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Effect of acetaminophen on liver microsomal drug-metabolizing enzyme *in vitro* in mice

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Acetaminophen (AAP) is widely used as an analgesic and an antipyretic, and no particular adverse reaction of the drug at therapeutic doses has been reported. However, large doses of AAP were found to cause hepatic necrosis in man [1, 2] and in several animal species [3]. Recently, some investigators have reported that AAP is metabolized to an active metabolite by liver microsomal cytochrome P-450 and the metabloite binds covalently to tissue macromolecules causing hepatic necrosis [4, 5]. In this regard, no spectral and kinetic studies of AAP have been elucidated.

The present study was undertaken, therefore, to determine the effect of AAP on the drug-metabolizing enzyme in mouse liver microsomes *in vitro*.

Male mice of ddY strain (18–21 g) were maintained on commercial chow and given tap water *ad lib.* Animals were fasted for about 18 hr prior to sacrifice. Throughout the present study, livers were pooled and used for each experiment. The animals were decapitated and the livers were perfused with 1.15% KCl *in situ* to remove the blood. The livers were then homogenized in 3 vol. of ice-cold 1.15% KCl in a Potter-Elvehjem-type glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 9000 *g* for 20 min and the resultant supernatant fraction was recentrifuged at 105,000 *g* for 1 hr in a Hitachi preparative ultracentrifuge, model 65P. The microsomal pellet was suspended in the isotonic KCl solution or 0.2 M phosphate buffer, pH 7.4. The resultant microsomal suspension was used as the enzyme preparation.

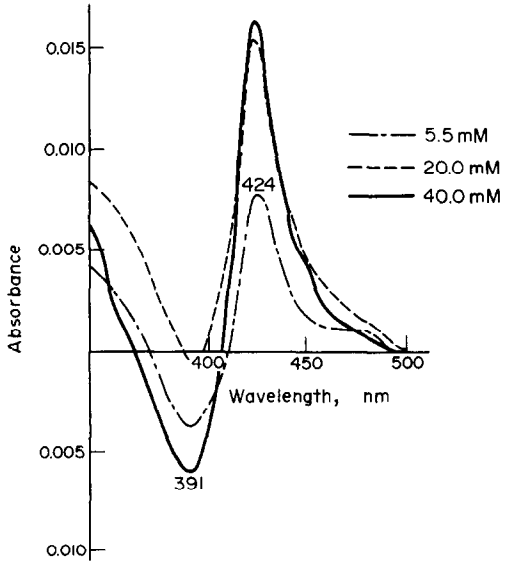

Fig. 1. Difference spectra produced by increasing concentrations of AAP in mouse liver microsomal suspension. Equal volumes of microsomal suspensions were placed in the reference and sample cuvettes. Difference spectra were recorded after the addition of 1 ml each of AAP and equal volumes of redistilled water to sample and reference cuvettes respectively. The microsomal protein concentration was 1.33 mg/incubation mixture.

Aniline hydroxylase activity was measured by determining the amount of *p*-aminophenol (PAP) formed using the method of Imai *et al.* [6]. The method could be also used for the determination of PAP in the presence of AAP. Ethylmorphine *N*-demethylase activity was measured by the determination of the amount of formaldehyde formed according to the method of Nash [7].

Cytochrome P-450 was assayed by the method of Omura and Sato [8]. Difference spectra were obtained at room temperature with a DW-2 Aminco–Chance spectrophotometer in the split beam mode. Absorbance changes at the peaks and troughs were determined relative to 500 nm. A description of the condition for obtaining difference spectra is given in the legends to the figures. Commercial aniline was redistilled under vacuum and the distillate was stored at about −10° under a nitrogen atmosphere. Other substrates were purchased from commercial sources and used without further purification.

The microsomal protein was determined according to the method of Lowry *et al.* [9].

As shown in Fig. 1, AAP produced a characteristic type II spectrum with a peak at 424 nm and a trough at 391 nm. In addition, AAP has two spectral dissociation constants (K_s), 3.82 and 22.7 mM (Fig. 2). Dixon plots of the inhibitory effect of AAP on aniline hydroxylation and ethylmorphine *N*-demethylation are illustrated in Fig. 3. Figure 3a shows that the nature of the inhibitory effect of AAP on aniline hydroxylation appears to be competitive, and the apparent K_i value is found to be 2.7 mM. On the other hand, Fig. 3b shows that the inhibitory effect of AAP on *N*-demethylation of ethylmorphine seems to be noncompetitive, and its K_i value is 5.4 mM.

