RADIOACTIVE ASSAY FOR ARYL HYDROCARBON HYDROXYLASE. IMPROVED METHOD AND BIOLOGICAL IMPORTANCE

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SUMMARY

By addition of two volumes of a 1M aqueous KOH/dimethylsulfoxide (15/85; v/v) mixture to the enzymatic incubation medium, it is possible to selectively extract the unmetabolized benzo(a)pyrene in hexane. Therefore, the radioactivity remaining in the water phase corresponds to all the *in vitro* synthesized metabolites. This isotopic method is very sensitive (2×10^{-11} moles) and is almost insensitive to the room lighting. The aryl hydrocarbon hydroxylase activities found with this method are 2,3 and 10 times higher in the liver, lung and kidney respectively compared to those obtained with the fluorimetric method.

INTRODUCTION

The first step in polycyclic hydrocarbon metabolism is catalyzed by the AHH or benzo(a)pyrene hydroxylase, a microsomal bound monooxygenase (1). It leads to the production of a variety of reactive arene oxides or epoxides which are further transformed into more polar hydrosoluble metabolites by both enzymatic and non-enzymatic processes (2). That these epoxides are most likely the ultimate or proximate carcinogens is supported by several experimental facts such as their mutagenic activity in bacteria or their transforming activity in cell culture (3).

Large differences have been demonstrated in the mutagenic as well as in the cytotoxic activities of a number of BP epoxides (4). As, on the other hand the pattern of metabolites produced from a single polycyclic hydrocarbon varies with a number of biological and biochemical parameters (5,6), it is very important to accurately measure (a) the total activity of the AHH and (b) the exact nature of all of the metabolites. In most of the published works, the AHH activity is measured by a fluorimetric method whose main advantage lies with its very high sensitivity (7). Nevertheless, this technique is also limited in several ways (See discussion in reference 8) and particularly

ABBREVIATIONS:

AHH, aryl hydrocarbon hydroxylase; BP, benzo(a)pyrene; HPLC, high performance liquid chromatography; DMSO, dimethylsulfoxide.

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by the fact that it only measures two of the *in vitro* synthesized metabolites, i.e. 3 and 9 hydroxybenzo(a)pyrene (9).

Recently, DePierre *et al.* (8) described a new isotopic assay based on the measurement of the water soluble metabolites whose results were generally two times higher than those obtained with the fluorimetric assay.

In this report, we describe a modified isotopic AHH assay which compared to the DePierre *et al.* (8) method presents two major advantages: (a) all the metabolites formed during the *in vitro* incubation are measured and (b) lower activities such as those found in the lung or in the kidney can accurately be measured.

MATERIALS AND METHODS

The source of most of the chemicals was indicated elsewhere (10). Benzo(a)pyrene and [^{3}H]-benzo(a)pyrene (1.2 Ci/mmol) were obtained from Fluka (Buchs, Switzerland) and I.R.E. (Fleurus, Belgium) respectively. 3-hydroxybenzo(a)pyrene, 9-hydroxybenzo(a)pyrene, 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene, 4,5-dihydro-4,5-dihydroxybenzo(a)pyrene, benzo(a)pyrene-3,6- and 6,12-diones were kindly donated by Professor F. Oesch (Mainz, Germany).

Labelled BP metabolites were prepared as follows. [^{3}H]-BP was incubated with liver 13,5000 x g supernatant under the optimal conditions described for the AHH assay. The metabolites were extracted and separated by HPLC (See below). The fractions corresponding to the different phenols, quinones, and dihydrodiols were respectively pooled, evaporated under nitrogen and stored dry under argon at -20° C.

Benzo(a)pyrene Purification: 5 mCi [^{3}H]-BP diluted with unlabelled BP to a specific radioactivity of 30 mCi/mmol was dissolved in 100 ml of hexane and extracted 5 times with 50 ml of aqueous 1M KOH/DMSO (65/85; v/v). After evaporation of the organic phase, under a stream of nitrogen, the residue was stored dry under argon at -20° C in a desiccator.

Enzymatic Sources: 200 g male Sprague-Dawley rats (Centre des Oncins, Lyon, France) were used for the preparation of the enzymatically active samples (13,500 x g supernatant) according to a previously described procedure (10).

