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EFFECT OF SOME 6-SUBSTITUTED BENZO[A]PYRENE DERIVATIVES UPON LIVER ENZYMES AND ASCORBIC ACID EXCRETION IN MICE

FRANCIS DEWHURST and DAVID A. KITCHEN
School of Biology, City of Leicester Polytechnic, P.O. Box 143, Leicester LE1 9BH, England

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Abstract—The effects of benzo[a]pyrene, 6-hydroxymethylbenzo[a]pyrene, benzo[a]-pyrene-6-carboxaldehyde, 6-methylbenzo[a]pyrene, benzo[a]pyrene-6-carbonitrile and benzo[a]pyrene-6-carbonamide on urinary ascorbic acid excretion and hepatic tyrosine transaminase, aniline hydroxylase, aminopyrine *N*-demethylase and hexobarbital metabolizing activity in mice were examined. The effect of 6-bromobenzo[a]pyrene on the hepatic enzymes was examined. None of the compounds altered aminopyrine *N*-demethylase or hexobarbital metabolizing activity whilst all produced a statistically significant rise in aniline hydroxylase activity. The tyrosine transaminase activity was increased by 6-hydroxymethylbenzo[a]pyrene but was unaffected by the other compounds. All the compounds raised urinary ascorbic acid excretion after an initial 3 day lag. The level of excretion remained elevated for about a week or more except for 6-hydroxy-methylbenzo[a]pyrene-treated animals which showed a high raised level of excretion for 1 day followed by a drop to a lower than normal level of excretion. The significance of these observations and their relation to other work was discussed.

MANY xenobiotics induce, in rodents, increases in both liver microsomal mixed function oxidase activity[1] and urinary ascorbic acid excretion.[2] These compounds fall broadly into two major classes. The nonpolar polycyclic aromatic hydrocarbons induce "drug metabolizing" enzyme activity within 24 hr, are quite selective in the enzymes they induce and after an initial lag of 3 days, produce raised ascorbic acid excretion which persists for about 2 weeks. In contrast chlorinated compounds and drugs such as phenobarbitone induce drug metabolizing enzyme activity only after repeated dosage over several days, are unselective in the enzymes they induce and give an immediate increase in ascorbic acid excretion which reaches its peak on the third day and has returned to normal by the fifth day after dosing. It has been suggested[3] that stimulation of ascorbic acid excretion may be used as a simple test for "drug metabolizing" enzyme induction. However, Aarts[4] has shown that puromycin and actinomycin D block stimulation of "drug metabolizing" enzyme activity but not ascorbic acid excretion. Adrenal corticosteroids can affect urinary ascorbic acid excretion[5] and also "drug metabolizing" enzyme activity.[1] Adrenal release of hydrocortisone induces increased activity of liver tyrosine transaminase in the mouse.[6]

We have recently synthesized a series of 6-substituted benzo[a]pyrene derivatives[7] and investigated their effect upon zoxazolamine paralysis and hexobarbital sleeping times in mice[8,9] as part of a systematic study of their biological activity. We found that most of the derivatives, like the parent hydrocarbon itself, shortened zoxazolamine times but had no effect upon hexobarbital times. The 6-hydroxymethyl derivative, and substances which might be metabolized to this compound, prolonged sleeping

and paralysis times, however. To investigate this further and to examine the relationship between enzyme induction and ascorbic acid excretion the effects of the 6-substituted benzo[a]pyrene derivatives upon tyrosine transaminase, ascorbic acid excretion and a number of mixed function oxidase enzymes were investigated.

MATERIALS AND METHODS

The 6-substituted benzo[a]pyrene derivatives were synthesized and purified as described by Dewhurst and Kitchen[7] and the animals dosed for the enzyme activity studies as described previously.[8] The mice were always injected at the same time of day to eliminate diurnal variations.

For the assay of tyrosine transaminase activity mice were killed 6 hr after injection, the livers removed, cooled in ice, individually weighed and homogenized in 4 vol. of ice-cold 0·14 M KCl solution. The homogenates were centrifuged at 3000 *g* for 10 min and the resultant supernatant recentrifuged at 31,000 *g* for 30 min at 0°.

