against human strain mycoplasma. Addition of 0.002% of MB to PPLO agar converts it to a medium which is highly selective for *Mycoplasma pneumoniae*. This modification is recommended for epidemiological surveys, for purification of cultures, and even for identification of *M. pneumoniae*. A test based on a color reaction between *M. pneumoniae* and TTC has been described. This test is recommended for confirming or establishing the identity of this species from other mycoplasma of the human respiratory tract.

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Enhancement of Ethanol Toxicity by Ethacrynic Acid. (30019)

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Ethacrynic acid [2,3-dichloro-4-(2-methylenebutyryl) phenoxy] acetic acid is a new potent saluretic-diuretic agent that was developed through a search for biologically active compounds that react selectively with sulfhydryl groups(1). Although ethacrynic acid probably most closely resembles the mercurial diuretics(2), its mechanism of action is thought to be qualitatively different from diuretics employed commonly.

During studies in our laboratory on the effect of ethacrynic acid on thiol groups it was noticed that mice treated with ethacrynic acid became much more susceptible to the toxic effects of ethanol. Doses of alcohol, non-lethal normally, were lethal to a large percentage of mice after treatment with this diuretic. This problem was further investigated because of the importance of sulfhydryl containing enzymes in the metabolism of ethanol, and the possible clinical implications of ethanol toxicity.

This paper presents data concerning the increased lethality of ethanol after ethacrynic acid treatment and preliminary studies on the mechanism of this action.

Methods. Male and female general purpose mice weighing 20-30 g were used in these experiments. Mice were generally treated subcutaneously (s.c.) with the drug prior to administration intraperitoneally (i.p.) of alcohol. Alcohol was administered as a volume/volume solution prepared such that the dose/kg of alcohol was brought to a volume of 10 ml with distilled water or saline. One-tenth ml of this solution was administered per 10 g of body weight.

Blood alcohol was determined by the alcohol dehydrogenase (AlDH) method of Bucher and Redetzki as described(3). Enzymes and cofactor were obtained from Calbiochem. Yeast AlDH was used as received. Equine liver AlDH was used after dialysis for 48 hours to remove residual ethanol.

TABLE I. Effect of Combination of Ethacrynic Acid and Ethanol on Lethality.

Ethanol treatment	% Lethality (7 days) Ethacrynic acid treatment	Saline treatment
5.0 ml/kg (3.95 g/kg)	100	90
3.75 " (2.96 ")	60	0
2.50 " (1.97 ")	40	0
1.25 " (.99 ")	0	0
.25 ml saline	0	0

Animals were treated with ethacrynic acid 100 mg/kg s.c. one hour prior to i.p. administration of alcohol. Each group consisted of 10 mice.

Ethacrynic acid (MK-595), kindly supplied by Merck, Sharp & Dohme, was dissolved in a small volume of 2% $NaHCO_3$ solution and brought to final volume with distilled water or saline. Other diuretics used were acetazolamide sodium (Diamox), chlorothiazide (Lyovac-Diuril), and mercaptomerin sodium (Thiomerin).

Results. Table I shows the lethality of ethacrynic acid (100 mg/kg) and a variety of doses of ethanol in mice. It can be seen that the combination of ethacrynic acid and alcohol greatly increased the percent lethality in these mice when compared to administration of either drug alone. Similar results were obtained after oral administration of ethacrynic acid followed by alcohol i.p.

Table II demonstrates that even very low doses of ethacrynic acid (3.13 mg/kg) resulted in death within 7 days for some of the animals treated with ethanol (3.16 g/kg). This dose of ethanol was selected because it is just below the lethal level. The 7 day LD_{50} for ethanol administered i.p. is 3.40 (3.60-3.21) g/kg with a slope of 1.06. Therefore, even a slight effect of the drug on toxicity is demonstrable after administration of 3.16 g/kg (4.0 ml/kg) ethanol. These data are in contrast to similar experiments utilizing acetaldehyde. Ethacrynic acid administered s.c. at comparable doses had no effect on the lethality of acetaldehyde (0.5-1.0 ml/kg) administered i.p.

TABLE II. Effect of Various Doses of Ethacrynic Acid on Lethality of 4 ml/kg Ethanol.

