AN ABSTRACT OF THE THESIS OF

Diane Leigh Bunting for the degree of Master of Science

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Title: AN EVALUATION OF BENZO(A)PYRENE METABOLISM IN AN OYSTER (OSTREA EDULIS)-BACTERIA SYSTEM

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Abstract approved: Michael C. Mix

The overall objective of this research project was to evaluate the metabolism of benzo(a)pyrene (BaP) by the European flat oyster (Ostrea edulis)-bacteria system. Individual oysters exist in nature as "systems" with commensal microorganisms and should be recognized as such when the metabolism of a specific environmental contaminant is being studied. Since bivalves are exposed to environmental pollutants that enter coastal waters, it is important to determine if the "natural" oyster system can detoxify those contaminants likely to cause biological damage.

Tritiated-BaP was incubated with whole animal multienzyme preparations of (1) oyster/bacteria and (2) bacteria-free oyster. Background controls were run with homogenizing buffer (no oyster tissue added), homogenizing buffer + antibiotics (no oyster tissue added) and autoclaved oyster/bacteria multienzyme systems. Extraction techniques allowed separation of four fractions: (1) unmodified ^{3}H-BaP plus simple non-conjugated derivatives; (2) ^{3}H-BaP-glucuronides and -sulfates; (3) other ^{3}H-BaP polar

derivatives susceptible to cleavage by base/acid hydrolysis; and (4) the remaining ^{3}H-BaP derivatives tightly bound and unaffected by previous treatment. Further separation of the unaltered ^{3}H-BaP and the simple non-conjugated derivatives was effected by thin-layer chromatography.

The results show a metabolic conversion of ^{3}H-BaP by both multienzyme systems to simple, non-conjugated ^{3}H-BaP derivatives and to other more polar ^{3}H-BaP derivatives which were not susceptible to cleavage by glucuronidase/sulfatase or hydrolysis treatment. Hydrolysis-susceptible ^{3}H-BaP metabolites were detected in the oyster/bacteria multienzyme preparations. Thus, it was not possible to clearly distinguish the metabolism of BaP by bacterial multienzymes from that of oyster multienzymes, but it does appear that the major proportion were contributed by oyster multienzymes.

There were several additional points of interest. The treatment of oysters with antibiotics prior to and during the ^{3}H-BaP incubation eliminates their indigenous bacterial flora as established by most-probable-number analyses of bacterial numbers. Further studies are required to determine whether or not metabolic conversions are due to the presence of bacterial cell-free enzymes. Also, the importance of establishing a background measure by an inviable enzyme incubation with the substrate is deemed critical as a result of this study. Finally, two oxidative degradation products are formed during the stustrate incubation, the subsequent organic extraction and/or the TLC preparation.

An Evaluation of Benzo(a)pyrene Metabolism in an Oyster (*Ostrea edulis*)-Bacteria System

by

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INTRODUCTION

Marine waters, especially estuaries, are receiving a tremendous influx of foreign organic compounds (i.e., xenobiotics). Clark and MacLeod (1977) estimate that 6.1 million metric tons of oil entered the marine environment in 1973. The sources of these petroleum hydrocarbons included land-based discharges, marine operations, marine accidents, offshore production, natural oil seeps, and atmospheric fall-out. The land-based discharges, from refineries, waste oils, runoff and sewage, are generally concentrated near the coastal waters that have great biological productivity (Ryther, 1969). The major component of waste oils are highly-refined oils containing the high boiling fractions and a corresponding high percentage of aromatic hydrocarbons (Clark and MacLeod, 1977). Most sedentary marine filter-feeding bivalve mollusks may be exposed to significant concentrations of potentially toxic aromatic hydrocarbons, as well as other xenobiotics, through acute and chronic exposure.

The metabolism of aromatic hydrocarbons is mediated by the cytochrome P-450-dependent mixed-function oxidase (MFO) system found in the endoplasmic reticulum of animal cells. The MFO system, also known as aryl hydrocarbon hydroxylase (AHH), catalyzes the formation of virtually all the first metabolic products by converting the aromatic hydrocarbon to an oxygen-containing epoxide (Sims and Grover, 1974; Figure 1), which is an intermediate in the formation of hydroxy derivatives. Some of these electrophilic intermediates are chemically

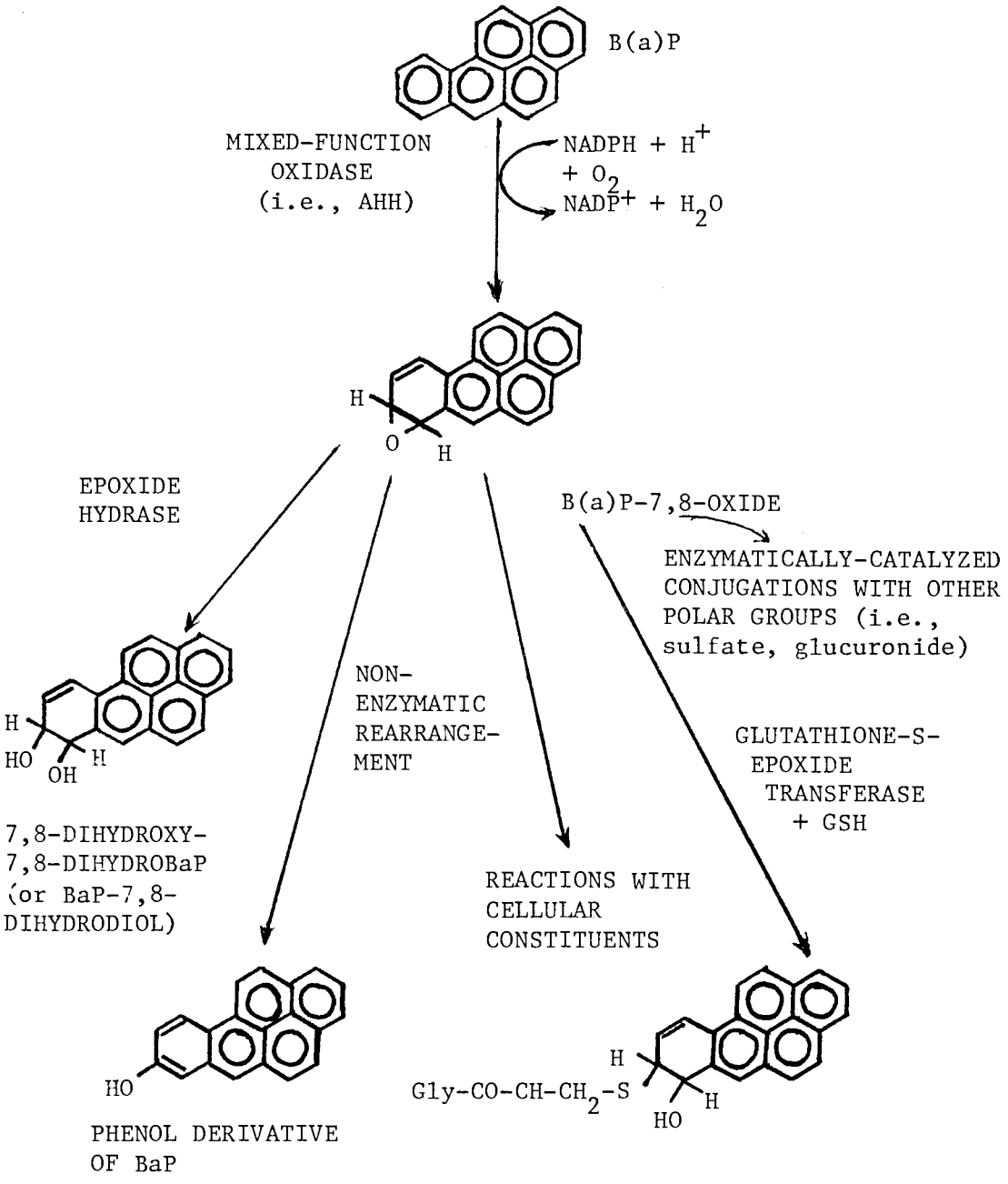


Figure 1. Reactions involved in the metabolism of benzo(a)pyrene by mammalian systems. Adapted from Keysell et al., 1975 and Varanasi and Malins, 1977.

reactive and bind covalently to cellular macromolecules, including protein, DNA and RNA (Sims and Grover, 1974; Pezzuto et al., 1976; Jernström et al., 1976; Jennette et al., 1977). Certain alkene and arene oxides also cause transformation of bacterial and mammalian cells in vitro and have been identified as ultimate carcinogens, mutagens, and cytotoxins (Sims and Grover, 1974; Wood et al., 1976). For benzo(a)pyrene (BaP), it appears that the isomeric 9,10-epoxy-7, 8-dihydro-7,8-dihydroxybenzo(a)pyrene, formed by further oxidative metabolism of 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene (Figure 1), is the ultimate mammalian carcinogens (Sims and Grover, 1974; Wislocki et al., 1976).

Figure 1 shows the enzymatic and nonenzymatic conversions of the BaP epoxide. The conjugation reactions, of epoxides and phenols with water-soluble substances, give rise to BaP derivatives of mercapturic acid precursors (i.e., glutathione conjugates), glucuronides, sulfates, and glycosides. These reactions are important because the toxic properties of the arene oxides are greatly reduced through the conversion of the epoxide group to polar derivates (Sims and Grover, 1974).

To summarize, most bivalves exist in coastal waters and are exposed to aromatic hydrocarbons. It is of interest to determine whether these shellfish possess the metabolic pathways to activate and/or detoxify aromatic hydrocarbons. Given these pathways, the question arises of whether or not mollusks are susceptible to cellular transformation after contact with activated carcinogens. If the animals do not possess the detoxification pathways, there are important and

perhaps serious public health implications; these commercially-important bivalves then could accumulate potential carcinogens or cytotoxins that can be activated by consumers (i.e., man). However, to date, there is uncertainty as to whether or not bivalves possess oxidative detoxification enzyme systems for aromatic hydrocarbons.

It has been assumed previously that bivalves do not have the capability to metabolize aromatic hydrocarbons. In a study by Lee's group (1972) with Mytilus edulis, mussels were found to accumulate significant concentrations of BaP when exposed to relatively small amounts (2 to 200 ppb) in the water. No metabolic products were detected by autoradiographic analysis of extracted lipids. In another study, the quahaug clam, Mercenaria mercenaria, was not shown to possess any of the oxidative detoxification pathways treated for by the substrates, p-nitroanisole, aminopyrine, hexobarbital, and the organophosphate, O-ethyl O-P-nitrophenyl phenylphosphonothivate (EPN) (Carlson, 1972). These experiments were run using: (1) whole homogenates of hepatopancreas, mantle, foot, and gills; (2) 9000 g supernatant fractions of hepatopancreas; and (3) 105,000 g microsomal and soluble fractions of hepatopancreas. The method of metabolite detection was spectrophotometric.

In recent years, other studies have tended to support these earlier studies. Dunn and Stich (1976) also concluded that the mussel, Mytilus edulis, was incapable of metabolizing BaP, or if any metabolism occurred, it ceased after the mussels' removal from water. The measurement of BaP in whole mussel extractions was made by fluorimetric analysis.

Three bivalve species, Mya arenaria, Mytilus edulis, and Ostrea edulis, were exposed to short-term (4 days) and long-term (6 years) oil pollution and subsequently shown to have no detectable BaP hydroxylase activity (Vandermeulen and Penrose, 1978). However, all test organisms contained detectable petroleum hydrocarbons in their tissues at the time of AHH assay. The AHH assay was made by a determination of BaP hydroxylation to 3-OH-BaP. Whole bivalve homogenates were incubated at 25°C with cofactor concentrations as established for mammalian cellular preparations by Nebert and Gelboin (1968). Thus, it was concluded that bivalves were not able to metabolize aromatic hydrocarbons by an AHH enzyme system, but that they were able to eliminate the majority of the hydrocarbon load by depuration.

Khan and co-workers (1972) and Stegeman and Teal (1973) introduced the first evidence that at least some bivalves possess MFO enzyme systems. Khan et al. (1972) demonstrated the epoxidation of aldrin (1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-endo-exo-1,4:5,8-dimethanonaphthalene) in the fresh-water mussel, Anodonta sp. A 4-hour in vivo exposure and a 1-hour in vitro assay of whole homogenate and four subcellular fractions were performed. The microsomal mixed-function oxidase activity was determined by gas chromatographic detection of the reaction product, dieldrin. It was also shown that the hepatopancreas microsomes contained the highest oxidase activity of all the separated organs.

MFO systems can also metabolize aliphatic hydrocarbons (Bend et al., 1977). The accumulation, release, and retention patterns of

petroleum hydrocarbons were monitored for the oyster using whole animal homogenates and gas-liquid chromatography analysis (Stegeman and Teal, 1973). Oysters were exposed to a seawater:fuel oil mixture and were regularly removed for analysis during the 50-day exposure. The gas chromatograms of the hydrocarbons extracted from the oysters were typified by the fairly rapid loss of straight-chain alkanes. One of the several explanations offered by the authors to account for the alkane degradation was that the oyster was partly responsible for the metabolism of alkanes contained in its tissues, thus indicating the presence of an AHH enzyme system.

In the past two years, two important papers have been published showing that bivalve mollusks are capable of metabolizing aromatic hydrocarbons. Anderson (1978) demonstrated AHH activity in the oyster, Crassostrea virginica, by using microsomal fractions of digestive gland homogenates. Specific in vitro incubation conditions were established which yielded optimal results for the oyster system; these conditions included cofactor requirements and concentrations, temperature-activity, oxygen requirements, time-activity, cellular and subcellular localization of the enzyme system. The resultant incubation conditions for C. virginica were quite different than those used for mammalian systems (Sims, 1966; Nebert and Gelboin, 1968). The BaP derivatives were separated and quantified by a radioisotopic technique, and were characterized as well by high-pressure liquid chromatography. The probable identities of the BaP metabolites were: 9,10-dihydrodiol; 4,5-dihydrodiol (or 7,8-dihydrodiol); 1,6-quinone (or 3,6-quinone); 3-hydroxyBaP; and an unknown monohydroxylated

derivative. Evidence was also presented that indicated the C. virginica AHH system was inducible by polychlorinated biphenyls (PCB).

As Figure 1 indicates, the enzyme, epoxide hydrase, is of interest because it catalyzes the conversion of epoxides, formed by cytochrome P-450-dependent metabolism, to diols. Generally, this biotransformation is a detoxification reaction. Glutathione S-transferases are also important conjugating enzymes, responsible for converting potentially toxic metabolic products to presumably less toxic and more water-soluble compounds. Bend and coworkers (1977) examined epoxide hydrase and glutathione S-transferase activities in the bivalves, Mya arenaria and Mytilus edulis using the substrates, styrene 7,8-oxide, octene 1,2-oxide, and B(a)P-4,5-oxide. Microsomal fractions of the hepatopancreas were tested. In vitro incubation conditions were derived for elasmobranch species and, except for temperature, were generally similar to those used with mammalian microsomal preparations. Assays for the metabolites were made by extraction or chromatographic separation, followed by radioactive liquid scintillation counts. The clam, M. arenaria, exhibited significant microsomal epoxide hydrase activity, whereas the mussel, M. edulis, showed measurable but low epoxide hydrase activity. Glutathione S-transferase activity was present in both mollusks but not in great amounts. The bivalve species were not tested for MFO activity.

Thus, the process of aromatic hydrocarbon metabolism by bivalve mollusks is not yet clearly established. Preliminary experiments and other research conducted in our Corvallis laboratory (Riley, 1978)

showed that when Ostrea edulis, the European flat oyster, was exposed to 90 ppb naphthalene in a flow-through seawater system, simple non-conjugated and conjugated derivatives of naphthalene were identified in the oyster tissue. Thus, naphthalene, an aromatic hydrocarbon, was being metabolized by oxidative detoxification enzymes present in the oyster system. This oyster system was composed of oyster and commensal microorganisms (i.e., possibly bacteria, yeast, and fungi). Based on these results, the oyster system, as it exists in nature, possesses the enzymatic capacity for detoxifying potentially toxic or carcinogenic aromatic hydrocarbons. However, it would be invalid to conclude from the preliminary experiments and the research by Riley (1978) that the oyster, O. edulis, possesses enzyme systems capable of metabolizing naphthalene and other aromatic hydrocarbons. It would be of interest and of value to determine whether the aromatic hydrocarbon metabolism could be attributed to oyster enzymes and/or to microbial enzymes.

There has been no published work on the yeast or fungi presence in the oyster mantle fluid or digestive system. There have been some studies performed on the commensal bacteria, showing that significant numbers of bacteria are found in the mantle fluid, in oyster tissue, and in whole oyster homogenates. Lovelace and colleagues (1968) demonstrated that the total viable aerobic heterotrophic bacterial populations numbered 10^3 to 10^4 per gram of oyster (C. virginica) gill tissue and 5×10^4 per ml oyster mantle fluid. These findings were consistent with other studies by Colwell and Liston (1960) and Vasconcelos et al. (1969) where 10^4 to 10^5 organisms

per ml whole oyster homogenate was recorded for C. gigas.

While no researcher has isolated the bacterial flora from the bivalve gut and examined it for AHH activity, there has been much work done on the biodegradation of petroleum by the bacteria in the water column and in the sediments. See Karrick (1977) for an excellent review of the vast literature on bacterial metabolism. Gibson (1976) demonstrated that bacteria can use naphthalene as an energy source and degrade it to CO_2 and water. Barnsley (1975) measured the degradation of benzo(a)pyrene by a number of bacterial species. Also, recently, intermediates were isolated in the metabolism of BaP by the mutant strain of the bacteria, Beijerinckia; the major product isolated was 9,10-dihydroxy-9,10-dihydroBaP, and the minor product was 7,8-dihydroxy-9,10-dihydroBaP (Gibson, 1976).

Thus, the microflora associated with the gut or mantle fluids may be important in the oxidative detoxification processes. Yet, surprisingly, the metabolic contribution by bacteria to the bivalve system has not been directly considered. When whole animal homogenates were used, the possible bacterial contribution to metabolism was ignored. When microsomal preparations were used, the possible bacterial contribution was negated because it was assumed that all the bacteria were centrifuged out (Lee, 1976). However, if any bacteria were lysed during the homogenate or microsomal preparation, the intracellular enzyme systems would be released and available for action on the aromatic hydrocarbon substrate. Also, bacteria and other microorganisms have been shown to secrete extracellular enzymes, which remain active after their release into the environment

(Karrick, 1977). In either the microsomal preparation or the whole animal homogenate, these viable cell-free enzymes, either of intra- or extracellular origin, may be present and may contribute to the metabolism of aromatic hydrocarbons.

Given the confusion over the ability of bivalves to metabolize aromatic hydrocarbons and given the lack of consideration which past researchers have given to the contributions of commensal microbial metabolism, this current research has significant investigative potential. The three primary objectives of this research were to provide answers to the following questions:

1. Is BaP metabolized by the "oyster system" (i.e., oyster and associated microorganisms)?
2. What role do oyster enzymes play?
3. What contributions do bacterial enzymes make?

MATERIALS AND METHODS

1. Animal Collection and Maintenance

The European flat oyster, Ostrea edulis, was utilized in this study for several reasons. During earlier studies in our laboratory, it was determined that O. edulis remained physiologically healthy, as indicated by the addition of new shell, in the available seawater system. Also it was possible to obtain oysters that were the same size and age. Finally, the characteristic flat shell overlying the gills and cloaca was easily excised prior to exposing the animals to experimental conditions.

Becker Associates (Newport, Oregon) originally obtained six-month old O. edulis from the International Shellfish, Inc. (Moss Landing, California) in June 1977. Approximately 200 of these oysters were given to W. C. Breese of the Oregon State University Marine Science Center (MSC); they were maintained in an outdoor flow-through seawater tank at the MSC from June, 1977 until acquisition for this study. Oysters were acquired from the MSC in late October at age 10 months and again in late January at age 13 months. They were transported on ice to Oregon State University (Corvallis, Oregon) where they were held for two to three weeks at 13° ± 1°C in an aerated synthetic seawater (28.5 °/oo), bottom-filtered aquarium. The oysters acquired in late October were used in the experimental multienzyme preparations (series C, A, and I, to be described), whereas the oysters acquired in January were used only in the autoclaved

oyster/bacteria series (series Au).

The Au samples served as a background control group. Because these samples were autoclaved, it was assumed that the Au series did not contain any viable enzyme systems, including the enzyme systems capable of metabolizing polycyclic aromatic hydrocarbons. It should be noted that the only oysters that were analyzed for their capability of metabolizing ^{3}H-BaP were animals which had the same environmental histories. This was important since recent findings indicate it may be possible to induce enzyme systems in oysters (Anderson, 1978).

After these oysters were kept in the Corvallis laboratory for two to three weeks, the dorsal shell overlaying the gills and cloaca was carefully removed. The oysters were returned to the same holding aquarium and monitored daily. If, within one week of shell excision, the mantle was re-extended and the animal had begun to deposit new shell material, the oyster was deemed healthy and was used within the next three days in an experiment.

2. Chemicals

Nicotinamide, $NADP^{+}$, glucose-6-phosphate, chloramphenicol, streptomycin, penicillin, and B-glucuronidase (Type H-1 from Helix pomatia, also containing sulfatase activity) were obtained from Sigma Chemical Co. Seawater was prepared from synthetic Instant Ocean sea salts (Aquarium Systems, Inc.). The ^{3}H-benzo(a)pyrene (^{3}H-BaP) (generally labeled; specific activity 20 Ci/mmole) was purchased from Amersham-Searle. Before use, the ^{3}H-BaP was purified by thin-layer chromatography in ethanol:toulene:water (17:4:4; v/v/v) solvent.

The ^{3}H-BaP band was identified under ultraviolet light, scraped, eluted with warm methanol, evaporated to dryness under N_2 and immediately redissolved in ethanol (Dunn and Stich, 1975).

3. Experimental Protocol

A. Preliminary preparations

In order to distinguish between the oyster contribution and the oyster-indigenous bacteria contribution to the metabolism of ^{3}H-BaP, precautions were taken against introducing extraneous bacteria into any part of the experimental system.

Prior to the three-day oyster pretreatment as described in section B, the four-liter Pyrex holding tanks were thoroughly washed, rinsed with distilled water and covered until use. The air hoses were rinsed with ethanol and blown dry, and new air stones were used.

The synthetic seawater was hydrated, the salinity adjusted to 28.5 °/oo and the seawater serated for 24 hours to remove any dissolved chlorine. Prior to its use in the holding tanks, the seawater was Millipore-filtered with 0.8 µm prefilters and then filtered with the 0.22 µm filters into autoclaved storage containers which were tightly capped and stored at 2°C. A few hours before use, the storage bottle was placed in the cold room and allowed to equilibrate to the experimental temperature (13° ± 1°C).

Section D contains details on the preparation of the multi-enzyme systems and background control samples. The seawater buffer used during homogenization was prepared by Millipore filtrations

outlined above, followed by autoclaving just prior to use. Thus, the seawater buffer (main constituent of the homogenizing buffer) was considered to be sterile. The buffer was equilibrated and stored at 2°C. All glassware and metal instruments used in the multienzyme system preparation and the subsequent two-hour ^{3}H-BaP incubation (section E) were sterilized by autoclaving and covered until use. The Potter-Elvehjen pestles were rinsed in absolute ethanol and warm oven-dried prior to each use.

During the two-hour incubation with the ^{3}H-BaP substrate, it was necessary that the incubation set-up did not introduce any bacteria into the samples. This was accomplished by first bubbling pumped air through a 20% NaOH trap and then sending it through sterile distilled water before it reached the samples. All aeration tubing had absolute ethanol blown through several hours before use and all glassware was previously autoclaved.

Most-probable-number (MPN) determinations of the bacterial populations were also made (section F). The plastic microplates and covers were sterilized first by a rinsing in absolute ethanol and then by an 18-hour exposure to a germicidal lamp. The 50-µl plastic tips for the automatic pipetor used in the MPN serial dilutions were also exposed to the germicidal lamp for 18 hours.

B. General procedures

Six experimental groups were utilized to address the question of whether or not BaP is metabolized by oysters and/or their indigenous bacteria populations. Three groups were multienzyme system

preparations containing either enzymes from oysters only or enzymes from oysters and bacteria (see Table 1; series C, series A, and series I). The other three groups were background control samples which were assumed to contain no viable enzyme systems. Thus, these samples provided a background measure of the physically- or chemically-produced derivatives of ^{3}H-BaP, as opposed to ^{3}H-BaP metabolites. As outlined in Table 2, these groups were series B (homogenizing buffer; no oyster tissue), series BA (homogenizing buffer plus antibiotics; no oyster tissue), and series Au (autoclaved oyster/bacteria homogenate).

Attempts were made to produce a bacteria-free oyster multi-enzyme system by the addition of antibiotics. Tables 1 and 2 describe the three-day pretreatment to which the oysters were exposed, with some groups filtering seawater and some groups filtering antibiotic-seawater.

The subsequent preparation of multienzyme systems and background control samples, followed by the two-hour incubation with the ^{3}H-BaP as outlined in Figure 2, was performed with specific homogenizing buffers (i.e., buffers with and without antibiotics). Tables 1 and 2 describe the buffer composition for each experimental series.

At the end of the two-hour incubation of the multienzyme systems (i.e., series C, A, and I) and the background control samples (i.e., series B, BA, and Au) with the ^{3}H-BaP substrate, aliquots from each sample were put on an MPN plate for determination of indigenous bacterial numbers, and the remaining sample was stored until metabolite analysis.

Table 1. The three treatments to which the oysters were exposed during the 3-day pretreatment and the subsequent multienzyme system preparation as an attempt to produce oyster/bacteria multienzyme preparations (series C) and bacteria-free oyster multienzyme systems (series A and I).

	Series C oysters	Series A oysters	Series I oysters
Oyster pretreatment (section III, C)	Oysters filtered sterile aerated seawater for 3 days.	Oysters filtered sterile aerated seawater containing: chloramphenicol (100 µg/ml) streptomycin (50 µg/ml) penicillin (50 µg/ml) for 3 days.	Oysters filtered sterile aerated seawater for 3 days.
	The seawater was changed daily.	The seawater-antibiotic mixture was changed daily.	The seawater was changed daily.
Multienzyme system preparation (section III, D)	As diagrammed in Fig. 2. Homogenizing buffer was a seawater buffer which included: (a) sterile, 28.5 °/oo seawater (pH: 7.5-7.7) (b) cofactors: nicotinamide (0.220 g/50 ml buffer) glucose-6-phosphate (0.0312 g/50 ml buffer) $NADP^+$ (0.0036 g/50 ml buffer)	As diagrammed in Fig. 2. Homogenizing buffer included: (1) the seawater buffer as described in Series C (i.e., sterile seawater and cofactors) and (2) antibiotics: chloramphenicol (100 µg/ml buffer) streptomycin (50 µg/ml buffer) penicillin (50 µg/ml buffer)	As diagrammed in Fig. 2. Homogenizing buffer included: (1) the seawater buffer as described in Series C (i.e., sterile seawater and cofactors) and (2) antibiotics: chloramphenicol (100 µg/ml buffer) streptomycin (50 µg/ml buffer) penicillin (50 µg/ml buffer)

Table 2. The treatment to which the background control samples (series B, BA, and Au) were exposed during the 3-day pretreatment and the subsequent background control system preparation.

	Series B (buffer)	Series BA (buffer + antibiotics)	Series Au (autoclaved oysters/bacteria)
Oyster pretreatment (section III, C)	No oysters were used, so no pretreatment was necessary	No oysters were used, so no pretreatment was necessary	Oysters filtered sterile aerated seawater for 3 days. The seawater was changed daily.
Background control system preparation (section III, D)	Because only buffer and no oysters were used, the multienzyme preparation outlined in Fig. 2 was unnecessary. 5.0 ml of homogenizing buffer was placed in a sterile 25-ml Erlenmeyer flask. Homogenizing buffer was a seawater buffer as described in Series C, Table 1 (i.e., sterile seawater and cofactors).	Because only buffer and no oysters were used, the multienzyme preparation outlined in Fig. 2 was unnecessary. 5.0 ml of homogenizing buffer was placed in a sterile 25-ml Erlenmeyer flask. Homogenizing buffer included: (1) the seawater buffer as described in Series C, Table 1 (i.e., sterile seawater and cofactors) and (2) antibiotics: chloramphenicol (100 µg/ml buffer) streptomycin (50 µg/ml buffer) penicillin (50 µg/ml buffer).	As diagrammed in Fig. 2. Homogenizing buffer was a seawater buffer as described in Series C, Table 1 (i.e., sterile seawater and cofactors).

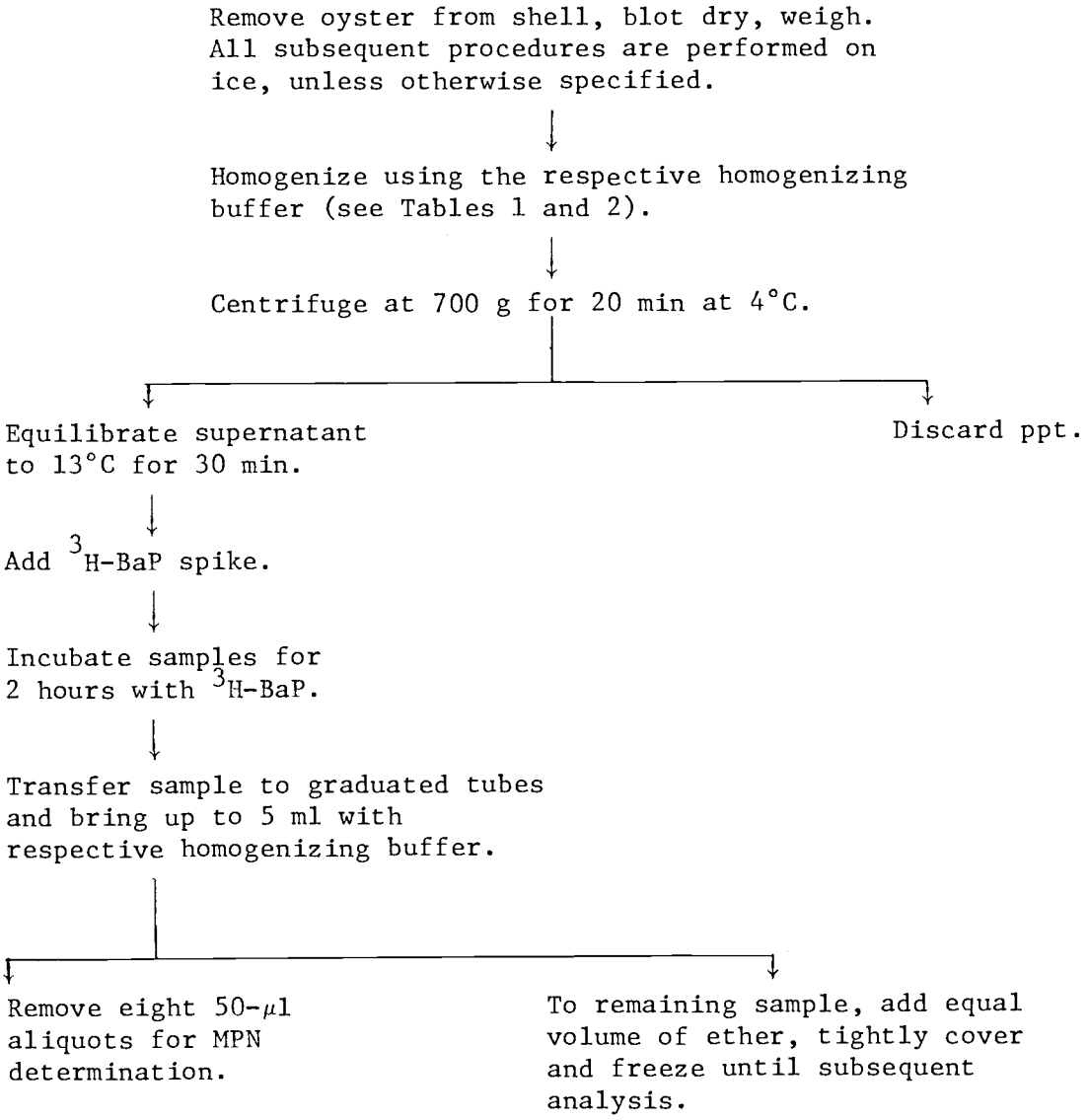


Figure 2. The experimental protocol for the preparation of the multienzyme systems (series C, A, and I) and the background control (series Au).