Since AAP was found to inhibit the metabolism of both type I and type II compounds, attempts were made to determine its effect on the difference spectra produced by

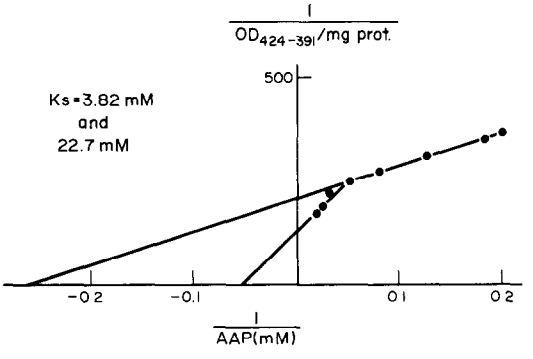

Fig. 2. Reciprocal plots of changes in absorbance/mg of microsomal protein at peak (424 nm) minus trough (391 nm) relative to 500 nm caused by the addition of AAP to microsomal suspensions. Equal volumes (2 ml) of microsomal suspension in 0.2 M phosphate buffer, pH 7.4, were placed in the reference and sample cuvettes. Difference spectra were recorded in the presence of 1 ml of AAP solution and 1 ml of redistilled water in the sample and reference cuvettes respectively. K_s values for AAP were 3.82 and 22.7 mM. The microsomal protein concentration was 1.33 mg/incubation mixture.

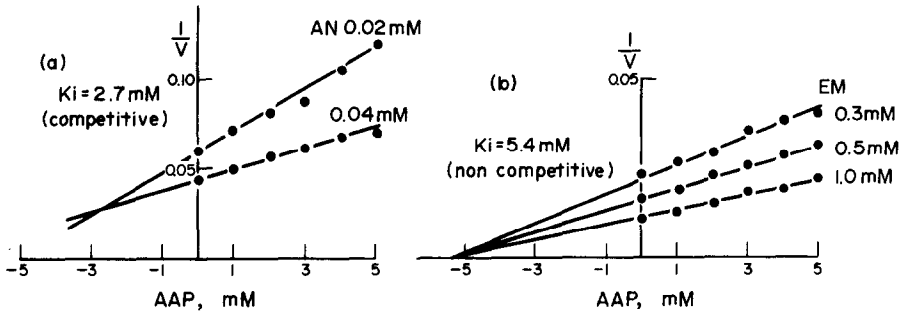

Fig. 3. Inhibition of ethylmorphine *N*-demethylation and aniline hydroxylation by AAP. The incubation mixture consisted of either ethylmorphine (0.3, 0.5 and 1.0 mM) or aniline (0.02 and 0.04 mM), liver microsomes, 0.1 mM EDTA, AAP ranging from 1.0 to 5.0 mM, 5 mM semicarbazide, pH 7.4, an NADPH-generating system (0.33 mM NADP, 8 mM glucose 6-phosphate, 6 mM $MgCl_2$ and 0.045 unit of glucose 6-phosphate dehydrogenase) and 0.3 ml of 0.2 M phosphate buffer, pH 7.4, in a final volume of 1.0 ml. In the case of aniline hydroxylation, redistilled water was used in place of semicarbazide. Incubations were carried out at 37° for 20 min aerobically. Protein concentrations of microsomes were 0.98 mg in Fig. 3a and 0.76 mg/incubation mixture in Fig. 3b respectively.

