enzyme may itself be composed of two components differing in their reactivities towards 2-bromo-2-phenylacetaldehyde although the possibility of two reactive groups on the enzyme being responsible for this differential loss of activity cannot be completely ruled out. The presence of more than two monoamine oxidases in rat liver mitochondria is in agreement with the results of electrophoretic separations.[3,4]

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Stimulation *in vitro* of microsomal aniline hydroxylation by 2,2′-bipyridine

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IN A PREVIOUS report[1] it was shown that acetone markedly stimulated the microsomal hydroxylation of aniline *in vitro* when added directly to incubation mixtures. It was also shown that acetone produced significant alterations in the kinetics of aniline hydroxylation and in susceptibility to inhibition by SKF 525-A and piperonyl butoxide. Furthermore, it appeared that acetone produced its enhancing effect by a mechanism different from that of ethyl isocyanide, a compound known to stimulate the microsomal hydroxylation of aniline.[2]

In a study of the oxidation of drugs by fishes, Buhler and Rasmusson[3] observed that 2,2′-bipyridine markedly stimulated the hydroxylation of aniline but produced a slight inhibition of the *O*-dealkylation of phenacetin. Since the magnitude of the stimulation of aniline hydroxylation by 2,2′-bipyridine was similar to that produced by acetone it suggested that both compounds, though chemically unrelated, might produce their stimulatory effects on aniline hydroxylation by similar mechanisms. The results presented in this paper suggest that acetone and 2,2′-bipyridine produce their stimulation of aniline hydroxylation by a similar mechanism and, furthermore, that this stimulation is not the result of an inhibition of microsomal lipid peroxidation.

EXPERIMENTAL

Male Sprague–Dawley rats (Camm Research Institute, Inc., Wayne, N.J.), weighing 75–125 g, were employed as experimental animals. Microsomal fractions were isolated as previously described from 0·25 M sucrose homogenates by differential centrifugation.[1]

Incubation mixtures contained 150 μmoles Tris chloride buffer (pH 7·4), 15 μmoles magnesium chloride, a NADPH-generating system consisting of either 20 μmoles glucose-6-phosphate, 1 μmole NADP and 1 EU glucose-6-phosphate dehydrogenase (yeast)* or 10 μmoles DL-isocitrate, 1 μmole NADP and 1 EU isocitrate dehydrogenase (pig heart),† varying amount of substrates and activators and 0·5 ml of the microsomal enzyme preparation in total volume of 3·0 ml. In experiments in which formaldehyde was sought as the product the reaction mixture also contained 3 μmoles of neutralized semicarbizide. Incubations were carried out at 37° with shaking in an atmosphere of air. Incubation times were 30 min when aniline served as the substrate and 10 min in the case of *N*-demethylation reactions; under these conditions product formation was a linear function of time.

p-Aminophenol was determined according to the method of Kato and Gillette,[4] formaldehyde was determined by the method of Nash[5] and protein was measured as described by Wolfson *et al.*[6] Lipid peroxidation was measured by the thiobarbituric acid reaction as described by Ernster and Nordenbrand.[7]

Enzyme kinetic constants were estimated as described by Cleland;[8] other statistical methods employed have been described by Steel and Torrie.[9] All calculations were performed by digital computer using Fortran programs.

RESULTS

As can be seen in Table 1, 2,2′-bipyridine produced a marked stimulation of the hydroxylation of aniline by rat liver microsomal fractions. Although the concentration of 2,2′-bipyridine producing maximal enhancement was somewhat variable, the greatest stimulation was usually observed at a

TABLE 1. EFFECT OF 2,2′-BIPYRIDINE AND CONGENERS ON MICROSOMAL ANILINE HYDROXYLASE ACTIVITY*

Compound†	% Enhancement or inhibition
2,2′-Bipyridine	279 ± 13(3)‡
1,10-Phenanthroline	102 ± 8 (3)
4,7-Diphenyl-1,10-phenanthroline	−5 ± 2 (3)
2,9-Dimethyl-4,7-diphenyl-1,10 phenanthroline (Bathocuproine)	−6 ± 2 (2)
2,9-Dimethyl-1,10-phenanthroline (Neocuproine)	−67 ± 5 (3)
5-Nitro-1, 10-phenanthroline	−80 ± 4 (2)

* Control rate of aniline hydroxylation was 0·18 mμmoles *p*-aminophenol/mg protein/min.
† Aniline concn, 1 mM; activator or inhibitor concn, 1 mM.
‡ Values are shown as mean ± standard error of the mean; the numbers in parentheses refer to the number of determinations.

concentration of 1 to 5 mM. Furthermore, Table 1 shows that 1,10-phenanthroline, an analogue of 2,2′bipyridine, also stimulated aniline hydroxylation but was somewhat less potent. Other analogues of 2,2′-bipyridine tested produced varying degrees of inhibition of aniline hydroxylation. Although all of these compounds are known to chelate either iron or copper under proper conditions (g) no obvious relationship is apparent between the ability to chelate these metals and the effect on aniline hydroxylation.

