INVESTIGATION OF ENZYMATIC SCREENING TESTS FOR MUTAGENS IN ENVIRONMENTAL POLLUTANTS FROM SYNFUEL OPERATIONS

by

J. J. Schmidt-Collerus, N. L. Couse, J. King and L. Leffler

Written and Prepared by: N. L. Couse

Denver Research Institute
University of Denver
Denver, Colorado 80208

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Project Officer

Norman Richards
Environmental Research Laboratory
U.S. Environmental Protection Agency
Sabin Island
Gulf Breeze, Florida 32561

ENVIRONMENTAL RESEARCH LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
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J.J. Schmidt-Collerus, N.L. Couse, J. King and L. Leffler

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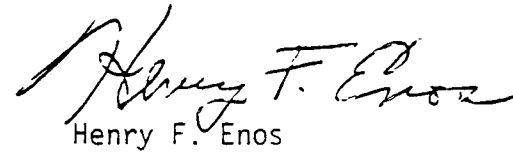
FOREWORD

The protection of our estuarine and coastal areas from damage caused by toxic organic pollutants requires that regulations restricting the introduction of these compounds into the environment be formulated on a sound scientific basis. Accurate information describing dose-response relationships for organisms and ecosystems under varying conditions is required. The Environmental Research Laboratory, Gulf Breeze, contributes to this information through research programs aimed at determining:

- the effects of toxic organic pollutants on individual species and communities of organisms;
- the effects of toxic organics on ecosystems processes and components;
- the significance of chemical carcinogens in the estuarine and marine environments;

Increasing pollution of aquatic environments has led to the development of biological assays designed to monitor toxic, mutagenic, and carcinogenic effects of contaminating chemicals. This report describes the investigation of a biochemical (in vitro) prescreen test for determining carcinogenic compounds.



Henry F. Enos
Director
Environmental Research Laboratory
Gulf Breeze, Florida

ABSTRACT

The objective of this research program was to develop an enzymatic screen for chemical carcinogens based on the selective in vitro stimulation of microsomal biphenyl-2-hydroxylase by known chemical carcinogens.

An attempt was made to repeat published work using a spectrophotofluorometric assay for biphenyl metabolites. It was found that this assay system is not valid for use with complex mixtures, and that metabolites must be separated from interfering compounds prior to quantitation. A high pressure liquid chromatography method was developed which permitted rapid separation of metabolites. Nanogram quantities of metabolites were detectable using this chromatographic separation in conjunction with a spectrophotofluorometric detector. Using this method, it was not possible to demonstrate in vitro stimulation of biphenyl-2-hydroxylase by chemical carcinogens.

Alternative assays were also examined. Terphenyl is metabolized to at least three different compounds by hamster microsomes. Further work is necessary to validate the utility of this substrate in an enzymatic screen for carcinogens. A marine protozoan, Parauronema acutum metabolizes biphenyl in vivo to 2- and 4-hydroxybiphenyl. This organism may provide a reliable, inexpensive source of biphenyl hydroxylase for an in vitro enzymatic assay system.

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ABBREVIATIONS AND SYMBOLS

ABBREVIATIONS

BaP----------benzo(a)pyrene

DMSO---------dimethylsulfoxide

HPLC---------high pressure liquid chromatography

MC----------3-methylcholanthrene

αNA----------α-naphthylamine

βNA----------β-naphthylamine

SA----------Safrole

SPF----------Spectrophotofluorometer

SYMBOLS

λ_{EM}----------emission wavelength

λ_{EX}----------excitation wavelength

CHEMICALS, SOURCES AND PURITY

This list does not constitute an endorsement of the manufacturers listed. It is included because purity and source may influence experimental results.

Biphenyl----------J.T. Baker, Ultrex, 99.99%

4,4'-Dihydroxybiphenyl--------Aldrich, 97%

2,2'-Dihydroxybiphenyl--------Aldrich, 99%

Dimethylsulfoxide----------J.T. Baker, Reagent grade

n-Heptane----------Burdick and Jackson Labs, UV grade, "distilled in glass"

n-Hexane----------Burdick and Jackson Labs, UV grade, "distilled in glass"

4-Hydroxybiphenyl----------Aldrich, 97%

2-Hydroxybiphenyl----------Aldrich, 99+%

Succinic acid----------J.T. Baker, 99.4%

p-Terphenyl----------Aldrich, 99+%

Tetrahydrofuran----------Burdick and Jackson Labs UV grade, "distilled in glass"

Tween 80----------Emulsion Engineering, Inc., Polysorbate 80, USP

ACKNOWLEDGEMENTS

We are greatly indebted to F. Krohlow and C. Burdick for invaluable technical assistance and to K. Gala and M. Shaffron for assistance with the high performance liquid chromatograph. We also wish to thank Dr. D. Lindmark for providing the P. acutum and advising us as to its growth characteristics.

SECTION 1

INTRODUCTION

GENERAL

Awareness of increasing chemical pollution of both aquatic and terrestrial environments has led to the development of biological assays designed to monitor toxic, mutagenic, and carcinogenic effects of industrial effluents. It is reasonable to assume that the potential hazards to the biosphere, and especially the marine environment which serves as the ultimate repository of the majority of pollutants, will continue to increase with expansion of technological productivity and with rapid and intensified developments in the area of alternate energy sources. Therefore, biological systems useful for screening effluents should be simple, sensitive, reliable, rapid and inexpensive. In addition, these systems should be directly useful for testing the effect of noxious materials upon the marine environment. Detection of harmful pollutants will permit detoxification of the material before release into the biosphere.

Current bioassays use a number of different systems including bacteria (Salmonella, Escherichia coli), yeast, Drosophila, mammalian cell cultures, and mice. A "tier" approach (Epler, 1976) to biological testing has been designed in order to screen material for potentially hazardous effects. The rationale of the tier system is to identify potentially harmful material by initial testing in relatively rapid and inexpensive assays prior to evaluation of effects in time-consuming and expensive in vivo mammalian systems. This approach begins with a microbiological assay and proceeds to other tiers (levels) of increasing

organismal complexity. The ultimate objective of the tier approach is to reduce the amount of time and number of tests which must be conducted in order to assess the potential biological hazard of a given material.

Tests using living organisms or cells suffer from a high sensitivty to toxic effects caused either by the active compound itself or by impurities present in complex mixtures. These toxic effects may thus be secondary, and may mask mutagenic or carcinogenic activities of the material. Complex mixtures therefore must often be fractionated prior to testing in order to reduce toxicity, and the number of tests to be conducted on a given sample may be extremely large. In addition, the spectrum of substances which can be effectively tested in a given system is frequently limited.

It would therefore be desirable to have available a rapid biochemical (in vitro) prescreen test of high sensitivity, broad spectrum of applicability, and simplicity which would be independent of secondary toxic effects. This type of system would permit rapid identification of compounds or fractions to be tested at higher tiers. Such a test would save considerable time and expense in monitoring effluents and their fractions for harmful effects.

Oxidative drug-metabolizing enzyme systems appear to fulfill the requirements for a biochemical assay for carcinogens and mutagens. These enzymes are associated with the microsomal fraction of a number of organisms, and have been shown to be selectively affected by known mutagens and carcinogens. A closer investigation of this effect could lead to the development of an in vitro system suitable for use as a rapid prescreen for mutagens and carcinogens.

MICROSOMAL BIPHENYL METABOLISM

Microsomal mixed function oxidases of a variety of animals are able to use biphenyl as a substrate for formation of hydroxybiphenyl compounds. In general, 4-hydroxybiphenyl is the major metabolite, and 2-hydroxybiphenyl is a secondary metabolite, with ratios of 26:1 to 2:1 being found depending on the specific species examined (Basu et al., 1971; Creaven et al., 1965; Willis and Addison, 1974).

It has been found by a number of investigators that biphenyl-2-hydroxylase is specifically stimulated by chemical carcinogens. This effect has been demonstrated in vivo in animals and in vitro using microsomes from both animals and plants. Administration of chemical carcinogens and mutagens to test animals resulted in a selective stimulation of biphenyl-2-hydroxylase as measured by an increase of 2- to 20-fold in the amount of 2-hydroxybiphenyl produced (Atlas and Nebert, 1976; Burke and Bridges, 1975; Burke and Prough, 1976; Friedman et al., 1972; Nebert et al., 1975; Tredger and Chhabra, 1976). The production of 4-hydroxybiphenyl was not affected by the treatment. The stimulation of biphenyl-2-hydroxylase by chemical carcinogens occurred in two phases: enzyme activation followed by enzyme induction (McPherson et al., 1976; Parke, 1976).

An in vitro system which permits determination of the activity of biphenyl-2-hydroxylase and biphenyl-4-hydroxylase was developed by McPherson and coworkers (1976). This system involves preparation of purified microsomes, incubation with an NADPH-regenerating system in the presence of biphenyl, extraction and spectrophotofluorometric determination of the 4- and 2-hydroxybiphenyl metabolites (Creaven et al., 1965).

In order to examine the effect of chemical carcinogens and mutagens on biphenyl hydroxylase in vitro, microsomes from animals or plants were preincubated with test compounds, biphenyl added, and the amount of 2- and 4-hydroxybiphenyl compared to control incubation mixtures. It was reported that in vitro preincubation of microsomes with known carcinogens resulted in a 60% to 300% increase in 2-hydroxybiphenyl production (Burke and Bridges, 1975; McPherson et al., 1976, 1975 a,b,c, 1974 a,b). Noncarcinogens had no effect on the hydroxylases, and biphenyl-4-hydroxylase was not affected by the carcinogens.

The biphenyl hydroxylase system which is apparently selectively stimulated by chemical carcinogens would appear to be ideal for the purpose of providing a preliminary screen for environmental carcinogens. The assay system is simple, the in vitro effects correlate well with the in vivo effects, and the reaction substrate (biphenyl) and products are themselves noncarcinogenic and easily detectable.

PROGRAM OBJECTIVES

The over-all program objectives include:

1. Compile a bibliography related to the biphenyl hydroxylase reaction and related subjects.
2. Investigate the utility of the biphenyl hydroxylase system as an enzymatic prescreen for chemical carcinogens and mutagens.
   a. Repeat the work of McPherson and coworkers with a small number of known carcinogens and non-carcinogens in order to validate the in vitro assay.
   b. Refine the test system:
      --- investigate other sources of microsomes
      --- examine the metabolism of other polyphenyls

--- study the metabolism of derivatives of biphenyl
--- examine algal biphenyl metabolism
--- compare the results of the biphenyl assay system with the Ames/Salmonella system for sensitivity to toxic effects and mutagenesis.

c. Validate the system for use with complex mixtures such as leachates from retorted oil shale.

d. Examine the ability of a marine protozoan, Parauronema acutum, to metabolize biphenyl.

SECTION 2

CONCLUSIONS

Attempts to reproduce the experimental data of others (Burke and Bridges, 1975; McPherson et al., 1976, 1975 a,b,c, 1974 a,b; Creaven et al., 1965) as a preliminary step in the development of an enzymatic screen for chemical carcinogens were negative. In all but one published report (McPherson et al., 1975a), the spectrophotofluorometric assay of Creaven and coworkers (1965) was used to determine in vitro production of 2- and 4-hydroxybiphenyl in complex mixtures without prior separation of the metabolites. It was found that this method cannot be used with complex mixtures containing fluorescent metabolites of carcinogens because these metabolites contribute fluorescence at the wavelength used to measure 2-hydroxybiphenyl. Our results are in agreement with the data of Tong and coworkers (1977).

Methods of separating biphenyl metabolites prior to quantification were investigated. High pressure liquid chromatography using a spectrophotofluorometric detector permitted reliable separation and quantification of metabolites. Using this method, it was not possible to repeat published work which demonstrated a stimulation of hepatic microsomal biphenyl-2-hydroxylase by chemical carcinogens. Microsomes were prepared from the same organisms used in previously published work (rat, hamster, and mouse liver; avocado mesocarp) as well as new sources (cauliflower, apple). Further experimentation will be necessary in order to reconcile the results presented in this report with the data of McPherson and coworkers (1975a) which demonstrated a 2.5 fold increase in 2-hydroxybiphenyl using ^{14}C-labeled biphenyl as a substrate.

Terphenyl is metabolized to a minimum of three different compounds by hamster hepatic microsomes. This substrate may prove useful as a supplement to biphenyl in an enzymatic screen for carcinogens. However, further experimentation is necessary to elucidate the nature of the metabolites and enzymes involved, and the effect of chemical carcinogens on metabolite production.

The marine protozoan, Parauronema acutum metabolizes biphenyl to 2- and 4-hydroxybiphenyl. It does not metabolize BaP (Lindmark, 1978). Preliminary experiments indicate that BaP at low concentrations may protect the organism from the lethal effects of biphenyl. This sytem may therefore prove useful in the study of carcinogen-induced membrane changes. In addition, hydroxylases of P. acutum are both soluble and membrane-bound (Lindmark, 1978). Parauronema acutum may prove to be a source of stable, easily recovered hydroxylases which could be used in an enzymatic screen for carcinogens when coupled with a carcinogen activating system.

SECTION 3

RECOMMENDATIONS

Because the in vivo stimulation of biphenyl-2-hydroxylase by chemical carcinogens is well documented, it would be useful to develop a rapid in vitro procedure using this system. Therefore, experiments should be performed using ^{14}C-labeled substrates and both TLC and HPLC separation techniques to determine whether or not biphenyl-2-hydroxylase is selectively stimulated in vitro by chemical carcinogens. In addition, simpler, more rapid methods of preparing plant or animal microsomes should be investigated. If the stimulation can be reliably demonstrated, further work should be performed to allow this system to be used as a routine assay.

Metabolism of terphenyls should be studied as a possible alternative or complement to biphenyl metabolism. The enzymatic system(s) responsible for terphenyl metabolism may prove to exhibit a different sensitivity to carcinogens or may be more stable than the biphenyl hydroxylases. This would prove extremely useful in the development of an enzymatic screen.

The metabolism of biphenyl by Parauronema acutum should be confirmed, and an investigation of the nature and stability of the enzymes involved should be carried out. Studies should be conducted to determine the response of these enzymes to activated and unactivated carcinogens both in vivo and in vitro. This organism could provide an excellent source of biphenyl-2-hydroxylase and biphenyl-4-hydroxylase for an enzymatic screen.

SECTION 4

ANALYTICAL METHODS

GENERAL

A number of different analytical approaches to quantitative measurement of 2- and 4-hydroxybiphenyl are possible. Initially the spectrophotofluorometric (SPF) method of Creaven and coworkers (1965) was employed in an effort to validate the assay system. This method was used in the majority of the in vitro work published by McPherson and coworkers, and has the advantage that metabolite separation is not necessary. It became apparent that the SPF method does not provide a valid measurement of the amount of 2-hydroxybiphenyl present when fluorescent carcinogens and their metabolites are present in reaction mixtures. Therefore two different techniques for separation of the metabolites were examined: thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC).

SPECTROPHOTOFLUOROMETRY

Background

Creaven and coworkers (1965) demonstrated that 2-hydroxybiphenyl and 4-hydroxybiphenyl can be determined fluorometrically in mixtures of the two compounds because the 2-isomer exhibits excited state ionization whereas the 4-isomer does not. At pH 2-9, 2-hydroxybiphenyl absorbs light in the unionized form (excitation wavelength = λ_{EX} = 295 nm), but emits the fluorescence of the anion (emission

wavelength = λ_{EM} = 415 nm) whereas 4-hydroxybiphenyl absorbs (λ_{EX} = 275 nm) and emits (λ_{EM} = 338 nm) in the unionized form. Figure 1 shows the excitation and emission spectra of the pure 2- and 4-hydroxybiphenyls determined using an Aminco Bowman spectrophotofluorometer equipped with a high pressure xenon lamp. The excitation and emission maxima of 290 nm and 412 nm for 2-hydroxybiphenyl, and 274 nm and 335 nm for 4-hydroxybiphenyl are within the limits of instrument variability of the values reported by Creaven and coworkers (1965) given above. The quantities of the two compounds present in a mixture are therefore determined fluorometrically at an acid pH using two different combinations of excitation and emission wavelengths.

Materials and Methods

All chemicals were either reagent grade or of the highest purity available. For detailed information concerning purity, see page ix.

Incubation of microsomes with substrate and test compounds was performed as described in Section 5. The method of extracting the biphenyl metabolites from microsome incubation mixtures used by Creaven and coworkers (1965) and all subsequent investigators was followed exactly. It is summarized in Figure 2.

