Benzo(a)pyrene Metabolites Formed by Plant Cells

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Benzo(a)pyrene, added to the nutrient solutions of cell suspension cultures of *Chenopodium rubrum*, was metabolized to oxygenated derivatives. With increasing time of incubation decreasing amounts of the added ^{14}C-radioactivity could be extracted with 70% methanol or be isolated as benzo(a)pyrene. The amount of metabolites formed increased with increasing time of incubation. After 48 hours most of the radioactivity was found in the extracted residue. These observations and some other indications give reason to believe that some of the metabolites are linked to proteins or nucleic acids.

Introduction

Polycyclic aromatic hydrocarbons are ubiquitous pollutants of the atmosphere, waterways and soil. They are also present in human food. Some of these compounds are known for causing cancer in experimental animals and are suspected to be carcinogenic for human beings. The uptake and metabolism of polycyclic aromatic hydrocarbons, especially of benzo(a)pyrene by plants, have been a subject of several investigations [1-5]. Experiments with cell cultures derived from vertebrates [6], with alveolar macrophages [7, 8] or bacteria [9] show that benzo(a)pyrene is metabolized to oxygenated derivatives. In this paper benzo(a)pyrene metabolites formed by plant cells are being reported.

Materials and Methods

Cell suspension cultures

Cell suspension cultures of *Chenopodium rubrum* were started from the corresponding callus cultures which had been derived from meristems of sterile grown seedlings. The culture methods have been described previously [10]. 2 grams of fresh cell material was inoculated in 40 ml of Murashige and Skoog liquid medium in 200 ml Erlenmeyer flasks and grown on a gyrotory shaker (Braun, Melsungen) at a revolution rate of 120 rpm. In order to know the growth behaviour of the *Chenopodium* cell culture a growth curve was drawn up. The changes in pH

and conductivity of the medium during the growth of the cell suspension cultures were recorded daily.

Measurement of growth: The cells from 5 separate culture flasks, containing 40 ml of culture each, were harvested daily by vacuum filtration, lyophilized and cell dry weight determined.

Measurement of pH and conductivity: After removal of cell material by vacuum filtration, conductivity of the medium was determined using the conductivity meter CDM 2 and conductivity cell CDC 104 (Radiometer, Kopenhagen). The same solutions were used for measurement of pH.

Chemicals

Labelled [7,10-^{14}C]benzo(a)pyrene was purchased from Amersham-Buchler, Braunschweig. Stock solutions containing 1 μCi benzo(a)pyrene/ml in ethylene glycol monomethyl ether were stored at $+ 4 \,°C$. Unlabelled benzo(a)pyrene (Carl Roth OHG, Karlsruhe) was dissolved in ethylene glycol monomethyl ether (0.5 mg/ml).

Experimental

Ten days after inoculation of cells 0.5 mg (5×10^{-5} M) labelled benzo(a)pyrene, dissolved in ethylene glycol monomethyl ether was added to the nutrient solutions. At that time the cell cultures were at the beginning of the statioary growth phase and at least took 2 more days to reach the maximum of the growth cycle. Depending on the experiment the incubation time lasted for 12, 24 or 48 hours. Thereafter cells were harvested by vacuum filtration, washed several times with destilled H_2O and lyophilized. Cell material as well as growth medium were analysed for benzo(a)pyrene and metabolites.

Aliquots of dry cell material were combusted in a Packard Tri-Carb Sample Oxidizer Model 306 and the radioactivity determined by liquid scintillation counting. The rest of the cell material was extracted with 70% methanol. After determination of the radioactivity the methanol extract was concentrated and this was furher extracted with ethyl acetate. The ethyl acetate extract was reduced to 1 ml volume. One part was used for thin-layer-chromatography (TLC) and the other for high pressure liquid chromatography (HPLC).

Thin Layer Chromatography: Aliquots of the ethyl acetate extracts were applied to thin layer plates (Kieselgel PF 254, Merck, Darmstadt, 1 mm layer) and developed in ether : *n*-hexane (60 : 40). Radioactive spots were detected with a TLC-Scanner (Berthold). Spots of interest were eluted with methanol and the compounds characterized by UV-spectra.

