

Fig. 1. Inhibition of phospholipid (●), protein (Δ), and RNA (○) synthesis by actinomycin D. Chick embryo fibroblasts were exposed to actinomycin D (0.5 μg/ml) for 30 minutes to 3 hours, washed, and incubated with ³²P_i (1 μc) for 30 minutes; the phospholipids were extracted, and the radioactivity was measured. Each value is the mean from three experiments.

barely affected. In cells treated with actinomycin D for up to 3 hours, phospholipid synthesis continued to be inhibited more than protein synthesis.

The phospholipids of treated and control cells were extracted and then separated by thin-layer chromatography (13); the concentration of each phospholipid and its content of ³²P were determined. Treatment with actinomycin decreased the incorporation of ³²P_i into all phospholipids; sphingomyelin was most affected (Table 1). There was no detectable effect on the phospholipid composition of the cells.

It seemed possible that the decrease in the incorporation of ³²P_i into phos-

pholipid might not represent a decrease in net phospholipid synthesis, but might be due either to an inhibition of phosphate uptake from the medium or to a decrease in the specific activity of intracellular adenosine triphosphate secondary to inhibition of RNA synthesis. We therefore investigated the incorporation of other precursors into phospholipid. In cells treated with actinomycin D for 4 hours, the incorporation of choline-C¹⁴ into phospholipid was decreased by 30 percent. When the phospholipids were separated by thin-layer chromatography, both sphingomyelin and lecithin were affected. Actinomycin D also caused a 35 percent decrease in the incorporation of glucose-C¹⁴ and glycerol-C¹⁴ into lipid-soluble material. With these precursors, neutral lipids which account for about one third of the total tissue lipid were present in the extract and were not separated from the phospholipid.

The mechanism by which actinomycin D inhibits phospholipid synthesis is unclear. Chick embryonic fibroblasts are rapidly differentiating into adult cells with extensive internal membranes, and actinomycin D may inhibit membrane formation. Alternatively, actinomycin D may inhibit the activity of an enzyme required for phospholipid synthesis.

In tissues treated with actinomycin D, a decrease in cellular function which is not directly related to a decline in RNA synthesis is often ascribed to a fall in protein synthesis subsequent to decreased RNA synthesis (2-4, 7, 8). Our finding that actinomycin D inhibits phospholipid synthesis in chick embryo fibroblasts more than it inhibits protein synthesis offers an alternative explanation for such findings.

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References and Notes

1. E. Reich, I. H. Goldberg, M. Rabinowitz, *Nature* **196**, 743 (1962); J. Hurwitz, J. J. Furth, M. Malamy, M. Alexander, *Proc. Nat. Acad. Sci. U.S.A.* **48**, 1222 (1962).
2. P. D. Cooper, *Virology* **28**, 663 (1966).
3. R. D. Barry, D. R. Ires, J. G. Cruickshank, *Nature* **194**, 1139 (1962).
4. P. J. Gomas, P. I. Tamm, S. Dales, R. M. Franklin, *Virology* **17**, 441 (1962).
5. M. R. Lunt and R. L. Sinsheimer, *J. Mol. Biol.* **18**, 541 (1966).
6. D. Korn, *J. Biol. Chem.* **242**, 160 (1966).
7. C. Yamada, A. J. Clark, M. E. Swendseid, *Science* **158**, 129 (1967).
8. D. Kennel, *J. Mol. Biol.* **9**, 789 (1964).
9. E. Reich, R. M. Franklin, A. J. Shatkin, E. L. Tatum, *Science* **134**, 556 (1961).
10. J. Taylor, *Virology* **25**, 340 (1965).
11. J. Folch, M. Lees, G. H. Sloane Stanley, *J. Biol. Chem.* **226**, 497 (1957).

12. G. Schmidt and S. J. Tannhauser, *ibid.* **161**, 83 (1945).
13. B. W. Uhlendorf, A. I. Holtz, M. B. Mock, D. S. Fredrickson, in *Proceedings of the Third International Conference on the Sphingolipidoses*, S. M. Aronson and B. W. Volk, Eds. (Pergamon Press, New York, 1967).
14. We thank R. Katzen and Mrs. D. Hughes for their technical assistance.