The 31,000 *g* supernatant was diluted 1:25 and 0·2 ml aliquots assayed for tyrosine transaminase activity using the method of Diamondstone,[10] duplicate determinations being carried out for each liver sample.

For microsomal studies mice were killed 24 hr after injection, the livers removed, chilled, weighed in pairs and homogenized in 2 vol. of ice-cold 0·25 M sucrose solution. After centrifuging for 10 min at 3000 *g* and for 30 min at 15,000 *g* the resultant supernatant was recentrifuged at 150,000 *g* for 60 min; all operations being carried out at 0–5°. The resultant microsomes were resuspended in 0·25 M sucrose so that 1 ml of suspension contained the microsomes from 1 g of liver. Microsomal protein was determined by the method of Itzhaki and Gill[11] using bovine serum albumin as standard. Enzyme incubation mixtures contained 1 ml of 150,000 *g* supernatant, microsome suspension (0·2 ml for aniline hydroxylase, 1 ml in other cases), 25 μM each of glucose-6-phosphate and $MgSO_4$, 100 μM of nicotinamide, 0·6 μM of NADP and substrate made up to a final volume of 5 ml with M/15, ph 7·3 phosphate buffer. After addition of substrate (3 μM, hexobarbital, 10 μM aniline or aminopyrine) the mixtures were incubated, with shaking for 1 hr. Aminopyrine *N*-demethylase activity was assayed by Gram *et al.*[12] modification of the method of Cochin and Axelrod,[13] aniline hydroxylase by the method of Kato and Gillette[14] and hexobarbital metabolism by the method of Cooper and Brodie.[15] All assays were carried out in duplicate and the mean of the readings recorded.

In the experiments on ascorbic acid excretion mice were maintained on a diet of evaporated milk and water (1:1) for 3 days prior to injection and during the course of the experiments. Mice were placed in pairs in metabolism cages and groups of 8 pairs taken per compound. Urine samples (24 hr) were collected (in darkness), into 2 ml of 40% w/v trichloroacetic acid. The urine samples were assayed for ascorbic acid content by the colorimetric dichlorophenolindophenol method of Howard and Constable.[15] Animals received a single dose of test compound as described previously[8] except that 0·5 mg of the 6-carboxaldehyde was given per animal (instead of 1·0 mg) due to the toxicity of the compound.

The substantial significance of results was determined by the application of Student's *t*-test.

Optical density measurements were made on a Unicam SP500 spectrophotometer and an M.S.E. superspeed 50 centrifuge was used for high speed centrifugation.

TABLE 1. EFFECT OF PRETREATMENT WITH 6-SUBSTITUTED BENZO[a]PYRENE DERIVATIVES UPON MOUSE LIVER MICROSOMAL PROTEIN, ANILINE HYDROXYLASE, AMINOPYRINE *N*-DEMETHYLASE AND HEXOBARBITAL METABOLIZING ENZYME ACTIVITY

Compound‡	Protein (mg/g liver)	Aniline metabolized (nmoles of *p*-aminophenol formed/mg microsomal protein/hr)	Aminopyrine metabolized (nmoles of formaldehyde formed/mg microsomal protein/hr)	Hexobarbital metabolized (nmoles/mg microsomal protein/hr
Benzo[a]pyrene	22·5 ± 2·9	68·0 ± 10·4*	10·01 ± 2·00	62·4 ± 11·6
6-Hydroxymethylbenzo[a]pyrene	22·4 ± 1·9	58·5 ± 8·8*	9·79 ± 2·16	55·8 ± 9·8
Benzo[a]pyrene-6-carboxaldehyde	21·7 ± 1·2	49·4 ± 4·2†	8·47 ± 0·64	69·3 ± 9·8
6-Methylbenzo[a]pyrene	22·7 ± 2·1	48·0 ± 4·1†	8·49 ± 1·09	57·6 ± 10·1
Benzo[a]pyrene-6-carbonitrile	22·6 ± 1·4	74·1 ± 11·4*	11·08 ± 0·30	63·8 ± 5·9
Benzo[a]pyrene-6-carbonamide	22·0 ± 3·8	67·2 ± 16·5*	9·22 ± 0·16	69·5 ± 22·2
6-Bromobenzo[a]pyrene	22·1 ± 0·7	51·9 ± 7·8†	11·69 ± 0·40	65·5 ± 16·1
Control	22·0 ± 2·8	41·0 ± 4·8	10·23 ± 1·75	67·1 ± 11·6

* Result significantly different from control ($P < 0·01$).

