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Kinetics and Biotransformation of Benzo(a)pyrene in *Chironomus riparius*

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Abstract. Uptake and depuration kinetics for benzo(a)pyrene (B(a)P) were determined for the midge *Chironomus riparius* (Diptera) with one and two compartment models. Nonfeeding animals were exposed to nominal 1.0 $\mu g \cdot L^{-1}$ ^{14}C- B(a)P for eight hr. Depuration over eight hr was determined in animals with and without substrate. The uptake rate constant was 214 ± 20 hr^{-1} ($X \pm SE$, $n = 3$), while elimination rate constants for the first four hr were 0.22 hr^{-1} (with substrate) and 0.06 hr^{-1} (without substrate). Biphasic depuration was observed with an initial rapid phase that lasted several hr. Approximately 10% of accumulated ^{14}C was associated with exoskeleton. As much as 50% of the accumulated B(a)P was transformed into polar compounds after one hr. Based on steady state ^{14}C concentration, an apparent bioconcentration factor of 650 was determined. The bioconcentration value based on B(a)P analysis was 200.

Polycyclic aromatic hydrocarbons (PAH) are a homologous series of fused-ring organic trace contaminants formed by natural synthetic processes (plant and microbial), natural pyrolytic processes (forest fires and volcanic activity), and man's activities such as manufacturing, fossil fuel combustion, and fuel conversion (Braunstein *et al* 1977; Harrison *et al* 1975; Suess 1976). Inputs to aquatic systems may be due to human activities, as found in east coast near-shore marine sediments (Hites *et al.* 1977), or of natural origin in sediments of pristine lakes (Brown and Starnes 1978). While PAH are degraded by a variety of physical and biological processes, concern centers on the carcinogenic nature of their transformation products and on projected increases in release. Many of these compounds can enter surface water directly in effluents or in urban and rural runoff, and lipophilic PAH compounds may concentrate in aquatic biota which represent a potential vector to man. Thus, the importance of developing predictive models of PAH fate in aquatic systems must be emphasized (Baughman and Lassiter 1978). Benzo(a)pyrene (B(a)P) is a ubiquitous carcinogenic PAH (Andelman and Snodgrass 1974). Larval midges (Chironomidae) are abundant benthic organisms which burrow in sediments and accumulate trace contaminants (Kawatski and Bittner 1975; Derr and Zabik 1972). Chironomids are a major food source for larger macroinvertebrates and smaller fish, and link both aquatic and terrestrial food chains through emergent adults.

The study of B(a)P kinetics in chironomids was conducted to (1) construct descriptive models of B(a)P uptake and elimination kinetics, (2) compare uptake and elimination coefficients determined by one and two compartment models, (3) determine the ability of chironomids to metabolize B(a)P and the effect of biotransformation on bioconcentration factor, (4) determine the contribution of exoskeleton on total bioaccumulation and (5) determine the effect of bottom substrate on depuration rate.

Materials and Methods

Uptake and Depuration

Chironomus riparius larvae were collected from a sewage outfall on Badfish Creek near Madison, WI, and reared in the laboratory for several generations. Cultures were maintained in 1-gallon glass jars that contained well aerated substrate of previously washed and fermented groung paper towels. Approximately one g of a mixture of dog biscuits, Tetramin® flake fish food shrimp pellets, and Cerophyll® (powdered nettle leaves) was added to the jars every three days during the three-week larval stage. After two days as pupae, adults emerged, mated and oviposited in the jars. Fourth instar larvae had a mean dry weight of 0.39 mg (SD = 0.2, n = 50) and a dry to wet weight ratio of 0.095.

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Benzo(a)pyrene (7,10[14]C) was used as purchased from Amersham/Searle in two lots, CFA472 Batch 26 (specific activity 60.7 mCi·mmole^{-1}) and Batch 29 (specific activity 21.7 mCi·mmole^{-1}). The specific activities were confirmed with a Varian Model 5000 high pressure liquid chromatography system. Radiochemical purity determined by thin layer chromatography was greater than 99%. All preparative, analytical, and experimental procedures were performed under gold fluorescent light ($\lambda \geq 500$ nm) to minimize photodegradation of B(a)P.