Ethacrynic acid (mg/kg)	% Lethality (7 days)
100	100
50	100
25	90
12.5	90
6.25	80
3.13	30

Animals were treated with ethacrynic acid s.c. one hour prior to administration i.p. of 4.0 ml/kg (3.16 g/kg) of ethanol. This dose of ethanol following treatment with 0.25 ml of 1% $NaHCO_3$ was nonlethal. 100 mg/kg of ethacrynic acid administered s.c. and not followed by ethanol was also nonlethal. Each group consisted of 10 mice.

Table III presents the concentration of a variety of diuretics which results in 50% inhibition of the alcohol dehydrogenase system described. Equine liver and yeast AlDH are presented for comparison. These values were obtained by plotting percent of control activity against the logarithm of the inhibitor concentration. It can be seen that ethacrynic acid is capable of inhibiting AlDH at about 10^{-3} M. It should be noted that the mercurial diuretic, mercaptomerin, is also a weak *in vitro* inhibitor of AlDH. Chlorothiazide is more than 10-fold less potent while acetazolamide had no effect on AlDH at any concentration tested. This *in vitro* data concerning mercaptomerin is not paralleled by an effect on *in vivo* studies on alcohol lethality. Animals treated with mercaptomerin (120 mg/kg) s.c. showed little or no change in lethality of 3.16 g/kg ethanol administered one hour subsequently. This dose is approximately 4 times the molar concentration of ethacrynic acid (15 mg/kg) which results in lethality for 90% of the animals treated subsequently with 3.16 g/kg ethanol. Similar negative results were obtained *in vivo* with chlorothiazide (60 mg/kg). Acetazolamide demonstrated no *in vivo* effect on alco-

TABLE III. Concentrations of Various Diuretics Necessary for 50% Inhibition of AlDH.

	Concentration for 50% inhibition	
	Liver AlDH	Yeast AlDH
Ethacrynic acid	5×10^{-3} M	2.6×10^{-3} M
Mercaptomerin sodium	5×10^{-3} M	2.5×10^{-3} M
Chlorothiazide	$>2.5 \times 10^{-2}$ M	2.5×10^{-2} M
Acetazolamide sodium	No effect at 5×10^{-2} M	

The enzyme reaction was measured by monitoring reduction of DPN at 340 mμ(4). The incubation system consisted of enzyme 10^{-6} M, DPN 10^{-5} M, ethanol 10^{-4} M brought to a final volume of 3 ml with 0.1 M phosphate buffer pH 7.4 containing 0.1 M semicarbazide.

TABLE IV. Venous Blood Ethanol Concentration After Prior Treatment of Dogs with Ethacrynic Acid.

Min after alcohol	Alcohol blood concentration (mg %) Ethacrynic acid treated	Control
30*	155 ± 65	87 ± 21
60*	96 ± 13	77 ± 9
120	75 ± 4	70 ± 5
180	65 ± 4	55 ± 8
240	51 ± 7	47 ± 12

Values presented are mean mg % alcohol plus or minus standard deviation at various times after alcohol administration. Asterisk indicates a significant difference from control.

Five dogs were treated with ethacrynic acid i.v. one hour prior to administration of 0.789 mg/kg of ethanol. The 4 control dogs received no drug treatment prior to ethanol.

hol lethality at doses as high as 500 mg/kg.

Table IV presents alcohol blood levels of dogs after prior treatment with ethacrynic acid and/or ethanol. Five dogs were treated with ethacrynic acid (5 mg/kg) intravenously (i.v.) one hour prior to administration i.v. of 50% ethanol (0.789 g/kg). The control dogs received no drug treatment prior to ethanol administration. All animals weighed approximately 12 kg. Venous blood was sampled 30 and 60 minutes after ethanol administration and then hourly for 4 hours. It can be seen that the values for ethanol in venous blood are significantly higher in ethacrynic acid treated than control animals during the first hour after alcohol administration. This increase could possibly be due to an effect on the metabolism of ethanol. However the data are also compatible with a change in total body water which might result from the potent natriuretic and diuretic action of ethacrynic acid.

Discussion. Data have been presented which indicate that ethacrynic acid is capable of increasing the lethality of ethanol. Ethanol is enzymatically oxidized to acetaldehyde which in turn is oxidized to acetic acid by alcohol and acetaldehyde dehydrogenase. An effect of ethacrynic acid upon AlDH might alter the lethality of ethanol. An effect of the diuretic on acetaldehyde dehydrogenase could alter the lethality of both ethanol and acetaldehyde. However, in this case only ethanol lethality was increased and no effects were seen with ethacrynic acid and acetaldehyde. Therefore, one possible mechanism of action might involve an inhibition by ethacrynic acid of the conversion of ethanol to acetaldehyde. This mechanism has been demonstrated for chlorpromazine(4) and partially explains the increased actions of alcohol after treatment with this tranquilizer.