6 February 1968

Dimethyl Sulfoxide: An Inhibitor of Liver Alcohol Dehydrogenase

Abstract. Dimethyl sulfoxide inhibits horse liver alcohol dehydrogenase. In the direction of aldehyde reduction, this inhibition is competitive with aldehyde, with an inhibition constant of 5×10^{-3} M. Dimethyl sulfoxide reacts with the binary complex consisting of enzyme and the reduced form of nicotinamide-adenine dinucleotide to form a highly fluorescent ternary complex, with a dissociation constant similar to the inhibition constant. The inhibition of aldehyde reduction can be interpreted as due to competition between aldehyde and dimethyl sulfoxide for the carbonyl binding site of the above-mentioned binary complex.

Dimethyl sulfoxide (DMSO), a by-product in the manufacture of paper, has recently gained notoriety as a potentially useful agent in the treatment of rheumatoid arthritis and scleroderma, and as a solvent for the topical administration of other drugs (1). Although DMSO has numerous biological activities, the chemical bases of these activities are unknown, and the specific interaction of DMSO with tissue constituents has not previously been reported. In the course of studies on the inhibition of dehydrogenases by thyroxine, we found that DMSO is an inhibitor of liver alcohol dehydrogenase (ADH). In this report we present some kinetic studies of this inhibition

Table 1. Effect of actinomycin on the incorporation of ³²P_i into various phospholipids. Cells were exposed to actinomycin D (0.5 μg/ml) for 2 hours, washed, and then incubated with ³²P_i (10 μc) for 30 minutes at 37°C. The cells from three 100-mm plates, each containing about 10⁸ cells, were pooled for each analysis.

Phospholipid	Phospholipid concentration (nmole phospholipid per mg protein)		Specific activity (count/min per nmole phospholipid)	
	Control	Actinomycin	Control	Actinomycin
Sphingomyelin	72	81	0.55	0.12
Lecithin	368	362	38	19
Phosphatidylserine	20	22	140	77
Phosphatidylinositol	66	59	345	162
Phosphatidylethanolamine	174	154	14	9.4
Unknown	29	16	78	64
Total	729	694		

Table 1. Fluorescent properties of free and bound NADH. All data were obtained in 0.1M tris HCl, pH 8.6, at 25°C.

Fluorescent species	Excitation maximum (nm)	Emission maximum (nm)	Relative quantum yield*
NADH	340	465	1
ADH-NADH	325	435	3
ADH-NADH-DMSO	325	435	10

* Quantum yields are expressed relative to the quantum yield of free NADH, which is taken as 1.

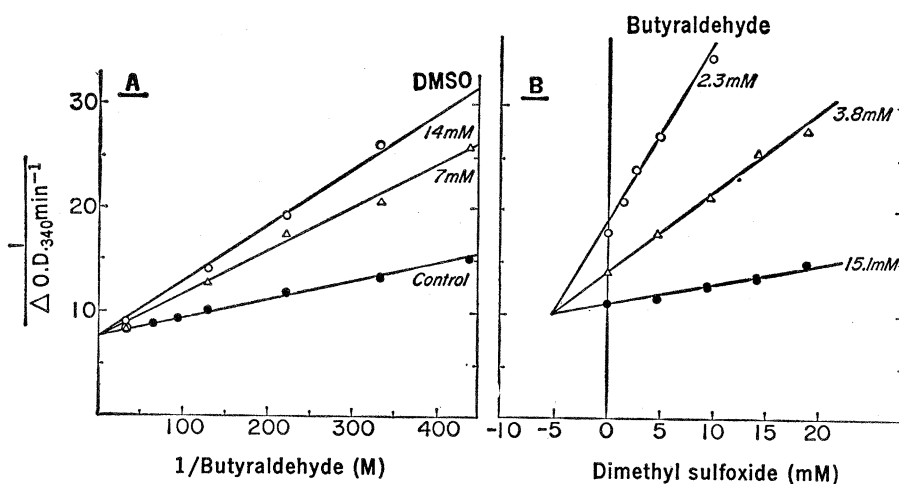


Fig. 1. Inhibition of alcohol dehydrogenase by DMSO. (A) Double reciprocal plot of butyraldehyde reduction, in absence of DMSO (control), at 7 mM, and at 14 mM DMSO. (B) Reciprocal plot of butyraldehyde reduction as a function of the concentration of DMSO at 2.3, 3.8, and 15 mM butyraldehyde. The three lines converge at a point representing $-K_i$.

and some fluorimetric observations of the ternary complex consisting of ADH, the reduced form of nicotinamide-adenine dinucleotide (NADH), and DMSO (2).

