WARREN D. JOHNSON*
PETER STOKES**
DONALD KAYE†

Department of Medicine, The New York Hospital—Cornell Medical Center, New York, New York 10021

THE EFFECT OF INTRAVENOUS ETHANOL ON THE BACTERICIDAL ACTIVITY OF HUMAN SERUM‡

Serious gram-negative bacterial infections (e.g. pneumonias caused by *Escherichia coli, Hemophilus influenzae,* and *Klebsiella pneumoniae*) are encountered with increased frequency in chronic alcoholics.[1-3]

Normal human serum is bactericidal for many strains of gram-negative bacteria.[4,5] A decrease in serum bactericidal activity might render the host more susceptible to infection caused by these organisms. Studies by Kaplan and Braude[6] on two human volunteers suggested that the bactericidal activity of serum was decreased against a strain of *Escherichia coli* and a strain of *Hemophilus influenzae* following ingestion of ethanol (ethyl alcohol).

The present study was undertaken to extend the studies of Kaplan and Braude and to determine if ethanol administered intravenously to normal human volunteers alters the bactericidal activity of serum.

METHODS

Subjects

Twelve healthy male volunteers, ranging in age from 20 to 39 years, were given a single intravenous infusion of 50 to 75 ml. of pyrogen-free absolute ethanol in 500 ml. pyrogen-free isotonic saline solution over a one hour period. All volunteers had fasted for 12 hours and had not ingested ethanol, or any medication during the preceding three day period. A single control infusion of saline was given to seven of these same volunteers at least one week before or one week after the ethanol infusion.

Serum from each volunteer was obtained immediately before the infusion, immediately after completion of the infusion, and 5 and 24 hours after the infusion. Blood was allowed to clot at 20°C. for one hour. Serum was separated and stored at —70°C.

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* Assistant Professor of Medicine.

** Associate Professor of Medicine in Psychiatry.

† Career Scientist of the Health Research Council of the City of New York under contract I-489. Associate Professor of Medicine. Present address, Chairman, Department of Medicine, Woman's Medical College of Pa., Philadelphia.

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and all determinations of bactericidal activity were made within two months. Ethanol levels were determined by the method of Kingsley and Current.[7] Acetaldehyde, the major initial metabolic product of ethanol,[8] was measured by the method of Stotz.[9]

Table 1 lists the age and weight of each volunteer and the ethanol concentrations achieved in serum.

Bacteria

Strains of *Escherichia coli, Hemophilus influenzae* and a strain of Citrobacter isolated from human sources were studied. Stock cultures were maintained by storing aliquots of an 18-hour broth culture at —20°C. *E. Coli* strain 1 (serotype 0111B4) was isolated from stool and *E. Coli* strain 2 (serotype 0139) and the strain of Citrobacter were isolated from urine of patients with urinary tract infection. *H. influenzae* strains 1 and 2 were type b and were isolated from spinal fluid.

Trypticase soy broth was used for *E. coli* and Citrobacter; Levinthal broth was used for *H. influenzae*. For each experiment an aliquot of the stock culture was subcultured in trypticase soy broth (*E. coli* and Citrobacter) or in Levinthal broth (*H. influenzae*). Inocula were prepared by diluting an 18-hour broth culture in isotonic saline solution.

Determination of serum bactericidal activity

Dilutions of each serum were prepared in trypticase soy broth in 10 percent steps (e.g., 90 percent serum, 80 percent serum, 70 percent serum etc.). Four sera were studied from each infusion: one obtained before the infusion, one immediately after the infusion, and sera 5 and 24 hours later. An inoculum of 10^6 to 10^7 bacteria suspended in 0.1 ml. of saline was added to 0.4 ml. of each serum dilution in tubes. The number of bacteria was determined before and after four hours incubation at 37°C. in a water bath.

Bacteria were enumerated by preparing serial 10-fold dilutions in saline solution and making pour plates (trypticase soy agar for *E. coli* and Citrobacter and Levinthal agar for *H. influenzae*). The number of viable bacteria was calculated by counting colonies after incubation of the plates for 24 to 48 hours at 37°C.

