Muscle Regeneration: Enhancement by Ethylene Inhalation (33640)

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Ethylenedinitramine (EDNA), a plant growth stimulator (1) and high impact explosive, ameliorates the symptoms of dietary and hereditary muscular dystrophy in laboratory animals (2). These beneficial effects have been correlated with enhanced muscle regeneration. In mechanically injured muscle, EDNA increases the regenerative yield most dramatically when its administration is timed to coincide with a certain critical period (3) before the new fibers appear. We speculated that subanesthetic concentrations of ethylene, too, might increase muscle regeneration.

Materials and Methods. Male mice, Re 129 *dydy*, 3 months of age, were paired in two groups, littermates being represented in each group. One group was exposed to ambient air and the other to USP grade ethylene. The cage containing the latter was enclosed in a gas-proof Saran bag. Ethylene–air mixtures were delivered at 1.8 liters/min. Animals inhaled ethylene continuously for 7 days, three at a concentration of 5.7% and the remaining four at 0.2%. Ethylene concentrations were determined at the Ethylene Analysis facilities of The Dow Chemical Company. Upon termination of exposure, animals were sacrificed and tibialis anterior muscle was dissected out, fixed in formalin, and paraffin sectioned serially and tangential to the anterior surface of the muscle belly. Sections were mounted in even rows convenient for random sampling. Slides were stained with Ehrlich's hematoxylin and eosin. Sampling constituted 10% of a given muscle and was carried out with the aid of a table of random numbers. Slides were examined for new muscle fibers (myotubes). Regeneration in a section was expressed as myotubes/muscle fibers. These ratios were analyzed on an SDS multiple language digital computer system. The program employed, in addition to performing conventional statistical computations, projects population parameters from sample variances for a population of a stated size. The projection was made for an infinitely large population.

Wounded muscle was studied using 3-month C57BL/6J males with birthdays ± 3 days of each other. Aseptic incisions, 1.5 × 2 mm were inflicted in the belly of the tibialis anterior, 3 mm from the origin. Exposures to air or ethylene were carried out in Saran enclosures as depicted in Fig. 1 (see legend for details). Groups of animals received: (a) air alone; (b) 0.1% USP grade ethylene, continuously; (c) 5.7% ethylene, continuously; (d) 5.7% ethylene from 0800–2000 EST and air at night; (e) air during the day and 5.7% ethylene at night. Tissues were retrieved for microscopic examination 146 hr after wounding. The rationale in choosing this time was determined by previous studies (4–7). Regeneration was expressed as 100 × (myotubes/cut muscle fibers). These data were analyzed on the computer by a matrix, row-column, two-way analysis of variance. Justification in using cut fibers as a common factor was established by performing linear regression analysis on myotubes versus cut fibers in 11 randomly selected sections from untreated wounds; the coefficient of correlation was 0.9.

Alternating 12-hr periods of light and darkness are maintained in our animal colonies as a standard procedure. New animals are acclimatized to this rhythm for a minimum of 2 weeks. In the present studies all animals had been maintained on the same light–dark regimen for 1 month prior to experimentation. The same schedule was maintained throughout the investigation. The importance of controlling light–dark rhythm in muscle regeneration is discussed elsewhere [cf. pp. 3–4, Ref. (3)].

Results. Dystrophic muscle. Ethylene increased regeneration in dystrophic muscle by