The extraction procedure for metabolite analysis (outlined in Figure 3) distinguished between ^{3}H-BaP and non-conjugated or conjugated ^{3}H-BaP derivatives by determining the amount of ^{3}H-activity associated with each of four extraction fractions (OE1, OE2, OE3, and AQ as seen in Figure 3). The OE1 fraction included both unmodified ^{3}H-BaP and simple non-conjugated derivatives; further separation of these compounds was effected by two thin-layer chromatography (TLC) systems. Quantifications of the ^{3}H-activity were derived for the extraction fractions and for the compounds separated by TLC.

By an examination of the ^{3}H-activity partitioning in the extraction fractions and the ^{3}H-labeled compounds separated by TLC, comparisons were made among the multienzyme systems (series C, A, and I) and the background control groups (series B, BA, and Au) to determine if metabolism of ^{3}H-BaP occurred.

This section has provided a general summary of the experimental methods. The following sections of the Materials and Methods will more fully detail the procedures.

C. Animal pretreatment

In order to distinguish between oyster and bacterial contributions to the metabolism of ^{3}H-BaP, an attempt was made to establish a bacteria-free oyster multienzyme system through the use of antibiotics. The antibiotics were first introduced to the oyster during a three-day pretreatment period. This was a three-day holding period when oysters, that had undergone dorsal shell excision over the gills

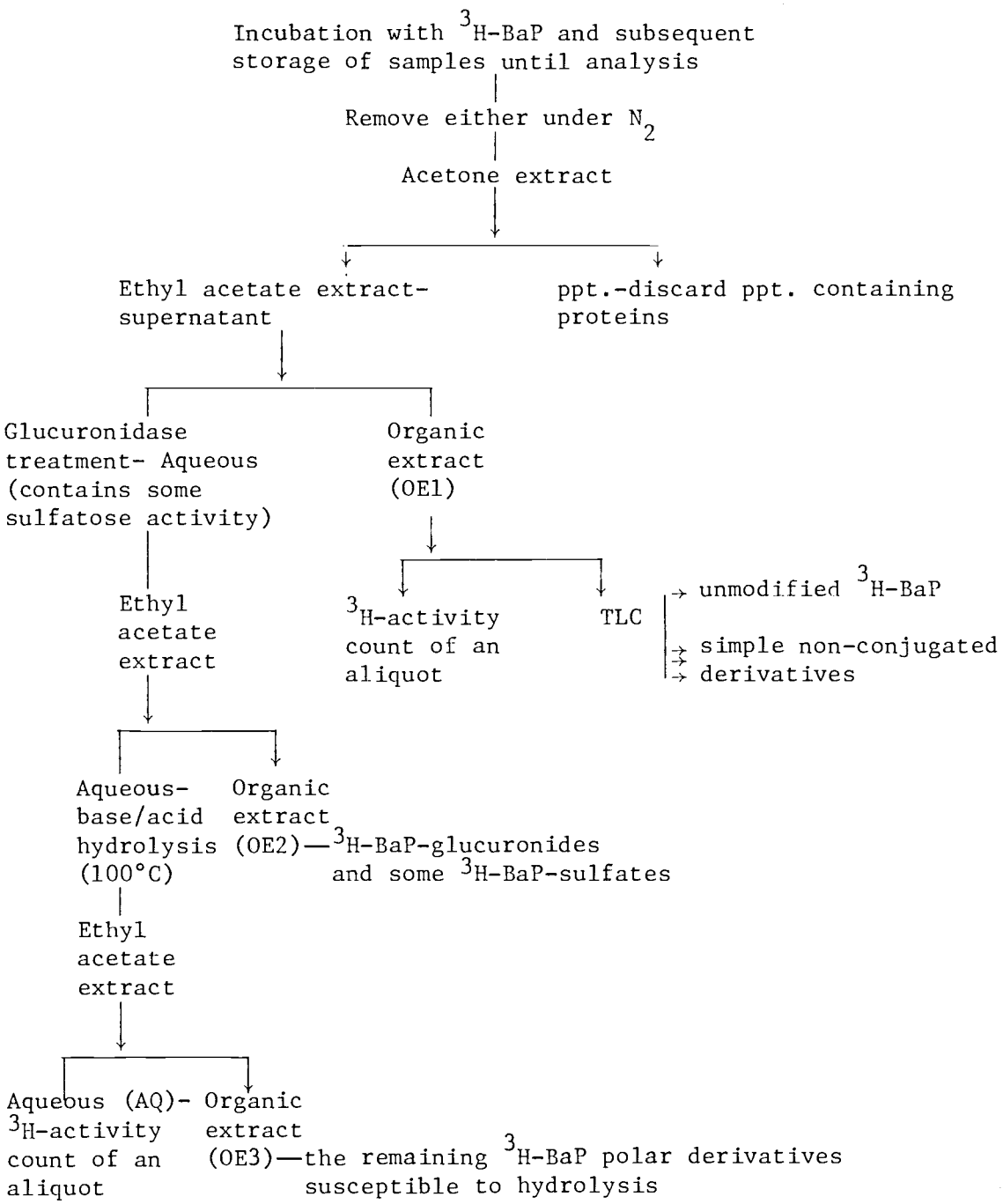


Figure 3. The fractionation protocol for separating the unmodified ^{3}H-BaP, the simple non-conjugated ^{3}H-BaP derivatives (i.e., phenols, dihydrodiols, quinones), the ^{3}H-BaP-glucuronides and -sulfates, the remaining ^{3}H-BaP-polar derivatives susceptible to hydrolysis and the remaining ^{3}H-activity unaffected by the previous treatments.

and cloaca, were exposed to either Millipore-filtered (0.22 μm) seawater or antibiotic-Millipore-filtered (0.22 μm) seawater (28.5 °/oo, 13° ± 1°C).

The experimental set-up was relatively simple. Each four-liter Pyrex tank was used as the holding container for two animals. Water was aerated by air stones and Silent Giant air pumps with the air flow mediated by C-clamps on the air hose. Two oysters of the same experimental group were placed in each tank which contained one liter of seawater with or without antibiotics (depending on the experimental treatment; see Tables 1 and 2). The holding water was changed daily. It was assumed that one liter of seawater was an adequate volume for the oysters to filter for 24 hours and still remain healthy. The three-day oyster pretreatment was conducted at 13° ± 1°C, which was the temperature at which the animals were acclimated for two weeks after their arrival into the Corvallis laboratory and also the temperature at which the ^{3}H-BaP metabolism assay was performed.

Six experimental groups were established: three multienzyme system preparations (series C, A, and I); and three background control groups (series B, BA, and Au). Tables 1 and 2 summarize the pretreatment conditions to which each group was exposed.

The first group, series C, received no antibiotic treatment to eliminate the indigenous bacterial flora. The oysters filtered Millipore-filtered (0.22 μm) aerated seawater for three days.

The second group, series A, was exposed to large amounts of antibiotics to reduce or eliminate the indigenous bacterial flora. Chloramphenicol (100 μg/ml), streptomycin (50 μg/ml) and penicillin

(50 µg/ml) were dissolved in the Millipore-filtered seawater and subsequently added to each holding tank for the three-day pretreatment. It was hoped that the numbers of indigenous bacteria could be greatly reduced or eliminated by exposing the oysters to this combination of antibiotics for three days and continuing the antibiotic exposure through the subsequent multienzyme preparation and ^{3}H-BaP incubation procedures (described in sections D and E) (Baross, personal communication).

There was one potential complication inherent in this method for reducing or eliminating the indigenous bacteria. If there were one or more strains of bacteria that were resistant to these three antibiotics, it is conceivable that this strain would normally be held to certain numbers by the normal bacterial flora. If most of the normal bacterial flora were eliminated or held in check by the antibiotics, the number of the antibiotic-resistant bacteria might increase exponentially. A pretreatment of three days would possibly be enough time for such a population development to occur. The series A oysters would then contain a large number of bacteria with a species-proportion atypical of the normal indigenous bacterial population and thus, an evaluation of ^{3}H-BaP metabolism would be made in an unusual oyster/bacteria multienzyme system.

In case this occurred in the series A oysters, a third experimental group (series I) was established. For the three-day pretreatment, these oysters were maintained in Millipore-filtered seawater which did not contain antibiotics. However, the three antibiotics were added subsequently during the multienzyme system preparation and

the ^{3}H-BaP incubation. This represented a compromise which might expose the oysters to the antibiotics for a sufficient time to reduce or eliminate the bacteria and yet not be of such long duration that a hypothetical resistant bacterial population could develop.

Thus, three multienzyme systems were exposed to three-day pretreatments as described (also summarized in Tables 1 and 2). To determine the baseline changes in ^{3}H-BaP due to the extraction techniques or non-enzymatic oxidation, three background control groups were established. Two of the groups contained no oyster tissue and so required no pretreatment. These were: (1) the homogenizing buffer, series B (section D and Table 2); and (2) the homogenizing buffer plus antibiotics, series BA (section D and Table 2). The third background control group, series Au, was an autoclaved oyster/bacteria system. The oysters used in this group were maintained in Millipore-filtered seawater for the three-day pretreatment (Table 2).

D. Preparation of multienzyme systems and background control systems

Sterile conditions were maintained throughout these procedures and the subsequent incubation with ^{3}H-BaP. Samples were kept on ice at all times during these preparations.

It was known that seawater served as a buffer between pH 7.5 and pH 8.2 (Welsh et al., 1968). Therefore, the basic homogenizing buffer for all samples was made up of: (1) autoclaved sterile seawater (28.5 ^{o}/oo, pH 7.5-7.7); and (2) the cofactors, nicotinamide (0.220 g/50 ml buffer), glucose-6-phosphate (0.03125 g/50 ml buffer),

and $NADP^+$ (0.00355 g/50 ml buffer) (Sims, 1966). Both series C (a multienzyme preparation) and Au (a background control series) were homogenized in the basic homogenizing buffer. Some of the experimental groups required antibiotic addition to the homogenizing buffer during this procedure. Chloramphenicol (100 µg/ml buffer), streptomycin (50 µg/ml buffer), and penicillin (50 µg/ml buffer) were added to the homogenizing medium used for series A and I.

Since series B and series BA did not contain any oyster tissue, the samples did not have to be homogenized and centrifuged. Each sample in the B series was made up of 5 ml homogenizing buffer. Five milliliters of the homogenizing buffer were added to each sample in the B series; the BA samples were made up of the homogenizing buffer plus the three antibiotics. At this point, these samples were considered ready for incubation with ^{3}H-BaP.

The multienzyme systems and the background control systems were prepared according to a modification of the methods of Popják (1969), as described below and as outlined in Figure 2 and Tables 1 and 2. Each oyster was removed from the shell, blotted dry and weighed immediately. The whole oyster was placed in a homogenizing tube with three mls of the respective ice-cold homogenizing buffer (Tables 1 and 2). The suspension was homogenized in a glass Teflon Potter-Elvehjem homogenizer with 35 strokes at 350 rpm. In order to avoid warming the preparation, the homogenizing tube was immersed in an ice-packed beaker. One additional ml of the respective homogenizing buffer was used to rinse remaining homogenate off the pestle and into the tube. The homogenate was then centrifuged at 700 g for 20 minutes at 4°C.

The supernatant was decanted carefully from the loosely-packet pellet with a Pasteur pipet into a 25-ml Erlenmeyer flask.

E. ^{3}H-BaP incubation with the multienzyme preparations and the background control series

The experimental set-up used for the two-hour ^{3}H-BaP incubation was cleaned and sterilized as previously described. Air was passed first through 20% NaOH for purification, then through autoclaved distilled water to remove any dissolved NaOH and finally into four Pasteur pipets, each of which was placed directly over the sample surface. An enzyme incubator provided the gentle shaking during the incubation. The incubation temperature for all samples was 13°C.

The ice-cold multienzyme preparations and background control samples were allowed to equilibrate for 30 minutes until they reached the incubation temperature. The reaction was initiated by adding 50 µl of ^{3}H-BaP (4-8 µCi) in ethanol. The exact amount of substrate each preparation received in the 50-µl spike was quantified by a liquid scintillation count of another 50-µl ^{3}H-BaP spike taken that same day. This amount varied among experimental runs due to evaporative losses of the ethanol during storage. Because the ^{3}H-activity associated with the 50-µl spike was quantified for each experimental run, the variation between spikes could be taken into consideration.

The preparations were shaken and air was blown over each sample (DePierre et al., 1975). After a two-hour incubation period, each sample was transferred to a 25-ml Pyrex tube and brought up to 5 ml with the respective homogenizing buffer. Eight 0.05-ml aliquots

were removed from each sample and placed separately in the wells of a MPN bacteria microplate (Rowe et al., 1977). Enzymatic reactions in the remaining samples were stopped by adding an equal volume of ether. The Pyrex tubes were covered with tin foil, tightly capped and the samples frozen until metabolite assays were performed.

F. MPN determination of bacterial populations

The design of this experiment necessitated estimating the bacteria numbers in multienzyme systems and background controls. A modification of the MPN method (Rowe et al., 1977) was chosen because of the relative ease with which bacteria numbers could be estimated. This procedure employed a microplate for each sample instead of a series of test tubes.

A 0.05-ml aliquot of sterile seawater buffer (28.5 $^{o}/oo$, 13°C, containing no cofactors or antibiotics) was placed into each of the 8 x 12 wells of a sterile microplate. Aliquots of the multienzyme system or the background control sample (0.05 ml) were pipetted into each of the first eight wells. Using a 0.05-ml automatic pipetor (Oxford Labs.), serial dilutions were performed by withdrawing 0.05 ml from the previously inoculated well and then moving to the adjacent well where the inoculate is dispensed and the pipet tip rinsed three times. This process was continued until serial dilutions were carried out over the plate for each original aliquot from the multienzyme or background control sample. The result was 12 two-fold serial dilutions with eight replicates for each dilution.

After inoculation, the plates were protected from contamination by sterile plastic covers and incubated at 13°C for 10 days. Several plates were monitored at three-day intervals from day 0 to day 20. There was no change in the number of wells containing bacteria after day 10.

At the end of this 10-day incubation period, each microplate was scored by microscopic examination of 0.001 to 0.005 ml from each wall. A well was scored positive when bacteria were detected. The particular MPN value (as derived from tables presented in Rowe et al., 1977), dilution factors and individual oyster wet weights (when applicable) were used to calculate the MPN per gram oyster wet weight for each sample.

G. Assay for BaP metabolism

1. Radioactive assay

The radioactivity assay (as outlined in Figure 3) employed a synthesis of published techniques. The multienzyme preparations and the background control samples were removed from the freezer, thawed, and the ether evaporated off under N_2. Each sample was treated with one-volume cold acetone and refrigerated for one hour to precipitate proteins (Stohls et al., 1976). The samples were then filtered through a coarsely-pored Buchner funnel to remove the proteins and the remaining acetone was removed by evaporation under N_2.

Each preparation was extracted three times with 1/2-volumes of ethyl acetate by thoroughly mixing with 30 inversions (Rasmussen and

Wang, 1974; Yang et al., 1975). These three 1/2-volumes were pooled and labeled as the organic extract 1 (OE1). During this first organic extraction, the relatively non-polar, unmodified ^{3}H-BaP and simple non-conjugated derivatives (i.e., simple hydroxy derivatives, diols, and quinones) were readily extracted from the aqueous preparation into the organic solvent, ethyl acetate. Each OE1 sample was brought up to 10 ml with ethyl acetate; a quantification of the amount of ^{3}H-activity in each OE1 fraction was made by liquid scintillation count of a 0.05 ml aliquot. The remaining OE1 fractions were stored frozen until subsequent separation of the ^{3}H-BaP and non-conjugated derivatives by thin-layer chromatography (section G, 2).

The remaining aqueous preparation was adjusted to pH 4.8 by the addition of 1 M acetate buffer, pH 4.8. Glucuronidase (containing some sulfatase activity) was added as 500 units per ml aqueous preparation, and toluene was layered over the top as a bacterial barrier (Buhler, personal communication). This mixture was agitated during the 14-hour incubation at 37°C. If any of the ^{3}H-BaP was enzymatically or chemically conjugated with glucuronides or sulfates, this treatment cleaved off the glucoronide/sulfate moieties and released the ^{3}H-BaP to be extracted out in the organic solvent (Bock et al., 1976). After the toluene was evaporated off under N_2, the aqueous preparation was extracted three times with 1/2-volumes of ethyl acetate. This second organic extraction (OE2) was transferred to a scintillation vial, evaporated to dryness under N_2, redissolved in 1-ml methanol, and counted by liquid scintillation.

After the second organic extraction was completed, the remaining aqueous preparation was hydrolyzed in 2 M NaOH for 10-15 minutes in a boiling water bath at 100°C (Keysell et al., 1975). The solutions were cooled, acidified with HCl and extracted three times with 1/2-volumes of ethyl acetate. Any ^{3}H-BaP-polar derivatives which had been unaffected by glucuronidase and yet were susceptible to hydrolysis, were cleaved and the ^{3}H-BaP was made available for extraction into the third organic extraction (OE3). The OE3 samples were evaporated to dryness under N_2 in scintillation vials, redissolved in 1-ml methanol and counted by liquid scintillation.

Any remaining activity in the aqueous preparation represented ^{3}H-BaP which was so tightly bound as to be resistant to hydrolysis, modified ^{3}H-BaP rings which were incorporated into other polar compounds or ^{3}H-labels which were displaced from the BaP and conjugated with polar compounds not susceptible to hydrolysis. A quantification of the residual activity in the aqueous phase was made by counting an aliquot by liquid scintillation.

The ^{3}H-activity in all fractions was quantified by liquid scintillation counting using PCS scintillation fluor (full-strength or 2:1 PCS:xylene) and a Packard Tri-Carb Liquid Scintillation Spectrometer. The total radioactivity in each sample was determined by computing quenching, machine efficiency, partitioning, and dilution. The data was presented as the μCi recovered in a particular fraction per gram oyster wet weight relative to the total μCi recovered for that sample per gram oyster wet weight.

2. Thin-layer chromatography

The first organic extraction (OE1) contained ^{3}H-BaP and simple non-conjugated derivatives. Thin-layer chromatography (TLC) provided the tool for separating these compounds. The two TLC systems were modifications of methods described by Sims (1967).

Each OE1 sample was evaporated to dryness under N_2 and redissolved in ≤ 0.1 ml methanol. Thin-layer chromatograms were prepared by coating glass plates (20 x 20 cm) with silica gel G of 0.25 mm thickness. The TLC plates were activated at 120°C for 20 minutes before use. The OE1 samples were streaked on the plates and developed for approximately 16 cm in the solvent system, benzene: hexane (1:1, v/v). This system carried most of the unaltered ^{3}H-BaP to the solvent front, leaving the simple non-conjugated derivatives at the origin (Sims, 1967; DePierre et al., 1975).

To detect ^{3}H-activity at the origin (i.e., indicating the presence of simple non-conjugated ^{3}H-BaP derivatives), the plates were radioscanned on a radiochromatogram scanner capable of reading 20 x 20 cm plates. All areas which corresponded to the observed peaks of ^{3}H-activity were scraped, and elution of the silica gel was made with warm methanol. The methanol eluant for the origin peak (i.e., ^{3}H-BaP-simple non-conjugates) was evaporated to dryness under N_2, redissolved in ≤ 0.01 ml methanol and chromatogramed in a second TLC system.

In the second TLC system, simple non-conjugated derivatives of ^{3}H-BaP were separated by the solvent system, benzene:ethanol (19:1,

v/v). Silica gel flexible plastic TLC plates (Kodak) were scribed in 5-cm lanes, and an origin sample from the first TLC was streaked and run in each 5-cm lane.

Scribing the plates in 5-cm lanes was performed for two reasons. The radiochromatogram scanner read 5-cm plates; thus, each sample could be scanned for ^{3}H-activity over the entire TLC plate. In addition, the TLC plate for each sample was cut in 5-mm strips and put in scintillation vials for counting, producting a quantification of the ^{3}H-activity distributed in peaks along the TLC plate. The 5-cm by 5-mm strips fitted easily into the scintillation vials.

The TLC plate from each sample was counted for ^{3}H-activity in 5-mm strips. The quantification of ^{3}H-activity distributed on the TLC plates (in dpm per g oyster wet weight) was graphed against the corresponding height on the TLC plate (in cm). These radioprofiles and the radioscans were compared, and Rf values for observed peaks were determined. In addition, quantifications of each peak were made in a form which allowed comparisons to be made among samples (i.e., μCi in each peak (total μCi in OE1)$^{-1}$/g oyster wet weight). Comparisons, using the Rf data and the ^{3}H-activity quantifications for the observed peaks, were made between the multienzyme systems (series C, A, and I) and the background control samples (series B, BA, and Au) to identify metabolites of ^{3}H-BaP by oysters and/or the indigenous bacteria.

RESULTS

Most-Probable Number (MPN) determination of the bacterial population size

The MPN results for the C, A, B/BA, and Au series oysters were straightforward (Table 3). None of the background control samples (B/BA and Au) contained detectable numbers of bacteria. The B/BA series showed no bacterial growth, demonstrating that sterile conditions were maintained throughout the ^{3}H-BaP incubation period and during the ten days when MPN plates were developed. The autoclaved oyster/bacteria series (Au) were also bacteria-free. Thus, it was assumed that the Au samples contained no viable enzyme systems from bacteria or oyster.

Series C oysters received no antibiotic treatment to control indigenous bacterial numbers. Table 3 indicates that there were bacterial populations present in each multienzyme sample. The MPN per gram oyster wet weight for each sample (Table 3) ranged approximately from 3×10^{5} to 2×10^{6}.

The antibiotic treatment for series A oysters (i.e., antibiotics added during both the three-day animal pretreatment and the subsequent multienzyme system preparation) successfully eliminated the bacterial flora in the oyster multienzyme system for the two-hour ^{3}H-BaP incubation (Table 3).

The MPN data for the series I multienzyme preparations was ambiguous (Figure 4). Antibiotics were not added to the series I oysters during the three-day pretreatment, but were included during

Table 3. The determination of commensal bacteria numbers as the most-probable-number (MPN) per g oyster wet weight.

Sample		MPN	Standard error	MPN (bacteria #) per oyster homogenate	Oyster g wet weight	MPN / g oyster wet wt.	Corresponding S.E.
Buffer series	B1	-				0	-
	B2	-				0	-
	BA1	-				0	-
	BA2	-				0	-
Autoclaved oyster/bacteria series	Au1	-				0	-
	Au2	-				0	-
	Au3	-				0	-
	Au4	-				0	-
Series C	C1	1.855	0.291	4.75×10^4	.4858	9.78×10^5	1.53×10^5
	C2	1.054	0.287	2.70×10^4	.4623	5.84×10^5	1.59×10^5
	C3	2.124	0.294	5.44×10^4	.2625	2.07×10^6	2.87×10^5
	C4	1.993	0.285	10.20×10^4	.3449	2.96×10^5	4.23×10^4
	C5	1.760	0.283	9.01×10^4	.2805	3.21×10^5	5.12×10^4
	C6	1.993	0.285	40.81×10^4	.2830	1.44×10^6	2.06×10^5
Series A	A1	-				0	-
	A2	-				0	-
	A3	-				0	-
	A4	-				0	-
	A5	-				0	-
	A6	-				0	-
Series I	I1	Sample eliminated from consideration.					
	I2	>2.921	-	$>119.6 \times 10^4$	.3890	$>3.08 \times 10^6$	-
	I3	>2.921	-	$>119.6 \times 10^4$	.3986	$>3.00 \times 10^6$	-
	I4	>2.921	-	$>119.6 \times 10^4$	.3198	$>3.74 \times 10^6$	-
	I5	-				0	-
	I6	-				0	-

		1	2	3	4	5	6	7	8	9	10	11	12
I1	A	-	+	+	+	+	+	+	+	+	+	+	-
	B	+	-	+	+	+	+	+	+	+	-	-	-
	C	-	+	+	+	+	+	+	+	+	+	+	+
	D	+	+	+	+	+	+	+	+	+	+	+	+
	E	-	-	+	+	+	+	+	+	+	+	-	-
	F	+	-	+	+	+	+	+	+	+	+	+	+
	G	-	+	+	+	+	+	+	+	+	+	-	-
	H	-	+	+	+	+	+	+	+	+	+	+	-

I2 The entire plate was positive.

I3 The entire plate was positive.

I4 The entire plate was positive.

		1	2	3	4	5	6	7	8	9	10	11	12
I5	A	-	-	-	+	+	+	+	+	+	-	-	-
	B	-	-	+	+	+	+	+	+	+	-	-	-
	C	-	-	+	+	+	+	+	+	-	-	-	-
	D	-	-	+	+	+	+	+	+	-	-	-	-
	E	-	-	+	+	+	+	+	+	+	+	-	-
	F	-	-	-	+	+	+	+	+	+	+	-	-
	G	-	-	+	+	+	+	+	+	-	-	-	-
	H	-	-	-	+	+	+	+	+	-	-	-	-
I6	A	-	-	+	+	+	+	+	-	-	-	-	-
	B	-	-	+	+	+	+	+	+	-	-	-	-
	C	-	-	+	+	+	+	+	+	-	-	-	-
	D	-	-	+	+	+	+	+	+	-	-	-	-
	E	-	-	-	+	+	+	+	+	-	-	-	-
	F	-	-	+	+	+	+	+	+	-	-	-	-
	G	-	-	+	+	+	+	+	+	-	-	-	-
	H	-	-	+	+	+	+	+	+	+	-	-	-

Figure 4. The MPN microplates, for series I, as scored positive for bacterial presence and negative for bacterial absence.

the subsequent multienzyme system preparation. For samples I2, I3, and I4, bacteria were detected at all concentrations on the MPN plate (Figure 4). The MPN method for determining bacterial numbers depends on diluting the number of bacteria to extinction; thus, it can only be concluded that the MPN of the indigenous bacterial population for samples I2, I3, and I4 was greater than 3.08×10^6 (Table 3).

The results for samples I5 and I6 were somewhat equivocal. It can be seen in Figure 4 that no bacteria were present in the first two wells (i.e., the wells containing the greatest concentration of both multienzyme preparation and antibiotics). However, bacteria were observed in adjacent wells which actually contained less of the sample. For reasons outlined in the Discussion, these two samples, I5 and I6, were considered to be bacteria-free multienzyme systems during the two-hour incubation with ^{3}H-BaP.

Because sample I1 had positive and negative first wells, the sample was eliminated from the study. It would be difficult to determine whether or not the multienzyme incubation mixture was bacteria-free.

To summarize, there were four discrete experimental systems acting upon the ^{3}H-BaP during the two-hour incubation. They were: (1) the buffer system (B and BA) without viable enzymes; (2) the autoclaved oyster/bacteria system (Au) without viable enzymes; (3) the oyster/bacteria multienzyme system (series C and samples I2, I3, and I4); and (4) the bacteria-free oyster multienzyme system (series A and Samples I5 and I6). All extraction and metabolite comparisons

were made after grouping the data into these four discrete experimental treatments.

Radioactive assay for ^{3}H-BaP metabolism

The results of the radioactive assay for ^{3}H-BaP metabolites were variable (Tables 4 and 5). The ^{3}H-BaP and simple non-conjugates (i.e., simple hydroxy derivatives, diols, and quinones) were counted in the first organic extraction (OE1). The second organic extraction (OE2) contained ^{3}H-BaP which was enzymatically or chemically conjugated with glucuronides or some sulfates. Any ^{3}H-BaP-polar derivatives which were susceptible to base/acid hydrolysis were quantified in the third organic extraction (OE3). The remaining aqueous sample (AQ) was counted for residual ^{3}H-activity not extractable by the above treatments. The radioactivity in the aqueous preparation represented ^{3}H-BaP which was so tightly bound as to be resistant to hydrolysis, modified ^{3}H-BaP rings which were incorporated into other polar compounds or ^{3}H-labels which were displaced from the BaP and conjugated with polar compounds not susceptible to hydrolysis. For the remainder of this section, the four extractions will be referred to by their abbreviations (OE1, OE2, OE3, AQ) and will connote the particular ^{3}H-BaP compounds described above.

The radioactivity assay quantified any chemical alterations in ^{3}H-BaP during the two-hour incubation with the four experimental preparations (i.e., 1. buffer/buffer + antibiotics system - B/BA; 2. autoclaved oyster/bacteria series - Au; 3. oyster/bacteria multienzyme system - series C and I2, I3, I4; 4. bacteria-free oyster

Table 4. Comparison of ^{3}H-activity partitioned in each organic extract and the remaining aqueous phase (i.e., OE1, OE2, OE3, and AQ) expressed as μCi recovered in the particular extraction relative to the total μCi recovered for the buffer series (B) and the buffer + antibiotics series (BA).

Sample		OE1-μCi rec / total μCi rec	OE2-μCi rec / total μCi rec	OE3-μCi rec / total μCi rec	AQ-μCi rec / total μCi rec
Buffer series	B1	.98732	.00155	.00151	.00960
	B2	.99032	.00269	.00125	.00574
Buffer + antibiotics series	BA1	.98772	.00314	.00329	.00584
	BA2	.98655	.00300	.00225	.00825

Table 5. Comparison of ^{3}H-activity partitioned in each organic extract and the remaining aqueous phase (i.e., OE1, OE2, OE3, and AQ) expressed as µCi recovered in the particular extraction per g oyster wet weight for the autoclaved oyster/bacteria background controls (series Au), the oyster/bacteria multienzyme systems (series C and I2-4), and the bacteria-free oyster multienzyme systems (series A and I5-6).

Sample		OE1-µCi rec/g total µCi rec/g	OE2-µCi rec/g total µCi rec/g	OE3-µCi rec/g total µCi rec/g	AQ-µCi rec/g total µCi rec/g
Autoclaved oyster/bacteria system	Au-1	.94751	.04781	.00142	.00315
	Au-2	.96783	.02853	.00101	.00264
	Au-3	.90414	.09054	.00147	.00385
	Au-4	.95555	.03477	.00473	.00494
Oyster/bacteria multienzyme preparation	C1	.93502	.00391	.00190	.05917
	C2	.94493	.00581	.00147	.04779
	C3	.82315	.11913	.00591	.05181
	C4	.82966	.09335	.00561	.07138
	C5	.88706	.05000	.00488	.05808
	C6	.91501	.00722	.00483	.07294
	I2	.81355	.07907	.01486	.09252
	I3	.82682	.07633	.01015	.08671
	I4	.83185	.07205	.02025	.07585
Bacteria-free oyster multienzyme system	A1	.91183	.01940	.00343	.06534
	A2	.91232	.00991	.00392	.07385
	A3	.91081	.01244	.00261	.07414
	A4	.92250	.01713	.00234	.05805
	A5	.90914	.01706	.00501	.06879
	A6	.90723	.01310	.00372	.07595
	I5	.89520	.02427	.00358	.07695
	I6	.87858	.04292	.00433	.07416

multienzyme preparation - series A and I5, I6). This ^{3}H-activity is expressed as μCi-^{3}H extracted in each of the four extractions per gram oyster wet weight relative to the total μCi-^{3}H recovered in all extractions for that sample per gram oyster wet weight [μCi-^{3}H recovered in each extraction (g wet wt)$^{-1}$/total μCi-^{3}H recovered in all fractions (g wet wt)$^{-1}$]. Henceforth, reference will be made to the particular extraction fraction being dealt with—μCi recovered (i.e., OE1-μCi recovered).

