Results are shown in Table I. Fractions II + III and IV have a considerable capacity to inactivate lupulone, whereas Fractions I and VI have only feeble inactivating powers. The description of these fractions(4) suggests that the lipoidal constituents or β-globulin might very likely be associated with the lupulone-inactivating properties, while albumin and fibrinogen are apparently eliminated as possibilities. Manometric experiments yielded essentially the same results as those shown in Table I. To test the importance of lipids in lupulone inactivation, Fraction IV was extracted for 24 hours with ether and the residue

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* Peptone, .5%; yeast extract, .15%; beef extract, .15%; dextrose, .1%; KH_2PO_4, .39%; NaCl, .35%; pH 6.8 with NaOH.

† Yeast extract, .50%; N-Z Amine, .2%; dextrose, .1%; NaCl, .35%; KH_2PO_4, .39%; pH, 6.8 with KOH.

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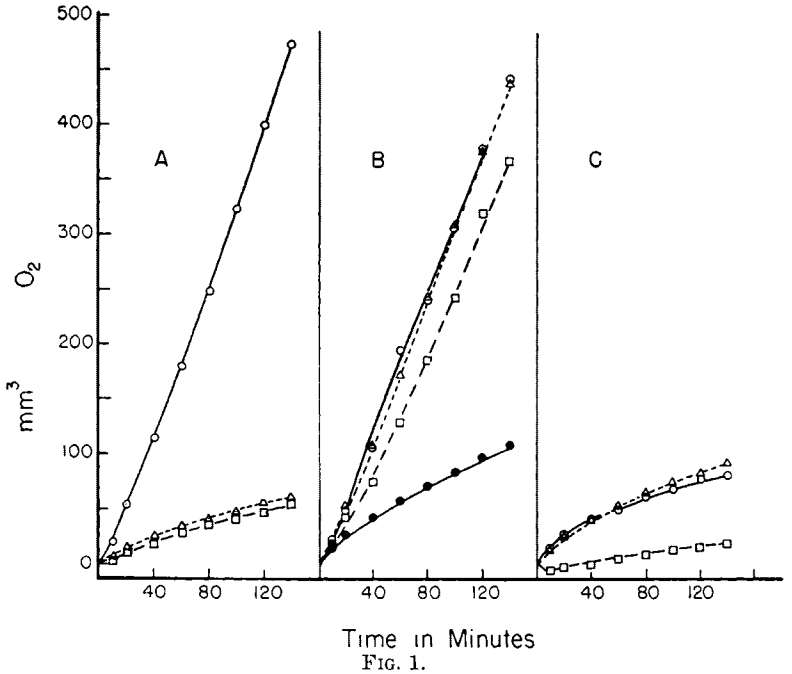

FIG. 1.

Influence of lecithin on the action of lupulone on the respiration of *B. subtilis*. A. Inhibitory action of lupulone on respiration of *B. subtilis*. △ endogenous respiration, ○ succinate substrate, □ succinate + lupulone. B. Influence of lecithin on inhibitory action of lupulone. ○ lecithin (.05%) + succinate, △ lecithin (.05%) + succinate + lupulone, □ lecithin (.01%) + succinate + lupulone, ● lecithin (.05%). C. Additional controls: △ lecithin (.05%) + lupulone, ○ lecithin (.01%) + lupulone, □ lecithin (.05%) + succinate + lupulone (cells heat killed). Lupulone concentration is 4 ppm in all cases.

tested manometrically. Its lupulone-inactivating capacity was found to be essentially unimpaired. Fraction IV was also autoclaved for 15 minutes at 15 lb pressure, and then dialyzed for 24 hours against running water. The non-dialyzable residue was still highly active. If, however, this autoclaved, dialyzed material were extracted with ether for 24 hours, practically all of its lupulone-inactivating properties were removed, while the ether extract possessed marked capacity to inactivate lupulone. These results suggested that lipoidal materials, probably bound to proteins, might be implicated.

