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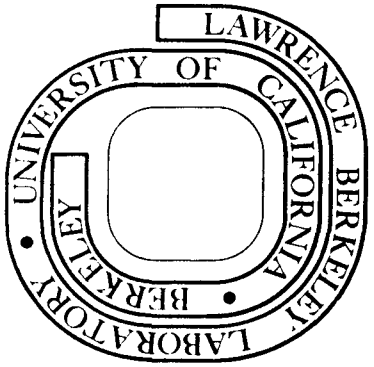

DISCLAIMER

SPECIFIC POSITIONS INVOLVED IN ENZYME CATALYZED COVALENT BINDING OF BENZO[a]PYRENE TO POLY(G)

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(3-methylcholanthrene/microsomes/monooxygenases/carcinogens/epoxides)

Running Title: Binding of Benzo[a]pyrene to Poly(G)

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ABSTRACT

Covalent binding of benzo[a]pyrene to poly(G) was studied with the use of a radioactive assay and specifically labeled substrates to define the role of the 1,3 and 6-positions of the hydrocarbon during this process. Binding was shown to be dependent on microsomes, NADPH, O_2 and poly(G). 7,8-Benzoflavone and 2',2'-diethylaminoethyl-2,2-diphenyl valerate were inhibitory whereas modulators of epoxide hydrase activity had little effect. ^{3}H and ^{14}C studies suggested a possible loss of 1-2 protons. Incorporation of [6-$^{3}H_1$]benzo[a]pyrene provided evidence that the 6-position of the hydrocarbon was not metabolized during covalent attachment to poly(G) and furthermore results with [1,3,6-$^{3}H_3$]benzo[a]pyrene suggest that the 1- and 3-positions may not be involved either. After scaling up of the standard assay 20-fold,characterization of the tritiated BaP-poly(G) complex was carried out by hydrolysis and subsequent chromatography. Thin layer chromatography of the isolated hydrolysis products treated with HCl or alkaline phosphatase indicated that the complex formed between BaP and poly(G) was covalently linked and composed of hydrocarbon-nucleotide(s .

Benzo[a]pyrene (BaP) when activated chemically and photochemically (1-3) or enzymically (4-6) will undergo covalent binding to nucleic acids and proteins. The hydrocarbon can be metabolized by microsomal cytochrome P-450 monooxygenases and it is these enzymes which are thought to activate polycyclic aromatic hydrocarbons (PAH) to bind to cellular macromolecules (7). Although P-450 monooxygenases occur primarily in endoplasmic reticulum, they have been reported in other subcellular fractions as well (8) and BaP binding to DNA has also been reported to be catalyzed by rat liver nuclei (9).

Although several positions on BaP have been proposed to be involved in covalent binding of the hydrocarbon to nucleic acids, very little evidence exists concerning this or the more fundamental question of the resultant chemical structure of the complex. The 4,5- (10) and the 7,8,9 and 10-positions (11) of BaP have been proposed as possible sites of activation. Evidence obtained from isotopic exchange studies led to the suggestion (12,13) that covalent linkage of BaP could occur at the 1,3 or 6-positions. Labeling studies (14) and the observation that 6-hydroxybenzo[a]pyrene (6-hydroxy BaP) covalently binds to DNA (15,16) supported this proposal.

Since this report describes experiments which cast doubt on the viability of this theory, it is apparent that alternative positions are involved in enzyme catalyzed covalent binding of PAH to nucleic acids.

Abbreviations: BaP, benzo[a]pyrene; 6-hydroxyBaP, 6-hydroxybenzo[a]-pyrene; PAH, polycyclic aromatic hydrocarbons; SKF525A, 2',2'-diethylaminoethyl-2,2-diphenyl valerate; 7,8-BF, 7,8-benzoflavone; Tris-sucrose, 0.05 M Tris-base + 0.2 M sucrose + 0.1 mM dithiothreitol (pH 7.5).