After the incubation period was completed, the reaction was terminated by addition of 1 ml of 4N HCl, and the mixture immediately extracted with n-heptane. The incubation procedure and heptane extraction were carried out in 20 ml glass tubes with teflon lined screw caps. After the initial centrifugation, the tubes were stored in the cold overnight. The samples were extracted and analyzed with a mini-

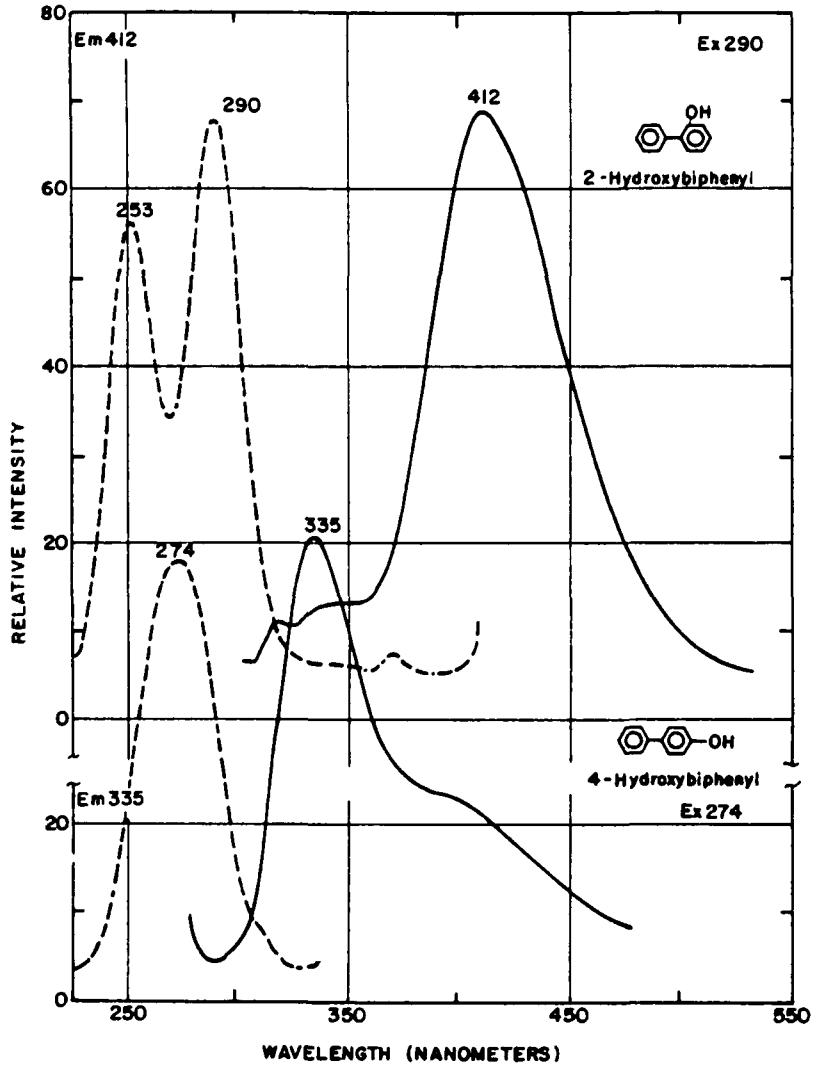


Figure 1. Excitation and emission spectra of 2- and 4-hydroxybiphenyl standards in 0.1N NaOH buffered to pH 5.5 with 0.1N succinic acid. The solid lines are emission spectra, and the dashed lines are excitation spectra. The spectrum for 2-hydroxybiphenyl is vertically offset and is shown in the upper portion in this and all following figures.

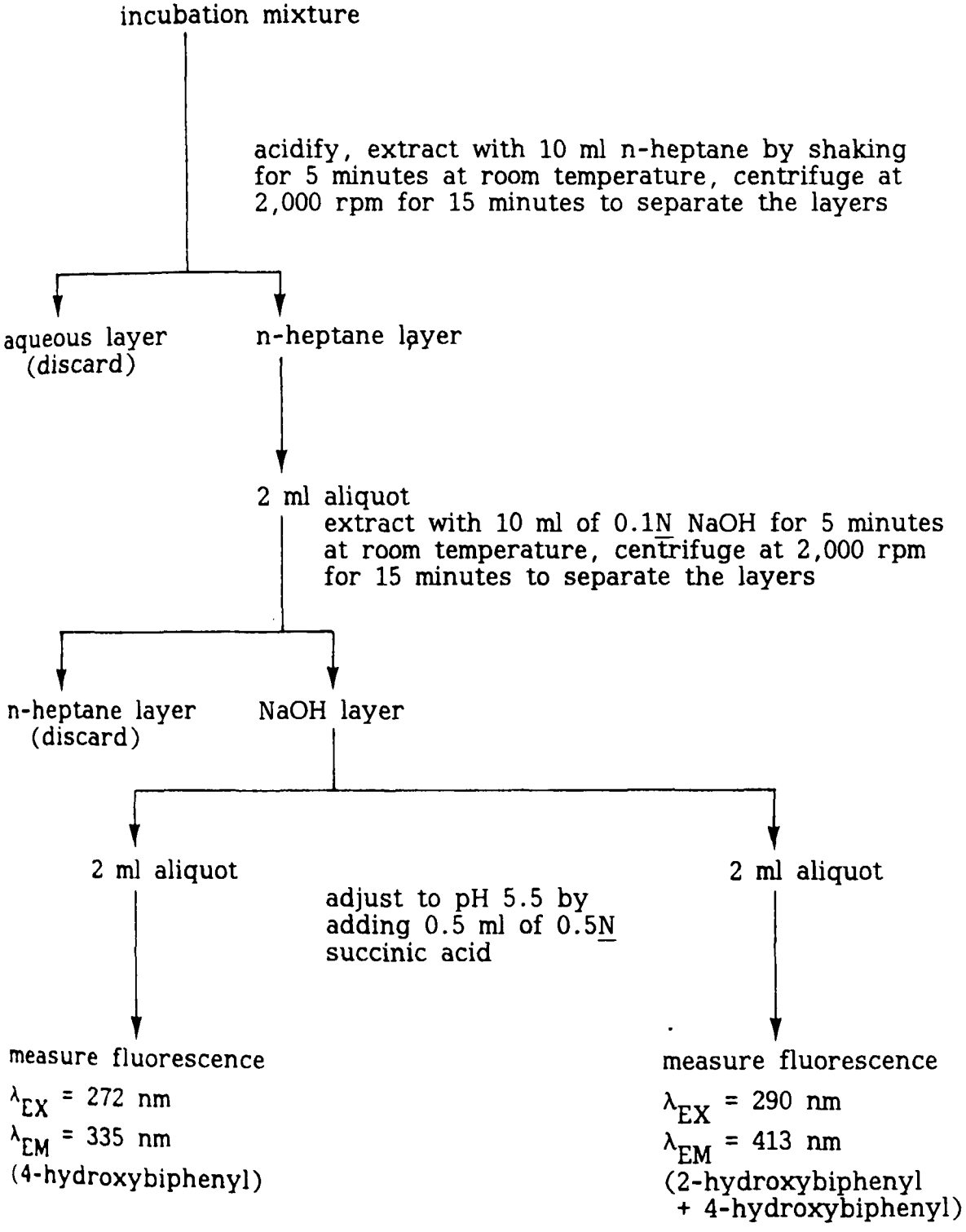


Figure 2. Extraction procedure for SPF analysis of 2- and 4-hydroxybiphenyl (Creaven et al., 1965).

mum of exposure to ultraviolet light. Therefore incandescent light bulbs were used when necessary.

The tubes containing the heptane extract were removed from storage and allowed to come to ambient temperature. The 2 ml aliquots of the n-heptane layer were transferred to 14 ml glass tubes with teflon lined screw caps. The tubes containing the remaining 8 ml of n-heptane were returned to cold storage. After extraction and centrifugation, the heptane was removed by pipetting and discarded. A 2 ml aliquot of the NaOH extract was transferred to a 5 ml quartz cuvette, and adjusted to pH 5.5 by addition of 0.5 ml of 0.5N succinic acid. Fluorescence was measured using an Aminco Bowman spectrophotofluorometer. Fluorescence of the 4-isomer was measured first because 4-hydroxybiphenyl was less stable in the basic NaOH solution than was 2-hydroxybiphenyl. The instrument was corrected for background solvent fluorescence using 2 ml of 0.1N NaOH to which 0.5 ml of 0.5N succinic acid was added.

Calibration curves were prepared using three standard solutions: 24.8 µg/ml of 4-hydroxybiphenyl, 6.0 µg/ml of 2-hydroxybiphenyl, and a mixture containing 24.8 µg/ml of 4-hydroxybiphenyl and 6.0 µg/ml of 2-hydroxybiphenyl. The standards were dissolved in an aqueous 5% (v/v) ethanol solution. Following HCl addition, 1 ml of each standard solution was added to each of three microsome incubation tubes from which the biphenyl substrate had been omitted. The solutions were then extracted as described in Figure 2. Three dilutions of each standard were used to construct quantitative calibration curves. A set of standard curves was constructed for each experiment. This method of preparing standard curves allows the calculation of absolute amounts

of metabolites present in the unknown reaction mixtures without the need to determine efficiency of extraction because all samples are treated in an identical manner.

The quantities of the two metabolites present in the extract were calculated according to the method of Creaven and coworkers (1965). The 2-isomer does not interfere with fluorometric determination of the 4-isomer. However, the 4-isomer contributes fluorescence at the wavelength used to measure 2-hydroxybiphenyl. Therefore, the following measurements and calculations must be performed:

1. Determine the percent fluorescence of the unknown solution using λ_{EX} = 274, λ_{EM} = 335 nm. Read the quantity of 4-hydroxybiphenyl present from the standard curve.
2. Determine the percent fluorescence of the 4-hydroxybiphenyl standard at λ_{EX} = 290, λ_{EM} = 412 nm.
3. Determine the percent fluorescence of the unknown solution using λ_{EX} = 290, λ_{EM} = 412 nm. This is the sum of the emissions of both isomers.
4. Calculate the amount of 2-hydroxybiphenyl present in the mixture from the equation (Creaven et al., 1965):

$$C = B - (Ax/y)$$

where: C = percent fluorescence of 2-hydroxybiphenyl at 412 nm

B = percent fluorescence of the mixture at 412 nm

A = percent fluroescence of the standard 4-hydroxybiphenyl solution at 412 nm

x = concentration of 4-hydroxybiphenyl in the unknown

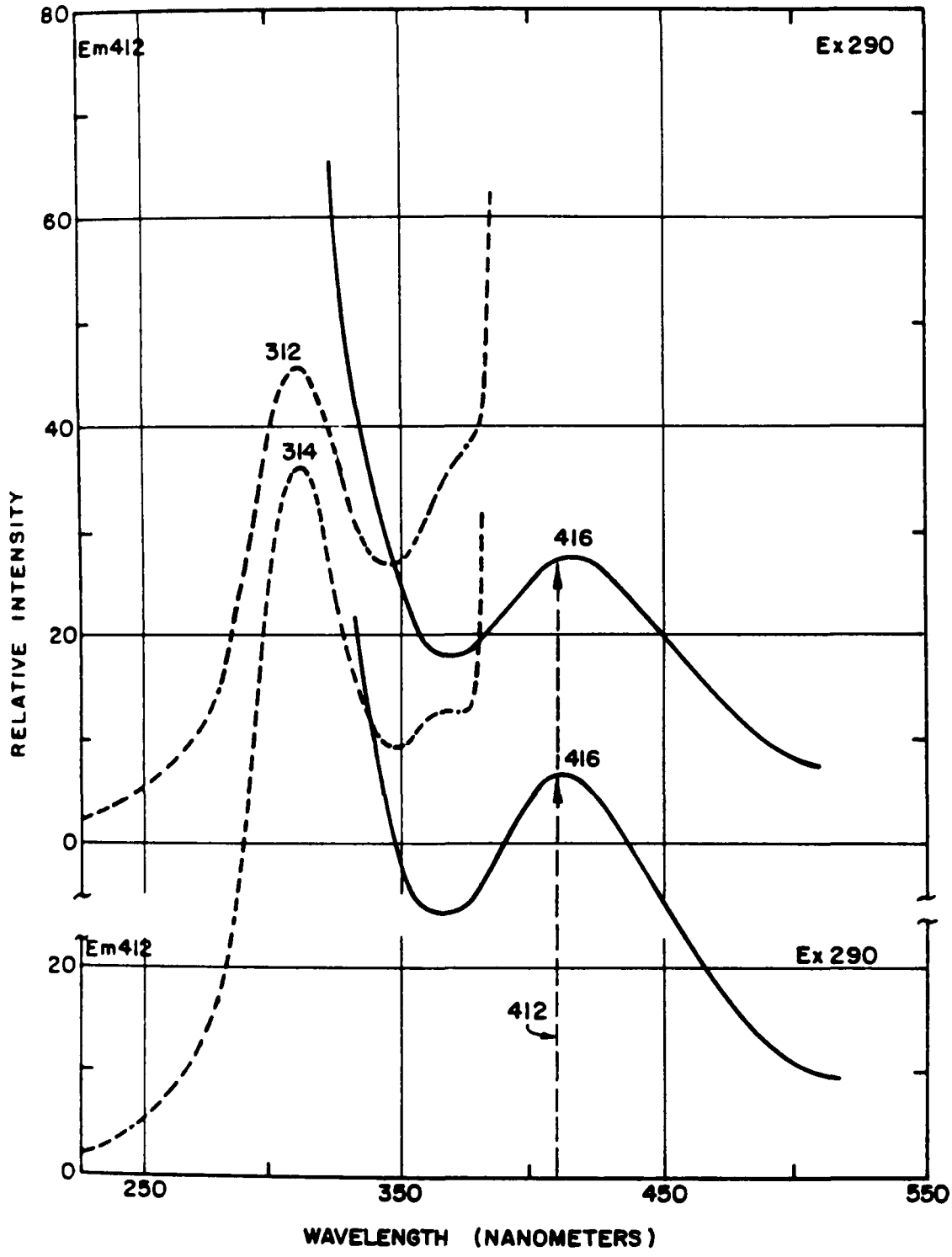
y = concentration of 4-hydroxybiphenyl in the standard

The concentration of 2-hydroxybiphenyl in the unknown mixture can then be determined from the standard curve.

Results

Because the results obtained using this method were anomalous, excitation and emission spectra of several different mixtures were examined.

Initially it was thought that the peanut oil present in the microsome incubation mixture might contribute fluorescence at the wavelengths used to measure the biphenyls. In addition, it was also possible that unaltered carcinogens such as benzo[a]pyrene (BaP) could interfere with the fluorescence measurements. Therefore, 0.5 ml of peanut oil and 0.5 ml of 1 mM BaP dissolved in peanut oil were dissolved in 10 ml each of n-heptane and extracted as described in Figure 2. The excitation and emission spectra of these extracts are shown in Figure 3. The results show that material extracted from peanut oil does fluoresce at the wavelength used to measure 2-hydroxybiphenyl. However, the peanut oil is present in all samples, including the standards, and this effect is therefore corrected for when the standard curves are constructed. The sample containing BaP in oil showed the same spectrum as the oil alone. Therefore unaltered BaP does not contribute to fluorescence at the wavelength used to determine the 2-isomer. In addition, BaP is apparently not hydroxylated during the extraction procedure.



igure 3. Excitation and emission spectra of material extracted from oil (bottom spectrum) and from BaP dissolved in oil (top spectrum).

Figure 4 shows excitation and emission spectra of material extracted from incubation mixtures containing purified hamster microsomes. As expected, both 2- and 4-hydroxybiphenyl were formed from the biphenyl (Figure 4A). However, the emission peak for 2-hydroxybiphenyl was shifted to 418 nm, and 4-hydroxybiphenyl appeared as a broad shoulder at 335 nm. Incubation mixtures containing BaP (Figure 4B) had a broad emission peak from 380 to 470 nm when excited at 290 nm. Material which was extracted from mixtures containing BaP and biphenyl (Figure 4C) again had the 418 nm peak, but lacked the 335 nm shoulder.

It is apparent that metabolites of BaP formed during incubation with the microsomes are coextracted with the hydroxylated biphenyls, and contribute significant fluorescence at the wavelength used to measure 2-hydroxybiphenyl. Determination of 4-hydroxybiphenyl is relatively unaffected by the fluorescence of these metabolites.

In the case of material extracted from incubation mixtures containing plant microsomes and biphenyl (Figure 5A), two emission optima were observed. Excitation at 290 nm gave an emission peak at 405 nm, and excitation at 272 nm gave an emission peak at 367 nm. A similar pattern was observed in material extracted from microsomes incubated with BaP in oil (Figure 5B) or methylcholanthrene (MC) in oil (Figure 6A). Incubation mixtures containing both carcinogen and biphenyl yielded material which had an increased fluorescence in the 405 to 410 nm region. The emission spectrum obtained by exciting at 272 nm showed essentially no discrete peaks in the case of BaP plus biphenyl (Figure 5C), and a broad shoulder (350 to 450 nm) in the case of MC plus biphenyl (Figure 6B).