RESULTS

Effect of infusion of ethanol on serum bactericidal activity

Method of analyzing data. All serum samples studied were bactericidal for all of the bacterial strains studied if a high concentration of serum was used. For example, a concentration of 90 percent serum from all samples resulted in over 100-fold reductions in recoverable bacteria for all strains. Therefore, to compare serum bactericidal activity before and after ethanol, lower concentrations of serum (i.e., 20 percent to 60 percent) were used in all experiments. Figure 1 depicts results from a typical experiment in which the fate of *E. coli* strain 1 was determined in different concentrations of serum from a subject (number 12) who received an infusion of ethanol. Each group of four sera in Figure 1 are in the following order: serum before

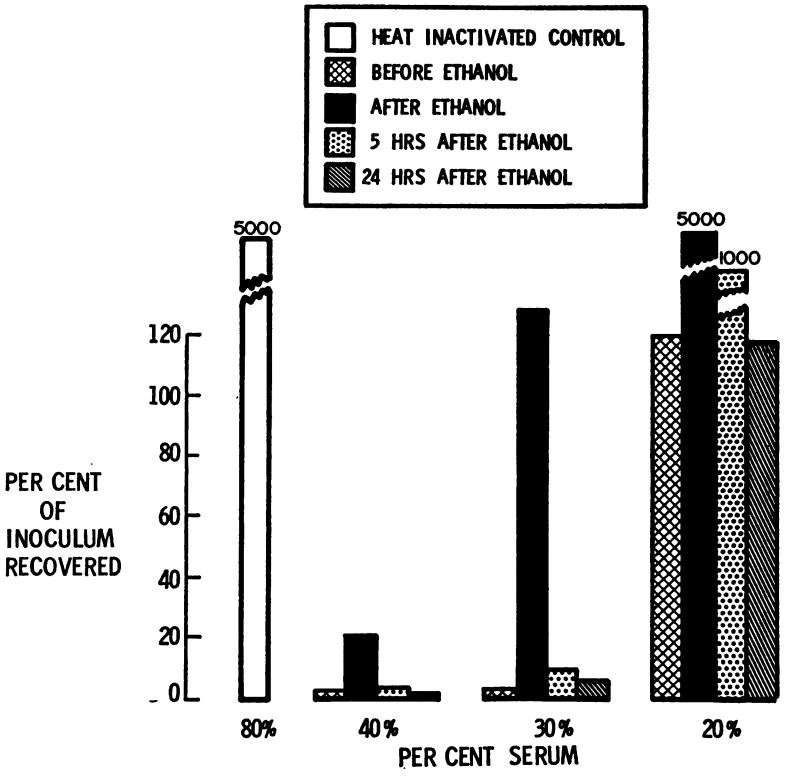

FIG. 1. Percent of inoculum recovered following incubation of *E. coli* strain 1 with multiple concentrations of serum from subject 12. Serum was obtained before infusion of ethanol, immediately after infusion of ethanol and 5 and 24 hours after infusion of ethanol. Heat inactivated pre-infusion serum was used as a control.

ethanol; serum immediately after the ethanol; and sera 5 and 24 hours after the infusion. Heat inactivated serum (80 percent concentration) served as a control. The percentage of the original inoculum recovered from each concentration of each serum is indicated.

It is clear that for each concentration of serum, the greatest number of bacteria was recovered from the serum obtained immediately after the infusion. As might be expected, fewer organisms were recovered when higher concentrations of serum were used and more organisms were recovered when lower concentrations of serum were used.

Pre-infusion serum from each subject was studied to determine the lowest concentration that would produce at least 50 percent reduction in the original inoculum after four hours incubation at 37° C. Once this "critical concentration" was determined, all four sera from each infusion were

studied at this concentration to compare bactericidal activity. For example, the "critical concentration" for the serum in Figure 1 was 30 percent. In all studies the "critical concentrations" ranged from 20 to 60 percent.

Most groups of sera were also studied at concentrations above and below the "critical concentration." For purposes of presenting the data only results obtained with the "critical concentration" of pre-infusion sera and sera obtained immediately after the infusion will be reported.

Reproducibility of methods. The reproducibility of the methods in quantitating bacteria was determined by plating many of the test sera in duplicate. The differences in the number of bacterial colonies on the duplicate plates did not alter the results of the experiments. Furthermore, a single serum was stored at −20° C. for two months and there was no change in bactericidal activity when weekly determinations were performed.

One subject (number 12) received a second infusion of ethanol one month after that noted in Table 1. Changes in serum bactericidal activity following the second ethanol infusion were similar to those observed after the first ethanol infusion.