MATERIALS AND METHODS

Chemicals. Sephadex LH-20 was a product of Pharmacia (Piscataway, N.J.). Ribonuclease T_1 (E.C.3.1.4.8), bovine spleen phosphodiesterase type I, alkaline phosphatase (E.C.3.1.3.1) type I, poly(G) and NADPH were supplied by Sigma (St. Louis, Mo.). 3-Methylcholanthrene was purchased from Calbiochem (LaJolla, CA) and used without further purification. BaP (Aldrich, San Leandro, CA) was applied and eluted from a neutral alumina column with Chromatoquality benzene (Matheson, Coleman,Bell, Norwood, OH). The hydrocarbon was then recrystallized from benzene-isopropanol and purity was ascertained by tlc and fluorescence spectroscopy. Samples of BaP-poly(G) were counted in a gel (Aquasol, New England Nuclear, Boston, Mass. plus 25% water) to avoid the necessity of hydrolyzing the nucleic acid. Controls showed the same dpm in a gel as for NaOH hydrolyzed samples. All solvents were redistilled.

Labeled substrates: [G-^{3}H]BaP and [7,10-$^{14}C_2$]BaP were obtained from Amersham-Searle (Arlington Heights, Ill.). [6-$^3H_1$]BaP and [1,3,6-$^3H_3$]-BaP were synthesized as described (17). The labeled compounds were purified by tlc prior to use. Chromatography was carried out on glass plates coated with 0.2 mm silica gel G and developed in a solvent system of benzene-ethanol 19:1.

Microsomes: Male Sprague-Dawley rats (Simonsen Laboratories, Gilroy, CA) weighing ~200 g were given 3-methylcholanthrene in corn oil by intraperitoneal injection at a dose of 25 mg/kg body weight 48 hrs before sacrifice. Animals were sacrificed by decapitation and the livers were immediately removed and chilled in ice cold 0.9% NaCl. After the tissue had been minced it was suspended in two volumes of 0.05 M Tris-base, 0.25 M sucrose, 0.1 mM dithiothreitol (pH 7.5) (Tris-sucrose) buffer and a cell extract was prepared with a glass-Teflon tissue homogenizer. The

extract was centrifuged at 1500 x g for 10 min, then 10,000 x g for 30 min and finally 100,000 x g for 90 min. The resultant pellet was washed twice by resuspension in Tris-sucrose and resedimentation. The 100,000 x g pellet, or microsomal fraction, was dispersed in Tris-sucrose at a protein concentration of 16 mg/ml.

Analytical procedures: Protein concentration was determined by the method of Lowry (18) with bovine serum albumin as reference standard. Counting of ^{3}H and ^{14}C was carried out on a Packard liquid scintillation spectrometer (Model 3380). $^{3}H/^{14}C$ ratios were determined from efficiency quench curves constructed with the use of sealed standards (New England Nuclear, Boston, Mass.). Poly(G) concentration was determined by ultraviolet absorption. Purity of the labeled hydrocarbons was also determined by fluorescence spectroscopy.

Binding assay Covalent binding of BaP to poly(G) was monitored by a radioactive assay. The standard reaction contained 6 nmoles BaP (labeled with 0.1 to 0.7 μCi ^{3}H or ^{14}C) 500 μg poly (G), 0.45 μmoles NADPH, 400 μg microsomal protein and 20 μl ethanol in a total volume of 1.5 ml phosphate buffer 0.01 M (pH 7.5). Assays were incubated for 30 min at 37°C and the reaction stopped by the addition of 3 ml phenol reagent (phenol 500 ml, m-cresol 70 ml, water 50 ml and 8-hydroxyquinoline 0.5 g). NaCl was added to bring its concentration to 0.1 M and the sample volume to 2 ml. The samples were then extracted by the method of Leaver and Key (19). The phenol phase was re-extracted with 2 ml 0.01 M phosphate buffer (pH 7.5), the aqueous phases combined and extracted twice with one volume of ethyl acetate. The aqueous phase was transferred to a screw cap test tube, two volumes of ethanol were added, and the sample treated in a boiling water bath for 5 min. The poly(G) precipitate was collected by

centrifugation and after dissolving it in 0.1 M NaCl the precipitation and heating were repeated. The final precipitate was taken up in 0.01 M phosphate buffer (pH 7.5) and aliquots of the samples were then analyzed for radioactivity and optical density at 252 nm. Binding experiments were carried out at least three times. Assays were scaled up 20-fold for the hydrolytic and chromatographic analysis of the complex.