Of special note in Table 6 are the large differences among samples regarding the fraction of total ^{3}H-recovery relative to the total ^{3}H-initially spiked. Thus, the experimental preparations were actually exposed to different amounts of the original spike. If the μCi ^{3}H-recovered in each extraction per gram were presented relative to the total μCi ^{3}H-BaP spiked, the comparison would be invalid because of the differences in the percent recovered for each sample (Table 6). The method selected for presenting the data (μCi ^{3}H-recovered in each extraction per g relative to the total μCi ^{3}H-recovered in all extractions/g) denotes the partitioning among four extractions of ^{3}H-BaP to which the animal was actually exposed.

Other researchers (e.g. DePierre et al., 1975) have subtracted the background activity of inviable enzyme systems from the radioactivity of viable enzyme systems in order to determine what fraction of the radioactivity in each extraction was due to metabolism of the compound under study. With regard to the present experiment (Table 5), attempts to subtract the autoclaved oyster/bacteria systems' extractions—μCi recovery (i.e., background) from the experimental

Table 6. Comparison of the total ^{3}H-activity recovered in all extracts of each sample per g oyster wet weight relative to the total ^{3}H-activity spiked in each sample per g oyster wet weight.

Sample		Total µCi recovered in all extracts / g wet wt	Total µCi spiked / g wet wt	Total µCi recovered in all extractions/g / total µCi spiked/g
Buffer series	B1	2.1259	3.8154	.55719
	B2	2.2316	3.8154	.58489
	BA1	2.0036	3.8154	.52513
	BA2	1.7327	3.8154	.45413
Autoclaved oyster/ bacteria series	Au-1	10.5463	10.5500	.99965
	Au-2	16.7261	16.9521	.98667
	Au-3	13.8556	15.6943	.88284
	Au-4	9.0615	9.1453	.99084
Oyster/ bacteria multienzyme preparations	C1	4.4766	6.5871	.67960
	C2	3.4738	6.9219	.50186
	C3	12.4588	40.000	.31147
	C4	9.2757	30.4436	.30468
	C5	7.6005	13.1194	.57933
	C6	7.7013	13.0035	.59225
	I2	7.7111	10.2314	.75367
	I3	7.1562	9.2323	.77513
	I4	9.5978	11.8786	.80799
Bacteria-free oyster systems	A1	5.3134	5.7741	.92021
	A2	6.0011	6.2943	.95342
	A3	6.7046	8.9559	.74862
	A4	7.1356	8.6995	.82023
	A5	23.1852	40.9357	.56638
	A6	21.9991	37.9610	.57952
	I5	10.9054	13.7109	.78611
	I6	11.9830	15.1191	.79257

multienzyme systems' μCi recovery in each extraction made the large individual sample variation apparent. In some cases, the Au sample's activity was greater than the activity of an experimental multienzyme sample. Rather than subtract the background, statistical comparisons were made among the four experimental groups to identify whether and where any differences occurred in the partitioning of activity in the extractions. This difference indicated the presence or absence of ^{3}H-BaP derivatives.

To statistically analyze the difference in the activity partitioning, t-tests and analyses of variance (ANOVA) were performed. The use of both these analyses assumed a homogeneity of variance among the samples; examination of Tables 4 and 5 showed that this assumption was not valid. For example, the variance for all OE1 fractions was much greater than the variance for the OE3 fractions. An arcsin transformation reduced these differences in variances and thus transformed the data so that t-tests and ANOVA's could be performed (Snedecor and Cochran, 1973).

Comparisons of the ^{3}H-activity partitioning within each of the extraction fractions (OE1, OE2, OE3, and AQ) revealed no significant differences between the buffer series (B) and the buffer + antibiotics series (BA) (t-test, $P > 0.05$). Since there was no difference between the B and BA groups, they were combined as one background control treatment (B/BA) for all subsequent analyses.

Thus, four experimental groups could be compared (B/BA, Au, C/I2-4, and A/I5-6) and the transformed data used in ANOVA's. The two-way analysis of variance (ANOVA-2) can only be performed on groups

of equal sample size. Since the experimental groups were of unequal sample size (B, n = 4; Au, n = 4; C/I2-4, n = 9; A/I5-6, n = 8), an unweighted mean was determined for each group and the ANOVA-2 was then performed.

The ANOVA-2 was used to compare the 4 x 4 matrix of the two main effects: (1) the experimental treatments (B/BA, Au, C/I2-4, A/I5-6); and (2) the ^{3}H-BaP derivatives seen in the four extractions (OE1, OE2, OE3, AQ) subjected to arcsin transformations. Because of a significant interactive value for (experimental treatments) x (extractions) ($F_{(9,84)} = 2.72$, $P < 0.01$), the F values for main effects were irrelevant and a clear statement could not be made about the significance of the two main effects.

A second ANOVA-2 compared a 2 x 4 matrix of: (1) the experimental treatments (C/I2-4 and A/I5-6); and (2) the ^{3}H-BaP derivatives seen in the four extractions (OE1, OE2, OE3, AQ) subjected to arcsin transformation. There was no difference between the oyster/bacteria multienzyme preparations (C/I2-4) and the bacteria-free oyster multienzyme systems (A/I5-6) with regard to ^{3}H-BaP metabolism ($F_{(1,60)} = 0.0727$, $P > 0.05$). There was a significant difference in the partitioning of ^{3}H-BaP or ^{3}H-BaP derivatives among the extractions ($F_{(3,60)} = 186.60$, $P < 0.005$). Visual examination confirmed this difference (Tables 4 and 5). It was obvious that the majority of ^{3}H-activity was extracted initially (OE1) as unmodified ^{3}H-BaP and simple non-conjugated derivatives with the remaining small fraction divided among the other three extractions (OE2, OE3, and AQ). The interaction value between treatment and metabolite partitioning was

not significant ($F_{(3,60)} = 0.5146$, $P > 0.05$).

To summarize, the use of ANOVA-2 on a 2 x 4 matrix showed that there was no difference between the ^{3}H-BaP metabolite separation for the two oyster multienzyme systems with and without the presence of bacteria (C/I2-4 and A/I5-6, respectively). However, this test made no comparison between the two multienzyme system groups and the two background control groups (i.e., the buffer series, B/BA, and the autoclaved oyster/bacteria group, Au). The ANOVA-2 which attempted to make this comparison with a 4 x 4 matrix showed a significant interactive value between the main effects, making any statement about the difference in the ^{3}H-activity partitioning among the four experimental groups irrelevant. Thus, the two-way analysis of variance could not be used to compare all four experimental treatments.

To avoid this problem of interaction between the two main effects which appeared in the ANOVA-2, the one-way analysis of variance (ANOVA-1), evaluating the difference among four experimental treatments for each extraction, was considered the best way to approach the question of any differences between experimental groups (i.e., analysis comparing all groups within one extraction column of Tables 2 and 3). Because the variances were similar for all experimental groups within one extraction column, it was necessary to transform the data. An ANOVA-1 was run on the four experimental groups (B/BA, Au, C/I2-4, A/I5-6) for each of the extractions, and it showed that a significant difference existed between at least two of the experimental groups for all extractions (OE1, OE2, OE3, and AQ

fractions) ($P < 0.05$).

To distinguish between which experimental treatments this significant difference was present, Student-Newman-Keules tests were run for each determination of significance identified by the ANOVA-1 (Table 7). For the first organic extraction (OE1) containing unmodified ^{3}H-BaP and simple non-conjugates, the oyster/bacteria multienzyme system and the bacteria-free oyster multienzyme preparation differed significantly in the amount of ^{3}H-activity recovered when compared to that recovered from the buffer series ($P < 0.01$). Also for OE1, the oyster/bacteria multienzyme system was significantly different ($P < 0.01$) than the autoclaved oyster/bacteria preparation.

Considering the OE2 which contained ^{3}H-BaP-glucuronides and -sulfates, the oyster/bacteria multienzyme preparation differed from the buffer preparation in the amounts of recovered ^{3}H-activity ($P < 0.05$). However, there was no significant difference between any two experimental groups for the second organic extraction at the 0.01 significance level.

There was no difference between any two groups at the 0.01 significance level for the OE3 fraction containing ^{3}H-BaP-polar derivatives susceptible to cleavage by hydrolysis. However, at the 0.05 significance level, the buffer series, the autoclaved oyster/bacteria preparation and the bacteria-free oyster multienzyme system were all significantly different than the oyster/bacteria multienzyme preparation in the amounts of ^{3}H-activity recovered.

For the ^{3}H-activity remaining in the aqueous phase (AQ), there were significant differences recorded ($P < 0.01$). The oyster/bacteria

Table 7. The results of Student-Newman-Keules determinations of significant difference between experimental treatments (i.e., series B/BA, Au, C/I2-4, and A/I5-6) for each of the extracts (OE1, OE2, OE3, and AQ). These differences are expressed at both the 1% and 5% levels of significance.

	At the 1% significance level	At the 5% significance level
OE1-µCi rec./g total µCi rec./g	Oyster/bacteria (C/I2-4) are significantly greater than buffer series (B/BA). Bacteria-free oyster (A/I5-6) Oyster/bacteria (C/I2-4) is significantly greater than autoclaved oyster/bacteria series (Au)	Same as $P < 0.01$
OE2-µCi rec./g total µCi rec./g	No significant difference	Oyster/bacteria (C/I2-4) is significantly greater than buffer series (B/BA)
OE3/µCi rec./g total µCi rec./g	No significant difference	Buffer (B/BA) Autoclaved oyster/bacteria (Au) Bacteria-free oyster (A/I5-6) are significantly less than oyster/bacteria series (C/I2-4)
AQ-µCi rec./g total µCi rec./g	Oyster/bacteria (C/I2-4) are significantly greater than buffer series (B/BA). Bacteria-free oyster (A/I5-6) Oyster/bacteria (C/I2-4) are significantly greater than autoclaved oyster/bacteria series (Au) Bacteria-free oyster (A/I5-6)	Same as $P < 0.01$

multienzyme preparation and the bacteria-free oyster multienzyme system were both significantly different than the buffer series and the autoclaved oyster/bacteria preparation in the amounts of ^{3}H-activity recovered.

The results, summarized in Table 7, indicate that there was more ^{3}H-activity found in the extraction fractions for the multienzyme systems (C/I2-4 and A/I5-6) than in the buffer (B/BA) or the autoclaved oyster/bacteria (Au) series. The buffer group contained only ^{3}H-BaP which had been modified due to physical or chemical changes. It was expected, and found, that these samples would differ from the multienzyme systems in associated ^{3}H-activity.

For all four extractions, the amount of ^{3}H-activity associated with the oyster/bacteria multienzyme system (C/I2-4) was significantly greater than that associated with the buffer series (B/BA) ($P < 0.05$). The autoclaved oyster/bacteria system was the background control for measuring any ^{3}H-BaP changes in an organics-rich system such as the oyster/bacteria homogenate. Some inherent problems with the autoclaved control group will be explained in the Discussion. If the autoclaved oyster/bacteria system is accepted as the background control and if the amount of associated ^{3}H-activity for a particular extraction fraction was less for the autoclaved oyster/bacteria series than that for a multienzyme system preparation, then it can be assumed that metabolism of ^{3}H-BaP to the compounds characterized by the particular extraction fraction occurred in the multienzyme system.

The unaltered ^{3}H-BaP and simple non-conjugated derivatives (OE1) were found in significantly greater amounts in the oyster/bacteria multienzyme systems than in the autoclaved oyster/bacteria samples ($P < 0.05$). The subsequent thin-layer chromatography separated the unmodified ^{3}H-BaP from the derivatives. It was only after this separation and quantification of ^{3}H-BaP simple non-conjugates that the question of ^{3}H-BaP metabolism by multienzyme systems could be addressed.

With regard to the presence of ^{3}H-BaP-glucuronides and -sulfates (OE2), neither multienzyme system was significantly different than the background control (i.e., Au series). This indicates that there was no metabolism of ^{3}H-BaP to the glucuronide- or sulfate-conjugated derivatives by the oyster/bacteria or bacteria-free oyster systems. A note of caution should be inserted; Table 5 reveals that the ^{3}H-activities recovered in the OE2 fraction for the Au samples were too high to be considered background. The implications of this finding will be considered in the Discussion.

The hydrolysis-susceptible polar derivatives of ^{3}H-BaP (OE3) were found in a significantly greater amount in the oyster/bacteria multienzyme series than in the autoclaved oyster/bacteria series. This indicates that ^{3}H-BaP was metabolized to polar derivatives by the oyster/bacteria multienzyme preparation. This difference was not seen between the bacteria-free oyster multienzyme system and the background controls (Au), implying that the metabolism of ^{3}H-BaP to hydrolysis-susceptible derivatives did not occur in an oyster multienzyme system without bacteria. However, the amount of ^{3}H-activity

associated with the oyster/bacteria multienzyme system was not significantly greater than that associated with the bacteria-free oyster system as would be expected if bacteria were making the metabolic contribution. Thus, any definitive trend indicating a greater occurrence of ^{3}H-BaP metabolism to hydrolysis-susceptible polar derivatives in one multienzyme system over the other multienzyme system is masked by the large variation between individual samples.

The ^{3}H-activity remaining in the aqueous phase (AQ) after these three organic extractions included polar derivatives in which ^{3}H-BaP or only the ^{3}H-label had been incorporated. The amount of ^{3}H-activity associated with both multienzyme systems (i.e., oyster/bacteria and bacteria-free oyster) was significantly greater than that associated with the autoclaved oyster/bacteria system ($P < 0.05$). This indicates that the ^{3}H-BaP was metabolized into these polar compounds by both multienzyme systems.

In summary, these results indicate the presence of ^{3}H-BaP metabolism in both multienzyme systems. The extraction procedure differentiated in the ^{3}H-activity associated with four fractions: (1) the unaltered ^{3}H-BaP and simple non-conjugated derivatives (OE1); (2) the ^{3}H-BaP-glucuronides and -sulfates (OE2); (3) the ^{3}H-BaP-polar derivatives susceptible to cleavage by hydrolysis; and (4) the ^{3}H-activity associated with the remaining aqueous phase (AQ) which was not susceptible to hydrolysis.

Thin-layer chromatography

The OE1 fraction contained unmodified ^{3}H-BaP and simple non-conjugated derivatives of ^{3}H-BaP. Thin-layer chromatography (TLC) was used to discriminate between the two.

The chromatographic techniques used in this research were described by Sims (1967) (see Materials and Methods). To recapitulate, the first TLC run on the OE1 fraction separated most of the unmodified ^{3}H-BaP from the ^{3}H-BaP derivatives which stayed at the origin. The plates were radioscanned to detect ^{3}H-activity at the origin. When ^{3}H-activity was observed, the origin was scraped, eluted and restreaked in a second TLC system capable of separating all the simple non-conjugates of ^{3}H-BaP. The second TLC plates were radioscanned for use in Rf determination and immediately cut in 5-mm strips for liquid scintillation counting. The ^{3}H-activity profiles over the TLC plates were graphed and the ^{3}H-activity for each peak was quantified.

The radioscans of the first preparative TLC were not quantified for radioactivity. Two typical examples of radioscans for the first TLC are seen in Figures 5 and 6. All scans included a peak of ^{3}H-activity at the origin (i.e., possible ^{3}H-BaP derivatives), a distribution of activity over the plate and a large peak of ^{3}H-activity at the solvent front (i.e., unmodified ^{3}H-BaP).

In some cases (for example, Figure 6), there appeared to be a mid-plate peak. To determine if this was ^{3}H-BaP, a metabolite or neither, the corresponding plate area was scraped, eluted and

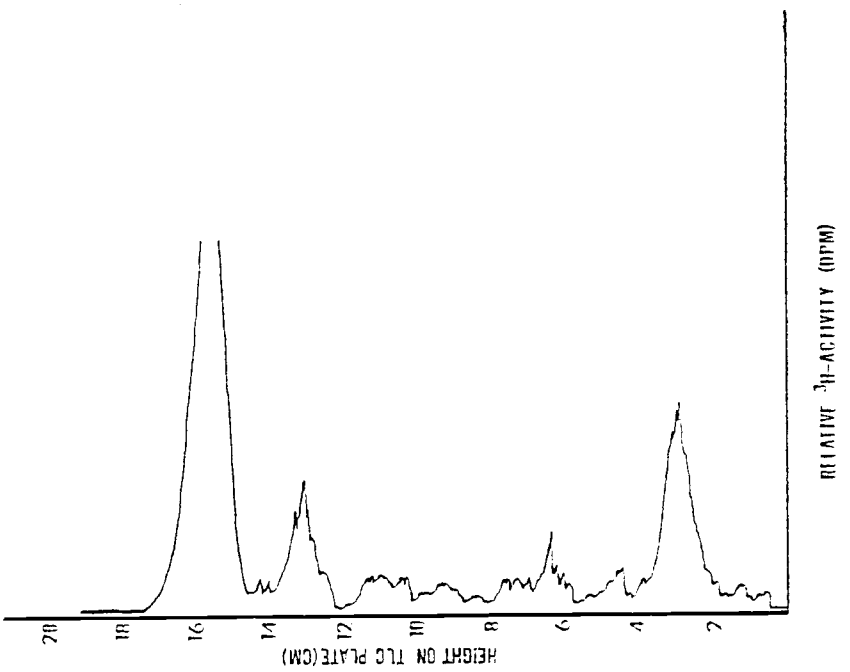


Figure 5. A typical radioscan of the first preparative TLC for sample B2.

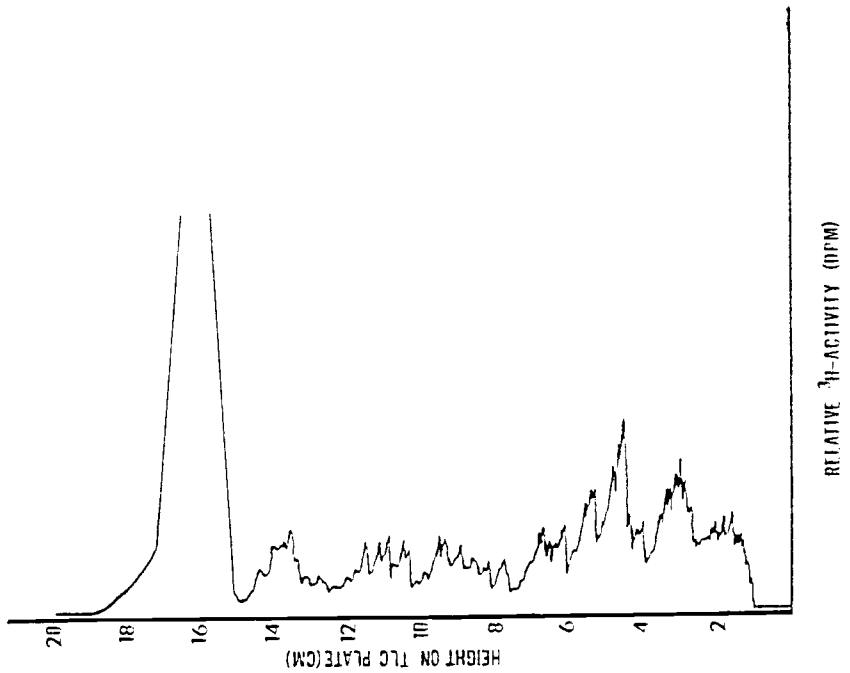


Figure 6. A typical radioscan of the first preparative TLC for sample C5.

restreaked in the second TLC system. The migration of the sample was found to correspond to that of ^{3}H-BaP.

The corresponding plate area of the origin peak which included all ^{3}H-BaP derivatives was scraped, eluted and restreaked in the second TLC system. The developed plates were then radioscanned; selected scans for each of the experimental treatments are shown subsequently (buffer series - Figures 7 and 8; autoclaved oyster/bacteria system - Figure 9; oyster/bacteria multienzyme preparation - Figures 10 and 11; bacteria-free oyster multienzyme system - figures 12 and 13).

The plates were then cut in 5-mm strips, placed in separate scintillation vials and counted. For each sample, radioactivity profiles were graphed by ^{3}H-disintegrations per minute (dpm) vs. the corresponding height on the TLC plate. The buffer series profiles are seen in Figures 14-17; the profiles for the autoclaved oyster/bacteria system in Figures 18-21; the oyster/bacteria multienzyme system preparation profiles in Figures 22-30; and the bacteria-free oyster multienzyme system profiles in Figures 31-38.

The radioactivity profile and radioscan for each sample were compared and Rf values for the peaks along with the corresponding peak area (in ^{3}H-dpm) were claculated (Table 8).

Upon examining both Rf data and the radioactivity profiles, there appeared to be seven peaks distributed up the plate with the origin being the first and the ^{3}H-BaP peak at the solvent front the seventh. This paper will only be concerned with the six peaks below the unaltered ^{3}H-BaP peak, and each peak was designated as one of these

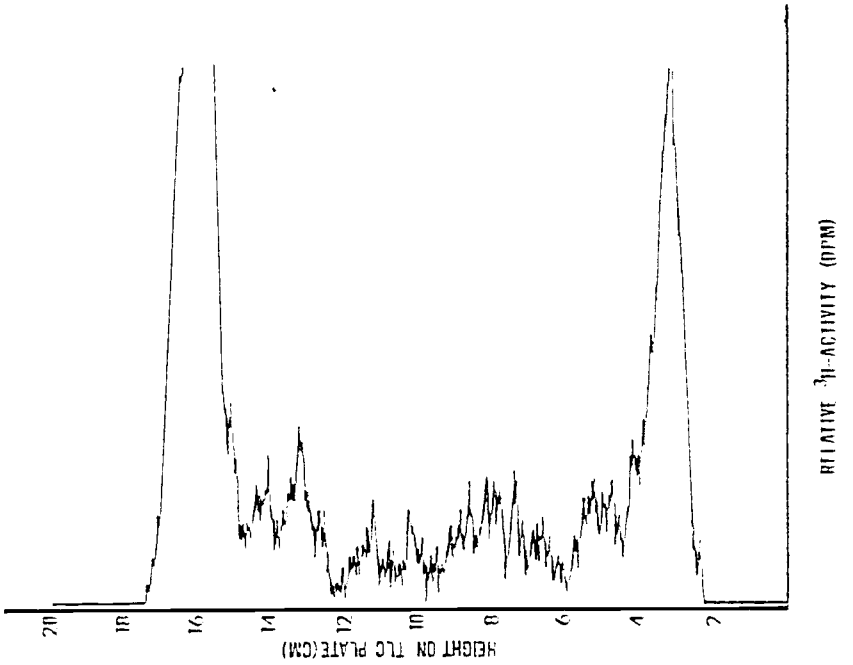


Figure 7. An example of the second TLC radioscan for the buffer series (sample B1).

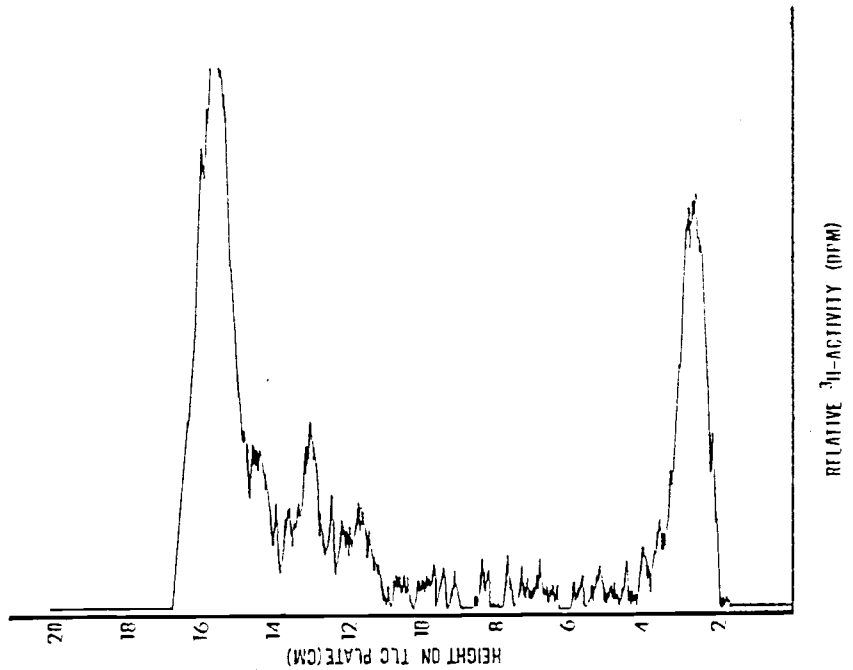


Figure 8. An example of the second TLC radioscan for the buffer + antibiotics series (sample BA1).

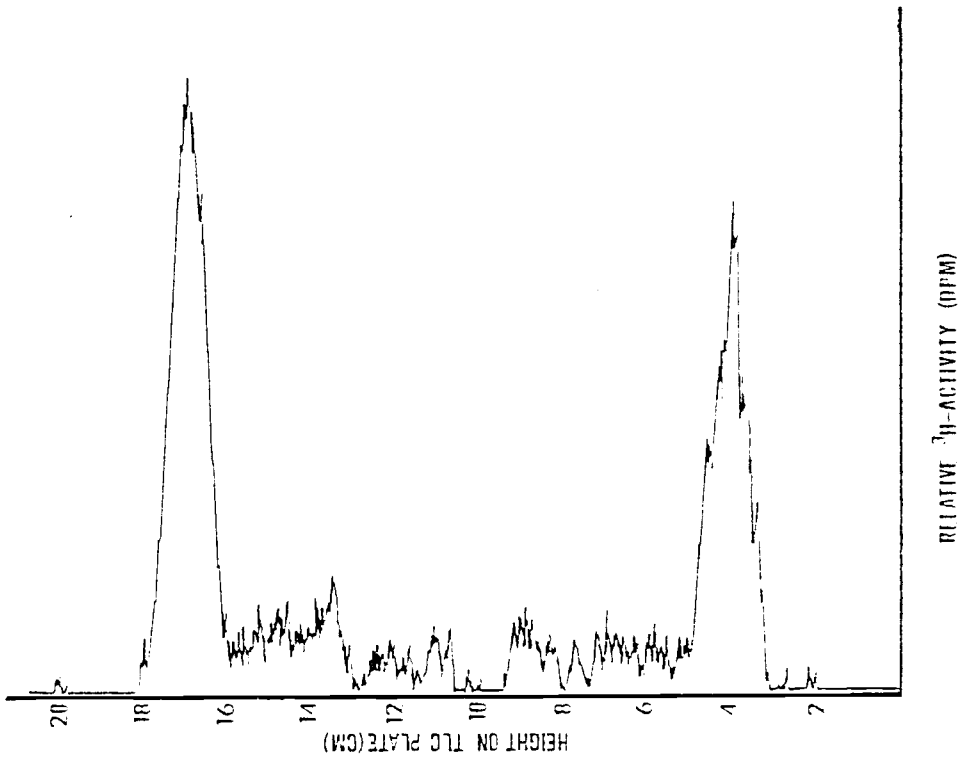


Figure 9. An example of the second TLC radioscan for the autoclaved oyster/bacteria series (sample Au4).

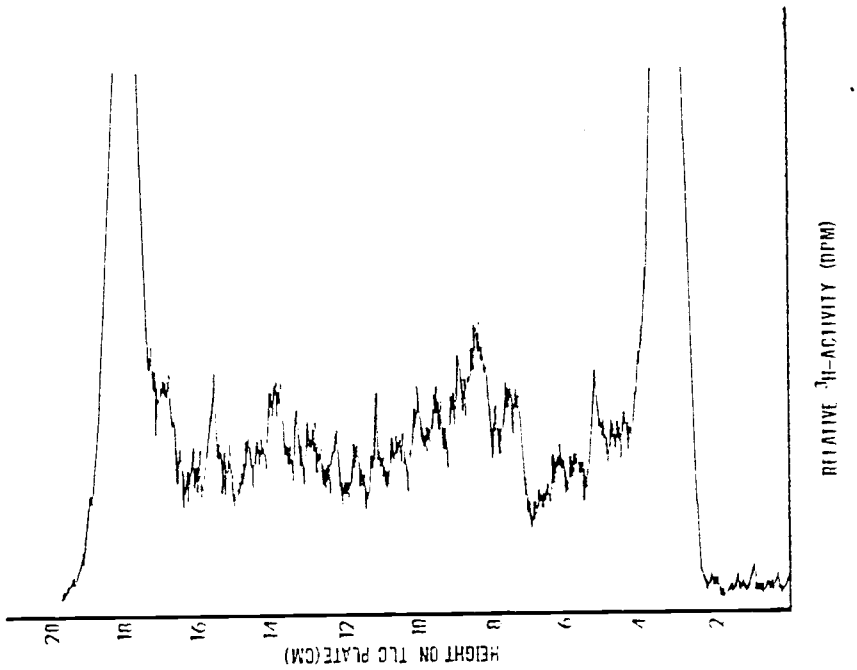


Figure 10. An example of the second TLC radioscan for the oyster/bacteria multienzyme preparations (sample C8).

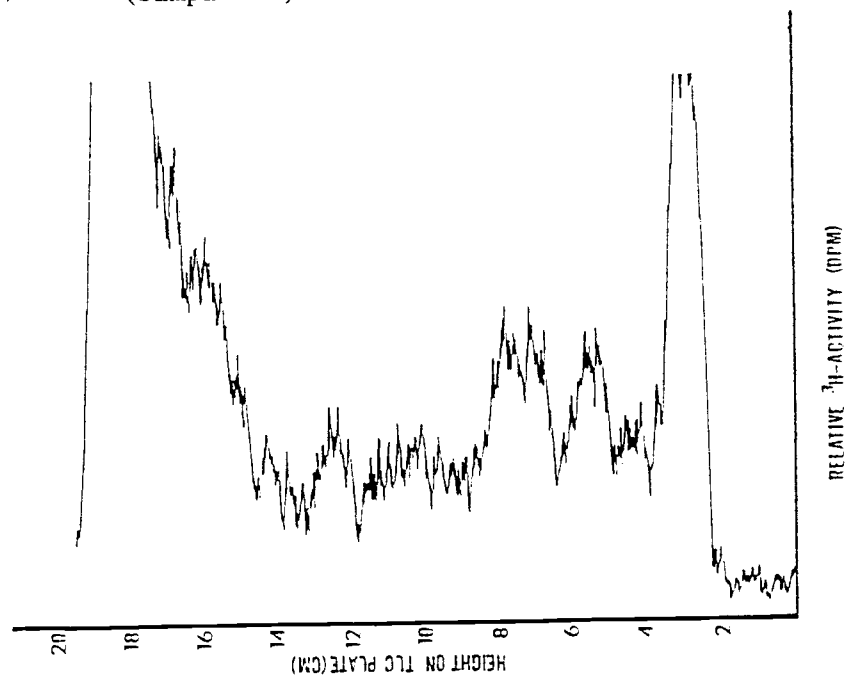


Figure 11. An example of the second TLC radioscan for the oyster/bacteria multienzyme systems (sample I2).

