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A COMPARISON OF SOME EFFECTS OF DIMETHYL SULPHOXIDE AND DIMETHYL SULPHONE ON RAT LIVER MICROSOMAL ENZYMES*

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Abstract—Dimethyl sulphone, while inactive as a hepatic microsomal enzyme stimulator when injected into rats, caused a significant increase in the enzymic formation of para-aminophenol when added *in vitro* to incubation systems containing microsomes plus cofactors plus aniline. This response, which was associated with changes in K_m and V_{max} for aniline metabolism *in vitro*, was similar to that obtained after treating rats with dimethyl sulphoxide (DMSO). However at least 10^6 times as much dimethyl sulphone had to be added *in vitro* to give a comparable response to that found after injection of effective doses of ^{14}C labeled DMSO. The DMSO treatment of rats was found to change the pH optimum of hepatic microsomal aniline hydroxylase from 7·1 to 6·7, and to increase the microsomal NADPH-cytochrome P-450 reductase activity. Neither of these results were found after *in vitro* addition of dimethyl sulphone to hepatic microsomes. The apparent additive effect on microsomal aniline para-hydroxylase of DMSO pretreatment of rats and dimethyl sulphone addition *in vitro* to microsomes also suggests that these two chemicals stimulate this enzyme by different mechanisms.

PREVIOUS work[1–3] has demonstrated that administration of dimethyl sulphoxide (DMSO) to rats caused a rapid and marked increase in the hydroxylation *in vitro* of monocyclic aromatic ring systems by rat hepatic microsomes. This stimulation of microsomal enzyme activity, although not shown by adding DMSO *in vitro* to microsomal incubation mixtures, occurred without any measurable increase in microsomal cytochrome P-450 levels[1] and was not prevented by treating the rats with actinomycin D or cycloheximide.[2] These results suggested that this effect of DMSO treatment was not a true enzyme induction, but that some modification of existing hepatic microsomal enzymes was involved.

However, the *in vivo* nature of this stimulation, together with the demonstration by Hucker *et al.*[4] that DMSO was partially metabolized to dimethyl sulphone when administered to rats, suggested that dimethyl sulphone or some similar metabolic product of DMSO might be involved in the observed stimulation of hepatic microsomal enzymes.

The following report, therefore, considers certain metabolic, kinetic, spectral and

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pH characteristics of the enzyme response obtained after *in vivo* administration of DMSO to rats and compares these with changes after the *in vitro* addition of dimethyl sulphone to rat liver microsomes.

METHODS AND MATERIALS

Animals. Male Sprague–Dawley rats weighing 170–190 g were used for all experiments and were allowed free access to Wayne Lab Blox and water up to the time of sacrifice. All animals were housed in suspended stainless steel cages under conditions of controlled temperature and lighting.

Animal pretreatment. Undiluted DMSO, at dosages of 2 ml/kg and dimethyl sulphone, as a 25 per cent aqueous solution, in the dosages indicated in the Results section, were administered i.p. twice a day for 3 days with the last dose 12 hr prior to sacrifice. Dimethyl sulphone was prepared from DMSO by the method of Douglas[5] and twice recrystallized from water before use.

Tissue preparation. All animals were killed by cervical dislocation between the hours of 5 to 6 a.m. to minimize the effects of variation in hepatic microsomal metabolism caused by circadian rhythm.[6] The livers were removed and immediately placed in 1·15 per cent KCl solution containing 0·005 M potassium phosphate, pH 7·5 (buffered KCl solution). All subsequent procedures were performed at 4°. The livers were weighed, minced with scissors and rinsed with buffered KCl solution.

The liver-mince was then homogenized with two volumes of buffered KCl solution in a motor-driven Potter homogenizer using a Teflon^{R} pestle. All treatment and control groups were homogenized under identical conditions with the same homogenizer. Each homogenate was then centrifuged at 9000 *g* for 20 min and the resultant post-mitochondrial supernatant aspirated with a syringe, taking care to avoid both the pellet at the bottom and the fatty layer at the top of the centrifuge tube. Microsomal fractions were prepared by centrifuging the 9000 *g* supernatants at approximately 100,000 *g* for 1 hr in a Beckman L2-65B preparative ultracentrifuge. The resultant pellet was resuspended in buffered KCl solution and then resedimented. These washed microsomes were finally resuspended in buffered KCl solution and assayed by the method of Lowry *et al.*[7] using bovine serum albumin as a standard.