Studies with E. coli strain 1. Studies with *E. coli* strain 1 in sera from the 12 subjects are shown in Figure 2. The percentage of the inoculum recovered from serum obtained before and immediately after infusion of ethanol was compared at the "critical concentration" of serum. In six

TABLE 1. SERUM ETHANOL CONCENTRATIONS IN 12 SUBJECTS

Subject	*Age (years)*	*Weight (pounds)*	*Serum concentrations of ethanol* (mg/100 ml.) Immediately after infusion*	*3 hours after infusion*	*5 hours after infusion*
1	23	135	145	62	
2	26	140	107		47
3	39		101		0
4	24	200	92		0
5	23	160	98	78	
6	26	220	78		12
7	22	155	73		0
8	20	165	61		0
9	23	159	60		0
10	29	190	45		0
11	24	195	41		0
12	24	154	37		0

* Only subject 1 had a detectible amount of ethanol present in his serum 24 hours after the infusion.

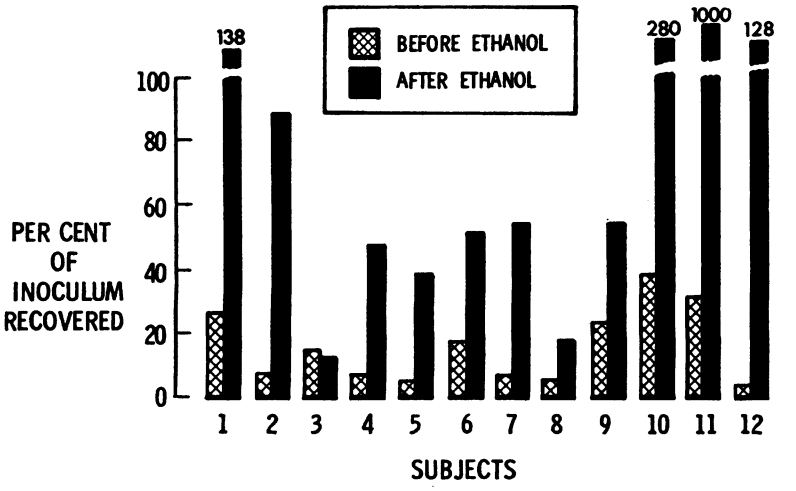

FIG. 2. Percent of inoculum recovered following incubation of *E. coli* strain 1 with serum obtained before and immediately after infusion of ethanol in 12 subjects.

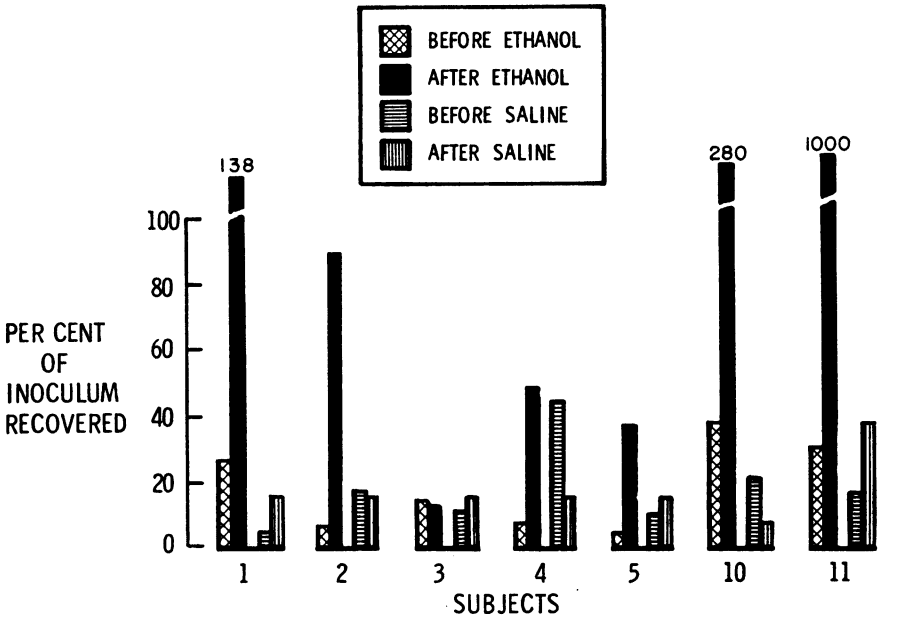

FIG. 3. Percent of inoculum recovered following incubation of *E. coli* strain 1 with serum obtained before and immediately after infusion of ethanol or saline in 7 subjects.

subjects determinations were repeated on two or more occasions with similar results.