Proteins: The protein concentration was determined according to Lowry *et al.* (11), bovine serum albumin being used as the standard.

Aryl Hydrocarbon Hydroxylase Assays: Regardless of the method (fluorimetric or radioactive) used, the incubation medium always consisted of NAD (0.43 mM), NADP (0.37 mM), glucose-6-phosphate (2.5 mM), glucose-6-phosphate dehydrogenase (1 I.U./ml), bovine serum albumin (0.8 mg/ml), Tris buffer (50 mM), $MgCl_2$ (5 mM), BP (0.08 mM) dissolved in 20 µl of acetone and the enzyme preparation (See below) in a final volume of 0.5 ml.

Blank values were obtained by incubating the substrate in the presence or absence of boiled enzymes (the two procedures gave similar results).
The incubation conditions for the different tissues were as follows:

liver and intestine: 10 min incubation at pH 7.6 with an enzyme preparation corresponding to 20 mg of fresh tissue (0.2 mg of protein for the liver, 0.4 mg of protein for the intestine) per ml of incubation medium.

lung: 20 min incubation at pH 8 with an enzyme preparation corresponding to 40 mg of fresh tissue (2.5 mg of protein) per ml of incubation medium.

kidney: 15 min of incubation at pH 7.6 with an enzyme preparation corresponding to 40 mg of fresh tissue (3 mg of protein) per ml.

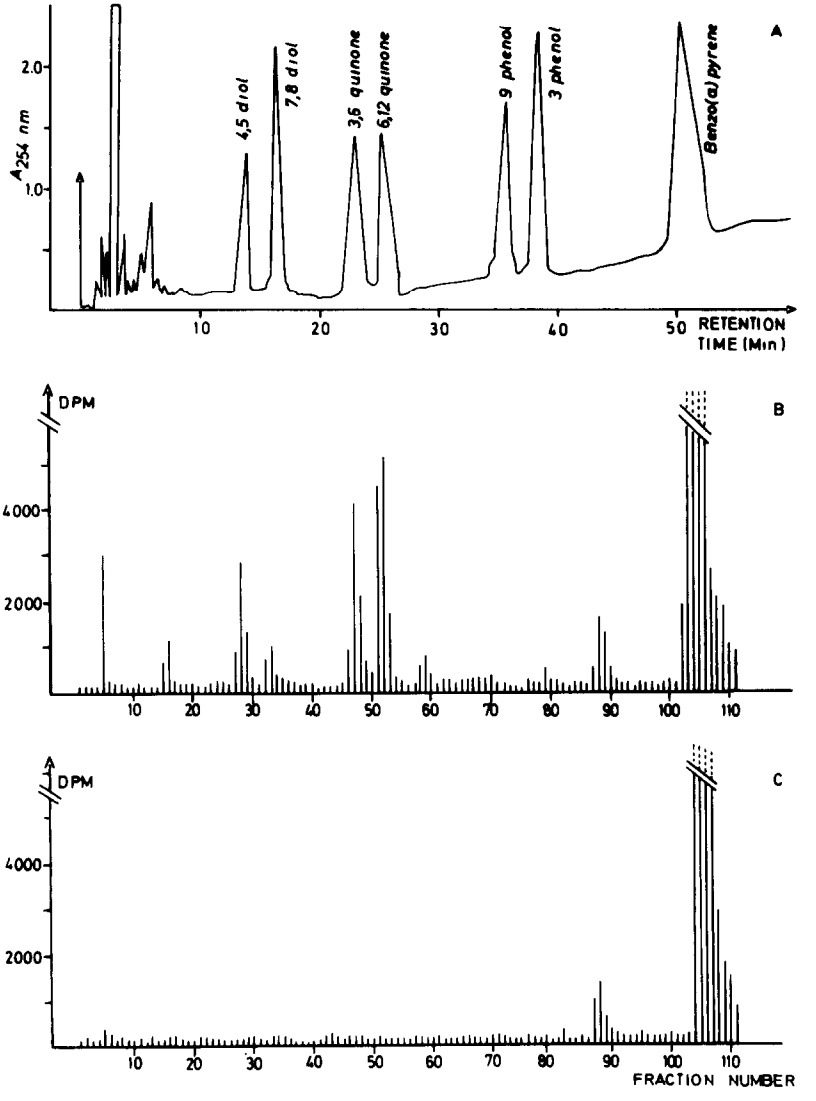

Figure 1: High performance liquid chromatography:

A. Absorption profile (254 nm) of a mixture of reference compounds.
B. Radioactive profile of the metabolites extracted in the hexane phase according to the DePierre method.
C. *Idem* B but according to our methodology.

