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BIOCHEMICAL EFFECTS ON KIDNEY OF EXPOSURE TO HIGH CONCENTRATIONS OF DIMETHYL SULPHOXIDE

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Abstract—Kidney preparations were exposed to up to 30% dimethyl sulphoxide at various temperatures following which the dimethyl sulphoxide was removed or diluted out, and metabolism was examined at 37°.

Respiration of slices and mitochondria and the P/O ratios and respiratory control index of mitochondria were little affected by 30% dimethyl sulphoxide at 4°.

Homogenate respiration and nuclear ATPase were inhibited. Anaerobic glycolysis of slices and homogenates were much reduced, due in part to activation of FDPase by dimethyl sulphoxide. Activation of FDPase, and inhibition of glycolysis could be prevented by adding urea or lysine with the dimethyl sulphoxide.

TRANSPLANTATION of kidney could be carried out more frequently if preservation of kidneys were possible at low temperatures as is now done with simpler tissues. Dimethyl sulphoxide (DMSO) has many of the properties required of an ideal preservative against freezing damage.[1] About 35% DMSO would be required to prevent separation of a phase of high salt concentration at −79°.[2] We have examined the effects of such concentrations on kidney. The main interest lies in residual abnormalities after all but traces of DMSO have been removed. This treatment did not lower oxygen consumption but affected ATPase and glycolysis. The latter appeared to be due to activation of FDPase. A means of preventing it has been found.

MATERIALS AND METHODS

Tissue preparations. Male Wistar rats, 250–350 g, were fed M.R.C. diet 41B *ad lib.* They were killed by cervical dislocation and kidneys were removed to the appropriate medium at 0°. Slices were prepared with a Stadie–Riggs[3] microtome. Medulla was removed. The standard medium for manometric determination[4] of oxygen consumption was that of Robinson.[5] Glycolysis was determined manometrically with the bicarbonate medium of Krebs and Henseleit.[6] Glucose (5·5 m-moles/l.) was present in both media. Glucose consumption by slices was determined[7] in Robinson's medium in the presence of 2 m-moles/l. glucose.

Homogenates were prepared from renal cortex by fine chopping and the use of a Potter–Elvehjem homogenizer (1200 rev./min) with a Teflon pestle (clearance 0·5 mm). Four to six passages were made. For studies of respiration the medium contained 70 m-moles/l. sucrose, 30 m-moles/l. tris, 10 m-moles/l. $MgCl_2$, 10 m-moles/l. KH_2PO_4, and 15 m-moles/l. glucose at pH 7·4. Anaerobic glycolysis was measured manometrically in the following medium; 70 m-moles/l. sucrose, 30 m-moles/l. $KHCO_3$, 10 m-moles/l. KH_2PO_4, 10 m-moles/l. $MgCl_2$ and 15 m-moles/l. glucose. After gassing

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with 95% N_2:5% CO_2 for 30 min at 0° the pH was 7·3–7·6. Lactic acid production was measured in 5 m-moles/l. KH_2PO_4, 50 m-moles/l. glycyl-glycine, 2 m-moles/l. $MgCl_2$, 30 m-moles/l. nicotinamide at pH 7·4. Aliquots (0·5 ml) of the homogenates were incubated at 37° with 0·1 ml of 10 m-moles/l. ADP, 10 m-moles/l. ATP, 10 m-moles/l. AMP and 10 m-moles/l. NAD^+ (in the above buffer) and 0·3 ml of 3·3 m-moles/l. azide under 100% N_2. The reaction was started by adding 0·1 ml of 100 m-moles/l. substrate in buffer, and was stopped, after 30 min at 37°, by the addition of 1 ml 10% trichloracetic acid. Aliquots of supernatant were analysed for l-lactate.

Subcellular fractionation for studies in which the integrity of mitochondria was not critical was carried out in 0·25 moles/l. sucrose or isotonic medium appropriate to the assay. The following conditions were used for separation: nuclear fraction 650 $g_{av.}$ for 10 min; mitochondrial fraction, 9200 $g_{av.}$ for 10 min; microsomal fraction, 36,000 $g_{av.}$ for 100 min. Sap refers to the final supernatant. Fractions were tested for completeness. Sap contained 5 per cent of the glucose-6-phosphatase, the remainder being in the microsomes. All the FDPase was in the sap. Oxygen consumption was over 95 per cent mitochondrial with a small amount in microsomes. The nuclear fraction had negligible oxygen consumption but density gradient centrifugation showed some contamination by membranes and erythrocytic fragments.

