A sensitive fluorometric assay for epoxide hydratase

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1. INTRODUCTION

It is now firmly established that a great number of xenobiotics, e.g., drugs, environmental pollutants and food additives can be metabolized by microsomal cytochrome P450-dependent monooxygenases to highly reactive epoxide intermediates [1,2]. Epoxide hydratase (EC 3.3.2.3.) has a dual role in the further metabolism of these epoxides. On the one hand, hydration of arene oxides to the chemically less reactive *trans*-dihydrodiols can be considered as a detoxification pathway, while on the other hand certain *trans*-dihydrodiols of PAH can be further oxidized by the monooxygenases to highly mutagenic and carcinogenic diol-epoxides [3,4].

Since inter-individual differences in carcinogen metabolism can result in different susceptibility to obtain carcinogen-induced neoplasm, it is of interest to detect genetic differences in activity of the various carcinogen metabolizing enzymes [5,6]. Human hair follicles have been suggested as a convenient biopsy-tissue for these investigations [7,8]. Here, we present a rapid and very sensitive fluorometric assay for EH, using 4,5-dihydro-epoxyBP as substrate, which is especially useful for hair follicles and for other small tissue samples with relatively low EH activities.

Abbreviations: EH, epoxide hydratase; PAH, polycyclic aromatic hydrocarbons; BP, benzo(a)pyrene; TCPO, trichloropropylene oxide; DMSO, dimethylsulfoxide

2. MATERIALS AND METHODS

2.1. *Chemicals*

4,5-Dihydro-epoxyBP and *trans*-4,5-dihydrodiol BP were obtained from the National Cancer Institute Chemical Repository (IIT Research Institute, Chicago IL). TCPO was purchased from Aldrich (Beerse).

2.2. *Biopsy material*

Human hair follicles were obtained from the scalp of normal volunteers (non-smokers). Only hair follicles with visible bulb and sheath were used. Human lung specimens, macroscopically free of tumour tissue, were obtained from lung resection operations. After coarsely mincing the tissue with scissors and rinsing in several changes of homogenisation buffer [0.1 M potassium phosphate (pH 7.9), 10% (v/v) glycerol and 1 mM EDTA], 20 g material was homogenised, first in a Waring blender (20 s at maximal speed) and then in a Potter S homogenizer (Braun). The homogenate was centrifuged at 10 000 × *g* for 1 h and 1/2 of the resulting supernatant was used for preparation of microsomes by centrifugation at 120 000 × *g* for 1 h. The microsomal pellet was resuspended in 0.1 M Tris (pH 9.5). Protein in the 10 000 × *g* supernatant and microsomes was adjusted to ~5 mg/ml.

2.3. *Assay procedure*

All assays were performed in triplicate. For each assay 15 hair follicles or 50 μl lung 10 000 × *g* supernatant or microsomes were added to 0.5 ml final vol. 0.1 M Tris (pH 9.5). The reaction was initiated by addition of the substrate in 10 μl DMSO (final

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conc. 100 μM) and carried out in a shaking water bath at 37°C. After the required incubation time, the reaction was stopped by addition of 3.5 ml petroleum ether (b.p. 40–60°C), shaking the tube on a Vortex mixer for 5 s and cooling in ice. Then 0.5 ml of DMSO was added and the tubes were mixed on a Vortex mixer for 60 s. After centrifugation at 5 000 × *g* for 2 min the petroleum ether fraction was removed and the extraction procedure repeated twice, resulting in removal of the unreacted substrate [9]. The product, *trans*-4,5-dihydrodiolBP, was then extracted into 1 ml ethylacetate by agitating on a Vortex mixer for 90 s. After separation of the phases by centrifugation at 5 000 × *g* for 5 min the ethylacetate was transferred to a new tube and the fluorescence measured in a Perkin Elmer 650-40 spectrofluorometer at an excitation wavelength of 312 nm and an emission wavelength of 388 nm. Known amounts of *trans*-4,5-dihydrodiolBP were used as standards. In routine experiments, quinine HBr in 0.1 N H_2SO_4 was used for the calibration of the fluorometer. DNA in the hair follicles was measured by the mithramycine technique [10] employing calf thymus DNA as a standard and protein in the lung samples was determined as in [11] using bovine serum albumin as a standard. Enzyme activities were expressed as pmol *trans*-4,5-dihydrodiolBP . μg $DNA^{-1} . h^{-1}$ and in the lung samples as nmol *trans*-4,5-dihydrodiolBP . mg $protein^{-1} . h^{-1}$.

