(sp) were subjected to cloning. Out of 51 sp plaques so examined, three yielded predominantly, on passage through CEF, unusually large-plaque (u) mutants. This mutant as well as the parent large-plaque (lp) and sp viruses were purified by consecutive clonings. Mixed infection of CEF with the sp and u viruses tended to exclude the sp virus. Contrarily, mixed infection with the lp virus and the u mutant tended to exclude the latter. Thus for maintenance of the u mutant, frequent clonings had to be done, because otherwise an incidental appearance of the parent type virus by back mutation seemed to result in exlusion of the u mutant virus. The u mutant as well as the sp virus was more sensitive to interferon than the lp virus. The reason why the interferon-sensitive u mutant can grow more rapidly and yield a higher amount of virus than the parent lp virus remains to be elucidated.

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The Effect of Dimethyl Sulfoxide (DMSO) on Cellular Systems (32943)

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Dimethyl sulfoxide (DMSO), a dipolar aprotic solvent with a high dielectric constant, differs from protic solvents such as water and alcohols because of its tendency to accept rather than donate protons. It has been used as a chemical solvent and a biological preservative (1,2), and also provides protection from radiation damage (3). It enhances the percutaneous penetration of several steroids (4), and the permeability of the frog skin to mannitol, urea, sodium, potassium, and chloride (5,6). However, it is possible that the effects are mainly caused by changes in osmotic activity of the bathing solution (5,6).

In this report the effects of DMSO at the cellular level are investigated. We found that DMSO inhibited the growth of L-cells, HeLa cells, *Escherichia coli*, Mengo virus, and bacteriophage T4. We could find no evidence that DMSO altered the permeability characteristics of any of these systems.

Materials and Methods. DMSO: spectroquality reagent from Matheson, Coleman and Bell was used. Maximum evaporative residue was 5 ppm.

Mammalian cells and tissue culture. HeLa strain S_3 and L-cell strain 929 were grown in a modified Eagle's medium custom-made by General Biochemicals with 20% horse serum (for HeLa) and 10% new born calf (for L-cells). The cells were maintained as monolayers on 60 × 15-mm plastic petri dishes (Falcon) and incubated at 37°C in a 5% CO_2 atmosphere.

Bacteria. Escherichia coli strain FW3A, Sm^r T^- L^- B^- M^- lac^- (y^-) was obtained from Dr. F. Neidhardt. *E. coli* strain P4X6, M^- L^- lac^+ and strain PA106, M^- lac^- were obtained from Dr. P. Margolin.

Virus. Mengo virus was obtained from Dr. R. Franklin, and passaged in L-cell monolayers in growth medium. The Beaudette strain of NDV was maintained by passage through embryonated eggs. The *r*II mutants *r*147 and

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r227 were originally supplied by Dr. S. Benzer. Glycerol dehydrogenase and sRNA were obtained from Nutritional Biochemicals Corporation and ribonuclease from Worthington Biochemical Company.

Growth rate determinations. Two circles, about 4 mm in diameter, were drawn on the bottom of tissue culture plates containing 4 ml of growth medium and the desired concentration of DMSO. Approximately 10^5 cells were added to each plate. Cells within the circles were counted 24 hours after plating and on successive days thereafter. Duplicate plates were used for each determination.

Autoradiography. The methods described by Toliver and Simon (7) were used.

In vitro *enzyme assays.* (a) Glycerol dehydrogenase was assayed by the method of Lin and Magasanik (8): 0.1 ml of $(NH_4)_2SO_4$ (1.0 M), 0.1 ml of nicotinamide adenine dinucleotide (NAD) 0.01 M, pH 7) and 0.1 ml of enzyme diluted in 0.01 M phosphate buffer (pH 7.4) were put into a test tube. Two minutes later 0.6 ml of 0.5 M carbonate–bicarbonate buffer (pH 10) and 1.8 ml of water or the desired concentration of DMSO was added, and the mixture was transferred into cuvettes. At time zero 0.3 ml of 1.0 *M* glycerol was added. Absorbance at 340 mμ was read at 30-sec intervals. The concentration of DMSO was calculated as the percentage of DMSO present in 3 ml of the reaction mixture (v/v).