For the preparation of tightly coupled mitochondria the following modifications of Schneider's[8] techniques were used. Chopped washed kidney was homogenized in 9 vol. of 0·25 moles/l. sucrose, 1 m-moles/l. tris, 0·1 m-moles/l. EDTA at pH 7·4. The homogenate was spun at 650 $g_{av.}$ for 10 min, the supernatant removed, the pellet resuspended and recentrifuged and supernatants pooled and spun at 9200 $g_{av.}$ for 10 min. The supernatant was removed with a Pasteur pipette and discarded. The pellet was resuspended and recentrifuged. The final pellet was resuspended so that the mitochondria from 1 g kidney were in 2 ml. This contained approximately 20 mg protein. All operations were at 0–2° using a M.S.E. Magnum centrifuge with high speed head attachments.

Oxygen consumption of mitochondria and P/O ratios were determined polarographically at 30° using a Rank oxygen electrode and Servoscribe potentiometric recorder. Metabolic rates were estimated according to Chance and Williams.[9] ADP was injected with an Agla micrometer syringe. The medium was 10 m-moles/l. of substrate and 0·2 ml of mitochondrial suspension (from 100 mg kidney) was injected.

Treatment of tissues with DMSO. In preparing DMSO-containing media the ionic composition was maintained—i.e. 50% (v/v) DMSO was prepared by adding an equal volume of twice concentrated medium. Cortical slices were placed in the required volume of DMSO-containing medium in Warburg flasks. After incubation at the required temperature cold medium was added to a final fluid volume of 2 ml, reducing the DMSO concentration to the required level. In some experiments slices were incubated in 10 vol. of DMSO-containing medium with shaking. After the required period the slices were removed, blotted and gently agitated for 10 min in 10 vol. saline at 0°. The slices were then blotted and transferred to appropriate medium for examination at 37°. Cell fractions were prepared by homogenizing slices which had been exposed to DMSO in this way.

Analytical methods. Conventional methods were used for phosphate,[10] protein by biuret with bovine serum albumin as reference, glucose by oxidase[7] and lactic acid with lactic dehydrogenase.[11]

Phosphorylated intermediates in anaerobic glycolysis were prepared from slices metabolizing glucose anaerobically in Robinson's medium containing 0·2 m-moles/l. azide. About 500 mg of cortical slices were incubated at 37° in 5 ml of medium for 0–30 min in 25 ml conical flasks. Perchloric acid at 0° was added to 5%. The slices were broken up in a Sorvall Omni-mixer and centrifuged at 0°. The supernatant was decanted, the pellet resuspended in 5% $HClO_4$ and recentrifuged. The supernatants were pooled and solid $KHCO_3$ slowly added until effervescence had ceased. The solution was chilled for 1 hr and centrifuged to remove the $KClO_4$. The supernatant was frozen at −20° and lyophilized overnight. The bulky white residue was dissolved in 200 m-moles/l. KCl, 20 m-moles/l. imidazole and 5 m-moles/l. $MgCl_2$, so that 1 g of kidney was in 10 ml. Glucose-6-phosphate, fructose-6-phosphate, fructose-1:6-diphosphate and ATP were estimated consecutively in one reaction cuvette. (ADP was measured separately). The appropriate enzymes and coenzymes were glucose-6-phosphate dehydrogenase and $NADP^+$, phosphoglucoseisomerase, partly purified FDP-ase, yeast hexokinase. The $E_{340\,nm}$ was measured in a SP 800 spectrophotometer with scale expander. Recoveries were 85–93 per cent.

ADP was estimated by the oxidation of NADH in the presence of phosphoenolpyruvate, pyruvate kinase and lactate dehydrogenase.

Enzyme determinations. Glycolytic enzymes were assayed at 37° in 50 m-moles/l. tris, pH 7·4 according to Wu and Racker[12] unless otherwise stated. Fructose-1:6-diphosphate was estimated by the phosphate-release method of Mendicino, Beaudreau, Hsu and Medicus[13] or by the $NADP^+$ reduction method of Underwood and Newsholme[14] as indicated in the text. The buffer in each system was 200 m-moles/l. KCl, 20 m-moles/l. imidazole and 5 m-moles/l. $MgCl_2$.