3. RESULTS

Since the assay has been primarily developed for the use of human hair follicles, optimal assay conditions have been determined for this tissue type. Fig.1 shows the excitation and fluorescence spectra of pure *trans*-4,5-dihydrodiolBP and the ethylacetate fraction after the enzyme reaction using 15 hair follicles. Comparison of both spectra shows that the

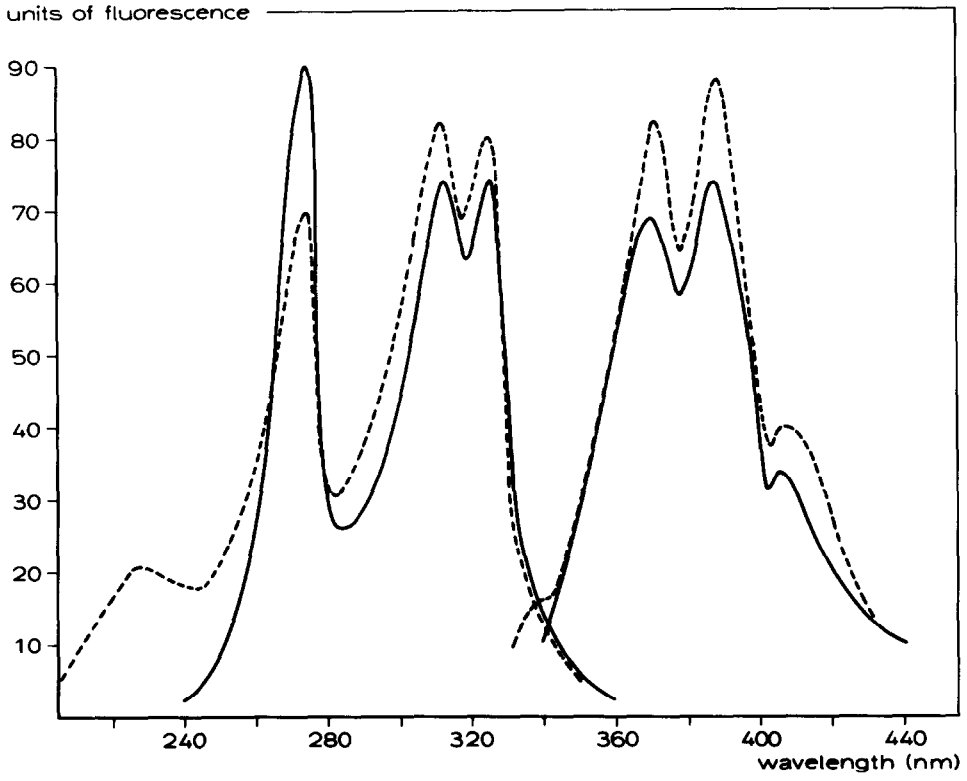

Fig.1. Excitation (left) and fluorescence (right) spectra of pure *trans*-4,5-dihydrodiolBP (dotted lines) and the ethylacetate extract after the enzymatic reaction using 15 hair follicles as in section 2 (solid line).