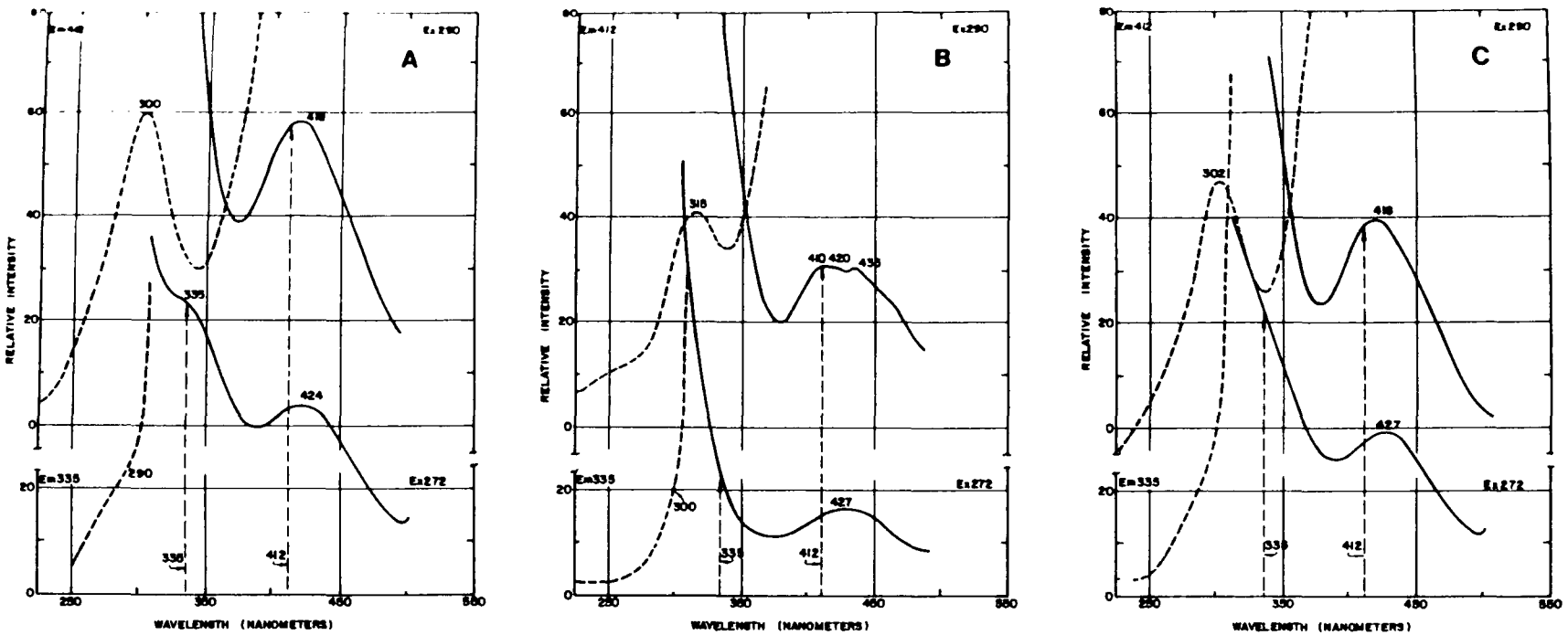


Figure 4. Excitation and emission spectra of material extracted from incubation mixtures containing purified hamster microsomes and (A) oil plus biphenyl; (B) BaP in oil; (C) BaP in oil plus biphenyl. The dashed vertical lines designate 335 and 412 nm, the wavelengths at which emission was measured in the fluorometric assay.

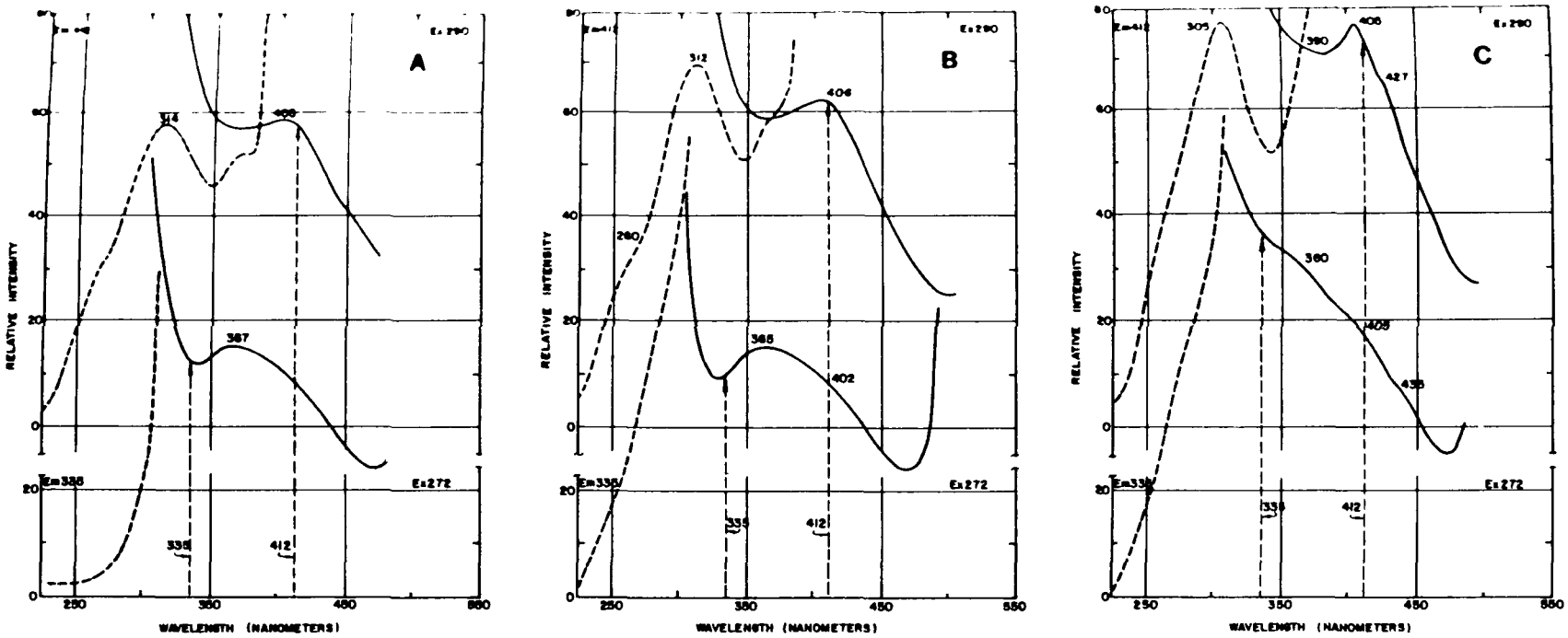


Figure 5. Excitation and emission spectra of material extracted from incubation mixtures containing purified cauliflower microsomes and (A) oil plus biphenyl; (B) BaP in oil; (C) BaP in oil plus biphenyl.

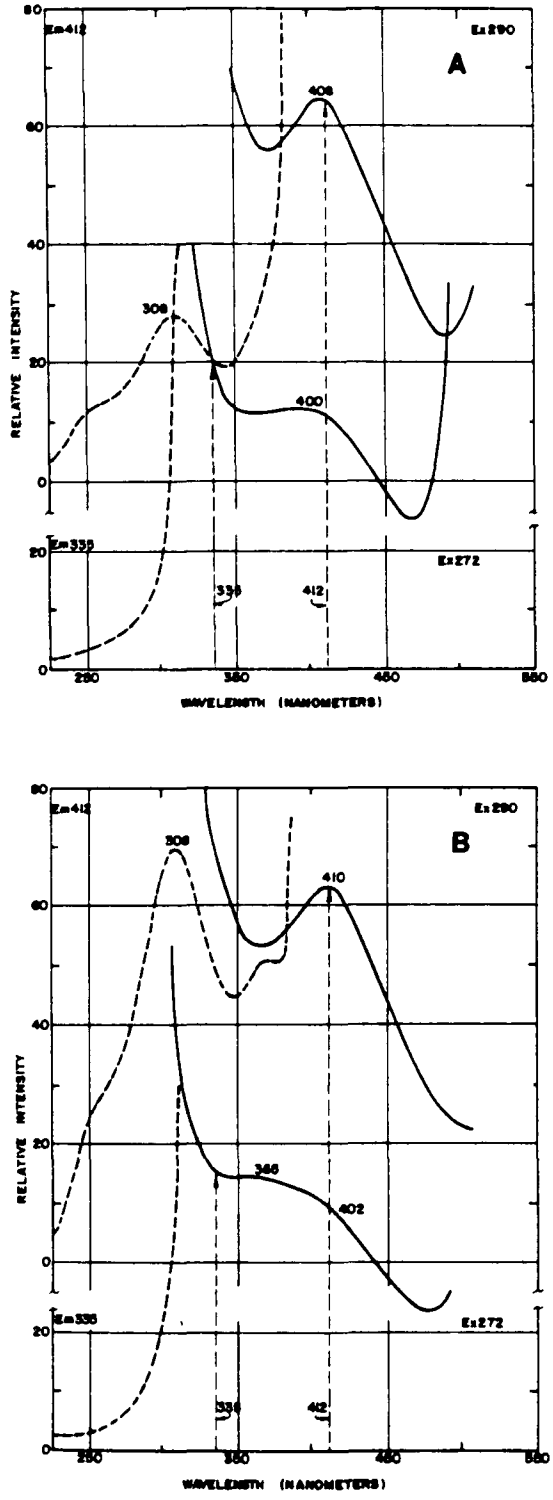


Figure 6. Excitation and emission spectra of material extracted from incubation mixtures containing purified cauliflower microsomes and (A) MC in oil; (B) MC in oil plus biphenyl.

Discussion

It is obvious that the spectrophotofluorometric method of Creaven and coworkers (1965) is not useful for determining hydroxybiphenyls in the presence of carcinogens whose metabolites fluoresce at the wavelengths used to measure the hydroxylated biphenyls. This conclusion agrees with the findings of Tong and coworkers (1977) which were published after the conclusion of the SPF work presented in this report.

It is therefore necessary to separate the metabolites of biphenyl from each other and from other fluorescent material extracted from incubation mixtures prior to making quantitative measurements. Two such possible separation methods were examined next.

THIN LAYER CHROMATOGRAPHY

Background

McPherson and coworkers (1975a) used thin layer chromatography (TLC) to separate ^{14}C-labeled metabolites of biphenyl. Initial experiments therefore employed the same silica gel substrate (HF_{254}) and solvent system (benzene:ethanol, 95:5, v/v) as these investigators in order to validate the separation technique. Subsequently other solvent systems were examined as a preliminary step in selecting the optimal solvents to be used in the high performance liquid chromatographic separation of metabolites on a silica gel column.

Materials and Methods

A slurry containing 30 g of silica gel HF_{254}, type 60 (EM Reagents), 96 ml deionized water, and 4 ml acetone was mixed in a Virtis blendor at high speed for 2 minutes. The slurry was spread using a Desaga spreader onto 20 × 20 cm glass plates to a thickness of 0.25 mm. The plates were air dried for at least 8 hours. Standards were dissolved in methanol. Plates were examined under a Mineralight UVS-54 lamp at a wavelength of 254 nm. All chemicals were reagent grade.

Results

Table 1 gives the Rf values obtained using a number of different solvent systems. As can be seen, several of the solvent systems gave good separation of the four hydroxylated biphenyls. Both p- and m-terphenyl were also examined in the benzene:ethanol (95:5) system, and were found to have the same Rf values as biphenyl.

Use of TLC to quantitate biphenyl metabolite production requires removal of the spot from the plate and analysis either by scintillation counting (McPherson et al. 1975a) of labeled material or fluorescence of unlabeled compounds. In order to examine the possibility of using fluorescence measurements, the silica gel alone was scraped from the plate, eluted with benzene, and fluorescence determined. It was found that material was eluted which would interfere with measurement of the hydroxylated biphenyls.

TABLE 1. SEPARATION OF BIPHENYLS BY TLC

Rf values for solvent systems[1]:

compound	µg	benzene: ethanol[3] 95:5	CCl_4: acetone 20:1	CCl_4: methanol 20:1	CCl_4: methanol 95:10	$CHCl_3$:n-hexane: methanol 45:30:5	dioxane: n-hexane 20:80	dioxane: iso-octane 20:80	dioxane: cyclohexan 20:80
biphenyl	14.3	0.71	0.54	0.58	0.63	0.72	0.57	0.45	0.49
2-hydroxybiphenyl	14.1	0.55	0.24	0.30	0.39	0.55	0.26	0.19	0.26
4-hydroxybiphenyl	19.3	0.38	0.12	0.15	0.27	0.39	0.19	0.14	0.19
2,2'-dihydroxybiphenyl	16.1	0.30	0.06	0.12	0.24	0.34	0.12	0.09	0.12
4,4'-dihydroxybiphenyl	10.3	0.12	0.01	0.02	0.08	0.10	0.04	0.03	0.05

compound	µg	THF 100%	THF: cyclohexane 50:50	THF: cyclohexane 10:90	THF: dioxane 10:90	THF: iso-octane 50:50	THF: iso-octane 10:90	THF: n-hexane 50:50	THF: n-hexane 10:90
biphenyl	14.3	0.63	0.52	0.39	0.63	0.53	0.44	0.61	0.55
2-hydroxybiphenyl	14.1	0.60	0.45	0.15	0.59	0.43	0.13	0.52	0.20
4-hydroxybiphenyl	19.3	0.60	0.41	0.08	0.57	0.39	0.08	0.48	0.12
2,2'-dihydroxybiphenyl	16.1	0.60	0.36	0.05	0.59	0.37	0.04	0.44	0.07
4,4'-dihydroxybiphenyl	10.3	0.59	0.35	0.04	0.62	0.36	0.02	0.37	0.03

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1 Each compound was placed in a separate spot. Abbreviations: CCl_4 = carbon tetrachloride, CCl_3 = chloroform, THF = tetrahydrofuran.

2 Total amount of material spotted on the plate.

3 Values are the average of four different determinations.

Discussion

Thin layer chromatography is a valid method for separating hydroxylated biphenyls. However, unless radioactive material is used, quantitation appears to be difficult. The method is not useful in conjunction with a routine assay system because the amount of material present cannot be determined directly on the plate. A more direct method for separation and quantitation of metabolites is required.

HIGH PRESSURE LIQUID CHROMATOGRAPHY

Background

High pressure liquid chromatography has recently been used to separate and identify polycyclic aromatic hydrocarbons in complex mixtures (Burchill et al., 1978; Dong and Locke, 1976; Thomas et al., 1978). This method combined with either an ultraviolet or fluorometric detection system would appear to provide several advantages as an analytical means for quantitating biphenyl metabolite production. Sample handling would be minimal because the n-heptane extract of the microsomal incubation mixture could be used directly. An SPF detection system would permit selective quantitative measurement of material at wavelengths specific for a given compound thus minimizing interferences. The SPF system also allows detection of very small amounts of material.

Materials and Methods

A Perkin-Elmer 220 high pressure liquid chromatograph equipped with an ultraviolet detector (254 nm) was used. The chroma-

tograph was attached to the spectrophotofluorometer by means of a 150 µl flow through cell having a 2 mm path length. Three different columns (Whatman) were assessed: Partisil 10, Partisil PSX 10/25 PAC, and Partisil ODS/2. All were 25 cm long × 4.6 mm inside diameter. The temperature was ambient. The location of compounds in the chromatographic fractions was recorded using a Linear Instrument Company strip chart recorder at a chart speed of 16 inches per hour. Ten microliters of the n-heptane extract of the microsome incubation mixtures was injected directly into the chromatograph. All chemicals were of the highest purity available, and all solvents were spectrophotometric grade, distilled in glass.

Results

Table 2 shows that the hydroxybiphenyls can be separated from each other and from biphenyl using HPLC. The best separation was obtained using a Partisil PSX 10/25 PAC column and a solvent system of THF:n-hexane of 15:85. The retention time of BaP under these conditions was 4.2 minutes.

The optimum fluorescence wavelengths for detection of the hydroxybiphenyls in this solvent system were determined, and the following standard conditions were therefore used in all subsequent analyses:

column:	Partisil PSX 10/25 PAC	flow rate:	2 ml/min.
solvent:	THF:n-hexane, 15:85	pressure:	300 psi
fluorescence detector:	λ_{EX} = 300 nm	range:	0.33
	λ_{EM} = 335 nm		

TABLE 2. SEPARATION OF BIPHENYL STANDARDS BY HPLC[1]

column	solvent system		flow rate (ml/min)	retention time (minutes) of: biphenyl	4-hydroxy-biphenyl	2-hydroxy-biphenyl
Partisil 10	dioxane:n-hexane	10:90	1	--	9.9	5.8
	THF:isooctane	15:85	1	--	6.8	4.4
		13:87	1	--	8.4	5.0
Partisil PSX	THF	100%	1	--	3.8	3.6
10/25 PAC	THF:n-hexane	50:50	1	--	6.0	5.5
		25:75	1	--	11.2	9.7
		15:85	1	--	21.4	17.6
		15:85	2	2.2	14.3[2]	10.5
Partisil ODS/2	methanol:water	80:20[3]	1	10.2	5.7	5.9
		85:15	1	7.6	4.6	4.2
		90:10[3]	1	9.2	6.2	4.4
		90:10[3]	0.5	9.8	6.5	4.4

[1] Except as noted, compounds were monitored with an ultraviolet light detector at 254 nm. A line indicates that the retention time was not determined.

