tissue support. To change the nutrient medium, the old medium was siphoned off and the new medium was added to the same petri dish. The composition of the basal medium, the plant growth regulator supplements optimal for initiation of adventitious bud formation and subsequent bud growth, and the culture environment have been described (*1*). The plant growth regulators used included auxins [NAA, indole-3-acetic acid (IAA), and indole-3-butyric acid (IBA)] and a cytokinin (BAP). To secure the production of adventitious buds on cotyledon cultures, combined cytokinin and auxin (5 μM BAP plus 0.5 to 5.0 nM NAA, or 5 μM BAP plus 2.5 to 5 μM each of IBA and IAA) was added to the basal nutrient medium. To obtain sufficient numbers of adventitious buds, a cultivation period of 4 to 6 weeks was necessary. Subsequently, bud growth was stimulated by replacing the medium containing plant growth regulators with basal medium. The continued presence of the growth regulators (especially auxin) resulted in inhibition of bud development because of competition by callus growth on tissue in direct contact with the nutrient. Removal of the growth regulators stopped callus growth and resulted in stem elongation and needle expansion of adventitious buds (Fig. 1a). Shoots large enough to be easily excised were separated from the callus mass and grown on basal medium.

To produce Douglas fir plantlets, the excised shoots (approximately 2 cm long) were placed in individual culture tubes (2.5 by 15 cm) containing 20 ml of agar-solidified nutrient medium and incubated at 19°C. The basal medium was identical to that used for bud formation except for the concentration of sucrose. Studies performed with varying concentrations of sucrose and NAA in the nutrient medium showed that the optimal concentrations of these two compounds for promotion of root formation were 0.5 percent sucrose and 0.25 μM NAA. With this medium, a high frequency (~ 80 percent) of plantlet production was obtained (Table 1). Failure to produce plantlets occurred when a higher concentration of either NAA or sucrose was used; high levels of NAA caused prolific callus growth at the basal end of the stem and excess sucrose reduced the vitality of the shoots. Approximately 4 weeks after the excised shoots were subjected to the rooting conditions, root primordia started to emerge from the cut surface; subsequent withdrawal of NAA from the medium resulted in rapid root elongation (Fig. 1c) and plantlet growth. When similar experiments were performed at an incubation temperature of 24°C, relatively few plantlets (~ 30 percent) were produced; furthermore, those produced were abnormal, exhibiting a discontinuity in their anatomical structure caused by a proliferation of friable callus at the transition region between stem and root (Fig. 1b). In contrast, the plantlets produced at 19°C had a normal morphological appearance (Fig. 1c) and were subsequently established in soil with > 90 percent survival.

Table 1. Effect of various concentrations of NAA and sucrose on regeneration of Douglas fir plantlets in culture. The basal nutrient medium (minus sucrose) was supplemented with NAA and sucrose at the concentrations indicated. The growth chamber was maintained at 19°C with a 16-hour photoperiod (200 foot-candles) followed by an 8-hour dark period.

NAA (μM)	Sucrose (%)	Shoots cultured	Shoots producing roots
2.50	0.5	10	0
0.50	0.5	10	0
0.25	0.5	10	8
0.25	1.0	10	4
0.25	3.0	10	1
0.25	5.0	10	0
0.25	8.0	10	0

These experimental results demonstrate that the mode of action of auxin (in this case, NAA) is influenced by changes in incubation temperature. Since cells cultured at the higher temperature (24°C) are expected to be metabolically more active than those cultured at the lower temperature (19°C), we conclude that auxin affects two different cell types. Thus, the hormonal effect of auxin (NAA) on cultured cells is expressed either as (i) stimulation of adventitious root formation at the lower temperature, or (ii) stimulation of unorganized cell proliferation at the elevated temperature.

Regeneration of plantlets in vitro has been reported for only two economically important conifer species, longleaf pine (*Pinus palustris* Mill.), for which no quantitative data were presented (*2*), and western hemlock (*Tsuga heterophylla*), for which an undefined rooting medium (soil) was used (*3*). In the work reported here, we used chemically defined media and achieved high-frequency regeneration of plantlets from tissues of Douglas fir. The reproducibility we obtained should encourage application of this method in tree improvement programs. We believe that this is an important step in developing tissue culture as a tool for use in the domestication of wild tree species.

