Chemical Reactions Producing Chrysene as an Artifact of Some K-Region Metabolites of Benzo(*a*)pyrene[1] (37359)

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Miller (1) has reported the formation of protein-bound derivatives of benzo(*a*)pyrene (B(*a*)P) (Fig. 1A) in the epidermal fraction of mouse skin. One of us (2) has reported the isolation and identification of chrysene (Fig. 1B) as a metabolite of B(*a*)P. During our early work (2) on whole-body metabolism, total viscera was extracted and chrysene was isolated after HCl treatment; however, no attempt was made to determine whether chrysene occurred as such in the protein or if it was produced by the chemical treatment.

During this early work on whole-body metabolism, it was suggested that chrysene could come from the degradation of, among others, protein-bound metabolites of B(*a*)P. According to Pullman and Pullman (3) the primary process in cancer induction is an addition reaction of a cellular component, for example, protein at the K-region. The double bond between carbon atoms 4 and 5 of B(*a*)P (Fig. 1A) is a chemically reactive phenanthrene double bond (K-region) where the addition of a protein may occur. Since chrysene (Fig. 1B) does not contain the K-region of B(*a*)P (Fig. 1A), it is possible that a K-region metabolite of B(*a*)P, perhaps bound to protein, underwent degradation to produce chrysene (2).

Gelboin *et al.* (4) and Hultin (5) demonstrated the binding of carcinogens to proteins in *in vitro* systems.

The objective of the present work was to isolate a sample of protein from an *in vitro* system metabolizing B(*a*)P (6) and to determine whether chrysene occurred in the protein as such as a metabolic product or if it was produced only after chemical degradation. Results of the present experiments showed that the chrysene arises as an artifact of the chemical procedure in contradiction to our earlier claim (2). A preliminary report of our work has appeared (7).

Material and Methods. Purification of B(a)P. B(*a*)P (Fig. 1A) (Aldrich Chemical Co., Milwaukee, WI), 3 g 98% pure, was dissolved in 1200 ml cyclohexane, pretreated with 12 ml concentrated H_2SO_4, and shaken with 10 ml 80% H_2SO_4 to remove basic impurities. (Without H_2SO_4 treatment the B(*a*)P shows a mass peak at *m/e* 279, probably due to the presence of dibenzcarbazole.) The B(*a*)P was then passed through an alumina (1500 g) column (11.5% water content) to retain some orange and brown material on top of the column. The bluish lower hydrocarbon band was extruded, extracted with acetone and benzene, solvents removed, and the residue was chromatographed, in the ascending way, in 50 mg quantities on 18 in. × 22 in. 3MM Whatman paper soaked in *N,N*-dimethyl formamide (8), with iso-octane as the solvent. During this stage, 7,8,9,10-tetrahydro-B(*a*)P, R_f 0.7–0.8, (greenish fluorescence) and 4,5-dihydro-B(*a*)P, R_f 0.25–0.35, (light blue fluorescence) moved ahead of B(*a*)P, R_f 0.1–0.2. The B(*a*)P was extracted with benzene at room temperature, the solvent was removed and the product was redissolved in benzene (1 g/100 ml), and 100 ml methanol were added. Then Darco G-60 (activated charcoal) 1 g/g B(*a*)P was added, stirred and filtered. The residue obtained after concentration of the solvent was crystallized thrice more from 1:1 benzene–

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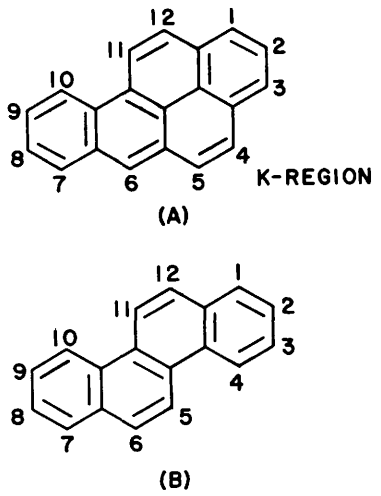

FIG. 1. (A) Chemical structure of benzo(*a*)pyrene showing the K-region between carbon atoms 4 and 5 as suggested by Pullman and Pullman (3). (B) Chemical structure of chrysene in which the K-region is not present.

methanol. The product was a light yellow solid with an mp of 175–176°. The mass spectrum of the B(*a*)P used in our biological experiments still contained traces of dihydro-, with peak at *m/e* 254, tetrahydro-B(*a*)P, *m/e* 256, and most likely monomethyl (B(*a*)P), *m/e* 266 but no peak for chrysene (structure shown in Fig. 1B).