(b) β-Galactosidase was measured by its ability to hydrolyze o-nitrophenyl-β-galactoside (ONPG) (9). Ten ml of *E. coli* strain BB was grown to 3×10^8 cells/ml in M9-glucose (a minimal medium containing (in gm per liter) Na_2HPO_4, 5.8; KH_2PO_4, 3.0; NaCl, 0.5; NH_4Cl, 1.0; glucose, 4.0; $MgSO_4$, 0.25; $FeCl_3$, 0.0016), centrifuged, and resuspended in 10 ml of M9-buffer (M9-glucose, with glucose omitted). The suspension was aerated at 37°C for 20 min to eliminate residual glucose, and 0.5 ml of 2% lactose added to induce the synthesis of β-galactosidase. Thirty min later, the enzyme was released by adding toluene and agitating the culture. One ml of the enzyme solution was added to 4 ml of 2.5×10^{-3} M ONPG diluted in H_2O and the desired concentration of DMSO. The change in optical density was read at 420 mμ.

(c) Ribonuclease (RNase) activity was determined by measuring release of oligonucleotide from sRNA (10). Six ml of an 0.4% solution of yeast sRNA in 0.05 M phosphate buffer (pH 7.2) was mixed with the desired concentration of DMSO and 0.6 ml of 5 μg/ml RNase solution. At various times, the undegraded RNA was precipitated by adding uranyl acetate (0.75% in 25% perchloric acid), and 30 min later, the precipitate was removed by centrifugation. The oligonucleotides remaining in the supernatant were measured by determining the optical density at 260 mμ.

Growth of a lactose permease negative E. coli *mutant in lactose.* Cells were grown in M9-buffer supplemented with 0.2% glycerol, and harvested during log phase. Two ml of cell suspension was added to 50 ml of M9-buffer containing 0.2% lactose and different concentrations of DMSO. Growth was measured as an increase in turbidity. Lac^+ and lac^- cells grown in lactose and glycerol served as controls.

Mengo virus growth curve. The growth of mengo virus was measured by infecting a series of L-cell monolayers with about 5 plaque forming units (pfu) of mengo virus per L-cell, adsorbing for 30 min at 37°C, and washing each plate three times with 2 ml of warm phosphate buffered saline. Four ml of cell growth medium containing the desired amount of DMSO was added, and the plates incubated at 37°C. The supernatant fluids were collected at various times postinfection, and assayed for mengo virus pfu.

Mengo virus assay. The plaque assay described by Dulbecco was used except that a starch overlay was employed in place of agar (11). The virus was diluted in Eagle's medium and 0.2 ml was added to each PBS-washed L-cell monolayer. After 30 min adsorption at 37°C, 5 ml of starch overlay was added to the plates. Plaques were counted 36–48 hours postinfection several hours after the staining the cells with 0.01% neutral red in Eagle's medium. Starch overlay was prepared by gradually adding a slurry of 10 gm of starch (starch hydrolyzed, Mann Re-

search Laboratories) in 20 ml of cold water to 54 ml of boiling water on a combination stirrer–hotplate with vigorous stirring. When the temperature of the starch solution fell to 60°C, a mixture containing 18.5 ml of 5× Eagle's and 7.5 ml new born calf serum at 45°C was added. The overlay was applied to the cells at temperatures below 50°C.

Growth and recombination studies with bacteriophage T4. For the one-step growth studies, *E. coli* strain BB was grown in tryptone broth to 5×10^8/ml and infected at a multiplicity of infection of 0.02. After a 5-min adsorption period the infected culture was diluted 10^4-fold into broth at 37°C containing the desired concentration of DMSO. The cells were lysed periodically with chloroform to release intracellular virus. For recombination studies, the bacteria were infected with 3 to 5 plaque forming units each of *r*147 and *r*227. After a 5-min adsorption period, the infected culture was diluted 40-fold into broth at 37°C containing various concentrations of DMSO. At intervals samples of the cells were lysed periodically with chloroform. In all cases, total virus was determined by assay on *E. coli* B, wild type recombinants on *E. coli* K12 (λ).

Results. 1. *Effect of DMSO on the growth of L-cells and HeLa cells.* The results for L-cells are shown in Fig. 1. The data for HeLa cells were similar, except that complete inhibition occurred in 2% rather than 3% DMSO. In both cell lines, only a twofold difference in concentration separated completely toxic and nontoxic doses. In a separate experiment, HeLa cells were treated with 12% DMSO for 5 hours; 20% of them survived the treatment and resumed normal growth.