Well water (pH 7.1) was aerated and centrifuged to remove particulates (>0.15 μ). Water was labeled in bulk (7-10 L) and dispensed into replicate test chambers after a 1 to 2 hr equilibration. Actual water concentrations were determined from calculations based on dpm·ml^{-1} and known B(a)P specific activity. Concentrations ranged between 0.6–1.5 μg B(a)P·L^{-1}. Uptake experiments (in triplicate) were performed in 1) 0.35 L staining dishes which contained 0.2 L of water and 20 chironomids; 2) 6.0 L aquaria which contained 1–2 L of water and 100 to 200 chironomids. Samples of 10 chironomids were taken after 0.25, 0.5, 1.0, 1.5, 2, 4, and 8 hr. Each staining dish provided a single sample of 10 chironomids for total ^{14}C and 10 for metabolite analysis. The biomass to volume ratios ranged from 0.020–0.039 mg dry wt·ml^{-1} water. The range of biomass to volume ratios in both uptake and depuration experiments was due to differences in water volume. The chironomid larvae were approximately the same size in all experiments. Depuration experiments were performed by transferring 100 to 200 chironomids labeled for eight hr into 6-L glass aquaria which contained 1 to 2 L of clean water. Ten chironomids as well as one ml of water were taken from the aquaria after 0.5, 1.0, 1.4, 2.0, 4, 8, 15, 24 and 48 hr. Mass to volume ratios ranged from 0.035 to 0.070 mg dry wt·ml^{-1} water. Addition of paper towel with associated microflora provided a food source, to avoid starvation effects, and a substrate allowing normal behavioral patterns. Substrate was not used in uptake experiments, because sorption and microbial biotransformation would have confounded exposure calculations.

Samples of 10 chironomids were combusted in a Packard Model 306 sample oxidizer and collected in a scintillation cocktail consisting of 17 ml Permafluor® and 5 ml Carbosorb®. Internal and external standards indicated a $^{14}CO_2$ recovery greater than 99% and no carryover. Water and solvent samples were placed directly into a premixed commercial cocktail (Research Products International 3a70B). All sample activities were measured with a Beckman Model LS8100 liquid scintillation counter and corrected for quench with internal and external standards and the sample channels ratio method.

Biotransformation

Animals and water were analyzed for ^{14}C-B(a)P and transformation products. Chironomids were assayed by a combination of solvent extraction, thin layer chromatography (TLC), and liquid scintillation counting (LSC). Thirty chironomids (10 from each replicate) were homogenized in a Ten Broeck tissue homogenizer with five drops of concentrated HCl. The acid homogenate was extracted sequentially by homogenizing with benzene (5 ml Nanograde®), diethylether (2 × 10 ml, anhydrous), and ethyl acetate (5 ml Nanograde). The organic solvents were combined, a 2 × 0.5 ml aliquot counted, and the remaining volume determined. The samples were dried with anhydrous sodium sulfate and the volume reduced to approximately 100 μl by rotary flash evaporation and final evaporation under a nitrogen stream. The samples were brought to 500 μl with diethylether and activity determined on a 25 μl aliquot. Recovery of ^{14}C-B(a)P from spiked chironomids was 92.3 ± 3%.