Incubation system. The system used was essentially the same as that described by Conney *et al.* (6). The following were used: glucose-6-phosphate, (Sigma Chemical Co.), 0.03 *M*, 200 ml; ATP (Sigma), 0.01 *M*, 200 ml; KCl, 2.0 *M*, 100 ml; Na/K PO_4 buffer, pH 7.5, 0.1 *M*, 500 ml; NADP (Sigma) 200 mg; NAD (Sigma) 200 mg; $MgCl_2$, 0.04 *M*, 100 ml; water 1350 ml, enzyme (500 ml 20,000*g* supernatant of 20% homogenate in 0.25 *M* sucrose). The livers of untreated female Wistar rats (weighing 150–180 g at sacrifice) were used. Final volume of mixture was three liters.

Protein was determined according to the Kalckar modification (9) of the method of Warburg and Christian. In each experiment, liver weight was kept constant. Purified B(*a*)P, 195 mg prewarmed at 37° in 50 ml distilled alcohol in a 100-ml flask, was decanted into the system, mixed carefully, and the mixture was distributed in 500 ml portions in 1 liter Erlenmeyer flasks, and incubated for one hour with gentle shaking, open to air but in dulled light. Two experiments were carried out.

A control experiment was run with a boiled enzyme preparation.

Processing the incubation mixture. The incubation mixture was poured into 3 liters of ice-cooled acetone containing 1 mg/ml $Na_2S_2O_4$ to inhibit air oxidation of the metabolites, 15 m*M* citric acid and 3 m*M* EDTA (pH 3.6). We have referred to $Na_2S_2O_4$, citric acid and EDTA as the additives. Both citric acid and EDTA were used to chelate magnesium and perhaps other metals picked up from laboratory dust. (Conney *et al.* (6) encountered difficulties in isolating their products in the presence of magnesium.) The mixture was centrifuged on a Sorvall RC2-B at 3000*g* for 10 min to separate the supernatant from the sediment. The sediment was extracted twice with 10 vol, each run, of 50% acetone containing the additives. The sediment was stored in a cold room for working up later. Acetone was distilled off from the supernatant; the aqueous portion was chilled in ice and dissolved protein (10, 11) was precipitated with 1 *N* $HClO_4$ at 0°. After standing one hour in ice, the solid was centrifuged off as above to yield the aqueous supernatant. This sediment was combined with the earlier one that was stored in the cold room and was extracted successively with 7 vol acetone, 7 vol chloroform and then 7 vol alcohol at 70° to remove lipids (12). (This extraction with organic solvents will also remove any free organic compounds.) The combined organic extract was called the lipid extract. To remove nucleic acids (10, 11, 13), the solid residue was stirred with the virtis homogenizer (The Virtis Company, Inc., Gardiner, NY) with 7 vol of 1 *N* $HClO_4$ for 5 min. Then the mixture was heated at 90° for 10 min, cooled in ice and centrifuged to remove the supernatant. The sediment was extracted for nucleic acids twice more.

The protein was suspended in 2 vol of water, neutralized with 10% NaOH solution, and then precipitated with 12 vol of alcohol

(86% final alcohol concentration). The precipitate was washed successively with 66% alcohol two times, with methanol once, with benzene once at 50°, and finally twice with ether.

The residue left was regarded as the protein wet weight (21 g). The protein was suspended in 10 vol of dioxane and treated with 21 ml 40% NaOH solution. With constant mechanical stirring, the protein suspension was treated with portions of powdered $NaIO_4$ (sodium metaperiodate, Fisher Scientific Co., St. Louis, MO). After 21 g $NaIO_4$ (same weight as that of the wet protein) had been added the mixture was stirred overnight at room temperature. Next morning the mixture was filtered and the solid on the filter paper was extracted with 4× 180 ml ether. The ether extract was divided into 4 parts and was used to extract the aqueous filtrate. The ether extract was washed successively with 20 ml water two times, with 20 ml 5% sodium thiosulfate solution three times, with 20 ml water two times and then dried over anhydrous sodium sulfate. After removal of solvent the residue (a few mg) obtained was chromatographed on 18 in. × 22 in. 3 MM Whatman paper in a 2-dimensional run. The first-dimension run (System 1) consisted of soaking the paper in *N,N*-dimethyl formamide, spotting the sample in one corner (corner of lines drawn at right angles to each other, 4 in. from either edge), drying the paper for 90 min, and running in the ascending direction with iso-octane for about 5–6 hr (8). After overnight drying the R_f values of all fluorescent and nonfluorescent bands were noted. For the second dimension (System 2) the paper was turned at right angles and run for 5–6 hr in cyclohexane(1)–methanol(10)–water(1) in the ascending direction. After overnight drying the R_f values of all fluorescent and nonfluorescent bands were recorded. All bands were cut out separately, extracted thrice with distilled benzene and thrice with distilled 95% alcohol and the solvents removed. Each band was again chromatographed in the ascending way on silica paper (SG81 paper Reeve Angel, NJ) with benzene (System 3) as the developing solvent (Raha, unpublished). After about 15-hr run, R_f values of all bands, fluorescent and nonfluorescent, were recorded. Each band was separately cut out, extracted into 2 ml 95% alcohol and ultraviolet spectrum recorded on the Cary 15 Spectrophotometer between 410 and 250 nm. Products were identified according to ultraviolet spectra described by Conney *et al.* (6), Sims (14), and Freidel and Orchin 15).

