DIMETHYL SULFOXIDE: ACTIVATION OF LYSOSOMES IN VITRO

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The ability of dimethyl sulfoxide (DMSO) to penetrate cellular membranes became evident during comparison with glycerol for its effect in preserving viable cells on freezing and subsequent thawing.[1] Additional interest in both the physiological and medical aspects of the action of DMSO was generated by the observations that it not only penetrates the skin,[2-6] but that it may also have a palliative or therapeutic effect on skeletal, muscular, or dermal inflammatory responses clinically described as bursitis, rheumatoid arthritis and gout, and scleroderma.[3-5]

The ability to be absorbed through the skin is by no means unique to DMSO, but the interesting point is the rapidity with which it penetrates membranous barriers and its apparent low toxicity in biological systems. These and numerous other observations[7] amply demonstrate the fact that DMSO exhibits not only special permeability properties, but special chemical and pharmacological properties as well which endow this molecule with important biological and clinical actions.

We here report additional properties of DMSO in the activation of lysosomes isolated from homogenates of rat-liver tissue as judged by increased activity of acid phosphatase.

Methods.—Cell fractionation: Cytoplasmic extracts containing lysosomes and mitochondria were obtained largely free of nuclei by centrifugation of 20% homogenates (w/v) in a refrigerated centrifuge.

Homogenates were prepared from livers of female Sprague-Dawley rats in 0.25 *M* sucrose using a Teflon/glass homogenizer (Kontes; Potter-Elvehjem) driven at approximately 1000 rpm. Homogenization was limited to 5 or 10 up-and-down strokes.

A nuclear fraction also containing smaller particles was obtained in a single sedimentation of 1210 *g* min (121 × *g*, 10 min). Next, a cytoplasmic extract, used for enzyme incubations, was prepared by a single sedimentation of the nuclear supernatant at 173,000 *g* min (17,300 × *g*, 10 min). The sediment was resuspended in various test media while the supernatant, still containing a number of particulates, was discarded.

For experiments in which the amount of acid phosphatase lost from the particulate fraction was estimated, an additional centrifugation of the cytoplasmic extract was made, following resuspension, at 1,032,500 *g* min (41,300 × *g*, 25 min).

Acid phosphatase assay: Reactions were started in timed sequence by addition of 0.1 ml of buffered substrate (0.25 *M* sodium-beta-glycerophosphate in 0.12 *M* acetate buffer) to 0.1 ml acetate buffer and 0.1 ml of 10% cytoplasmic extract at a final pH of 5.8. The reaction period was 10 min.

Reactions were terminated in timed sequence by addition of 0.1 ml of 30% trichloroacetic acid (TCA). Reaction tubes remained on ice for 30 min following addition of TCA, and the precipitates were removed by centrifugation (1,000 × *g*, 5 min). The concentration of DMSO in resuspended fractions was 25%. Threefold dilution for enzyme assay reduced this to 8.3%.

Acid phosphatase activity was measured following resuspension of cytoplasmic extracts under five conditions: (1) in 0.25 *M* sucrose alone; (2) 0.25 *M* sucrose + 25% DMSO; (3) water; (4) water + 25% DMSO; (5) water + 0.1% Triton X-100 (nonionic detergent obtained from Sigma).

Controls included standard 0-time tubes for each experimental tube, a check for linearity of reaction over a severalfold concentration range of enzyme, and stability of pH during the reaction period.

Enzyme activity was expressed as micromoles of phosphorus hydrolyzed/mg protein/hr, or as per cent of total activity.

The activity of acid phosphatase, previously made soluble by Triton X-100, was not affected by 25% DMSO. Concentrations above 45% DMSO caused increasing inhibition of acid phosphatase.

Protein was estimated using the Folin-Ciocalteu phenol reagent by modification of a micromethod.[8] Phosphorus was estimated by a modification of the method of Chen *et al.*[9]

Changes in particle size were estimated as changes in optical density of lysosomal-mitochondrial suspensions measured at 520 mμ in a spectrophotometer. In these experiments DMSO was used at 10% and 25% concentrations.

Results.—Activation of lysosomes by DMSO: Pellets in 25 per cent DMSO–0.25 *M* sucrose mixtures showed an approximate twofold increase in activity compared with pellets resuspended in sucrose alone (Fig. 1). The activity of pellets resuspended in 0.1 per cent Triton-water[10] or in DMSO-water mixtures was greatest, fivefold more than pellets resuspended in sucrose alone, while pellets resuspended in water alone exhibited some fourfold greater activity than those in sucrose alone. The activity of acid phosphatase in water resuspensions was significantly lower than in DMSO-water or in Triton-water mixtures.