Hydrolysis: The phosphodiester linkages of poly(G) were hydrolyzed enzymatically or chemically. The enzyme hydrolysis was carried out at 37°C in 0.1 M Tris-base containing 2 mM EDTA (pH 7.5). Incubation of the BaP-poly(G) complex for 3-6 hrs with ribonuclease T_1 (10,000 units/mg nucleic acid) was then followed by an additional 20-24 hrs after adding spleen phosphodiesterase (0.3 units/mg nucleic acid). Chemical hydrolysis was done with 0.1 N NaOH at 37°C for 20-24 hrs.

The glycosidic bond of the nucleic acid was hydrolyzed by heating samples in a boiling water bath for 3 hrs in the presence of 0.1 N HCl.

Phosphomonoester hydrolysis was accomplished by incubating samples for 1 hr at 37°C with alkaline phosphatase in 0.1 M sodium carbonate (pH 8.5).

Sephadex chromatography: The enzyme and base hydrolyzed BaP-poly(G) complex were chromatographed on columns of LH-20 with a discontinuous gradient of 0.01 M sodium phosphate (pH 7.5) and ethanol. The BaP-nucleotide(s) which eluted well after the solvent front were analyzed by tlc after treatment with alkaline phosphatase and HCl.

Thin layer chromatography: After hydrolysis samples of the BaP-poly(G) complex were reduced in volume with nitrogen and applied in 50% ethanol-water to thin layers of silica gel G coated on glass

plates (0.25 mm). Chromatography was carried out in a solvent system of butanol-acetone-water (60:38: 2 v/v/v). Standards were visualized with iodine, fluorescence or with a fluorescent indicator. Fractions of the tlc plate were then scraped and radioactivity measured directly in a gelled scintillant.

RESULTS AND DISCUSSION

Covalent attachment of BaP to poly(G) was not significant in the absence of microsomes, NADPH, or O_2 (Table I). These results are consistent with the proposal that PAH are activated by microsomal monooxygenases (5,7), since these enzymes are known to require O_2 and NADPH. Only trace amounts of ^3H were isolated with assays carried out in the absence of poly(G), indicating little covalent attachment of the hydrocarbon to endogenous nucleic acid. The P-450 monooxygenase inhibitor 2',2'-diethylaminoethyl-2,2-diphenyl valerate (SKF525A) (2) and an inhibitor of aryl hydrocarbon hydroxylase activity, 7,8-benzoflavone (7,8-BF) (21), interfered with binding (Table I). Microsomes from rats not pretreated with 3-methylcholanthrene and thus containing low levels of aryl hydrocarbon hydroxylase, weakly activated BaP. When rat liver microsomes are incubated with NADPH under aerobic conditions, endogenous lipid peroxidation occurs concomitant with cytochrome P-450 destruction (22). In further support of cytochrome P-450 participation in activating BaP was the observation that pre-incubation of microsomes with NADPH, i.e., in the absence of BaP, resulted in a marked reduction of binding (Table I).

Epoxide hydrase inhibitors have been reported to stimulate covalent binding of PAH to nucleic acids, possibly by accumulating epxoide intermediates (23-25). However, when assays were carried out with epoxide hydrase inhibitors, trichloropropene oxide and cyclohexene oxide (26), binding was slightly decreased while the use of glycidol and styrene oxide had very little effect (Table II). If a diol-epoxide were an intermediate, ashas been proposed (11), hydrase inhibition would decrease diol formation and thus binding. Results obtained here do not rule out a possible role of epoxide hydrases in binding, but how the enzymes are involved remains to be established.