The effect on FDPase of pyridoxal phosphate and borohydride reduction was determined according to Marcus and Hubert.[15] Enzyme activity was determined by the phosphate-release method.[13]

ATPase was estimated by phosphate release in a medium of 80 m-moles/l. tris, 5 m-moles/l. $MgCl_2$, 40 m-moles/l. NaCl, 6·7 m-moles/l. KCl and 2·7 m-moles/l. ATP.

Gel filtration. Columns (2·5 by 18 or 25 cm) of Sephadex G-200 in FDPase buffer were set up at room temperature. Kidney sap (2 ml, 25%) was eluted at 10–16 ml/hr with a head of 15–25 cm H_2O. Fractions (2 ml) were collected at 4°.

Reagents. All chemicals were Analar grade and obtained from British Drug Houses Ltd., Poole, Dorset, or from Koch–Light Limited, Colnbrook, Bucks. DMSO was puriss grade, over 99 per cent from Koch–Light Limited. It was redistilled at normal pressure. Biochemicals and enzyme preparations were obtained from Sigma Chemical Company, St. Louis, U.S.A.

RESULTS

Respiration. DMSO (5%) had a small (11–12%) inhibitory effect on oxygen consumption of kidney slices. To determine the lasting effects of DMSO kidney slices were exposed to medium containing DMSO and then transferred to new medium without DMSO for estimation of respiration at 37°. Exposure of slices to DMSO at 37° and 20° produced inhibition of subsequent respiration (Fig. 1) but the effect was negligible, even with 30% DMSO, if the exposure was at 4°.

In the presence of DMSO at 37° respiration of homogenates was inhibited 11, 42 and 62 by 10, 20 and 30 per cent DMSO, respectively. To determine residual effects

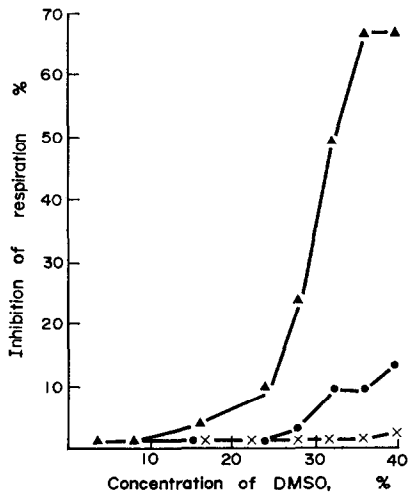

FIG. 1. Inhibition of respiration of cortical slices following exposure to DMSO at 37° (▲), 20° (●) and 4°(×). Each entry is the mean of eight determinations.

slices were exposed to 32% DMSO and then removed, homogenized and respiration determined at 37°. Exposure for 10 min at 37°, 20°, and 4°, produced inhibitions of 78, 67 and 51 per cent respectively.

The mechanism by which exposure to DMSO reduced respiration of homogenate was examined in more detail. The respiration of normal homogenate is greater than the sum of the respirations of separated nuclei (about 35 per cent of the whole) and of separated cytoplasm (about 15 per cent of the whole), a considerable increment (about 50 per cent) resulting from the interaction of the two. An experiment was carried out to determine whether the inhibiting effect of DMSO was predominantly on nuclear or cytoplasmic fractions. Nuclei and cytoplasmic fractions (pellet and supernatant at 650 $g_{av.}$) were separated from homogenates of normal and DMSO-treated kidney slices. Homogenates were then reconstituted in the four possible combinations (Table 1). The reduction in Q_{O_2} due to DMSO treatment largely resulted from an effect on

TABLE 1. OXYGEN CONSUMPTION OF HOMOGENATES RECONSTITUTED FROM NUCLEAR AND CYTOPLASMIC FRACTIONS OF CONTROL AND DMSO-TREATED KIDNEYS

Reconstructed preparation	Q_{O_2} (m-moles/g/hr)	Inhibition (%)
Control nuclei + control cytoplasm	31·2	0
Control nuclei + DMSO-treated cytoplasm	31·6	−1
DMSO-treated nuclei + control cytoplasm	14·4	54
DMSO-treated nuclei + DMSO-treated cytoplasm	15·3	51

Cortical slices were exposed to 32% DMSO for 10 min at 4°. They were cleared of DMSO, homogenized and spun at 650 $g_{av.}$ for 10 min to obtain nuclear and cytoplasmic fractions, which were separated and reconstituted as shown. Oxygen consumption was determined manometrically in the presence of 20 m-moles/l. glucose.

nuclei, which failed to stimulate when combined with cytoplasm. As ADP is required for maximal respiration the ATPase activity of nuclei and cytoplasm of normal renal cortex was determined. Nuclei accounted for about 70 per cent of the total activity. DMSO-treatment of slices, especially at 37°, strongly inhibited ATPase of homogenate (Fig. 2).

