A SIMPLE ASSAY OF ARYL HYDROCARBON HYDROXYLASE IN CULTURED HUMAN LYMPHOCYTES

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SUMMARY. A novel technique is described for assay of aryl hydrocarbon hydroxylase in cultured human lymphocytes. The sensitivity is equal to that of previous methods, but this method requires fewer manipulations. One million lymphocytes are incubated for one hour with 2 micrograms of benzo(a)pyrene in a glass cuvette. The reaction is stopped by addition of neutral formalin and the cell suspension is alkalinized with NaOH. Fluorescence intensity of the suspension is measured with excitation at 465 nm and emission at 520 nm.

INTRODUCTION. AHH is an intracellular membrane bound enzyme complex which can metabolize xenobiotic chemicals such as polycyclic aromatic hydrocarbons to more polar derivatives. This inducible enzyme complex has been implicated in chemical carcinogenesis in man (1). If the activity or inducibility of AHH in human tissues is related to cancer risk in man, then a rapid and simple method of assay of AHH in human tissue is needed. Previous studies employed BP as substrate and measured the rate of formation of the alkali-soluble BPOH by fluorometry (2). A radiometric assay of AHH was introduced by Diamond (3) which

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Abbreviations: AHH, aryl hydrocarbon hydroxylase; BP, benzo(a)pyrene; BPOH, monohydroxybenzo(a)pyrene; BA, benz(a)anthracene; EMSAD buffer is 25 mM EPPS (Sigma), 3 mM $MgCl_2$, 125 mM sodium chloride, 0.1% bovine serum albumin and 10 mM dextrose at pH 8.0.

measured the accumulation of water-soluble metabolites in tissue culture over several hours. A more direct assay reported by Dehnen, et al. (4) eliminated the need for organic extractions but required triethylamine to quench the fluorescence of BP during the measurement of the phenolic metabolites (a mercury lamp was employed in that procedure for excitation with the 436 nm line). Kouri, et al. (5) measured AHH in single cells by a microfluorometric procedure. That method is not widely used today, possibly due to the technical difficulties inherent in quantitative fluorescence microscopy.

We have developed a rapid and direct assay for AHH which is similar to that of Dehnen, et al. (4) but provides a high level of sensitivity and resolution. The assay does not require solvent extraction, exogenous NADPH, or the noxious triethylamine. This procedure is particularly applicable to rapid, automated single-cell analysis of AHH in human cells (Harry Tyrer et al.- manuscript in preparation).

MATERIALS AND METHODS. Cultures of human peripheral lymphocytes were prepared by ficoll-Hypaque^{R} sedimentation and the mononuclear leukocytes (mostly lymphocytes) were resuspended in Joklik's modified MEM culture medium (F-13) which contained 15% heat-inactivated fetal calf serum, 1% reconstituted phytohemagglutinin and pokeweed mitogens and 50 units/ml heparin (culture reagents were purchased from Grand Island Biologicals). The final cell concentration was 0.5×10^6 cells per ml. Cultures were maintained in 250 ml Falcon plastic culture flasks or in sterile glass scintillation vials with the depth of the medium 3-4 mm. Half of the cultures contained 10 μM benz(a)anthracene (BA).

After 96 hours of culture at 37° under 5% CO_2 in air, the cell suspensions were centrifuged gently in conical centrifuge tubes for 3 mins at 100 x g and the supernatant medium was carefully aspirated. The cells were resuspended in EMSAD buffer to a concentration of 0.5×10^6 cells per ml. One ml aliquots of cells were placed into 13 x 100 mm disposable glass culture tubes, and each tube received 2.0 μg of BP in 0.010 ml methanol. All subsequent steps were performed under subdued yellow light or darkness. The cell suspensions were incubated for 60 mins at 37° except as indicated. After the incubation 0.2 ml of 37% neutral formalin was added to each tube with gentle swirling, and the tubes were allowed to stand at room temperature for at

