Cyclopenta[c,d]pyrene: A highly mutagenic polycyclic aromatic hydrocarbon

(*Salmonella* test/arene oxides/environmental carcinogens/molecular orbital theory)

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ABSTRACT   A polycyclic aromatic hydrocarbon recently isolated from carbon black and identified as cyclopenta[c,d]-pyrene (CPP) is highly mutagenic. By the criteria of the *Salmonella*/mammalian-microsome mutagenicity test, the mutagenic potency of CPP is equalled by only two other naturally occurring polycyclic aromatic hydrocarbons—benzo[a]pyrene and dibenz[a,c]anthracene. The potent mutagenicity of CPP is noteworthy for two reasons: (*i*) CPP is a mutagenic polycyclic aromatic hydrocarbon without a "bay-region" and (*ii*) there is evidence that it is distributed widely in the environment. On the basis of experimental observations and perturbational molecular orbital calculations we propose that a mutagenic metabolite of CPP will be the 3,4-oxide. The carbonium ion derived from opening of CPP 3,4-oxide is identical to that derived from opening of benzo[a]pyrene 7,8-diol-9,10-oxide, the metabolite now thought to be an ultimate mutagenic and carcinogenic species.

There is circumstantial evidence that initial steps in chemical carcinogenesis with polycyclic aromatic hydrocarbons (PAH) involve metabolism to chemically reactive species by microsomal mixed-function oxidases (1) and the modification of nucleic acid bases by adduct formation (2–5). Much of the recent work on PAH metabolism and carcinogenicity has focused on benzo[a]pyrene (BzP) and has led to the identification of many polar products of microsomal enzyme metabolism including the particularly reactive diol epoxide, BzP-7,8-diol-9,10-oxide (3), which accounts for most of the material covalently bound to DNA (2–4). This metabolite is suspected of being an ultimate carcinogen via an S_N1 reaction with DNA of the benzylic carbonium ion derived from epoxide opening (6–8). This carbonium ion intermediate is the basis of a model proposed by Jerina and coworkers (9) to explain in chemical terms the biological activity of PAH consisting of fused aromatic six-membered rings. They suggest that epoxide formation on a saturated, angular benzo-ring adjacent to a "bay region" is a principal determinant of PAH activity because of the energetically favorable opening of "bay region" epoxides to reactive benzylic carbonium ions. The geometric "bay region" feature is exemplified by the area enclosed between the C(10) and C(11) positions of BzP (Fig. 1B).

We became interested in a PAH, cyclopenta[c,d]pyrene (CPP) (Fig. 1A), recently identified as a component of carbon black (10, 11), automobile exhaust (12), and atmospheric soot (ref. 13; Gold, unpublished observation) and reported to be carcinogenic to mice (14). We have begun an investigation of the biological activity and metabolism of CPP and report here that it is a potent mutagen by the criteria of the *Salmonella*/mammalian-microsome mutagenicity test (15). "Bay region" theory does not explain the activity of CPP, which contains a

fused five-membered ring and lacks the geometric "bay region" feature; however, we propose, on the basis of experimental observation and perturbational molecular orbital calculations, that CPP can be metabolized to a benzylic carbonium ion similar to that responsible for the biological activity of BzP 7,8-diol-9,10-oxide. Thus, the concept of the benzylic carbonium ion intermediate as an important characteristic of biologically active PAH would be extended to more generalized PAH structures. Studies of CPP activity and metabolism may contribute much to our understanding of the structural basis for PAH mutagenicity and carcinogenicity.

MATERIALS AND METHODS

CPP was obtained by preparative liquid chromatography of 120 mg of PAH fraction of carbon black extract (10) on a size B Lobar (EM Laboratories, Inc.) silica column attached to a Perkin–Elmer Model 1250 high-pressure liquid chromatograph equipped with a refractive index detector. The material was eluted at 23° with 3% methylene chloride in hexane at 1.5 ml/min and 4-ml fractions were collected automatically. Fractions 50–55 were combined to yield 8 mg of CPP, which was further purified by recrystallization from hexane to constant melting point. The melting point (176°), UV λ_{max}(log ϵ) [374(4.16), 354(4.25), and 285(4.37) nm], and mass spectrum were all consistent with data previously reported for pure CPP.