[2] The retention times for 2- and 4-hydroxybiphenyl are an average of three separate determinations.

[3] Compounds were monitored fluorometrically at λ_{EX} = 300 nm, λ_{EM} = 335 nm.

An example of the chromatogram of 2- and 4-hydroxybiphenyl standards obtained using this system is shown in Figure 7.

The separation of 2- and 4-hydroxybiphenyl in an extract from an incubation mixture containing purified hamster microsomes is shown in Figure 8. The positions of the 2- and 4-isomers are clearly defined, and well separated from earlier peaks (Figure 8A). Most of the early peaks represent material extracted from the complex incubation mixture as is shown in Figure 8B.

Using this system, 2- and 4-hydroxybiphenyl can be identified unequivocably, and there is no interference from metabolites of the carcinogens added to the incubation mixtures (Figures 9 and 10). In order to be certain that this was true, an extract of an incubation mixture containing purified hamster microsomes, BaP in oil and biphenyl was chromatographed using the standard conditions except that λ_{EM} was 422 nm. This is the optimum wavelength for detection of potential BaP metabolites. Figure 11 shows that there are no BaP metabolites at the retention times of the hydroxybiphenyls.

In order to ascertain whether the supernatant oxygen present during the extraction of the incubation mixture could produce oxidation products which would interfere with the detection of the hydroxybiphenyl compounds, a standard solution of BaP was exposed to air and sunlight and subsequently chromatographed. The conditions were standard with the exception that the emission wavelength was 422 nm. It can be seen (Figure 12) that even under these extreme conditions, only traces of materials have been formed which have retention times similar to those of the hydroxybiphenyls.

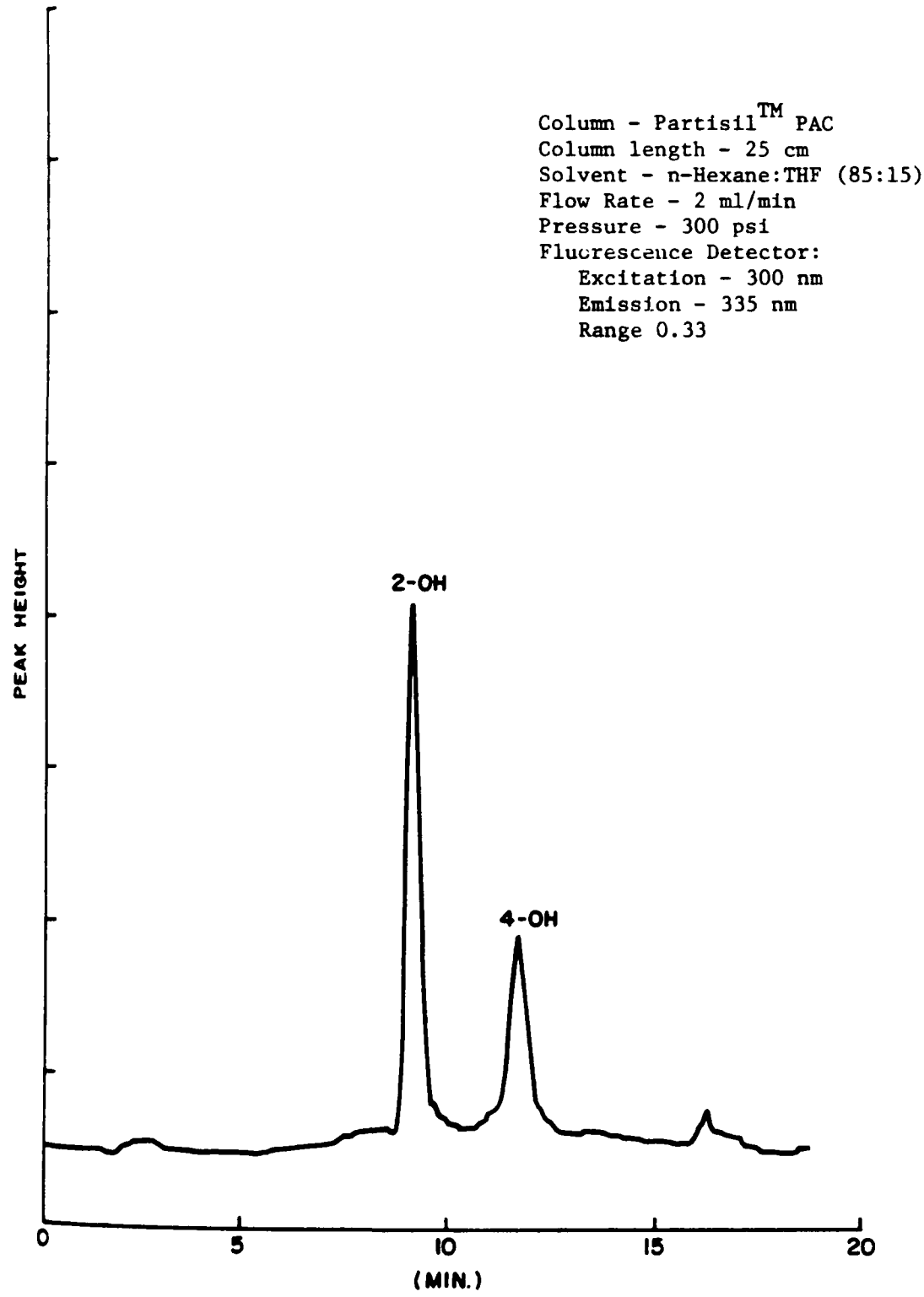

Figure 7. Separation of 2- and 4-hydroxybiphenyl standards by HPLC. The sample contained 2.4 ng of each standard.

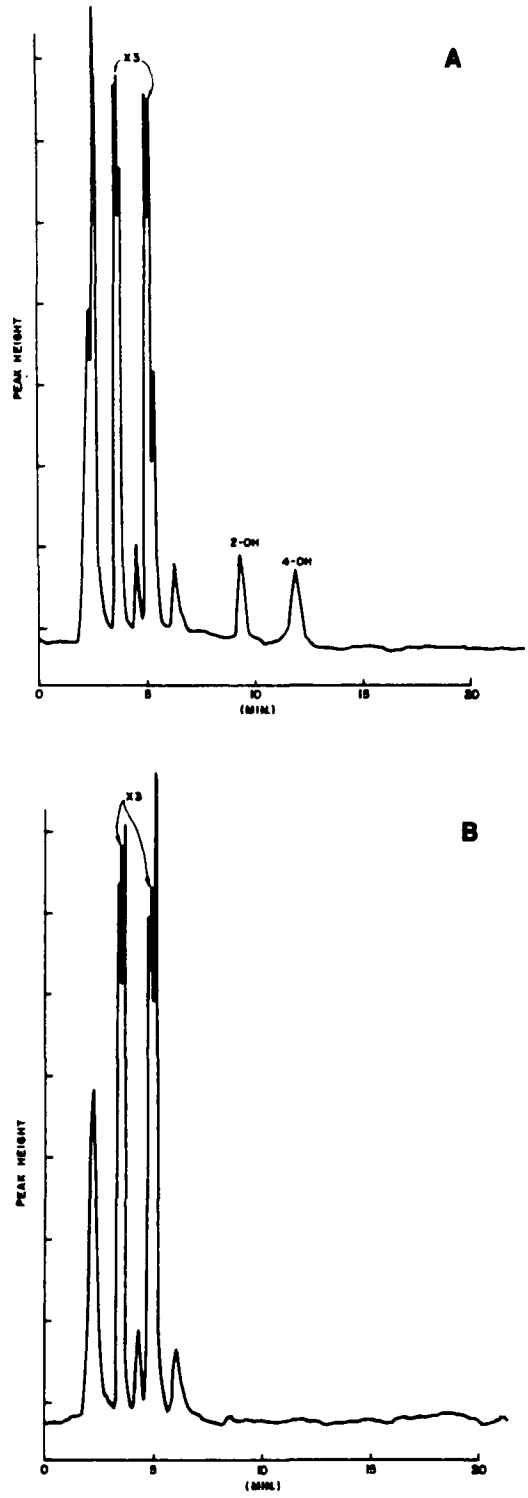

Figure 8. Material separated by HPLC and obtained from reaction mixtures containing purified hamster microsomes and (A) oil plus biphenyl; (B) oil alone.

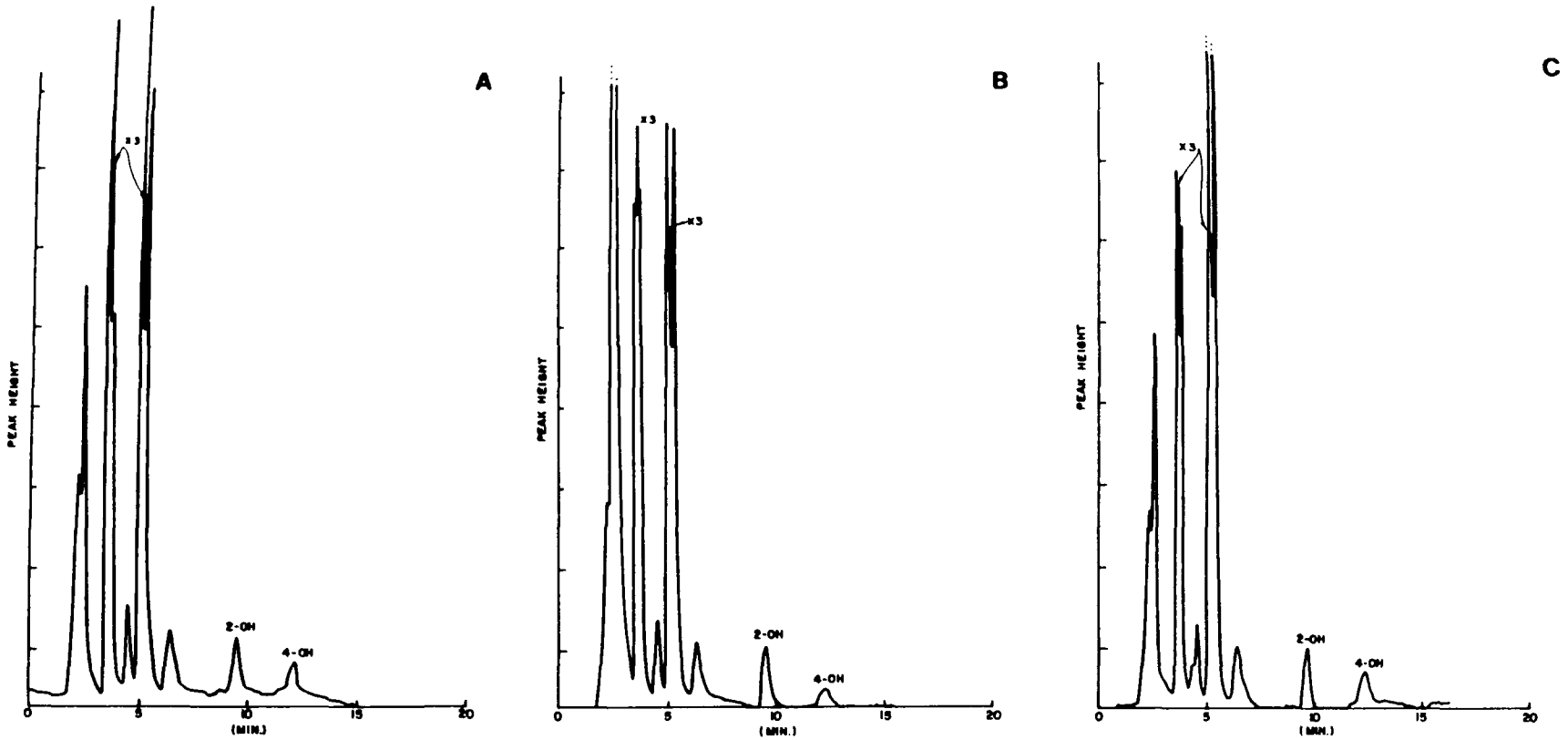

Figure 9. HPCL of material extracted from purified hamster microsomes incubated with biphenyl and (A) BaP in oil; (B) safrole in oil; (C) 3-methylcholanthrene in oil.

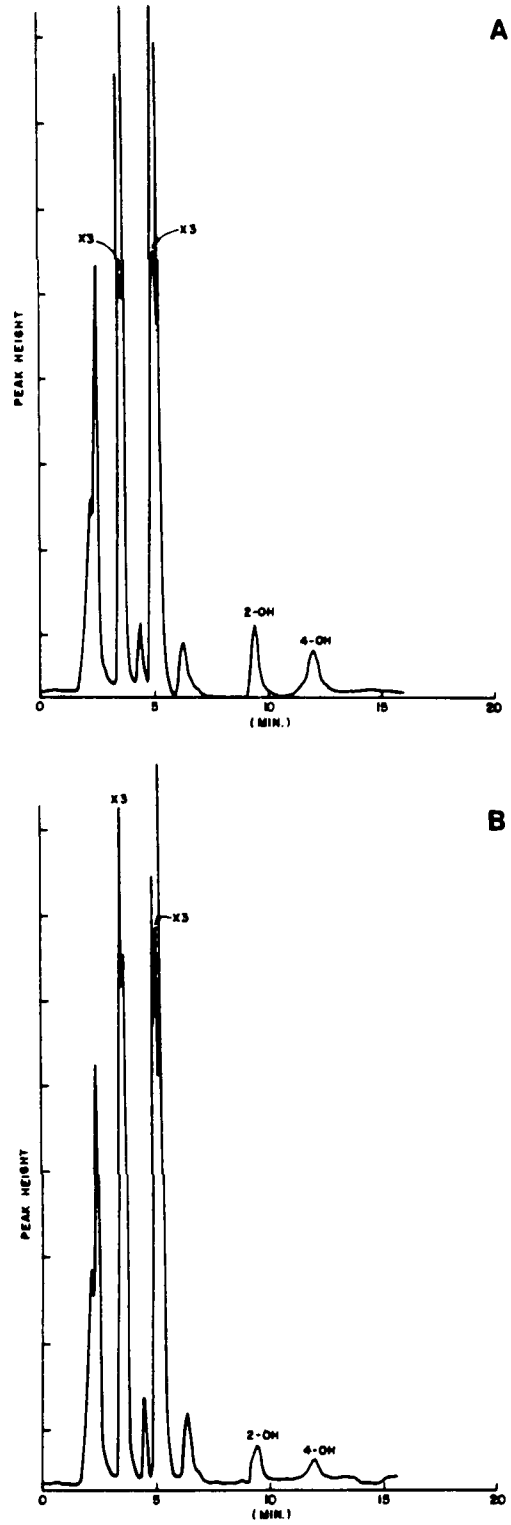

Figure 10. HPLC of material extracted from purified hamster microsomes incubated with biphenyl and (A) α-naphthylamine in oil; (B) β-naphthylamine in oil.