Samples were spotted onto thin layer plates and chromatographed in pentane:diethylether (9:1) in an unsaturated system. Developed plates were divided into five sections corresponding to B(a)P, hydroxylated metabolites, the origin, and two unknowns. The sections were scraped from the plate and ^{14}C activity measured. Chironomid extracts were also analyzed by high pressure liquid chromatography. All samples were kept frozen (−20°C) prior to analysis. Water from an eight-hr uptake experiment was analyzed for metabolites by 2-dimensional TLC. pH was adjusted to 4.0 with glacial acetic acid and water was passed through 100 ml of wet XAD-4 resin precleaned by the method of Garnas (1975). No breakthrough of radioactivity was detected by counting aliquots of water which passed through the resin column. The resin column was eluted sequentially with 250 ml each of diethylether and acetone. The solvents were combined and one ml subsamples were used to determine ^{14}C activity. Solvents were dried by the addition of 50 ml petroleum ether followed by passage over anhydrous sodium sulfate. Volume was reduced as previously described and metabolites were isolated by 2-dimensional TLC with pentane:ether (9:1) and toluene:methylene chloride:methanol (25:10:1) (Pitts *et al.* 1978). The spots were identified with UV light and quantified by LSC. Standards of B(a)P metabolites for TLC co-chromatography were obtained from the National Cancer Institues' Standard Chemical Reference Repository. Water was also analyzed for B(a)P by high pressure liquid chromatography (HPLC). After chironomids were removed, 200 ml water samples were taken from uptake aquaria and uptake controls (no chironomids) and extracted with hexane (3 × 50 ml). The three hexane extracts from each water sample were combined and dried with anhydrous sodium sulfate. Extract volumes were reduced to <0.25 ml as previously described, diluted to 0.5 ml with methanol and analyzed by a Varian Model 5000 HPLC system with a 254 nm fixed wavelength detector. Recovery of ^{14}C-B(a)P from spiked water was 77.6 ± 6%. Separations were made with a Micro-Pak MCH-10 reverse-phase column (35 cm long) equipped with a Whatman guard column of Co·Pel C_{18}ODS on 35 μm particles using gradient programmed elution conditions at 28°C (Johnson *et al.* 1977). The gradient was from 75% acetonitrile:25% water to 90% acetonitrile. Acetonitrile (90%) was pumped through the column for five min before recreating the initial conditions. Chironomid extracts were analyzed by HPLC with a Varian Fluorichrome detector with 7-54 and 7-60 excitation filters (bandpass 250-390 nm, peak 360 nm) and 4-76 and 3-72 emission filters (bandpass 430-650 nm, peak 525 nm). Gradient elution was from 30% acetonitrile to 90% acetonitrile in water at 2% min^{-1}.

Data Analysis

Distribution of B(a)P and metabolites in a static uptake test is represented schematically in Figure 1. The data supported and assumptions of 1) constant water B(a)P concentration, 2) low water metabolite concentration ($K_3 \approx 0$). Under these conditions, bioconcentration of total ^{14}C was described by equation 1

$$\frac{dC_a}{dt} = K_1 C_w - (K_{-1} + K_{-3}) C_a \qquad (1)$$

where

C_a = ^{14}C concentration in animal (ng/g wet wt)
C_w = ^{14}C concentration in water (ng/ml)
K_1 = B(a)P uptake rate constant (hr^{-1})
K_{-1} = B(a)P depuration rate constant (hr^{-1})
K_{-3} = Metabolite depuration rate constant (hr^{-1}).

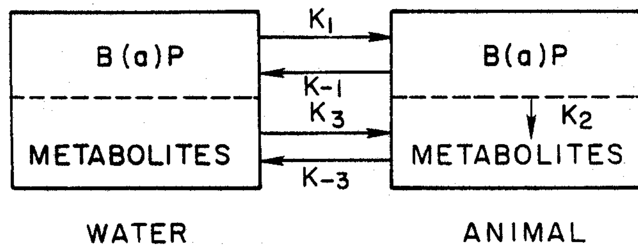

Fig. 1. Distribution of B(a)P and metabolites in static uptake test

Table 1. Bioaccumulation parameter estimates for total ^{14}C in *Chironomus riparius* using one- and two-compartment models

Model and assumptions	Parameter estimates		Depuration half-life (hr)
One compartment	K_1	K_{-1}	
$C_a = (K_1/K_{-1})\, C_w\, (1 - e^{-K_{-1}t})$	214 ± 20	0.22 ± 0.04	3.1
Assumes constant infusion			
B(a)P concentration units ($ng \cdot g^{-1}$ wet weight in animal; $ng \cdot ml^{-1}$ in water)[a]			
Two compartment	K_1	K_{-1}	
$Q_a = [(K_1 \cdot Q_{total})/(K_1 + K_{-1})](1 - e^{-(K_1+K_{-1})t})$	0.039 ± 0.003[b]	0.15 ± 0.001	4.8
Assumes constant B(a)P mass balance within experimental system			

[a] Assumes density of water is $1\ g \cdot ml^{-1}$
[b] In concentration units $0.039 \pm 0.003\ hr^{-1} = 190 \pm 15\ hr^{-1}$, which compares favorably with one compartment model
All parameter estimates = Mean ± SE, for pooled data from triplicate experiments
K_1 = Uptake rate constant (hr^{-1}); K_{-1} = Depuration rate constant (hr^{-1}); Q_a = Mass ^{14}C as B(a)P (ng) in experimental system; t = hr