High Pressure Liquid Chromatography: For high pressure liquid chromatography, an instrument from Waters (2 pump model 6000, solvent programmer model 660) with a fluorescence detector from Dupont (model 836), was used. Column packing: Nucleosil-5-C 18 (Macherey and Nagel). Column dimensions: 200×4 mm. A part of the ethyl acetate extract was evaporated to dryness and redissolved in 20 μl methanol. Aliquots of 5 to 10 μl were injected for separation by HPLC. The separation of metabolites was accomplished by elution (1 ml/min) with a methanol : water gradient ranging from initial 30% : 70% to a final concentration of 70% : 30%. Column temperature 31 °C. Fractions of 0.5 ml were sampled and mixed with 2.5 ml scintillator (Xylol : Triton 100 : ethylene glycol monomethyl ester 600 : 300 : 100 + 8 g PPO and 0.6 g dimethyl-POPOP as the counting medium. The radioactivity was determined in Mini-vials in a Packard 2425 liquid scintillation counter.

Results

Growth cycle of Chenopodium rubrum cell culture

In order to characterize the applied cell suspension culture or *Chenopodium rubrum* a growth curve was drawn up (Fig. 1).

The increase in biomass within a growth period of 12 days was more than 7.5 fold of the incubated amount. Beyond the 13th day of culture cell dry weight decreased. While the pH except for an increase during the end of the stationary growth phase, remained nearly constant througout, a considerable change in the conductivity was observed.
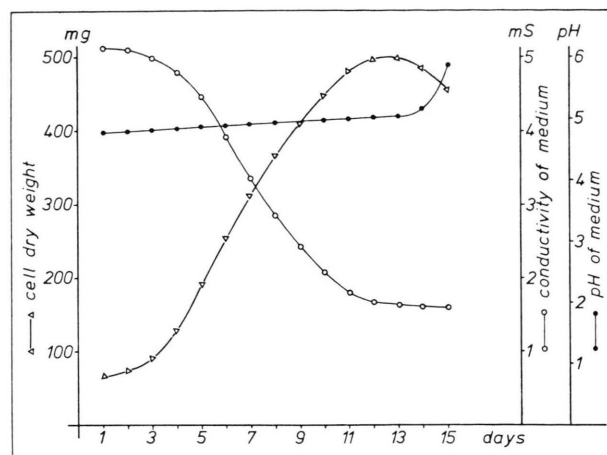

Fig. 1. Changes in cell dry weight, pH and conductivity of the medium of *Chenopodium rubrum* cell suspension cultures for a growth period of 15 days.

The curve of conductivity is virtually a mirror image of the growth curve; during the stationary phase, however, the conductivity of the medium remained constant. By measuring the conductivity of the medium it was thus possible to start the incubation with benzo(a)pyrene always at a defined stage of growth.

Metabolism of benzo(a)pyrene in Chenopodium cell cultures

Previous experiments with *Chenopodium* cell cultures showed that most of the activity of the added benzo(a)pyrene was found in water-soluble compounds. Only $3-6\%$ was detected as benzo(a)-pyrene. In order to study the turnover of benzo(a)-pyrene incubation was stopped after 12, 24 and 48 hour intervals. 0.5 mg benzo(a)pyrene (1 μCi) dissolved in 1 ml ethylene glycol monomethyl ether was added to each of the nutrient solutions of the *Chenopodium* cultures. After harvesting of cells distribution of the radioactivity was determined in the different fractions. The data are presented in Table I.

The time course study demonstrates very clearly the pattern of uptake and metabolism of benzo(a)-pyrene in *Chenopodium* cultures. After 12 hours of incubation 1/4 of the radioactivity could still be detected in the nutrient solution. The whole of radioactivity in the dried cell material could be extracted with 70% methanol and again with ethyl acetate. TL-Chromatograms showed that this activity was present as benzo(a)pyrene. Only 3% metabo-

Table I. Distribution of the added radioactivity in different fractions of *Chenopodium* cell suspension cultures after 12, 24 and 48 hours of incubation with ^{14}C-labelled benzo (a)-pyrene.

fractions	Percent of radioactivity in after		
	12 hours [%]	24 hours [%]	48 hours [%]
Applied radioactivity	100	100	100
Residual activity in culture medium	22.8	9.6	5.8
Dried cell material	63.6	79.0	90.3
Methanol extract	55.2	43.1	38.3
Ethyl acetate extract	49.6	28.9	20.5
Benzo (a) pyrene	45.5	22.0	8.7
Metabolites	2.9	6.9	8.9
Extracted residue	2.7	29.3	53.0

lites had been formed during this time. There was very little radioactivity in the extracted residue. After 24 hours of incubation, however, 80% of the applied radioactivity was assimilated by the cells, but only a little more than half of this activity was extractable with methanol. As compared to the values at 12 hours only a smaller amount of activity could be extracted with ethyl acetate from the water phase of the methanol extract. The activity in the metabolite fraction increased significantly as could be demonstrated by thin layer chromatography. After 48 hours of incubation the cells absorbed more than 90% of the applied radioactivity. Only 1/3 of this activity could be extracted with methanol the balance remaining in the extracted residue. The part which could be determined in the form of benzo (a) pyrene decreased to about 9%, while nearly the same amount of activity was detectable in the metabolite fraction.