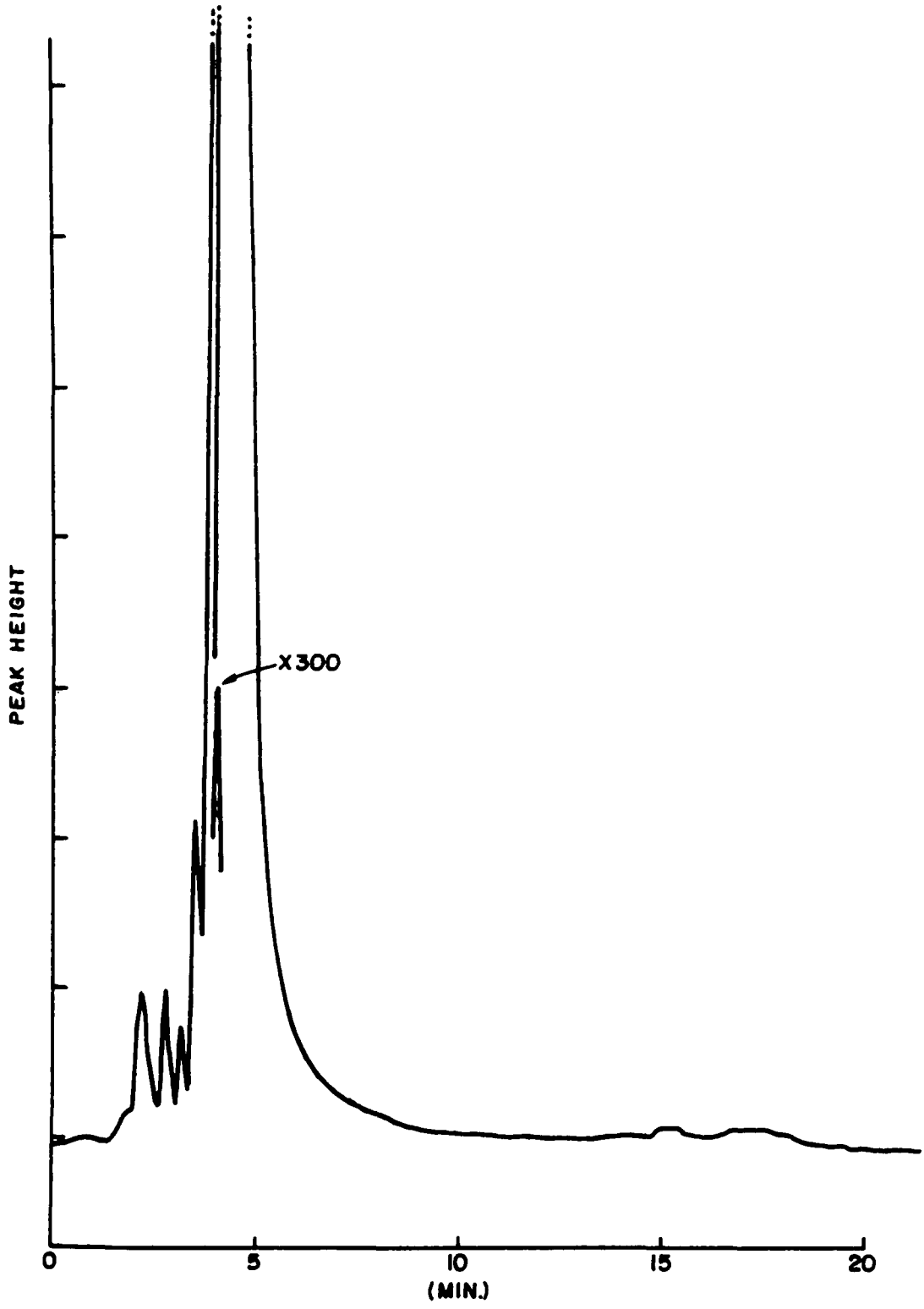

Figure 11. HPLC of material extracted from purified hamster microsomes incubated with BaP in oil. The emission wavelength was optimum for detection of BaP metabolites.

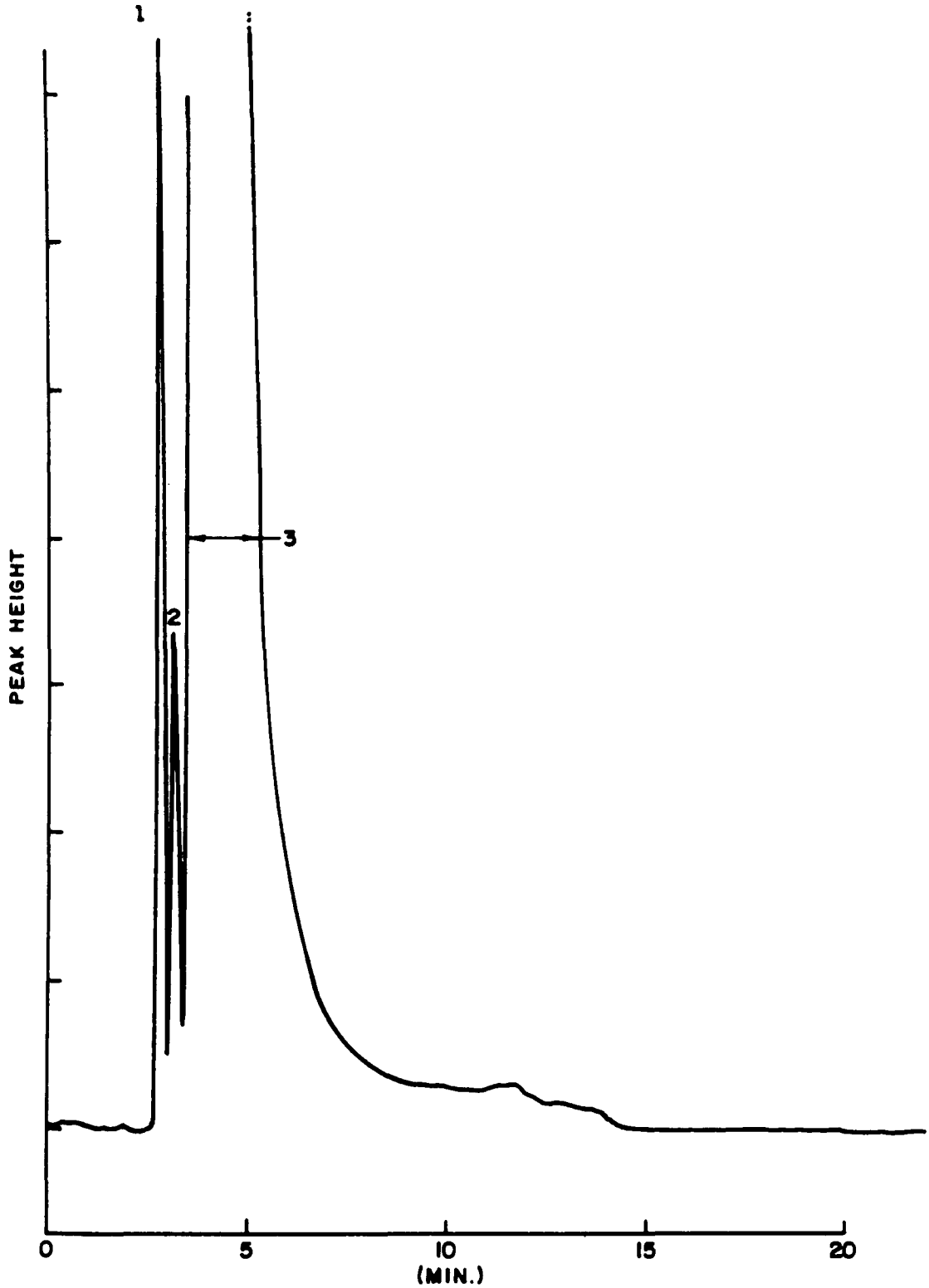

Figure 12. HPLC of a standard BaP solution exposed to oxygen and light for several hours.

Discussion

HPLC combined with SPF would appear to be the analytical method of choice for determining the amounts of 2- and 4-hydroxybiphenyl produced in complex reaction mixtures. Because of problems with the Partisil PSX 10/25 PAC column in terms of stability, it may be necessary to use the Partisil ODS/2 column for long term studies.

SECTION 5

ENZYMATIC HYDROXYLATION EXPERIMENTS

GENERAL

A second major objective of this work was the duplication of *in vitro* stimulation of biphenyl-2-hydroxylase by chemical carcinogens as described by others.

The *in vivo* stimulation of microsomal biphenyl-2- and 4-hydroxylase by chemical carcinogens is well documented for a number of animals (Creaven *et al.*, 1965; Atlas and Nebert, 1975; Hook *et al.*, 1975; Burke and Prough, 1976; Burke and Bridges, 1975). The *in vitro* stimulation of biphenyl-2-hydroxylase has also been reported for both animal and plant microsomes (Creaven *et al.*, 1965; McPherson *et al.*, 1976, 1975 a,b,c 1974 a,b; Tredger *et al.*, 1976; Tong *et al.*, 1977).

We have examined the ability of chemical carcinogens to stimulate production of 2-hydroxybiphenyl *in vitro* using both plant and animal microsomes. The first series of investigations were carried out to duplicate the experiments and data reported by McPherson, Bridges and Parke (1976) on the *in vitro* effects of benzopyrene and safrole on biphenyl-2-hydroxylase and other drug-metabolizing enzymes using liver microsomal extracts and microsomes from avocado pear (*Persea americana*) (McPerson *et al.*, 1975b). The method used by these authors was followed exactly.

The investigation of substrates other than biphenyl was also initiated. Because of the somewhat labile nature of the biphenyl-2-

hydroxylase, it was thought that a higher order polyphenyl substrate such as terphenyl might provide useful information.

ENZYMATIC METHODS

Chemicals

Benzo(a)pyrene (BaP) and safrole (SA) were purchased from Aldrich Chemical Company, α-naphthylamine (α-NA) and β-naphthylamine (β-NA) were from Sigma, and 20-methylcholanthrene (MC) was from K&K Chemicals. All were of the highest purity available. These compounds were dissolved in peanut oil (Planter's) to provide stock solutions at 1 mM. Biphenyl was dissolved in 1.5% (w/v) Tween 80 and 1.15% (w/v) KCl to make a 13 mM stock solution. Stock solutions of the hydroxylated standards were made in 5% (v/v) aqueous ethanol at the following concentrations: 4-hydroxybiphenyl, 0.146 μmoles/ml; 2-hydroxybiphenyl 0.0342 μmoles/ml; 2-hydroxybiphenyl and 4-hydroxybiphenyl, 0.146 μmoles/ml and 0.146 μmoles/ml, respectively. A thin layer chromatogram of the substrate and standard compounds at 0.1 mg material per spot showed no impurities in the biphenyl or 2-hydroxybiphenyl, but trace amounts of biphenyl and 2-hydroxybiphenyl in the 4-hydroxybiphenyl standard. Meta-terphenyl was recrystallized, and p-terphenyl (99+%) was purchased from Aldrich Chemical Co. Stock solutions at 13 mM in Tween 80 and 1.15% KCl were made as for biphenyl.

Animals

Swiss-Webster mice (mean weight 37.9 g), Sprague-Dawley rats (mean weight 172.5 g) and Syrian hamsters (mean weight 118.7 g)

were obtained from commercial breeders. Water and food (Wayne Lab Blocks) were provided ad libitum. Animals were sacrificed between 8:30 and 10:30 a.m. by decapitation.

Preparation of Hepatic Microsomes

Microsomes were prepared by the method of McPherson and coworkers (1976). The livers were rapidly removed into cold buffered KCl (1.15% w/v KCl, 0.3 M NaH_2PO_4, 0.3 M K_2HPO_4, pH 7.6), blotted, weighed, and placed in fresh cold buffered KCl. The weighed livers were homogenized with a motor-driven teflon pestle using 10 strokes of 10 seconds each at 1,200 rpm. The homogenate was diluted with cold buffered KCl to 250 mg tissue per ml of homogenate and centrifuged (2°C) for 10 minutes at 15,000 g. In one case, this low speed pellet was resuspended in buffered KCl at 25 mg protein/ml and used in a hydroxylation experiment. The low speed supernatant was decanted and centrifuged. The supernatant was discarded and the pellet washed with cold buffered KCl, resuspended in cold buffered KCl, and again centrifuged (2°C) for 60 minutes at 104,000 g. The final pellets were resuspended in cold buffered KCl at a protein concentration of 10 mg/ml. Protein was determined by the method of Lowry and co-workers (1951).

Preparation of Plant Microsomes

Plant Microsomes were prepared according to the method of McPherson and coworkers (1975b). Plant material was obtained 24 hours prior to use and stored in the cold. Cauliflower heads were soaked in cold water for 1 hour before storage to rehydrate the tissue.

The mesocarp portion of both avocado and apple were used; rosettes of cauliflower were shaved from the head. The tissue was weighed, placed in cold phosphate buffer (0.1 M NaH_2PO_4, pH 7.4), and homogenized in either a Virtis homogenizer or Waring blender. Tissues were homogenized at 0.5 to 2 g tissue per ml phosphate buffer. The homogenate was filtered through muslin and centrifuged (2°C) for 20 minutes at 13,500 g. The supernatant was decanted and centrifuged (2°C) for 90 minutes at 80,000 g. The pellets were resuspended in cold phosphate buffer and adjusted to 1-10 mg/ml protein with cold buffered KCl. Protein was determined by the method of Lowry and coworkers (1951).

Hydroxylation Reactions

Hydroxylation reactions were performed according to the method of McPherson and coworkers (1976, 1975c). All reactions were carried out at 37°C in a shaking water bath at 100 cpm. The microsomal mixtures were warmed for 60 seconds after addition of the NADPH-regenerating system. The tenfold concentrated NADPH-regenerating system consisted of: glucose-6-phosphate dehydrogenase, 20 IU/ml; glucose-6-phosphate, 25 mM; NADP, 5 mM; $MgSO_4$ 0.5 mM, dissolved in buffered KCl. In the case of the crude homogenates, low speed supernatants and pellets, and plant microsomes, 1.8 ml of the preparation was used directly. The final protein concentration in the 2 ml reaction mixture for these preparations is given in Table 3. Four-tenths ml of the first high speed pellets and purified animal microsomes (second high speed pellets) at 10 mg protein/ml was added to 1.4 ml of cold buffered KCl to provide a final protein concentration of 2 mg/ml in the 2 ml reaction mixture.

TABLE 3. PROTEIN CONCENTRATIONS IN HYDROXYLATION EXPERIMENTS

Preparation	Protein Concentration[1] (mg/ml)
Mouse Homogenate	29.7
Rat Homogenate	27.0
Hamster Homogenate	29.7; 35.9
Mouse Low Speed Pellet	22.5
Hamster Low Speed Supernatant	13.3
Avocado Microsomes	5.8
Apple Microsomes	1.0
Cauliflower Microsomes	5.0; 9.1
Cauliflower Low Speed Supernatant	2.2

[1] Two numbers indicate the concentrations in two separate experiments. Protein is given as the final concentration in 2 ml of reaction mixture.

Each tube received 0.2 ml of the ten-fold concentrated NADPH-regenerating system. Five-tenths ml of test compound in oil, or oil alone, was added and incubated for 10 minutes. Biphenyl or terphenyl (0.3 ml of 13 mM) was added, and incubation continued for an additional 5 minutes. The reaction was terminated by the addition of 1 ml of 4 M HCl to each tube.

The incubation mixtures used in each hydroxylation experiment are given in Table 4.

Following the addition of HCl (and standards where indicated), the tubes were immediately extracted with n-heptane as described in a previous section of this report.

Separation and Analysis of Metabolites

In the case of the SPF determinations, standard curves were contructed for each different biological preparation and for every ex-

TABLE 4. INCUBATION MIXTURES USED IN HYDROXYLATION REACTIONS[1]

Microsomal System[2]				
No. 1	+ Oil		+ HCl[3]	
No. 2	+ Oil	+ Biphenyl[4]	+ HCl	
No. 3	+ Test Compound In Oil		+ HCl	
No. 4	+ Test Compound In Oil	+ Biphenyl	+ HCl	
No. 5		+ Biphenyl	+ HCl	+ Test Compound *In Oil*
No. 6	+ Oil		+ HCl	+ 2- or 4-Hydroxy-biphenyl + biphenyl

[1] Materials are listed in order of addition from left to right. The microsomal system and HCl were added to all tubes. A blank space indicates no addition, but continued incubation.

[2] Microsomes or homogenate fractions plus NADPH-regenerating system.

[3] HCl was added at the end of the incubation period to terminate the reaction.

[4] Terphenyl was added in place of biphenyl in some experiments. Tube #6 was omitted in these experiments.

periment using dilutions from the tubes containing known concentrations of 2-hydroxybiphenyl or 4-hydroxybiphenyl.

Quantitation of metabolites using the HPLC method was accomplished by constructing standard curves using three different concentrations of 2- and 4-hydroxybiphenyl in the range of 1 to 5 ng per injection.

Terphenyl metabolites were examined at several excitation-emission wavelengths.

BIPHENYL HYDROXYLATION

Experimental Results Using Fluorometric Analysis (SPF)

The results obtained from a relatively large number of experiments carried out to either attempt to duplicate the results published by others or to obtain an initial evaluation of other microsomal extracts in conjunction with the biphenyl substrate are summarized in Tables 5 and 6.

Table 5 shows the results of three different hydroxylation experiments using various fractions of hepatic homogenates from mice, rats, and hamsters. The quantity of 2-hydroxybiphenyl and 4-hydroxybiphenyl present in each of the reaction mixtures was determined fluorometrically by the method of Creaven and coworkers (1965). Benzo(a)pyrene (BaP) was included in all experiments as a known carcinogen, and where possible, α-Naphthylamine (α-NA) was included as an example of a non-carcinogen. All other test compounds are known carcinogens (McCann, et al., 1975).