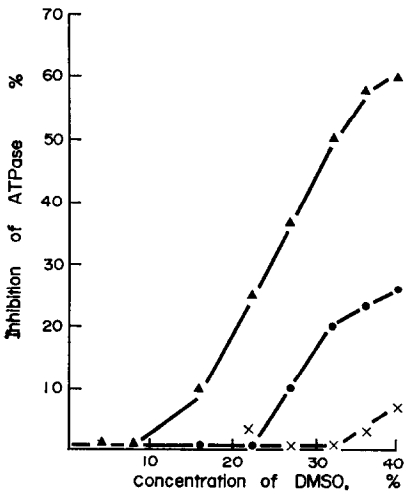

FIG. 2. Inhibition of ATPase activity of homogenates prepared from cortical slices which had been exposed to DMSO for 20 min at 37° (▲), 20° (●), and 4°(×). The figures are the means of eight replicates. Slices were homogenized in 0·25 moles/l. sucrose.

Oxidative phosphorylation and respiratory control. Mitochondrial respiration was estimated after storage at 4° in 35% DMSO. After 10–120 min the medium was diluted to bring DMSO concentration to 2%. The control contained sucrose (250 m-moles/l.) in place of DMSO. Even 10 min in DMSO lowered the Q_{O_2} by 9 per cent in the presence of ADP and 16 per cent in its absence. (A part of the inhibition may have been due to 2 per cent DMSO present during measurement.) These inhibitions rose to 25 per cent after 30 min exposure to 35% DMSO. Oxidative phosphorylation, with 2-oxoglutarate (10 m-moles/l.) as substrate, was unaffected and the respiratory control index was well maintained. Respiratory control was less well preserved in sucrose after periods of 1 and 2 hr than in DMSO.

Glycolysis. Slices of renal cortex were exposed to DMSO following which the slices were washed and added to fresh bicarbonate medium containing 5·5 m-moles/l. glucose. Glycolysis was greatly reduced. The protective effect of lowering the temperature of exposure from 20° to 4°, which had been noted with respiration, was entirely absent with anaerobic glycolysis (Fig. 3). It was immaterial whether the slices were aerobic or anaerobic during exposure to DMSO. The time required for the DMSO to produce the lasting effect was examined by reducing the period of exposure to DMSO to 10 min, which was the least time in which penetration of DMSO would occur (as indicated by the rate of development of inhibition of glycolysis). The inhibition produced by 32% DMSO in 10 min accounted for most of that shown in Fig. 3. Longer exposure at 4° or 37° did not increase the inhibition (53 and 76 per cent, respectively).

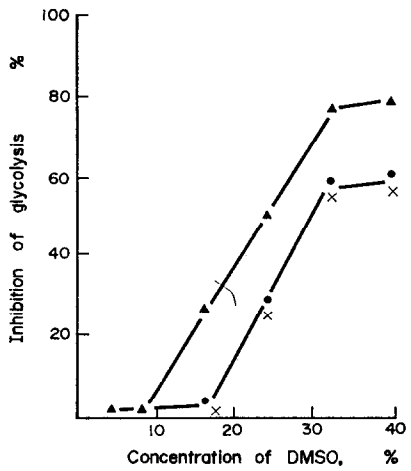

FIG. 3. Inhibition of anaerobic glycolysis of cortical slices after exposure to DMSO for 20 min at 37° (▲), 20° (●), and 4°(×). Each entry is the mean of eight replicates.

However the inhibition due to exposure at 20° depended on time of exposure rising from 57 per cent at 10 min to 78 per cent at 120 min. Exposure of slices to DMSO (32 per cent; 10 min; 4°) also produced 50 per cent inhibition of anaerobic glucolysis of homogenates prepared from them.

In order to plan control experiments it was necessary to find out whether DMSO in the glycolytic medium affected glycolysis of homogenate (Table 2). There was a 3-fold stimulation at about 20 per cent DMSO and inhibition above 40 per cent. Thus there were contrasting effects—glycolysis being approximately tripled in the presence of DMSO (Table 2), but if the DMSO were then removed glycolysis dropped to about half the initial rate for untreated kidney (Fig. 3).