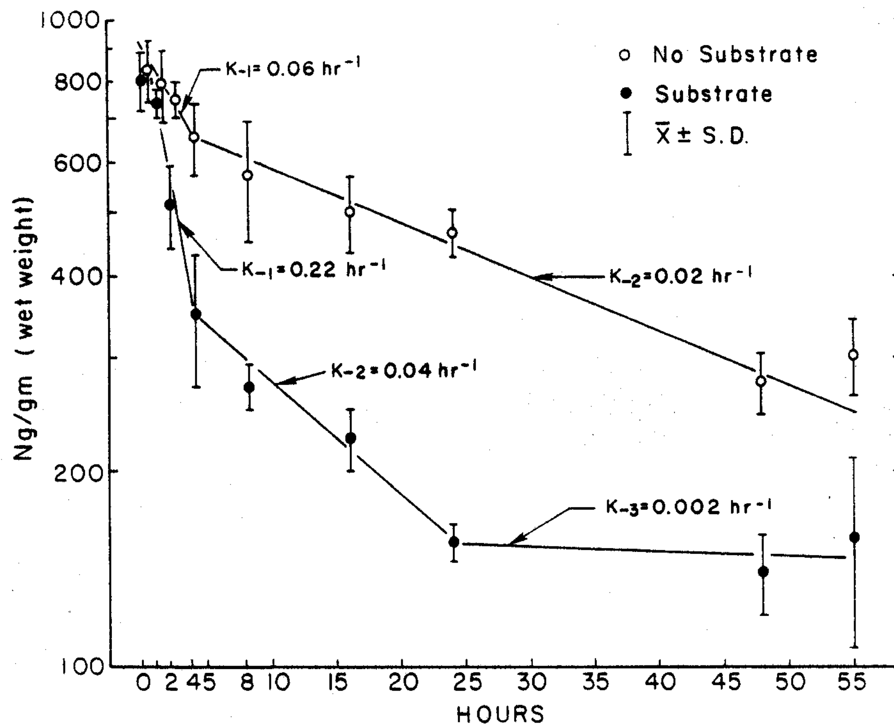

Fig. 2. Depuration of ^{14}C by *C. riparius* with and without substrate. Concentration expressed as ng B(a)P$\cdot g^{-1}$, (wet weight) chironomid, assuming all ^{14}C is B(a)P. Each point is the mean of three determinations ± SD

Low metabolite concentration in water and high metabolite concentration in chironomids suggested that $K_{-3} << K_{-1}$, and a single overall depuration rate constant designated K_{-1} was assumed. Equation 2 is the integral form of equation 1 using these assumptions

$$C_a = (K_1/K_{-1})\, C_w\, (1 - e^{-K_{-1}t}). \quad (2)$$

This is the familiar first order equilibrium model, and at steady state, the bioconcentratiön factor (BCF) can be calculated as shown in equation 3

$$Ca/Cw = K_1/K_{-1} = BCF. \quad (3)$$

A simple two compartment model (equation 4) which did not assume constant water concentration but does assume constant mass within the system was also used to estimate K_1 and K_{-1} (Giesy *et al.* 1980)

$$Q_a = [(K_1 \cdot Q_{total})/(K_1/K_{-1})] (1 - e^{-(K_1+K_{-1})t}) \quad (4)$$

where

Q_a = mass of ^{14}C in animals
Q_{total} = total mass of ^{14}C in experimental system.

Large concentrations of metabolites in chironomids indicated a large biotransformation rate. There were insufficient data to merit independent estimates of K_2 using a model analogous to equation 1 for chironomid metabolite accumulation. The importance of biotransformation was shown instead by comparing calculated BCF (K_1/K_{-1}) with steady state total ^{14}C and B(a)P. Loss of ^{14}C in depuration experiments was biphasic. Rate coefficients were estimated from semi-log plots by linear least-square fits. Rate constants, steady state concentrations and asymptotic 95% confidence limits were estimated by the Marquardt iterative least squares procedure (Procedure NLIN, Statistical Analysis System, Barr *et al.* 1979).