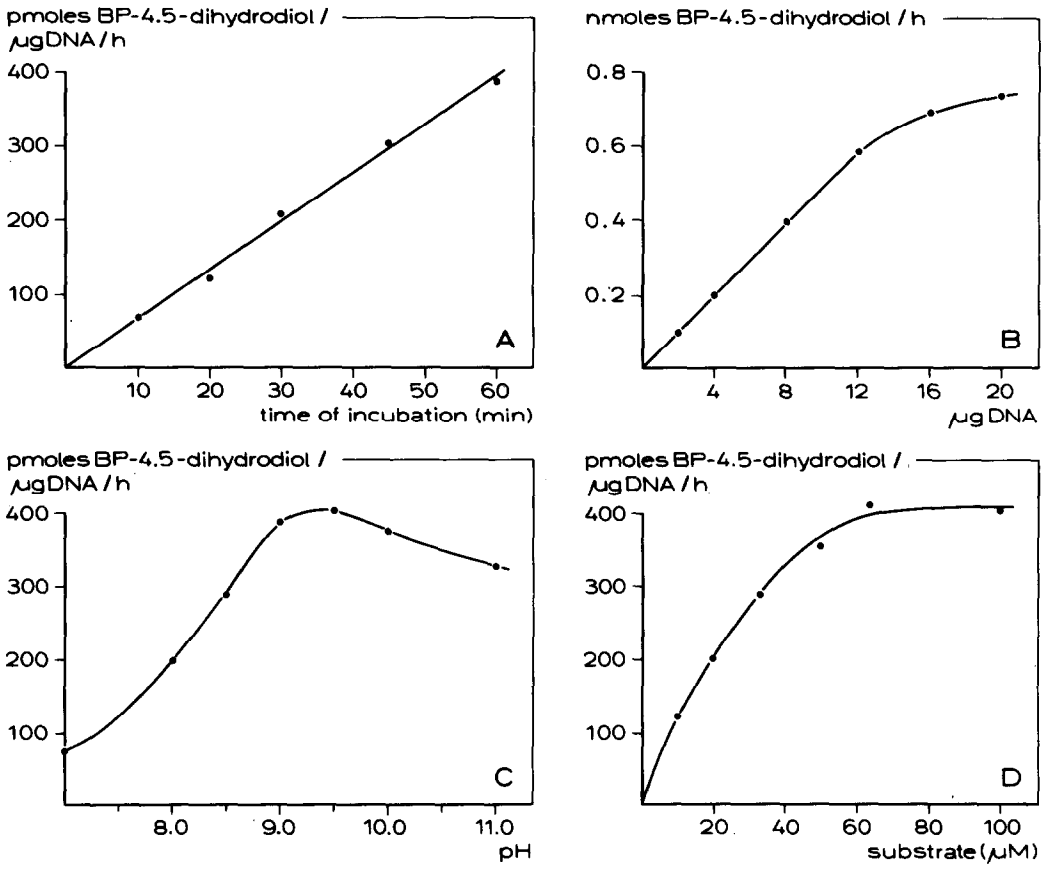

Fig.2. Kinetics of the hydration of 4,5-dihydro-epoxyBP by human hair follicles. All data are the mean of triplicate incubations: (A) time–velocity relation (using 15 hair follicles corresponding to ~7.5 µg DNA; 100 µM substrate); (B) DNA–velocity relation (60 min incubation; 100 µM substrate;) (C) pH–velocity relation (60 min incubation; 15 hair follicles; 100 µM substrate); (D) substrate–velocity relation (60 min incubation; 15 hair follicles).

fluorescence in the extract is almost entirely due to *trans*-4,5-dihydrodiolBP. Addition of TCPO (1.8 mM) results in almost complete inhibition of enzyme activity (table 1). The enzymic nature is further demonstrated by the absence of hydration in boiled hair follicles and at 0°C. The reaction is linear with time for at least up to 60 min and with [DNA] up to 12 µg, corresponding to about 25 hair follicles

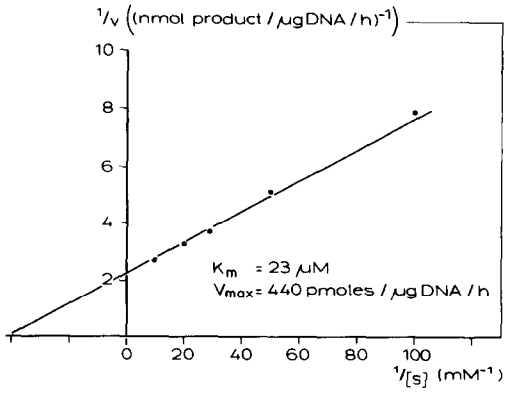

Fig.3. Double-reciprocal plot of the variation of the rate of enzymatic 4,5-dihydro-epoxyBP hydration as a function of substrate concentration. Triplicate assays were performed with 15 hair follicles and by incubation for 60 min.

Table 1

EH levels in various incubation systems

Samples	Activity
Human lung	
10 000 × *g* supernatant[a]	10.9
microsomes[a]	72.6
Human hair follicles[b]	440
+ 1.8 mM TCPO[b]	33
− 4,5-dihydro-epoxyBP[b]	0
incubation at 0°C[b]	0
boiled[b]	10

[a] Activity expressed as nmol 4,5-dihydrodiolBP . mg protein^{-1} . h^{-1}

[b] Activity expressed as pmol 4,5-dihydrodiolBP . μg DNA^{-1} . h^{-1}

Assays were done as in section 2

(fig.2A,B). Since with 15 hair follicles enzymatic rates are 3–6-times as high as those of the blank without enzyme, a number of 15 hair follicles has been used routinely. Optimal enzyme activity was reached at pH of 9.5 (fig.2C). Substrate concentrations exceeding 100 μM did not result in any increase in enzyme activity (fig.2D). From a Lineweaver-Burk plot (fig.3) the K_m and V_{max} can be determined for EH in hair follicles. The app. K_m is 23 μM, a value higher than that for lung microsomes (8 μM) which could be caused by substrate depletion due to binding to non-specific sites in the hair follicles.

Using human lung preparations the reaction proceeds linearly with time for 20 min with microsomes (0.5 mg protein/ml) and for 60 min with 10 000 × *g* supernatant (0.5 mg protein/ml). With a standard incubation time of 30 min linearity with protein is maintained up to 0.6 mg protein/ml with lung microsomes and up to 1.3 mg protein/ml with lung 10 000 × *g* supernatant.