The extracts from growth mediums and especially those from the cell material were subjected to further analysis by thin layer chromatography and high pressure liquid chromatography. On the thin layer plates up to 5 metabolites could be detected besides benzo (a) pyrene. In order to characterize and identify these metabolites they were analysed by HPLC. This technique has recently been shown to be a sensitive and rapid method [11] for the detection of benzo (a) pyrene metabolites. The ethyl acetate extracts were concentrated to 1 ml and 10 μl of this injected into the column. The separation of benzo (a) pyrene metabolites by high pressure liquid chromatography and the distribution of the radioactivity is shown in Fig. 2.

In juxtaposition the chromatograms and the distribution of radioactivity obviously show that after 12 hours of incubation 2 compounds could be detected as quinones and 2 as phenols while there were no diols. After 24 hours of incubation a number of fluorescent peaks appeared in the region of the diols. One of these compounds increased in concentration after 48 hours of incubation while one of the compounds in the quinone fraction decreased. The peaks of activity followed the same distribution as could be seen in the fluorescence chromatograms. With increasing time of incubation an increase of activity was also detectable in the polar compound fraction which contained most of the water soluble derivatives. The fluorescent peaks in the beginning of the chromatogram probably originated from compounds extracted from the plant material.

In Fig. 3 two chromatograms of benzo (a) pyrene metabolites have been compared. Both chromatograms were run under identical conditions. One is the already known chromatogram of metabolites formed by plant cells after 24 hours of incubation. The other shows the separation of metabolites formed by liver microsomes and which have been characterized by comparing them with authentic compounds as 3 hydroxy-benzo (a) pyrene, quinones in 1,6 and 3,6 positions and dihydro-dihydroxy-benzo (a) pyrenes in 4,5, 7,8 and 9,10 positions. In comparing these two chromatograms one could conclude that the *Chenopodium* cells form metabolites which are to some extent quantitatively and qualitatively identical but in parts different to metabolites formed by liver microsomes. The plant cells, however, appear to metabolize benzo (a) pyrene to more polar compounds.

Discussion

The study of growth behaviour of *Chenopodium rubrum* cells made it possible to determine the physiological stage of the culture at which benzo (a) pyrene should be added. Taking parsley and soybean cultures Hahlbrock and Kuhlen [12] also found that conductivity of the medium could be used to regulate the growth of plant cells in continuous cultures.