2. *Effect of DMSO on DNA synthesis of HeLa cells.* HeLa cells were labeled with thymidine-^{3}H during the last hour of a 2-hour and a 5-hour DMSO pulse. The frequency of surviving cells ranged from 100 to 20% depending on the DMSO concentration. At *all* concentrations, 45% of the survivors were synthesizing DNA. The data of Fig. 2 show that the *rate* of synthesis decreased with increasing DMSO concentration. Since a normal proportion of the cells were synthesizing DNA, it appears that the only effect of DMSO on DNA metabolism is to decrease its rate of synthesis.

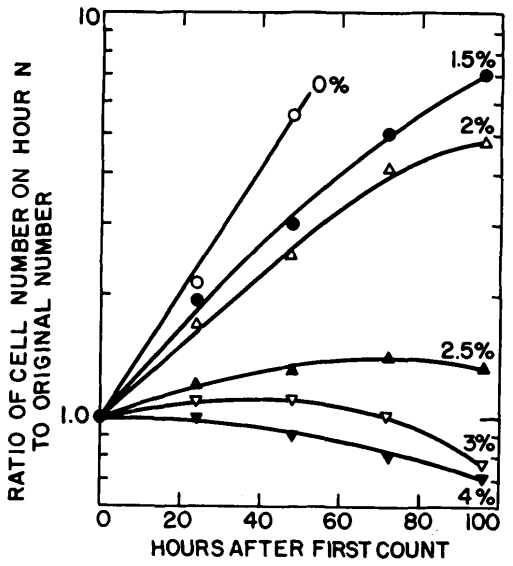

FIG. 1. Growth curves for L-cells in DMSO: 7.5 × 10^4 cells were plated into the indicated concentration of DMSO; the number of cells in each circle was counted daily; the medium was changed every 48 hours. The ordinate is the ratio of the cell count on a given day to the original cell count. The abscissa is days after the first cell count. The concentration of DMSO (v/v) for each treatment is indicated on the graph.

3. *Effect of DMSO on several* in vitro *enzyme systems.* To determine if the main site of action of DMSO was on DNA synthesis, several enzyme systems were tested *in vitro*. In the yeast-sRNA–RNase system, (Figs. 3 and 4), the initial reaction rate *increased* linearly in proportion to the amount of DMSO in the reaction mixture. The average velocity between the fourth and the ninth min was also enhanced and reached its maximum in 12% DMSO, and then declined. Apparently, DMSO activated the entire system in the early part of the reaction. Perhaps the configuration of yeast-sRNA was changed into a form which was more reactive than the native molecule. This suggestion is supported by the fact that in 50% DMSO, sRNA had a 12% greater absorbance than the control.

There was a 20% increase in relative activity of β-galactosidase in 24% DMSO,

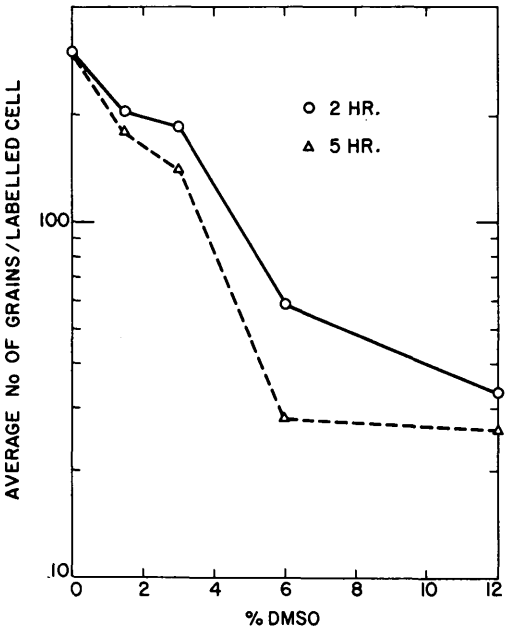

FIG. 2. HeLa cells were treated with DMSO-containing medium of the indicated concentration for either 2 or 5 hours. During the last hour of DMSO treatment, 0.4 μC/ml of thymidine 1-1^{3}H was added to each plate. At the end of the DMSO treatment the cells were fixed as described in "Materials and Methods". The number of grains per labeled cell represents an average grain count from 20 labeled cells.