The radioactivity present in fractions 87-91 is also present in the non incubated samples.

The technical procedure for the fluorimetric assay has been described elsewhere (7), results being expressed in nmol of 3-hydroxybenzo(a)pyrene formed.

For the isotopic assay, the enzymatic reaction, realized in a large conical tube was initiated by addition of the enzyme preparation. The incubation was stopped by the addition of 1 ml KOH (0.15 M in 85% DMSO). The unmetabolized substrate was extracted in 5 ml hexane (5 min on a revolving agitator, 40 rpm). After centrifugation, the upper and the interphases were eliminated and the remaining aqueous phase was extracted again in a similar manner. Finally, 0.5 ml of the aqueous phase was acidified by addition of an equal volume of HCL (N), diluted with 10 ml of Instagel (Packard, Downers Grove, Ill., U.S.A.) and counted by liquid scintillation. The enzymatic activity was expressed in nmol of total metabolites formed per min and per g of wet tissue (or per mg protein).

High Performance Liquid Chromatography: After incubation of [^{3}H]-BP under the standard conditions described above, the metabolites were extracted as by Selkirk *et al.* (12) and separated on a Waters (Model ALC 202) HPLC equipped with a microbound pack C 18 column.

Elution was realized at room temperature by a methanol-water gradient from 65/35 to 85/15; v/v) with a constant flow (1.5 ml/min). The absorbtion of the effluent was monitored at 254 nm through a 12 µl flow cell and 0.5 ml fractions were collected. Identification of peaks was obtained by compiling the literature and comparing their retention time with the available standard references.

RESULTS AND DISCUSSION

In the isotopic method described by DePierre *et al.* (8), the enzymatic reaction is stopped by the addition of alcoholic KOH and the unmetabolized substrate is then extracted by hexane. Nevertheless, when analyzed by HPLC, it became clear that in addition to BP, this organic phase contained significant amounts of dihydrodiols and quinones (Figure 1B). Therefore, only a fraction of the total enzymatic activity could be appreciated by measuring the water phase.

To improve this isotopic assay, we have modified the polarity of the water phase before hexane extraction. Thus, by adding different amounts of DMSO, the partition coefficients of the different metabolites as well as the substrate were severely changed (Table 1). Optimal conditions, (57% DMSO) were selected, i.e. those allowing the highest recovery of the different metabolites in the water phase (over 95%) with a minimal contamination by the unmetabolized substrate (0.6%). In fact, after two successive hexane extractions, only 0.04% (or 300 to 400 cpm) of the incubated BP was left over in the water. Figure 1C illustrates that, under those experimental conditions, there were no longer any metabolites appearing in the organic phase.

The addition of a lipophilic enzymatic substrate to an aqueous incubation medium is always a problem. We successfully used detergents for several steroid hydroxylase assays (10,13,14), but were unable to apply the same method for the AHH assay as the detergents also modified the partition coefficient of the unmetabolized BP between the water and the organic phase. Organic solvents

TABLE 1: EXTRACTION COEFFICIENTS BETWEEN THE AQUEOUS PHASE AND HEXANE AS A FUNCTION OF DMSO CONCENTRATION IN THE AQUEOUS PHASE

NATURE OF COMPOUNDS	DMSO (%)								DEPIERRE METHOD
	0	30	50	55	57	60	70	80	
[^{3}H]benzopyrene	0.05	0.1	0.3	0.4	0.6	0.9	3.6	16	0.3
[^{3}H]dihydrodiols	26	59	87	94	96	97	98	99	63
[^{3}H]quinones	7	30	72	89	92	95	97	99	41
[^{3}H]phenols	89	96	98	98	99	99	99	99	99

The different groups of compounds purified by HPLC were introduced into a 0.5 ml incubation medium (without enzyme) in 20 µl of acetone. 150 µl of KOH (N) was added together with water and DMSO in order to obtain the desired concentrations of DMSO in 1.5 ml (or more for the highest concentrations). This aqueous phase was extracted by 5 ml hexane as in the AHH assay and the radioactivity was counted in both phases.

were thus utilized to introduce the substrate into the enzyme reaction mixture. Many of these are known as *in vitro* inhibitors of the monooxygenase activities. Consequently, we tested a number of them and found that acetone and acetonitrile were the best solvents. The enzymatic activity was only 65% of that obtained in the presence of those two substrate carriers when DMSO was used, 55% with ethylene glycol, 40% with ethanol and methanol, 20% with propanol and 7% with butanol.