TABLE 5. EFFECT OF TEST COMPOUNDS ON PRODUCTION OF 4-HYDROXYBIPHENYL AND 2-HYDROXYBIPHENYL BY LIVER FRACTIONS

Fraction	Animal	Reaction Mixture	n Mole/Min/mg Protein 4-Hydroxybiphenyl	2-Hydroxybiphenyl	Corrected[1]
Crude Homogenate	Mouse	Oil	0.007	1.05	
		BaP	0.014	1.41	
		Oil + Biphenyl	0.026	2.55	
		Biphenyl + BaP[2]	0.033	1.03	
		BaP + Biphenyl	0.005	1.58	0.17
	Rat	Oil	0.016	0.19	
		BaP	-0-	0.54	
		Oil + Biphenyl	0.018	0.34	
		Biphenyl + BaP	0.024	0.86	
		Bap + Biphenyl	0.011	0.92	0.38
	Hamster[3]	Oil	0.004	0.19	
		BaP	-0-	0.37	
		SA	0.006	-0-	
		Oil + Biphenyl	0.022	0.29	
		Biphenyl + BaP	0.018	0.22	
		Biphenyl + SA	0.034	0.005	
		BaP + Biphenyl	0.007	0.78	0.41
		SA + Biphenyl	0.021	-0-	-0-
Low Speed Supernatant	Hamster	Oil	0.016	0.008	
		BaP	0.011	0.020	
		Oil + Biphenyl	-0-	0.020	
		Biphenyl + BaP	0.142	0.122	
		BaP + Biphenyl	0.055	0.075	0.055
Low Speed Pellet	Mouse	Oil	0.005	0.003	
		BaP	0.009	0.008	
		Oil + Biphenyl	0.032	0.005	
		Biphenyl + BaP	0.045	0.006	
		BaP + Biphenyl	0.032	0.008	-0-

TABLE 5 (continued)

Fraction	Animal	Reaction Mixture	n Mole/Min/mg Protein 4-Hydroxybiphenyl	2-Hydroxybiphenyl	Corrected[1]
First High Speed Pellet	Hamster	Oil	--	--	
		BaP	-0-	0.56	
		SA	-0-	0.07	
		MC	-0-	0.37	
		Oil + Biphenyl	0.47	0.26	
		Biphenyl + BaP	--	--	
		Biphenyl + SA	1.58	0.85	
		Biphenyl + MC	1.32	1.43	
		BaP + Biphenyl	0.51	0.54	-0-
		SA + Biphenyl	0.66	0.32	0.25
		MC + Biphenyl	0.56	0.65	0.28
Second High Speed Pellet	Mouse[3]	Oil	0.21	0.06	
		BaP	0.09	0.10	
		SA	0.82	-0-	
		MC	0.29	0.12	
		αNA	0.26	0.07	
		βNA	0.07	0.29	
		Oil + Biphenyl	2.54	0.008	
		Biphenyl + BaP	2.23	0.13	
		Biphenyl + SA	3.85	-0-	
		Biphenyl + MC	2.68	0.26	
		Biphenyl + αNA	3.12	0.03	
		Biphenyl + βNA	3.38	0.04	
		BaP + Biphenyl	2.86	0.034	-0-
		SA + Biphenyl	1.15	0.07	0.07
		MC + Biphenyl	4.00	0.03	-0-
		αNA + Biphenyl	2.97	-0-	-0-
		βNA + Biphenyl	1.10	0.28	-0-
	Rat	Oil	0.54	-0-	
		BaP	0.13	0.10	
		Oil + Biphenyl	1.25	0.015	
		Biphenyl + BaP	1.10	0.044	
		BaP + Biphenyl	1.01	0.074	-0-

TABLE 5 (continued)

Fraction	Animal	Reaction Mixture	n Mole/Min/mg Protein 4-Hydroxybiphenyl	2-Hydroxybiphenyl	Corrected[1]
Second High Speed Pellet	Hamster[4]	Oil	0.12	0.008	
		BaP	0.15	0.20	
		SA	0.62	0.06	
		MC	0.25	0.42	
		αNA	0.21	0.13	
		βNA	0.71	0.58	
		Oil + Biphenyl	0.93	0.17	
		Biphenyl + BaP	1.46	0.40	
		Biphenyl + SA	2.08	0.23	
		Biphenyl + MC	1.29	0.44	
		Biphenyl + αNA	1.57	0.68	
		Biphenyl + βNA	1.72	0.65	
		BaP + Biphenyl	0.78	0.32	0.12
		SA + Biphenyl[5]	1.07	0.18	0.12
		MC + Biphenyl[5]	1.03	0.30	-0-
		αNA + Biphenyl[5]	1.04	0.28	0.15
		βNA + Biphenyl[5]	0.78	0.56	-0-

[1] The contribution of test compound metabolites as determined in reaction mixtures containing test compound alone was subtracted from the quantity of 2-hydoxybiphenyl apparently present in the test compound plus biphenyl reaction mixtures.

[2] When the test compound follows the substrate, it was added to the reaction mixture after the addition of HCl.

[3] Results are the average of two experiments for the oil, oil and biphenyl, and BaP mixtures.

[4] Results are the average of two experiments, 2 to 3 replicates for the oil, oil and biphenyl, and BaP mixtures.

[5] Results are the average of two replicates in one experiment.

TABLE 6. EFFECT OF TEST COMPOUNDS ON PRODUCTION OF 4-HYDROXYBIPHENYL AND 2-HYDROXYBIPHENYL BY PLANT MICROSOMES

Fraction	Plant	Reaction Mixture	n Mole/Min/mg Protein			
			4-Hydroxybiphenyl	Corrected[1]	2-Hydroxybiphenyl	Corrected[1]
Low Speed Supernatant	Cauliflower	Oil	0.53		0.27	
		BaP	0.80		0.39	
		SA	0.80		0.18	
		Oil + Biphenyl	0.27		0.18	
		Biphenyl + BaP[2]	0.49		0.66	
		BaP + Biphenyl	0.43	-0-	0.31	-0-
		SA + Biphenyl	0.61	-0-	0.12	-0-
Purified Microsomes	Cauliflower[3]	Oil	0.18		0.085	
		BaP	0.21		0.075	
		SA	0.25		0.032	
		MC	0.036		0.039	
		Oil + Biphenyl	0.15		0.003	
		Biphenyl + BaP	0.065		0.026	
		Biphenyl + SA	0.081		-0-	
		Biphenyl + MC	0.048		0.27	
		BaP + Biphenyl	0.12	-0-	0.032	-0-
		SA + Biphenyl	0.21	-0-	0.012	-0-
		MC + Biphenyl	0.048	0.012	0.036	-0-
	Avocado	Oil	0.102		0.010	
		BaP	0.082		0.031	
		Oil + Biphenyl	0.077		0.005	
		Biphenyl + BaP	0.113		0.031	
		BaP + Biphenyl	0.087	0.005	0.005	-0-
	Apple	Oil	0.34		-0-	
		BaP	0.31		0.31	
		Oil + Biphenyl	0.54		-0-	
		Biphenyl + BaP	0.57		0.20	
		BaP + Biphenyl	0.43	0.12	-0-	-0-

[1] The contribution of test compound metabolites as determined in reaction mixtures containing test compound alone was subtracted from the quantity of 2- or 4-hydroxybiphenyl apparently present in the test compound plus biphenyl reaction mixtures.

[2] When the test compound follows the substrate, it was added to the reaction mixture after the addition of HCl.

[3] All except MC are the average of results from two experiments.

It is apparent that the oil which serves as the solvent for the test compounds can contribute fluorescence at the wavelengths used to measure both hydroxylated biphenyls. However, the oil is included in all reaction tubes, including those containing the standard concentrations of the hydroxylated biphenyls, and is therefore corrected for when the standard curves are constructed. Of more significance is the observation that incubation mixtures containing the test compound alone show fluorescence at the wavelength used to determine 2-hydroxybiphenyl, and to a lesser extent at the wavelength used to determine 4-hydroxybiphenyl. In essentially each case, the apparent increase in the amount of 2-hydroxybiphenyl produced in the presence of the test compound was accounted for by the contribution of the test compound.

The effect of various test compounds on 4- and 2-hydroxybiphenyl production by plant microsomes was also examined fluorometrically because McPherson and coworkers (1975c) had reported a stimulation of 2-hydroxybiphenyl production by 3,4-benzopyrene in avocado microsome reaction mixtures. The results are presented in Table 6. In this case, the test compound contributed significantly to the determination of both 2- and 4-hydroxybiphenyl.

Experimental Results Using High Pressure Liquid Chromatography (HPLC)

Because of the inconsistent and erratic results obtained using the fluorometric analysis by Creaven et al. (1965), an alternate method of analysis was investigated. Quantitative HPLC was used to determine the amount of 2- and 4-hydroxybiphenyl present in incubation mixtures of hamster microsomes (first and second high speed pellet). Figure 7 shows that this method provides a complete separation of the two hy-

droxylated biphenyls in a known mixture. Figures 9 and 10 show typical chromatograms obtained from incubation mixtures containing test compounds and biphenyl. The test compounds did not contribute fluorescent material to the 2- and 4-hydroxybiphenyl peaks under the conditions of this assay. Table 7 presents quantitative data obtained from these chromatograms. It can be seen that using this method, the amounts of 2- and 4-hydroxybiphenyl produced in the reaction containing substrate alone agree with the amounts determined using the fluorometric method (Table 5). However, production of 2-hydroxybiphenyl was not stimulated by preincubation with test compounds.

In the case of BaP, an attempt was made to determine whether the test compound itself or its metabolites were contributing to the fluorescence at the 2-hydroxybiphenyl wavelength using the HPLC method. Samples from reaction mixtures containing BaP were chromatographed on the HPLC and examined at the excitation-emission couples of: Ex 300, Em 405 and Ex 300, Em 422. Although BaP itself was detected at these wavelengths (average retention time 5.6 minutes), no metabolites could be detected. It is possible that the metabolites were present in undetectable amounts or that they eluted with the early material.

Discussion

Using a high pressure liquid chromatography system which permits unequivocal identification of hydroxylated biphenyls, it has been possible to demonstrate, in contrast to the findings of others (Creaven et al., 1965; McPherson et al., 1976, 1975 a,b,c, 1974 a,b; Tredger et al., 1976; Tong et al., 1977), that the amount of 2-

TABLE 7. EFFECT OF TEST COMPOUNDS ON PRODUCTION OF 4- AND 2-HYDROXYBIPHENYL AS DETERMINED BY QUANTITATIVE HPLC

Reaction Mixture[1]	n Mole/Min/mg-Protein 4-Hydroxybiphenyl	2-Hydroxybiphenyl	Ratio of 4-OH/2-OH
First High Speed Pellet			
Oil + Biphenyl	0.64	0.16	4.0
BaP + Biphenyl	0.36	0.14	2.6
SA + Biphenyl	0.26	0.20	1.3
MC + Biphenyl	0.55	0.21	2.6
BaP	0.00	0.00	--
Second High Speed Pellet			
Oil + Biphenyl	0.59	0.22	2.7
BaP + Biphenyl	0.26	0.15	1.7
SA + Biphenyl	0.19	0.17	1.1
MC + Biphenyl	0.24	0.16	1.5
αNA + Biphenyl	0.30	0.18	1.7
βNA + Biphenyl	0.14	0.09	1.6
Oil	0.00	0.00	--
BaP	0.04	0.02	--

[1] Hamster microsomes were used.

hydroxybiphenyl remains constant and the amount of 4-hydroxybiphenyl decreases in the presence of carcinogens and non-carcinogens using animal microsome incubation mixtures. Similarly, plant microsome mixtures show an apparent total lack of hydroxylation of biphenyl in the presence of carcinogens. The test compound or its metabolites contribute fluorescence at the wavelengths used to measure the hydroxylated biphenyls thus causing an apparent increase in 2-hydroxybiphenyl when the fluorometric method of Creaven and coworkers (1965) is used. Burke and coworkers (1977) and Tong and coworkers (1977) have recently suggested that this may be a problem when using this assay.

McPherson, Bridges and Parke (1975a), using radioactive biphenyl and TLC separation methods reported a 2.5 fold stimulation of biphenyl-2-hydroxylase by BaP in vitro. Using HPLC methods, this result was not duplicated and it is not possible at this time to explain this discrepancy.

TERPHENYL METABOLISM

Results

Purified hamster microsomes were used to examine the in vitro metabolites of terphenyl and the effect of carcinogens on their production. Incubation conditions were identical to those used for biphenyl. The reaction mixtures were extracted with 10 ml of n-heptane, and 10 µl of this was injected into the HPLC.

Because the fluorescence spectra of possible metabolites was not known, a number of different excitation-emission wavelengths were used to examine the chromatograms for possible terphenyl metabolites.

In each case, unaltered m-terphenyl was observed at an average retention time of 3.1 minutes. A total of three different m-terphenyl metabolites were detected at two different excitation-emission wavelength pairs (Table 8).

In an initial experiment, a reaction mixture containing m-terphenyl as a substrate was preincubated for 10 minutes with BaP. The n-heptane extract was chromatographed and examined at λ_{EX} = 270, λ_{EM} = 350. Figure 13 shows that preincubation with BaP caused a loss of the metabolite having the 15 minute retention time, and an apparent decrease in the second (17 minute) metabolite.

This experiment was repeated using several test compounds and m-terphenyl as the substrate. Because the chemical nature of the metabolites is not known, it was not possible to construct standard curves. An auto-oxidation control mixture containing m-terphenyl without microsomes was included in the experiment. The chromatogram

TABLE 8. METABOLITES OF m-TERPHENYL PRODUCED BY PURIFIED HAMSTER MICROSOMES

wavelength (nm)	Peak heights (mm)[1] at retention times of: 15.1 - 15.3 min.	17.2 - 17.3 min.	18.9 min.
λ_{EX} = 270, λ_{EM} = 360	5.8	14.8	--[2]
λ_{EX} = 250, λ_{EM} = 360	2.2	5.5	--
λ_{EX} = 270, λ_{EM} = 350	5.4	15.0	--
λ_{EX} = 300, λ_{EM} = 360	--	7.0	9.0
λ_{EX} = 300, λ_{EM} = 335	--	3.0	4.0

1 Peak widths at half-height were identical for each metabolite.

2 Not present.

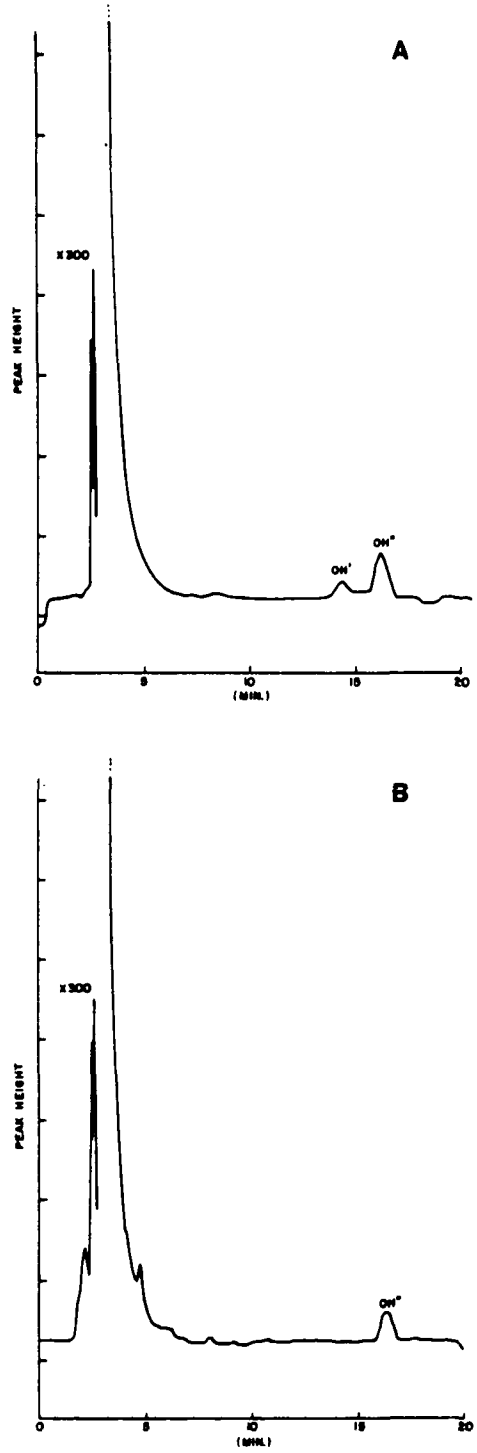

Figure 13. Material separated by HPLC obtained from reaction mixtures containing purified hamster microsomes and (A) oil plus m-terphenyl; (B) BaP in oil plus m-terphenyl. The excitation wavelength was 270 nm and the emission wavelength was 350 nm.

of the n-heptane extract of this mixture showed no metabolites. In addition, incubation mixtures containing test compounds without terphenyl showed no material at the retention times of the terphenyl metabolites.

Table 9 presents the results of this experiment. The last five reaction mixtures in the table are those in which the oil or test compound in oil was added follwing termination of the reaction with HCl. In general, metabolite production in these mixtures was higher than when the oil was added during the incubation. This suggests that the oil may inhibit the oxidative metabolism to some extent.

The results presented in Table 9 do not substantiate those obtained in the first experiment. There was apparently no effect, or a general decrease in metabolite production in the presence of all test compounds in the second experiment. The major difference between the two experiments was that the incubation of the microsome reaction mixtures was conducted in normal laboratory light (fluorescent) in the first experiment, whereas incubations were performed in dim yellow light in the second experiment. It is interesting to speculate that these conditions alter the sensitivity of the enzyme(s) to test compounds.