Drug metabolism and cytochrome b_5 *assays.* Microsomal suspensions from either treated or control groups were adjusted to 5 mg protein/ml and 0·5 ml of this suspension was added to 0·25 ml of the 100,000 *g* supernatant (soluble fraction) from control animals, 12·5 μmoles of glucose-6-phosphate, 12·5 μmoles of $MgSO_4$, 2·5 μmoles of NADP and 250 μmoles of Hepes*/NaOH (pH 7·0) together with 10 μmoles of aniline.HCl or 5 μmoles of para-nitroanisole in a total volume of 2·5 ml.

Para-hydroxylation of aniline was measured by the method of Kato and Gillette[8] as modified by Gram *et al.*,[9] and the *O*-demethylation of paranitroanisole was estimated by the method of Netter and Seidel.[10]

Incubations were carried out under oxygen at 37° for either 20 or 30 min (20 min used in Table 2), in a Dubnoff metabolic shaker. A marble was added to each beaker to improve mixing of the incubating solutions.[11] Cytochrome b_5 was estimated by the method of Omura and Sato.[12] In these determinations all microsomes were resuspended in 0·1 M Hepes (pH 7·0) and the spectra recorded at room temperature

* Hepes is a buffer with the chemical name: *N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulphonic acid.

on a Shimadzu Model MPS-50L recording spectrophotometer. A molar extinction coefficient of 185,000 was used for calculation of microsomal cytochrome b_5 content.

NADPH oxidase, NADPH cytochrome c *reductase, and NADPH cytochrome P-450 reductase assays.* These assays were performed in a Gilford 2000 spectrophotometer using 10 × 10 mm silica cuvettes containing 3 ml of reaction mixture. In all cases the initial linear phase was used to calculate the rate of each reaction.

NADPH oxidase activity was determined by the method of Gillette *et al.*[13] A microsomal protein concentration of 1·67 mg/ml was used and after the addition of 0·21 mM NADPH, the decrease in the extinction at 340 nm was measured at 20°. A molar extinction coefficient of 6220 was used in these calculations.

NADPH cytochrome *c* reductase was determined by a modification of the method described by Phillips and Langdon.[14] The assay system contained 0·1 mg microsomal protein/ml; 0·1 mM NADPH; 0·3 mM KCN; 0·05 mM cytochrome *c* (Sigma Chemical Co., Type VI) and 0·1 M Hepes buffer, pH 7·0, at 20°. Reduction of cytochrome *c* was followed at 550 nm. A molar extinction coefficient of 18,500 was used for calculations.

NADPH cytochrome P-450 reductase activity was determined as described by Holtzman *et al.*[15] except that: (1) 0·12 M Hepes buffer, pH 7·0 was bubbled with dithionite-scrubbed nitrogen at 37° for 60 min. (2) Microsomal suspensions were resuspended in buffered KCl to a protein concentration of 18 mg/ml and bubbled with nitrogen at 37° for 5 min. 0·5 ml of this suspension was then diluted up to 3 ml with the nitrogen-gassed Hepes buffer, and the resultant suspension bubbled with CO at 20° for 10 min before transferring to an Aminco anaerobic spectrophotometric cuvette. Final protein concentration was 3 mg/ml. (3) 0·4 mM NADPH was added instead of the NADPH generating system.

A molar extinction coefficient of 91,000[12] was used to calculate the number of nmoles of cytochrome P-450 reduced/min/mg of microsomal protein.

Measurement of substrate-induced microsomal difference spectra. The type II difference spectra were obtained by the consecutive addition of aniline to microsomes (3·0 mg protein/ml or 1·5 mg protein/ml) in 0·1 M Hepes buffer of pH 6·7 or 7·1. With the addition of substrate to the sample cuvette, a corresponding volume of water was added to the reference cuvette. The total volume added to any one cuvette was 0·06 ml.

The difference in extinction between the trough at 395 nm and the peak at 430 nm was designated ΔE. The difference spectra were determined at room temperature using a Shimadzu MPS-50L spectrophotometer.

Estimation of microsomal-bound DMSO and/or its metabolites. Rats (190 g) were injected twice a day with 2μc of [^{14}C]DMSO, specific activity 5 μc/ml (New England Nuclear Corporation, Boston, Mass.). This dose of 2 ml DMSO/kg was given twice a day for 3 days with the last dose 12 hr prior to sacrifice.