Our experiments with *Chenopodium rubrum* cell suspension cultures showed that with increasing time of incubation the uptake of benzo (a) pyrene also increased. With increasing time of incubation
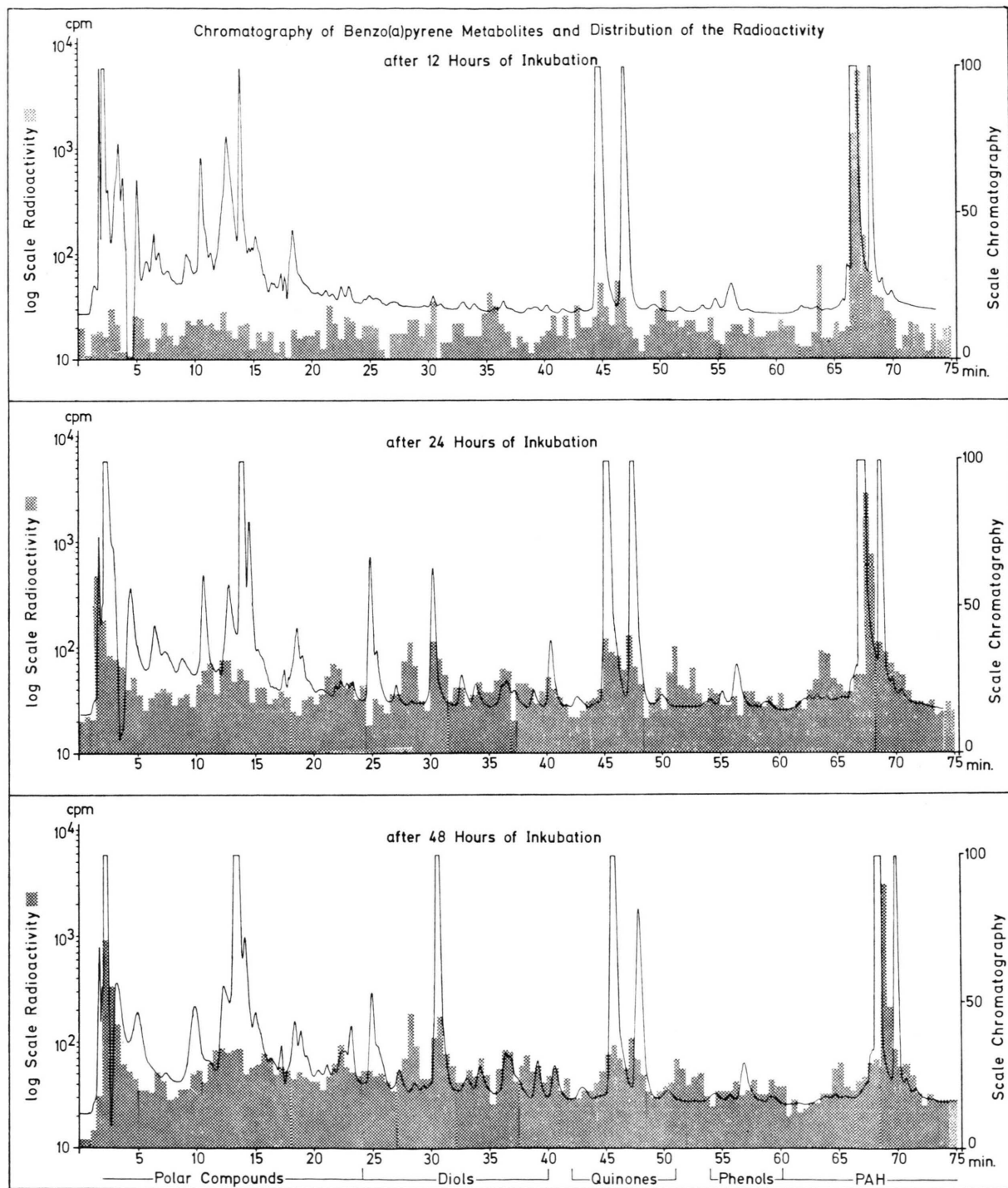
Fig. 2. Chromatography of benzo (a) pyrene metabolites formed by plant cells and distribution of radioactivity in elution fractions of high pressure liquid chromatography after 12, 24 and 48 hours of incubation. Fluorescence: Excitation 325– 385 nm, Emission above 408 nm. PAH = polycyclic aromatic hydrocarbons.
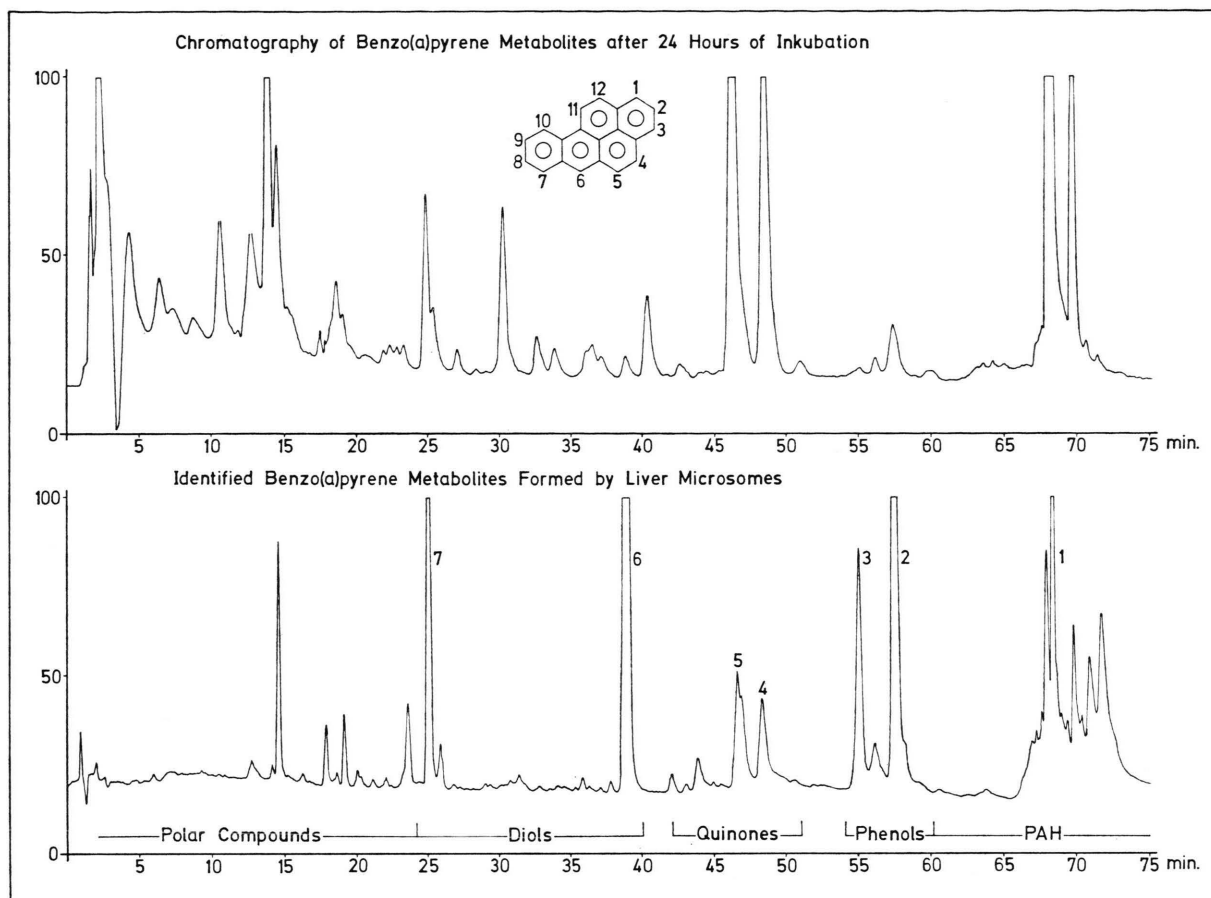
Fig. 3. Chromatography of benzo (a) pyrene metabolites after 24 hours of incubation as compared to chromatography of identified benzo (a) pyrene metabolites formed by liver microsomes. 1) benzo (a) pyrene; 2) 3-hydroxybenzo (a) pyrene; 3) 9-hydroxybenzo (a) pyrene; 4) benzo (a) pyrene-3,6-quinoe; 5) benzo (a) pyrene-1,6-quinone; 6) 7,8-dihydro-7,8-dihydroxybenzo-(a) pyrene; 7) 9,10-dihydro-9,10-dihydroxybenzo (a) pyrene.