In studies with substrates other than aniline, it was found that 2,2′-bipyridine produced inhibition

* One enzyme unit (EU) will reduce 1 μmole of NADP per min at pH 7·4 at 25°.
† One enzyme unit (EU) will convert 1 μmole of isocitrate to α-ketoglutarate per min at pH 7·4 at 25°.

of the *N*-demethylation of both ethylmorphine and aminopyrine and caused a consistent stimulation of the *N*-demethylation of *N*-methylaniline and *N*,*N*-dimethylaniline.

In view of the earlier report[1] of the stimulation of aniline hydroxylation by acetone it was of interest to establish whether both acetone and 2,2′-bipyridine produced their stimulatory effect in a similar manner. It can be seen in Table 2 that the effects of acetone and 2,2′-bipyridine are not additive. A slight depression of the stimulatory effect of the acetone was observed in the presence of 2,2′-bipyridine.

TABLE 2. EFFECT OF ACETONE AND 2,2′-BIPYRIDINE ON MICROSOMAL ANILINE HYDROXYLATION

Acetone concn (M)	2,2′-Bipyridine concn (mM)	% Enhancement*
0·45	None	25 ± 30(3)†
0·90	None	190 ± 24(3)
1·35	None	97 ± 19(3)
None	5	186 ± 20(3)
0·45	5	238 ± 26(3)
0·90	5	168 ± 26(3)
1·35	5	63 ± 20(3)

* Control rate of aniline hydroxylation was 0·36 mμmoles *p*-aminophenol/mg protein/min at a substrate concentration of 1 mM.

† Values are shown as the mean ± standard error of the mean; the numbers in parentheses refer to the number of determinations.

TABLE 3. EFFECT OF ACTIVATORS AND ANTIOXIDANTS ON MICROSOMAL LIPID PEROXIDATION AND ANILINE HYDROXYLATION

Addition*	Lipid peroxidation (O.D. 535 mμ)	Aniline hydroxylation (mμmoles *p*-aminophenol/mg protein/min)
Experiment 1		
None	0·67	0·00
Aniline, 1 mM	0·14	0·70
Aniline, 1 mM + 2,2′-bipyridine, 5 mM	0·00	1·76
2,2′-Bipyridine, 5 mM	0·10	0·00
Aniline, 1 mM + acetone, 1·35 M	0·10	2·25
Acetone, 1·35 M	0·66	0·00
Experiment 2		
None	0·22	0·00
Aniline, 1 mM	0·10	0·47
Aniline, 1 mM + Mn^{++}, 0·5 mM	0·00	0·38
Mn^{++}, 0·5 mM	0·00	0·00
Aniline, 1 mM + EDTA, 1 mM	0·00	0·32
EDTA, 1 mM	0·01	0·00

* The incubation mixtures were prepared as described in the Experimental section and contained the additions shown in the table.

It was also of interest to determine the effect of 2,2′-bipyridine on the kinetics of aniline hydroxylation. In the presence of 5 mM 2,2′-bipyridine the Michaelis constant (K_m) increased from a control value of $2{\cdot}68 \pm 0{\cdot}96 \times 10^{-4}$ M to $4{\cdot}06 \pm 1{\cdot}08 \times 10^{-4}$ M ($n = 4$). This change was not significant ($P > 0{\cdot}1$). However, the same concentration of 2,2′-bipyridine effected an increase in the maximal velocity (V_{max}) of the reaction from a control value of 0·68 ± 0·09 mμmoles *p*-aminophenol/mg protein/min to 2·30 ± 0·23 mμmoles *p*-aminophenol/mg protein/min. This increase in the V_{max} was highly significant ($P < 0{\cdot}001$).

In view of the studies of Lewis *et al.*[10] on the relationship between microsomal NAPDH-coupled lipid peroxidation and aldrin epoxidation, it was of particular interest to determine the effect of acetone and 2,2′-bipyridine on lipid peroxidation. Table 3 shows the results of these experiments. It can be seen that while both aniline and 2,2′-bipyridine produce a marked decrease in microsomal lipid peroxidation, and Mn^{++} and EDTA essentially abolish it, acetone alone or in combination with aniline had no pronounced effect. Under the conditions of this experiment both acetone and 2,2′-bipyridine enhanced the hydroxylation of aniline; Mn^{++} and EDTA, on the other hand, were slightly inhibitory.

DISCUSSION

The activation *in vitro* of microsomal drug metabolizing enzymes was first reported by Imai and Sato[2] who showed that ethyl isocyanide produced both inhibition and stimulation of the microsomal hydroxylation of aniline. The inhibitory effect was shown to be due to a competition between oxygen and ethyl isocyanide for cytochrome P-450. The mechanism of the stimulation was not elucidated. More recently, Anders[1] has reported that only acetone and 2-pentanone, out of many carbonyl compounds tested, produced a marked stimulation of aniline hydroxylation. Again the mechanism of the stimulation was not determined. Buhler and Rasmusson[3] observed that the chelating agent, 2,2′-bipyridine, produced a marked enhancement of aniline hydroxylation by hepatic microsomal fractions of male rainbow trout but did no experiments to characterize the nature of this enhancement.