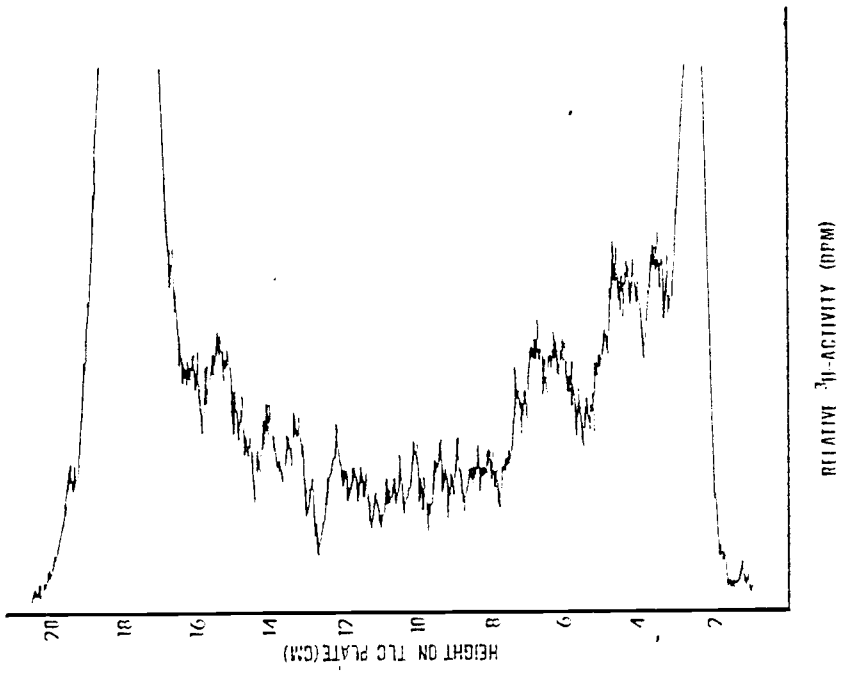


Figure 12. An example of the second TLC radioscan for the bacteria-free oyster multienzyme systems (sample A8).

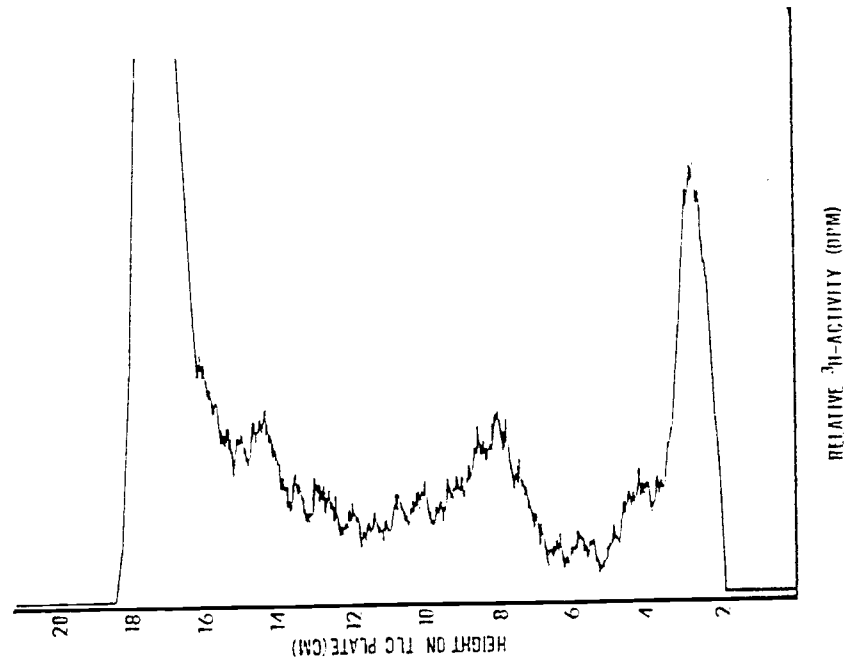
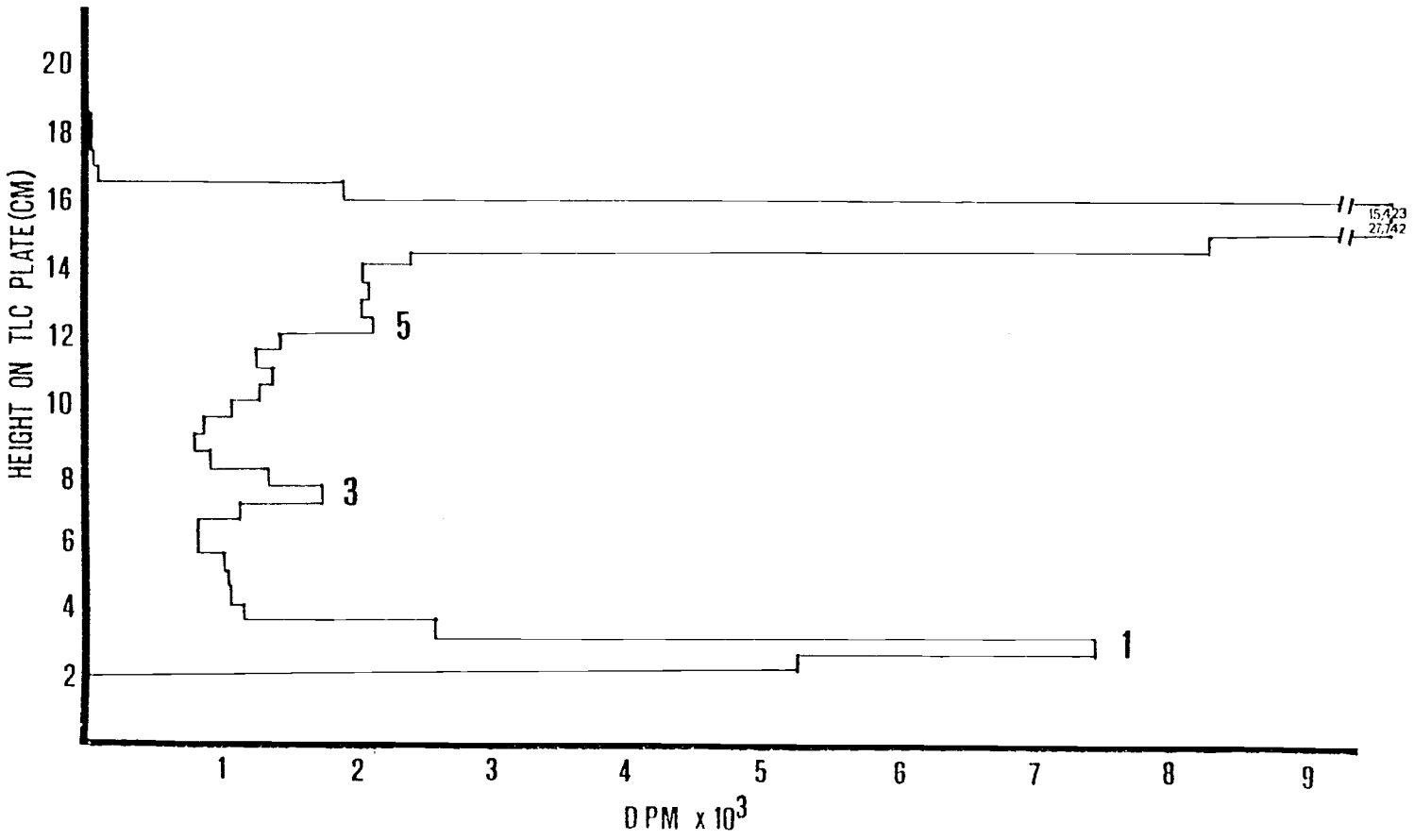


Figure 13. An example of the second TLC radioscan for the bacteria-free oyster multienzyme systems (sample I6).

Figure 14. The radioprofile of the ^{3}H-activity (in dpm x 10^{3}) associated with a height on the second TLC plate (in cm) for the buffer series (sample B1). All radioprofiles (Figures 19-38; 39-41) were derived from liquid scintillation counts of each 5-mm strip on the TLC plate (from 2 cm to 20 cm). The ^{3}H-BaP peak can be seen at the top of the plate. Peak #1 represents the ^{3}H-activity remaining at the origin. All other peaks of the simple non-conjugated ^{3}H-BaP derivatives are numbered (2 through 6).


HEIGHT ON TLC PLATE (CM)
20
18
16
14
12
10
8
6
4
2
1
2
3
4
5
6
7
8
9
DPM x 10^3
15,423
27,742
5
3
1

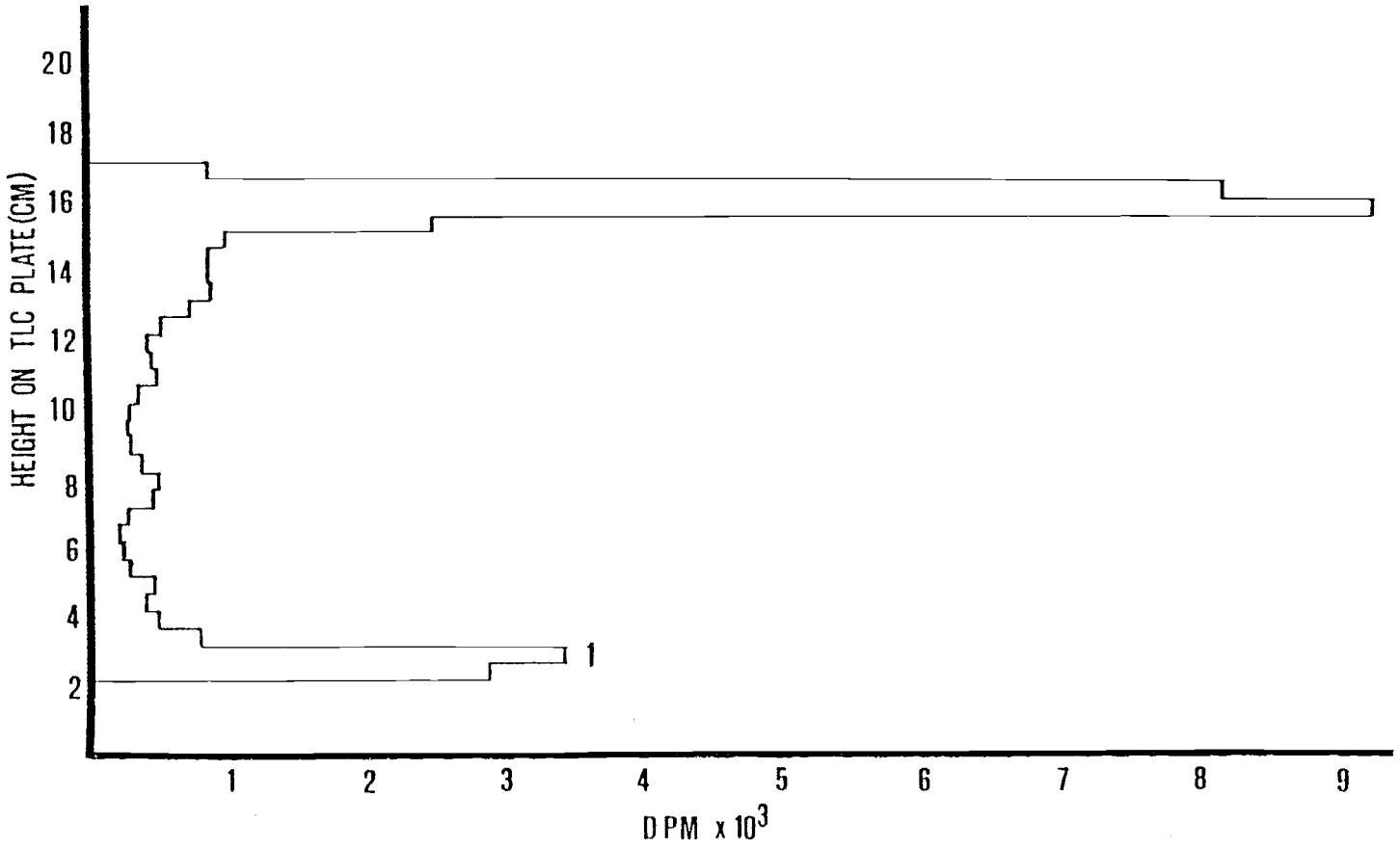


Figure 15. The radioprofile of the ^{3}H-activity (in dpm x 10^{3}) associated with a height on the second TLC plate (in cm) for the buffer series (sample B2).

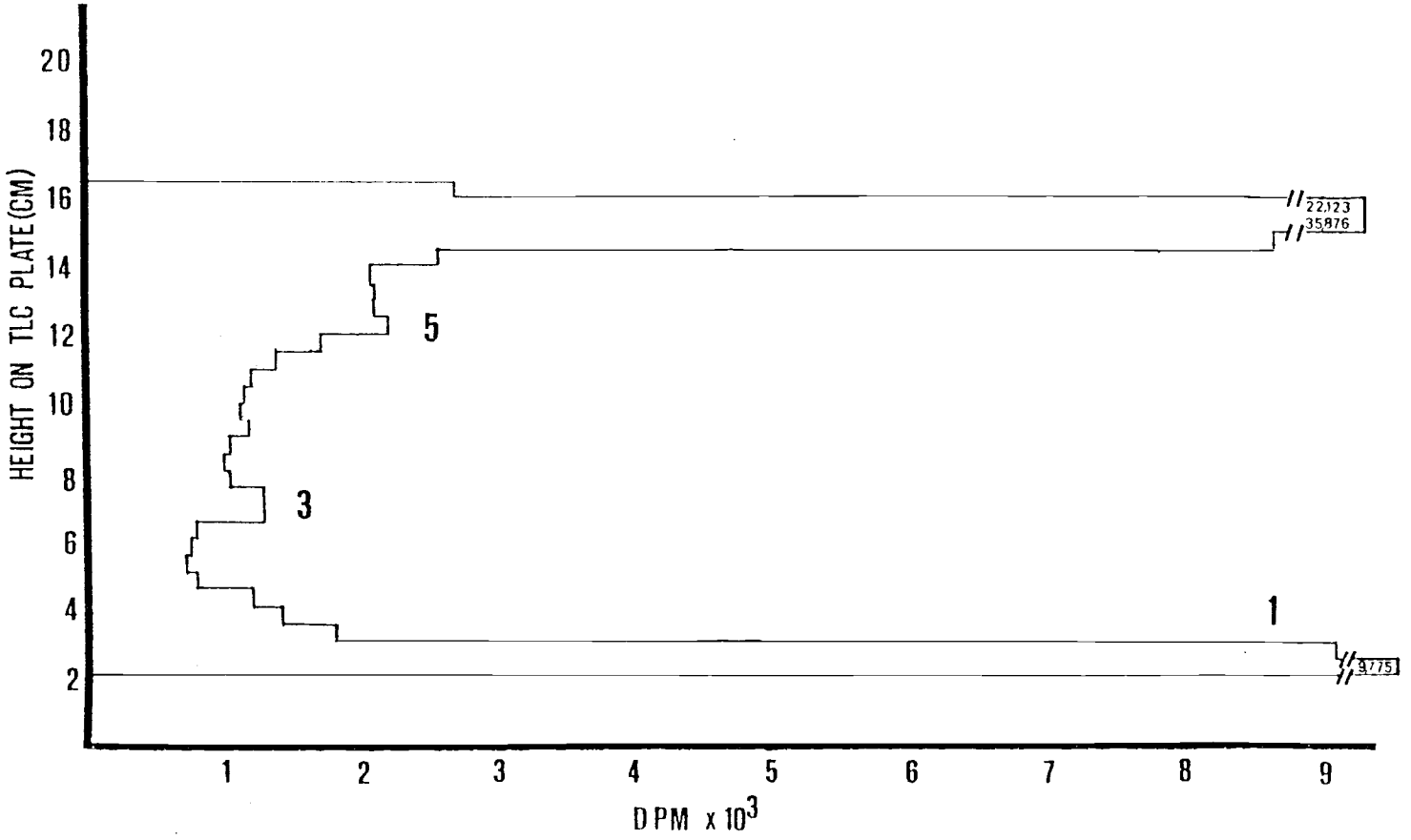


Figure 16. The radioprofile of the ^{3}H-activity (in dpm x 10^{3}) associated with a height on the second TLC plate (in cm) for the buffer + antibiotic series (sample BA1).

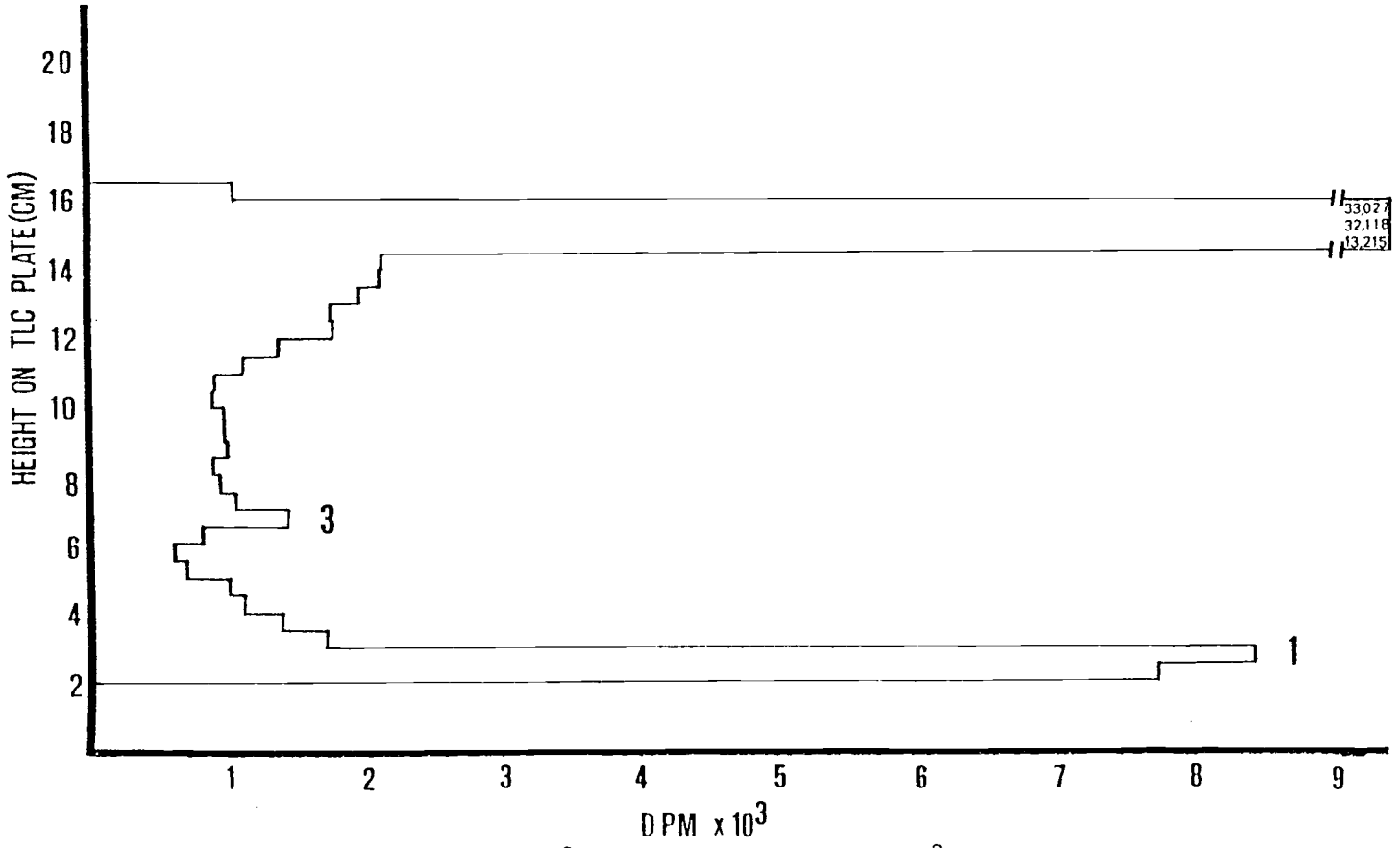


Figure 17. The radioprofile of the ^{3}H-activity (in dpm x 10^{3}) associated with a height on the second TLC plate (in cm) for the buffer + antibiotic series (sample BA2).

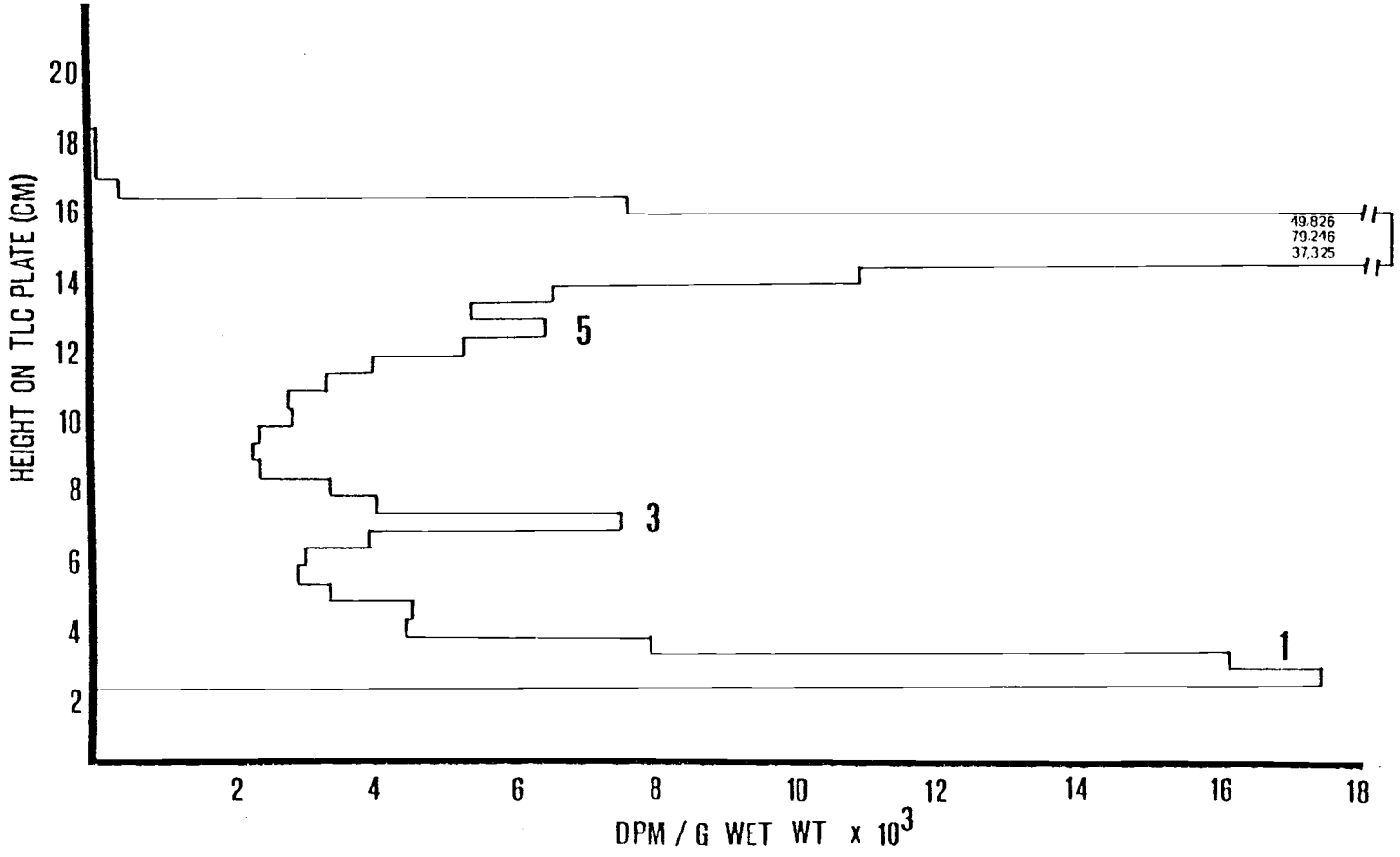


Figure 18. The radioprofile of the ^{3}H-activity (in dpm per g oyster wet wt x 10^{3}) associated with a height on the second TLC plate (in cm) for the background controls, the autoclaved oyster/bacteria systems (sample Aul).

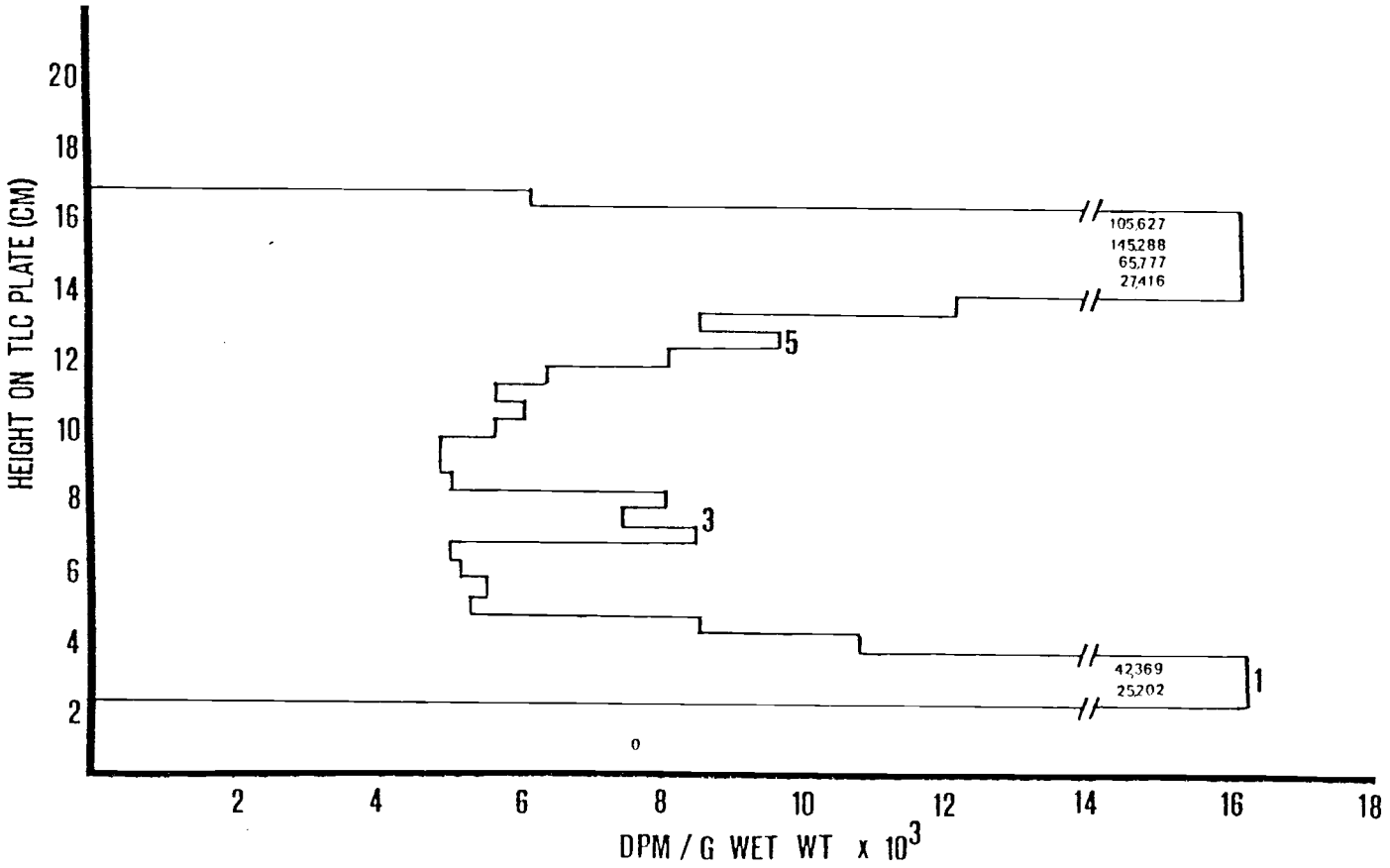


Figure 19. The radioprofile of the ^{3}H-activity (in dpm per g oyster wet wt x 10^{3}) associated with a height on the second TLC plate (in cm) for the background controls, the autoclaved oyster/bacteria systems (sample Au2).

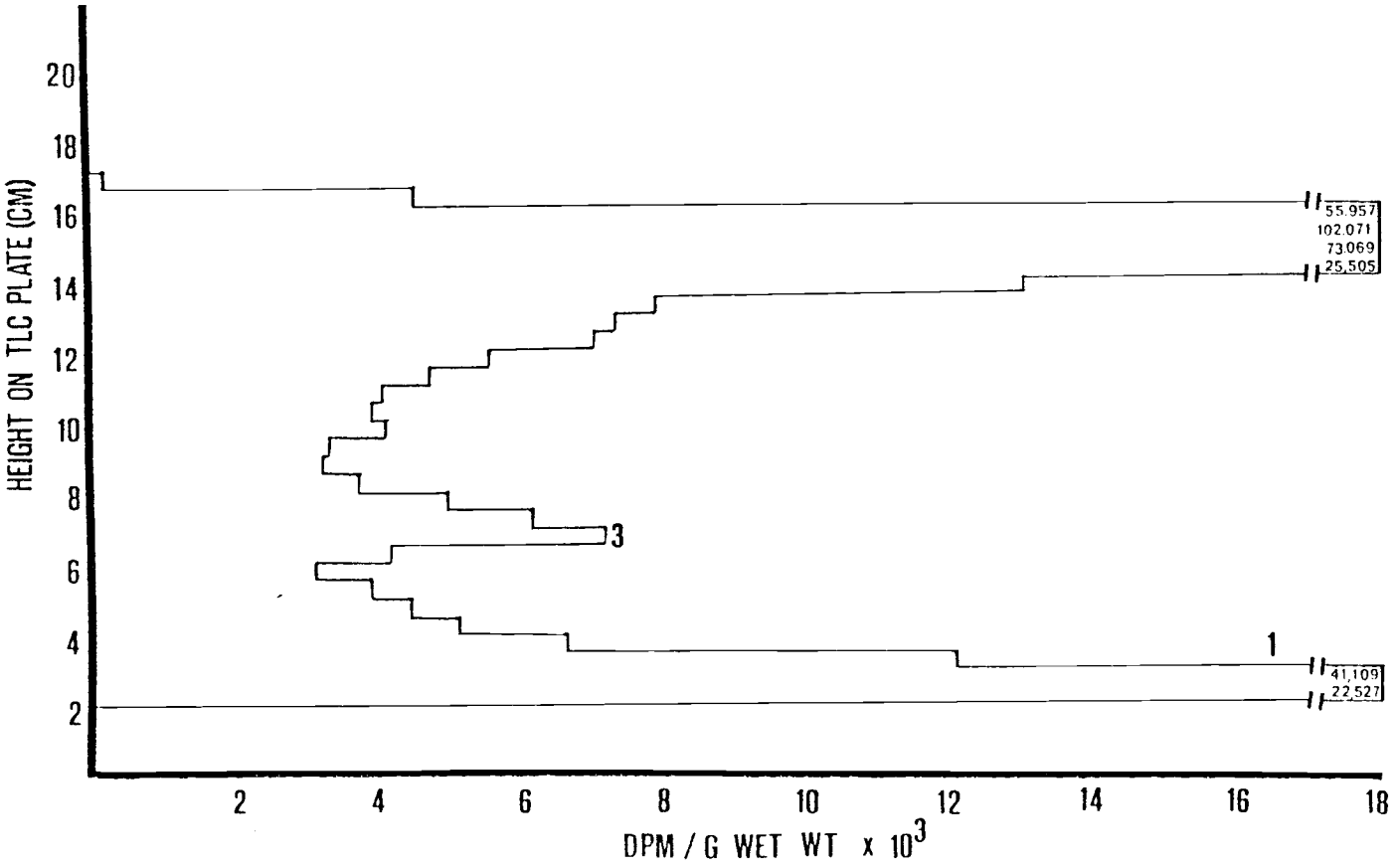


Figure 20. The radioprofile of the ^{3}H-activity (in dpm per g oyster wet wt x 10^{3}) associated with a height on the second TLC plate (in cm) for the background controls, the autoclaved oyster/bacteria systems (sample Au3).