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Diazepam Maintenance of Alcohol Preference During Alcohol Withdrawal

Abstract. *After forced intragastric intubation of alcohol, rats will show a greatly increased tendency to self-administer alcohol in a free-choice situation. Diazepam (Valium) dosage (5 milligrams per kilogram of body weight) during the period of withdrawal serves to maintain undiminished such alcohol self-administration. Without such diazepam dosage the tendency to self-administer alcohol returns to control levels.*

Although there is at present little evidence that most pharmacological treatment is of benefit in the treatment of alcoholism (*1*), some success has been claimed with the use of benzodiazepines (*2*). In an effort to investigate the use of such drugs, we administered diazepam (Valium, injectable, Hoffmann–La Roche) to rats in which elevated intakes of alcohol had been produced (*3–5*) by forced intragastric intubation of ethanol through implanted gastric fistulas. Once the period of forced intubation was over, the rats were given a choice between two neutral flavors. The choice of one of the flavors was paired with direct intragastric intubation of an equal volume of 20 percent alcohol (*3, 4*). Whereas rats not pretreated with alcohol tend to avoid the flavor paired with it, rats pretreated with alcohol will drink 70 to 80 percent of the fluid paired with alco-

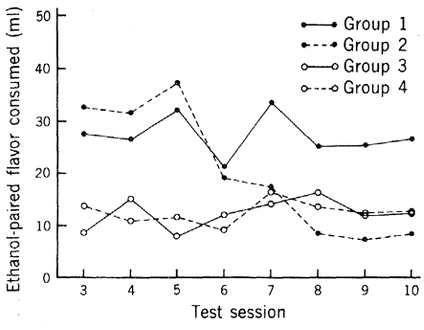

Fig. 1. Consumption of the ethanol-paired flavor (almond) by each of the four experimental groups during each of the choice test sessions. Group 1: forced intubation of ethanol (to induce dependence) followed by diazepam. Group 2: forced intubation of ethanol (to induce dependence) followed by treatment with water. Group 3: forced intubation of water followed by treatment with diazepam. Group 4: forced intubation of water followed by treatment with water.

hol (*4*). The percentage of alcohol-paired flavor drunk depends on the amount of alcohol injected during the initial treatment. We expected that diazepam administration during the period of alcohol withdrawal would reduce the volume of alcohol-paired flavor chosen by rats that had been subjected to forced alcohol intubation.

Thirty-two naive, male albino rats of the Charles River (Sprague-Dawley) strain served as subjects. Preoperative body weights were 500 to 600 g; rats were assigned to one of four groups on the basis of weight, so that the groups would have equal mean weights. Rats were housed singly in Plexiglas cages (18 by 28 by 38 cm) with sawdust-covered floors. Food was freely available throughout the experiment, and water was freely available until the forced intubation was completed, at which time it was removed from the home cages until the experiment was finished.

All animals had gastric tubes implanted (*3*, *4*) and were then allowed to recover for 7 days in their home cages. Pretreatment for rats in groups 1 and 2 ($N = 8$ in each group) consisted of forced intubation of 40 percent ethanol (by volume, 95 percent ethanol in tap water) every 6 hours for 7 days. The daily ethanol dosage was increased by 1 g per kilogram of body weight each day, from a dosage of 6 g/kg on day 1 to a dosage of 10 g/kg on day 5. The 10 g/kg dosage level was then repeated on the last 2 days of intubation. Pretreatment for rats in groups 3 and 4 ($N = 8$ in each group) was identical, except that tap water was intubated instead of ethanol. Rats were weighed daily, and each day's volume of ethanol to be intubated was calculated on the basis of the previous day's weight for each rat. Water was removed from the home cages 6 hours after the last intubation, and ethanol preference testing was begun after 24 hours of water deprivation.

Testing was conducted in test cages connected to counterinjection pumps (*3*, *4*). When the rat drank one flavored-water solution (0.5 percent Schilling almond flavoring), an amount of 20 percent ethanol equal to the amount drunk was automatically counterinjected through the fistula. When the rat drank another flavored-water solution (0.5 percent Schilling banana flavoring), an amount of tap water equal to the amount drunk was automatically counterinjected. We have found these flavored-water solutions to be equally preferred by this strain of rats. In half of the eight test cages, almond was on the left and banana on the right, and in the other half banana was on the left and almond on the right. A given rat was always tested in the same cage. Test sessions were conducted every 12 hours for 5 days, for a total of ten test sessions. During the first test session only the banana-flavored-water solution was available, with water counterinjection. During the second test session only the almond-flavored-water solution was available, with alcohol counterinjected. During sessions 3 through 10, both flavors were present, and the rats could drink as much of either or both solutions as they desired. Rats were left in the test cages until they had drunk nothing for 2 minutes, and test sessions lasted 5 to 10 minutes once the rats became familiar with the situation.