The effect of various resuspending media upon swelling of isolated particle fractions was measured in several test media. Particles resuspended in sucrose alone or in DMSO-sucrose mixtures underwent only gradual changes in optical density, showing a decrease of approximately 15 per cent over a 20-minute period. No detectable changes in the particle suspension could be observed in the light microscope. Suspensions of particles in water or in DMSO-water mixtures underwent a more rapid and extensive loss in optical density, to approximately 35 per cent of starting values. Examination by light microscopy revealed swelling of some particulates presumed to be mitochondria, but not specifically identified. Suspensions of particles in Triton X-100–water mixtures showed an immediate drop in optical density to approximately 10 per cent that of starting values.

Release of acid phosphatase from lysosomes: The release of acid phosphatase from lysosomes to the supernatant fluid was measured in order to distinguish the effects of treatments which caused enhanced enzyme activity within the lysosomes from those which also resulted in leakage of enzyme from intact or from ruptured particles. The results of these experiments are shown in Table 1. When lysosomes were resuspended in 0.25 *M* sucrose alone, only 3 per cent of the total acid phosphatase activity appeared in the supernatant fraction. Following resuspension in 25 per cent DMSO–0.25 *M* sucrose mixtures, approximately 15 per cent of total activity appeared in the supernatant, an increase of 12 per cent compared with particles suspended in sucrose alone. This amount of enzyme release can be attributed to the action of DMSO in the absence of osmotic stress. Great increases in the phosphatase activity appearing in nonsedimentable form were seen, however, when lysosomes were resuspended either in water alone or in 25 per cent DMSO–water mixtures. In these cases, over 60 per cent of total acid phosphatase activity was found in the supernatant fraction. A similar but less pronounced movement of protein from particulate to supernatant fraction also occurred. Of additional interest may be the slight protective effect against protein solubilization shown by DMSO–water mixtures as compared with water alone. The amount of protein appearing in the supernatant (Table 1) seems to indicate that acid phosphatase leaks from the particulates at a faster rate than does protein when an osmotic stress is applied, although it should be pointed out that this interpretation