In 11 of the 12 subjects there was an increase in the percentage of the inoculum recovered in the post-infusion serum as compared with the pre-infusion serum, indicating a decrease in bactericidal activity following ethanol. Subject 3 was the sole exception. In 10 of the 12 subjects there was at least a 3-fold difference in the number of bacteria recovered from the pre- and post-ethanol infusion sera. In three subjects (numbers 2, 11, and 12) the difference was greater than 10-fold. The results from sera obtained 5 and 24 hours after the infusion are not shown as in all subjects the bactericidal activity was returning toward normal by these times.

In 7 of the 12 subjects, control infusions with saline were administered at least one week before or after ethanol. Figure 3 shows serum bactericidal activity against *E. coli* strain 1 before and after infusion of ethanol and before and after infusion of saline in these seven subjects. In six of the

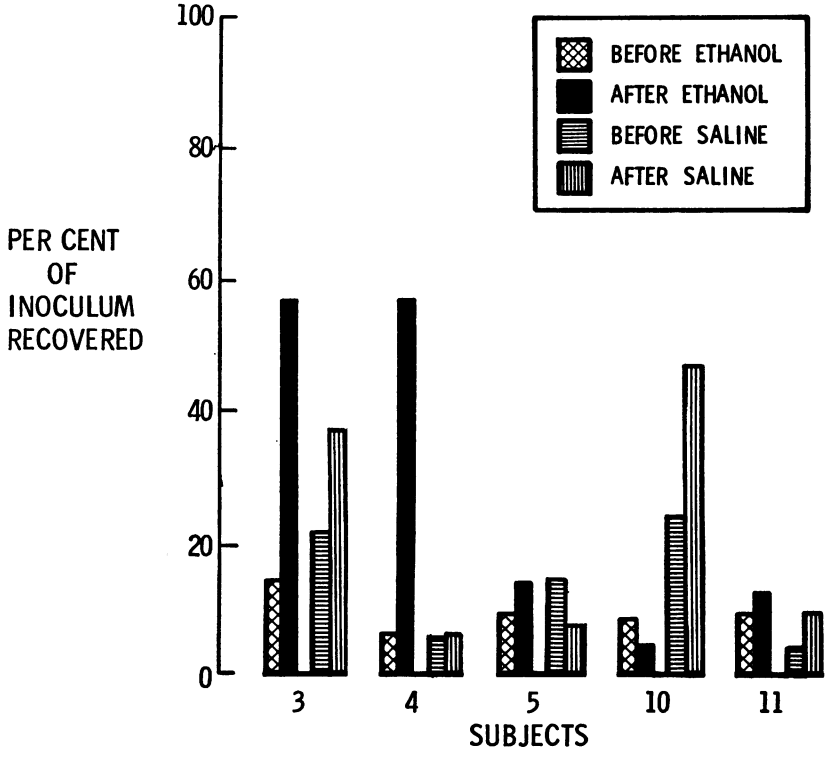

FIG. 4. Percent of inoculum recovered following incubation of *E. coli* strain 2 with serum obtained before and immediately after infusion of ethanol or saline in 5 subjects.

seven ethanol studies there was at least a 5-fold difference in the number of organisms recovered from the pre-infusion serum as compared with the post-infusion serum, all in the direction of a decrease in bactericidal activity following ethanol. None of the saline experiments showed even a 3-fold difference in the number of organisms recovered. There was also no uniform pattern in the change of bactericidal activity following saline. In two subjects (numbers 1 and 11) there was a small change in the direction of decreased bactericidal activity and in two subjects (numbers 4 and 10) there was a change in the direction of increased activity. In the remaining three subjects saline infusion produced essentially no change. The decrease in bactericidal activity after ethanol as compared with after saline was significant by chi square analysis ($P < .01$).

Studies with E. coli strain 2. Sera from five subjects receiving both ethanol and saline were tested against *E. coli* strain 2 in the same manner as with *E. coli* strain 1. Although the results were not as striking as with *E. coli* strain 1, the same trend was observed (Fig. 4). That is, bactericidal activity was decreased after ethanol but was not appreciably altered by infusion of saline. In two of the five ethanol studies (subjects 3 and 4) there was at least a 4-fold difference in the number of organisms recovered from the pre-infusion serum as compared with the post-infusion serum, both in the direction of a decrease in bactericidal activity following ethanol. None of the saline experiments showed even a 2-fold difference in the number of organisms recovered.

