PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **186**, 139–141 (1987)

Changes in Ethanol Concentration during Incubation in Multiwell Tissue Culture Trays[1] (42593)

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Abstract. Ethanol can have direct effects in tissue culture and is often used as a solvent. Analysis of these effects will require a precise knowledge of the concentration over time in the particular system employed. We measured the disappearance rate of ethanol from multiwell culture trays (open system) containing single or multiple concentrations of ethanol over a 48-hr time period. The ethanol concentration was also measured at 72 hr in stoppered Erlenmeyer flasks (closed system). In multiwell culture trays, 1 and 0.3% ethanol (V/V) evaporated with a $t_{1/2}$ of 6 to 12 hr. Media containing no ethanol but adjacent to wells with 1% ethanol accumulated ethanol with a peak of 0.2% at 12 hr. The evaporation of 0.3% ethanol was slower from wells adjacent to those containing 1% ethanol. At 72 hr, stoppered Erlenmeyer flasks, which originally contained 1% ethanol, still had a concentration of 0.85%. Since both evaporation and transfer can occur in an open system, it is necessary to specify precise conditions or measure concentrations in such systems. Alternatively, a closed system in which the concentration of ethanol is maintained can be employed.

Ethanol is used as a diluent for numerous organic substances in *in vitro* culture systems. Compounds such as vitamin D and corticosteroids are more soluble and stable in the presence of ethanol. In the course of our experiments on the effects of ethanol on bone formation and resorption using organ culture, we decided to assess the disappearance rate and the possibility of transfer of ethanol from wells containing high to low concentrations in the multiwell plates typically employed in tissue culture experiments.

Materials and Methods. Several strategies were used to obtain time- and dose-related evaporation curves. Ethanol (pharmco ethyl alcohol dehydrated 200 proof) was added to prepared tissue culture medium (BGJb, Fitton-Jackson Modification, GIBCO, Grand Island, NY) with 1 mg/ml bovine serum albumin at concentrations of 0.3 and 1% (V/V). Using sterile techniques, 1 ml of media plus the effector was individually placed in 12 well-covered tissue culture trays (Costar Mark 11, Cambridge, MA). To assess both evaporation and transfer of ethanol, adjacent rows contained control media (no ethanol) 0.3 or 1% ethanol. Tissue culture trays were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. At 0, 6, 12, 24, 36, and 48 hr, media were removed, put into sealed plastic vials, and refrigerated until analysis of ethanol content was performed. To assess the possible effects of evaporation alone in this system, multiple trays were prepared which contained only media with similar ethanol concentrations (0.3 or 1%). At 0, 12, 24, and 48 hr, entire trays were removed from the incubator and 100-μl aliquots were taken from each well to be sampled for ethanol concentrations. In the closed system, 25-ml Erlenmeyer flasks with 2 ml of media containing 1% ethanol were gassed with 5% CO_2 in air and immediately closed with a tight-fitting rubber cap and incubated at 37°C for 72 hr.

Three methods were used to determine the ethanol alcohol content of the sampled media. The gas chromatographic method for assessment of alcohol content used is a modification of the procedure of Jain (1). The ethanol sample is diluted (1:5) and injected into a Hewlett/Packard gas chromatograph equipped with a flame ionization detector. There is no extraction or distillation required with this procedure and detection of less than 10 μg of alcohol per

[1] A preliminary report of this work was presented at the 8th Annual Meeting of the American Society for Bone and Mineral Research, Anaheim, CA, June 1986.

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0037-9727/87 $1.50

milliliter is possible. Two separate alcohol dehydrogenase methods were used and results were highly comparable. The first method employed, the Dupont ACA Discrete Clinical Analyzer, is a modification of the standard alcohol dehydrogenase enzymatic procedure originally described by Bonnichsen and Lundgren (2). Alcohol dehydrogenase catalyzes the oxidation of ethanol to acetaldehyde with the simultaneous reduction of nicotinamide-adenine dinucleotide (NAD). An alkaline pH and an aldehyde-trapping agent forces the reaction to 1 mole of NADH for each mole of alcohol present. The absorbance due to NADH is determined by absorption spectrophometry at 340 n*M*. The second alcohol dehydrogenase method is a colorimetric assay using iodonitrotetrazolium violet and fluorescein as indicators and is a modification of the NADH-generating system. The lowest measurable level of alcohol by all methods is 6 μg/ml. The assays for measuring ethanol levels were equally sensitive and correlated closely for paired determinations. The average ratio of ethanol concentration by enzymatic assay to that by gas chromatography was 0.98 with a coefficient of variation of 7%.