Both nuclear and supernatant (650 $g_{av.}$) fractions showed a lasting inhibitory effect of 50–60 per cent. Further studies were limited to the glycolytic system of the cytoplasmic fraction. Slices of cortex were incubated in Robinson's medium with 32%

TABLE 2. RATE OF ANAEROBIC GLYCOLYSIS OF KIDNEY HOMOGENATES IN THE PRESENCE OF DMSO AT 37°

DMSO Concentration (%)	Rate of glycolysis (μmoles CO_2/hr/g)	Control (%)
0	11·8	100
10	15·9	135
20	36·2	307
30	21·7	184
40	12·0	102
50	2·7	23

Kidneys were homogenized and glycolysis was determined manometrically by CO_2 evolution.

DMSO for 10 min at 4°. The slices were then washed, homogenized, and the nuclei and debris were removed by spinning at 650 $g_{av.}$ for 10 min. In order to determine which enzymatic stage in glycolysis might be rate-limiting, lactate formation by control and by DMSO-treated homogenates was measured with a number of intermediates from the glycolytic pathway. The main limitations introduced by DMSO treatment were the phosphorylation of glucose and the utilization of G-6-P and F-6-P (Table 3). No inhibition appeared with FDP.

TABLE 3. LACTATE PRODUCTION FROM INTERMEDIATES OF GLYCOLYSIS BY HOMOGENATES OF CORTICAL SLICES WHICH HAD BEEN TREATED WITH 32% DMSO FOR 10 min AT 4°

Substrate	Lactate production (μmoles/hr/g)	
	Untreated kidney	DMSO-treated kidney
Glucose	16·8 ± 0·9	3·0 ± 0·4
G-6-P	101·1 ± 8·3	75·9 ± 5·6
F-6-P	106·9 ± 7·2	75·5 ± 5·6
FDP	112·5 ± 9·3	104·1 ± 5·6
glyceraldehyde-3-phosphate	120·0 ± 10·3	113·5 ± 10·2
diphosphoglycerate	78·7 ± 8·7	75·3 ± 10·3
3-phosphogylycerate	78·2 ± 3·2	75·9 ± 7·3
phosphoenolpyruvate	78·2 ± 2·7	83·6 ± 3·6
pyruvate	78·1 ± 7·9	75·3 ± 6·3

Cortical slices were exposed to DMSO, cleared of DMSO and homogenized in the medium for determination of lactate. Substrate was 10 m-moles/l. Lactate was determined enzymatically. Figures are the mean and SD of eight replicates from 16 rats.

A second method of identifying the inhibited stage of glycolysis was to compare the concentration of intermediates, after a period of incubation with glucose at 37°, in control slices and those which had been exposed to 32% DMSO for 10 min at 4°. Four intermediates were estimated after 5 min of glycolysis (Fig. 4). A "cross-over" point occurred between F-6-P and FDP. After 30 min glycolysis all four intermediates were at a third to a quarter of the control values and no "cross-over" was seen. DMSO treatment also affected ATP concentration. In control slices this fell from 0·24 m-moles/kg at 5 min to 0·17 m-moles/kg at 30 min, while treated slices contained 0·20 m-moles/kg at 5 min and 0·05 m-moles/kg at 30 min. At 5 min treated slices had unaltered ADP and inorganic phosphate. In a further experiment the amount of ATP was determined at 5-min intervals. In slices which had been exposed to 32% DMSO for 10 min at 4° ATP concentration fell linearly from an initial value of 0·20 m-moles/kg to 0·05 at 30 min. Control slices maintained the ATP concentration for 15 min at 0·24 m-moles/kg after which it fell to 0·18 at 30 min. In view of the failure of control and treated slices to maintain ATP concentration after 15 min, the amounts of intermediates were not examined further as they would probably be ATP-dependent.

A number of enzyme activities were determined in slices following exposure to

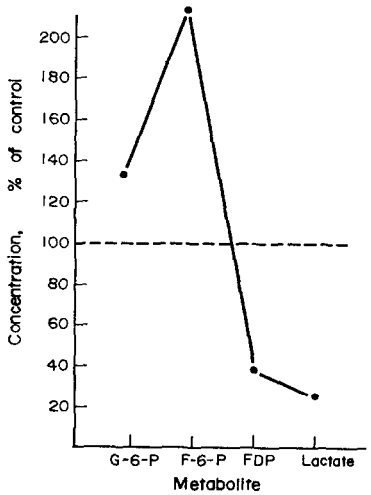

FIG. 4. Intracellular concentration of glycolytic intermediates in cortical slices during anaerobic glycolysis after exposure to 32% DMSO for 10 min at 4°. Each entry is the mean of duplicate determinations made 5 min after glycolysis began.