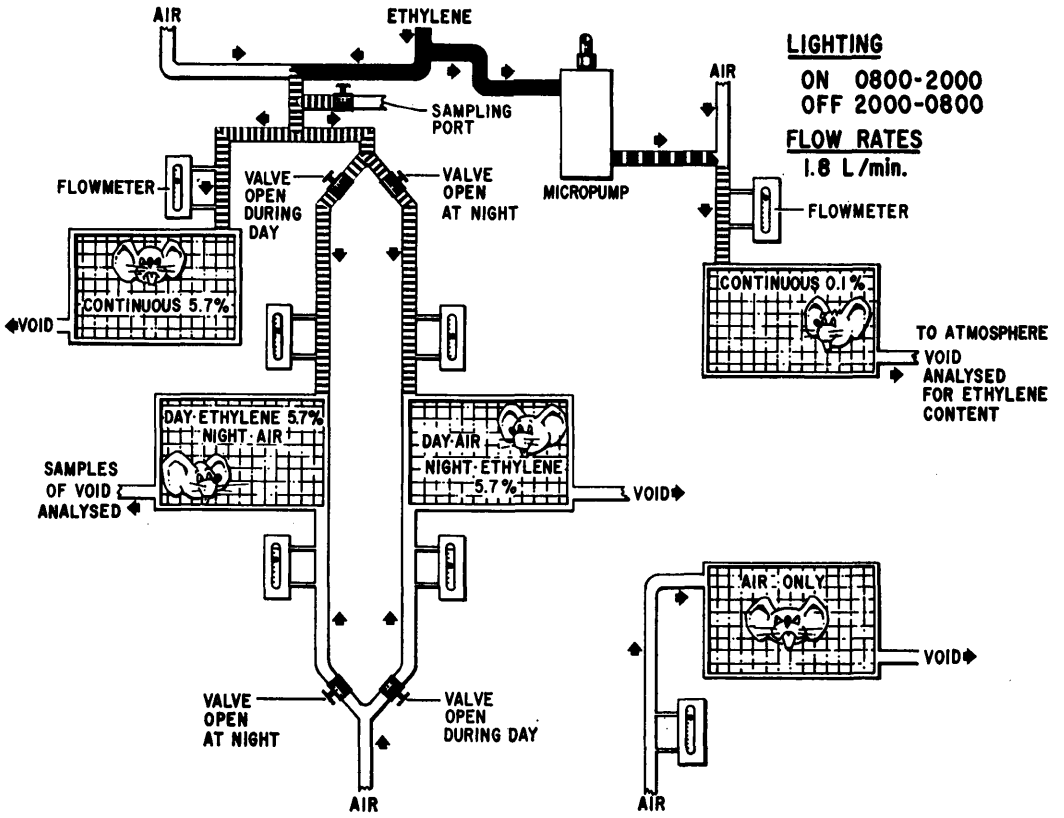

FIG. 1. Schematic illustration of exposures: air was driven into the system by pumps and ethylene, USP grade, from a cylinder; cages were enclosed in Saran bags; gas flow was regulated in each line by a regulator valve, not shown in the illustration. Each flow meter had been calibrated previously. The concentration of ethylene was determined both from samples taken at the sample port and from the various void outlets. It was necessary to employ a micropump to deliver the 0.1% concentration of ethylene illustrated in the upper right. Tubing was of Tygon, joints of poly propylene, and sealing was accomplished with vinyl tape; electrical connections were sealed to minimize fire hazard. The concentrations of ethylene employed were far below the anesthetic range.

some 3.5-fold (Table I). Projecting to an infinitely large population and employing the most conservative estimates—ethylene would enhance regeneration in dystrophic muscle by some 35% (see last row of Table I). Reversing the order of comparison—lower limits of controls against upper limits of experimentals—the maximum theoretical increase in yield attributable to ethylene under the conditions of these experiments would be of the order of some six times.

TABLE I. Enhancement of Regeneration in Dystrophic Mouse Muscle during Ethylene Inhalation.

	Control	Ethylene
Sample size	11	6
Sample mean ± SD	0.114 ± 0.05[a]	0.408 ± 0.06[a]
Variance	0.002	0.003
SE of mean	0.015	0.026
Confidence limits[b] (99.99 level)	0.08–0.20	0.31–0.51

[a] Regenerating myotubes/nonregenerating muscle fibers.

[b] For a population asymptotically approaching infinity.

Wounded muscle. These results are compiled in Table II. Ethylene had no detectable influence on regeneration at 0.1% concentration. Each of the schedules involving 5.7% ethylene led to increased regeneration but of significantly different levels. Continuous exposure produced an elevation of 57%. The

TABLE II. Effects of Ethylene Inhalation on Regeneration in Wounded, Nondystrophic Muscle.

Gas[a]		Regeneration (100 × myotubes/cut fibers; means ± SD)[b]	Analysis of variance[c] (significance level) versus	
Day	Night		Air alone	Ethylene at night
Air	Air	103 ± 16	—	0.01
0.1	0.1	101 ± 6	insignificant	0.01
5.7	5.7	156 ± 10	0.01	0.01
5.7	Air	123 ± 18	0.05	0.01
Air	5.7	240 ± 76	0.01	—

[a] Numbers refer to percentage of ethylene in air.

[b] Coefficient of correlation between myotubes and cut muscle fibers in normal regeneration is 0.9 as determined by regression analysis.

[c] Based upon matrical computed two-way analysis of variance using rows for replicates, columns for variates; numbers represent significance level.

daytime exposure schedule led to an increase of 23%. Discontinuous, nighttime exposure enhanced regeneration 2.4-fold. Differences between the three groups were significant (cf. last column in Table II).