Results and Discussion

Data from triplicate uptake experiments were fit to one compartment and two compartment models (Table 1). Kinetics of ^{14}C accumulation were accurately described by the one compartment model. Bioaccumulation was rapid and steady state activity was approached after eight hr. The uptake rate constant (K_1) for the one compartment model was 214 $\pm$ 20 hr^{-1} when calculated using water and animal concentrations. A one compartment model is only appropriate if water concentration is constant. When calculated from activity measurements, water concentration was 1.38 $\pm$ 0.32 $ng \cdot ml^{-1}$ ($\bar{X}$ $\pm$ SE, n = 3) initially and did not decrease significantly during the course of the experiment (1.11 $\pm$ 0.25 $ng \cdot ml^{-1}$ after eight hr). Analysis by TLC and HPLC indicated no measurable amounts of non-B(a)P materials in the control water (no chironomids) or through two hr in water containing chironomids. After eight hr, water containing chironomids had 86% of ^{14}C as B(a)P and 14% as unidentified metabolites. Therefore, water activity through eight hr was a good measure of B(a)P concentration. If water concentration changes significantly, and observed losses are into the animals, a two compartment model is required. Two compartments models have a major advantage in not requiring a constant source term ;however, a knowledge of total mass is required. They are used in experimental systems where mass additions can be controlled but may be of limited use in natural systems with unknown mass additions. The depuration rate constant (K_{-1} = 0.22 $\pm$ 0.04 hr^{-1}) was estimated by the one-compartment model from data collected during the exposure phase. The time required for loss of 50% of ^{14}C was between 3 and 4 hr. The two compartment model estimate of the same constant (K_{-1} = 0.15 $\pm$ 0.001 hr^{-1}) was in good agreement. Depuration rate constants were also estimated by placing exposed animals in clean water (Figure 2). A semi-log plot of depuration shows a slope change between four and five hr, which suggests biphasic depuration. Animals in chambers containing substrate depurated about 60% of initial activity within four hr. The initial depuration rate constant (K_{-1} = 0.22 hr^{-1}) agrees well with the overall depuration rate constant calculated from the accumulation data. The initial depuration includes a component due to K_{-1} from 4 to 24 hr. This component was comparatively small (K_{-1} = 0.04 hr^{-1}) and accounted for 22% of initial activity released. Between 24 and 55 hr, 19% of the maximum ^{14}C concentration attained remained as bound B(a)P and metabolites. Animals exposed to clean water in the absence of substrate also exhibited biphasic depuration, but the rates were consistently slower. Approximately 23% of the initial activity was rapidly released. The slower depuration was not complete when the experiment was terminated after 55 hr. As noted, the initial depuration rate constant was similar to the overall depuration constant derived from a monophasic model and for B(a)P the monophasic model was adequate to summarize the data. This initial rate constant gives the best overall estimate of net flux of B(a)P and metabolites from chironomids for ecological fate studies. The mechanism by which substrate increased depuration in chironomids in unknown but may involve increased biotransformation of B(a)P related to gut processes, an active role by bacterial food, behavioral changes by chironomids on the paper towel substrate, or increased gut contents into which the ^{14}C labeled compounds can partition. Simple clearance of gut contents was probably not important in observed initial depuration since animals were starved for eight hr prior to exposure and for eight hr during exposure. More rapid depuration by feeding animals may be due to partitioning of B(a)P from the animal into uncontaminated food. Synthesis and excretion of the peritrophic membrane facilitates excretion of DDT by mosquito larvae (Abedi and Brown 1961) and may be important. Only 10% of activity in chironomids was associated with exoskeleton (Figure 3). Therefore, surface losses were probably not important in the observed initial depuration rates. Exoskeleton may influence initial uptake rates in chironomids since nearly 50% of activity at 0.5 was in the exoskeleton.