Rats in groups 1 and 3 were given diazepam (5 mg/kg) intragastrically via the fistula 2.5 hours prior to each test session (*6*). Rats in groups 2 and 4 were treated identically, except that tap water was administered instead of diazepam. The amounts of each flavored-water solution drunk were recorded for each rat at the end of the last eight test sessions.

Figure 1 presents the volume of alcohol-paired flavored water drunk by each of the four groups of rats during each of the eight choice-test sessions (3 through 10). A one-way analysis of variance was used to compare the four groups on each of the following measures: (i) total volume of fluid drunk during the eight choice-test sessions, (ii) volume of alcohol self-administered during the first four choice-test sessions (3 through 6); and (iii) volume of alcohol self-administered during the last four choice-test sessions (7 through 10). Where the overall F test was significant, individual comparisons were made between groups by use of the Newman-Keuls procedure.

The total volume of flavored water drunk did not differ significantly among the groups although individual variation was great, which indicates that no difference in overall thirst existed among the four groups of rats. Alcohol self-administration during the first four choice-test sessions did differ significantly among the groups [F (3, 28) = 6.79, $P < .01$]; individual comparisons revealed that the two alcohol-pretreated groups each self-administered significantly more alcohol than did the two water-pretreated groups, differences between groups 1 and 3 being significant at $P < .05$, while differences between groups 1 and 4, 2 and 3, and 2 and 4 were all significant at $P < .01$.

Alcohol self-administration during the last four test sessions (7 through 10) showed a significant overall difference [F (3, 28) = 3.23, $P < .05$]. Individual comparisons confirmed the change in alcohol intake by group 2 (Fig. 1), group 2 now being not significantly different from either group 3 or 4 and actually having a lower mean intake. Group 1, however, continued to self-administer significantly higher volumes of alcohol, with differences between groups 2 and 1, 1 and 3, and 1 and 4 all being significant ($P < .05$).

The most parsimonious explanation of these results is that diazepam maintains the dependence that had been established by the forced intubation of ethanol. Group 2 rats drank substantial amounts of alcohol in the early test sessions, but then their consumption declined, as the animals "dried out." Group 1 rats, on the other hand, although they started off drinking somewhat less than group 2, maintained their consumption throughout the test sessions and showed no decline at all. It has been suggested that diazepam has an amnesic effect on the rat (*7*), an effect that could explain the group differences because the diazepam-treated group 1 might forget the aversive consequences of drinking the alcohol-paired flavor. But the behavior of groups 3 and 4, which were not pretreated with alcohol, contradicts this hypothesis, as both of these groups drank little of the alcohol-paired flavor; no differences are seen between group 3, which was treated with diazepam, and group 4, which was treated with water only.

As a result of diazepam administration, the tendency to select alcohol may undergo a diminution which is tenuous at

best. However to offset this possible advantage, diazepam administration definitely acts to maintain the tendency to select alcohol. Such a result, if confirmed at the clinical level, has important implications for the pharmacological treatment of alcoholism. Diazepam is a drug which is very widely prescribed, often for alcoholism, which has assumed major proportions as a health hazard in this country. The use of diazepam to treat human alcoholism may be counterproductive.

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Erythrocyte Lipids in Heterozygous Carriers of Duchenne Muscular Dystrophy

Abstract. *Erythrocyte membranes from heterozygous carriers of Duchenne muscular dystrophy exhibit a diminished amount of palmitoleic acid when compared to membranes from normal subjects. A similar, but more variable, diminution is observed in the case of patients with this disorder. The change in fatty acid composition appears related to a low membrane triglyceride content and may provide both a possible technique for carrier detection and a clue regarding pathogenesis.*

Muscular dystrophy of the Duchenne type is transmitted in a recessive, sex-linked fashion, and the majority of affected children are borne by mothers who are heterozygous carriers. Rational genetic counseling requires reliable detection of such individuals, and the most commonly employed criterion, increased concentrations of creatine phosphokinase (E.C. 2.7.3.2) in the serum, fails to identify about 30 percent of the carriers examined (*1*). Therefore, alternative methods for detecting carriers are required. Moreover, the occurrence of a heterozygous dystrophic condition, in which the phenotype is generally functionally normal, allows the study of the pathogenesis of the disease under conditions that avoid a background of general deterioration of physical condition and abortive regeneration of muscle tissue. Here we report that the erythrocyte membrane lipids are altered in heterozygous individuals as well as in their hemizygous, affected offspring.