Mutagenicity tests were performed with the histidine auxotrophs of *Salmonella typhimurium* developed by Ames and his colleagues (15). All strains were checked routinely for the relevant phenotypes (UV sensitivity, crystal violet sensitivity, and, for strains TA98 and TA100, ampicillin resistance). Strain TA1535 carries the base-pair mutation *hisG46* and strains TA1537 and TA1538 carry the frameshift mutations *hisC3076* and *hisD3052*, respectively. TA98 and TA100 are the plasmid pKM101-bearing derivatives of TA1538 and TA1535, respectively. Liver homogenates (S9) were prepared from Aroclor 1254 induced rats (Spraque–Dawley males) as described by Ames *et al.* (15).

RESULTS AND DISCUSSION

The mutagenicity of CPP was determined by assaying for induction of histidine revertants among the standard set of *S. typhimurium* histidine auxotroph tester strains. Significant and roughly equivalent mutagenic activity was detected in the presence of a liver homogenate (S9) with strains TA98, TA100, TA1537, and TA1538 (Table 1). TA1535 was not reverted by CPP.

The spectrum of CPP activity on the tester strains can be compared to that of BzP. BzP is 10 times more active as a mu-

Abbreviations: BzP, benzo[a]pyrene; PAH, polycyclic aromatic hydrocarbons; CPP, cyclopenta[c,d]pyrene.
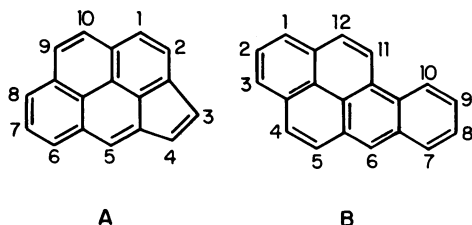
FIG. 1.   (A) Cyclopenta[c,d]pyrene; (B) benzo[a]pyrene.

tagen for TA100 than for either TA1537 or TA1538 (Fig. 2 and ref. 16). In contrast, CPP is strongly mutagenic for all these strains (Table 1). CPP may be classified as a frameshift mutagen since it causes reversion of TA1537 and TA1538, which carry frameshift mutations (15).

BzP and CPP are not mutagenic for the base-pair mutant TA1535 though these PAH cause TA100, the plasmid-pKM101-bearing derivative of TA1535, strongly to revert. Although the frameshift mutagenic activity of BzP is enhanced by 3.5-fold (16) in the plasmid-bearing derivative of TA1538, TA98, the frameshift mutagenic activity of CPP is not enhanced by the plasmid (compare TA1538 with TA98, Table 1). McCann *et al.* (16) have noted that the activity of some reactive frameshift mutagens, such as 2-aminoanthracene and 2-nitrosofluorene, is stimulated by the presence of the pKM101 plasmid in the base-pair mutant but not the frameshift mutant TA1538. CPP, an unsubstituted PAH, appears to be a reactive frameshift mutagen of this type.