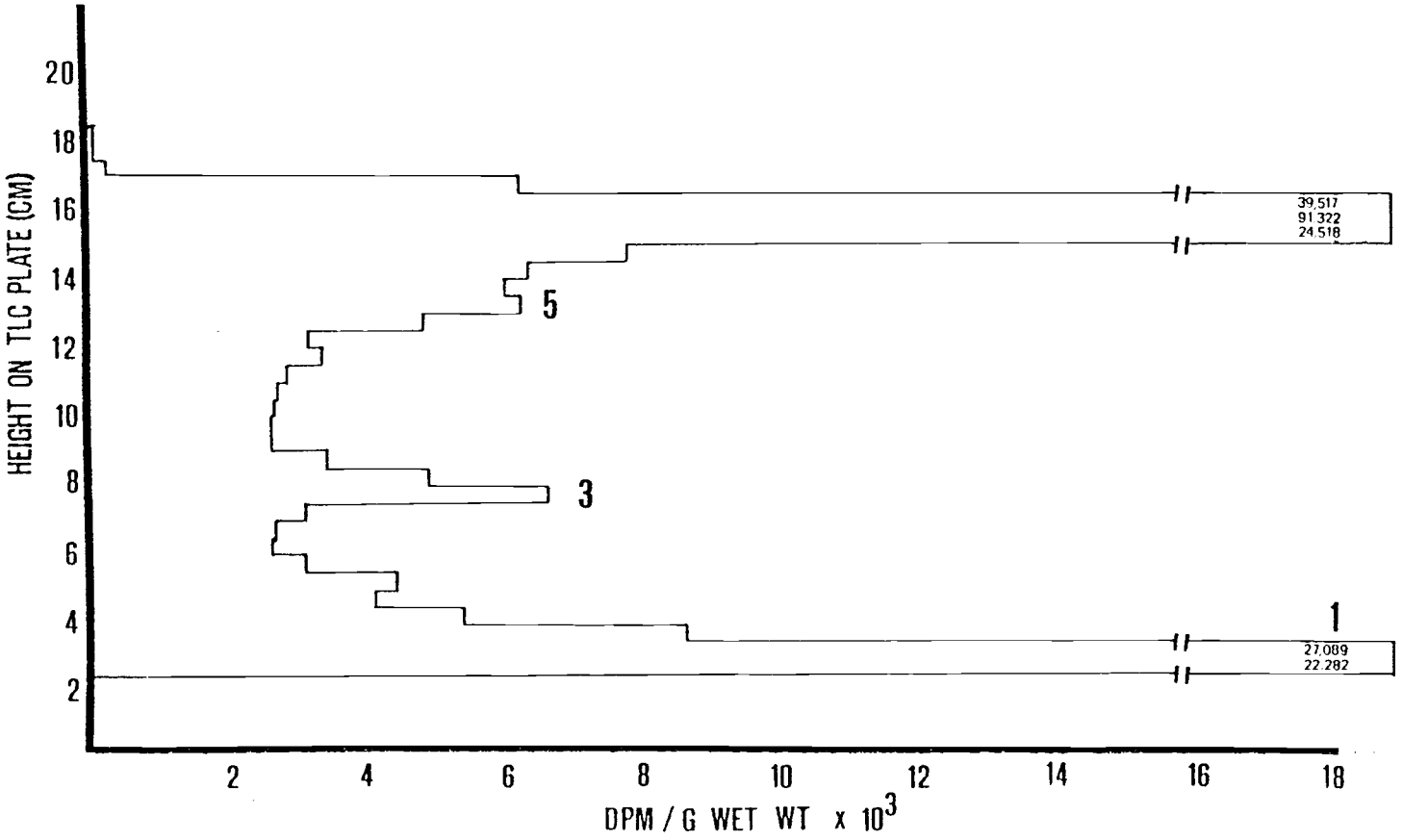


Figure 21. The radioprofile of the ^{3}H-activity (in dpm per g oyster wet wt x 10^{3}) associated with a height on the second TLC plate (in cm) for the background controls, the autoclaved oyster/bacteria systems (sample Au4).

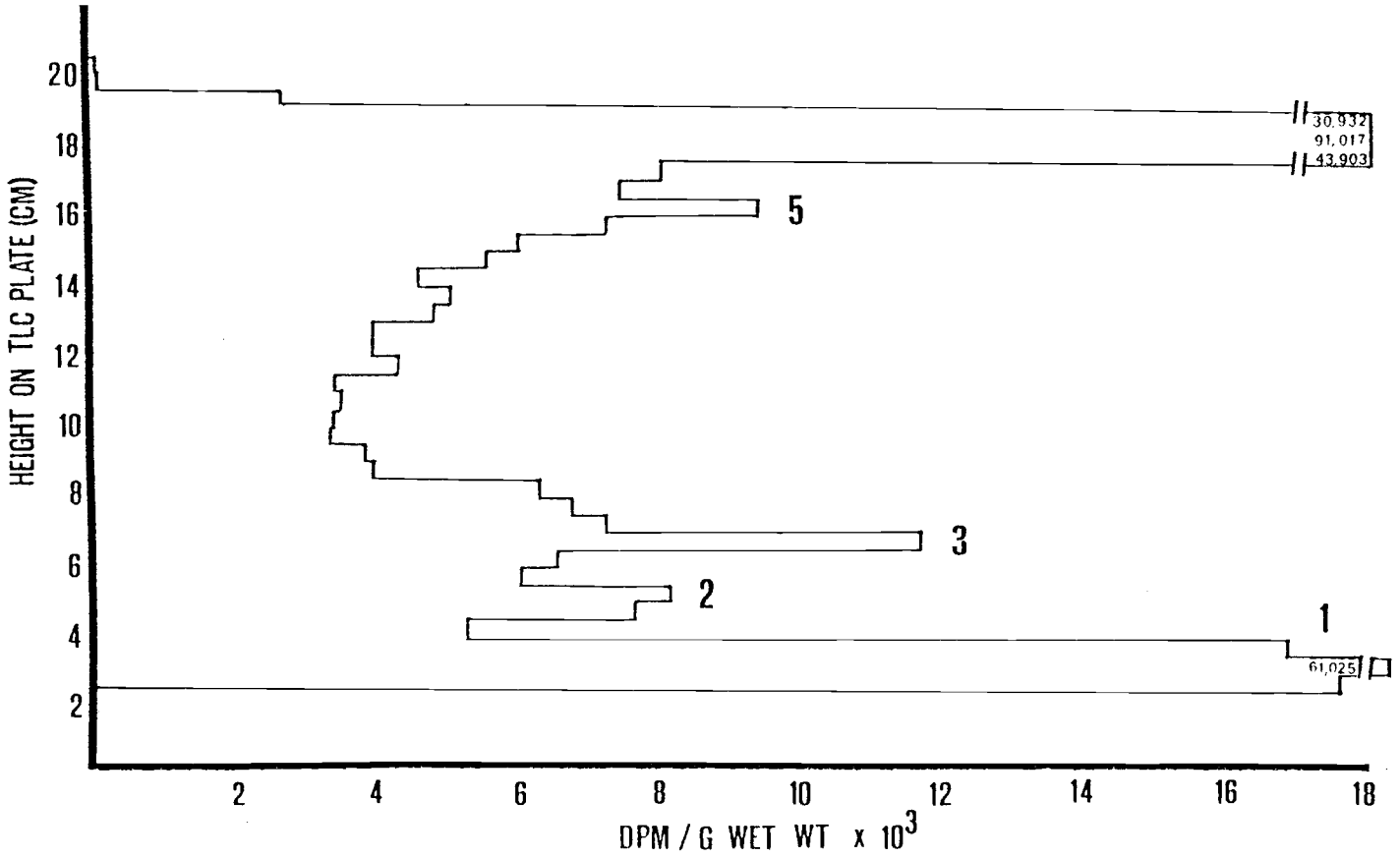


Figure 22. The radioprofile of the ^{3}H-activity (in dpm per g oyster wet wt x 10^3) associated with a height on the second TLC plate (in cm) for the oyster/bacteria multienzyme systems (sample C1).

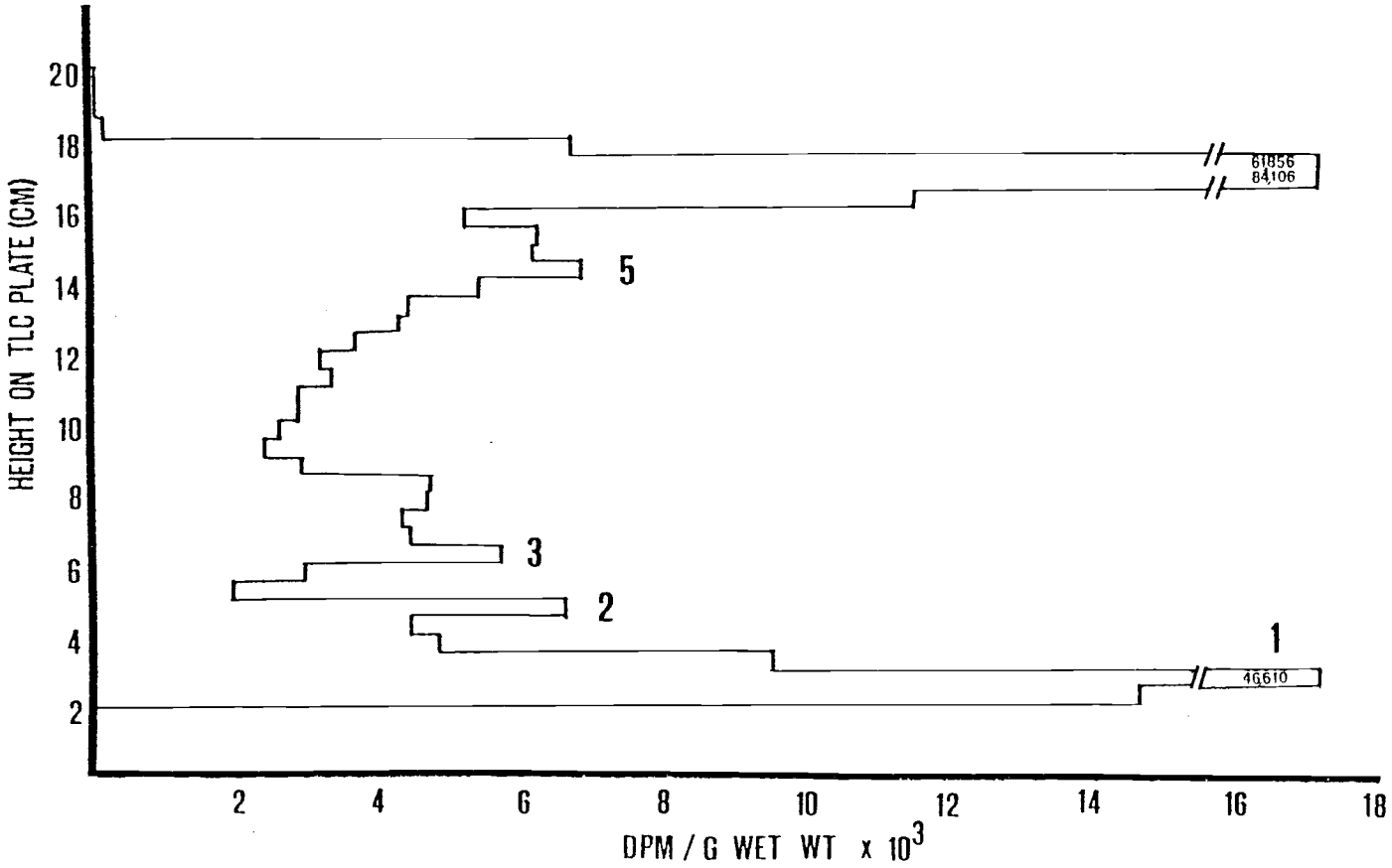


Figure 23. The radioprofile of the ^{3}H-activity (in dpm per g oyster wet wt x 10^{3}) associated with a height on the second TLC plate (in cm) for the oyster/bacteria multienzyme systems (Sample C2).

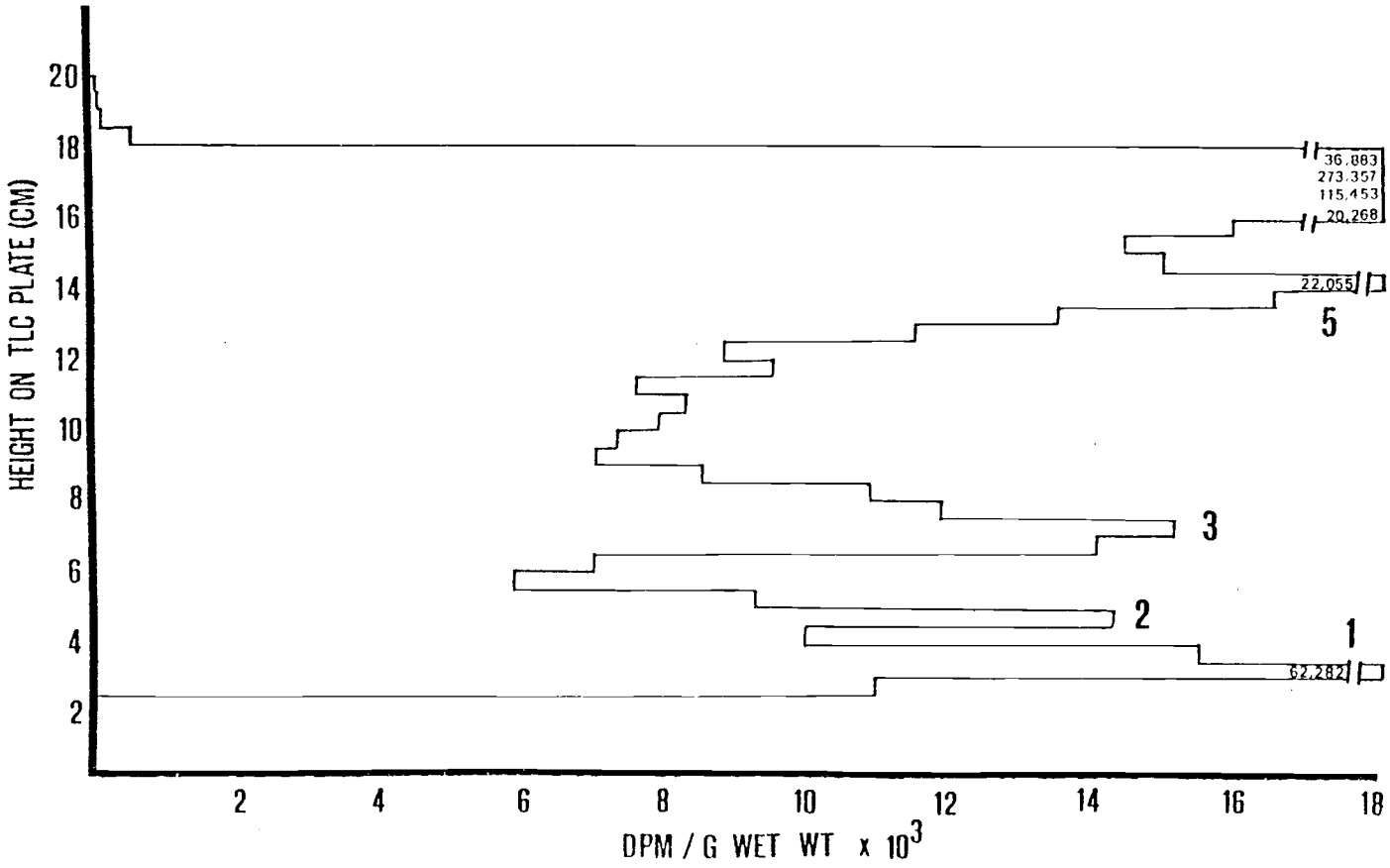


Figure 24. The radioprofile of the ^{3}H-activity (in dpm per g oyster wet wt x 10^3) associated with a height on the second TLC plate (in cm) for the oyster/bacteria multienzyme systems (sample C3).

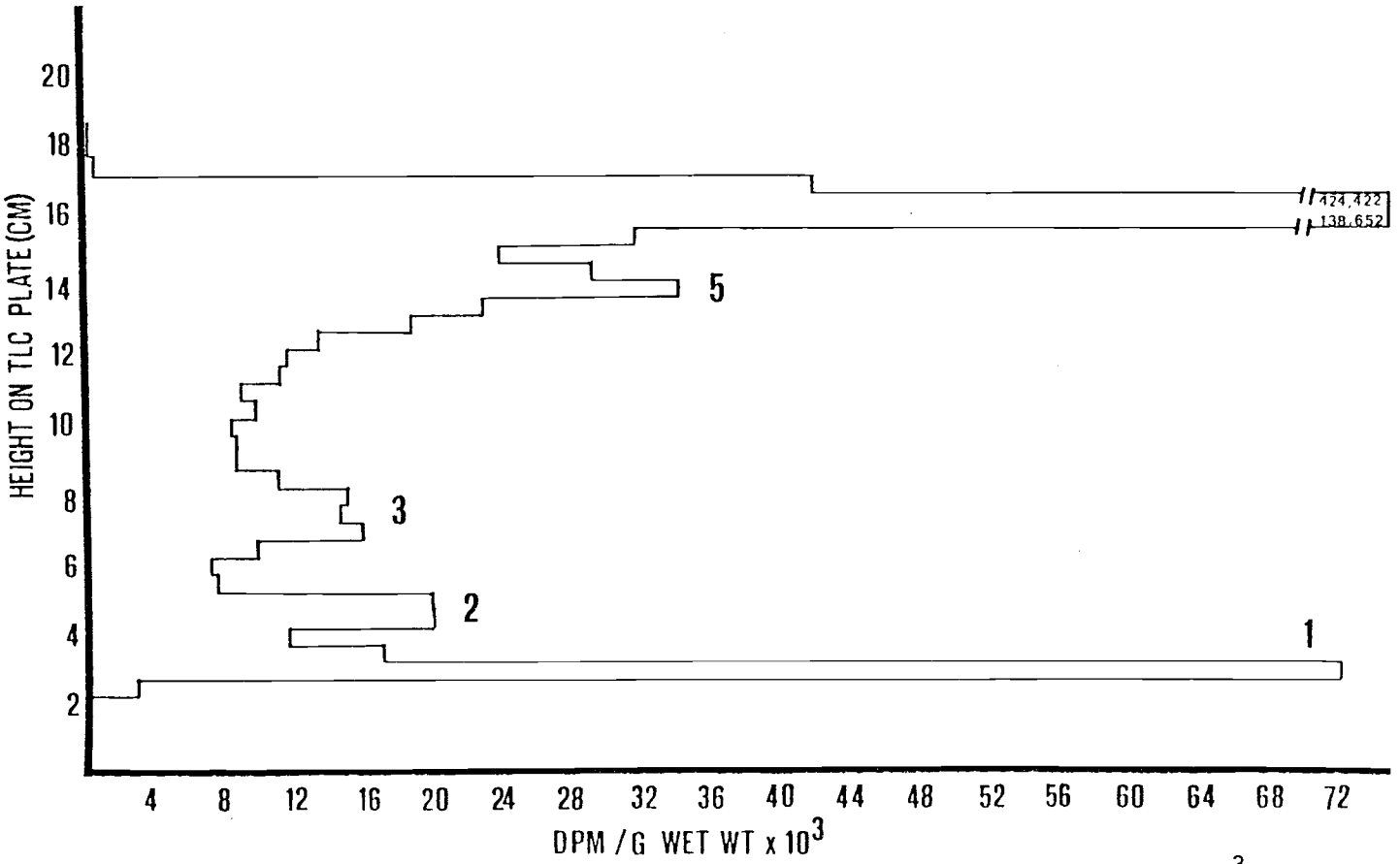


Figure 25. The radioprofile of the ^{3}H-activity (in dpm per g oyster wet wt x 10^{3}) associated with a height on the second TLC plate (in cm) for the oyster/bacteria multienzyme systems (sample C4).

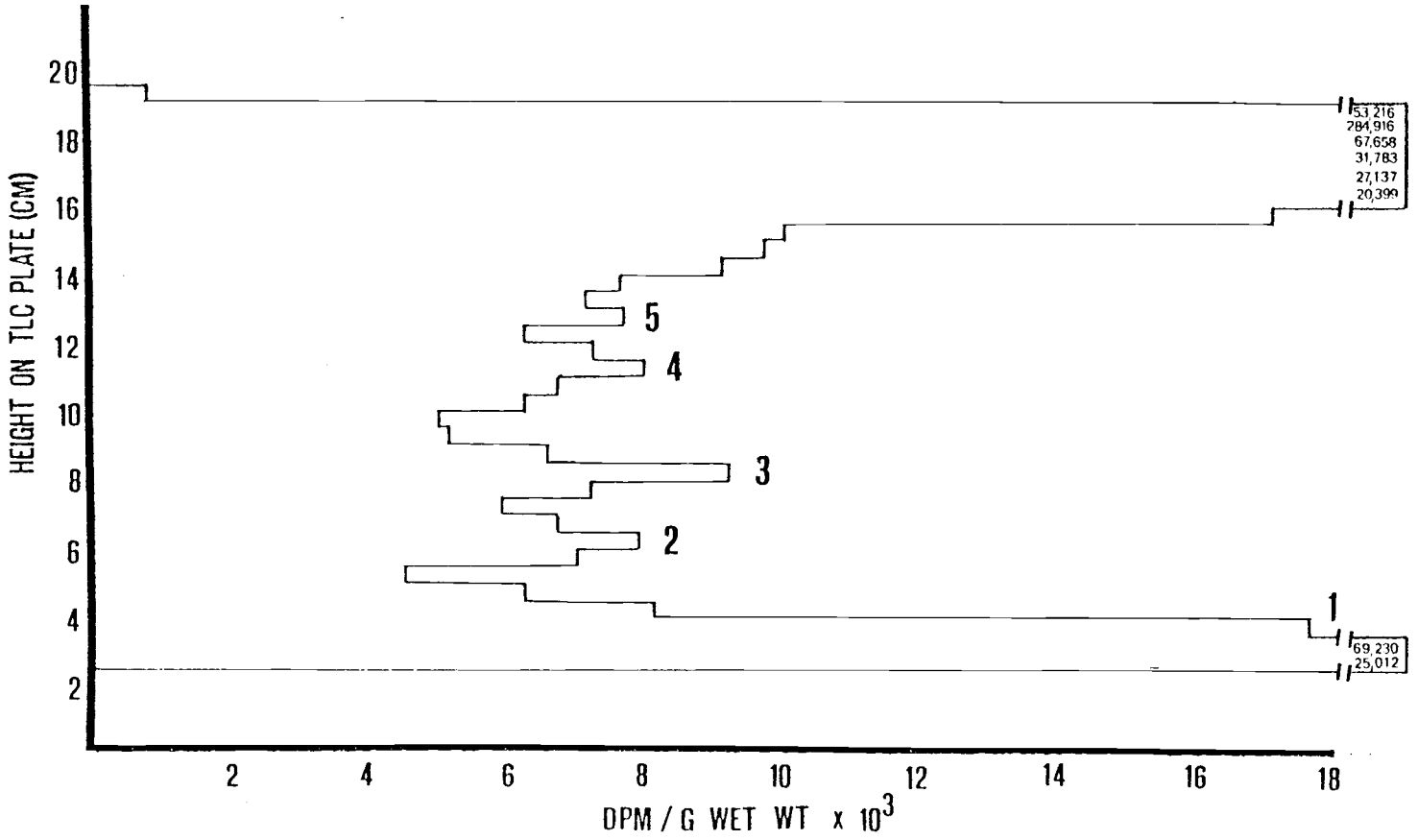


Figure 26. The radioprofile of the ^{3}H-activity (in dpm per g oyster wet wt x 10^3) associated with a height on the second TLC plate (in cm) for the oyster/bacteria multienzyme systems (sample C5).

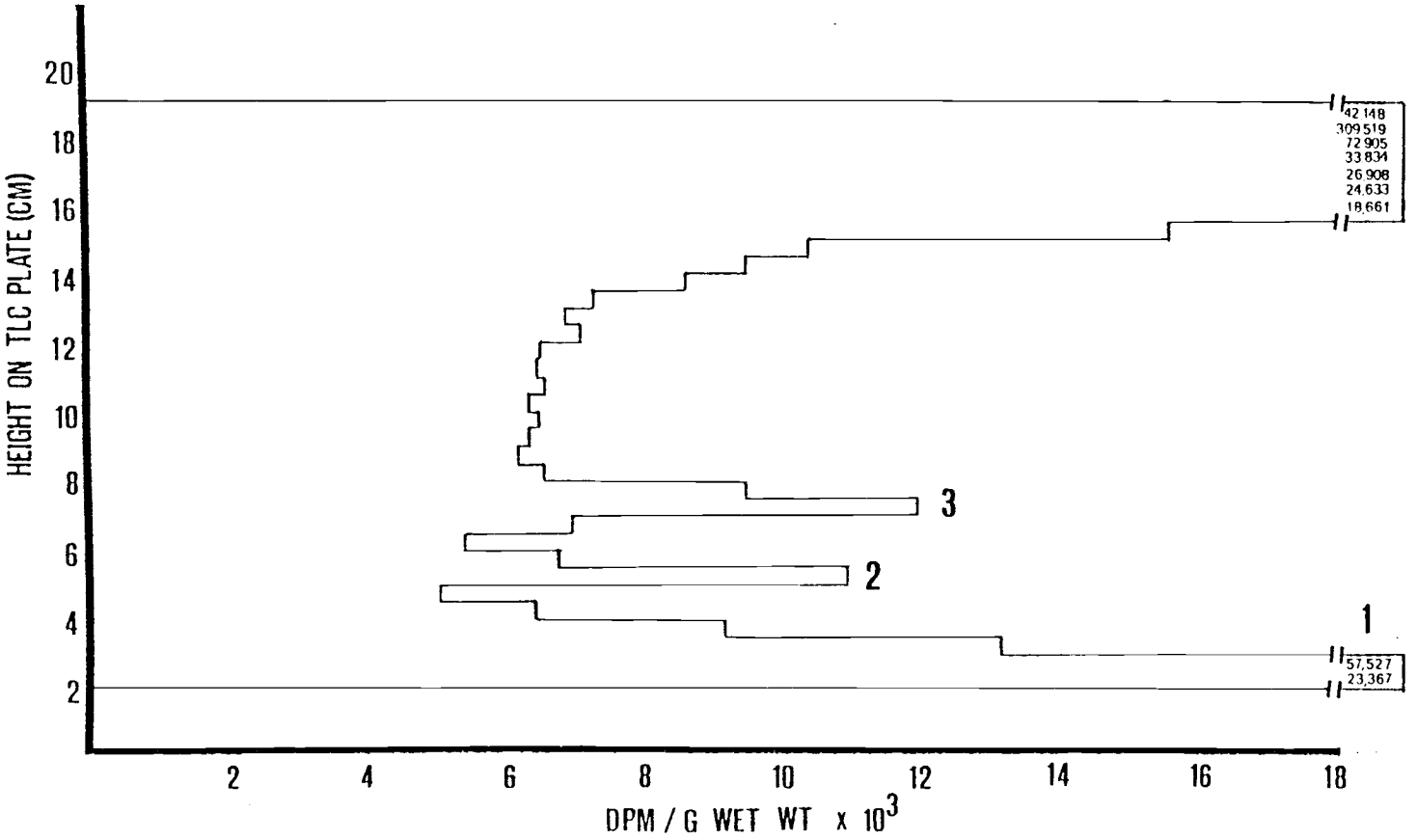


Figure 27. The radioprofile of the ^{3}H-activity (in dpm per g oyster wet wt x 10^{3}) associated with a height on the second TLC plate (in cm) for the oyster/bacteria multienzyme systems (sample C6).

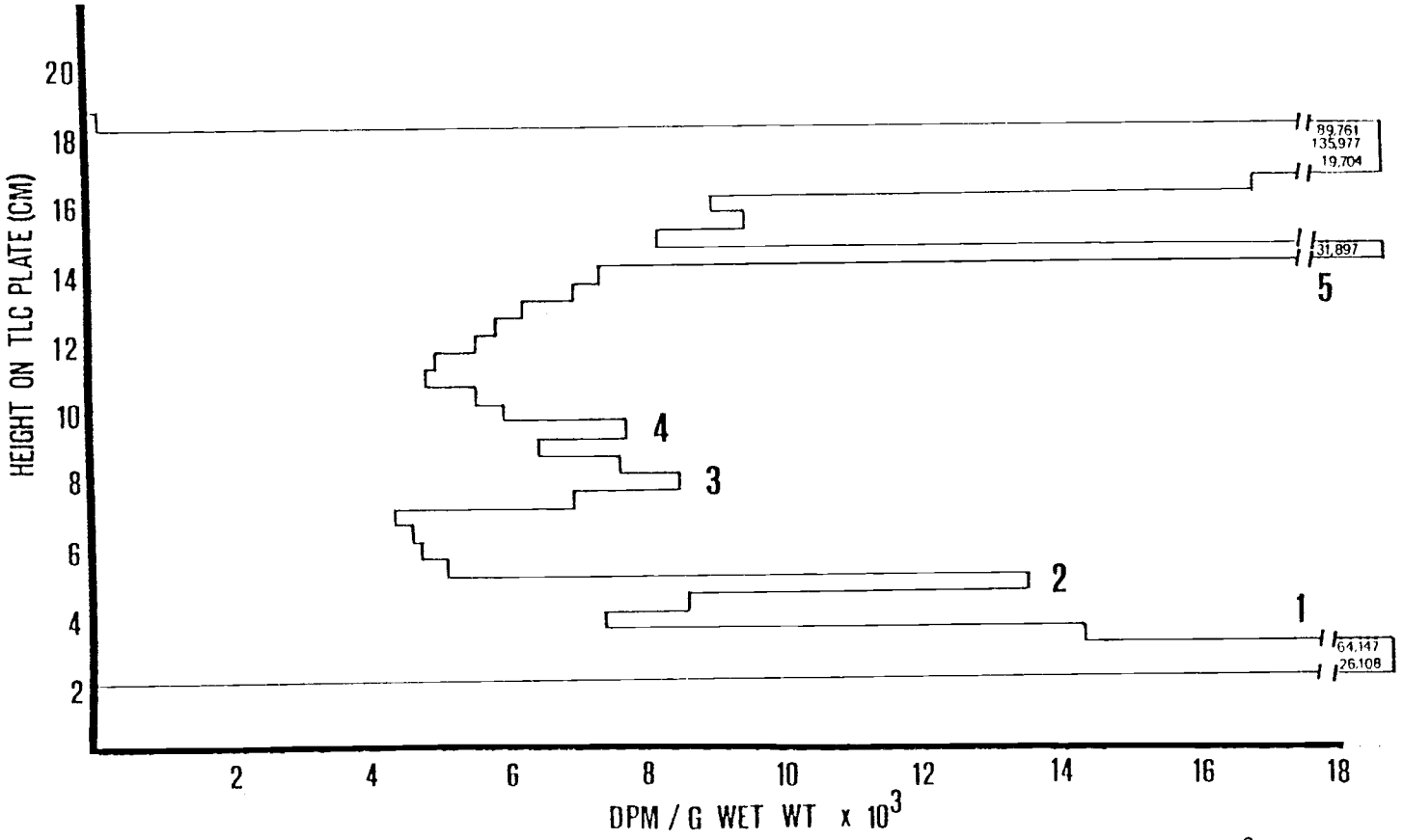


Figure 28. The radioprofile of the ^{3}H-activity (in dpm per g oyster wet wt x 10^3) associated with a height on the second TLC plate (in cm) for the oyster/bacteria multienzyme systems (sample I2).