Results and Discussion. The protein was prepared by extensive washing with organic solvents. Periodate cleavage of this protein, in the presence of a strong base (NaOH), yielded 19.5 μg chrysene (Fig. 2, Spectrum a). R_f values of the produced chrysene were: 0.30 (System 1), 0.47 (System 2), and 0.93 (System 3).

In the second experiment NaOH was omitted and chrysene was not produced.

In the boiled enzyme control, chrysene could not be produced by $NaOH/NaIO_4$ treatment. The possibility was, therefore, suggested that a metabolically produced sub-

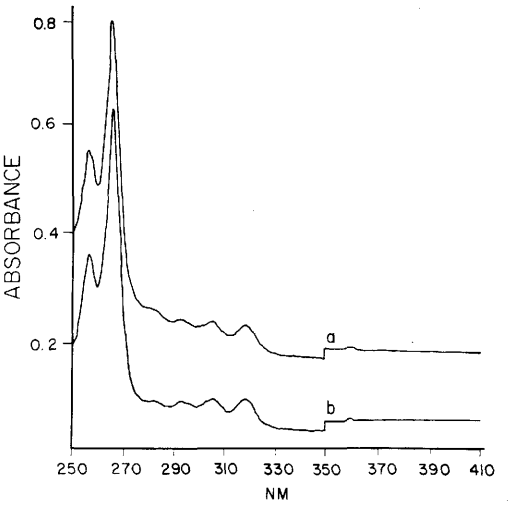

FIG. 2. Chrysene chemically produced from a protein-bound metabolite(s) of B(*a*)P. After running the sample on silica (SG-81) paper the chromatographic band at R_f 0.93 was cut out, extracted with 16 ml of 95% alcohol, and the spectrum taken. From the peaks recorded in spectrum (a) the degradation product of the B(*a*)P metabolite(s) appeared to be chrysene (15). This was confirmed by superimposing spectrum (b) taken from a known sample of chrysene. An absorbance of 1 at 267 nm = 1.52 μg/ml chrysene (8).

strate was used by NaOH and $NaIO_4$ to yield the chrysene.

No intermediates were sought in the conversion of B(*a*)P to chrysene, so far. However, according to some authors an epoxide may result metabolically (16–18) from a hydrocarbon which would react with a protein to produce a protein-bound trans-dihydrodiol (19).

In the present series of experiments NaOH could have released the 4,5-dihydrodiol from protein-bound metabolite with, perhaps, structural inversion and then $NaIO_4$ produced chrysene dialdehyde (20, 21). It is possible that in *in vivo* studies (2) dialdehyde is produced by a mechanism involving oxidation in the absence of $NaIO_4$. The aldehyde can undergo decarbonylation by alkali (22), acids (23), and even free radicals (24) to a hydrocarbon. Thus, perhaps, chrysene dialdehyde yielded chrysene.

The aqueous supernatant and the lipid extract in both experiments contained 1-OH, 4- or 5-OH (25), 3-OH, 8-OH, and perhaps other hydroxy-B(*a*)Ps as reported by others (6, 14). Also present were 4,5-dihydrodiol (25) and perhaps some other dihydrodiol (14). Free B(*a*)P, 12–20 mg, was recovered in each experiment. Lipid extract did not contain chrysene.

In the boiled enzyme control, 147.6 mg B(*a*)P, 591 μg 6, 12-dione (26) and some other diones were recovered. From the lipid extract, after saponification with 2 *N* alcoholic KOH, 61 μg of what appeared to be 1-OH-B(*a*)P (14) was isolated. The standard used was 3-OH-B(*a*)P (a gift from Dr. H. L. Falk). Conney *et al.* (6) also suggested that some of the products could result due to chemical treatments.

For *in vivo* studies (2) female Swiss mice were used. In the present series of *in vitro* studies female Wistar rats were used. In both studies chrysene was obtained. The possibility, therefore, does exist that the same substrate, free or bound or both, is converted into chrysene. The metabolic formation of the substrate is, therefore, perhaps not dependent on species. To determine species dependence, more species should be studied.

Summary. Alkali catalyzed periodate cleavage of what seems to be a protein-bound metabolite of B(*a*)P produced chrysene.

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