To test this hypothesis, lecithin and cephalin prepared from eggs were tested manometrically and found highly effective in reducing lupulone inhibition, as was fresh egg yolk. The following compounds were tested and found void of activity: cholesterol, egg albumin, yeast autolysate, NZ-case, and choline, as well as lauric, myristic, and palmitic acids. The influence of varying quantities of lecithin on the inhibition of respiration of *B. subtilis* by lupulone is shown in Fig. 1.

That lecithin was allowing succinate to be metabolized, and not merely providing the cells with a substrate oxidizable via a resistant metabolic pathway, was demonstrated by omitting succinate in parallel cups. It is apparent that oxygen uptake is markedly greater in the presence of succinate. The slow acceleration of oxygen uptake at the lowest phosphatide concentration suggests that lupulone inhibition is neutralized more slowly, but that cells are still capable of metabolism once the lupulone has been "inactivated": *i.e.*, lupulone poisoning is reversible. This was more clearly demonstrated by adding plasma from a second side-arm of the Warburg vessels after various periods of lupulone inhibition. It was found that lupulone inhibition was completely reversible, even

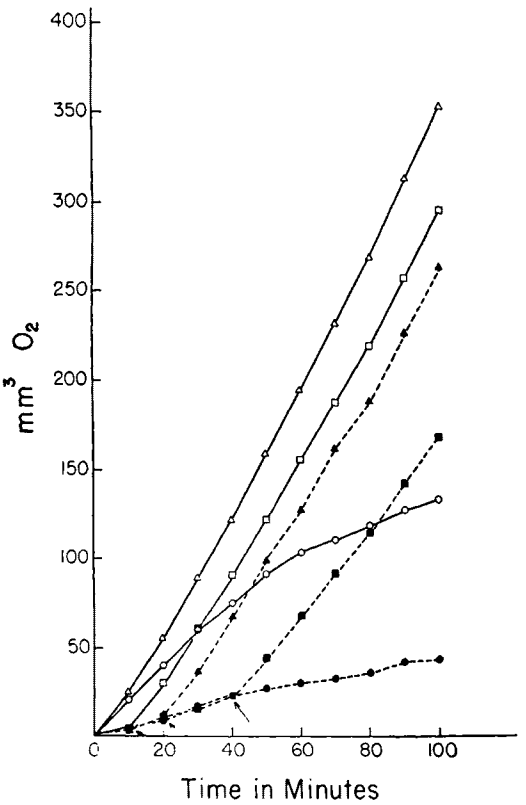

FIG. 2.

Reversibility of lupulone inhibition by the subsequent addition of plasma. ● Oxidation of succinate in the presence of lupulone. △ Plasma added at 0 minutes, □, ▲, ■ plasma added at time indicated by arrow, ○ oxidation of plasma in the presence of lupulone (succinate omitted). Final concentration of plasma in each vessel is 2.5%. Lupulone concentration is 4 ppm.

after 40 minutes (See Fig. 2).

In an effort to demonstrate more convincingly that the phospholipids are responsible for the lupulone-inactivating properties of plasma, we attempted to remove them from whole plasma. Conventional methods for the extraction of phospholipids generally involve treatment with alcohol-ether, which renders the proteins insoluble and thus makes impossible further evaluation of the residue. However, we were able to remove considerable amounts of lipid phosphorus by emulsifying plasma with two volumes of chloroform, freezing at —30°C, and discarding the chloroform fraction. By repeating this process six times, it was possible to extract 31% of the total P, or about 45% of the lipid phosphorus, as determined by the method of Schmidt *et al.* (5). The neutralizing capacity of this extracted plasma was then compared with that of normal plasma by the manometric technic. The results shown in Table II indicate that about half of the neutralizing capacity of the plasma had been lost. Controls lacking succinate, but containing lupulone and plasma, demonstrate that the extractable materials increase the oxidation of succinate in the presence of lupulone. These results were verified in a growth experiment, using *S. fecalis* as described earlier. The results shown in Table III clearly indicate a marked reduction in neutralizing capacity of the expected order of magnitude.