† Result significantly different from control ($P = 0·05$–$0·01$).

‡ Animals each received, i.p., 0·5 ml of arachis oil containing 1 mg of test compound except in the case of the hydroxymethyl derivative, when 0·5 mg was given, and the controls which received arachis oil only. Results are expressed as the mean value obtained with at least eight mice ± S. E. M.

RESULTS

None of the compounds affected liver microsomal protein content, *in vitro* aminopyrine or hexobarbital metabolism but all increased aniline hydroxylase activity (Table 1). Liver tyrosine transaminase activity was increased 3-fold by 6-hydroxymethylbenzo[a]pyrene but was unaffected by the other compounds (Table 2). All the compounds produced an increase in ascorbic acid excretion (Table 3). The usual lag, followed by prolonged increased ascorbic acid excretion, produced by polycyclic aromatic hydrocarbons was observed in every case except with the 6-hydroxymethyl derivative. This compound gave an increased excretion of short duration, similar to the effect produced by phenobarbitone, after a time lag and followed by a significant fall in excretion. The effect was of a type not previously reported. Amongst the other compounds the 6-carbonamide and 6-carbonitrile derivatives were much less effective inducers than the rest.

TABLE 2. EFFECT OF SOME 6-SUBSTITUTED BENZO[a]PYRENE DERIVATIVES UPON LIVER TYROSINE TRANSAMINASE ACTIVITY IN THE MOUSE*

Compound	Tyrosine transaminase activity (μmoles product/g liver/30 min)
Benzo[a]pyrene	13·5 ± 1·7
6-Hydoxymethylbenzo[a]pyrene	46·3 ± 28·3†
Benzo[a]pyrene-6-carboxaldehyde	14·0 ± 2·9
6-Methylbenzo[a]pyrene	15·5 ± 4·2
Benzo[a]pyrene-6-carbonitrile	14·2 ± 1·9
Benzo[a]pyrene-6-carbonamide	16·1 ± 2·7
6-Bromobenzo[a]pyrene	17·3 ± 1·8
Control	14·6 ± 3·5

* Dose levels are as described in Table 1 and results are expressed as the mean value obtained with at least four mice ± S. E. M.
† Result significantly different from control ($P < 0{\cdot}001$).

DISCUSSION

It has been reported by Hansen and Fouts[17] that benzo[a]pyrene does not increase aniline hydroxylase and a number of other microsomal mixed function oxidase activities in mice. They state, with regard to the well-established ability of benzo[a]-pyrene to induce microsomal mixed function oxidase activity in the rat, that "there seemed to be an animal species difference in response to this hydrocarbon". Their observation that methylcholanthrene increased mixed function oxidase activity in their mice establishes that they do respond to some polycyclic aromatic hydrocarbons. Hansen and Fouts[18] had previously observed that in mice benzo[a]pyrene had no effect upon hexobarbital sleeping times and did not produce a consistent statistically significant shortening of zoxazolamine paralysis times. In contrast Abernathy *et al.*[18] reported benzo[a]pyrene shortened both paralysis and sleeping times in mice. We[8] have previously observed shortening of zoxazolamine paralysis times by benzo[a]-pyrene but found it without effect upon hexobarbital sleeping times. Creaven and Parke[20] observed stimulation of biphenyl hydroxylation both *in vivo* and *in vitro* by benzo[a]pyrene in mice. In the present study we have observed benzo[a]pyrene to