Hepatic microsomes prepared in the usual manner were then resuspended to a protein concentration of 10 mg/ml, and 0·5 ml and 1 ml-aliquots were suspended in 15 ml Dioxan Scintillation liquid containing 100 g of naphthalene, 7 g of 2,5-diphenyloxazole (PPO), 0·3 g of 1,4-bis-2-(4-methyl-5-phenyloxazoyl)-benzene (dimethyl-POPOP), and 30 g of Carbosil per litre. Radioactivity was determined in a Packard TriCarb Model 3310 Liquid Scintillation Spectrophotometer, with quenching corrected by utilizing an automatic external standard.

Statistical analysis of the difference between control and treated groups was determined by Students *t*-test.[16]

RESULTS

Responses to dimethyl sulphone. Microsomes isolated from the livers of rats injected with aqueous solutions of dimethyl sulphone at dosages of 2·5 g/kg at 6, 12 and 24 hr before sacrifice, or at 1 g/kg twice a day for 3 days prior to sacrifice, failed to show any evidence of altered (increased or decreased) aniline or *p*-nitroanisole metabolism. However, the addition of 0·5 M dimethyl sulphone to incubating systems containing aniline, cofactors, and hepatic microsomes from control rats showed significant elevation of para-aminophenol formation as compared with no addition of dimethyl sulphone.

TABLE 1. MICROSOMAL ANILINE AND PARA-NITROANISOLE METABOLISM AFTER DMSO PRETREATMENT OF RATS *v.* THE ADDITION *in vitro* OF DIMETHYL SULPHONE TO HEPATIC MICROSOMES FROM UNTREATED RATS

Treatment group	Aniline metabolism	para-Nitroanisole metabolism
Control	40·8 ± 2·6	42·1 ± 1·8
DMSO-treated*	107·7 ± 4·5‡	73·4 ± 2·7‡
Dimethyl sulphone† added *in vitro* to controls	105·2 ± 5·8‡	26·5 ± 1·5‡

* Treated (i.p.) with 2 ml DMSO/kg twice a day for 3 days; the last dose was 12 hr before sacrifice.
† Dimethyl sulphone (final concentration was 0·5 M) was added to microsomes from untreated rats immediately prior to incubation.
All values expressed as nmoles of product formed per mg microsomal protein per 30 min. Mean ± S.E.; $n = 6$.
‡ Significantly different from appropriate controls ($P < 0·05$).

Table 1 shows the magnitude of this response to addition *in vitro* of dimethyl sulphone as compared with enzyme activity in microsomes from rats treated with 2 ml DMSO/kg twice a day for 3 days. The response of aniline hydroxylase to dimethyl sulphone, which was measurable at *in vitro* concentrations of dimethyl sulphone above 0·05 M, was essentially linear to 0·35 M; the maximal practical amount of the sulphone that could normally be added to the incubating system was about 0·5 M. Increasing rates of para-aminophenol formation (increases were not linear above 0·5 M) were recorded up to the highest concentrations (0·625 M) of dimethyl sulphone studied. The stimulation by dimethyl sulphone was shown to be enzymic in character as substitution of boiled microsomes, or the deletion of cofactors or soluble fraction, abolished para-aminophenol formation.

DMSO treatment of rats has been shown to stimulate the *in vitro* hepatic microsomal metabolism of para-nitroanisole to para-nitrophenol.[2] However, as is shown in Table 1, the addition of 0·5 M dimethyl sulphone to hepatic microsomes from untreated, control rats resulted in a decrease in para-nitrophenol formation.

pH optimum for aniline para-hydroxylase. In view of the recent demonstration[17] that benzpyrene treatment of rats caused a shift in the pH of optimal activity *in vitro* for hepatic microsomal aniline para-hydroxylase, the influence of DMSO treatment of rats on this enzyme was considered. Figure 1 shows the effect of pH on *in vitro* hepatic microsomal aniline para-hydroxylase from both control and DMSO-treated animals. The pH optimum for aniline metabolism by hepatic microsomes from DMSO-treated rats was 6·7 as compared with the pH optimum of 7·1 for microsomes from control rats.