Structural studies showed that only two compounds, 2,2′-bipyridine and 1,10-phenanthroline, produced substantial stimulation. Although all of the compounds tested are known to chelate various metal ions under proper conditions the stimulatory action appears to be distinct from any chelating ability since closely related analogues produced only no effect or inhibition. Further evidence for a lack of relationship between stimulation and chelation is found in reported experiments in which chelating agents not structurally related to 2,2′-bipyridine, such as 8-hydroxyquinoline and EDTA, produced only minimal alterations in the rate of aniline hydroxylation.[3,11]

Previously it had been shown that acetone and ethyl isocyanide stimulate aniline hydroxylation by dissimilar mechanisms.[1] It was, therefore, of interest to determine whether acetone and 2,2′-bipyridine produced their stimulatory action by the same mechanism. To test this possibility experiments were conducted in which both acetone and 2,2′-bipyridine were included in the same reaction mixture. If similar mechanisms were involved the two agents should compete for the activating mechanism and the results would not be additive. If, on the other hand, there is no competition for the activating mechanism, additive effects would be expected. The results shown in Table 2 indicate that acetone and 2,2′-bipyridine do indeed compete for the same activating mechanism since no additive effect was obtained. Indeed, a slight blockade of the stimulation was noted when both agents were incubated together. It should be pointed out, however, that an alternative explanation is available from the data shown in Table 2. Since there is no additivity of the effects of acetone and 2,2′-bipyridine it is possible that acetone serves to block or prevent the enhancing effect of 2,2′-bipyridine rather than competing for the same mechanism. If a simple competition was involved one might expect additivity of results at low acetone concentrations depending on the relative affinity of the two agents for the enhancing mechanism. The data do not permit a conclusive choice to be made between these two interpretations. From the results obtained with ethyl isocyanide and acetone[1] it can be concluded that 2,2′-bipyridine and ethyl isocyanide produce their enhancements by different mechanisms. Further evidence for a mechanism of activation common to both acetone and 2,2′-bipyridine can be seen in the kinetic experiments since both agents tend to cause an increase in the K_m for aniline hydroxylation.

Lewis *et al.*[10] have shown that compounds inhibiting microsomal NADPH-linked lipid peroxidation produce a concomitant increase in the microsomal mixed-function epoxidation of aldrin. Such compounds include 2,2′-bipyridine, as well as Mn^{++} and Co^{++} ions, 2,6-di-*tert*-butyl-*p*-cresol (BHT), EDTA and 3,4-dihydroxyphenylalanine (DOPA). These workers concluded that microsomal epoxidation and lipid peroxidation compete for a common intermediate. These experiments pose the question of whether the stimulatory effect of acetone and 2,2′-bipyridine might be explained by an inhibition of lipid peroxidation accompanied by an increase in aniline hydroxylation. The results of experiments designed to test this hypothesis are shown in Table 3. The finding that aniline inhibits lipid peroxidation is in agreement with the observation of Gram and Fouts.[12] The inhibition of lipid peroxidation by 2,2′-bipyridine, Mn^{++} and EDTA likewise confirms the findings of Lewis *et al.*[10]

Acetone, on the other hand, was without effect on lipid peroxidation. Since Mn^{++} and EDTA inhibit lipid peroxidation but fail to stimulate aniline hydroxylation it would appear that inhibition of lipid peroxidation by 2,2′-bipyridine is probably not involved in the stimulation of microsomal aniline hydroxylation. Support for this contention is found in the results obtained with acetone where no inhibition of lipid peroxidation is observed yet stimulation of hydroxylation occurs.

The mechanism by which 2,2′-bipyridine stimulates microsomal aniline hydroxylation is unknown. As was suggested[1] for the stimulation of aniline hydroxylation by acetone, it is possible that 2,2′-bipyridine produces its effect by unmasking additional active sites or in some other manner altering the properties of aniline hydroxylase so that an increased rate is obtained. Acetone and 2,2′-bipyridine might also promote the penetration of aniline into microsomal particles by a simple solvent effect. It is difficult, however, to rationalize this type of mechanism with the activator specificity and the alteration of kinetics, in the case of acetone. It would be expected that similar compounds would exert similar solvent effects. As a possible explanation for the enhancement of aniline hydroxylation by acetone, it was suggested that the activator could exert its effect by promoting a more rapid breakdown of an enzyme–activator–substrate complex relative to that of an enzyme–substrate complex.[1] A similar proposal has been made by Imai and Sato.[13] 2,2′-Bipyridine might exert its effects in a related manner.

It is perhaps significant that ethyl isocyanide, acetone and 2,2′-bipyridine all stimulate the metabolism of aniline which has been shown to be a Type II substrate.[14] Furthermore, Type II substrates produce an inhibition of cytochrome P-450 reduction by NADPH.[15] It is, therefore, possible to speculate that the mechanism of activation is related to P-450 reduction, the stimulation being the result of a reversal of the intrinsic inhibition of P-450 reduction by Type II substrates.

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