The S9 dependence of CPP mutagenesis is displayed and compared to that of BzP in Fig. 2. Two features are striking: (*i*) the amount of S9 that yields the maximum level of CPP mutagenesis, 3 μl per plate, is about $^1/_{10}$ the amount needed for maximal BzP mutagenesis; and (*ii*) the extent of CPP mutagenicity, as a function of increasing S9 concentration, rises and falls sharply and is similar for both strains TA100 and TA1537. The biochemical basis for the S9 dependence profile is not known.
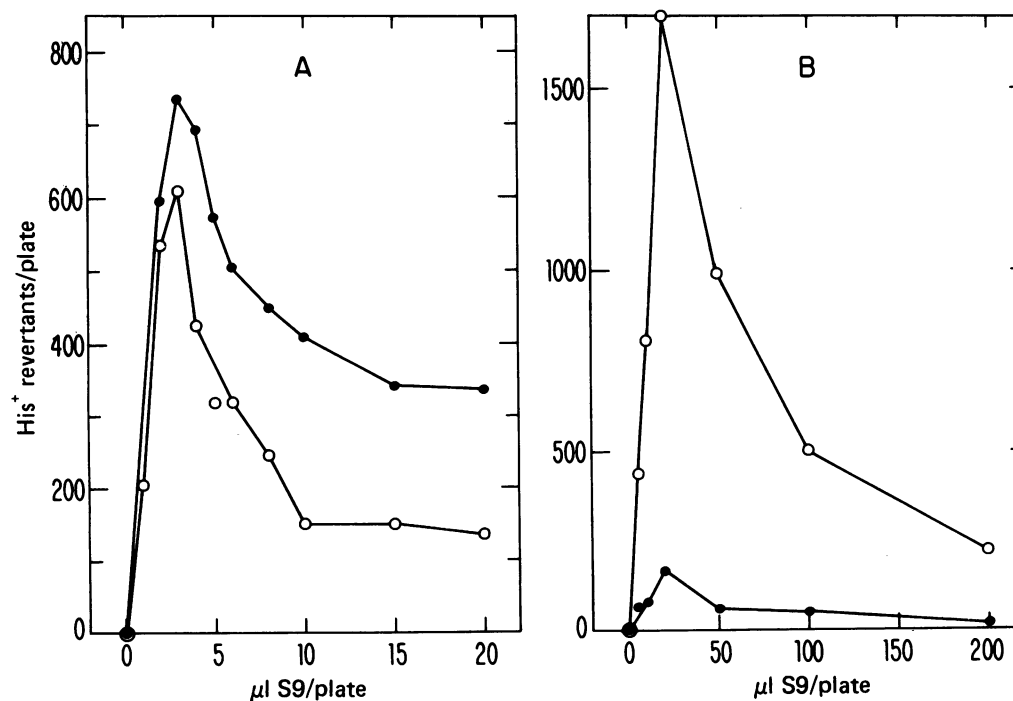
Table 1.   Comparison of CPP-induced mutagenesis in *S. typhimurium* strains

Addition	Histidine revertants per plate				
	TA98	TA100	TA1537	TA1538	TA1535
S9	26	95	13	21	14
S9 + 1 μg CPP	470	523	945	675	10

Liver homogenate (S9) (4.5 μl per plate) (3 μl per plate for TA1535 plates) was used to determine the mutagenicity of CPP in this experiment. The spontaneous reversion incidence indicated is the average of three plates (two plates for TA1535). The CPP-induced reversion incidence is from the linear portion of dose-response curves.

The mutagenic activity of CPP on strain TA1537 was linearly dependent on CPP concentration, at the optimal S9 level, up to 1 μg per plate. The mutagenic potency of CPP, determined from the slope of the line (calculated by linear regression through the origin), was 174 revertants per nmol. For strain TA100 we determined a mutagenic potency of 80 for CPP. These levels of mutagenicity in the *Salmonella* assay are comparable to the values of 58, 121, and 175 reported for strain TA100 for 3-methylcholanthrene, BzP, and dibenz[a,c]anthracene, respectively (17). Thus, CPP is a highly mutagenic PAH. Though a quantitative relationship between mutagenicity and carcinogenicity is difficult to establish (18), there is evidence that mutagenic potency, as defined by the criteria of the *Salmonella* assay, permits an estimate of the carcinogenic potency of a chemical (19).