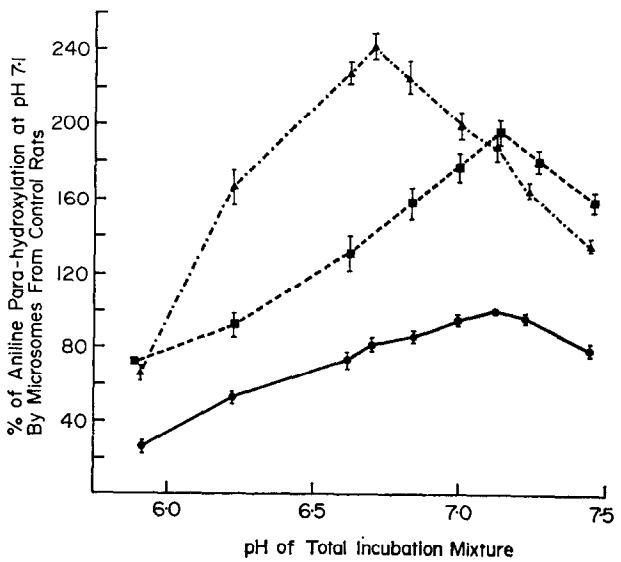

FIG. 1. pH optima for aniline *p*-hydroxylation by hepatic microsomes from control rats (data points connected by solid line); by microsomes from control rats to which 0·5 M dimethyl sulphone was added *in vitro* (data points connected by dashed line); and by microsomes from DMSO-pretreated rats (data points connected by dot-dashed line). Incubation conditions are given in Methods. Data are normalized so that each point is expressed as a percentage of the rate of *p*-hydroxylation of aniline obtained with microsomes from control rats at pH 7·1. This rate was 39·2 ± 2·6 nmoles *p*-aminophenol formed per mg microsomal protein per 30 min (mean ± S.E.). Each point is the mean of at least five experiments, except for control microsomes with 0·5 M added dimethyl sulphone which was the mean of three experiments. The average of the pH measured in the incubation mixture is given as a point without indication of variability (for the sake of clarity). However, no final pH varied from the point on the figure by more than 0·1 pH unit from one experiment to another.

To help establish if the response obtained with DMSO pretreatment of rats was the same as that found after the addition *in vitro* of dimethyl sulphone to the incubating solution, the effect of pH was also examined on aniline para-hydroxylase in the presence and absence of added 0·5 M dimethyl sulphone. This addition of dimethyl sulphone did not shift the pH optimum value from that of the control series.

Kinetic and spectral characteristics of the aniline-microsome interactions. Because DMSO treatment of rats caused a shift in pH optimum, the K_m, V_{max}, spectral dissociation constant (K_s) and ΔE_{max} values, were measured (with aniline as substrate) at both pH 6·7 and 7·1 using microsomes from both DMSO-stimulated and control rats.

The results (Table 2) show a 3-fold increase in V_{max}, together with an approximately 2-fold increase in K_m values for aniline hydroxylase in microsomes from DMSO-treated as compared with control rats. The differences were significant at both pH values. However, the K_s and ΔE_{max} values for the aniline–microsome difference spectrum with hepatic microsomes from DMSO-treated rats were not significantly different from the K_s and ΔE_{max} using microsomes isolated from untreated control rats.

TABLE 2. THE EFFECT OF pH, AND DMSO PRETREATMENT OF RATS, OR DIMETHYL SULPHONE ADDITION *in vitro* ON THE APPARENT K_m AND V_{max} OF ANILINE *p*-HYDROXYLATION, OR ON THE K_s AND ΔE_{max} RESULTING FROM THE ADDITION OF ANILINE TO RAT HEPATIC MICROSOMES

Treatment group	pH	K_m*	V_{max}*	K_s*	ΔE_{max}*
Control	7·1	0·063 ± 0·003	1·6 ± 0·1	1·1 ± 0·1	21·2 ± 1·5
Control	6·7	0·065 ± 0·003	1·3 ± 0·1	1·0 ± 0·1	20·3 ± 1·6
DMSO-treated†	7·1	0·125 ± 0·009	4·8 ± 0·2§	1·1 ± 0·2	20·3 ± 1·4
DMSO-treated†	6·7	0·133 ± 0·010	5·1 ± 0·2§	1·1 ± 0·1	22·1 ± 1·3
Dimethyl sulphone added *in vitro* to controls‡	7·1	0·144 ± 0·010	4·3 ± 0·3§	See text	See text

* Incubations were performed with 1 mg microsomal protein per ml for 20 min; aniline concentration in the incubation mixture was 0·02 to 2 mM.

K_m and K_s are expressed as mM; mean ± S.E., $n = 4$.

V_{max} expressed as nmoles *p*-aminophenol formed per mg microsomal protein per minute; mean ± S.E., $n = 4$.

ΔE_{max} expressed as $E_{395-435}$ ($\times 10^3$) per mg microsomal protein; mean ± S.E., $n = 4$.