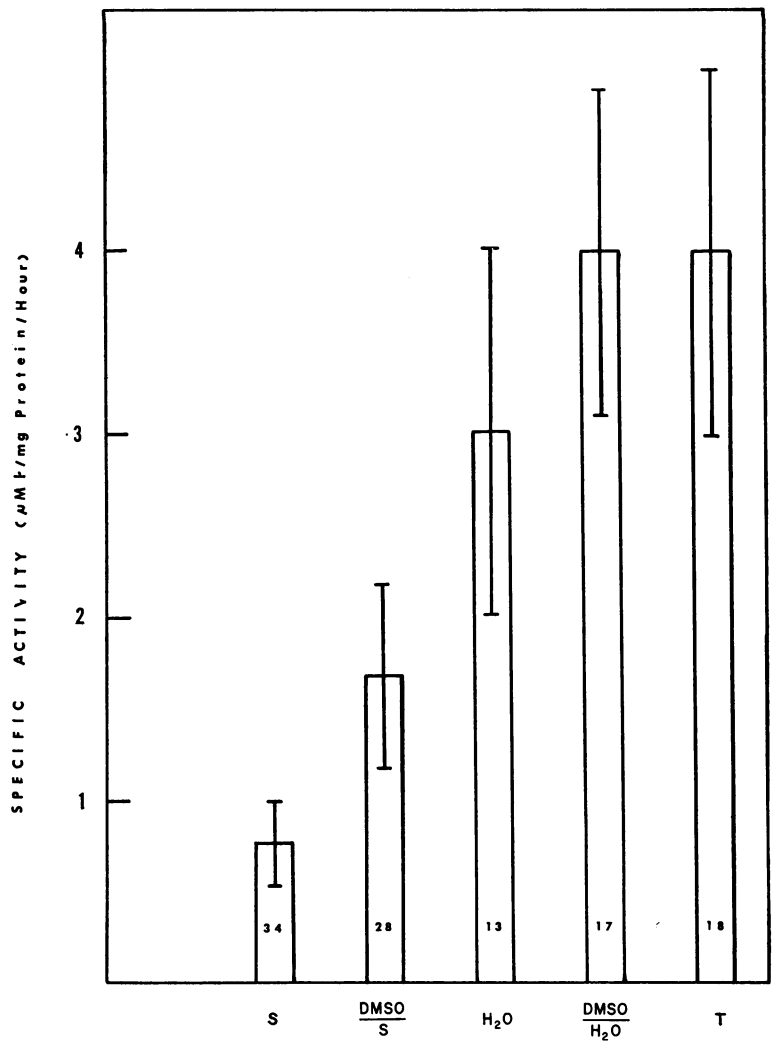

FIG. 1.—Specific activity of acid phosphatase in cytoplasmic extracts of rat-liver homogenates resuspended as follows: S = 0.25 *M* sucrose; DMSO/S = 25% dimethyl sulfoxide in sucrose; H_2O = distilled water; DMSO/H_2O = 25% dimethyl sulfoxide in water; T = 0.1% Triton X-100 in water. Activity is in micromoles phosphorus/mg protein/hr.

Figures within the bars indicate the number of experiments for each test condition. The vertical lines represent standard deviation from the mean. The difference between T and DMSO/H_2O is not significant ($P > 0.1$). Differences between all other pairs are significant; for T and H_2O, P is between 0.01 and 0.025; for all others P is less than 0.005.

may be complicated by the presence of numbers of mitochondria in the lysosomal resuspensions.

Discussion.—Our experiments have shown that dimethyl sulfoxide, used at concentrations which protect cells against damage due to freezing and subsequent thawing, can bring rat-liver lysosomes, *in vitro*, into greater activity as measured by increased activity of acid phosphatase. The presence of DMSO in sufficient con-

TABLE 1

RELEASE OF ACID PHOSPHATASE FROM LYSOSOMES TO SUPERNATANT FLUID

Exptl. conditions	No.	Per Cent of Cytoplasmic Extract Phosphorus in supernatant*	Protein in supernatant†	Relative specific solubilization‡
0.25 *M* sucrose	8	3	11	1
25% DMSO–0.25 *M* sucrose	8	15	17	7
H_2O	7	64	42	45
25% DMSO–H_2O	4	69	34	67

Release of acid phosphatase from lysosomes to supernatant fluid. Cytoplasmic extracts were resuspended under the conditions listed in the table, then centrifuged again and tested for acid phosphatase activity in the resulting pellets and supernatants.

* Mean values: micromoles of phosphorus hydrolyzed/ml supernatant/hr divided by micromoles of phosphorus hydrolyzed/ml cytoplasmic extract in DMSO–H_2O multiplied by 100.

† Mean values: mg protein/ml supernatant divided by mg protein/ml cytoplasmic extract multiplied by 100.

‡ Specific activity (micromoles of phosphorus hydrolyzed/mg protein/hr) for each supernatant divided by specific activity of sucrose supernatant.

centration apparently increases the permeability of rat-liver lysosomes to the sodium-beta-glycerophosphate used as substrate, thereby reducing the latency of acid phosphatase. This is evident from the fact that DMSO can bring lysosomes into 40 per cent of total activity, in the absence of osmotic stress, while liberating only 15 per cent of total phosphatase to the supernatant fraction. At the concentration employed, this effect was a characteristic feature of the interaction of DMSO with hepatic lysosomes. A change in accessibility of enzyme to substrate already within the lysosome is not ruled out. To our knowledge this is the first report of a large reduction in lysosomal latency during homogenate assay not accompanied by a corresponding release of enzyme from the particulates.

The use of relatively unpurified lysosomal-mitochondrial fractions in these experiments does not detract from the interpretation that it is acid phosphatase of lysosomal origin alone which is being affected by DMSO. The bulk of evidence clearly shows that acid phosphatase is localized exclusively to the lysosomes in rat liver. By means of the special technique involving intravenous injection of Triton WR-1339, Wattiaux *et al.*[11] have accomplished virtually total separation of lysosomes from mitochondria as indicated by almost total separation of acid phosphatase activity from cytochrome oxidase, and by the lack of mitochondrial profiles in electron micrographs of the lysosomal fraction. In addition to this biochemical evidence for the unitary localization of acid phosphatase, numerous cytochemical investigations at the level of electron microscopy have shown acid phosphatase reaction product localized within dense bodies of liver parenchymal cells and never in mitochondria (e.g., Essner and Novikoff,[12] Holt and Hicks,[13] Sabatini *et al.*,[14] Novikoff[15]).