4. DISCUSSION

A wide range of assays for EH have been described including radiometric [12], photometric [13], gas chromatographic [14] and liquid chromatographic assays [15]. Each of these assays has its own limitations for application in large population studies such as the use of radioactive substrates, low sensitivity (photometric assay), the need for derivatization (gas chromatography) or tedious procedures (liquid chromatography). The continuous fluorometric assay developed in [16], although very sensitive, is limited by the fact that at higher substrate concentrations (> 10 μM) the absorbance of the solution is not sufficiently low to avoid quenching and by the photodecomposition of substrate and product. The fluorometric endpoint assay described here combines the efficient separation of substrate and product with the sensitive fluorometric determination of the product in the ethylacetate fraction. In the assay, EH is measured using an epoxide of PAH, a class of chemicals in which the crucial role of EH in activation and inactivation of biologically active intermediates, is obvious. Moreover, 4,5-dihydro-epoxyBP has a relatively very low spontaneous rate of hydrolysis. Although we have used 4,5-dihydro-epoxyBP obtained as a gift from the National Cancer Institute, the compound can be synthesized relatively easily from BP [17]. The K_m-values obtained with this substrate (8 μM for human lung microsomes and 23 μM for human hair follicles) are much lower than those reported for, e.g., styrene oxide, using human and guinea pig liver preparations [18,19]. This is consistent with the idea that EH has evolved as a response to the continuous presence of PAH as a result of incomplete combustion. The described assay offers the opportunity to investigate the possible interrelationship between EH levels and susceptibility to develop cancer upon exposition to PAH.

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REFERENCES

[1] Sims, P. and Grover, P.L. (1974) Adv. Cancer Res. 20, 165–274.

[2] Jerina, D.M. and Daly, J.W. (1974) Science 185, 573–582.

[3] Buening, M.K., Wislocki, P.G., Levin, W., Yagi, H., Thakker, D.R., Akagi, H., Koreeda, M., Jerina, D.M. and Conney, A.H. (1978) Proc. Natl. Acad. Sci. USA 75, 5358–5361.
[4] Slaga, T.J., Bracken, W.J., Gleason, G., Levin, W., Yagi, H., Jerina, D.M. and Conney, A.H. (1979) Cancer Res. 39, 67–71.
[5] Autrup, H., Grafstrom, R.C., Brugh, M., Lechner, J.F., Haugen, A., Trump, B.J. and Harris, C.C. (1982) Cancer Res. 42, 934–938.
[6] McLemore, T.L., Martin, R.R., Busbee, D.L., Richie, R.C., Springer, R.R., Topell, K.L. and Cantrell, E.T. (1977) Cancer Res. 37, 1175–1181.
[7] Vermorken, A.J.M. and Bloemendal, H. (1979) in: Tumour Markers: Impact and Prospects (Boelsma, E. and Rümke, Ph. eds) pp. 305–326, Elsevier Biomedical, Amsterdam, New York.
[8] Hukkelhoven, M.W.A.C., Vermorken, A.J.M., Vromans, E. and Bloemendal, H. (1982) Clin. Genet. 21, 53–58.
[9] Schmassmann, H.U., Glatt, H.R. and Oesch, F. (1976) Anal. Biochem. 74, 94–104.
[10] Hukkelhoven, M.W.A.C., Vromans, E., Markslag, A.M.G. and Vermorken, A.J.M. (1981) Anticancer Res. 1, 341–344.
[11] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[12] Oesch, F., Bentley, P., Platt, K.L. and Golan, M.D. (1980) Arch. Biochem. Biophys. 199, 538–544.
[13] Watabi, T. and Akamatsu, K. (1974) Biochem. Pharmacol. 3, 2839–2844.
[14] Duverger–van Bogaert, M., Noël, G., Rollman, B., Cumps, J., Roberfroid, M. and Mercier, M. (1978) Biochim. Biophys. Acta 526, 77–84.
[15] Ross, M.S.F., Lines, D.S., Brain, K.R. and Stevens, R.G. (1978) Anal. Biochem. 87, 267–271.
[16] Dansette, P.M., DuBois, G.C. and Jerina, D.M. (1979) Anal. Biochem. 97, 340–345.
[17] Dansette, P. and Jerina, D.M. (1974) J. Am. Chem. Soc. 96, 1224–1225.
[18] Oesch, F., Thoenen, H. and Fahrlaender, H. (1974) Biochem. Pharmacol. 23, 1307–1317.
[19] Oesch, F. and Daly, J. (1971) Biochim. Biophys. Acta 227, 692–697.