† Rats received (i.p.) 2 ml DMSO/kg, twice a day for 3 days; the last dose was 12 hr before sacrifice.

‡ Dimethyl sulphone (final concentration = 0·5 M) was added to microsomes from untreated rats immediately before incubation.

§ Significantly different from appropriate controls, $P < 0·05$.

Table 2 also shows the effects of added dimethyl sulphone on the kinetics of aniline hydroxylation; the K_m and V_{max} were increased at least 2-fold. Experiments to measure the effects of dimethyl sulphone on aniline–microsome spectral interactions were performed, but a different strain of rats (Charles River, male 200 g) and different equipment had to be used. These changes resulted in K_s and ΔE_{max} values which were slightly different from those listed in Table 2. The results were, however, consistent and indicated that dimethyl sulphone had no effect on either K_s or ΔE_{max} (control microsomes gave a $K_s = 1·3$ and 1·2 mM and $\Delta E_{max} = 29·4$ and 30 O.D. units $\times 10^{-3}$ while in the presence of either 0·25 or 0·5 M dimethyl sulphone $K_s = 1·2$ and 1·1 mM and $\Delta E_{max} = 33·3$ and 31 O.D. units $\times 10^{-3}$; values for two separate experiments).

Effect of DMSO treatment on electron transport systems in rat hepatic microsomes. Even though DMSO treatment of rats has been shown not to change the levels of cytochrome P-450 in hepatic microsomes.[1,2] the DMSO-caused increases in metabolism of some substrates might influence some enzyme activities of the microsomal electron transport system.

This was not the case with either NADPH oxidase or NADPH-cytochrome *c* reductase (Table 3). However, Gigon *et al.*[18] have proposed that the rate-limiting step in several hepatic microsomal oxidations is NADPH-cytochrome P-450 reductase,

TABLE 3. THE ACTIVITY OF NADPH OXIDASE, NADPH-CYTOCHROME *c* REDUCTASE, AND AMOUNT OF CYTOCHROME b_5 IN HEPATIC MICROSOMES ISOLATED FROM RATS TREATED WITH DMSO

Treated group	NADPH oxidase*	Cytochrome *c*† reductase	Cytochrome b_5‡
Control	19·7 ± 0·4	47·4 ± 2·1	0·50 ± 0·04
DMSO-treated§	21·0 ± 0·3	49·8 ± 0·8	0·51 ± 0·03

* Values expressed as nmoles NADPH oxidized per minute per mg microsomal protein.
† Values expressed as nmoles cytochrome *c* reduced per minute per mg microsomal protein.
‡ Values expressed as nmoles of cytochrome b_5 per mg of microsomal protein.
All values are mean ± S.E., $n = 4$.
§ Rats received (i.p.) 2 ml DMSO/kg, twice a day for 3 days; the last dose was 12 hr before sacrifice.

and as shown (Table 4) a significant increase in the activity of this enzyme was found in microsomes from DMSO-treated rats. Again to establish if a relationship exists between the DMSO (*in vivo*) and dimethyl sulphone (*in vitro*) responses, 0·25 M or 0·5 M dimethyl sulphone was added to control microsomal preparations and P-450-reductase assays were made. The results (Table 4) showed that 0·25 M dimethyl sulphone produced a significant fall in reductase activity. With 0·5 M dimethyl sulphone, the inhibition of P-450-reductase was so marked that linearity of initial reaction rate was lost and results could not be calculated (data not given).

The possibility was considered that another type of cytochrome P-450 was being formed as a result of DMSO treatment of rats, such as has been proposed by Sladek and Mannering [19, 20] to occur after 3-methylcholanthrene treatment of rats. However, pH dependence of the ethylisocyanide difference spectra of reduced microsomes was examined by the method of Sladek and Mannering[21] and showed "cross over" values of pH 7·7 with microsomes from either DMSO-treated or control rats.