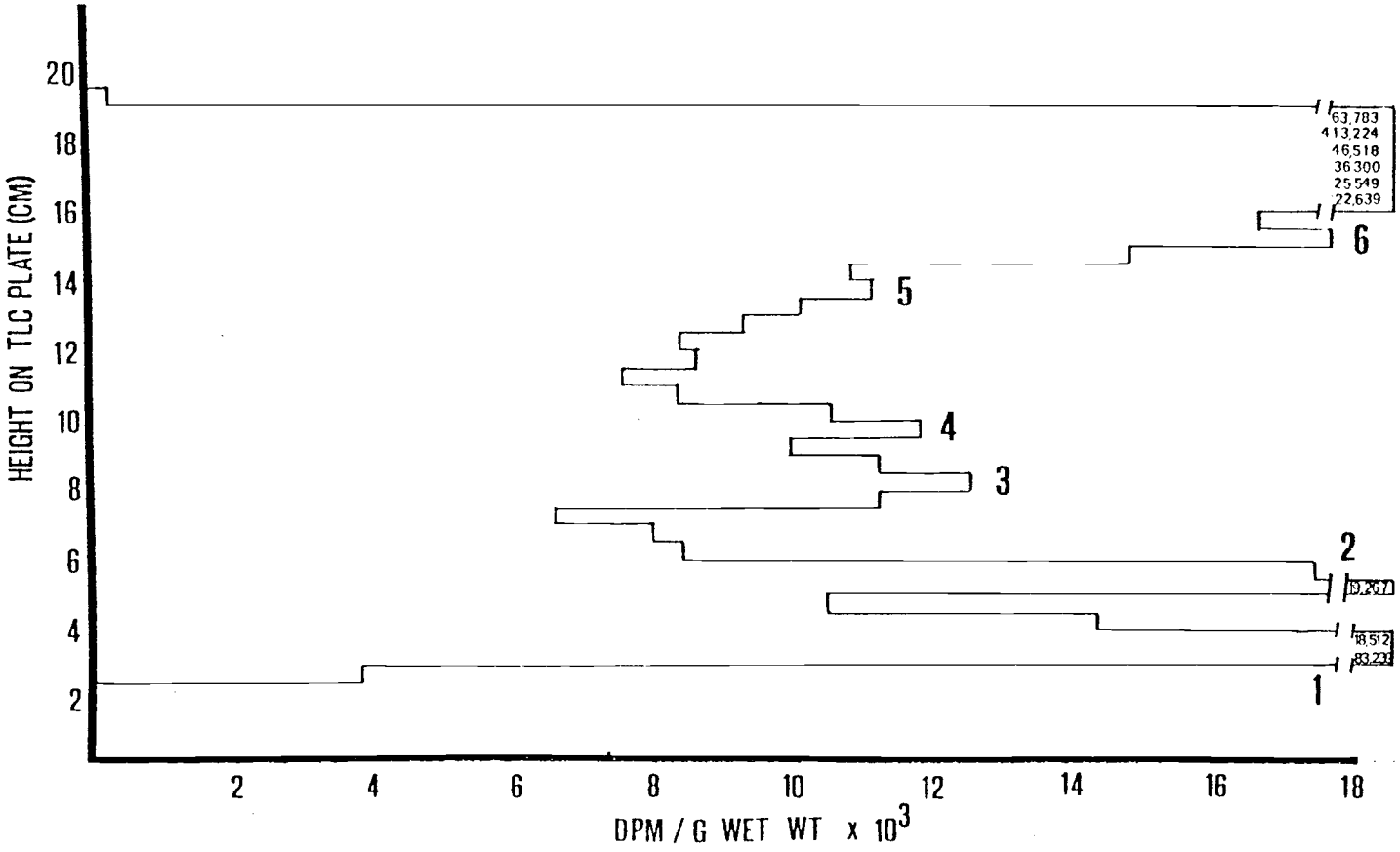


Figure 29. The radioprofile of the ^{3}H-activity (in dpm per g oyster wet weight x 10^{3}) associated with a height on the second TLC plate (in cm) for the oyster/bacteria multienzyme systems (sample I3).

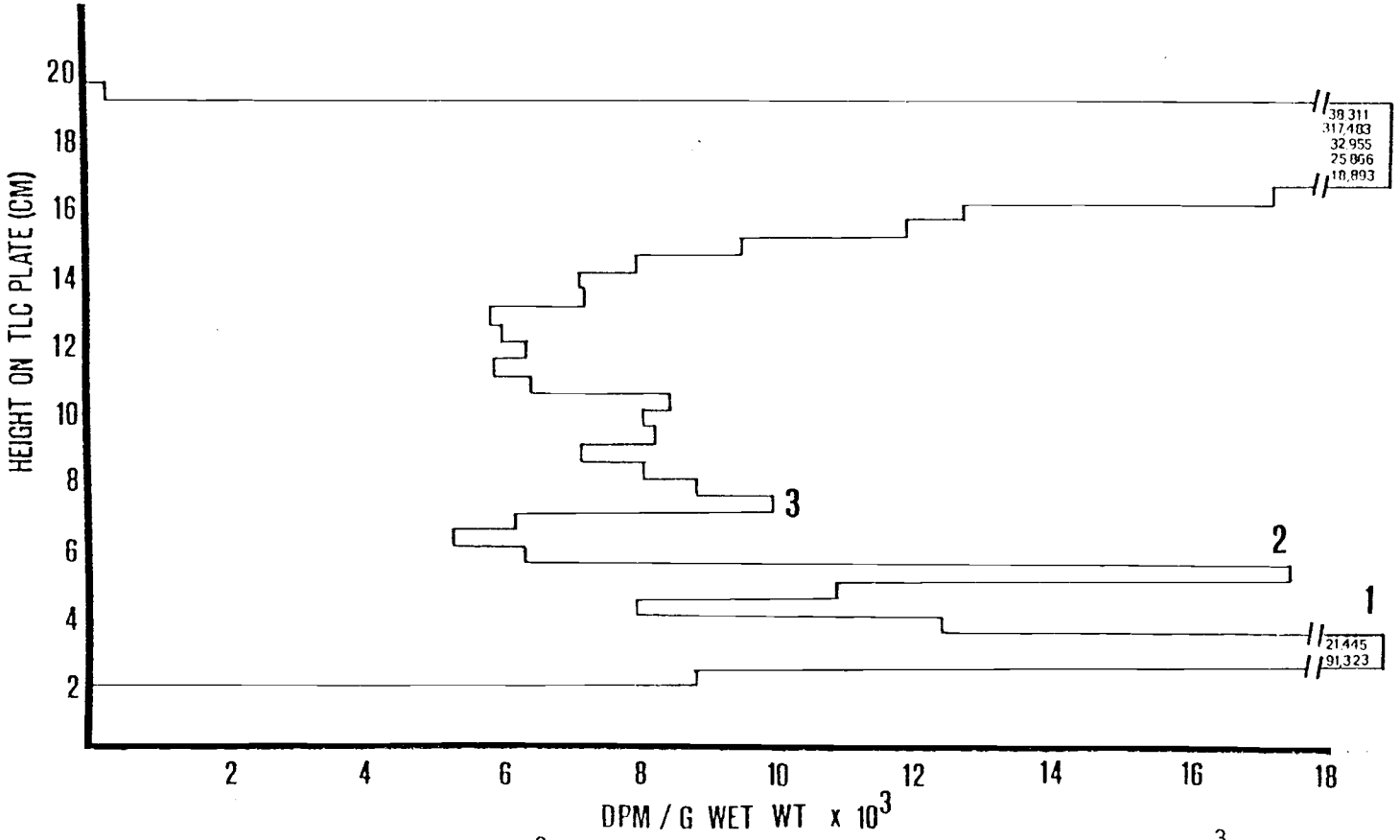


Figure 30. The radioprofile of the ^{3}H-activity (in dpm per g oyster wet wt x 10^{3}) associated with a height on the second TLC plate (in cm) for the oyster/bacteria multienzyme systems (sample I4).

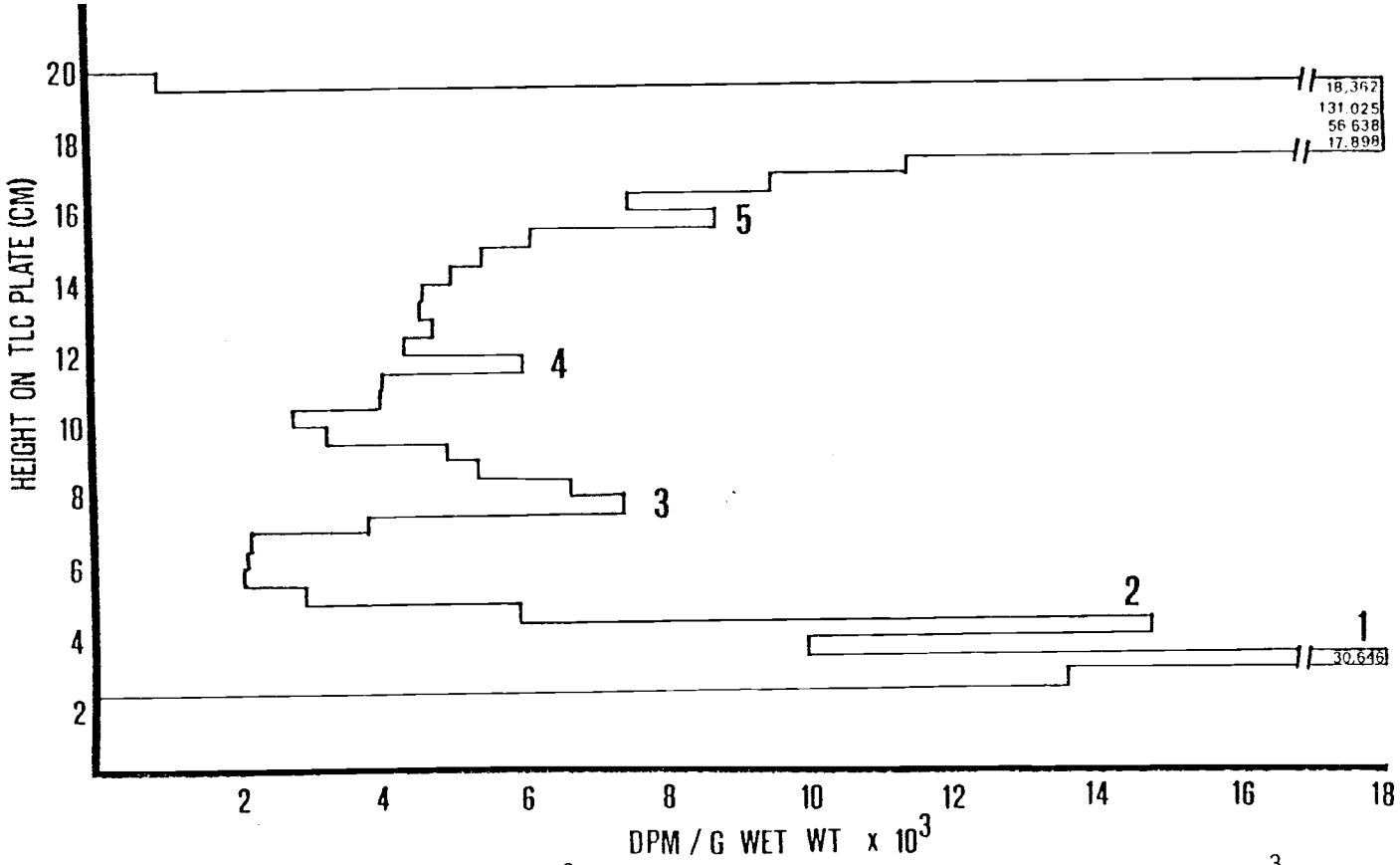


Figure 31. The radioprofile of the ^{3}H-activity (in dpm per g oyster wet wt x 10^{3}) associated with a height on the second TLC plate (in cm) for the bacteria-free oyster multi-enzyme systems (sample A1).

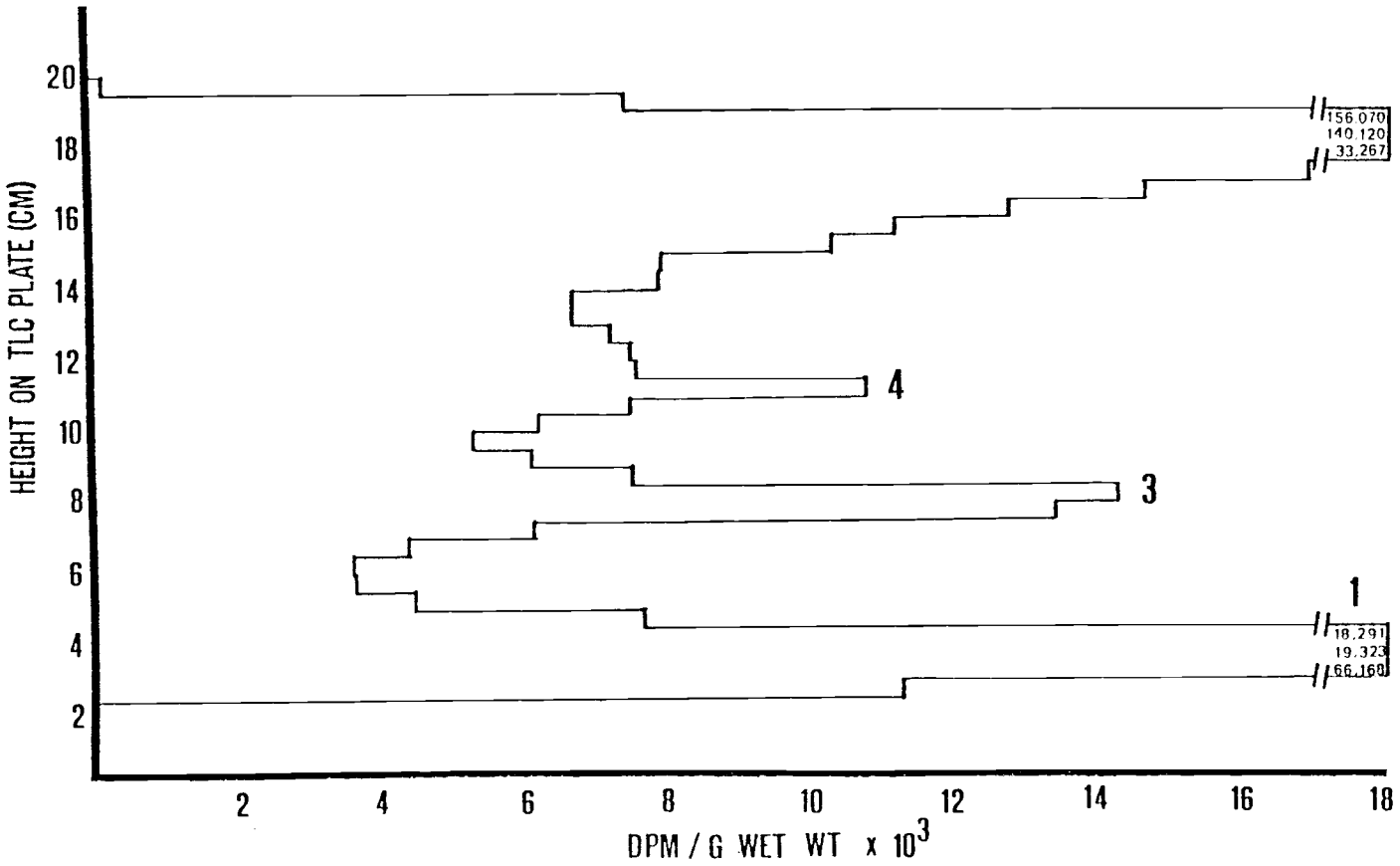


Figure 32. The radioprofile of the ^{3}H-activity (in dpm per g oyster wet wt x 10^{3}) associated with a height on the second TLC plate (in cm) for the bacteria-free oyster multi-enzyme systems (sample A2).

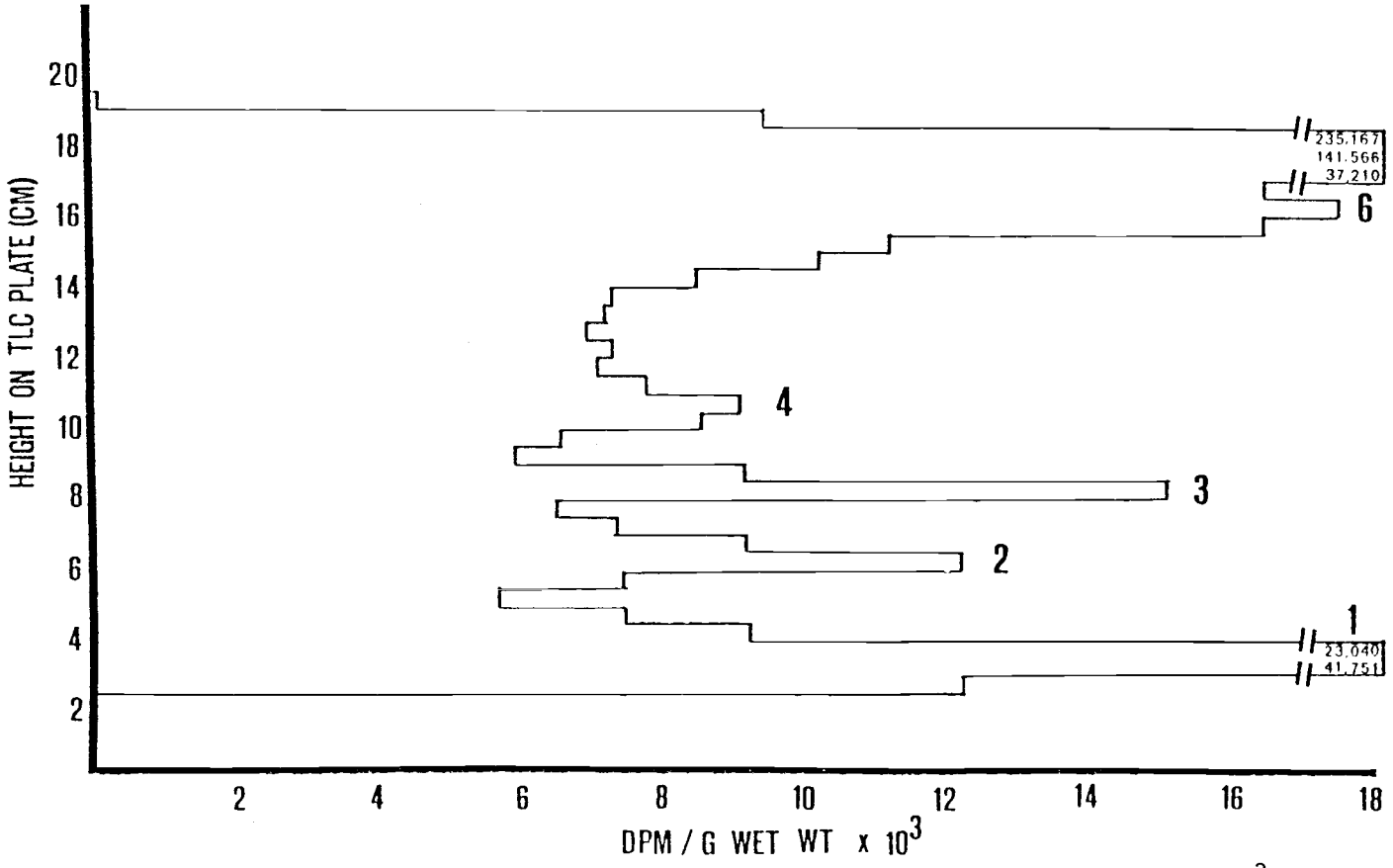


Figure 33. The radioprofile of the ^{3}H-activity (in dpm per g oyster wet wt x 10^3) associated with a height on the second TLC plate (in cm) for the bacteria-free oyster multi-enzyme systems (sample A3).

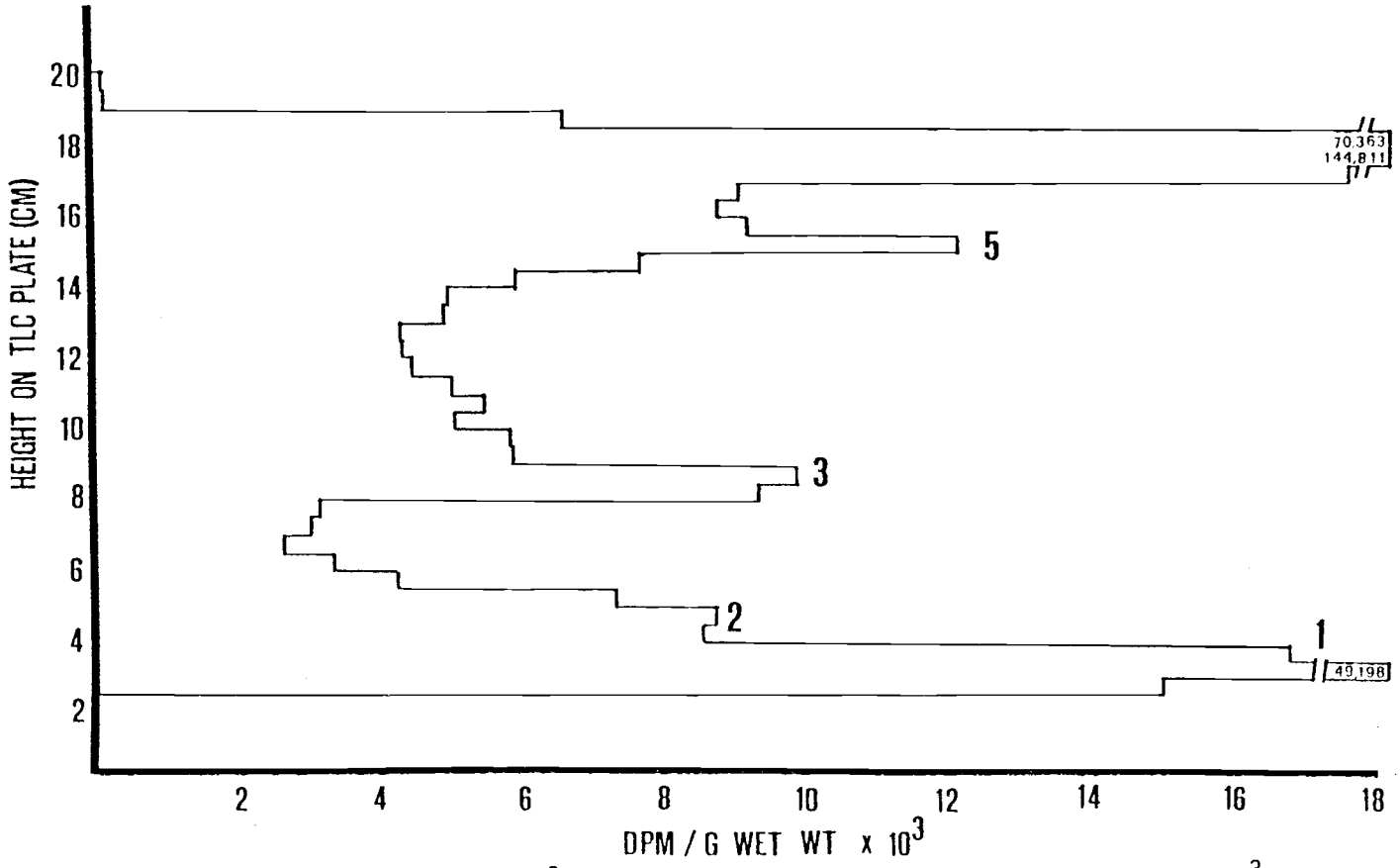


Figure 34. The radioprofile of the ^{3}H-activity (in dpm per g oyster wet wt x 10^3) associated with a height on the second TLC plate (in cm) for the bacteria-free oyster multi-enzyme systems (sample A4).

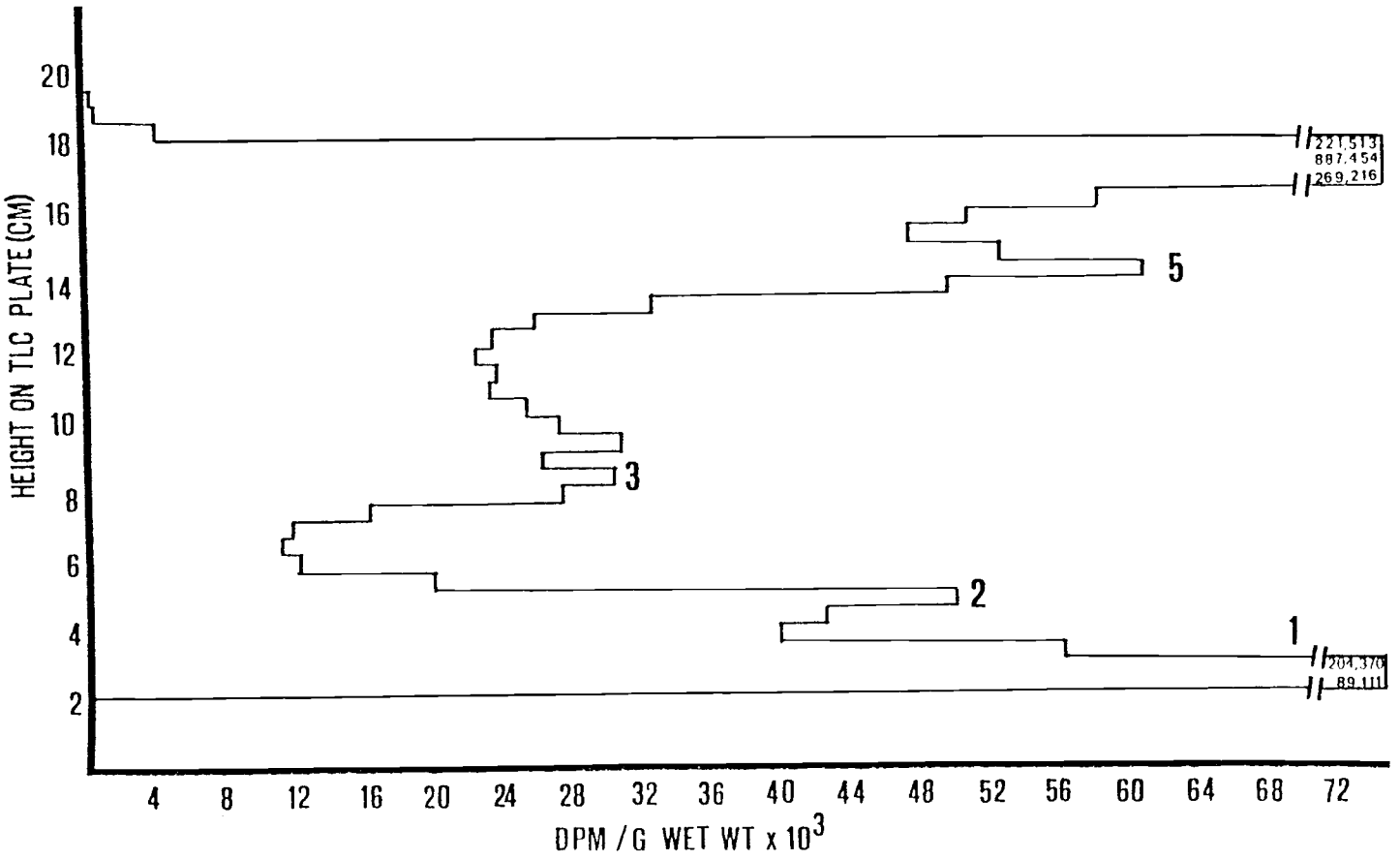


Figure 35. The radioprofile of the ^{3}H-activity (in dpm per g oysters wet wt x 10^{3}) associated with a height on the second TLC plate (in cm) for the bacteria-free oyster multi-enzyme systems (sample A5).

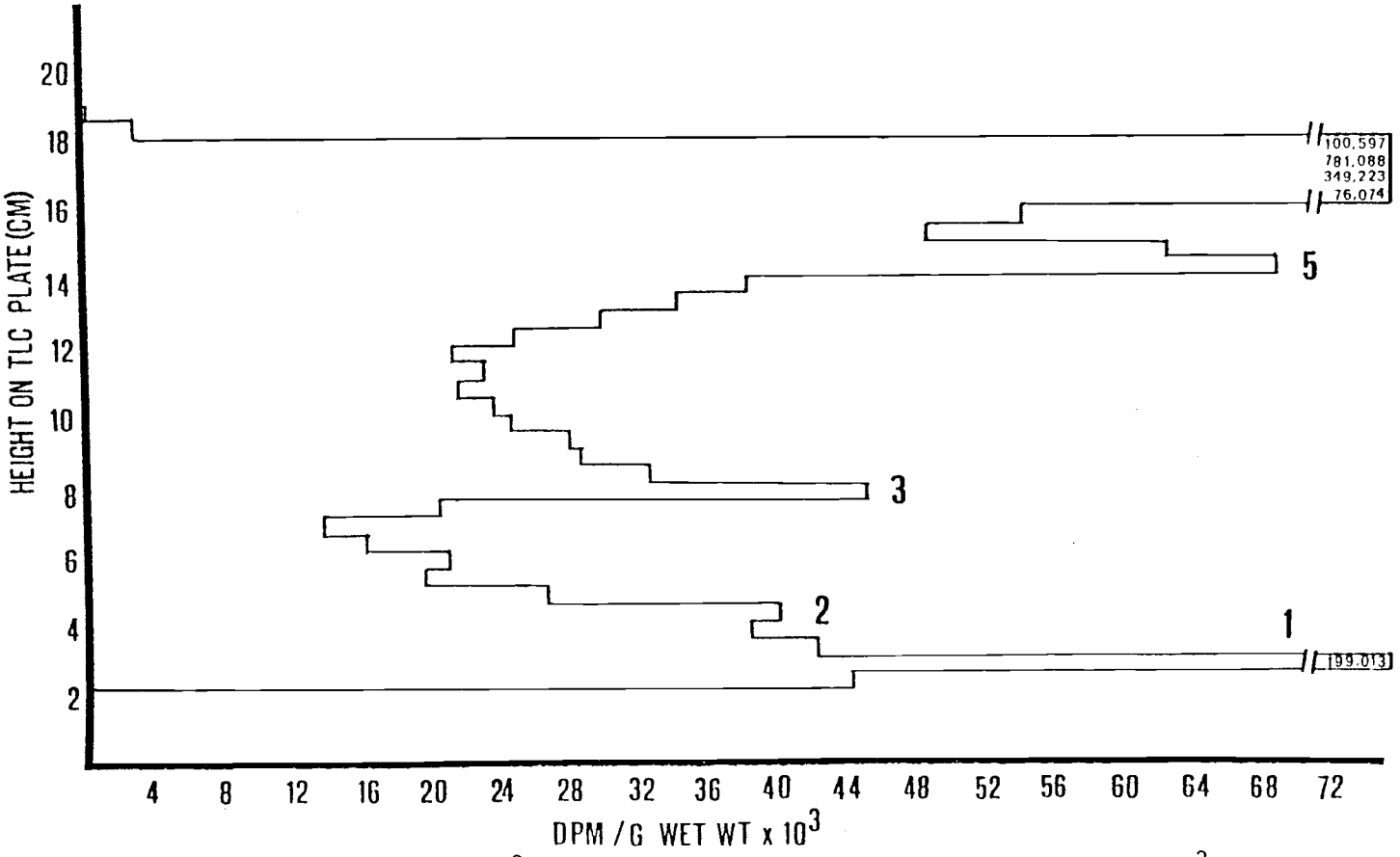


Figure 36. The radioprofile of the ^{3}H-activity (in dpm per g oyster wet wt x 10^{3}) associated with a height on the second TLC plate (in cm) for the bacteria free oyster multi-enzyme systems (sample A6).