Under the optimal conditions of our modified isotopic assay (see methods) it is possible to measure the AHH activity, not only in the liver, but also in the extra hepatic tissues as well as in other low activity samples such as cells in culture. The lower limit of sensitivity of the assay was estimated to be about 2.10^{-11} moles of total metabolites formed per ml of incubation medium, i.e. about 5 times less sensitive than that of the fluorimetric assay. The accuracy of the isotopic method is also very good, results on duplicate samples usually being within 5% of the mean value.

We have then measured the AHH activity in three different tissues by our new isotopic assay and compared the results with those obtained by the fluorimetric and by the original isotopic methods. Table 2 shows that in all the tissues, the highest enzymatic activities were always measured with the new

TABLE 2: LIVER, LUNG AND KIDNEY AHH ACTIVITIES MEASURED BY VARIOUS METHODS UNDER DIFFERENT LIGHTING CONDITIONS

ORGAN	METHOD	AHH ACTIVITY		
		DARKNESS	NATURAL LIGHT (without sun)	ARTIFICIAL LIGHT (under neon)
LIVER	Fluorimetric	596	499	356
	"DePierre"	814	862	816
	This assay	1002	986	945
KIDNEY	Fluorimetric	6.8	3.8	1.2
	"DePierre"	27.6	26.5	27.3
	This assay	52.4	47.9	55.8
LUNG	Fluorimetric	6.6	4.5	2.9
	"DePierre"	11.0	14.8	13.7
	This assay	18.5	20.2	17.7

The enzyme activities are expressed in nmol/g tissue x h and represent the mean of three separate determinations on the same tissular preparation. The incubation conditions are described in the methods section.

isotopic method. Compared to the very widely used fluorimetric method, it is worth emphasizing that, if in the liver the AHH activity was about two times higher, it was three and ten times higher in the lung and in the kidney respectively. Those differences were not caused by the formation of non-enzymatically produced water soluble metabolites or by unspecific release of tritium in the water phase. In fact, whatever the method used, the enzymatic activities could be similarly inhibited by carbon monoxide or by various monooxygenase inhibitors, such as α-naphtoflavone, SKF-525, metyrapone or diphenyloxazole (data not shown).

Those results support the hypothesis that the polycyclic hydrocarbon metabolism is not only qualitatively but also quantitatively different in the liver compared to the lung or the kidney. This is in accord with recent results showing that the pattern of metabolites is very different in the lung compared to the liver, less phenols and more dihydrodiols being formed with the former enzymatic preparations (15,16).

Another advantage of the isotopic method is also demonstrated in Table 2.

Many polycyclic hydrocarbons are photosensitive and easily transformed by non-enzymatic reactions. This is particularly critical for the fluorimetric method which only measures one or a small number of the metabolites and which, opposed to the isotopic method, showed a critical sensitivity to the room lighting during the manipulations.

On a theoretical basis, the results obtained by the isotopic method should better reflect the AHH activity, as all the metabolites are solubilized in the aqueous alkaline phase enriched with DMSO. In addition, as the tritium replaced during the enzymatic oxidation is released in the medium as a molecule of water (17) and is thus counted together with the radioactivity remaining on the metabolites, the specific radioactivity of the substrate can be used to accurately calculate the enzyme activity in terms of moles of product synthesized during the incubation.

It is well documented that the pattern (or type) of polycyclic hydrocarbon metabolites produced by mammalian cells varies extensively with the tissues, animal species or the physiological status of the animals (5,6). The best analytical method should thus, measure the formation of all of the metabolites. HPLC would, of course, fulfill those requirements, but would, nevertheless, be difficult to apply for large scale analyses. This new isotopic method might be a compromise in the routine estimation of the AHH activity in animal tissues.

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