To determine the extent to which BaP is metabolized during its activation and covalent attachment to poly(G) ^{3}H and ^{14}C studies were carried out. When ^{3}H and ^{14}C labeled BaP was used in the standard assay, 4-16% of the ^{3}H was lost (Table III). This corresponds to a loss of 1-2 protons, assuming even distribution of ^{3}H on the hydrocarbon. Similar losses have been reported to occur in a chemical activation system (27). To test the involvement of the 6-position, assays were carried out using [6-$^{3}H_1$]BaP (Table III). More ^{3}H from the [6-$^{3}H_1$]BaP was incorporated into poly(G) than from [G-^{3}H] BaP. Label lost from the [G-^{3}H]BaP species is consistent with results obtained in the ^{3}H and ^{14}C experiments (Table III). Thus, the 6-position of BaP must not have been metabolized. A similar conclusion was reached recently by a more indirect approach (28). Label is also retained from the 1- or 3-positions of the hydrocarbon during activation (Table III). An assessment of this result is complicated since the 1- and 3-positions could participate in the NIH shift, resulting in retention of significant amounts of label (29). These retentions

are usually not quantitative (29) as was the case of [1,3,6-$^{3}H_{3}$]BaP and, therefore, the 1- and 3-positions of BaP were probably not metabolized in the assays carried out here. On the other hand, the 6-position of BaP cannot participate in the NIH shift. Although it is clear that 6-hydroxyBaP can covalently attach to nucleic acids in vitro (15,16), these results show that the 6-position is not metabolized during enzyme activated binding of the hydrocarbon to poly(G).

After scaling up the standard assay characterization of the tritiated BaP-poly(G) complex was carried out by hydrolysis and subsequent chromatography.

Hydrolysis of the phosphodiester linkages of the BaP-poly(G) complex was carried out enzymatically with ribonuclease T_1 and bovine spleen phosphodiesterase or with 0.1 N NaOH. Samples were then chromatographed on columns of LH-20 (Fig. 1). The presence of guanine was detected only at or near the solvent front where controls indicated elution of poly(G) and 5'-GMP occurred. Tritium eluted as two bands, one at the solvent front and a hydrophobic band which came off with 50% ethanol-buffer. BaP itself is not eluted under the conditions used in Fig. 1. Column profiles resulted in recovery of 75% of the tritium and 80% of the absorbance units applied. Only 25% of the recovered tritium chromatographed with hydrolyzed nucleotide(s) when the reaction was carried out enzymatically, and 80% when the reaction was carried out with NaOH. This result suggests the possibility of incomplete enzyme hydrolysis. Evidence for ribonuclease T_1 resistant oligonucleotides bound with chemically activated BaP has been reported (27). Support for this view was obtained from alkaline phosphatase and HCl treatment of the hydrolyzed BaP-poly(G) complex and subsequent tlc.

The material in peak 2, Fig 1 was pooled (fractions 13-18) and an aliquot was run on tlc before and after treatment with HCl and alkaline phosphatase. The plate was then profiled for tritium (Fig. 2a). The untreated material remained at the origin, while treatment with acid or alkaline phosphtase resulted in tritium migrating as a very broad band with an R_f value of 0.6-0.8. The experiment was repeated using an enzyme hydrolyzed BaP-poly(G) complex (pooled fractions 13-18) from peak 1, Fig. 1. Although the tritium migrated after acid treatment, alkaline phosphatase had no effect. HCl treatment probably resulted in glysodic bond cleavage which would explain the resulting mobility of tritium on tlc from both enzyme and base hydrolyzed complexes. Tritium mobility changed after alkaline phosphatase treatment from the chemically derived hydrolysis products, but not those from enzymes, suggesting that base hydrolysis produced BaP-mononucleotides while the enzyme procedure yielded BaP-oligonucleotides. Recent studies have shown that enzyme digests of carcinogen-DNA complexes chromatographed on LH-20 result in most of the radioactive hydrocarbon eluting at the solvent front(11). These results could also reflect a resistance to hydrolysis which would explain why most of the radioactivity eluted with oligonucleotides.