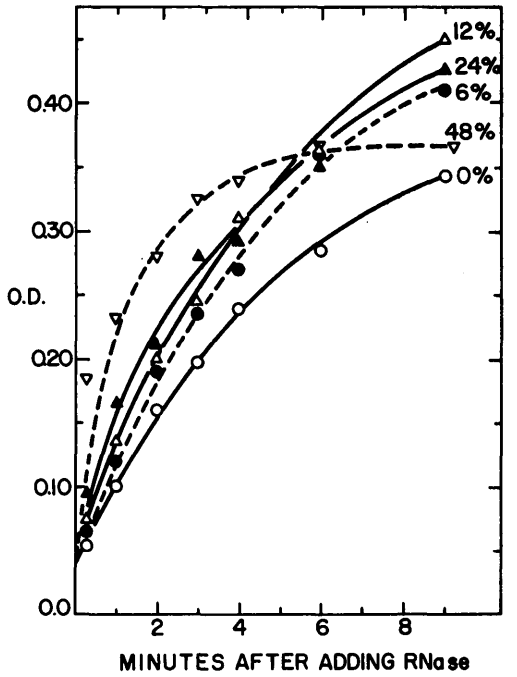

FIG. 3. The effect of DMSO on the activity of the SRNA RNase system. The conditions are described in "Methods". The abscissa is minutes after adding RNase (5 μg/ml) into the substrate (4 mg of sRNA/ml). The ordinate is the optical density at 260 mμ after correction for the control. The concentration of DMSO is indicated on the graph.

while glycerol dehydrogenase retained only 50% relative activity in 6% DMSO. Table I summarizes these results.

4. *Effect of DMSO on the growth of Mengo virus.* The data of Fig. 5 shows that the yield of virus decreased as the DMSO concentration increased. At a concentration of 8% DMSO, the eclipse period was increased by 2 hours, while at a concentration of 10% DMSO, no virus was released. Control experiments eliminated the possibility that DMSO sensitized the virus to heat inactivation. In fact the fraction of virus surviving in L-cell growth medium after 24 hours at 37°C, increased from 0.65% in controls, to 12% in the presence of 12% DMSO.

5. *Effect of DMSO on* E. coli *and bacteriophage T4.* While division of *E. coli* is inhibited by DMSO, it can tolerate a much higher concentration than mammalian cells. In 12% DMSO, the bacteria can grow in-

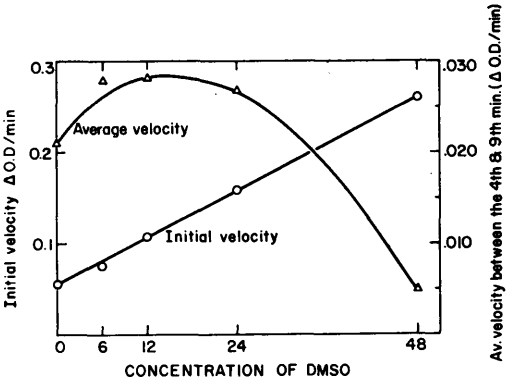

FIG. 4. The initial velocity and average velocity of the reaction of sRNA–RNase. The data was taken from Fig. 3. The initial rate of the reaction was measured by taking the tangent of each activity curve during the first minute. The ordinate on the left is the initial velocity (OD/min). The ordinate on the right is the average velocity between the fourth and ninth min and the scale is expanded by a factor of 10.

TABLE I. Effect of DMSO on the Activity of Glycerol Dehydrogenase and β-Galactosidase.

Concentration of DMSO (v/v)	Relative activity of glycerol dehydrogenase[a]	β-Galactosidase[b]
0	1	1
2	.81	—
3	—	1.025
4	.70	—
6	.53	1.10
12	.43	1.18
24	.27	1.20

[a] The rate at room temperature (24°C) is expressed as the change in optical density at 340 mμ/min (details in "Materials and Methods"). Relative activity was obtained by dividing the activity at each DMSO concentration by the activity of the control (no DMSO).