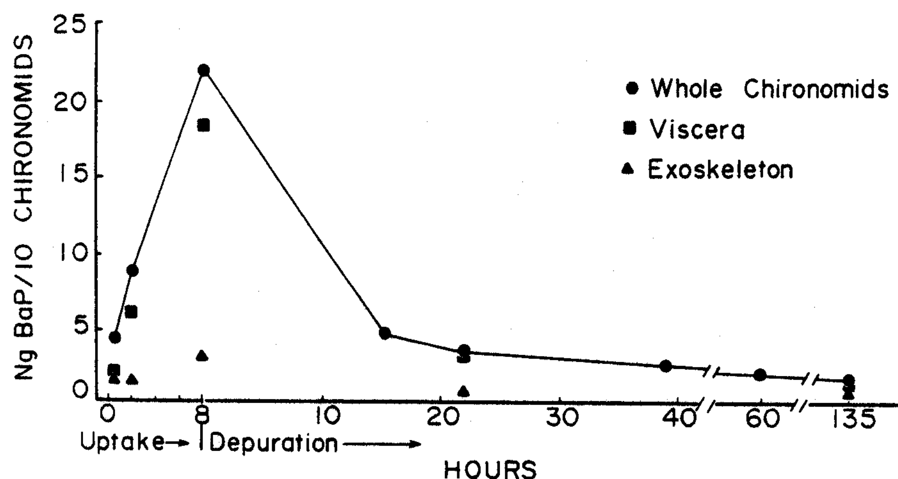

Fig. 3. Uptake and loss of ^{14}C in whole *C. riparius*, exoskeleton and viscera. Each point represents the mean of three determinations ± SD

Table 2. Biotransformation of ^{14}C-B(a)P by *Chironomus riparius*

Hr	% Recovery[a]	^{14}C-B(a)P (ng·g^{-1}) TLC	HPLC	% Metabolites[b]	Biotransformation rate (nmoles·g^{-1}·hr^{-1}) TLC	HPLC
0.5	142 ± 33[c]	767 ± 137	N.D.	57 ± 9	7.4 ± 3.0	N.D.
1.0	103 ± 23	1166 ± 199	N.D.	43 ± 2	3.6 ± 0.7	N.D.
2.0	97 ± 13	1349 ± 217	2180 ± 286	57 ± 2	3.6 ± 0.9	1.9 ± 0.7
4.0	80 ± 4	1935 ± 115	2372 ± 115	60 ± 2	2.7 ± 0.3	2.2 ± 0.3
8.0	77 ± 6	2085 ± 291	2760 ± 286	72 ± 2	2.7 ± 0.6	2.4 ± 0.9

[a] DPM of sample extracted/DPM of combusted sample
[b] DPM Total − DPM B(a)P/DPM Total × 100
[c] Mean ± SE, n = 3

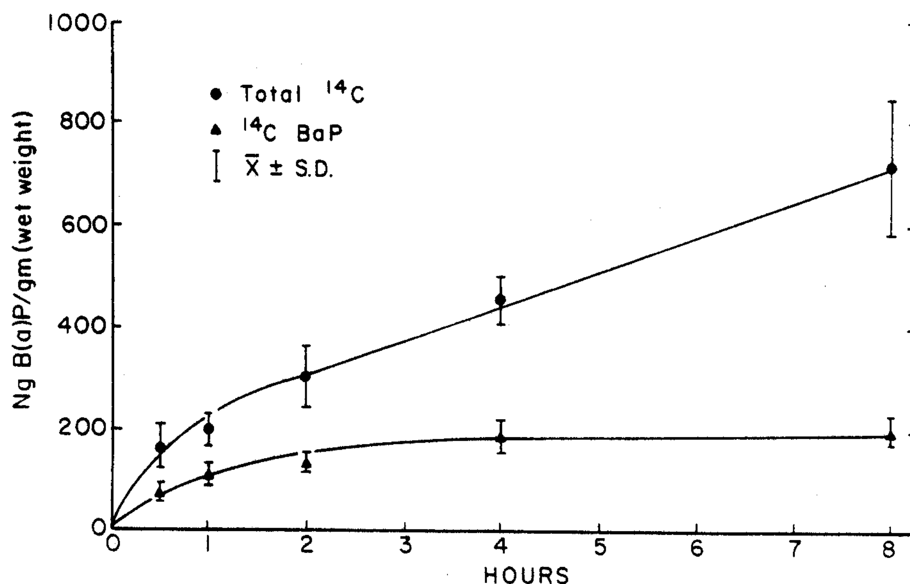

Fig. 4. Accumulation of ^{14}C B(a)P (△) as determined by TLC, and B(a)P plus metabolites (○) expressed as ng B(a)P·g^{-1} (wet weight) chironomid. Each point represents the mean of three determinations ± SD

Biotransformation

Information on biotransformation is important in hazard assessment, because some metabolites of B(a)P are mutagenic and carcinogenic (Lehr *et al.* 1978). The biotransformation of B(a)P by *C. riparius* was rapid. After one hr, 43 ± 2% $\bar{X}$ ± SE) of chironomid ^{14}C activity existed as non-B(a)P metabolites, as determined by TLC, and after eight hr, 72 ± 2% was non-B(a)P) (Table 2 and Figure 4).