Studies with strains of H. influenzae. Sera from 5 subjects receiving both ethanol and saline were tested with *H. influenzae* strain 1. As shown in Figure 5 there was no consistent change in bactericidal activity after infusion of ethanol or saline. After ethanol one subject (number 4) demonstrated a 4-fold difference in the number of organisms recovered in the direction of decreased bactericidal activity and two subjects (numbers 5 and 10) demonstrated 8 and 2-fold differences respectively in the direction of increased bactericidal activity. Following saline there were no increases in bactericidal activity but three subjects (numbers 3, 4, and 5) demonstrated appreciable decreases in bactericidal activity (i.e. 6-fold, 5-fold and 13-fold increases respectively in the number of bacteria recovered).

Sera from two subjects (numbers 10 and 11) were tested with *H. influenzae* strain 2; non-viability of the strain prevented testing of additional sera. In contrast to the results with *H. influenzae* strain 1, there was a striking decrease in bactericidal activity in the serum of both subjects following ethanol as demonstrated by a 33-fold increase in the number of bacteria recovered from serum of subject 10 after ethanol and a 4-fold increase in the number of bacteria recovered from serum of subject 11. There

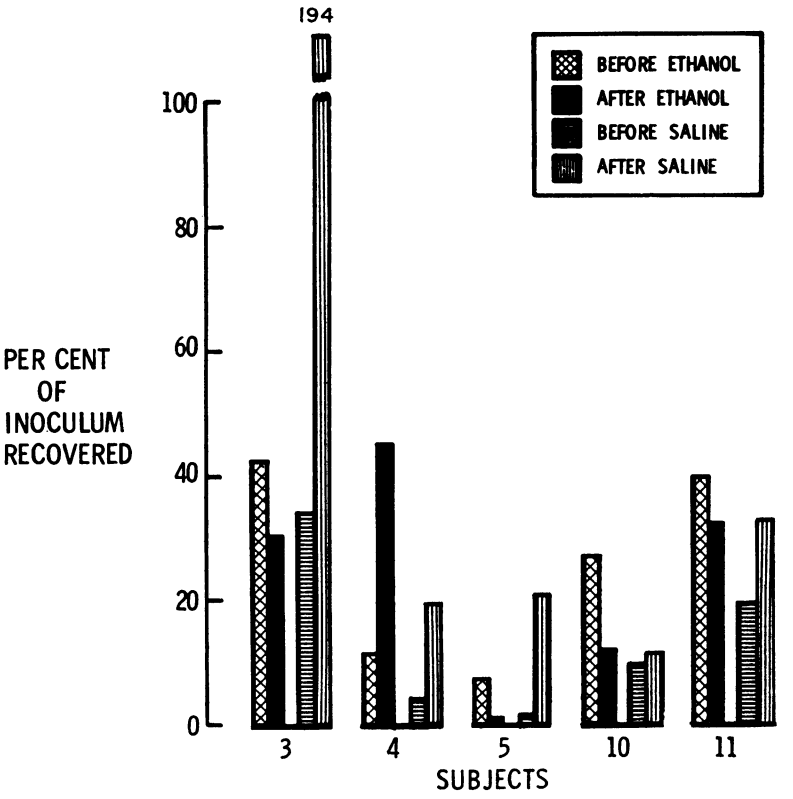

FIG. 5. Percent of inoculum recovered following incubation of *H. influenzae* strain 1 with serum obtained before and immediately after infusion of ethanol or saline in 5 subjects.

was no change in the number of recoverable organisms after infusion of saline.

Studies with a strain of Citrobacter. Sera from five subjects who received infusions of both ethanol and saline solution and from two other subjects who received only saline infusions were tested with a strain of Citrobacter. As shown in Figure 6, all five of the subjects receiving ethanol demonstrated a decrease in bactericidal activity as shown by an increase in the number of Citrobacter recovered from the post-infusion sera as compared with the preinfusion sera. In four of the five subjects (numbers 3, 4, 5, and 11) there was over a 3-fold difference. Two of the seven saline subjects (numbers 10 and 11) showed comparable decreases in bactericidal activity after saline infusion and one subject (number 4) demonstrated a