Although the penetration of DMSO into lysosomes was not measured directly, it is unlikely that this molecule, much lower in molecular weight than glycerophosphate, could fail to permeate the lysosomes when glycerophosphate itself does so. Whether or not DMSO can promote the penetration of substrates for other lysosomal enzymes, particularly the high-molecular-weight substrates for cathepsins or nucleases, is not known. The observation has been made, however, that DMSO promotes the interaction, if not the penetration, of steroids with lysosomes.[16] The release of small amounts of acid phosphatase from lysosomes to the supernatant in the presence of DMSO might suggest that the permeability of lysosomes to protein can be affected by DMSO. Two considerations make such an interpre-

tation doubtful, however. The presence of significant amounts of mitochondria prevents relating the loss to lysosomes specifically, and the question as to whether some releasable protein may coat the surface of lysosomes has yet to be resolved.

In spite of the release of some acid phosphatase from lysosomes in these experiments, there are reasons to suggest that the effect of DMSO, at the concentrations employed, may not permanently alter the lysosomal membrane. First, DMSO appears to penetrate rat-liver lysosomes with little or no osmotic stress, a conclusion which is supported by the fact that activity of lysosomes under conditions of strong osmotic stress (water) is much greater than activity in DMSO sucrose mixtures. Lysosomes suspended in DMSO water mixtures show even greater activity. This indicates that DMSO may be working independently of water in exerting its activating effect. Such a suggestion is strengthened by the fact that the activity of lysosomes due to DMSO alone, when added to that due to water alone, gives the level of activation found in DMSO water mixtures (Fig. 1). A second indication that DMSO does not grossly alter the structure of biological membranes can be seen in its interaction with isolated mitochondria. When frozen and thawed in 10 per cent DMSO, both animal[17] and plant[18] mitochondria retain the linkage between oxidation and phosphorylation ordinarily uncoupled when isolated mitochondria are frozen and thawed. Third, in a detailed study of the effects of DMSO on hemolysis of human erythrocytes, no leakage of hemoglobin was reported for concentrations up to 40 per cent, with a small degree of protection noted at approximately 25 per cent DMSO.[19] Finally, our observations which indicate no change in particle size when pellets are resuspended in either 10 per cent or 25 per cent DMSO–0.25 *M* sucrose mixtures give further evidence that osmotic effects are minimal or lacking at concentrations of DMSO used in these experiments.

That the lysosomes in our preparations maintained properties as originally described by de Duve and associates[20] is evident in the similarity of response to Triton X-100[10] and to the osmotic effect of water,[21] as well as in the closely similar values (19–20%) for the free activity of homogenates.[11]

The mechanism by which DMSO penetrates membranous barriers is unknown. Several significant facts relating to the penetration of biological membranes by DMSO have, however, been noted. There is no irreversible damage done to membranes at low or moderate concentrations of DMSO, as deduced by Kligman[6] from reversible permeability changes induced by DMSO in human skin. The action by DMSO of increasing permeability is not likely to be mediated by osmotic effects, for reasons already discussed. Penetration in biological systems can probably be accounted for on the basis of the chemical properties of DMSO. These include not only the ability to promote solvation of a large variety of inorganic and organic molecules, but to catalyze a number of their reactions as well.[22] These properties, and the fact that DMSO must be used in high concentration (over 1 molar), suggest to us that it must form a continuous solvent phase which promotes the permeation of membranes, a view consistent with observations of the effect of DMSO on permeability of human skin.[6] The ability of DMSO to sequester water and to induce the formation of hydrogen bonds[22] tempts us to speculate that its mechanism of action may depend in part upon its ability to cause local, reversible dislocations of lipid and protein components of cell membranes.

We have now demonstrated, *in vitro*, enhanced activity of an enzyme within

lysosomes brought about by interaction with dimethyl sulfoxide. The question as to whether the action of DMSO on lysosomes *in vivo* can be related to its diverse physiological effects, and the mechanism of action of DMSO upon cells and organelles are currently under study in our laboratory.

Summary.—Dimethyl sulfoxide causes activation of rat-liver lysosomes *in vitro* as measured by increased acid phosphatase activity. Lysosomes suspended in mixtures of dimethyl sulfoxide and sucrose appear osmotically stable and are brought into 40 per cent of total activity while losing only 15 per cent of total acid phosphatase to the supernatant.

Aspects of the biological activity of DMSO and a mechanism for its effect on permeability of lysosomes are discussed.

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