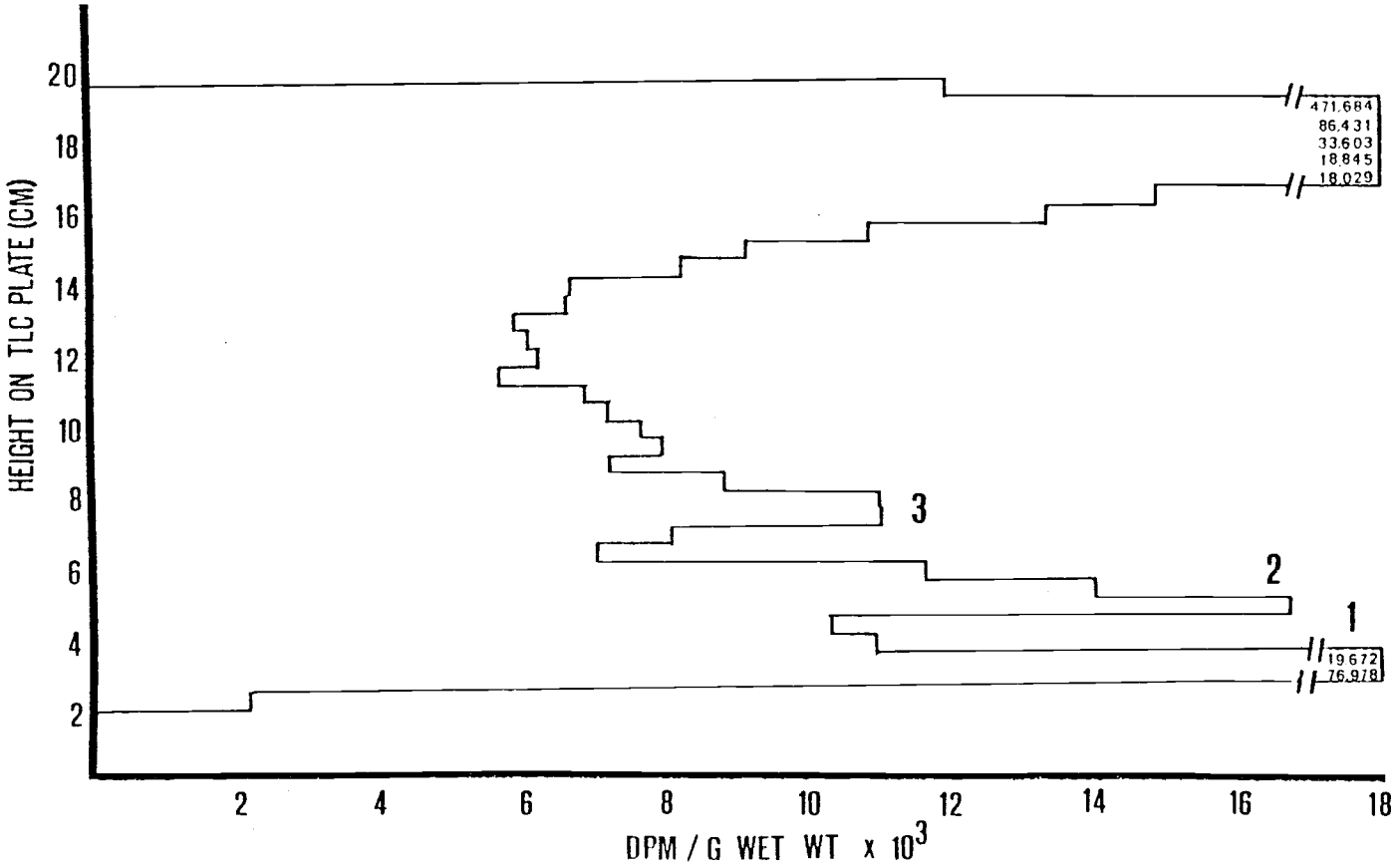


Figure 37. The radioprofile of the ^{3}H-activity (in dpm per g oyster wet wt x 10^{3}) associated with a height on the second TLC plate (in cm) for the bacteria-free oyster multi-enzyme systems (sample I5).

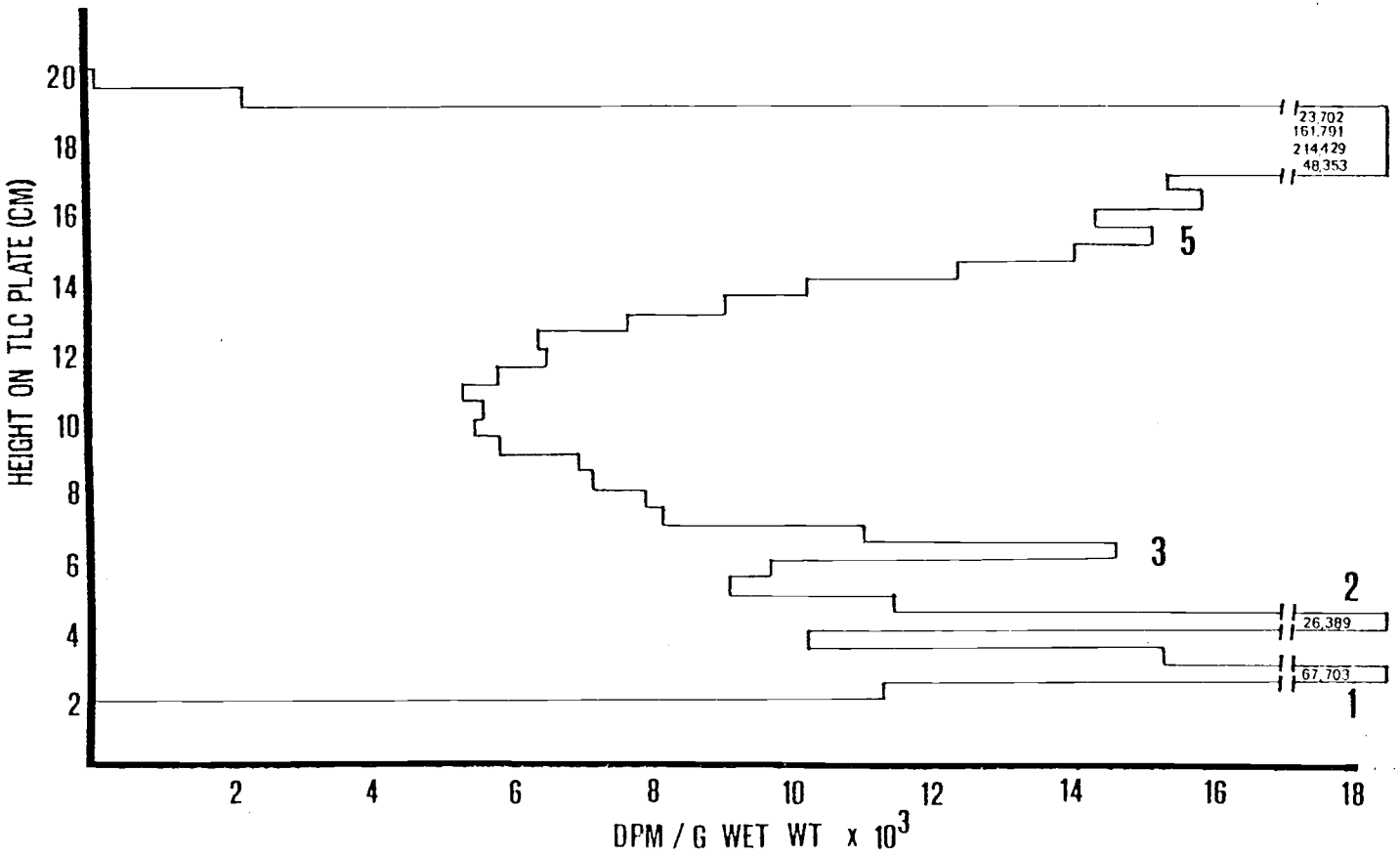


Figure 38. The radioprofile of the ^{3}H-activity (in dpm per g oyster wet wt x 10^{3}) associated with a height on the second TLC plate (in cm) for the bacteria-free oyster multi-enzyme systems (sample I6).

Table 8. Comparison of the ^{3}H-BaP-simple non-conjugated derivatives separated by TLC. Peak designations are shown with the corresponding Rf values and ^{3}H-activities.

Sample		Rf's	DPM's in peak	Peak designation	$\frac{\mu Ci \text{ in peak}}{\mu Ci \text{ total OEl}}$ /g wet wt
Buffer series	B1	0.0000	13,499	#1	2.8970×10^{-3}
		0.4070	1,864	3	$.4000 \times 10^{-3}$
		0.7019	1,330	5	$.2854 \times 10^{-3}$
	B2	0.0000	6,345	#1	1.2933×10^{-3}
	BA1	0.0000	21,339	#1	4.8571×10^{-3}
		0.3026	762	3	$.1734 \times 10^{-3}$
		0.6449	725	5	$.1650 \times 10^{-3}$
	BA2	0.0000	17,018	#1	4.4845×10^{-3}
		0.2391	866	3	$.2282 \times 10$
Autoclaved oyster/ bacteria series	Au1	0.000	34,678	#1	11.9158×10^{-3}
		0.2468	8,081	3	2.7767×10^{-3}
		0.6429	3,082	5	1.0590×10^{-3}
	Au2	0.0000	93,266	#1	47.8041×10^{-3}
		0.2744	8,974	3	4.5997×10^{-3}
		0.6075	2,838	5	1.4547×10^{-3}
	Au3	0.0000	72,124	#1	43.7749×10^{-3}
		0.2950	10,468	3	6.3534×10^{-3}
	Au4	0.0000	55,196	#1	16.4578×10^{-3}
		0.2509	7,452	3	2.2222×10^{-3}
		0.6182	1,412	5	$.4210 \times 10$
Oyster/ bacteria multi-enzyme system	C1	0.0000	87,692	#1	39.9875×10^{-3}
		0.1392	6,117	2	2.7893×10^{-3}
		0.1917	17,418	3	7.9425×10^{-3}
		0.7596	3,216	5	1.4664×10^{-3}
	C2	0.0000	66,804	#1	42.8940×10^{-3}
		0.0760	4,660	2	2.9921×10^{-3}
		0.2374	9,094	3	5.8391×10^{-3}
		0.7035	5,437	5	3.4910×10^{-3}
	C3	0.0000	73,765	#1	47.0199×10^{-3}
		0.0349	7,698	2	4.9069×10^{-3}
		0.2602	25,578	3	16.3040×10^{-3}
		0.6627	15,155	5	9.6602×10^{-3}
	C4	0.0000	76,589	#1	37.6859×10^{-3}
		0.0793	20,684	2	10.1780×10^{-3}
		0.2726	27,345	3	13.4551×10^{-3}
		0.6201	22,298	5	10.9716×10^{-3}
	C5	0.0000	114,939	#1	94.6754×10

Table 8. Continued.

Sample		Rf's	DPM's in peak	Peak designation	$\frac{\mu Ci \text{ in peak}}{\mu Ci \text{ total OE1}}$ /g wet wt
	C5	0.1693	5,994	2	5.0898×10^{-3}
		0.2761	6,433	3	5.4629×10^{-3}
		0.5583	3,224	4	2.7376×10^{-3}
		0.6564	926	5	0.7859×10^{-3}
	C6	0.0000	97,059	#1	77.4672×10^{-3}
		0.1396	7,255	2	5.7905×10^{-3}
		0.2567	10,370	3	8.2768×10^{-3}
Oyster/	I2	0.0000	93,502	#1	44.3676×10^{-3}
bacteria		0.1013	9,602	2	4.5562×10^{-3}
multi-		0.3101	8,234	3	3.9071×10^{-3}
enzyme		0.4367	3,220	4	1.5279×10^{-3}
system		0.7358	24,095	5	11.4331×10^{-3}
(cont.)	I3	0.0000	98,886	#1	47.3820×10^{-3}
		0.1411	17,469	2	8.3704×10^{-3}
		0.3055	15,050	3	7.2113×10^{-3}
		0.4049	7,323	4	3.5089×10^{-3}
		0.6429	640	5	0.3067×10^{-3}
		0.7791	1,978	6	0.9478×10^{-3}
	I4	0.0000	118,088	#1	65.1450×10^{-3}
		0.1302	14,059	2	7.7559×10^{-3}
		0.3728	14,552	3	8.2078×10^{-3}
	A1	0.0000	34,261	#1	10.3675×10^{-3}
		0.0651	6,806	2	2.0603×10^{-3}
		0.3181	10,328	3	3.1264×10^{-3}
		0.4795	1,833	4	0.5549×10^{-3}
		0.8048	1,908	5	0.5776×10^{-3}
	A2	0.0000	111,645	#1	35.5385×10^{-3}
Bacteria-		0.2928	23,411	3	7.4521×10^{-3}
free		0.4789	8,402	4	2.6745×10^{-3}
oyster	A3	0.0000	79,371	#1	$29,6457 \times 10^{-3}$
multi-		0.1601	6,580	2	2.4577×10^{-3}
enzyme		0.3049	11,850	3	4.4261×10^{-3}
system		0.4558	4,937	4	1.8440×10^{-3}
		0.8466	6,339	6	2.3677×10^{-3}
	A4	0.0000	67,163	#1	21.9582×10^{-3}
		0.0725	3,309	2	1.0818×10^{-3}
		0.3587	6,514	3	2.1297×10^{-3}
		0.7509	4,889	5	1.5984×10^{-3}

Table 8. Continued.

Sample		Rf's	DPM's in peak	Peak designation	$\frac{\mu Ci \text{ in peak}}{\mu Ci \text{ total OEl}}$ /g wet wt
	A5	0.0000	289,983	#1	94.1896×10^{-3}
		0.1006	32,663	2	11.5262×10^{-3}
		0.3229	49,359	3	16.0323×10^{-3}
		0.6928	43,132	5	14.010×10^{-3}
Bacteria-	A6	0.0000	227,789	#1	67.1976×10^{-3}
free		0.1018	8,080	2	2.3836×10^{-3}
oyster		0.3341	56,527	3	16.675×10^{-3}
multi-		0.7213	44,490	5	13.125×10^{-3}
enzyme	I5	0.0000	89,132	#1	7.0887×10^{-3}
system		0.1359	16,355	2	10.4751×10^{-3}
(cont.)		0.2941	7,890	3	5.0535×10^{-3}
	I6	0.0000	78,940	#1	57.0108×10^{-3}
		0.1200	18,488	2	13.3521×10^{-3}
		0.2389	9,378	3	6.7728×10^{-3}
		0.7371	928	5	0.6702×10^{-3}

(Table 8).

A particular Rf value denoted one ^{3}H-BaP-simple non-conjugated compound. By placing all the Rf values in only six peaks, the assumption was made that there were five compounds separated by the solvent system and that there were some compounds which do not migrate in this TLC system but remain at the origin. Within each of the four experimental groups, the Rf values for all samples showing a particular peak (i.e., a particular ^{3}H-BaP non-conjugated derivative) were grouped and the means calculated (Table 9). A particular peak's Rf mean for one experimental group was not significantly higher or lower than that same peak's Rf mean for any other experimental group (ANOVA-1, $P > 0.05$ for peaks 1, 3, and 5; t-test, $P > 0.05$ for peaks 2 and 4). The ANOVA-1 tests were used when four groups were compared and t-tests when two groups were compared. These results supported the grouping of peaks into the six ^{3}H-BaP derivatives, as given.

Based on the radioactivity profiles (Figures 14-38), a quantification of ^{3}H-activity was made for each peak (Table 8). An average baseline measurement was taken from the profile and the ^{3}H-activity in that peak (in dpm's) was calculated. This quantification, µCi in the particular peak relative to the µCi in the total OE1 sample, expressed the amount of ^{3}H-BaP which was modified to this derivative relative to the total unmodified ^{3}H-BaP and simple non-conjugated derivatives. In order to make valid comparisons among animals of different weights, this number was expressed relative to one-gram of oyster wet weight (i.e., µCi in peak $\cdot$ (µCi total OE1)$^{-1}$/g wet wt). These quantifications were grouped for each of the four experimental

Table 9. For each of the four experimental groups (i.e., B/BA, Au, C/I2-4, and A/I5-6), the means of the Rf values are included for all samples showing a particular peak (numbered 1 through 6 on the radioscans).

Experimental treatments		Peak #1 Rf (origin)	Peak #2 Rf	Peak #3 Rf	Peak #4 Rf	Peak #5 Rf	Peak #6 Rf
Buffer series	B/BA	0.000 SD = — n = 4		0.3162 SD = 0.848 n = 3		0.6734 SD = .0403 n = 2	
Autoclaved oyster/bacteria systems	Au	0.0000 SD = — n = 4		0.2668 SD = .0224 n = 4		0.6229 SD = .0182 n = 3	
Oyster/bacteria multienzyme preparations	C and I2-4	0.0000 SD = — n = 9	0.1123 SD = .0424 n = 9	0.2765 SD = .0497 n = 9	0.4666 SD = .0810 n = 3	0.6830 SD = .0513 n = 7	0.7991 SD = — n = 1
Bacteria-free oyster multienzyme systems	A and I5-6	0.0000 SD = — n = 8	0.1080 SD = .0337 n = 7	0.3081 SD = .0354 n = 8	0.4724 SD = .0128 n = 3	0.7414 SD = .0415 n = 5	0.8466 SD = — n = 1

treatments according to the peak designation and the means calculated (Table 10). Extremely large standard deviations were observed and these will be considered in the Discussion.

Of special note, the peak 1 quantifications for B samples (buffer) and BA samples (buffer + antibiotics) appeared to be different (Table 10). This was also noted in the profiles shown in Figures 19-22. However, they did not differ significantly (t-test, $P > 0.05$), so they were considered as one treatment (i.e., B/BA) for subsequent analyses.

As seen in Tables 9 and 10, the buffer series (B/BA) and the autoclaved oyster/bacteria series (Au) showed a maximum of three peaks below the solvent front (i.e., peak 1 - origin, peak 3 and peak 5) whereas both multienzyme preparations (C/I2-4 and A/I5-6) showed a maximum of six peaks below the ^{3}H-BaP peak at the solvent front.

Since no ^{3}H-BaP metabolism could occur in an enzyme-free preparation (i.e., B/BA and Au), it was surprising to find simple non-conjugated derivatives of ^{3}H-BaP in the buffer series and the autoclaved oyster/bacteria series. However, it can be seen in Table 9 that peaks 3 and 5 were present in most of the B/BA and Au samples. The appearance of these ^{3}H-BaP derivatives indicated the presence of contaminants in the original ^{3}H-BaP spike and/or oxidation products of ^{3}H-BaP produced by the experimental technique during the original two-hour ^{3}H-BaP incubation or by the subsequent extraction procedure. The extraction technique combined widely-accepted procedures and since it would necessitate always running a background control series to compare with the experimental, the ability to distinguish between contamination and/or oxidation was important.

Table 10. For each of the four experimental groups (i.e., B/BA, Au, C/I2-4, and A/I5-6), the means of the ^{3}H-activities are included for all samples showing a particular peak (numbered 1 through 6 on the radioscans). The ^{3}H-activities are expressed as the μCi in each peak relative to the μCi in the first organic extraction (OE1) per g oyster wet weight.

Experimental treatments		$\frac{\mu Ci \text{ in each peak}}{\mu Ci \text{ in total OE1}}$ / g wet weight					
		Peak #1 (Origin)	Peak #2	Peak #3	Peak #4	Peak #5	Peak #6
Buffer series	B	2.0852×10^{-3} SD=1.1340×10^{-3} n = 2					
	BA	4.6708×10^{-3} SD=0.2635×10^{-3} n = 2					
	B/BA total	3.3830×10^{-3} SD=1.6319×10^{-3} n = 4		0.2672×10^{-3} SD=0.1182×10^{-3} n = 3		0.2252×10^{-3} SD=0.0851×10^{-3} n = 2	
Autoclaved oyster/bacteria systems	Au	29.9882×10^{-3} SD=18.4134×10^{-3} n = 4		3.9879×10^{-3} SD=1.8757×10^{-3} n = 4		0.9782×10^{-3} SD=0.5215×10^{-3} n = 3	
Oys/bac multi-enzyme preps.	C and I2-4	55.1805×10^{-3} SD=19.5459×10^{-3} n = 9	5.8254×10^{-3} SD=2.4847×10^{-3} n = 9	8.4918×10^{-3} SD=3.9543×10^{-3} n = 9	2.5914×10^{-3} SD=0.9985×10^{-3} n = 3	5.4449×10^{-3} SD=5.0319×10^{-3} n = 7	0.9478×10^{-3} SD = — n = 1
Bac-free multi-enzyme systems	A and I5-6	46.6246×10^{-3} SD=27.3528×10^{-3} n = 8	6.1909×10^{-3} SD=5.3181×10^{-3} n = 7	8.3068×10^{-3} SD=6.4882×10^{-3} n = 6	1.6911×10^{-3} SD=1.0680×10^{-3} n = 3	5.9962×10^{-3} SD=6.9301 n = 5	2.3677×10^{-3} SD = — n = 1

The ^{3}H-BaP which was used as the original spike was developed by TLC to distinguish any contaminants. Radioscans were made and the plates subsequently counted in 5-mm strips by liquid scintillation. Radioactivity profiles are graphed in Figures 39-41. There appeared to be a small mid-plate peak whose Rf corresponded to that of peak 3 in all the other experimental groups (Figures 39-41, Table 11). Because of this peak's appearance in the original ^{3}H-BaP spike and its smaller size compared to the corresponding peak in the B/BA series for a comparable amount of ^{3}H-activity on the plates (Figures 19-23, Tables 8 and 10), it seems likely to be an oxidation product associated with the natural breakdown of ^{3}H-BaP with time. Possibly, the process was accelerated by the aeration and agitation which the experimental systems received during the two-hour incubation with ^{3}H-BaP, or by the steps of the extraction procedure for the OE1 fraction and the subsequent preparation for TLC. Hence, more of the non-conjugated ^{3}H-BaP derivative (i.e., seen as peak 3) would be expected, and was observed, in the experimental systems than in the original ^{3}H-BaP spikes.

Since there was little or no non-conjugated ^{3}H-BaP derivative corresponding to peak 5 in the original ^{3}H-BaP spike profiles, the assumption was made that this was also an oxidation product, intrinsically associated with the experimental procedure.

Furthermore, peaks of ^{3}H-activity remained at the origins of the ^{3}H-BaP spikes (Figures 39-41), indicating ^{3}H-activity that was not in a form which migrated in this solvent system. That could mean either more polar ^{3}H-BaP derivatives than the simple

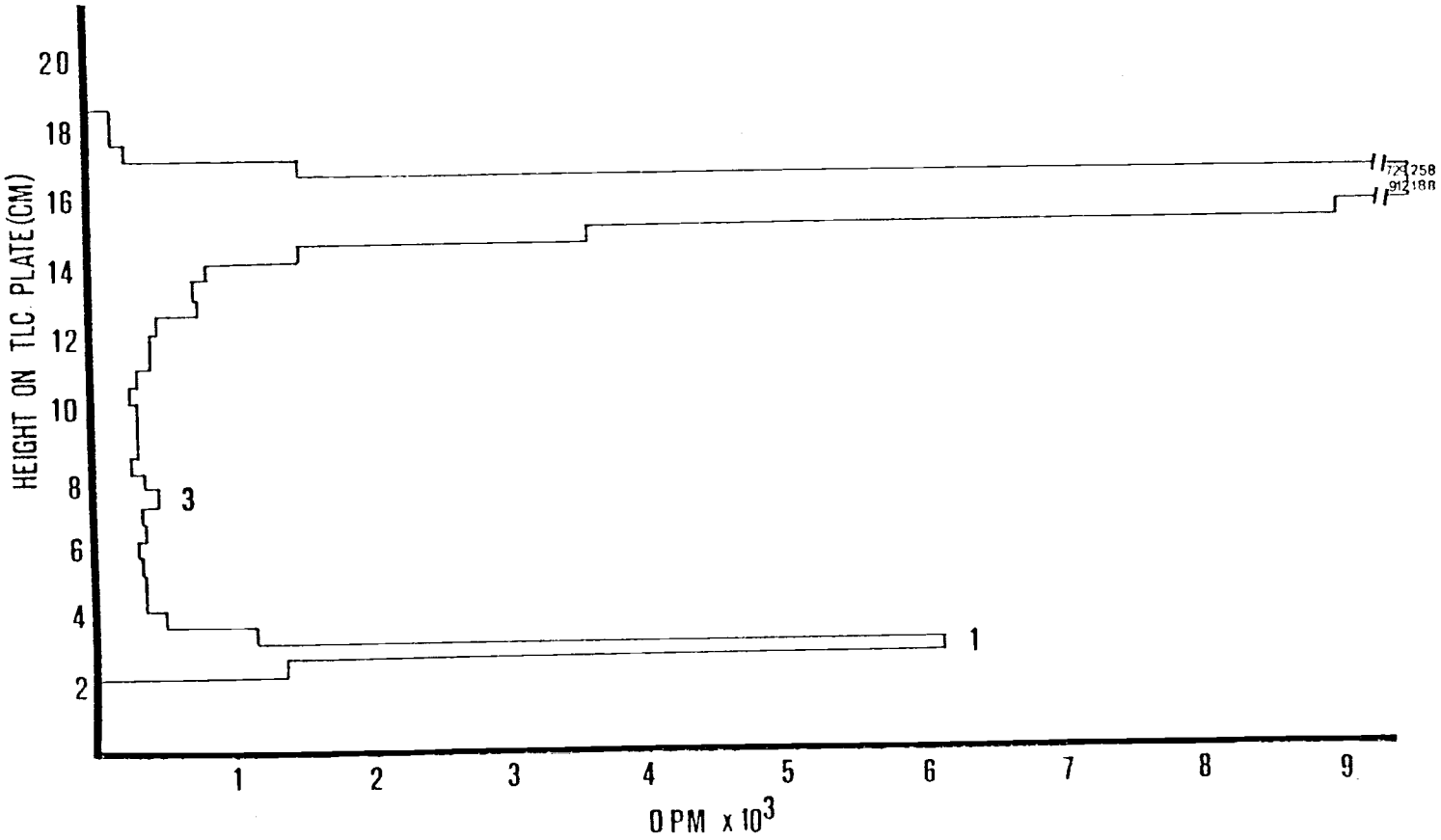


Figure 30. The radioprofile of the ^{3}H-activity$_3$(in dpm x 10^3) associated with a height on the TLC plate (in cm) for the original ^{3}H-BaP spikes (sample BaP-1). The ^{3}H-BaP peak can be seen at the top of the plate. Peak #1 represents the ^{3}H-activity remaining at the origin. The midplate peak (#3) represents a contaminant which is probably an oxidative degradation product.

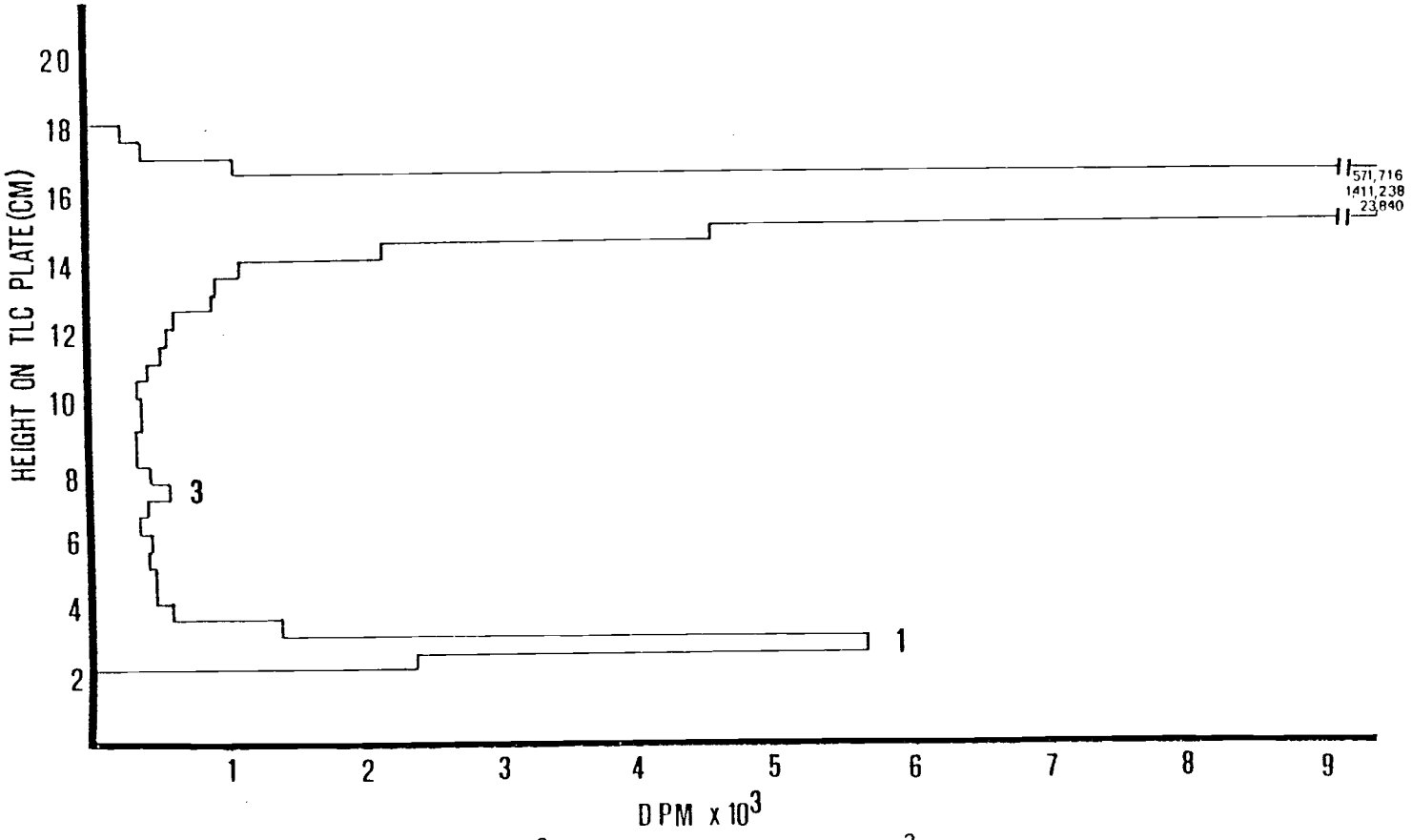


Figure 40. The radioprofile of the ^{3}H-activity (in dpm x 10^{3}) associated with a height on the TLC plate (in cm) for the original ^{3}H-BaP spikes (sample BaP-2).