32% DMSO (for 10 min at 4°), and removal of the DMSO. Two enzymes, phosphoglucoseisomerase and fructosediphosphatase were elevated by 25 and 57 per cent, respectively, while the following were within 11 per cent of control values: hexokinase, glucose-6-phosphatase, phosphofructokinase, aldolase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase and lactic dehydrogenase.

Fructosediphosphatase. The possibility that the lasting effect of DMSO on glycolysis might be due to activation of FDPase led to an examination of the activity of rat kidney cortex. There was marked activity between pH 7·4 and 9·6 in contrast to rat liver homogenate which has a pH optimum at 7·4.[14] The sap accounted for 86 per cent of the activity[13] of the whole homogenate. Gel filtration of normal sap gave two peaks of FDPase activity[13] corresponding to molecular weights of 130,000 and 73,000. The sap from kidney slices which had been treated with DMSO, showed enhanced activity in the region of 73,000. The plot of activity with substrate concentration was sigmoid and neither enzyme fraction showed Michaelis–Menten kinetics. The enzyme of lower molecular weight showed much greater increase in activity[14] at FDP concentrations greater than 0·1 m-moles/l. The enzyme of higher molecular weight was markedly inhibited[14] by AMP: 49 per cent by 0·1 m-moles/l. and 73 per cent by 1 m-mole/l. The corresponding inhibitions for the smaller enzyme were 8 and 27 per cent. As DMSO-treatment enhanced the activity of the second peak and as the activity of this peak was little inhibited by AMP, the effect of DMSO treatment on sensitivity to AMP was determined. Sap was prepared from treated and control slices obtained from three rats. The FDPase activity[13] was determined with and without 0·4 m-moles/AMP. Although the absolute activity varied between animals, the DMSO-treatment reduced AMP inhibition of FDPase by about 35 per cent, in each experiment.

The activity of FDPase could also be enhanced by DMSO *in vitro*. As a preliminary the inhibition of the enzyme was determined in the presence of DMSO. This increased

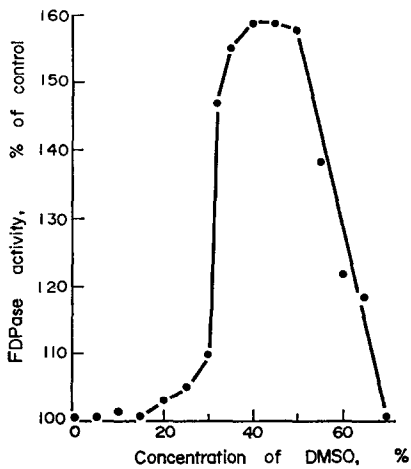

FIG. 5. Activation of FDPase of kidney sap by exposure to DMSO at 4° for 10 min. Before estimation of FDPase activity the sap was diluted to bring the DMSO concentration to 2%. Each entry is the mean of 12 determinations.

from 15 per cent at 5% DMSO to 97 per cent with 33% DMSO. To determine the residual effects sap was prepared from normal kidneys and increasing amounts of DMSO were added, at 4°. After 10 min the mixtures were diluted to 1–2% DMSO, for estimation of FDPase activity. Marked activation was produced by exposure to concentrations above 30% DMSO, but this fell away above 50% DMSO (Fig. 5).

The addition of DMSO to sap produced turbidity and the enzyme could be spun off. In a typical experiment varying amounts of DMSO were added, at 4°, to 10% kidney sap which was spun at 700 $g_{av.}$ after 10 min standing. The FDPase activity of the supernatant, after dilution, was determined by the phosphate-release method.[13] Although there was no loss of activity from the supernatant up to 30% DMSO, above this the activity in the supernatants fell to 50 per cent at 40% DMSO and 3 per cent at 50% DMSO. Thus the aggregation of the enzyme occurred over the range of DMSO concentration which activated the enzyme. The relation between turbidity and activation was examined with a number of solvents. These were added to 5% sap to a concentration of 30% for 10 min at 4°, following which the solvent was diluted to 1 per cent and the FDPase activity was assayed. Only DMSO and dimethyl formamide produced turbidity and activation. Methanol, ethanol, acetone and dimethyl acetamide produced turbidity without change in activity. Ethylene and propylene glycols and glycerol affected neither. Sulpholane inhibited but produced no turbidity.