The chromatographic data demonstrates that microsomal enzymes catalyze covalent complex formation between BaP and poly(G). Binding has also been shown to proceed in the absence of metabolism at the 1,3 and 6-positions. Thus, it is likely that one or more of the positions 2,4,5 and 7 through 12 are involved in the binding of the hydrocarbon to poly(G). The 4- and 5-positions have recently been

questioned (25). Therefore, it is more likely that the 7,8,9- and/or 10-positions are involved, and evidence supporting this view has been obtained (30).

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Table I

Assay conditions, necessary cofactors and effect of inhibitors on binding of BaP to poly(G)

Assay conditions	pmoles BaP bound/500 µg poly(G)*
complete	125 ± 8
minus O_2	30 ± 6
minus NADPH	2 ± 1
minus poly(G)	5 ± 1
minus microsomes	< 1
non-induced microsomes	10 ± 2
10 minutes preincubation without BaP	70 ± 4
plus 9×10^{-4} M SKF525A	92 ± 5
plus 5×10^{-6} M 7,8-BF	13 ± 2

* Average of duplicate assays

Table II

Effect of epoxide hydrase inhibitors and activators on binding of BaP to poly(G)

Assay conditions	pmoles BaP bound/500 μg poly(G)*
complete	102 ± 6
1×10^{-3} M trichloropropene oxide[a]	86 ± 2
1×10^{-3} M styrene oxide[b]	92.5 ± 2.5
1×10^{-3} M cyclohexene oxide[c]	85 ± 3
1×10^{-3} M glycidol[d]	95 ± 1

*, average of duplicate assays

[a] An inhibitor of both epoxide hydrase and aryl hydrocarbon hydroxylase activities (23,26)

[b] A substrate for epoxide hydrase (26)

[c] An inhibitor of epoxide hydrase (26)

[d] An activator of epoxide hydrase (26)

Table III

Binding of Specifically Labeled BaP to Poly(G)

A

Tritium Loss from [G-^{3}H] BaP

Labeled substrate*	$^3H/^{14}C$ ratio		% ^3H loss
	substrate	BaP bound to Poly(G)	
[G-^{3}H] + [^{14}C] BaP	8.33	7.49 ± 0.52	10 ± 6

B

Tritium Retention from [6-^{3}H] and [1,3,6-$^3H_3$] BaP

Labeled substrate*	pmoles hydrocarbon bound/ 500 µg Poly(G)
0.83 µCi [G-^{3}H] BaP	112 ± 6
0.28 µCi [6-^{3}H] BaP	119 ± 4
0.65 µCi [1,3,6-$^3H_3$] BaP	129 ± 3

Approximately 10% of the tritium from generally labeled BaP was lost in comparison to the ^{14}C hydrocarbon (part A) and only a small amount was lost from [G-^{3}H]BaP compared to the [6-] and [1,3,6-] labeled hydrocarbons. Thus, the amount of label incorporated was essentially the same for the ^{14}C and [6-] and [1,3,6-$^3H_3$] hydrocarbons, resulting in total tritium retention during covalent binding to poly(G).

* Each assay contained a total of 6 nmoles of BaP.

FIGURE LEGENDS

Fig. 1. Samples of [G-^{3}H]BaP-poly(G) were hydrolyzed as described under "Materials and Methods" and chromatographed on columns of LH-20. Two ml fractions were collected from the columns which were eluted with 0.01 M sodium phosphate (pH 7.5) and ethanol. Fractions 13-18 from both profiles were combined and reduced in volume for further analysis. Samples were hydrolyzed with (a) enzymes and (b) 0.1 N NaOH.

Fig. 2. Aliquots of the combined fractions 13-18 from columns profiled in Fig. 1 were treated with alkaline phosphatase or HCl as described under "Materials and Methods" and applied to tlc plates. After development, fractions were scraped into scintillation vials and counted to determine changes in mobility. Samples were derived from [G-^{3}H]BaP-poly(G) hydrolyzed with (a) 0.1 N NaOH and (b) enzymes.