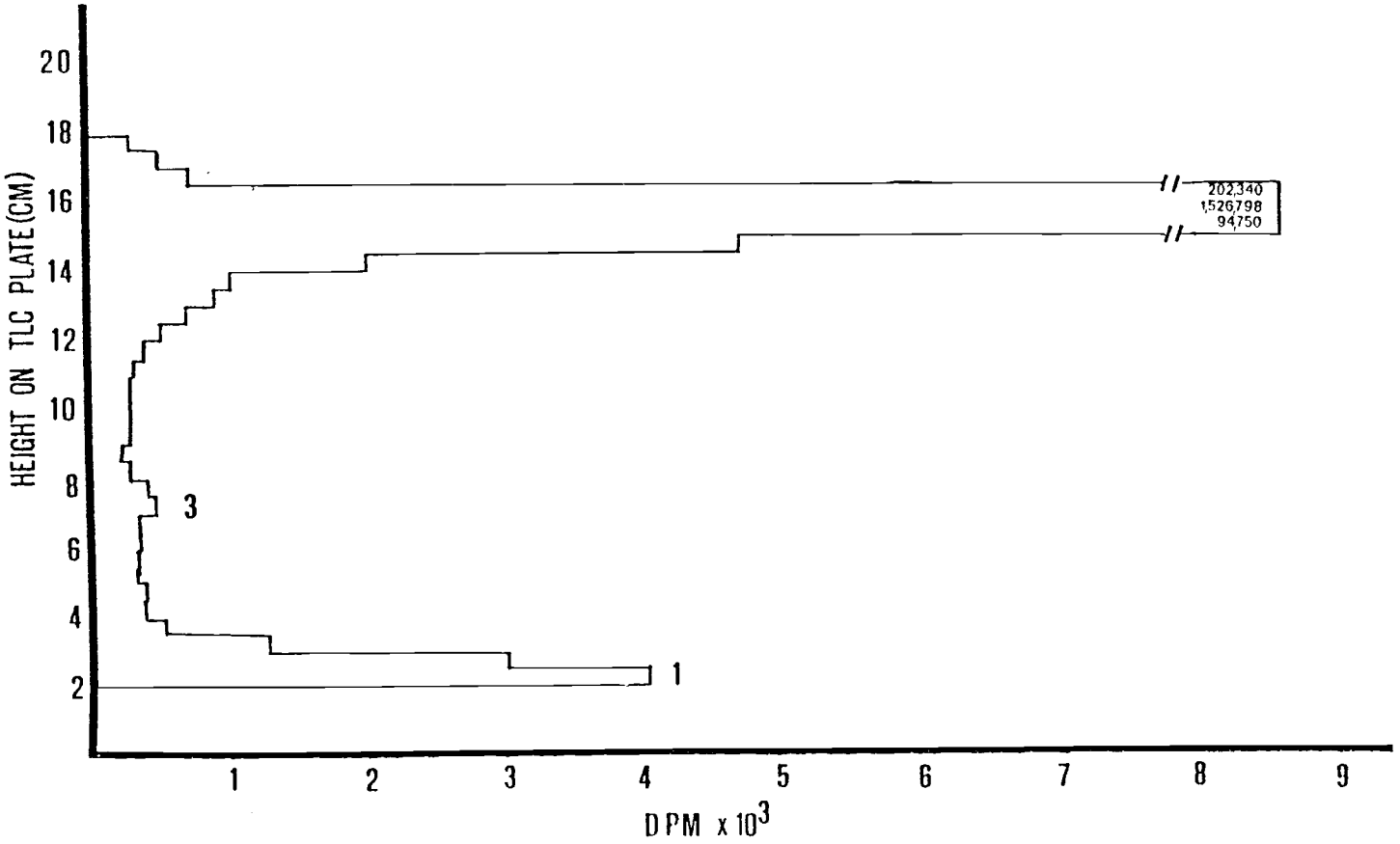


Figure 41. The radioprofile of the ^{3}H-activity (in dpm x 10^{3}) associated with a height on the TLC plate (in cm) for the original ^{3}H-BaP spikes (sample BaP-3).

Table 11. Comparison of the thin-layer chromatograms for the original ^{3}H-BaP spikes. Peak designations are shown with the corresponding Rf values and ^{3}H-activities.

Sample		Rf's	DPM's in peak	Peak designation	µCi in peak
^{3}H-BaP spikes-check for contamination-tion	^{3}H-BaP1	0.0000	8,344	#1	3.7586×10^{-3}
		0.3025	122	3	0.0550×10^{-3}
	^{3}H-BaP2	0.0000	8,474	#1	3.8171×10^{-3}
		0.3084	376	3	0.1694×10^{-3}
	^{3}H-BaP3	0.0000	9,002	#1	4.0550×10^{-3}
		0.3125	238	3	0.1072×10^{-3}

non-conjugates or a possible association of the ^{3}H-activity with the silica gel on the TLC plate. The significance of the appearance of the three peaks (1, 3, and 5) in the background control preparations will be discussed in the next section.

It should be noted that even if the three peaks represent ^{3}H-BaP derivatives which were oxidation products formed during the experimental procedures, these same compounds could also be ^{3}H-BaP metabolites produced by the oyster/bacteria multienzyme system or the bacteria-free oyster multienzyme system. They would be considered to be metabolites if any of the three peaks were observed in greater quantity in the multienzyme preparations than in the background control systems. Statistical comparisons were performed by one-way analyses of variance for the quantification of each peak (Table 8) among the four experimental groups (B/BA, Au, C/I2-4, A/I5-6). The only significant difference between at least two experimental groups was found for peak 1 at the origin ($F_{(3,21)} = 6.31$, $P < 0.01$) and peak 3 ($F_{(3,20)} = 3.54$, $0.01 < P < 0.05$).

Student-Newman-Keules tests were used to distinguish which two groups differed significantly in the amount of ^{3}H-activity associated with the particular peak. With regard to the ^{3}H-activity that remained at the origin (i.e., peak 1), the amount of such activity found in both the oyster/bacteria multienzyme systems (C/I2-4) and the bacteria-free oyster multienzyme preparations (A/I5-6) was significantly greater than the buffer series (B/BA) ($P < 0.01$). This same difference was also noted for the ^{3}H-BaP non-conjugated derivatives characterized by peak 3; the ^{3}H-activity associated with

the oyster/bacteria and the bacteria-free oyster multienzyme preparations was greater than the ^{3}H-activity associated with the buffer series ($0.01 < P < 0.05$).

No such differences were observed between the two multienzyme systems and the autoclaved oyster/bacteria system (Au), or between one multienzyme system and the other. The two multienzyme system preparations (C/I2-4 and A/I5-6) and the autoclaved oyster/bacteria system differed from the buffer series in that the former three were rich in organics from oyster or oyster/bacteria homogenates. Possibly, more ^{3}H-BaP was changed into simple non-conjugated derivatives in an organic-rich system than a seawater buffer system. However, this interpretation should be tempered by the fact that there were no significant differences noted between the autoclaved oyster/bacteria system (i.e., an organics-rich system) and the buffer series nor between the autoclaved oyster/bacteria system (i.e., an enzyme-free system) and the two multienzyme preparations. Thus, the results of this quantitative comparison for the compounds characterized by peaks 1, 3, and 5 were not definitive.

When visual examination and comparisons between the background control samples (Figures 19-26) and the multienzyme preparations (Figures 27-43) were made, it was apparent that the TLC profiles of the multienzyme preparations were much more complicated than those of the background control series. Once these peaks were designated as certain ^{3}H-BaP non-conjugated derivatives and characterized by certain Rf and peak designations (Table 8), there appeared to be three more compounds present in the multienzyme

preparations (peaks 2, 4, and 6) than in the background control systems. As summarized in Table 9, peak 6 was only observed twice; once each in the oyster/bacteria and the bacteria-free oyster multienzyme systems. The compound characterized by peak 4 was observed in approximately one-third of all the multienzyme samples, and the metabolite associated with peak 2 was present in most of the multienzyme preparations.

In summary, there were no apparent differences between the oyster/bacteria multienzyme system and the bacteria-free oyster multienzyme system with regard to the presence/absence of a metabolite or with regard to the different amounts of a metabolite present. In both multienzyme systems, the original ^{3}H-BaP was metabolized to at least three simple non-conjugated derivatives.

DISCUSSION

Microsomal MFO activity has been demonstrated in mammals, many other vertebrates and various invertebrates, including marine crustaceans (Sims and Grover, 1974; Corner et al., 1976; Lee et al., 1976). The question of MFO activity in bivalves has been addressed, but there has been no clear determination as to whether or not they can metabolize aromatic hydrocarbons (Lee et al., 1972; Kahl et al., 1972; Dunn and Stich, 1976; Bend et al., 1977; Anderson, 1978; Vandermeulen and Penrose, 1978). These researchers have addressed the problem of bivalve metabolism without considering the potential metabolic contributions by indigenous microorganisms. Other researchers (reviewed by Karrick, 1977) have shown that yeast, fungi and bacteria in the marine water column and sediments can metabolize aromatic hydrocarbons, including BaP. There have also been studies demonstrating the presence of bacteria in Crassostrea sp. mantle fluid, digestive tissues and whole animal homogenates (Colwell and Liston, 1960; Lovelace et al., 1968; Vasconcelos et al., 1969).

To date, however, there have been no studies designed to evaluate the capacity of the bivalve-indigenous bacterial "system" to metabolize aromatic hydrocarbons. Schwarz and his colleagues (1976) examined the intestinal microfauna of certain deep-sea amphipods and found strong activity in the oxidative metabolism of soluble starch, urea and acetyl-glucosamine. Aromatic hydrocarbons were not included in the substrates for these studies, but the presence of such activity indicates the possible metabolism of

aromatic hydrocarbons by indigenous microorganisms. In addition, it may be relevant that bacteria can secrete viable cell-free enzymes into their environment (Karrick, 1977). Thus, it would not be surprising to find these cell-free enzymes in a whole-animal or tissue homogenate which had undergone only low-speed centrifugation. If a microsomal fraction is prepared instead, the ultra-centrifugations would eliminate bacteria; nevertheless, it is possible that cell-free enzymes of bacterial origin could become associated with the bivalve microsomal fraction.

The natural system (oyster and the commensal microorganisms) is continuously exposed to environmental contaminants from coastal receiving waters. It is important to determine whether this natural system can detoxify such contaminants. It is also of interest to discriminate between the metabolic contributions of oyster enzymes and bacterial enzymes. Within this context, the present research examined BaP metabolism by the natural system (i.e., the oyster/bacteria multienzyme system) and by the bacteria-free oyster multienzyme system.

Two antibiotic treatments, series A and I, were used to produce bacteria-free oyster multienzyme systems (Table 2). The MPN results for the series I were somewhat equivocal. Samples I2, I3 and I4 showed bacteria present at all dilutions; the bacteria were not diluted to extinction (Figure 4). The calculated MPN's per g oyster wet weight (Table 3) are within the range for bacterial numbers in oysters (Vasconcelos et al., 1969). The serial dilutions for each replicate on a plate (8 replicates by 12 two-fold dilutions)

were completed before the next replicate was begun, reducing the chances of contaminating all the replicates. It was assumed that the MPN's for samples I2, I3 and I4 reflected a large bacterial population size of the samples. However, the possibility that the MPN microplates were contaminated cannot be ruled out.

For samples I5 and I6, the first two wells for all replicates were bacteria-free, but other, more dilute, wells contained bacteria (Figure 4). This series had received antibiotics only during the multienzyme system preparation (about 3 to 4 hours) and the subsequent ^{3}H-BaP incubation (2 hours). Streptomycin acts as an inhibitor of protein synthesis on the 30S ribosomal units; chloramphenicol inhibits protein synthesis on the 70S ribosomal units; and penicillin inhibits cell wall biosynthesis (Lehninger, 1975). All viable bacteria were affected (i.e., killed or unable to reproduce) during the two-hour incubation with ^{3}H-BaP. Their population size was affected by these antibiotics at the full-strength concentration in the original sample, at the two-fold dilution (well #1 of each replicate) and at the four-fold dilution (well #2 of each replicate). Thus, no viable bacteria were present to metabolize the ^{3}H-BaP during the incubation, and no bacteria could reproduce in the first two wells of the microplate--the wells containing the greatest concentration of oyster sample and also antibiotics. In wells 3-12 for each replicate, the antibiotics, as well as the multienzyme preparations, were being diluted. If any of the bacteria were successful in reproducing during the 10-day development period of the MPN plate, there obviously was not sufficient concentration of antibiotics to inhibit the reproducing

bacteria, and the well would be scored positive on examination. Because the bacteria were inhibited by the antibiotics concentration of the original sample, the oyster multienzyme preparations for samples I5 and I6 were considered bacteria-free.

The results of the series I studies indicate that it may be desirable to pretreat the oyster with large concentrations of antibiotics during a relatively lengthy exposure period as was done for series A oysters (i.e., a three-day pretreatment prior to the multienzyme system preparation and ^{3}H-BaP incubation). Since there was no bacterial growth observed on series A - MPN microplates, it can be assumed that during the three-day pretreatment, there were enough antibiotics to inhibit growth and the resultant preparations were bacteria-free.

Methodology developed for mammalian systems has predominately been utilized to determine the presence or absence of MFO activity in bivalve molluscs. These methods include fluorimetric identification of BaP and metabolites (Dunn and Stich, 1976; Vandermeulen and Penrose, 1978), spectrophotometric analysis (Carlson, 1972), autoradiographic inspection of extracted lipids (Lee et al., 1972) and gas chromatographic analysis (Kahn et al., 1972; Stegeman and Teal, 1973). Anderson (1978) contended that a radioisotopic method is more sensitive and capable of quantifying the organic solvent-soluble compounds (i.e., unmodified labeled BaP and simple non-conjugated metabolites) and the water-soluble BaP metabolites which are not quantified by the previous procedures. Anderson (1978) further separated and characterized the organic extractions by high

pressure liquid chromatography (HPLC), a relatively new technique for efficiently separating simple non-conjugated BaP metabolites (Selkirk et al., 1974).

Anderson's (1978) point is important. However, he only made one organic extraction, and thus quantified only two fractions, the simple non-conjugated metabolites, containing phenols, diols and quinones, and the remaining water-soluble polar metabolites. It is possible to further characterize the water-soluble metabolites, as was done in the present study. By treating the sample first with glucuronidase (also containing sulfatase activity) followed by base/acid hydrolysis, it was possible to gain more information about the nature of the water-soluble metabolites and thus, indirectly, about the BaP-metabolizing enzyme systems (Bock et al., 1976; Keysell et al., 1975).

In the present study, thin-layer chromatography was utilized, instead of HPLC, to separate the simple non-conjugated ^{3}H-BaP derivatives. An HPLC system was not available to this researcher, and Sims (1966) had detailed an adequate TLC separation procedure for these BaP metabolites. There are two disadvantages of TLC separation which are eliminated by the HPLC. During the TLC, the ^{3}H-BaP migrates with the solvent front, thus moving over the entire plate. There are problems with tailing of ^{3}H-BaP (i.e., ^{3}H-activity associates with the silica gel along the entire height of the TLC plate), thereby potentially masking the activity associated with metabolites in the middle of the plate. In the present study, radioscans and radioprofiles (from liquid scintillation counts of

5-mm plate strips) were compared to detect any small metabolite peaks. Still, some of the metabolites produced in small quantities may have been masked by the background ^{3}H-activity along the plate height (Figures 22-38), and this may help account for the appearance of certain metabolites in some samples and not others (Table 9). In HPLC, the BaP comes off the column last, succeeding the simple non-conjugates; thus, there is no tailing effect.

Second advantage of HPLC over TLC is that the peak Rf values are more consistent in repeated uses of the same HPLC system than are repeated runs in the TLC system. Once the HPLC is set up, internal conditions, and subsequently, the metabolite separations are fairly constant. Separate TLC runs require new solvent mixtures and potentially alter the experimental conditions unless all samples are developed on the same day. This may partially account both for the variability observed for the Rf values of the same peak (note the standard deviations in Table 9) and for the variability noted for the ^{3}H-activity quantifications of the peaks characterizing the same derivative (Table 10). If this variability is due to experimental techniques, then it would be reduced by HPLC analysis. Even considering the weaknesses of TLC separation, the radioisotopic technique, further characterized by the thin-layer chromatography, is definitely a method of choice; it is sensitive and can potentially produce more information regarding the metabolism of benzo(a)pyrene.

A few points, concerning this radioisotopic technique, were noted in the Results and will be discussed in greater detail. Table 6 indicates that there were large variations among samples

regarding the fraction of total ^{3}H-recovered relative to the total ^{3}H-initially spiked as ^{3}H-BaP. The samples experienced differential losses. The buffer series (B/BA) consistently had an average of 50% recovery, while the autoclaved oyster/bacteria series (Au) had recoveries of approximately 97%. During the two-hour incubation, the preparations were aerated and agitated, and the ^{3}H-BaP may have been more easily evaporated from the buffer series (B/BA) than the Au series. Au samples were essentially autoclaved "organic soups"; and the ^{3}H-BaP may have associated with the available organics.

There were also considerable variations in the percent recoveries among the multienzyme samples (C/I2-4 and A/I5-6) (Table 6). The reasons for such variations are unknown. Because the percent recoveries varied to such a great degree between samples, it cannot be determined how much of the original ^{3}H-BaP spike the multienzyme systems actually encountered prior to the evaporative loss. Given that the percent recoveries were variable, if the ^{3}H-activity associated with a particular extraction was stated relative to the total ^{3}H-BaP spiked, the metabolite comparisons (as ^{3}H-activity associated with a particular extraction fraction) among samples would be meaningless. It is known, however, that each multienzyme sample was exposed to at least that amount of ^{3}H-BaP which was recovered in all extraction fractions (OE1 + OE2 + OE3 + AQ). It would be consistent between samples and possible to identify what fraction of this total ^{3}H-activity recovered was partitioned as, for example, hydrolysis-susceptible derivatives (OE3). The data is therefore presented as μCi ^{3}H-activity in a

particular extraction per g oyster wet weight relative to total μCi ^{3}H-activity recovered in all extraction fractions.

It was necessary to run background controls during the radioisotopic and TLC assays to identify physical/chemical alterations of the ^{3}H-BaP and, thus, provide an accurate measure of the metabolism by the multienzyme preparations. Most researchers have recognized this necessity and utilized a zero-time background measure by adding the stopping mixture before the substrate (zero-time incubation) (e.g., Anderson, 1978). In the present work, the background was determined by incubating the ^{3}H-BaP with an inviable multienzyme preparation (i.e., previously autoclaved samples). Such a treatment provided a background measure of any physical/chemical alterations produced by agitation and aeration during the two-hour incubation, as well as those produced by the extraction procedures and TLC methods.

However, there is one inherent problem in utilizing autoclaved samples as background measures. Autoclaving may have denatured molecules and exposed polar groups to which the ^{3}H-BaP could become non-enzymatically associated. The unexpectedly large amount of ^{3}H-activity associated with the OE2 fraction shows that perhaps the glucuronide moieties and/or free sulfate groups were made available for association with ^{3}H-BaP by autoclaving. The high background measure of ^{3}H-activity associated with the OE2 fractions for the Au samples makes it difficult to draw any final conclusions about the presence of glucuronide- and sulfate-conjugating enzymes in both multienzyme systems.

While recognizing that some problems may be inherent in the use of autoclaved systems as background measures, the Au series does represent a maximum estimate of non-enzymatically-produced ^{3}H-BaP derivatives for each of the four extractions. If the ^{3}H-activity associated with one of the multienzyme systems is significantly greater than that activity associated with the Au series, then it should be valid to conclude that metabolism of ^{3}H-BaP occurred in the multienzyme system. The evidence for the presence of BaP metabolic pathways in the oyster/indigenous bacteria systems will be discussed later.

The results of the TLC used in this study indicate that it may be essential to use inviable multienzyme systems as background measures rather than zero-time incubation controls. TLC radio-profiles of the original ^{3}H-BaP spikes (Figures 39-41) identified a small contaminant peak, a simple non-conjugated derivative characterized as peak 3. The buffer series and the autoclaved oyster/bacteria systems also showed the presence of this ^{3}H-BaP derivative but in much greater amounts ($P < 0.01$) (Tables 10 and 11). A second ^{3}H-BaP derivative did not appear as a contaminant in the TLC of the original ^{3}H-BaP spike but did appear on some of the TLC's for the background control series (series B/BA and Au) (Tables 9 and 11). This suggests that the two oxidative degradation products were produced by the incubation conditions (two-hour incubation with aeration and agitation), the subsequent extraction with ethyl acetate and/or the sample preparation for TLC. For this study, it was not possible to conclude where degradation of the

^{3}H-BaP had occurred. All samples were kept out of direct light when being used and were always stored in the dark; nevertheless, the observed degradation could have been caused by photo-oxidation. Also, evaporations for the TLC preparations were made under N_2 to reduce the chance of oxidative degradation. It is possible that the degradation occurred during the aeration of the incubation period. Thus, before investigators can assume that zero-time background controls represent an accurate background measure of physical/chemical alterations of BaP, inviable enzyme system incubations must first be run simultaneously with the zero-time incubations to see that they do not differ in the background measure. If there is no observed difference, only then can the more convenient zero-time controls be used. DePierre and co-workers (1975) justified the use of zero-time incubations for rat liver homogenates. It is not yet specified in the literature that researchers working with bivalve systems have taken this into consideration.

The thin-layer chromatograms also showed a peak of ^{3}H-activity that remained at the origin for all samples, including the ^{3}H-BaP original spikes (summarized in Tables 9, 10 and 11). This was probably ^{3}H-activity which became associated with the silica gel at the origin as the sample was applied to the plate. On visual examination, it appeared that a greater amount of ^{3}H-activity was associated with the origin for the buffer + antibiotics series (BA) than that for the buffer series (B). Statistical comparison did not confirm this observation. It would be interesting to examine more samples to see whether the presence of antibiotics does or

does not have an effect on the amount of ^{3}H-activity remaining at the origin, attempting to establish whether or not the ^{3}H-BaP or the ^{3}H-label associates with the antibiotics.

The ^{3}H-activity at the origin (peak 1) was present in significantly greater amounts for the multienzyme preparation (C/I2-4 and A/I5-6) than for the buffer series (B/BA) ($P < 0.01$) (Tables 10 and 11). This larger peak at the origin for the multienzyme systems possibly represents some extracted ^{3}H-BaP polar derivatives from the aqueous sample as well as the association of ^{3}H-activity with the silica gel. The organic solvent, ethyl acetate, does absorb some water, and also dissolves slightly in water (Stecher, 1968). Therefore, it is conceivable that some hydrophilic polar derivatives, which would remain at the origin in the TLC system, were extracted into the first organic extraction (OE1).

Another carry-over from the application of mammalian methodology to bivalve systems has been the consideration of cofactor requirements and concentrations. This study utilized the cofactor combination given by Sims (1966) for rat-liver homogenates; other studies (e.g., Vandermeulen and Penrose, 1978) have relied on the protocol of Nebert and Gelboin (1968) for mammalian cell preparations. Recently, Anderson (1978) has shown that such combinations and concentrations of cofactors inhibit AHH activity in C. virginica, and he offered a new incubation mixture for use in molluscan systems. Anderson (1978) also found that C. virginica AHH activity was maximized at 30°C. It should be noted that this

oyster species is constantly exposed to such temperatures during the summer months. The present study employed an incubation temperature of 13°C (a typical Oregon estuarine temperature) in order to maintain a normal bacterial flora for metabolic action upon the substrate.

When evaluating the ^{3}H-BaP metabolism by the multienzyme systems, the considerable variation among samples in the multienzyme systems (i.e., oyster/bacteria and bacteria-free oyster series) is striking, both in the ^{3}H-activity partitioned among the four extractions and in the quantifications of the peaks characterizing ^{3}H-BaP simple non-conjugated derivatives. This variation among animals of the same species for MFO activities has been observed previously (Bend et al., 1977; Varanasi and Malins, 1977). The variation may be a reflection of the large individual differences in a wild population. It should also be emphasized that even though the oysters used in this study were the same age, were from the same supply source, and had always experienced the same holding conditions, the individuals may have had different environmental histories. Oysters can close their valves and remain isolated from their environment for long periods. Thus, it cannot be assumed that all individuals were exposed to the same past environmental conditions, including exposure to environmental contaminants. Anderson (1978) provided indirect evidence that AHH activity in C. virginica is induced by exposure to PCB's. If this AHH induction is also assumed for O. edulis, then some individuals may have had partially-induced enzymatic activities from previous exposure to

ubiquitous environmental contaminants which resulted in different metabolic responses to the ^{3}H-BaP substrate.

The large variation among individuals may also be due to the fact that multienzyme preparations were made from whole oyster homogenates; the preparation had been homogenized and then centrifuged at 700 g to eliminate cell debris. This method offered a working preparation that did not eliminate or select any enzyme systems associated with particular subcellular fractions thereby resulting in a multienzyme system (Popjak, 1969). When the present research was begun, there were no studies indicating that the oxidative detoxification enzymes in the oyster were restricted to one organ or to one subcellular fraction. Also, it was apparent from the literature that if metabolites of BaP were to be found, the quantities would probably be small. The rationale for utilizing a whole oyster multienzyme preparation was to maximize the metabolic contributions from various organs and subcellular fractions. If metabolism was observed, then subsequent studies could specify the sites of concentrated AHH activity. Anderson (1978) found high AHH activity only in the digestive gland for C. virginica. He suggested that the digestive gland activity is diluted to less than detectable amounts when whole animal homogenates are used. In addition, the AHH enzyme system may be exposed to the digestive proteolytic enzymes or endogenous MFO inhibitors such as those associated with the gut contents and with certain natural pigments in other invertebrates (Anderson, 1978). Exposure of the multienzyme preparation to any of the factors (which reduce AHH activity)

would result in inconsistent preparations, and may account for overall individual variations. To summarize, the large variations among samples may have been caused by individual differences within a wild population, differential exposure to proteolytic digestive enzymes and MFO inhibitors, and/or partially-induced enzymatic activities for some animals and not others.

Even with the considerable variability among animals in the partitioning of ^{3}H-BaP metabolites, the present study does indicate that the oyster "system" has the capability of metabolizing benzo(a)-pyrene. Based on the TLC of the OE1 fraction, there appears to be two, and possibly three, simple non-conjugated metabolites produced by the multienzyme systems (Table 9).

The most common metabolite (peak 2) is characterized by an average Rf value of 0.11. Based on the table of Rf values and probable metabolite identities, derived by Sims (1967), this metabolite chromatographs close to 7,8-dihydrodiol (Rf 0.15). Since the table by Sims is not complete for all possible metabolites, and since the 9,10-dihydrodiol is not characterized by an Rf value in this solvent system, it must be stated that this study suggests a probable identity of 7,8-dihydrodiol, with 9,10-dihydrodiol remaining as an alternative possibility. Anderson (1978) identified one of the BaP metabolites of C. virginica to be either 4,5-dihydrodiol or 7,8-dihydrodiol.

The second metabolite (peak 4) is characterized by an average value of 0.47. This compound appears to correspond to BaP-3,6-quinone (Rf 0.45; Sims, 1967). In C. virginica, Anderson (1978)

noted also the presence of a metabolite which he identified as either BaP-1,6-quinone or BaP-3,6-quinone. Sims and Grover (1974) stated that it is not known whether quinones are produced enzymatically from unsubstituted aromatic hydrocarbons or if they arise from chemical oxidations during the working-up procedures. Since the derivative was not seen in the background controls, this experiment suggests an enzymatic origin of the 3,6-quinone by the O. edulis/ indigenous bacteria multienzyme systems.

The third non-conjugated derivative separated by TLC in this study (Rf 0.82) was present in only two samples of multienzyme preparations (Table 9; peak 6). None of the compounds identified by Sims (1967) have a corresponding Rf value. Since the peak was only observed twice and since it appeared on the shoulder of the unmodified ^{3}H-BaP peak, it is assumed to be an accumulation of ^{3}H-activity from the BaP tailing effect discussed previously.

In the present study, peak 3, characterized by the average Rf 0.29, is found in all treatments and is assumed to be an oxidative product formed by physical/chemical alterations. The BaP-11,12-dihydrodiol (Rf 0.28) in Sims' work (1967) most closely chromatographs with the derivative in the present research; the 4,5-dihydrodiol (Rf 0.25) is similar enough that it cannot be eliminated. The second oxidative degradation product formed during this analysis (Rf 0.68) corresponds to various phenols (i.e., 6-OH-BaP - Rf 0.65; 1-OH-BaP - Rf 0.62; 7-OH-BaP - Rf 0.62; 5-OH-BaP - Rf 0.61; 12-OH-BaP - Rf 0.60). Interestingly, Sims (1967) points out that upon chemical oxidation of BaP with OsO_4,

two products (cis-4,5-dihydrodiol and cis-11,12-dihydrodiol) were formed. In addition, Anderson (1978) noted the presence of 3-OH-BaP and another unknown monohydroxylated derivative as metabolites of C. virginica.

Based on the remaining three extractions (OE2, OE3 and AQ), further evidence is presented which indicates that the oyster/bacteria multienzyme system metabolizes ^{3}H-BaP to polar derivatives. It cannot be conclusively stated that there are no glucuronide- and sulfate-conjugating enzymes in either multienzyme system since the activity associated with the background measure (series Au) was so high. Further studies are needed to determine whether the oyster/bacteria system possesses these enzymatic capabilities. There is a significant amount of ^{3}H-BaP metabolism to hydrolysis-susceptible polar derivatives demonstrated for the oyster/bacteria multienzyme system. Finally, for both multienzyme preparations, ^{3}H-BaP was metabolized into polar derivatives which had not been affected by the previous glucronidase (OE2) and hydrolysis treatments (OE3) and into which the ^{3}H-BaP or the ^{3}H-label was incorporated.

This present study shows that the two multienzyme preparations (the oyster/bacteria and the bacteria-free oyster) possess the enzymatic capability to metabolize benzo(a)pyrene. In the antibiotic-treated, bacteria-free oyster multienzyme series, it can be assumed that the possible metabolic contribution by bacterial cell-free enzymes has been greatly reduced, but it is not possible to conclude that the metabolic contribution is solely oyster in origin. The finding that the amounts of any metabolite or the

amounts of activity associated with a metabolic fraction do not differ significantly between the bacteria-present and bacteria-free multienzyme systems indicates that the oyster multienzyme system contributes the majority of metabolites. The only exception may occur when the oyster/bacteria system metabolizes ^{3}H-BaP to hydrolysis-susceptible polar derivatives while the bacteria-free oyster system does not.

It should also be mentioned that oyster system, as it exists in nature, may contain other microorganisms such as fungi and yeast. The possibility that these other microorganisms may be contributing enzymatically to the system cannot be eliminated based on this study or on any other available literature.

This research has provided some insights about the direction of future experiments. It is suggested that the sensitive radioisotopic techniques, supplemented by quantitative TLC or, if available, HPLC be used. There should definitely be attempts by the investigators to maximize the information gained about the nature of the metabolites by various successive extractions. Once the radioactivity has been found to be present in a certain extration fraction, then chromatographic techniques can be employed for further separation and qualification of metabolites.

If it is important to eliminate bacteria and their associated cell-free enzymes in a bivalve system, then the antibiotics-pretreatment and the use of the microsomal fraction (if Anderson's findings (1978) with C. virginica are found to be applicable to other bivalves) would appear appropriate. The reduction may be

accomplished solely by using a microsomal fraction. An experiment, investigating any difference in metabolic activity for a microsomal fraction and an antibiotics-pretreated fraction, would determine the value of the antibiotics-pretreatment.

Finally, the identification of metabolites is important, but other research should be directed at characterizing the enzyme systems involved in bivalve detoxification mechanisms (Bend et al., 1977).

CONCLUSIONS

1. It was not possible to distinguish between oyster and bacterial metabolism of aromatic hydrocarbons in the oyster-bacteria system. This may not be of paramount importance since the oyster exists in nature as a system with commensal microorganisms. It should, however, be recognized as a "system" by researchers.

2. An antibiotics treatment has been found which is successful in producing a bacteria-free oyster multienzyme system for a two-hour incubation with the substrate.

3. Two oxidative degradation products, identified as simple non-conjugated ^{3}H-BaP derivatives, were found during either the incubation period (two-hour incubation with aeration and agitation), the subsequent extraction with ehtyl acetate and/or the sample preparation for TLC.

4. Before investigators can assume that zero-time background controls represent an accurate background measure of physical/chemical alterations of BaP, inviable enzyme system incubations must first be run simultaneously with the zero-time incubations to determine that they do not differ in the measure of background. If there are no observed differences, then the more convenient zero-time controls can be used.

5. In the present study, the oyster system has been shown to metabolize benzo(a)pyrene. Two simple non-conjugated derivatives were characterized by TLC. The ^{3}H-activity associated with the remaining aqueous phase indicated the presence of ^{3}H-BaP which was

so tightly bound as to be resistant to hydrolysis, modified ^{3}H-BaP rings which were incorporated into other polar compound and/or ^{3}H-labels which were displaced from the BaP and conjugated with polar compounds not susceptible to hydrolysis. In addition, the oyster/bacteria multienzyme system appeared to metabolize ^{3}H-BaP to hydrolysis-susceptible polar derivatives.

6. Considerable variation was observed, and can be expected, in the metabolic responses of individuals to the substrate, ^{3}H-BaP.

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