[b] The rate at room temperature (24°C) is expressed as the change in optical density at 420 mμ (details in "Materials and Methods"). Relative activity was obtained by dividing the activity at each DMSO concentration by the activity of the control (no DMSO).

definitely in a tryptose broth medium although with a greatly increased doubling time. In 6% DMSO growth was nearly normal. When either 6% or 8% DMSO was present in the bacterial culture during phage growth, the yield was ⅓ to ¼ of the control and was virtually zero at a concentration of 12%. The data of Table II shows that the decrease in phage yield and the delay in burst time were directly proportional to the concentration of DMSO. Table II also shows the results of a cross between two *r*II mutants. There was a small increase in the percentage of recombination in the presence of 8% DMSO. This probably reflects pool size differences, rather than a direct effect of DMSO on the recombination process.

6. *Effect of DMSO on the permeability of HeLa and* E. coli *cells.* Jacob showed that Evans blue dissolved in 15% DMSO penetrated through the urinary bladder mucous membrane in dogs within 4 hours (12). The dye was not taken up by HeLa cells treated with 1, 3, 6, or 12% DMSO for as long as 24 hours. Cells killed by UV-irradiation, however, took up the dye readily. A permease negative lac⁻ (y⁻), strain of *E. coli* was not induced for β-galactosidase after incubation in the presence of IPTG and DMSO. Lac⁻ (y⁻) cells fed lactose as the sole carbon source failed to grow either in the presence or absence of DMSO.

Discussion. We have shown that DMSO retards the growth of HeLa cells, L-cells, *E. coli,* Mengo virus, and bacteriophage T4. Similar findings have been made for poliovirus and HeLa cells by Hellman *et al.* (13), under somewhat different conditions. In HeLa cells and L-cells only a twofold difference in DMSO concentration separates completely toxic and nontoxic doses. Perhaps when the DMSO concentration reached a critical level, certain vital functions stopped and the cell died. The results of Fig. 1 show that the effect of a low concentration of DMSO increased

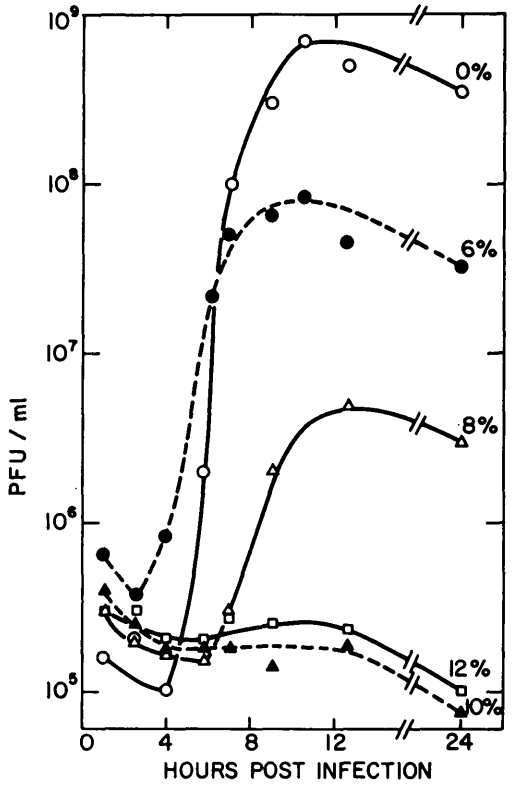

FIG. 5. Mengo virus growth in DMSO; 2 × 10^6 L-cells were infected by Mengo virus at an MOI of 10. Unadsorbed viruses were washed off after a 30-min adsorption period and various concentrations of DMSO in growth medium was added. The percentage of DMSO in the growth medium is indicated on the graph.

TABLE II. Effect of DMSO on Bacteriophage T4 Growth and Recombination.

Concentration of DMSO	Yield of phage/infected bacterium[a]	Latent period[a]	Frequency of recombination[b]
0	27	18	1.0
3	20	20	1.3
6	15	23	1.5
8	13	27	1.7
10	4	35	1.3
12	0.8	—	0.4

[a] Results are taken from a one-step growth curve (see "Materials and Methods" for details). The latent period is measured from the time of infection to the middle of the exponential rise period.