Measurement of hepatic microsomal-bound DMSO and/or its metabolites. The concentration of dimethyl sulphone required to be added to the incubating aniline para-hydroxylase microsomal system (0·05–0·5 M) to produce measurable stimulation of this pathway, was well in excess of that which would be expected to be associated

TABLE 4. NADPH-CYTOCHROME P-450 REDUCTASE ACTIVITY IN HEPATIC MICROSOMES FROM CONTROL RATS IN THE PRESENCE OR ABSENCE OF ADDED DIMETHYL SULPHONE *v.* NADPH-CYTOCHROME P-450 REDUCTASE ACTIVITY IN HEPATIC MICROSOMES FROM DMSO-TREATED RATS

Control	DMSO-treated*	Dimethyl sulphone† added *in vitro*
5·17 ± 0·35	8·28 ± 0·50‡	3·3 ± 0·40§

* Rats received (i.p.) 2 ml DMSO/kg, twice a day for 3 days; the last dose was 12 hr prior to sacrifice.
† Dimethyl sulphone (final concentration was 0·25 M) was added to microsomes from untreated rats immediately before incubation.
‡ Significantly different from control $P < 0·01$.
§ Significantly different from control $P < 0·025$.
Values expressed as nmoles cytochrome P-450 reduced per minute per mg of microsomal protein, mean ± S.E., $n = 4$.

with the isolated hepatic microsomes from DMSO-treated rats. To establish the amount of dimethyl sulphone that might be associated with washed hepatic microsomes from DMSO-treated rats, four rats were injected with a dose of 2 ml [^{14}C] DMSO/kg (specific activity = 5 μc/ml) twice a day for 3 days with the last dose 12 hr before sacrifice as described in the Methods section. Background (blank) counts were obtained by preparing microsomes from rats treated with the same dose of non-radioactive DMSO. The microsomal radioactivity in [^{14}C]DMSO-treated rats indicated such a low level of material associated with the microsomes that it was not practical for us to resolve it into DMSO and metabolites. However, assuming all the radioactivity was associated with dimethyl sulphone, we calculated the levels of ^{14}C present were equivalent to $4{\cdot}2 \times 10^{-4}$ nmoles dimethyl sulphone/mg of microsomal protein (which was equivalent to $5{\cdot}7 \times 10^{-4}$ nmoles of dimethyl sulphone/nmole of cytochrome P-450). This should be compared with the 500 nmoles dimethyl sulphone/mg microsomal protein added in the 0·5 M dimethyl sulphone incubation mixture, i.e. there is a 10^6 difference in the amounts of dimethyl sulphone which must be present in the *in vitro* aniline hydroxylase systems to give a comparable enzyme activity response when comparing control rat liver microsomes plus dimethyl sulphone added *in vitro* with microsomes from DMSO-treated rats.

Dimethyl sulphone as the enzyme modifier. It has been previously shown[1] that the stimulation of microsomal aniline hydroxylase by DMSO treatment of rats, though rapid, lags about 2 hr behind contact of DMSO with the liver. As it has been demonstrated[2] that the DMSO-induced increase in microsomal activity is probably not due to new protein synthesis, it seemed possible that this delayed onset of stimulation might be due to the enzymic conversion of DMSO to dimethyl sulphone, and that this sulphone modified the existing enzyme protein, causing the observed stimulation. Once this enzymic modification was achieved, the presence of the dimethyl sulphone might not be essential, but might in some cases, as shown above, even prove to be inhibitory.

To establish the validity of such a hypothesis, two experiments were performed.

(1) As it was established that the rate of dimethyl sulphoxide stimulation of aniline hydroxylase was linear with time,[1] microsomes were preincubated with dimethyl sulphone for 30 min prior to addition to the incubation reactants. Stimulation obtained with a range of dimethyl sulphone concentrations from 0·05 to 0·5 M were then compared with enzyme activities in microsome solutions treated identically except that dimethyl sulphone was added with the incubation reactants (at zero time).

(2) 0·5 M Dimethyl sulphone was allowed to stand in contact with microsomes from untreated rats at 25° for 60 min. These microsomes were then resedimented and washed to remove residual dimethyl sulphone, and the aniline metabolizing capacity of these microsomes was compared with that of control microsomes stored similarly, but without added dimethyl sulphone.

In neither experiment was there any elevation in the rate of para-aminophenol formation associated with the dimethyl sulphone pretreatment as compared with enzyme activities found in the appropriate control series.

Additional response of aniline hydroxylase to dimethyl sulphone added to microsomes from DMSO pretreated rats. Concentrations of 0·05–0·625 M dimethyl sulphone were added to incubation mixtures containing aniline, cofactors and microsomes

from DMSO-treated and control (untreated) rats. The results are plotted (Fig. 2) as formation of para-aminophenol against dimethyl sulphone concentration. There was a progressive increase in para-aminophenol formation with increased amounts of sulphone which was parallel with microsomes from control and treated animals. Both groups showed a fall in rate of increase of enzyme activity at concentrations of dimethyl sulphone in excess of 0·35 M.