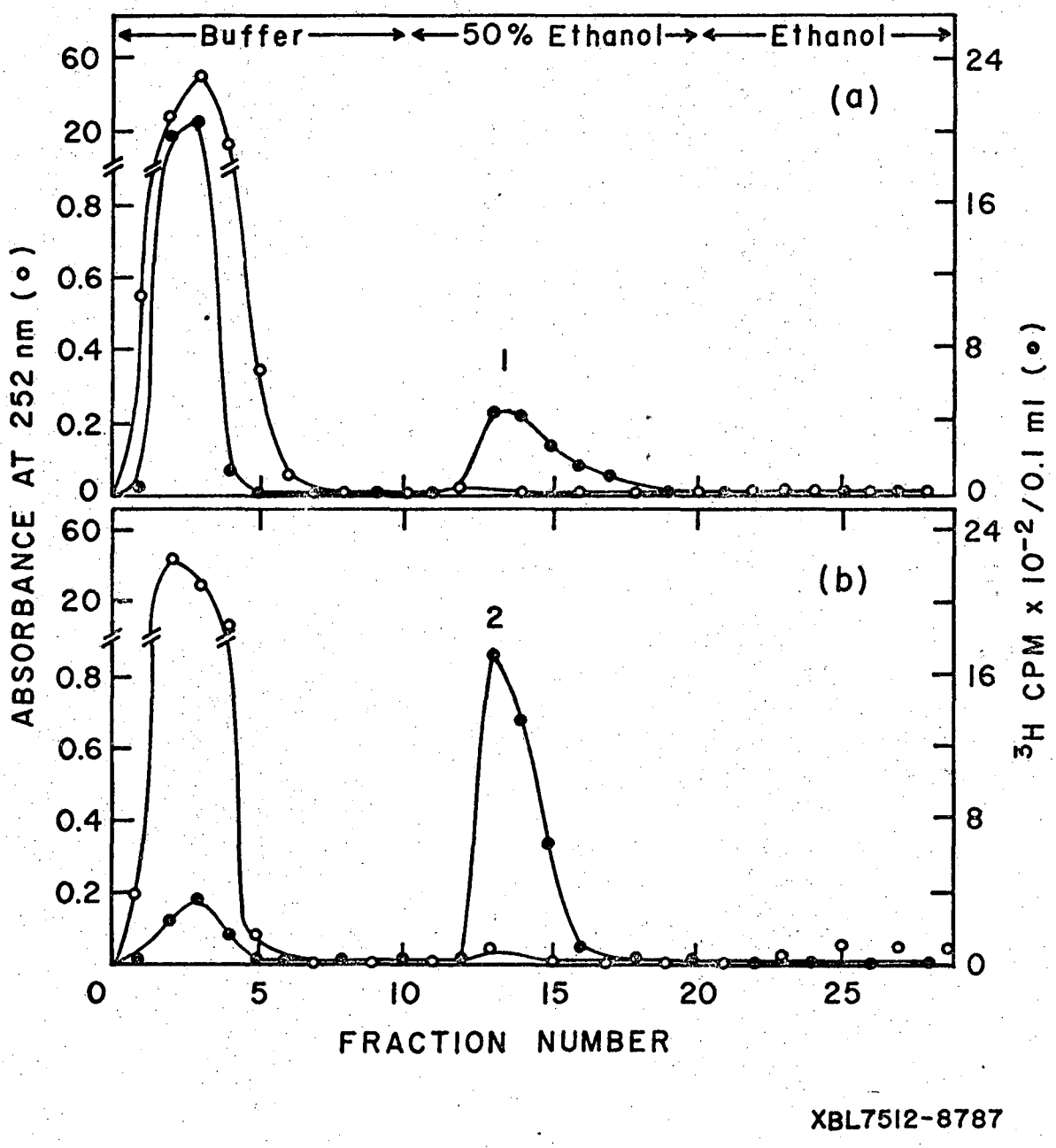
Buffer
50% Ethanol
Ethanol
(a)
(b)
1
2
ABSORBANCE AT 252 nm (•)
^{3}H CPM x 10^{-2}/0.1 ml (•)
FRACTION NUMBER
XBL7512-8787