Kinetic experiments were performed by measuring the initial rate of change of optical density (O.D.) at 340 nm in the presence of the appropriate coenzyme and substrate. Fluorescence emission spectra were recorded on a Turner spectrofluorometer, model 210. This instrument gives corrected fluorescence emission spectra, in quanta per unit bandwidth. All experiments were performed in 0.1M tris HCl, pH 8.6, at 25°C.

Dimethyl sulfoxide inhibits the reduction of butyraldehyde by horse liver ADH. Figure 1 illustrates the effect of varying concentrations of DMSO and butyraldehyde on the rate of butyraldehyde reduction; the effect of DMSO is competitive with respect to butyraldehyde concentration [from the data in Fig. 1B, an inhibition constant (K_i) of $5 \times 10^{-3}M$ was determined]. The inhibition is noncompetitive with respect to NADH. The reverse reaction, the oxidation of ethanol, *n*-butanol, or cyclohexanol by NAD, is also inhibited by DMSO. In this direction, inhibition is noncompetitive with respect to the alcohol; the K_i against butanol was $10^{-2}M$. The reason for the difference in K_i is unknown. Under the conditions of our experiments, DMSO is not a substrate for liver ADH, with either NAD or NADH as coenzyme.

The interaction of DMSO with ADH and NADH was also investigated by fluorescence spectroscopy. When

NADH binds to ADH, its fluorescence intensity is enhanced, and its fluorescence emission spectrum is shifted to shorter wavelengths (3). In the presence of DMSO, the fluorescence intensity of ADH-bound NADH increases more than threefold, with little or no further change in its fluorescence emission spectrum. In this respect DMSO behaves like a substrate. Typical fluorescence emission spectra of free NADH,

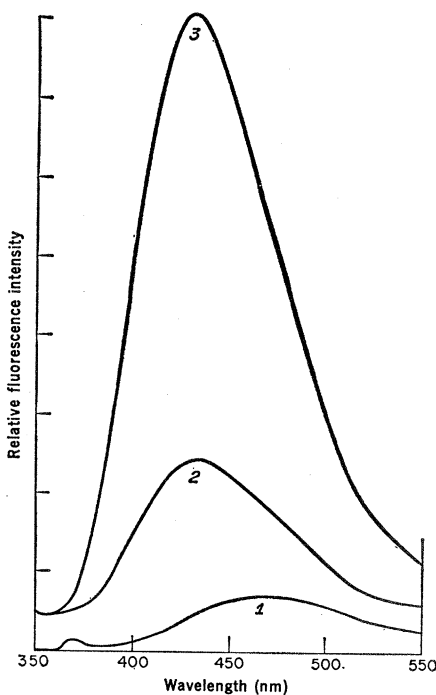


Fig. 2. Fluorescence emission spectra. Curve 1, NADH ($6 \times 10^{-6}M$). Curve 2, NADH ($6 \times 10^{-6}M$) plus ADH ($10^{-5}M$). Curve 3, NADH ($6 \times 10^{-6}M$), ADH ($10^{-5}M$), plus DMSO ($10^{-3}M$). All samples were in 0.1M tris HCl, pH 8.6, at 25°C. Excitation wavelength was 328 nm.

ADH-bound NADH, and the ADH-NADH-DMSO ternary complex are shown in Fig. 2; some of the pertinent spectral data are also presented in Table 1. From measurements of the fluorescence enhancement of ADH-bound NADH as a function of the concentration of DMSO, the equilibrium constant for the dissociation of DMSO from the ternary complex was estimated to be $4 \times 10^{-3}M$. Since the dissociation constant is similar to the K_i for aldehyde reduction, it is likely that formation of the ADH-NADH-DMSO ternary complex is a major factor in the inhibition.