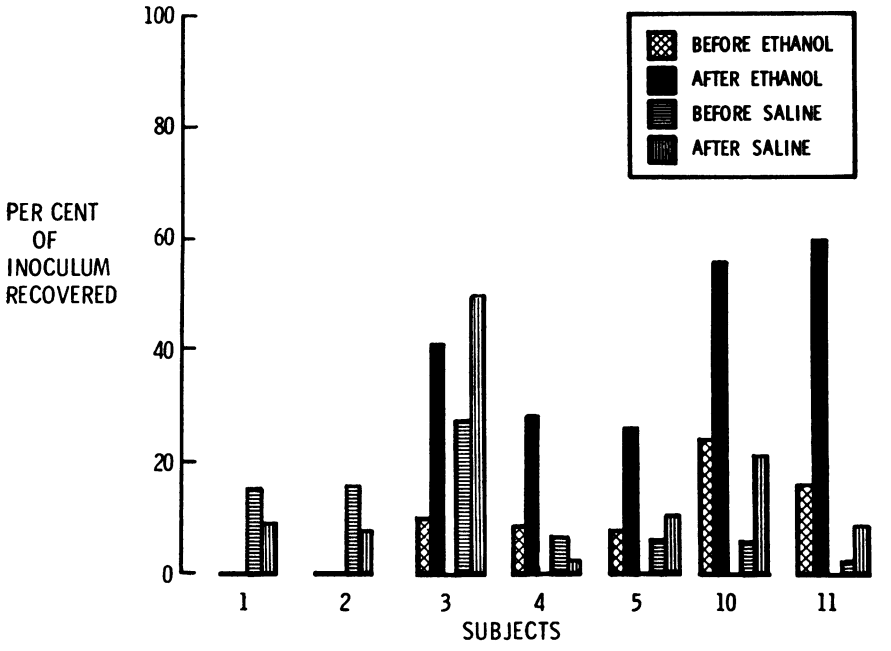

FIG. 6. Percent of inoculum recovered following incubation of Citrobacter with serum obtained before and immediately after infusion of ethanol and saline in 5 subjects. Two more subjects received only saline infusions.

comparable increase in bactericidal activity. The remaining four saline subjects showed no appreciable change in bactericidal activity.

Investigations into the mechanism of the change in bactericidal activity following ethanol

Studies were undertaken to determine the mechanism involved in the changes in bactericidal activity of serum following ethanol. *E. coli* strain 1 was used in all of these studies.

The decrease in bactericidal activity of serum against *E. coli* strain 1 was not related to the presence of ethanol in serum *per se*. Addition of ethanol to serum *in vitro* in concentrations up to 500 mg. per 100 ml. (more than three times greater than the highest concentration achieved in any subject) did not alter bactericidal activity of the serum. Furthermore, the rate of multiplication of *E. coli* strain 1 in trypticase soy broth was not affected by the addition of 500 mg. per 100 ml. There was no significant correlation between *in vivo* serum ethanol levels and decreases in bactericidal activity. The correlation coefficient was .52 ($P > .05$) for the

correlation of increasing ethanol levels and bactericidal activity against *E. coli* strain 1.

Addition of freshly distilled acetaldehyde to serum *in vitro* in concentrations up to 200 micrograms per 100 ml. did not alter bactericidal activity of serum. Similar concentrations of acetaldehyde also did not affect the rate of multiplication of *E. coli* strain 1 in trypticase soy broth. Two hundred micrograms per 100 ml. is more than three times greater than the highest concentration present in the serum of any subject (*in vivo* concentrations ranged from 19 to 67 micrograms per 100 ml.)

The decrease in bactericidal activity was not related to changes in the serum concentration of magnesium, calcium, potassium, sodium, or chloride ions. The concentrations of these electrolytes in the post-ethanol infusion sera were equal to those of the pre-infusion sera. All sera were diluted in trypticase soy broth which had the following electrolyte composition: magnesium 28 μg per ml., calcium 45 μg per ml., potassium 30 mEq per liter, sodium 140 mEq per liter, and chloride 90 mEq per liter. The levels of magnesium and calcium in serum and in trypticase soy broth are within the range that provides for optimal bactericidal activity.[10,11] Furthermore, as all sera from a subject were compared at the same dilutions in trypticase soy broth, the final concentrations of these electrolytes were equal.

Serum lysozyme activity was determined by the lysoplate assay method of Osserman and Lawlor[12] and found to be equal in both pre- and post-ethanol infusion sera.

Antibody against *E. coli* strain 1 in the pre- and post-ethanol sera of subject 7 was titrated by a modification of the bactericidal method of Muschel and Treffers[10,11] and Landy, Michael, and Whitby.[5] This method is based on providing excess complement and serially diluting the serum to be assayed for antibody. Subject 7 was selected because his serum showed a striking decrease in bactericidal activity against *E. coli* strain 1 following infusion of ethanol. In order to remove antibody with preservation of complement activity, normal serum was absorbed twice at 4° C. with 2 percent suspensions of heat-killed *E. coli* strain 1 for 18 hours each time. Absorbed serum in a concentration of 80 percent in saline did not inhibit multiplication of an inoculum of either 10^3 or 10^6 *E. coli* strain 1 per milliliter when incubated at 37° C. for 4 hours. One-tenth ml. of absorbed serum was added to tubes, each containing 0.1 ml. of undiluted pre- or post-ethanol serum or 0.1 ml. of two-fold dilutions of pre- or post-ethanol serum diluted in trypticase soy broth. An inoculum of 10^7 *E. coli* strain 1 suspended in 0.1 ml. of saline was added to each tube and the final volume brought to 0.5 ml. by adding 0.2 ml. trypticase soy broth. The control tube contained 0.1 ml. absorbed serum, 0.1 ml. bacterial inoculum and