Discussion. Ethylene, like EDNA (2), enhances regeneration in wounded and dystrophic skeletal muscle. Like EDNA the degree of enhancement depended on the timing of application (3). The mechanisms common to both are not as obvious as might be suggested by their names. Ethylene is not a derivative of EDNA and the configurational attributes of the two molecules in the monomeric state present no immediately obvious clues (8). There are certain facts, however, that suggest outlines for a plausible working hypothesis.

The EDNA interacts *in vivo* with chromatin; RNA and protein moieties appear to contribute to the integrity of the reaction product (9). Ethylene reacts with RNA (10). Both bind metals (8, 11). Given a ligand, metal-substituted ethylene can polymerize, and the same is probably true of EDNA. Ethylene and EDNA might, as a result of metal binding, form ligand-dependent oligomers that: (a) are not identical but have common features that in turn complement the same sets of receptor sites in the chromatin complex; (b) exert their influence by steric means; and (c) owe their like geometric features to metal binding. It is emphasized that this explanation is speculative.

At anesthetic concentrations ethylene can induce leukopenia (12); but in the low range employed in our experiments no leukocytic changes occur after as long as 90 continuous days of exposure (13). Inhibition of cholinesterase activity was reported in the latter work. This observation might parallel the well-known ripening effects of ethylene on plants (14). We bring up these points because some of our experiments indicate that ethylene had negative as well as positive effects on muscle regeneration. This was not immediately obvious and became evident only upon careful comparison of the values for continuous and discontinuous exposure schedules. While 5.7% ethylene inhalation stimulated an increase in myotube formation in each of the three schedules, the comparative results do not reflect simple addition. Continuous exposure was less efficient than the discontinuous nighttime regimen. Simple addition would require that the values from continuous experiments be greater than or, at the minimum, equal to nighttime values. The number of myotubes that developed in response to continuous inhalation, therefore, must contain a negative component, hidden in the overall positive character of the final outcome. It is convenient to regard the results of each schedule as representing plus and minus terms summed algebraically over a range from zero to some same positive value. Then, reasoning from the enhancement in each schedule, the pluses outweighed the

minuses and neither discontinuous schedule contained all the elements represented in continuous exposure: nighttime was deficient in minus components and the daytime regimen did not reflect a full complement of pluses. The logic of this is easily accommodated on temporal grounds, and it follows quite simply that plus and minus factors occur at different periods in early regeneration. Early regeneration—the interval before myotubes appear—is by no means a homogeneous phase of the process. Different events correspond with the night- and daytime inhalation schedules. Inhalation of ethylene during the nighttime schedule occurred during waves of DNA synthesis (3). In the daytime schedule ethylene "missed" the waves of replication, but "hit" mitosis per se (7) as well as an interval (around 72 hr) of extreme sensitivity to actinomycin D (4).

The point made in the last paragraph is not that ethylene exerts its net effect on regeneration by enhancing DNA synthesis and suppressing transcription or mitosis, but that these well-characterized initial phases of the regenerative process are, at the minimum, concomitants of whatever it is that ethylene affects pharmacodynamically. Events such as replication, mitosis, etc., can serve as useful guideposts in designing future investigations. Given, for example, a comprehensive knowledge of synchrony in dystrophic muscle, a schedule might be devised to enhance regeneration several orders beyond those we observed.

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1. Mees, G. C., J. Exptl. Botany **16**, 48 (1963).
2. Pietsch, P. and Harrold, J., Nature **213**, 601 (1967).
3. Pietsch, P. and McCollister, S. B., Nature **208**, 1170 (1965).
4. Pietsch, P., Nature **203**, 117 (1964).
5. Lash, J. L., Holtzer, H., and Swift, H., Anat. Record **129**, 679 (1957).
6. Godman, G. C., J. Morphol. **100**, 27 (1957).
7. Pietsch, P., Anat. Record **139**, 167 (1961).
8. Allentoff, N. and Wright, G. F., Acta Cryst. **6**, 1 (1953).
9. Holmsen, T. W., Plant Physiol. Suppl. **41**, 145 (1966).
10. Shimokawa, K. and Kasai, Z., Agr. Biol. Chem. (Tokyo) **32**, 680 (1968).
11. Basolo, F. and Pearson, R. G., "A Study of Metal Binding." New York (1960).
12. Aldrete, J. A. and Virtue, R. W., *in* "Toxicity of Anesthetics" (B. R. Fink, ed.), p. 105. Williams and Wilkins, Baltimore, Maryland (1968).
13. Krasovitskaya, M. L and Malyrova, L. K., Gigiena i Sanit. **33**, 7 (1968).
14. Lieberman, M. and Mapson, M., Nature **204**, 343 (1964).

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