We have reported the occurrence of structural abnormalities in erythrocytes from mice and humans with various forms of muscular dystrophy (*2*). This alteration, an increased proportion of echinocytes, was also observed in several carriers of Duchenne dystrophy, suggesting that it might be used in the detection of carriers. Although the observations were confirmed in other laboratories (*3*), it became apparent that cell shape is too variable to permit reliable diagnosis or carrier detection (*4*).

On the basis of these and other abnormalities, we suggested that muscular dystrophies might be associated with a systemic membrane defect (*5*), and a similar suggestion was made by Appel and associates [see (*6*)], who reported that membrane protein kinase activity and membrane fluidity were altered in erythrocytes. A number of other abnormalities of the erythrocyte membrane have been observed in patients and carriers, and some of them have been suggested as having possible diagnostic utility. For instance, Percy and Miller (*7*) demonstrated decreased membrane deformability in cells obtained from patients and carriers of Duchenne muscular dystrophy. It appeared likely that such a varied collection of membrane alterations reflected some abnormality in membrane composition. If the abnormality were closely related to the primary genetic defect, then its identification would be of importance, both because it would provide information about the enzymic basis of the disease, and because it might be a more reliable measure of genotype than its later consequences, thus providing a carrier test of greater certainty.

Since a number of the membrane alterations noted above might reflect conditions in the lipid phase of membranes, we searched for changes in the lipid composition of erythrocyte membranes obtained from normal individuals, carriers, and patients. Kalofoutis *et al.* (*8*) reported that the concentrations of the major

Table 1. Total fatty acids of erythrocyte ghosts from patients with Duchenne muscular dystrophy, heterozygous carriers, and normal subjects. For this report, carriers are defined as mothers of a dystrophic son who have, in their family, in addition, at least one occurrence of dystrophy in their own or the preceding generation. The fatty acids listed are, with the exception of palmitoleic, those present in highest concentrations; 18 others were measured but they showed no significant differences between normal, carrier, and patient populations. The ghosts were prepared from washed erythrocytes by osmotic lysis in a medium containing 10 m*M* tris buffer, *p*H 7.4, and 1 m*M* $MgCl_2$. The cells were washed three times in isotonic saline; in other experiments it was evident that variations in the number of washes (from two to five) did not affect the results obtained. The ghosts were washed and suspended in distilled water at a concentration of about 4 mg of protein per milliliter and twice extracted with eight volumes of a mixture of chloroform and methanol (2 : 1). The chloroform phases were pooled and evaporated to dryness under nitrogen. The extract was incubated at 70°C for 30 minutes in 3 percent KOH in methanol; this was again extracted with petroleum ether, and the residue was acidified with HCl. The fatty acids were extracted with petroleum ether and their methyl esters prepared by incubation at 70°C for 30 minutes with borontrifluoride-methanol (Sigma). The esters were extracted with petroleum ether and evaporated to dryness under nitrogen. Methyl esters of fatty acids were estimated using a Perkin-Elmer model 3920 gas chromatograph equipped with 12 percent diethylene glycol succinate (DEGS) dual columns and a temperature program from 150° to 220°C at 2° per minute. Peaks were assigned through comparison with known standards and measured either manually (height and width) or with a CSI computing integrator. The number of carbon atoms relative to the number of double bonds is indicated beside each acid.

Fatty acid	Total fatty acids (%)		
	Normal (N = 16)	Carrier (N = 10)	Patient (N = 17)
Palmitic (16 : 0)	26.9 ± 1.6	26.0 ± 1.8	25.5 ± 1.2
Palmitoleic (16 : 1)	4.9 ± 1.1	1.4 ± 0.1*	1.2 ± 0.4*
Stearic (18 : 0)	13.9 ± 1.1	16.0 ± 2.3	17.9 ± 1.3
Oleic (18 : 1)	17.7 ± 0.7	15.7 ± 1.2	15.2 ± 0.8
Linoleic (18 : 2)	11.7 ± 1.0	12.5 ± 1.2	10.1 ± 0.7
Arachidonic (20 : 4)	10.0 ± 1.2	11.0 ± 0.8	10.6 ± 1.1

*Significant to $P < .005$ when compared with normal; carriers and patients did not differ significantly.