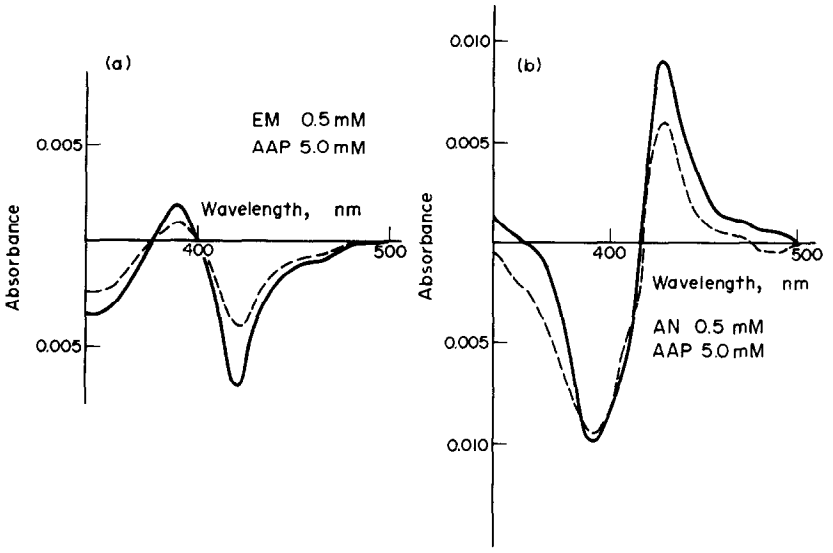

Fig. 4. Effect of AAP on the difference spectra of aniline–microsome and ethylmorphine–microsome complexes. Figure 4 (panels a and b) was obtained with either 0.5 mM ethylmorphine or 0.5 mM aniline in the sample cuvette and 5 mM AAP in both the sample and reference cuvettes. The concentrations of microsomal protein used with the substrates were 1.73 mg for ethylmorphine and 1.87 mg/incubation mixture for aniline.

Table 1. Effect of AAP on the duration of zoxazolamine paralysis and the content of cytochrome P-450*

AAP treatment (mg/kg, i.p.)	Time (hr)	Paralysis† (min)	P	Cytochrome P-450 content‡	P
None		64.4 ± 3.3 (27)		0.656 ± 0.061	
200	24.0	76.6 ± 4.6 (15)	<0.05	0.331 ± 0.039	<0.05
500	24.0	94.4 ± 6.9 (16)	<0.01	0.303 ± 0.034	<0.01

* Male mice weighing 16–22 g received zoxazolamine 100 mg/kg, i.p. in 0.2 N HCl solution 24 after AAP pretreatment at doses of 200 and 500 mg/kg in 0.9% saline solution. Control animals received the 0.9% saline solution as a vehicle of AAP and, later, 0.2 N HCl instead of zoxazolamine solution. The values represent mean ± S. E. Animals were sacrificed 24 hour after AAP administration, and control animals received saline solution. Figures in parentheses indicate the number of animals used in each experiment.

† Duration of paralysis was measured from the injection of zoxazolamine to the time the animals regained righting reflex.

‡ Values are expressed as nmoles/mg of protein.

ethylmorphine and aniline. As illustrated in Fig. 4, difference spectra of ethylmorphine (0.5 mM) and aniline (0.5 mM) were found to decrease when AAP (5 mM) was added to the cuvette.

The inhibitory effect of PAP formed from AAP on the hydroxylation of aniline was examined, because AAP is known to be hydrolyzed to form PAP by liver microsomal esterase [10]. No significant inhibition of hydroxylation of aniline (0.01, 0.03 and 0.04 mM) by PAP in five concentrations (3, 5, 10, 20 and 30 μM) was observed (Fig. 5). These findings indicate that aniline hydroxylation was inhibited by AAP itself, not its metabolite, PAP.

Experiments were conducted to determine whether the duration of paralysis produced by zoxazolamine, a type II compound [11], may be changed by pretreatment with AAP. Zoxazolamine (100 mg/kg, i.p.) was administered to mice 24 hr after AAP treatment (200 mg/kg or 500 mg/kg, i.p.), and the duration of loss of righting reflex was used as an index of paralysis. The data in Table 1 show that significant prolongation of paralysis was observed when zoxazolamine was injected 24 hr after AAP pretreatment even at a dose of 200 mg/kg. Table 1 also shows that the content of cytochrome P-450 of mouse liver microsomes treated with AAP at doses of 200 and 500 mg/kg was decreased to 50 per cent ($P < 0.05$) and 46 per cent ($P < 0.01$) of control level respectively. These results suggest that duration of paralysis produced by zoxazolamine was due to a decrease of cytochrome P-450 content, not to competitive inhibition of AAP and zoxazolamine.

It is known that large oral doses of AAP cause hepatic necrosis [1–3]. Recently, Jollow *et al.* [4] and Potter *et al.* [5] reported that AAP is oxidized to *N*-hydroxyacetanilide by liver microsomal cytochrome P-450. This reactive metabolite is postulated to bind to tissue macromolecules. Mice were shown to be more susceptible to AAP-induced necrosis than rats [4], and thus mice were chosen for use in this study.