0.3 ml. trypticase soy broth. The tubes were incubated for four hours at 37° C. and the number of bacteria determined.

As shown in Table 2, the number of bacteria in the test sera are expressed as the percent of the bacteria in the control tube. These results were plotted on probit log paper (paper No. 358-22, Keuffel and Esser Co., N.Y.) as probits of the percent bacteria in the control tube against the log of the amount of test serum at each dilution studied.[5,10,11] To compare the antibody content of the pre- and post-ethanol sera, the amount of serum in milliliters which resulted in 50 percent survival was read off a line, connecting the points. Utilizing this method, it was determined that 0.0063 ml. of pre-infusion serum and 0.0079 ml. of post-infusion serum was required to reduce the number of recoverable organisms to 50 percent of the control.

TABLE 2. TITRATION OF BACTERICIDAL ANTIBODY IN PRE- AND POST-ETHANOL SERUM

Test serum	*Dilution*	*Reaction mixture*	*Amount (ml.)*	*Surviving bacteria (percent of control)*
pre-ethanol	undiluted	TS*	0.1	0.8
post-ethanol	undiluted	AS**	0.1	2.9
		B†	0.1	
		TSB‡	0.2	
pre-ethanol	1:2	TS	0.1	5.2
post-ethanol	1:2	AS	0.1	9.4
		B	0.1	
		TSB	0.2	
pre-ethanol	1:4	TS	0.1	7.7
post-ethanol	1:4	AS	0.1	24.5
		B	0.1	
		TSB	0.2	
pre-ethanol	1:8	TS	0.1	38.7
post-ethanol	1:8	AS	0.1	35.5
		B	0.1	
		TSB	0.2	
control		AS	0.1	
		B	0.1	100
		TSB	0.3	

* Test serum (dilutions in trypticase soy broth).
** Absorbed serum.
†Bacteria (10^7 *E. coli* strain 1 in 0.1 ml saline).
‡ Trypticase soy broth.

Complement activity in the serum of subject 7 was titrated by a similar bactericidal method,[5,10,11] which is based on providing excess antibody and serially diluting the serum to be assayed for complement activity. The complement activity of normal serum was eliminated by heating at 56° C. for 30 minutes. Heat-inactivated serum in a concentration of 80 percent in saline did not inhibit multiplication of either 10^8 or 10^6 *E. coli* strain 1 per milliliter when incubated at 37° C. for four hours. Similarly there was no inhibition of multiplication when 40 percent heat-inactivated serum was tested in combination with 40 percent absorbed serum (absorbed with *E. coli* strain 1).

One-tenth ml. of heat-inactivated serum was added to tubes, each containing 0.1 ml. of undiluted pre- or post-ethanol serum or 0.1 ml. of two-fold dilutions of pre- or post-ethanol serum diluted in trypticase soy broth. An inoculum of 10^7 *E. coli* strain 1 suspended in 0.1 ml. saline was added to each tube and studies performed as already described for titration of antibody. The control tube contained 0.1 ml. heat-inactivated serum, 0.1 ml. bacterial inoculum and 0.3 ml. trypticase soy broth. The results are

TABLE 3. TITRATION OF BACTERICIDAL COMPLEMENT IN PRE- AND POST-ETHANOL SERUM

Test serum	*Dilution*	*Reaction mixture*	*Amount (ml.)*	*Surviving bacteria (percent of control)*
pre-ethanol	1:4	TS*	0.1	3.3
post-ethanol	1:4	HS**	0.1	3.45
		B†	0.1	
		TSB‡	0.2	
pre-ethanol	1:8	TS	0.1	17.2
post-ethanol	1:8	HS	0.1	13
		B	0.1	
		TSB	0.2	
pre-ethanol	1:16	TS	0.1	92.2
post-ethanol	1:16	HS	0.1	90.6
		B	0.1	
		TSB	0.2	
control		HS	0.1	100
		B	0.1	
		TSB	0.3	

* Test serum (dilutions in trypticase soy broth).
** Heat-inactivated serum.
† Bacteria (10^7 *E. coli* strain 1 in 0.1 ml saline).
‡ Trypticase soy broth.

shown in Table 3. Using probit analysis, it was determined that 0.0093 ml. of pre-infusion serum and 0.0085 ml. of post-infusion serum was required to reduce the number of recoverable organisms to 50 percent of the control.