Ethacrynic acid has been shown to be a rather weak inhibitor of alcohol dehydrogenase. Fifty percent inhibition was obtained at a concentration of about 10^{-3} M. This effect might explain the increased blood alcohol levels demonstrated in dogs after prior treatment with ethacrynic acid. However, the mercurial diuretic, mercaptomerin also an *in vitro* inhibitor of AlDH was much less effective than ethacrynic acid at comparable dosage in altering *in vivo* alcohol lethality. Chlorothiazide is a much weaker *in vitro* inhibitor of AlDH, and acetazolamide possessed no *in vitro* inhibitory effect. Neither of these diuretics show any significant effect *in vivo* on alcohol toxicity at the doses investigated.

Although alcohol dehydrogenase, both yeast and liver, contains thiol groups and is inhibited by sulfhydryl inhibiting agents(5), and ethacrynic acid has also been demonstrated to bind sulfhydryl groups(2), it does not appear likely that an inhibition of AlDH by ethacrynic acid can be the sole explanation for these data. Another possibility might be that ethacrynic acid, a potent natriuretic agent, and ethanol which is known to inhibit the release of antidiuretic hormone result in a dehydration with abnormal salt loss. These effects could result in a decreased extracellular volume and therefore greater intracellular concentrations of ethanol in animals treated with ethacrynic acid. The observed increase in blood alcohol of ethacrynic acid treated dogs would be compatible with either mechanism.

The clinical implications of these observations are obvious. Serious consequences could occur if patients taking ethacrynic acid became more susceptible to ethanol. It is clear that a person deeply intoxicated is close to the toxic level of alcohol, and an effect only slightly greater could be very hazardous.

Summary. Treatment with ethacrynic acid increases the lethality of ethanol in mice. Dogs treated with ethacrynic acid and etha-

nol have higher venous blood alcohol concentrations than those given alcohol alone. Ethacrynic acid has been demonstrated to be a weak *in vitro* inhibitor of alcohol dehydrogenase. Mechanism of action and possible clinical significance have also been discussed.

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Physical and Chemical Properties of H-1 Virus. I. pH and Heat Stability of the Hemagglutinating Property.* (30020)

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H-1 virus, one of a group of small spherical agents, was isolated by Toolan(1) from fractions of human tumor tissue (HEp-1) grown in conditioned rats. At least 2 other H-viruses are known: H-3, derived from another human tumor, HEp-3, also grown in rats, and H-B, isolated directly from human placental tissue. H-3 is similar to Kilham's RV(2). All the H-viruses produce a specific deformity in hamsters injected when newborn, but they can be distinguished from one another antigenically and by their different agglutination patterns with red cells from various species of animals. Since its first isolation, H-1 virus has been obtained repeatedly from human tumors received from the operating room and from products of human conception, when 3 to 4 blind passages in newborn hamsters are done(3). It is also this virus that is most active in production of deformities in hamsters infected *in utero*(4).

The activity of the H-viruses can be measured in 2 ways: infectivity in newborn hamsters and agglutination of guinea pig red cells. Data(5,6) have indicated that the virus itself is the hemagglutinating agent. The present paper reports on the stability of the hemagglutinating property of H-1 virus to ranges of heat and pH.

Materials and methods. Source of virus for these experiments was the liver of hamsters injected within 3 days after birth with the sixth hamster passage of H-1 virus. Livers from moribund hamsters were excised, minced, diluted to 10% (w/v) with sterile distilled water, homogenized in a Waring blendor, and filtered through a Selas porcelain candle filter, porosity 0.03. The filtrate was partially purified by centrifugation in a Beckman-Spinco ultracentrifuge in a 40 rotor at 30,000 rpm for 5 hours. The pellet, resuspended in distilled water, contained more than 67% of the initial hemagglutinating activity and about 8% of the original protein, as determined by the Lowry method(7). This final virus preparation had a titer of 1280 hemagglutinating units (HA units) with guinea pig red cells.

Hemagglutination assays were run as follows: equal parts of dilutions of virus and 0.4% guinea pig red cells were mixed. Phosphate-buffered saline (PBS), pH 7.2, was used as the diluent and suspending fluid. The mixtures were stored overnight in the cold room at 5°C, and the end-point was read the

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