Spectral and kinetic studies with mouse liver microsomal suspensions demonstrated that AAP produces typical type II difference spectra possessing two dissociation constants. These findings suggest the possibility that AAP may be bound to different sites of the molecule of one cytochrome P-450 or to two species of cytochrome P-450, since evidence for the existence of multiple forms of cytochrome P-450 has been presented [12, 13].

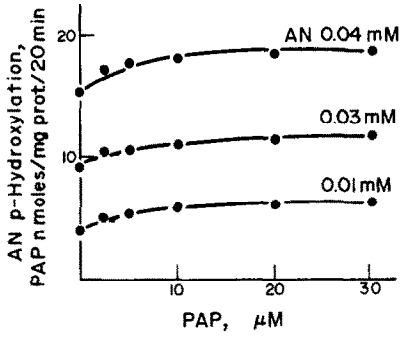

Fig. 5. Effect of *p*-aminophenol on aniline hydroxylation. Experimental details of the incubation mixture were described in Fig. 3 except that aniline in three concentrations (0.01, 0.03 and 0.04 mM) was used as substrate and *p*-aminophenol ranging from 3 to 30 μM was used as an inhibitor in place of AAP. The concentration of PAP added to the incubation mixture was subtracted from the PAP determined by assay. Microsomal protein was 0.91 mg/incubation mixture.

Ethylmorphine and aniline were chosen as model compounds of type I and type II substrate to test AAP as a possible inhibitor of drug metabolism *in vitro*. AAP exhibited noncompetitive inhibition for ethylmorphine *N*-demethylation and competitive inhibition for aniline hydroxylation (Fig. 3). In addition, the decrease in difference spectra of ethylmorphine and aniline caused by adding AAP may support the data obtained on the inhibitory effects of AAP on the metabolism of aniline and ethylmorphine (Fig. 4).

AAP is hydrolyzed to PAP by liver microsomal hydrolase, and it is possible to speculate that the inhibition of aniline metabolism by the addition of AAP may be caused by PAP. No inhibition of *p*-hydroxylation of aniline by PAP was observed (Fig. 5).

It is well known that zoxazolamine is oxidized by liver cytochrome P-450 to produce an inactive metabolite [14]. The present studies demonstrate that AAP prolongs the duration of paralysis produced by zoxazolamine and decreases the content of cytochrome P-450 simultaneously. Therefore, AAP-induced prolongation of the duration of zoxazolamine paralysis may be due to the decrease of cytochrome P-450 content rather than competitive inhibition of zoxazolamine with AAP.

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REFERENCES

1. L. F. Prescott, N. Wright, P. Roscoe and S. S. Brown and S. S. Brown, *Lancet* **1**, 519 (1971).
2. R. Clark, R. P. H. Tompson, V. Borirakchanyavat, B. Widdop, A. R. Davidson, R. Goulding and R. Williams, *Lancet* **1**, 66 (1973).
3. J. R. Mitchell, D. J. Jollow, W. Z. Potter, D. C. Davis, J. R. Gillette and B. B. Brodie, *J. Pharmac. exp. Ther.* **187**, 185 (1973).
4. D. J. Jollow, J. R. Mitchell, W. Z. Potter, D. C. Davis, J. R. Gillette and B. B. Brodie, *J. Pharmac. exp. Ther.* **187**, 195 (1973).
5. W. Z. Potter, D. C. Davis, J. R. Mitchell, D. J. Jollow, J. R. Gillette and B. B. Brodie, *J. Pharmac. exp. Ther.* **187**, 203 (1973).
6. Y. Imai, A. Ito and R. Sato, *J. Biochem., Tokyo* **60**, 417 (1966).
7. N. Nash, *Biochem. J.* **55**, 416 (1953).
8. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
9. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
10. K. Krisch, *Biochem. Z.* **337**, 546 (1963).
11. W. C. Lubamy, H. B. Kostenbauder and S. A. Staochansky, *Res. Commun. Chem. Path. Pharmac.* **8**, 75 (1974).
12. D. Ryan, A. Y. H. Lu, S. West and W. Levin, *J. biol. Chem.* **250**, 2157 (1975).
13. I. Schuster, C. Fleschurz and I. Helm, *Eur. J. Biochem.* **51**, 511 (1975).
14. A. H. Conney, C. Davidson, R. Gastel and J. J. Burns, *J. Pharmac. exp. Ther.* **154**, 310 (1960).