As DMSO inhibited FDPase by its presence and activated it when removed, the speed with which the latter took place was examined. Activation was not instantaneous. Thirty sec after dilution the activity was still 50 per cent inhibited and control activity was reached after 5 min. Full development of activity had occurred 10 min after dilution of kidney sap containing 32% DMSO to 1% DMSO at 4°, in the absence of AMP. If assayed in 0·1 m-moles/l. AMP about half the enhancement took place in the first 10 min, there was little change from 10 to 30 min, followed by a rise between 30 and 50 min when the experiment was stopped.

As FDPase is regarded as one of the control steps in gluconeogenesis glucose synthesis was measured. Slices (control and DMSO-treated) were incubated at 37° in Krebs–Henseleit[6] solution containing 20 m-moles/l. of substrate. After 1 hr the glucose in the medium was estimated. In each instance gluconeogenesis was greatly reduced by pre-treatment with DMSO: 63 per cent from glycerol, 82 per cent from pyruvate and 53 per cent from L-glutamate.

Prevention of enzyme activation. The effects of adding amides at the same time as DMSO was examined. Kidney sap was made to 4·6 moles/l. DMSO, with varying concentrations of amide from 0·28 to 4·6 moles/l. added simultaneously. The mixture stood for 10 min at 4°. The sap was then diluted 30 times and FDPase activity estimated.[14] Urea (1·15 moles/l.), sulphamide (1·15 moles/l.), formamide (2·3 moles/l.), glycinamide (3·5 moles/l.) and acetamide (4·6 moles/l.) prevented enzyme activation by 4·6 moles/l. DMSO. The most active compound tested was lysine which was effective at 0·46 moles/l. Thiourea (4·6 moles/l.) and thioacetamide (4·6 moles/l.) had no effect.

The effect of shielding the lysine residues by treatment with pyridoxal phosphate and sodium borohydride was examined. This was known[15] to activate pig liver enzyme and to remove the AMP inhibition. We found that DMSO would not further activate the enzyme which had been treated in this way. In addition, if sap was treated with DMSO at pH 10·65, the pK of the ϵ-amino group of lysine, activation did not occur.

Protection of glycolysis. An experiment was carried out to determine whether urea would also protect kidney slices against the lasting inhibitory effect of DMSO on glycolysis. In order to do this, aerobically and anaerobically, glycolysis was measured by glucose consumption rather than acid production. Exposure to DMSO greatly lowered glucose consumption anaerobically, and slightly increased it aerobically (Table 4). Both effects were abolished by adding urea with the DMSO. Similarly DMSO abolished the urea-stimulated aerobic glucose consumption and anaerobic inhibition. It was also noted that after incubation the control medium was opalescent,

TABLE 4. GLUCOSE CONSUMPTION BY CORTICAL SLICES AFTER EXPOSURE TO 32% DMSO IN THE PRESENCE AND ABSENCE OF UREA

Addition during pretreatment	Glucose consumption (μmoles/hr/g dry cortex)	
	Aerobic	Anaerobic
None	47·7 ± 3·6	64·6 ± 5·6
DMSO	52·7 ± 7·2	17·9 ± 3·3
Urea	71·4 ± 10·0	40·6 ± 6·9
DMSO + Urea	49·7 ± 4·6	62·4 ± 7·9

Cortical slices were exposed to DMSO or urea or both at 4·6 m-moles/l. for 10 min at 4°. They were then washed and transferred to Robinson's medium containing 2 m-moles/l. glucose. Aerobic conditions were 100% O_2, anaerobic were 100% N_2 and 1 m-mole/l. azide in the medium. Glucose was eliminated enzymatically. Results are expressed as means ± S.D. of replicates from 10 rats.

that containing DMSO was slightly turbid, while urea produced an extremely turbid medium. In contrast the medium containing DMSO and urea was transparent. Thus urea and DMSO suppress the deleterious effects of each other.

DISCUSSION

These studies emphasize the relative non-toxicity of DMSO. We have confirmed[16–20] that many biochemical systems are altered when DMSO is present in high concentrations, for example, oxygen consumption, glycolysis and FDPase. Most processes studied have been inhibited but glycolysis of homogenates was stimulated in the presence of DMSO (Table 2). As DMSO inhibited glycolysis of slices it may have interfered with glucose transport into cells.