TABLE 3. EFFECT OF PRETREATMENT WITH 6-SUBSTITUTED BENZO[a]PYRENE DERIVATIVES UPON THE URINARY EXCRETION OF ASCORBIC ACID BY MICE

	Ascorbic acid excreted μg/mouse/day									
Days after injection	1	2	3	4	5	8	9	10	11	15
Benzo[a]pyrene-treated animals	140 ± 27	129 ± 34	135 ± 28	344 ± 87*	406 ± 72*	471 ± 106*	414 ± 79*	345* ± 89*	304 ± 25*	238* ± 16*
6-Hydroxymethylbenzo[a] pyrene-treated animals‡	136 ± 24	134 ± 32	173 ± 33	430 ± 96*	175 ± 41	81 ± 50†	120 ± 49	128* ± 27	113 ± 26	141 ± 36
Benzo[a]pyrene-6-carboxaldehyde-treated animals‡	144 ± 38	128 ± 29	144 ± 34	192 ± 39†	262 ± 74*	423 ± 75*	369 ± 70*	379 ± 76*	330 ± 63*	287 ± 76*
6-Methylbenzo[a]pyrene-treated animals	148 ± 26	130 ± 22	166 ± 31	244 ± 62*	337 ± 50*	350 ± 36*	333 ± 41*	332 ± 31*	317 ± 34*	308 ± 61*
Benzo[a]pyrene-6-carbonitrile-treated animals	137 ± 31	135 ± 40	141 ± 28	164 ± 30	215 ± 73	268 ± 63*	276 ± 61*	202 ± 52	189 ± 38	157 ± 30
Benzo[a]pyrene-6-carbonamide-treated animals	135 ± 36	138 ± 30	135 ± 32	190 ± 65	243 ± 56*	247 ± 53*	212 ± 42†	189 ± 59	158 ± 42	140 ± 21
Control animals	134 ± 27	135 ± 28	149 ± 29	139 ± 30	150 ± 15	153 ± 12	141 ± 32	141 ± 27	150 ± 22	145 ± 18

All results are expressed as the mean value obtained with at least sixteen mice ± S. E. M. and 1 mg of compound, in 0·5 ml of arachis oil, administered per mouse unless otherwise stated. Control animals received arachis oil only.

* Significantly different from control values $P < 0{\cdot}02$.

† Significantly different from control values $P = 0{\cdot}05$–$0{\cdot}02$.

‡ Each animal received 0·5 mg of compound.

increase aniline hydroxylase activity but not aminopyrine demethylase or hexobarbital metabolism in mice. These observations indicate that our mice respond to benzo[a]-pyrene in the same way as rats but we have observed, in agreement with Creaven and Parke,[20] that a smaller increase in activity is produced in mice than in rats. It may be significant that both we and Creaven and Parke[20] used female mice and examined them after 24 hr whilst Hansen and Fouts[17] used male mice and examined them after 64–72 hr. Jori *et al.*[21] have shown that a daily rhythmic variation occurs in mixed function oxidase activity in rats. Abernathy *et al.*[19] using male mice gave doses of about a quarter the amount of benzo[a]pyrene used by the other authors,[8,17,20] dosed their animals daily for 3 days and examined them 24 hr later. These differences in experimental conditions make comparison of results difficult but it seems reasonable to conclude that benzo[a]pyrene acts like other polycyclic aromatic hydrocarbon enzyme inducers in most strains of mice and that a species difference in response does not exist between rats and mice. The above results do suggest the existence of strain differences in mice, however.