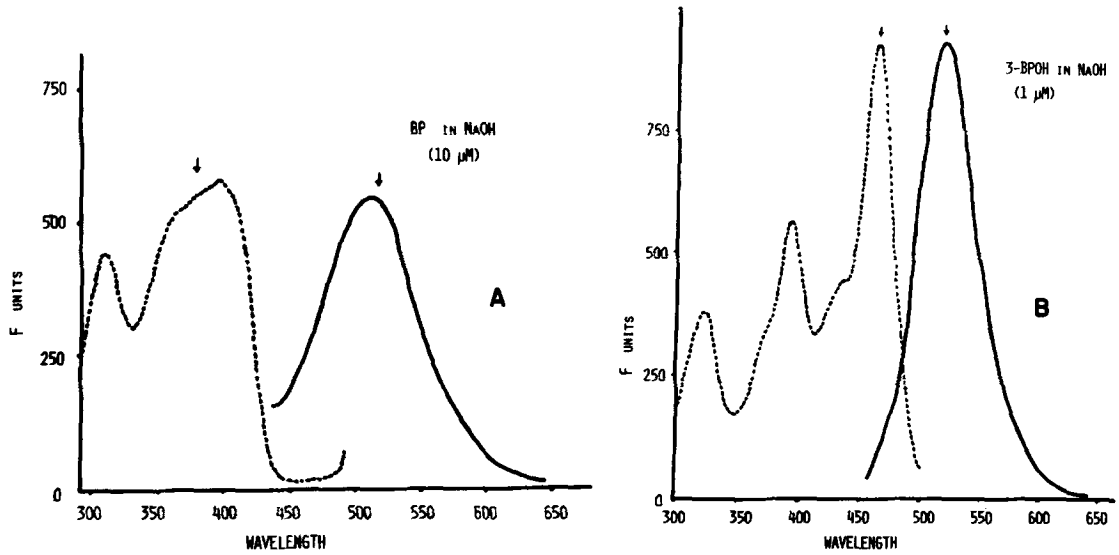
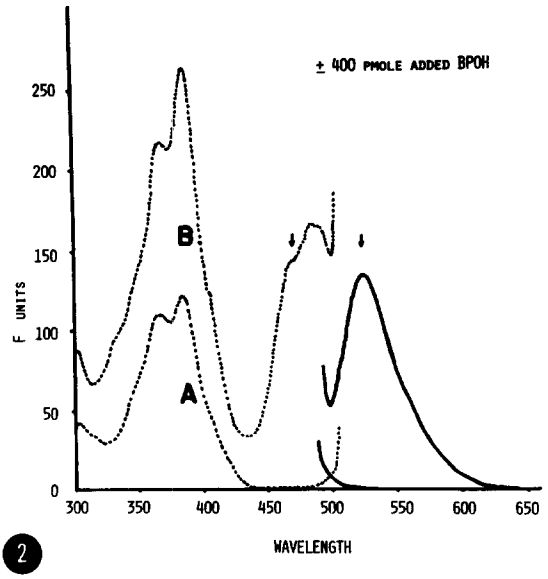


Figure 1. Fluorescence spectra of BP and 3-BPOH in 1N NaOH. a) BP. b) 3-BPOH. The small arrows indicate fixed wavelengths of either excitation or emission while scanning the other.

least five mins (Complete fixation is required to prevent flocculation). To each tube was then added 1.0 ml of 1N NaOH with vortex-mixing to suspend the fixed cells. The fluorescence of each suspension was measured in an Aminco-Bowman Model 8931A spectrophotofluorometer with a 0.5 mm excitation slit and a 1.0 mm emission slit. Excitation was at 465 nm with emission measured at 520 nm. A standard concentration curve was prepared by adding varying amounts of authentic 3-BPOH to 2.2 ml of fixed alkaline cells which had received 2.0 μg BP. A working standard of 1 μg/ml of quinine sulfate in 0.1 M H_2SO_4 was used daily to calibrate the instrument.

RESULTS AND DISCUSSION. The fluorescence spectra of BP in NaOH are shown in figure 1a. In alkaline solution a green component of BP fluorescence may be seen if excitation is below 425 nm but not at 465 nm. In contrast, 3-BPOH has a strong green fluorescence when excited at 465 nm (figure 1b). Under instrumental settings of 465 nm excitation and 520 nm emission small amounts of 3-BPOH may be detected in the presence of large amounts of BP. Figure 2 shows the spectra of a simulated reaction mixture containing BP and 3-BPOH.