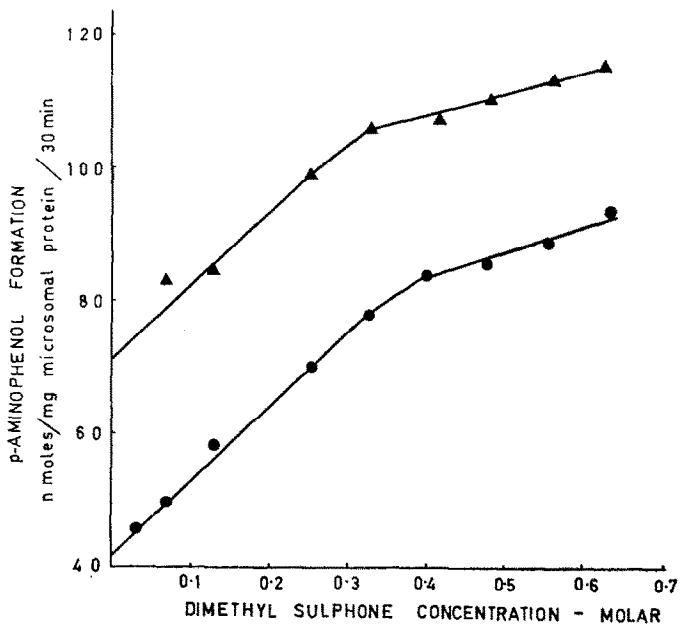

FIG. 2. Response of aniline *p*-hydroxylation in hepatic microsomes from control (●——●) and DMSO-treated rats (▲——▲) to dimethyl sulphone added *in vitro* to the incubation mixture. Incubation conditions are given in Methods. Microsomes for each experiment were from a pool of three rat livers. DMSO-treated rats were given i.p. 2 ml DMSO/kg twice a day for 3 days before sacrifice. Results are the average of two separate experiments.

DMSO pretreatment of rats with a dose of 2 ml DMSO/kg has been shown[1] to give maximal stimulation of hepatic microsomal aniline parahydroxylase activity. The results of the above "combination" experiment therefore suggested that stimulation of aniline hydroxylase by dimethyl sulphone added *in vitro* may be caused by a different mechanism than that stimulation seen after DMSO treatment of the animals. Additive enzyme activity responses should not be obtained if the mechanism of stimulation by two compounds is the same and a maximum effect has been produced by one of the stimulators.

Comparison of acetone and dimethyl sulphone stimulation of aniline hydroxylase. Dimethyl sulphone stimulation *in vitro* of aniline para-hydroxylation appeared to be similar to that described by Anders[22] for acetone. To examine this possibility, acetone and dimethyl sulphone in concentrations of 0·25 and 0·5 M were added alone or in various combinations to incubation mixtures containing aniline plus cofactors plus microsomes from control rats.

TABLE 5. EFFECT OF DIMETHYL SULPHONE AND ACETONE ADDED *in vitro* ON MICROSOMAL ANILINE HYDROXYLATION

Dimethyl sulphone concentration (M)	Acetone concentration (M)	Enhancement* (%)
0·25	—	70 ± 10
0·5	—	112 ± 18
—	0·25	127 ± 15
—	0·5	214 ± 15
0·5	0·5	236 ± 31
0·5	0·25	220 ± 25
0·25	0·5	263 ± 33
0·25	0·25	179 ± 23

* Control rate of aniline para-hydroxylation was 39·2 nmoles/mg microsomal prot./30 min at a substrate concentration of 4 mM aniline.HCl.
Values shown are mean ± S.E., $n = 3$.

The results (Table 5) demonstrated stimulation by both substances, There was an additive response of enzyme activity at the lower concentrations of the two stimulators present together, but when 0·5 M of both acetone and dimethyl sulphone were added, lower enzyme activities than expected were seen. This suggested that at the higher concentrations inhibition was occurring, perhaps due to competition between acetone and dimethyl sulphone for the same activation mechanisms.