decreasing amounts of the radioactivity could be extracted with methanol from the dried cell material. After 48 hours most of the radioactivity remained in the residue. With increasing time of incubation less and less of the radioactivity of the methanol extract could be extracted with ethyl acetate, and likewise less radioactivity could be isolated in form of benzo (a) pyrene. In contrast, with increasing time of incubation, the radioactivity in the metabolite fraction and in the extracted residue increased. Previous experiments [5] demonstrated that the radioactive material of the extracted residue could be hydrolized with 2 N methanolic potassium hydroxide and be isolated as water-soluble compounds. Furthermore parts of this activity when being extracted with buffer solutions could be precipitated with alcohol in protein fractions. This possibly indicates that some of the water-soluble derivatives are linked with proteins and nucleic acids.

Using high pressure liquid chromatography with gradient elution benzo (a) pyrene and its metabolites could be readily separated. This rapid assay yields separation without the necessity of derivatization and has been successfully used for this purpose by various workers [8, 11]. In the elution chromatogram the benzo (a) pyrene metabolites formed by *Chenopodium* plant cells appear in the same fractions as known metabolites formed by liver microsomes [8]. This indicates that the *Chenopodium* cells also form oxygenated derivates. The present knowledge about the pathways of metabolism of benzo (a) pyrene in animal tissues and cell cultures is summarized by Dehnen [8]. First of all monoaxygenases react with

the benzo(a)pyrene molecule and epoxides are formed [13, 14]. The epoxides in various position of the molecule are different in stability and reactivity and link with nucleic acids and proteins [15-17]. This may be an explanation for the increasing radioactivity in the residue and decreasing radioactivity extractable with methanol from cell material or with ethyl acetate from methanol extract with time. So far no other plants tested (wheat, potatoes, soybean, peas) except those of the family of Chenopodiaceae have been shown to form such metabolites. But the fact, that some plant species are able to metabolize polycyclic aromatic hydrocarbons like benzo(a)-pyrene, is of important relevance for evironmental hygiene. In further experiments we will test other varieties of Chenopodiaceae (Atriplex and Beta).

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