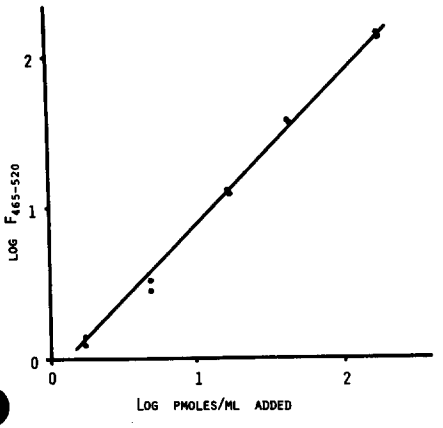


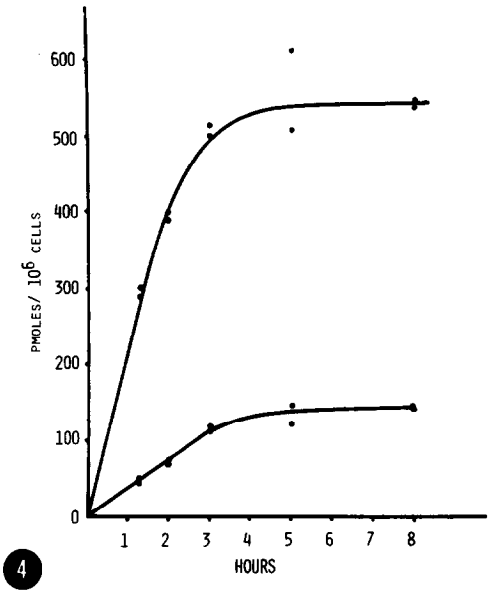
Figure 2. Fluorescence spectra of simulated reaction mixture. The cuvette contained 10^6 cells, 2 µg BP, 3.4% formalin, and 0.45N NaOH. a) without 3-BPOH. b) with 400 pmoles 3-BPOH.

Figure 3. Concentration of 3-BPOH vs fluorescence intensity. Cuvettes contained 10^6 cells, 2 µg BP, 3.4% formalin, and 0.45N NaOH with varying amounts of 3-BPOH in 2.2 ml.

The fluorescence intensity is proportional to metabolite concentration over a range exceeding 4-400 pmoles 3-BPOH in 2.2 ml (figure 3). The AHH reaction in whole cells is linear with time for 2-3 hours (figure 4). The plateau effect is consistent with the concept of secondary metabolism of 3-BPOH (7,8). The plateau is not due entirely to deterioration of the preparation, since cells held in EMSAD buffer for 4 hours at 37° prior to adding BP lost only about half of their activity (figure 5).

When 1 mg NADPH was added to the reaction mixture, the activity was increased by only 12%, indicating that the intact cell preparation was competent to generate its own reducing equivalents. By the trypan blue exclusion test, we found that the suspension contained 90% or greater viable cells.

After standing in the alkaline solution for two hours, the



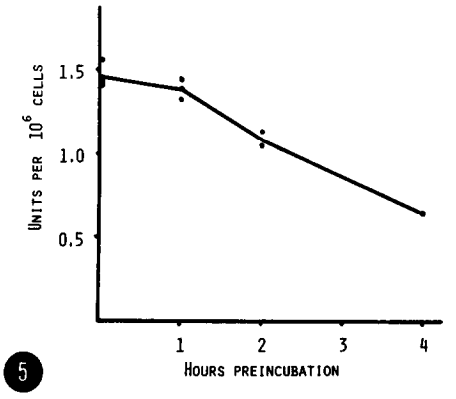


Figure 4. Time course of AHH reaction in control and BA-induced lymphocytes. Activity is expressed as fluorescence equivalent to 3-BPOH per 10^6 cells.

Figure 5. Effect of storage in EMSAD buffer prior to AHH assay. BA-induced lymphocytes were stored in EMSAD buffer @ 37° for varying times prior to adding BP for an additional 60 mins.

metabolite retains maximal fluorescence. This is in contrast to the fading within 15 mins noted in the previous assay (2). The metabolite was found associated with the cellular material rather than free in the alkaline medium as determined by centrifuging and resuspending whole or sonicated cells.

Cell growth and AHH activity was the same whether cells were grown in plastic T-flasks or in glass scintillation vials. The important characteristics were that the cell concentration be 0.3-0.5 million per ml and the depth of medium be 0.3-0.5 mm. This is consistent with the findings of Gurtoo et al. (9).

This assay provides a sensitive and rapid method of measuring AHH in human tissue without the variability introduced by

multiple extractions (2) or high background fluorescence levels (4). Not all the metabolites are measured as in certain other methods (10), but measurement of fluorescent phenols may, nevertheless, reflect the relative activity of AHH in individuals.

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