A preliminary experiment was performed in which p-terphenyl was incubated with purified hamster microsomes, and an n-heptane extract prepared. The excitation and emission spectra of n-heptane extracts of both m- and p-terphenyl substrates incubated with purified hamster microsomes are shown in Figures 14 and 15. Extracts of mixtures containing either substrate without microsomes did not show emission peaks under these excitation-emission conditions. It can be seen that p-terphenyl is also metabolized by the microsomes to produce an emission spectrum very similar to that of the metabolized m-terphenyl.

TABLE 9. METABOLITES OF m-TERPHENYL PRODUCED IN THE PRESENCE OF CARCINOGENS

	area (nm^2) of peaks at retention times (min) of:					
	λ_{EX}=270 nm, λ_{EM}=360 nm			λ_{EX}=300 nm, λ_{EM}=360 nm		
reaction mixture[1]	15.0	17.1	18.9	14.9	17.0	18.6
oil + terphenyl + HCl[2]	104	310	72	54	72	206
BaP/oil + terphenyl + HCl	103	245	46	64	76	197
αNA/oil + terphenyl + HCl	88	200	38	53	68	188
βNA/oil + terphenyl + HCl	79	208	34	68	76	198
MC/oil + terphenyl + HCl	93	240	42	68	78	202
terphenyl + HCl + oil[3]	112	298	44	75	94	280
terphenyl + HCl + BaP/oil	122	292	34	82	118	245
terphenyl + HCl + αNA/oil	110	372	49	82	94	240
terphenyl + HCl + βNA/oil	140	435	66	88	106	312
terphenyl + HCl + MC/oil	158	371	59	105	128	299

[1] Relevant compounds are given in the order of addition to the incubation mixtures.

[2] For the first five reaction mixtures, each number is the average of results obtained from two different mixtures.

[3] For the last five reaction mixtures, each number was obtained from one incubation mixture.

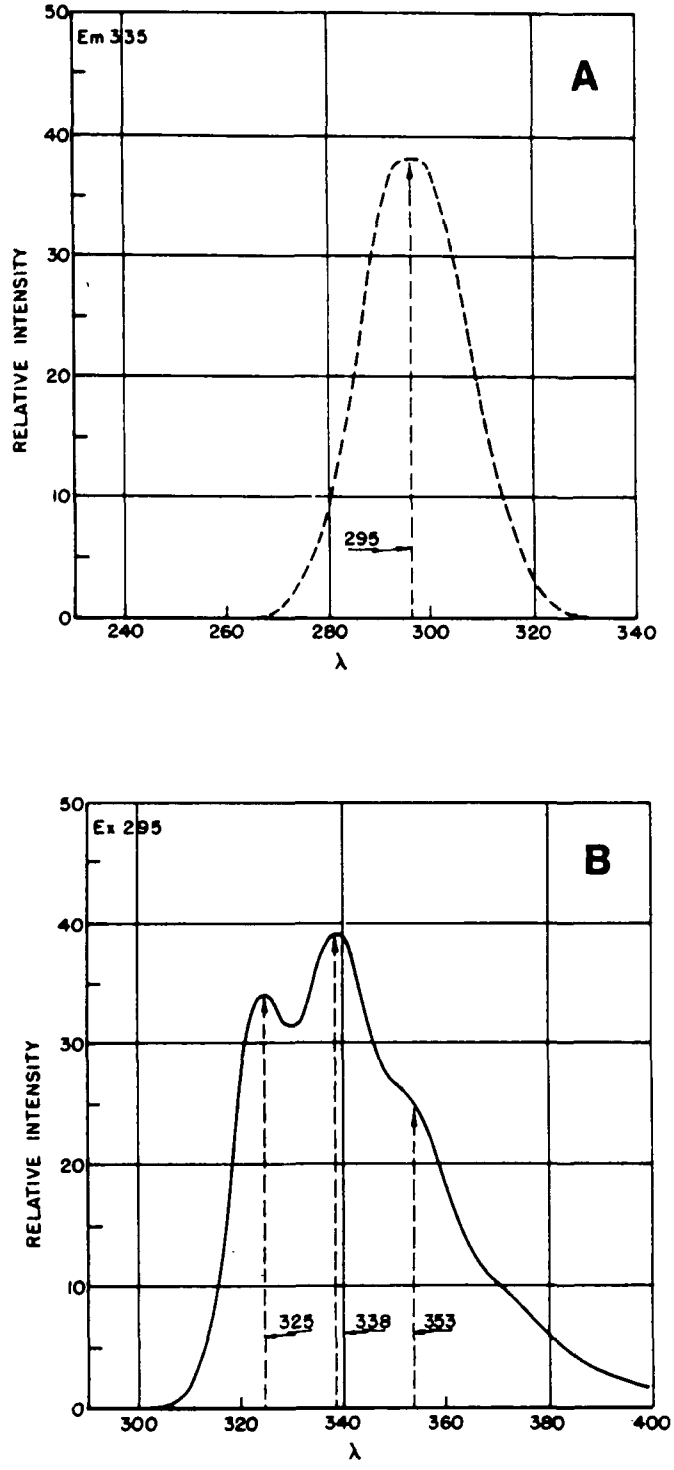

Figure 14. Excitation and emission spectra of an n-heptane extract of purified hamster microsomes incubated with m-terphenyl. (A) Excitation spectrum with emission measured at 335 nm; (B) emission spectrum with excitation at 295 nm; λ = wavelength in nm.

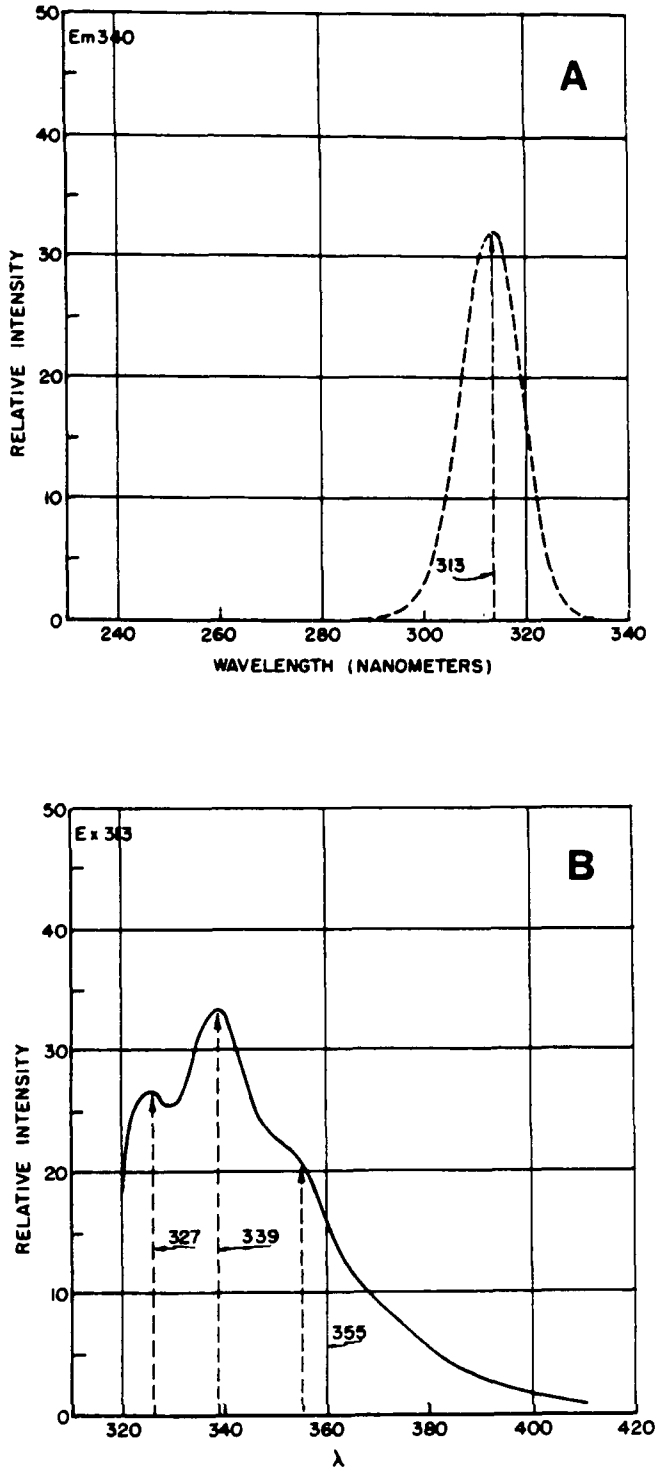

Figure 15. Excitation and emission spectra of n-heptane extracts of purified hamster microsomes incubated with p-terphenyl. (A) Excitation spectrum with emission measured at 340 nm; (B) emission spectrum with excitation at 313 nm; λ = wavelength in nm.

Discussion

Meta-terphenyl is metabolized in vitro by purified hamster microsomes to a minimum of three compounds. Preliminary data suggests that, under some conditions, the production of at least one of these metabolites may be inhibited by carcinogens such as BaP. Further experimentation is necessary in order to validate this result and determine the conditions under which inhibition occurs. Para-terphenyl is also metabolized by hamster microsomes, but the number and nature of the metabolites is not known. Further investigation of this substrate may provide information complementary to that obtained with the m-terphenyl substrate.

SECTION 6

METABOLISM OF BIPHENYL BY PARAURONEMA ACUTUM

GENERAL

Previous work (Schmidt-Collerus and Tame, unpublished) has shown that algae such as Chlorella pyranoidosa exhibit rapid oxidative metabolism of polycyclic aromatic hydrocarbons. This indicates that these organisms possess very effective oxidative enzymes. It is believed that similar strong activity may be present in marine algae and possibly ciliates. The use of algae or ciliates as a source of biphenyl hydroxylases has several advantages: (1) they can be prepared rapidly and inexpensively, (2) because of the large variety of these organisms, hydroxylases specific for various substrates or new enzymes sensitive to various classes of mutagens or carcinogens may be found, and (3) the enzymes in these organisms may not be membrane-bound, and may therefore be more useful in an assay system than microsomal enzymes.

Parauronema acutum was selected for study because it is a marine protozoan which can be grown in defined culture in the laboratory, and therefore is a possible indicator of pollution in the marine environment. In addition, P. acutum has been shown to activate 2-aminofluorene but not BaP, and the enzyme(s) responsible is distributed among soluble and particulate fractions (Lindmark, 1978). The hydroxylases of this organism may therefore be more stable than those which are microsome-associated.

Preliminary experiments in this laboratory examined the ability of this protozoan to metabolize biphenyl *in vivo*.

MATERIALS AND METHODS

Growth Medium

P. acutum was maintained in still culture in 50 ml sterile baffle flasks containing 10 ml of medium at 22°C in the dark. The growth medium (Lindmark, D. G., personal communication) consisted of: 20 mg asolectin, 20 mg cephalin, 20 mg Tween 80, 75 ml artificial sea water (sp. gr. 1.023), 1 g protease peptone, 1 g trypticase, 0.1 g yeast nucleic acid, and 1 ml vitamin mix per 100 ml final volume. The pH was adjusted to 7.2, and the medium sterilized by autoclaving for 15 minutes at 121°C. The vitamin mix consisted of: 0.001 µg/ml biotin, 1.0 µg/ml calcium pantothenate, 0.5 µg/ml each of folic acid, nicotinamide, pyridoxal-HCl and riboflavin, 1.5 µg/ml thiamine-HCl, and 0.01 µg/ml DL-thioctic acid.

Substrate and Test Compounds

A stock solution of biphenyl in dimethylsulfoxide (DMSO) was prepared at 200 mM. Biphenyl (20 mM) in Tween 80 was prepared by dissolving 30.8 mg of biphenyl in 0.15 g Tween 80 in a water bath with heating. *P. acutum* growth medium (10 ml) was then added slowly to form an emulsion. For Tween 80 controls in which the biphenyl was omitted, 0.15 g of Tween 80 was added to 10 ml of growth medium to provide a stock solution. The DMSO carrier was reagent grade. The BaP was prepared at 20 mM in DMSO. The 2- and 4-hydroxybiphenyl

standards were prepared at 24.8 ng/ml each in aqueous 5% (v/v) ethanol.

Experimental Procedure

Cultures were prepared by adding 0.5 ml of an inoculum culture to 10 ml of medium in sterile 50 ml baffle flasks. Cultures were incubated without shaking in the dark at 25°C or 22°C.

Cells were fixed in 10% formaldehyde and counted daily using a hemocytometer. Viabilities were determined on duplicate, unfixed samples. Test compounds were added when the cell density was approximately 1×10^5 cells per ml. Duplicates were made for each treatment. After addition of test compounds, cells were counted daily until the untreated cultures entered the decline phase of the growth curve. At that time, 2.5 ml of 8N HCl was added to each flask to terminate the experiment. Biphenyl, 4-hydroxybiphenyl and 2-hydroxybiphenyl were added to untreated cultures after HCl addition. These served as extraction controls. All manipulations following HCl addition were carried out in dim yellow light.

Following HCl addition, a 5 ml aliquot of each culture was transferred into each of two glass tubes equipped with teflon-lined screw caps. Microscopic examination of the HCl-treated culture showed that not all cells were lysed by acidification. Therefore, one of the replicates of each culture was frozen (-80°C) and thawed twice to ensure disruption of the cells. The solutions were then each extracted with 10 ml of n-heptane by shaking at room temperature for 10 minutes. Samples were centrifuged at 2,000 rpm for 10 minutes to separate the layers, and stored at 4°C in the dark. The heptane layer was removed and concentrated to approximately 0.5 ml under a nitrogen stream.

HPLC Analysis

The concentrated n-heptane extracts were analyzed for biphenyl metabolites using HPLC combined with SPF as described previously.

RESULTS

The effect of addition of biphenyl on growth of P. acutum was examined at both 22 and 25°C. In addition, the effect of the carrier (DMSO or Tween 80) on the ability of the organism to respond to biphenyl was also examined.

Figure 16 shows that, at 22°C biphenyl dissolved in DMSO at final concentrations in the culture of above 0.2 mM caused immediate death and lysis of the cells. The lower concentration allowed normal growth of the culture in terms of cell counts. The loss in viability of the cultures was caused by the biphenyl and not the DMSO carrier. However, when Tween 80 was the carrier, the lethal effect at higher concentrations of biphenyl was decreased.

Figures 17 and 18 show the effect of biphenyl at 25°C. In this case there appeared to be no difference between DMSO or Tween 80, with cultures being unaffected by 0.2 mM concentrations of biphenyl. Extracts of these cultures were examined by HPLC-SPF in order to quantitate the metabolites produced. The results are shown in Table 10. Numbers are provided for those extracts in which metabolites were detected. It can be seen that both 2- and 4-hydroxybiphenyl were produced, and that neither carrier nor medium produced material which interfered with metabolite determination. The results obtained

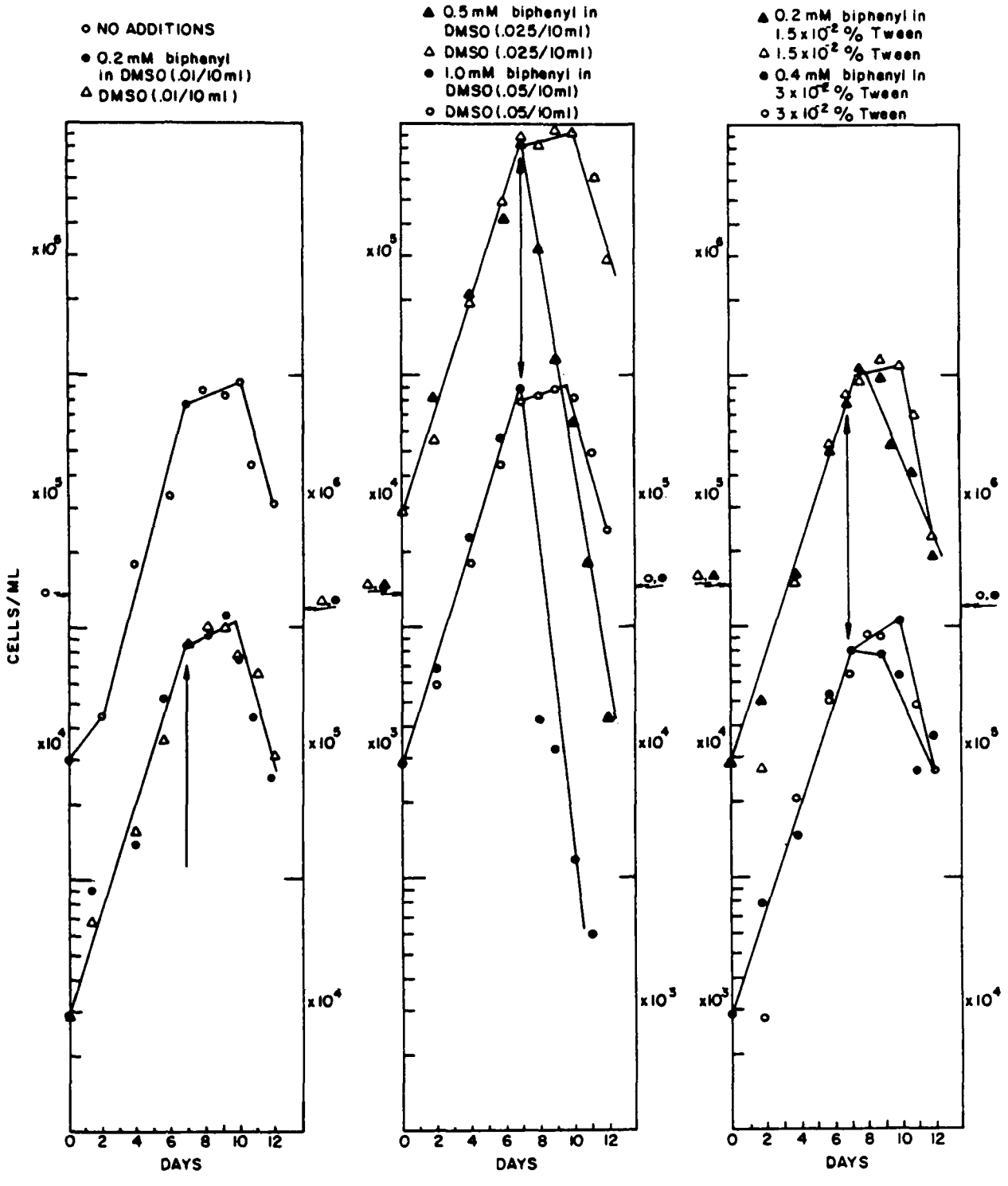

Figure 16. Growth of Parauronema acutum in the presence of biphenyl at 22°C. The arrows indicate time of addition of biphenyl or carrier (7 days), and each point is the mean of the cell counts from at least two different cultures in one experiment.