Results. The change in ethanol concentration in multiwell culture trays containing different initial concentrations is shown in Fig. 1. Initially, the half-life of 1% (V/V) ethanol was approximately 6 hr (closed circles). Control media containing no ethanol but situated next to culture wells with 1% ethanol showed a peak concentration of 0.2% at 12 hr followed by a decline to 0.01% at 48 hr. The rate of disappearance of 0.3% ethanol (Fig. 1) from wells adjacent to 1% ethanol was slow compared to trays containing 0.3% ethanol only (Table 1). Multiwell culture trays containing a single concentration of ethanol or no ethanol were removed from incubation at time 0, 12, 24, and 48 hr and analyzed by both gas chromatographic and alcohol dehydrogenase methods (Table 1). Control media did not accumulate ethanol from the environment in the incubator. The $t_{1/2}$ for 1% ethanol was 12 hr. At 48 hr, levels of ethanol in the media were too low for accurate measurement. Stoppered Erlenmeyer flasks which initially contained 1% ethanol had a concentration of 0.85% at 72 hr (data not shown).

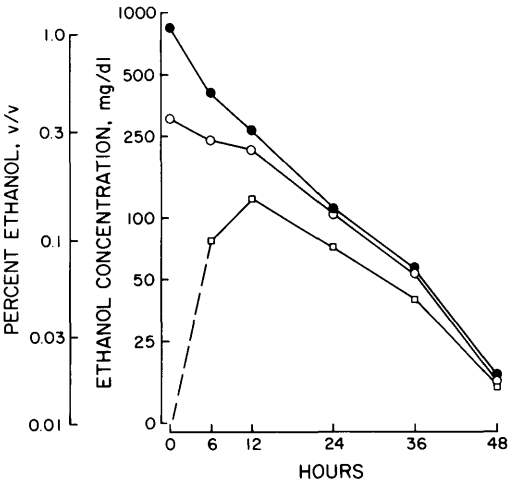

FIG. 1. Rate of evaporation of ethanol from multiwell culture plates (open system). Values are means of duplicate determination. Closed circles (●) represent 1% ethanol, open circles (○) represent 0.3% ethanol, and open squares (□) represent control media with no ethanol.

Discussion. Ethanol has been reported to have both inhibitory and stimulatory effects on cell proliferation and function even at low concentrations. Meagher and co-workers reported that red blood cell proliferation is suppressed by 0.05 to 0.2% ethanol in cell culture (3). Farley and co-workers (4) suggest that 0.03% ethanol in tissue cultures can increase bone cell proliferation and that 0.3% ethanol is inhibitory. Hynie and co-workers reported an increased cAMP accumulation in lymphocytes treated with 0.2% ethanol (5). Other studies have shown indirect effects of ethanol which can block the enhancing effects of known mitogens such as sodium fluoride and skeletal growth factor (4), or hormones such as insulin and glucocorticoids (6). If these studies were carried out in multiwell culture plates, their interpretation could be complicated by evaporation, resulting in changing rather than continuous ethanol exposure, or transfer, such that controls accumulate ethanol. It is important to note that while there is significant transfer from adjacent wells containing high concentrations of ethanol to wells containing lower concentrations or no ethanol, there is no transfer to separate culture trays from the incubator environment. However, there is still rapid disappearance of ethanol

TABLE I. THE CHANGE IN CONCENTRATION OF ETHANOL FROM MULTIWELL CULTURE TRAYS EACH CONTAINING A SINGLE CONCENTRATION

	Ethanol concentration (mg/dl, % V/V)			
Group	0	12	24	48
Control	n.d.	n.d.	n.d.	n.d.
1% Ethanol	765 (0.98)	379 (0.48)	135 (0.17)	34 (0.04)
0.3% Ethanol	268 (0.3)	159 (0.20)	63 (0.07)	25 (0.03)

Note. Mean values for ethanol often are presented in μg/dl or in parentheses as % V/V (n.d. = less than 0.001%).

from multiwell culture trays, all of which contain the same initial concentrations. While we have not been able to find any formal studies of ethanol disappearance rates in culture, there are other reports of loss of ethanol in open systems. For example, Levine *et al.* report loss of ethanol from 30 μM petri dishes at concentrations of 0.2 to 2% with $t_{1/2}$ varying from 20 to 45 hr (7). To determine the effect of ethanol on tissues in culture precisely, it may be important to maintain a constant environment which cannot be obtained using multiwell culture trays. Sealed culture flasks can obviate this problem but may result in other technical problems. The use of open systems with a changing ethanol concentrations, but without the complication of varying concentrations in the same multiwell plate, may still be appropriate for the study of ethanol effects since the intake of ethanol is intermittent *in vivo* and results in similarly changing serum concentrations.

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Received April 2, 1987. P.S.E.B.M. 1987, Vol. 186.
Accepted June 16, 1987.