The interaction of DMSO with the ADH-NADH binary complex is reasonably specific. Similar concentrations of DMSO had no effect on the fluorescence of free ADH, free NADH, or NADH bound to glutamic, malic, lactic, glyceraldehyde 3-phosphate, or yeast alcohol dehydrogenases. These concentrations of DMSO do not inhibit lactic or glutamic dehydrogenase (4). The concentrations of DMSO which inhibit liver ADH are well below those which cause significant changes in the structure of the solvent. Yeast alcohol dehydrogenase is slightly inhibited by much higher concentrations of DMSO ($> 0.1M$); this effect was not studied further. Finally, dimethyl sulfone does not inhibit horse liver ADH, nor does it augment the fluorescence of ADH-bound NADH.

Because DMSO inhibition is competitive with aldehyde concentrations, and because the K_i for DMSO determined kinetically is very close to the dissociation constant of the ternary complex determined fluorimetrically, it is likely that DMSO binds at the carbonyl binding site of the ADH-NADH complex. This carbonyl binding site is thought to include the enzyme-bound zinc; DMSO is known to form complexes with zinc salts (5). In these complexes, zinc is thought to bind to the oxygen atom of DMSO. Dimethyl sulfoxide can be considered an analog of acetone, which is a substrate for liver ADH (6) and not yeast ADH. Although the acetone molecule is planar and DMSO is pyramidal, consideration of DMSO as a substrate analog helps to rationalize the specificity of its interaction with dehydrogenases.

In many studies, experimental animals have been given more than 1 g of DMSO per kilogram of body weight (1). Since DMSO is widely distributed throughout total body water (7),

it would not be surprising if these doses produced intracellular concentrations of DMSO sufficient to inhibit ADH. Dimethyl sulfoxide is hepatotoxic in experimental animals (8) and in humans (9). Its use has been associated with hyperbilirubinemia and elevated serum transaminase levels. Under certain circumstances, DMSO can potentiate the toxic effects of ethanol (10). It is not known whether inhibition of liver ADH underlies these effects or any of the other biological activities of DMSO.

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References and Notes

1. C. D. Leake, Ed., "Biological actions of dimethyl sulfoxide," *Ann. N.Y. Acad. Sci.* **141**, Art. 1, p. 1 (1967).
2. Horse liver ADH was purchased from Worthington Biochemical Corp., and NAD and NADH from Sigma Chemical Co.; DMSO and butyraldehyde were obtained from Eastman Kodak Co. The butyraldehyde was freshly distilled with one drop of concentrated H_2SO_4 before use.
3. P. D. Boyer and H. Theorell, *Acta Chem. Scand.* **10**, 447 (1956).
4. D. H. Rammner, *Ann. N.Y. Acad. Sci.* **141**, 291 (1967).
5. F. A. Cotton and R. Francis, *J. Amer. Chem. Soc.* **82**, 2986 (1960).
6. A. Witter, *Acta Chem. Scand.* **14**, 1717 (1960).
7. C. W. Denko, R. M. Goodman, R. Miller, T. Donovan, *Ann. N.Y. Acad. Sci.* **141**, 77 (1967).
8. E. R. Smith, Z. Hadidian, M. M. Mason, *ibid.*, p. 96.
9. D. M. Bachman, D. R. Bottomly, Y. Shimazaki, *Arthritis Rheum.* **7**, 725 (1964).
10. H. J. Mallach, *Ann. N.Y. Acad. Sci.* **141**, 457 (1967).