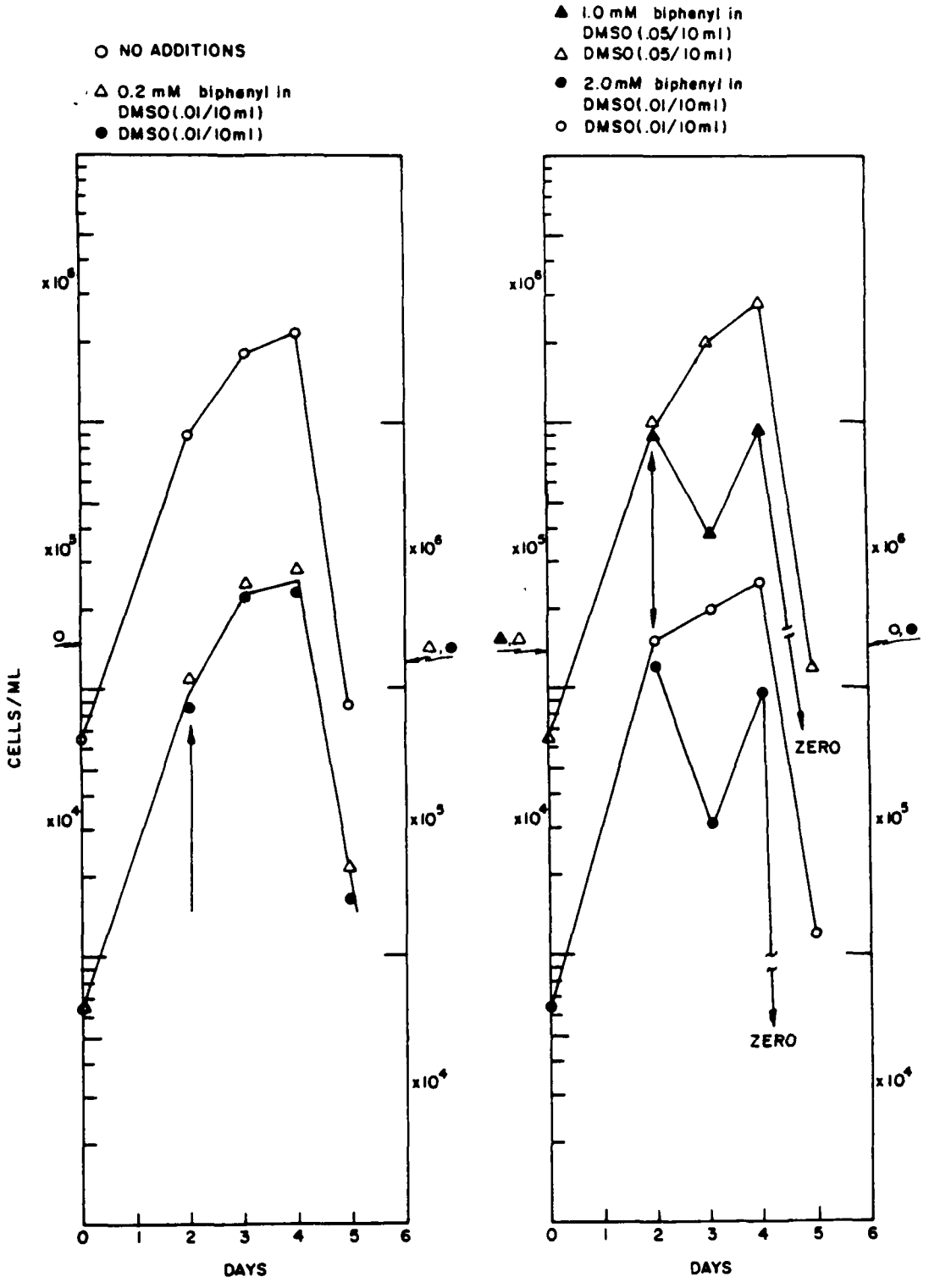

Figure 17. Growth of Parauronema acutum at 25°C in the presence of biphenyl dissolved in DMSO. The arrows indicate time of addition of biphenyl or carrier (2 days), and each point is the mean of the cell counts from at least two different cultures in one experiment.

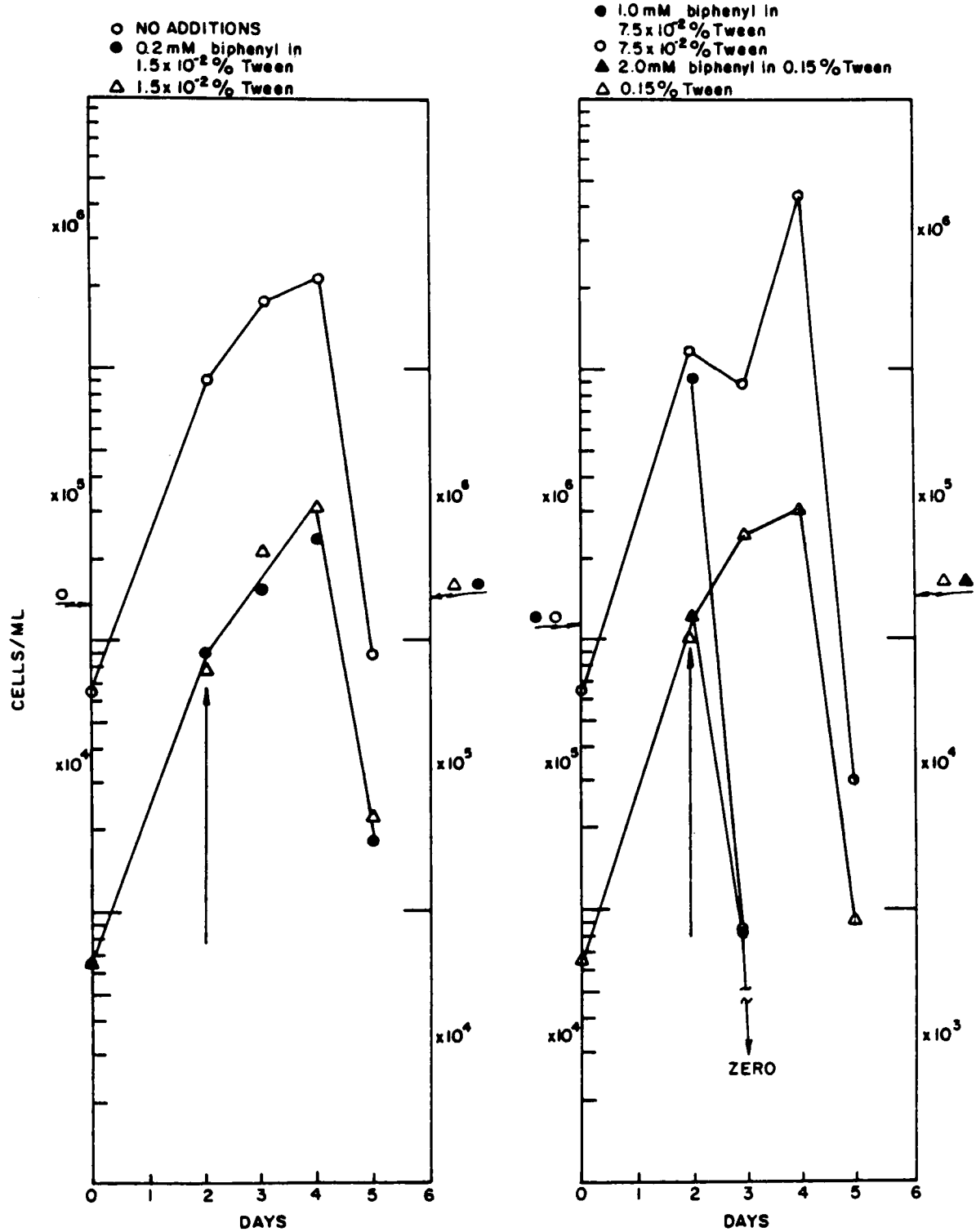

Figure 18. Growth of Parauronema acutum at 25°C in the presence of biphenyl dissolved in Tween 80. The arrows indicate time of addition of biphenyl or carrier (2 days), and each point is the mean of the cell counts from at least two different cultures in one experiment.

TABLE 10. QUANTITIES OF 2- AND 4-HYDROXYBIPHENYL PRESENT IN EXTRACTS OF PARAURONEMA ACUTUM CULTURES[1]

additions to incubation mixture	treatment before extraction	ng 2-hydroxy-biphenyl	ng 4-hydroxy-biphenyl	ratio 4-OH/ 2-OH
cells + medium[2]	none	-0-	-0-	--
	frozen	-0-	-0-	--
0.2 mM biphenyl in DMSO (.01/10)[3]	none	0.43	1.26	2.9
	frozen	0.44	0.98	2.2
1.0 mM biphenyl in DMSO (.05/10)	none	0.43	0.60	1.4
	frozen	0.40	3.24	8.1
DMSO (.01/10)	none	-0-	1.06	--
	frozen	-0-	-0-	--
DMSO (.05/10)	none	-0-	-0-	--
	frozen	.10	-0-	--
0.2 mM biphenyl in 1.5×10^{-2}% Tween[2]	none	0.75	1.38	1.8
	frozen	0.80	3.12	3.9
1.0 mM biphenyl in 7.5×10^{-2}% Tween[2]	none	0.34	0.40	1.2
	frozen	0.20	1.05	5.2
1.5×10^{-2}% Tween	none	-0-	-0-	--
	frozen	-0-	-0-	--
7.5×10^{-2}% Tween	none	-0-	-0-	--
	frozen	0.35	0.45	--

1 Cultures were grown at 25°C. Growth data are presented in Figures 17 and 18.

2 Numbers are the average obtained from two different culture flasks. All others represent one flask.

3 Numbers in parentheses indicate the volume (ml) of biphenyl in DMSO or DMSO alone added to the 10 ml of medium.

using Tween 80 as a carrier seem to indicate that the 4-hydroxybiphenyl metabolite may be located intracellularly in a form which is released by freezing and thawing the cells.

Figure 19 shows the effect of addition of BaP and biphenyl on culture growth. The data for cultures in which DMSO alone in the appropriate amounts was added in the place of test compounds were identical to the data obtained for growth in the absence of additions, and have been omitted. It is apparent that addition of BaP alone at 5 days has no effect on growth of the cultures. In this experiment, addition of 0.2 mM biphenyl caused death of the culture after a short lag. In the case of BaP and biphenyl addition, it appears that the rate of death of the cultures is decreased. It is known (Lindmark, 1978) that BaP is not metabolized by P. acutum. However, BaP may interact with the pellicle in such a way as to allow it to metabolize, and therefore detoxify, biphenyl more readily.

DISCUSSION

Parauronema acutum metabolizes biphenyl to form 2- and 4-hydroxybiphenyls. This organism is therefore a likely source of hydroxylases for in vitro studies of the effects of carcinogens. In addition, BaP may interact with the pellicle of the organism to allow it to more efficiently metabolize biphenyl.

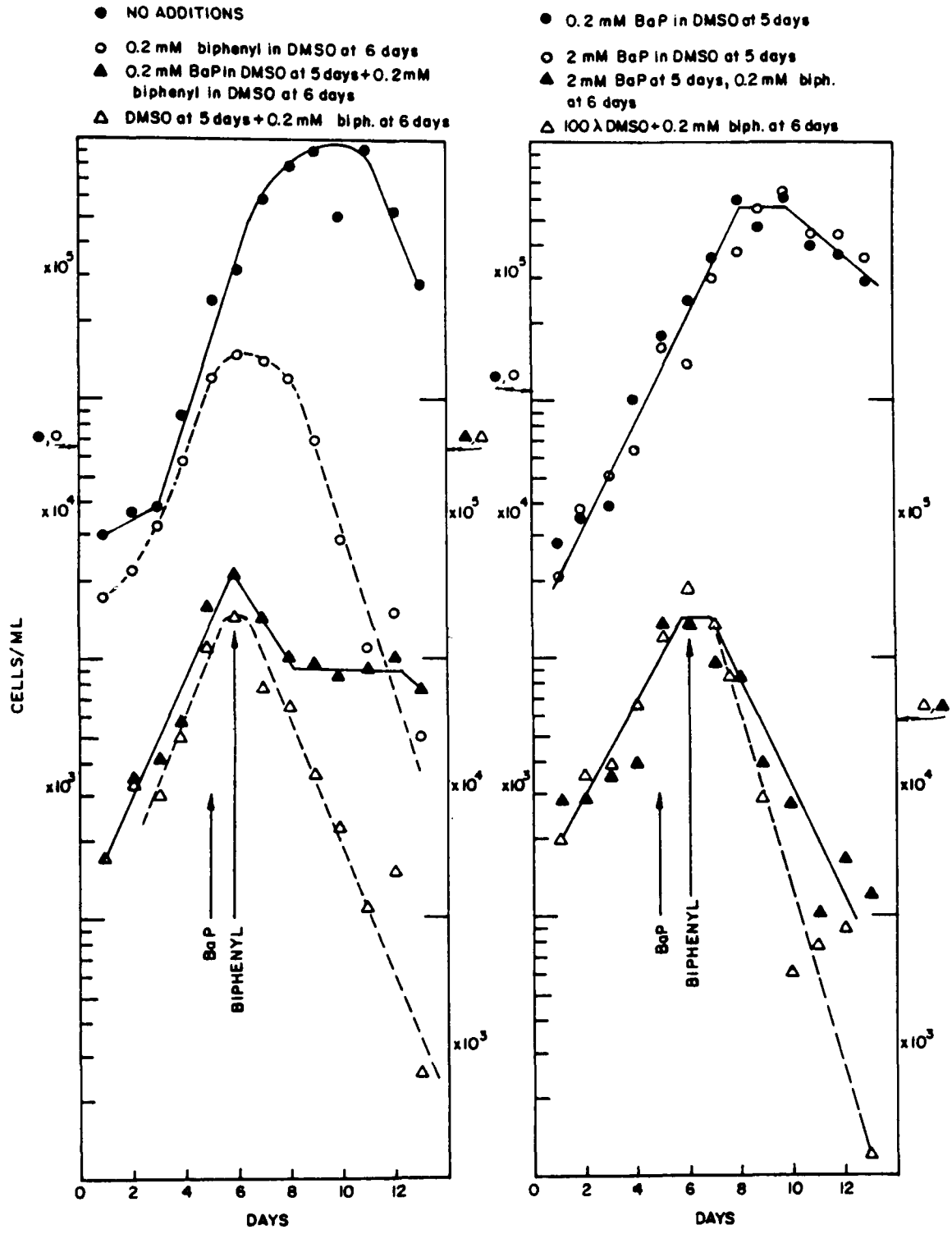

Figure 19. Growth of Parauronema acutum in BaP and biphenyl at 25°C. The arrows indicate time of addition of test compounds, and each point is the mean of the cell counts from at least two different cultures in one experiment.

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