Since the "bay region" theory criteria for activity do not apply to CPP, the structural basis for its potent mutagenicity merits consideration. The two most extensively documented examples of active "bay region" epoxides are BzP-7,8-diol-9,10-oxide (2, 3, 9, 20) and benz[a]anthracene-3,4-diol-1,2-oxide (21). The high chemical reactivity of these epoxides, based on the ease of opening to a benzylic carbonium ion (6–9), and their high biological activity are ultimately determined by electronic

FIG. 2.   S9 dependence of CPP and BzP mutagenicity in *S. typhimurium*. One microgram of CPP (A) or 2 μg of BzP (B) was added to each plate. The number of revertants obtained without mutagen have been subtracted from each plate count. ●, Strain TA1537; ○, strain TA100.
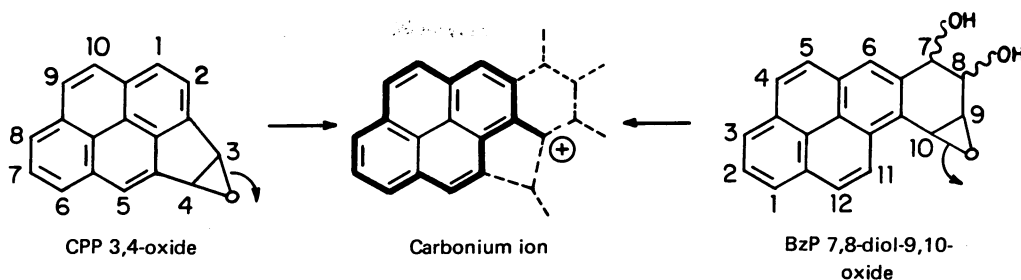
FIG. 3. Benzylic carbonium ion resulting from the opening of CPP 3,4-oxide or BzP 7,8-diol-9,10-oxide.

structure. It is reasonable to assume that the potent mutagenicity of CPP results from metabolic formation of a highly reactive epoxide and, therefore, our results imply that PAH epoxides of high reactivity need not contain the geometric "bay region" feature.

The likelihood of metabolic formation of CPP 3,4-oxide is supported by Hückel bond orders indicating a strong localization of the 3,4 double bond. The results of perturbational molecular orbital calculations (22) indicate that an addition across the ethylene bridge is favored since the resulting loss of resonance energy is smaller than for analogous additions that proceed readily across the 9,10 positions of phenanthrene or the 4,5 positions of pyrene. Reactivity of the ethylene bridge of CPP is confirmed by the experimental observation (10, 11) that catalytic hydrogenation occurs readily and exclusively at the ethylene bridge.

The energetically favorable opening of the 3,4-oxide should proceed via breakage of the O-C(3) bond, yielding the same type of benzylic carbonium ion derived from the opening of BzP-7,8-diol-9,10-oxide (Fig. 3). In the Hückel molecular orbital approximation, which considers only the unsaturated portions of the carbon skeleton, the two cations are identical. Hence, CPP 3,4-oxide should undergo reactions with DNA analogous to BzP-7,8-diol-9,10-oxide. We therefore predict that CPP 3,4-oxide will be a highly mutagenic metabolite of CPP.

In view of its mutagenic potency and reported carcinogenicity (14), the widespread environmental distribution of CPP is worth noting. CPP has been demonstrated in soots of wood, coal, kerosene, and benzene flames (23; 24). It is equal in abundance to BzP, in soots from coal and wood and 20 times more abundant in soots from kerosene. Furthermore, CPP has been identified in a sample of urban airborne particulates (13, 25) and is a major PAH component of automobile exhaust (12). We have recently been able to identify CPP in samples of air-borne particulates from Kingston, the site of a large coal-fired generating station (unpublished observations). CPP also occurs in varying amounts (1-65 μg/g) in carbon black (11) used in tire manufacture and could account for the total mutagenicity of the PAH extract of a carbon black sample (unpublished observations).

The correlation between PAH mutagenicity and carcinogenicity (17) and the likely widespread environmental distribution of CPP (12, 13, 25) requires inquiry into its possible role as a human carcinogen.

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