Fig. 1

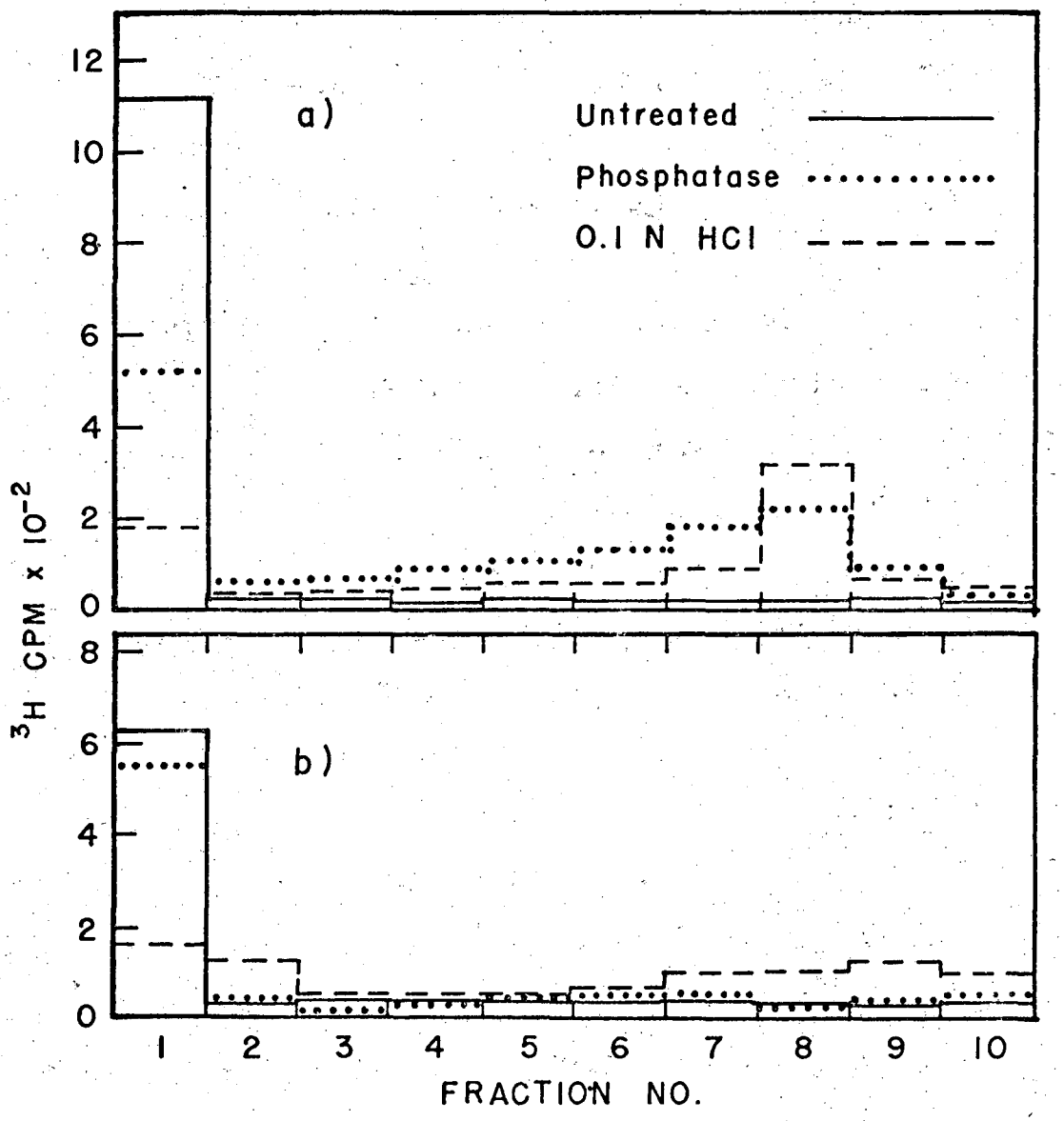

Fig. 2

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