23 February 1968

Microcirculation: Loss of an Enzyme Activity in Chickens with Hereditary Muscular Dystrophy

Abstract. *Histochemical localization of an alkaline phosphatase, with α -naphthyl phosphate used as substrate, shows that activity in breast muscle from normal chickens is restricted to the microvasculature. In chickens with hereditary muscular dystrophy, this enzyme activity disappears from capillaries and small arterioles before degeneration of muscle fibers is detectable. This loss is retarded in myopathic chickens that have received oxygen therapy.*

Continuous oxygen therapy results in a functional improvement during early stages of myopathy (1) in chickens with hereditary muscular dystrophy. Birds which show a functional improvement have muscle which appears more normal than dystrophic, as viewed histologically (2). Patterns of soluble protein and lactic dehydrogenase isoenzymes are also shifted toward normal (3). Because of apparent vascular abnormalities in dystrophic muscle, as seen both grossly and histologically (2), we further investigated the microcirculation in this myopathy. Normally, the control of blood flow to skeletal muscle is under greater control and is more closely associated with local metabolic activity than it is in any other organ except the heart (4). It has been suggested that an alkaline phosphatase located in the microvasculature of skeletal muscle may function in the autoregulation of blood supply (5). Our results show that there is an early loss of this enzyme activity in the microvasculature of dystrophic muscle, and they support the concept that an early vascular lesion contributes significantly to the progress of the myopathy.

We used the modified azo dye tech-

nique of Gomori (6), since its use in the study of this enzyme activity in muscle arterioles and capillaries has been thoroughly described (5, 7). The enzyme (or enzymes) that hydrolyzes α -naphthyl phosphate in skeletal muscle is localized almost entirely in the endothelial cells of small arterioles and capillaries (5, 7).

Normal chicks and those with hereditary dystrophy (8) were raised in a 70 percent oxygen environment from 1 day of age (1, 2). At least three chicks of each genotype raised in such an environment, and an equal number raised in the normal atmosphere, were exam-

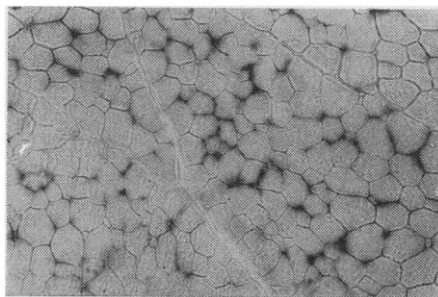


Fig. 1. Alkaline phosphatase activity in the microvasculature of breast muscle from a 4-week-old normal chicken ($\times 390$).

ined at 1, 2, 3, 4, 5, 7, and 11 weeks of age. Pieces of breast muscle (1 cm^2) were dissected from the midsternal region and rapidly frozen in a mixture of dry ice and acetone. In some cases, sections were also taken through the distal third of the gastrocnemius. The samples were then cross-sectioned at $16\text{ }\mu$ in a cryostat at -20°C and incubated for 30 minutes at 20°C (9).

Throughout the age range studied, there appeared to be no quantitative or qualitative differences in enzymatic activity between normal chickens raised in 21 percent oxygen and normal chickens raised in 70 percent oxygen. At 1 and 2 weeks of age, two types of staining were visible. A very dark, but sharply defined, stain was present at the location of small arterioles and capillaries between muscle fibers. Sections containing larger vessels showed that the stain was confined to the endothelial cells. Some vessels were not stained, but these were venules or the distal ends of capillaries (5, 7). Sections from 1-week-old and some 2-week-old chicks had, in addition to the vascular reaction, a somewhat lighter, but very diffuse, stain between and around the muscle fibers. This stain was usually heaviest around areas of connective tissue. Newly formed collagen fibers have a high alkaline phosphatase activity (10), as do proliferating cells and those with high metabolic activity (11). This diffuse stain, which was seen for a short period of time right after hatching, likely resulted from either or both of these sources. After 2 weeks of age, the reaction consisted only of the type which was confined to the blood vessels (Fig. 1).

The staining pattern was strikingly different in the dystrophic muscle. The diffuse stain seen in muscle from chicks 1 and 2 weeks old was much more intense than normal and persisted in most sections from dystrophic chicks up to 4 weeks old. It was essentially absent from muscle of chicks more than 4 weeks old, although some faint staining was occasionally seen in some localized areas at later ages. The number of blood vessels that stain in breast muscle from 1-week-old dystrophic chicks appeared to be less than that in normal muscle, but this cannot be said with certainty because the heavy diffuse stain in the dystrophic muscle may have masked some staining of vessels. Staining of small vessels was virtually absent from muscle of chicks more than 2 weeks old (Fig. 2). Larger arterioles did retain the