The large percentage of metabolites was not due to B(a)P oxidation on TLC plates as evidenced by a 92.3 ± 3% recovery of ^{14}C-B(a)P from spiked chironomid controls. Biotransformation of organic compounds is common for chironomids (Kawatski and Bittner 1975 Estenik and Collins 1979). The rate of biotransformation, calculated from metabolite activity and known B(a)P specific activity, was 3.2 ± 0.5 nmoles·g dry weight^{-1}·hr^{-1} (Table 2) and compares favorably, with that for conversion of aldrin to dieldrin (1.44 nmoles·g^{-1}·hr^{-1}) (Estenik and Collins 1979). The large biotransformation rate at 0.5 hr is due to sampling time errors, as suggested by the large standard error. While the data are not sufficient for careful statistical analysis, it was concluded that there was no apparent change in biotransformation rate with time. The rapid biotransformation observed will cause the bioconcentration factor (BCF) reported in terms of parent compound to be very low; Lu *et al.* (1977) reported a BCF of "0" for B(a)P in fish due to complete biotransformation. The high uptake rate constant and BCF reported here in terms of ^{14}C suggest very large accumulations of B(a)P metabolites, some of which may be carcinogenic. Accumulation of metabolites may be the most important aspect of environmental risk assessment for certain PAH; therefore the reason for reporting both an apparent BCF (total ^{14}C) and a true BCF (parent compound only).

The major metabolites depurated into water during the uptake phase were polar compounds. Using co-chromatography of authentic standards, the major metabolite was 3-hydroxy-B(a)P and represented 4.4% of water activity after eight hr. This compound has also been reported as the major metabolite in mammals (Nebert and Gelboin 1968). The 3-hydroxy B(a)P was fluorescent blue on the original TLC plate, but became a fluorescent red air oxidation product on co-chromatography plates. Small amounts of three other metabolites were detected. These had relative abundances of I) 0.35, II) 0.06 and III) 0.015 compared to 3-hydroxy B(a)P. Metabolite I had an R_f value corresponding to 7-hydroxy-B(a)P in the original 2-dimensional TLC plate, but apparently degraded and did not move from the origin after transfer and cochromatography with a standard. Metabolites II and III were tentatively identified as the 9,10- and 7,8-dihydrodiols of B(a)P respectively. In general, the metabolites were quite labile and degraded relatively rapidly, even in the dark at −20°C under organic solvent. Some of the polar compounds observed may have resulted from degradation during storage of both water and chironomid extracts. Rapid analysis should be made after sampling.

It was assumed that all ^{14}C was B(a)P and an apparent BCF (ng B(a)P·g^{-1} wet weight/ng B(a)P·ml^{-1}) of 650 was calculated from chironomid steady state activity at eight hr. An estimate of 970 was obtained using the ratio K_1/K_{-1}. The presence of substantial quantities of non-B(a)P materials in chironomids (Figure 4, Table 2) indicated that biotransformation was significant, and the actual BCF was about 200. B(a)P was at apparent steady state after 4 to 8 hr (Figure 4). Lu *et al.* (1977) reported a similar low BCF of 107−149 for B(a)P in mosquito larvae, with only 46% of the activity present as parent compound. Southworth *et al.* (1978) predicted a BCF of 13,000 for B(a)P in *Daphnia*, calculated from the octanol-water partition coefficient, but assumed no biotransformation. The difference in BCF between *Daphnia* and both chironomids and mosquito larvae was probably related to active biotransformation and excretion processes in the latter.

These studies demonstrate that donor controlled linear compartment models may be used to predict steady state bioaccumulation of B(a)P and metabolites (total ^{14}C) in *Chironomus riparius*. However, the BCF for parent compound is reduced by a factor of three relative to that of ^{14}C by active biotransformation of B(a)P in this species. The frequently used correlations between octanol-water partition coefficient and bioaccumulation may not be adequate for predictions of B(a)P fate in many species.

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