DISCUSSION

The proposition that more than one form of aniline hydroxylase exists in hepatic microsomes has been made by Wada *et al.*[23] on the basis of prednisolone inhibition studies; by Anders[22] on the basis of results obtained with acetone added *in vitro* to microsomes; and by Rickert and Fouts[17] as a result of changes in the kinetics and pH optima of the aniline hydroxylase system isolated from rats pretreated with benzpyrene. It would appear that enzyme responses to prednisolone or acetone are due to effects on existing enzyme proteins rather than the synthesis of a new protein. A modification of an existing protein has also been proposed[1,2] for the DMSO-mediated stimulation of hepatic microsomal aniline hydroxylase since response to DMSO could not be blocked by animal treatment with Actinomycin D or cycloheximide.[2] However, the inability of DMSO to stimulate enzymic activity when DMSO was added *in vitro* prevented the interpretation that DMSO and acetone act in the same way. The *in vivo* nature of this microsomal response to DMSO could indicate that either DMSO modified some secondary mechanism which then produced the stimulation of aniline hydroxylase, or that DMSO itself was altered, and the product of this alteration was the stimulating agent.

Work by Hucker *et al.*[4] has shown that 10–20 per cent of the DMSO administered to rats is metabolized to dimethyl sulphone. The results we obtained by addition *in vitro* of this metabolic product to the microsomal aniline hydroxylase system could support the second of these two possibilities, i.e. that dimethyl sulphone was the actual stimulator of hepatic aniline hydroxylase resulting from DMSO treatment of rats.

However, while the apparent K_m and V_{max} for aniline hydroxylase showed the same general trends in both systems (control microsomes plus sulphone *v.* microsomes from DMSO-treated rats) the similarity of response applied only to the aniline hydroxylase system, since inhibition by dimethyl sulphone added *in vitro* contrasted with stimulation in microsomes from DMSO-treated rats with regard to para-nitroanisole demethylase and NADPH-cytochrome P-450 reductase. These differences in response to DMSO treatment of animals *v.* dimethyl sulphone added *in vitro* might have been explained on the basis that some structural modification of the microsomal enzyme system was effected by the dimethyl sulphone but that once this had occurred (either *in vivo* or *in vitro*) the presence of dimethyl sulphone was no longer required, and in fact in high concentration was inhibitory to some enzymes. Such a proposition could also explain the lack of correlation between the maximum amount of dimethyl sulphone that could be present in hepatic microsomes after DMSO pretreatment of rats and the 10^6 times greater amount of dimethyl sulphone which was required to be added *in vitro* to produce a comparable aniline hydroxylase response. However, the lack of stimulation of hepatic aniline hydroxylase after pretreating rats with dimethyl sulphone together with the failure to find an increase in activity after microsomal preparations had been incubated with dimethyl sulphone (and then washed free of dimethyl sulphone) prior to the addition of aniline did not support such a hypothesis.

Further, hepatic microsomes isolated from rats treated with maximally effective doses of DMSO were able to show a further progressive increase in aniline hydroxylase with increasing dimethyl sulphone concentration added *in vitro*, together with the parallel nature of this response to sulphone in microsomes from DMSO-treated as compared with control rats, leads to the conclusion that the mechanisms of enzyme stimulations by DMSO *in vivo* and dimethyl sulphone *in vitro* are not the same.

There are, however, several features of the aniline hydroxylase response to dimethyl sulphone added *in vitro* that are similar to those obtained with acetone addition *in vitro*. These are: stimulation of microsomal aniline hydroxylase associated with increases in K_m and V_{max} for aniline as substrate, and inhibition of microsomal para-nitroanisole metabolism.

The 2-fold increase in NADPH-cytochrome P-450 reductase activity found in microsomes from DMSO-treated as compared with control rats was of the same magnitude as the increase in aniline hydroxylase levels, but the previous observations[1, 2] that the metabolism of all microsomal drug substrates tested were not stimulated by DMSO treatment suggested that increase in NADPH-cytochrome P-450 reductase activity was only part of the DMSO-mediated response. While the DMSO-caused shift in pH optimum for aniline hydroxylase activity from pH 7·1 to 6·7 was small, it was quite reproducible. This pH optimum shift, together with the results of Rickert and Fouts,[17] suggests that there could be three different microsomal enzymes, or at least different sites, for hepatic microsomal aniline para-hydroxylation, two of them becoming the dominant ones after appropriate drug treatments (low pH optimum after DMSO; high pH optimum after benzpyrene). The K_m and V_{max} for aniline hydroxylase were increased as a result of DMSO stimulation which is in agreement with the changes found as a result of other drug treatments,[17, 22] but the DMSO administration apparently did not cause marked changes in the capacity (K_s) of microsomal protein to bind aniline. Nevertheless, the results support the proposal[2]

that after DMSO treatment of rats there is an enzyme with different kinetic and pH properties, and which is distinct from the enzyme normally dominant in the hepatic microsomes of untreated rats.

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