[b] Results are from a separate experiment performed under slightly different conditions as described in "Materials and Methods."

with time and Fig. 2 that the rate of DNA synthesis in individual HeLa cells decreased in proportion to the amount of DMSO added. The results of the effect of DMSO on *in vitro* enzyme systems: RNase, glycerol dehydrogenase, and β-galactosidase, suggest that all of the cited results may reflect a general alteration of cellular reaction rates beyond the capacity of intracellular homeostatic mechanisms to correct.

The mechanism by which DMSO affects the various enzyme systems has not been studied and probably varies in each case. For example, since DMSO at high concentrations denatures DNA and RNA (14) and since we demonstrated a hyperchomic shift with the sRNA we used, it is possible that even at low concentrations, DMSO permits the sRNA to assume a more open, less hydrogen bonded configuration. Alternatively, in the presence of DMSO, the enzymes themselves may be denatured. Since the native form of biopolymers is surrounded by the ordered arrays of water molecules, substitution or removal of the hydration sheath of these substances by DMSO would be expected to alter their configuration (15). Although the site of action of DMSO in the enzyme systems studied is not clear, the present results are sufficient to show that DMSO certainly would disturb the enzymatic balance of the cell.

Similar results have been found by others who have studied the effect of DMSO on various enzyme systems. Rammler found that concentrations of less than 10% DMSO enhanced the activity of snail phosphomonoesterase, trypsin, and alanine dehydrogenase, and inhibited the activity of *E. coli* phosphomonoesterase, peroxidase, catalase, and glutamate dehydrogenase. In addition he found that it could alter the specificity of an "allosteric protein" such as glutamate dehydrogenase (16). These results, combined with ours, suggest two possible interpretations for the *in vivo* effect of the compound. (i) A whole complex of enzyme activities are altered, the affect becoming more severe as the DMSO concentration increases; at intermediate levels the cell growth is first slowed, and finally stopped. Ultimately "unbalanced growth" of some kind causes the cell to die. The rapid death of some cells during short-term pulse experiments with high concentrations of DMSO is probably due to osmotic effects. (ii) The cells can adjust their metabolism to allow for the relatively small changes in enzyme activity which occur at biologically effective doses of DMSO; however, some critical enzyme is extremely sensitive to DMSO and when it is no longer active, cell growth ceases. Either hypothesis will explain the results with the viruses since their growth is to a large extent dependent upon the metabolism of the cell. At present it is not possible to choose between them.

Although it has been demonstrated that DMSO is a penetrant-carrier on animal skins (5,6), we were unable to demonstrate this on the cellular level. In contrast to the study of Fowler and Zabin (17), our studies showed that permeaseless strains of *E. coli* could not be induced for β-galactosidase and would not

grow on lactose in the presence of DMSO. Similarly we were unable to demonstrate the DMSO mediated uptake of Evans blue into HeLa cells despite the claims of Jacob (12) that it occurs *in vitro*. Hellman *et al.* also failed to demonstrate an increased permability of HeLa cells (13), while Dixon *et al.* failed to demonstrate any increase in cellular penetration in work with whole animals (18). The report of Amstey and Parkman (19) that DMSO increases the infectivity of infectious polio virus RNA probably does not demonstrate an increase in cellular permability to the RNA, since the maximum efficiency of infectivity which he reports, 10^{-3}, is no more than is found in other systems in which DMSO is omitted (20). Similar findings have been made by Chang in L-cells (21), and Lau in HeLa cells (22). In both cases the maximum efficiency was below 10^{-3} and probably reflected improved protection of adsorbed RNA from various nucleases than an actual increase in uptake.

In conclusion, the *in vivo* effects of DMSO are attributed to its ability to alter the efficiency of various enzyme systems, and hence disturb cell metabolism and inhibit virus synthesis. Since a cell is a highly integrated system, it is reasonable to assume that if many enzyme reactions proceed at slightly abnormal rates, or if one critical system is strongly out of balance, an overall reduction in cell, and virus growth would result.

Summary. The growth of HeLa cells was strongly inhibited by 2%, L-cells by 3%, and *E. coli* (in broth) by 12% DMSO. The growth of Mengo virus in L-cells, and bacteriophage T4 in *E. coli* was markedly inhibited by similar concentrations of DMSO. *In vitro* studies showed that DMSO slightly increased RNase and β-galactosidase activity and markedly decreased that of glycerol dehydrogenase. It is suggested that the *in vivo* effects of DMSO are primarily due to its ability to alter enzyme reaction rates.

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