Discussion. Although the work presented here strongly indicates that the serum phosphatides can inactivate lupulone and that extraction of lipid phosphorus is closely correlated with loss of lupulone-inactivating ability, it must be emphasized that we have not obtained evidence demonstrating that the phospholipids are *solely* responsible for the antagonistic effect of serum on the antibiosis

TABLE II. Influence of Normal and Extracted Plasma* on Inhibition of Respiration of *B. subtilis* by Lupulone.

Cup contents (other than buffer, cells and KOH)	μl O_2 uptake at 120 min.
—	266
Succinate	885
Succinate + lupulone†	71
Succinate + lupulone† + normal plasma‡ (5%)	1004
Succinate + lupulone† + normal plasma‡ (2.5%)	915
Succinate + lupulone† + normal plasma‡ (1.67%)	660
Normal plasma (2.5%) + lupulone	436
Normal plasma (1.67%) + lupulone	374
Succinate + lupulone + extracted plasma (5%)	826
Succinate + lupulone + extracted plasma (2.5%)	576
Succinate + lupulone + extracted plasma (1.67%)	333
Extracted plasma (5%) + lupulone	558
Extracted plasma (2.5%) + lupulone	382

* Residue after 6 chloroform extractions.

† Lupulone concentration is 4 ppm in all vessels where employed.

‡ Figures in parentheses refer to concentration of plasma in vessel.

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TABLE III. Influence of Normal and Extracted Plasma* on Growth of *S. fecalis* in the Presence of Lupulone.

Lupulone	Total growth ($\log G_{initial} - \log G_{final}$) Normal plasma	Extracted plasma
--	.418	.421
5 ppm	.309	.284
10 ppm	.258	.194
20 ppm	.186	.046

* Residue after 6 chloroform extractions.

of lupulone. In fact, rough calculations comparing the antagonistic action of plasma with that of purified egg phosphatides indicate that the latter are only about one-tenth as active as might be anticipated. Substances in serum other than phospholipids may have considerable lupulone-inactivating property, or egg phosphatides may be less active than plasma phosphatides, or purified phosphatides may be less active than phosphatides occurring in the natural state (presumably as lipo-proteins).

The fact that lupulone inhibition was found to be reversible by the subsequent addition of plasma is evidence of the bacteriostatic nature of the lupulone antibiosis and contrasts with the irreversible action of detergents(6), where only prior or simultaneous addition of phosphatides is effective.

Summary. 1. Lecithin and cephalin are capable of neutralizing the inhibiting properties of lupulone. 2. Evidence is presented indicating that the antagonistic effect of serum on the bacteriostatic action of lupulone is due, at least in part, to its phosphatide content. 3. The inhibition of respiration by lupulone may be reversed by the subsequent addition of plasma.

The authors wish to thank Elizabeth A. McComb of this laboratory for the determinations of phosphorus.

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Variations in Anti-Hyaluronidase Titre of Normal Human Serum with Age and Sex.* (18447)

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In the course of studies on the serum anti-hyaluronidase in leukemia, and lymphosarcoma, by means of the viscosimetric method, it became necessary to reinvestigate the anti-hyaluronidase titres of normal human serum. Studies of the anti-hyaluronidase titre measured by the turbidimetric method, have shown that appreciable variations in the titre occur between certain age groups(1,2), and between sexes(2). However, none of the studies dealing with viscosity(3-7) have noted any variations in the titre due to sex, within the age groups studied. As the present data indicate, the anti-hyaluronidase titre in the serum of the normal female is appreciably higher than that of the normal male.

Method. The viscosity method as reported by Haas(3) has been used. The anti-hyaluronidase titre is expressed as the percent inhibition of the hyaluronidase activity by 0.02 ml of serum.

Results. The data are summarized in

* Aided by a grant from the Charles R. Blakely Fund of the National Research Council.

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