The ascorbic acid excretion studies showed polycyclic aromatic compounds inducing mixed function oxidase activity also induced increased urinary ascorbic acid excretion in mice. These results are in agreement with studies on a range of inducers in rats and there are no indications of a difference in species response to benzo[a]pyrene. With regard to the use of ascorbic acid excretion as a test for mixed function oxidase induction comparison of Tables 1 and 3 indicates a much clearer response is obtained from ascorbic acid measurements than from studies on aniline hydroxylase. It would be interesting to see the effect of benzo[a]pyrene upon ascorbic acid excretion in Hansen and Fouts strain of mice. It would be premature to use ascorbic acid excretion as an index of enzyme induction until a wider range of compounds has been studied, however. It should be noted that the 6-carbonamide and the 6-carbonitrile derivatives are relatively poor inducers of ascorbic acid excretion but are relatively potent in shortening zoxazolamine times[8] and in inducing aniline hydroxylase. Enzyme induction precedes the increase in ascorbic acid excretion by many hours, which also requires explanation. There is other evidence of a relationship between mixed function oxidase activity and ascorbic acid. Lycorine[22] has been shown to inhibit both ascorbic acid biosynthesis and mixed function oxidase activity in rats. Studies[23] on scorbutic guinea-pigs showed them to have reduced aniline hydroxylase and aminopyrine demethylase activities whilst injection of dehydroascorbic acid increased these activities in an hour even in the presence of an inhibitor of protein synthesis, ethionine. The level of cytochrome P-450 was also depressed in scorbutic guinea-pigs but rose 48 hr after injection of dehydroascorbic acid, ethionine blocking this rise. The observation[24] that ascorbic acid stimulates RNA synthesis in plant tissues with release of nuclear protein and possible removal of inhibitory control of protein synthesis is of interest in this context. The selective induction of aniline hydroxylase but not aminopyrine *N*-demethylase activity by the hydrocarbons indicates that the relationship between ascorbic acid excretion and enzyme induction is neither simple nor direct, however.

The tyrosine transaminase studies indicate that, leaving aside 6-hydromethylbenzo-[a]pyrene, adrenal release of hydrocortisone is not an explanation for either mixed function oxidase induction or raised ascorbic acid excretion. This is consistent with Aarts[4] observations on barbital effects on ascorbic acid excretion (and with enzyme induction studies in organ and tissue culture[25,26]). The increase in tyrosine transamin-

ase activity produced by 6-hydroxymethylbenzo[a]pyrene implies that this compound causes adrenal release of hydrocortisone. It is well established[27] that 7-hydroxymethyl-12-methylbenz[a]anthracene (produced via metabolism) is responsible for the adrenal changes following the administration of 7,12-dimethylbenz[a]anthracene to rats. These changes include a significant lowering of adrenal corticosteroid concentration[28] and are accompanied by a rise in tyrosine transaminase activity 5 hr after injection.[29] The two hydroxymethyl derivatives have a structural resemblance to each other and our results show that the 12 methyl group of the benz[a]anthracene can be replaced by an aromatic ring in the benzo[a]pyrene derivative without loss of biological activity. 6-methylbenzo[a]pyrene has no effect upon tyrosine transaminase activity in mice but the analogous compound 7,12-dimethylbenz[a]anthracene is active in rats. The explanation of this probably lies in quantitative differences in metabolism in rats and mice. It is known there are marked species differences in the response of rodents to 7,12-dimethylbenz[a]anthracene[30] and that the rat is unusually vulnerable.[31]

The observation that 6-hydroxymethylbenzo[a]pyrene induced microsomal aniline hydroxylase activity was surprising as our previous work[8,9] had shown that this compound prolonged zoxazolamine and hexobarbital times, which implied inhibition of metabolism *in vivo*.

Recently other studies in our laboratory (to be published when complete) have shown pretreatment with 6-hydroxymethylbenzo[a]pyrene lead to increased *in vitro* zoxazolamine metabolism and aromatic polycyclic hydroxylase activity. The results emphasize that sleeping and paralysis time measurements require caution in interpretation. 6-Hydroxymethylbenzo[a]pyrene may have a depressant action on the central nervous system but the animals seem normal after injection and the sleeping and paralysis time measurements are made 24 hr later when considerable metabolism and excretion will have occurred. Another possible explanation is that as this compound appears to cause release of adrenal hydrocortisone (which may explain the unusual effects on ascorbic acid excretion), progesterone and related steroids may also be released. Jori *et al.*[28] have shown the progesterone will prolong pentabarbital sleeping times and progesterone can be used as an anaesthetic in rodents.[29]

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