After DMSO was removed some of the processes were restored, especially if the exposure had been at 4°. The respiration of kidney slices (Fig. 1), and mitochondrial Q_{O_2}, P/O ratio and the respiratory control index were normal. It may be important, however, that slices respire at about half the rate of the perfused kidney.[21]

The Q_{O_2} of homogenates prepared from slices exposed to DMSO remained seriously affected, and the defect appeared to be in the nuclei (Table 1). As the ATPase of treated homogenates (Fig. 2) was low and nuclei were found to have 68 per cent of the ATPase activity of homogenates of untreated slices, the rate limiting factor may have been the generation of inorganic phosphate or of ADP. Wheeler and Whittam[22] have studied the nuclear ATPase of kidney. The maintenance of respiration in treated slices suggests that the defect observed in homogenates may not be a major one in intact cells. It may be exaggerated by the relatively low oxygen consumption of homogenates (about 20 per cent) compared with slices. Possibly there are other causes of the reduced respiration, for at 20° ATPase activity was little affected (Fig. 2).

Unlike respiration glycolysis of slices (Fig. 3) was inhibited even after exposure to DMSO at 4°. Since inhibition of glycolysis was equally marked in homogenates and slices the rate limiting factor could not have been entry of substrate into cells. We have studied cytoplasmic (non-nuclear) glycolysis in some detail because of the difficulties associated with nuclear studies, although glycolysis is known to be a function of nuclei.[23–25]

The main effect on cytoplasmic anaerobic glycolysis can be explained by increased FDPase which would be expected to reduce the flow of metabolites through FDP to triose. This was supported by three lines of evidence—rates of glycolysis with different substrates (Table 3), determination of enzymes after DMSO treatment, and determination of the amounts of glycolytic intermediates in slices (Fig. 4). The activation of FDPase could be produced by treating partly purified enzyme with DMSO (Fig. 5) and activation was associated with loss of one of the main allosteric properties of FDPase—inhibition by AMP. There have been few studies of kidney FDPase. Mendicino *et al.*[13] purified pig kidney FDPase and estimated the molecular weight as 129,500. They found a high proportion of basic amino acids. Mendicino and Vasarhely[26] noted the inhibition by AMP and the pH optimum of 9·2, which is different from that of the rat liver enzyme.[14,27] Enser *et al.*[28] have shown an immunological similarity between liver and kidney enzymes but the muscle enzyme did not cross-react. The gel filtration experiments and the study of AMP inhibition suggest that DMSO activation is associated with an increase of protein which has about half the size of the previously reported enzyme. The smaller form is less inhibited by AMP. As

our experiments did not show a large drop in the fraction of 130,000 mol. wt. one cannot conclude that the larger form is a dimer which dissociates under the influence of DMSO, though the evidence is suggestive. Another novel feature was that DMSO inhibited FDPase by its presence and the activation occurred on DMSO removal, taking about 10 min to reach maximum.

The explanation that glycolysis was inhibited by FDPase activation is strengthened by the demonstration that both phenomena were prevented by adding urea at the same time as DMSO. Two reasons led us to determine whether urea would protect the enzyme against activation by DMSO. Firstly, the association between DMSO-treatment and aggregation (turbidity) suggested, on an empirical basis, that a protein solubilizing agent might have an effect. Secondly, on theoretical grounds there was the possibility that the action of DMSO was mediated by DMSO hydrogen bonding with polar side chains, or the peptide links of the protein. Urea, with four hydrogens available for bonding with the oxygen of DMSO, seemed a suitable agent to test. Its effectiveness led us to try other amides. The comparatively greater effectiveness of lysine could be due to a competition between it and the ϵ-amino groups of the enzyme for DMSO. Fernando and Pontremoli[29] have shown that kidney FDPase contains a high proportion of lysine residues which account for most of the free amino groups. The effect of blocking ϵ-amino groups with pyridoxal phosphate and of exposing the enzyme to DMSO at pH 10·65 also suggests that lysine residues are involved in reaction with DMSO.

There may be other major metabolic results of exposure to DMSO. This is suggested by the reduced gluconeogenesis from glycerol, pyruvate and l-glutamate, which could not be explained by activation of FDPase. Possibly glycerokinase or pyruvate carboxylase are altered. Phosphorylation of glucose (Table 3) may be reduced but since hexokinase is intact the effect may be secondary to a low ATP resulting from the "futile" ATPase cycle of FDPase and fructokinase.

The immediate implication of these studies, for transplantation, is that attempts should be made to preserve organs with DMSO and an amide or lysine. Acetamide has been shown to be a cryo-protective agent itself[30] and it would have the advantage that residual amounts could be metabolized.

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