DISCUSSION

Kaplan and Braude[6] administered ethanol orally to two volunteers and measured bactericidal activity of serum against a strain of *H. influenzae* and a strain of *E. coli.* A decrease in bactericidal activity against *H. influenzae* occurred in one patient while ethanol was present in the serum while in the other subject a decrease in bactericidal activity was not observed until 16 hours afterwards, at which time ethanol was no longer present in the serum. One subject demonstrated no change in serum bactericidal activity against a strain of *E. coli* while in the other subject a decrease in bactericidal activity was observed both during and 16 hours after ingestion of ethanol. Serum from four acutely intoxicated males was found by Kaplan and Braude[6] to have "virtually no bactericidal effect upon the growth of *H. influenzae* but only one of the three tested with *E. coli* allowed its propagation." These subjects were not studied while sober.

In the present studies ethanol was administered intravenously and infusions of saline were used as a control. Serum obtained immediately following an infusion of ethanol demonstrated decreases in bactericidal activity in most subjects against one strain of *E. coli,* one strain of *H. influenzae,* and a strain of Citrobacter. However, bactericidal activity against another strain of *E. coli* and another strain of *H. influenzae* was decreased in only a minority of subjects. In contrast to the observations of Kaplan and Braude[6] the decrease in bactericidal activity was transient and was returning toward normal when determined 5 and 24 hours after the ethanol infusion.

The decrease in bactericidal activity observed in the present studies could be demonstrated only if sera were tested in concentrations of 20 to 60 percent. When higher concentrations of serum were studied, no decrease in bactericidal activity could be demonstrated in post-ethanol sera. Some of the differences between the present studies and those of Kaplan and Braude may be explained by the possibility that ethanol has a more pronounced effect on the antibacterial activity of serum of alcoholics or cirrhotics than on serum of normal subjects. Another possibility is that there may be differences related to oral versus intravenous administration of ethanol.

The mechanism by which ethanol decreases serum bactericidal activity is unknown. The decrease in bactericidal activity of serum following ethanol infusion was not related to the presence of ethanol or acetaldehyde in the

serum *per se*. Furthermore, there was not a significant correlation between the serum ethanol level of the volunteers and the degree to which bactericidal activity was decreased. Infusion of ethanol did not alter serum lysozyme activity or concentrations of magnesium, calcium, potassium, sodium, or chloride. As the bactericidal effect of serum against gram-negative bacteria is related to the presence of antibody and complement,[4] it seemed most likely that the decrease in the bactericidal activity of post-ethanol serum was related to a decrease in the activity of bactericidal complement or antibody. However, the difference in bactericidal complement and antibody levels of the pre- and post-ethanol infusion sera did not exceed 25 percent, which is the inherent error of the assay method use.[5] It is still possible that alterations in the activity of bactericidal complement or antibody accounted for the experimental results, but were not of sufficient magnitude to be detected by present assay methods.

It is not known if the increase in serious infections caused by gram-negative bacteria in chronic alcoholics is related to the decrease in bactericidal activity of serum observed following administration of ethanol. However, it is reasonable to postulate that the changes in serum antibacterial activity as well as abnormalities in leukocyte mobilization and the possible decrease in phagocytosis following administration of alcohol[13] increase the susceptibility of the alcoholic to infection.

SUMMARY

The intravenous administration of ethanol to normal volunteers decreased the serum bactericidal activity of a majority of subjects against a strain of *E. coli*, a strain of *H. influenzae*, and a strain of Citrobacter. However, bactericidal activity against another strain of *E. coli* and another strain of *H. influenzae* was decreased in only a few subjects. Infusion of saline in the same volunteers did not produce an equivalent change in bactericidal activity. The decrease in bactericidal activity was transient and was returning toward normal when determined 5 and 24 hours after the ethanol infusion.

The decrease in bactericidal activity was small and could be demonstrated only by testing diluted serum. In no subject was bactericidal activity abolished by ethanol infusion. The decrease in serum bactericidal activity could not be attributed to changes in serum lysozyme, serum electrolytes, or alterations of bactericidal antibody or complement levels.

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