

Benzo(a)pyrene-Triglyceride Adduct : a Potential Molecular Biomarker for Carcinogen Exposure

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Abstract

In this study, we demonstrated the *in vitro* and *in vivo* formation of carcinogen-lipid adduct and its correlation with DNA or protein adducts. The lipids from serum or hepatocyte membranes of Sprague-Dawley rats, human serum, and standard major lipids were *in vitro* reacted with benzo[a]pyrene (BP) and BP metabolites. 7,8-Dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE-I), an ultimate carcinogenic form of BP, was covalently bound to triglyceride (TG). BPDE-I-TG adducts isolated by thin-layer chromatography (TLC) were further detected by high performance liquid chromatography (HPLC). TGs, including triolein, tripalmitin and tristearin, showed positive reactions with BPDE-I. However, cholesterol, phospholipids (phosphatidylcholine, phosphatidyl-ethanolamine, phosphatidyl-inositol and sphingomyelin) and nonesterified fatty acids (palmitic acid, oleic acid, linoleic acid and stearic acid) did not react with BPDE-I. In addition, other BP metabolites (BP-phenols and -diols) did not react with TG, which TG appeared to be the most reactive lipid yet studied with respect to its ability to form an adduct with BPDE-I. There was a clear-cut dose-related formation of [1,3-³H]BPDE-I-lipid adduct *in vitro* between TG and [1,3-³H]BPDE-I. In an animal study, BPDE-I-TG was also formed in the serum of rats orally treated with BP (25 mg/rat). Also, obvious correlations between [³H]BP related-biomolecule adducts (DNA, protein) or lipid damage and the BPDE-I-TG adduct were obtained in various tissues of mice i.p. treated with [³H]BP. These data suggest

that TG can form an adduct with BPDE-I, as do other macromolecules (DNA, RNA, and protein). Therefore, a carcinogen-lipid adduct would be a useful biomarker for chemical carcinogenesis research and cancer risk assessment.

Introduction

Exposure to carcinogens can cause damage to various biomolecules such as DNA, RNA and protein in the body, and such damage has been used as a biomarker (1). Biomarkers are defined as cellular, biochemical, or molecular alterations in biological specimens of fluids, cells, or tissues, and have been developed and applied to cancer research and risk assessment (2). In chemical carcinogenesis, the formation of a carcinogen-DNA adduct is a critical step and is therefore considered an important biomarker during the initiation stage (3). Carcinogen-DNA adduct has been measured in human tissues to predict exposure levels of carcinogens as well as to investigate the cause of carcinogenesis (4). However, the analysis of DNA-adducts in human tissues has limitations in terms of sampling, sensitivity and specificity depending on the analytical methods (reviewed in Ref. 5 and 6).

Lipids are good sources of energy in the body and are composed of cellular membrane and tissue. There is some evidence that lipids could be involved in carcinogenesis. About one-half of all cancer patients show a syndrome of cachexia, characterized by the loss of adipose tissue and skeletal muscle mass (reviewed in Ref. 7). Lipids are easily peroxidized to lipid peroxides by free radicals (8), and malonedialdehyde (MDA) is used to estimate lipid damage during lipid peroxidation. Lipid peroxidation products are also bound to DNA in

the human liver and leukocyte (9,10). In recent studies, changes in the fatty acid composition of plasma lipids were characterized in patients with malignancy by nuclear magnetic resonance and gas-liquid chromatography (11,12), and lipid analysis was also performed in smooth muscle tumors (13). In breast cancer patients, serum triglyceride, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL) and very-low density lipoprotein (VLDL) levels were quantified in order to evaluate the changes in serum lipids and lipoproteins in the early and advanced stages of the disease. These studies were undertaken to assess the lipid changes due to the presence of malignancy and to investigate the usefulness of lipid changes for the diagnosis and prognosis of malignancy or metastasis (14,15). Prelabeling-chase experiments with [¹⁴C]-arachidonic acid in human gastric cancer cells showed that arachidonic acid was preferentially incorporated into the triglycerides followed by a transfer to phospholipids (16). If lipids are damaged by exposure to carcinogens, their biosynthesis and metabolism would be influenced in the body. This leads to the question of whether lipid damage can be used as a biomarker or play any role in the process of chemical carcinogenesis.

For the above stated reasons, this study was undertaken to investigate whether benzo(a)pyrene-diol-epoxide (BPDE) can directly damage lipids to form BPDE-lipid adducts *in vitro* and *in vivo*. The correlation between DNA or protein adducts and BPDE-lipid adducts was also investigated.

Materials and methods

Chemicals

Triglyceride, triolein, tripalmitin, tristearin, phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, sphingomyelin, cholesterol, palmitic acid, oleic acid, linoleic acid, stearic acid and benzo[a]pyrene were purchased from Sigma Chemical Co. (St. Louis, MO). The triglyceride assay kit was also purchased from the Sigma Diagnostics Co. (\pm)-r-7,t-8-Dihydroxy-t-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene and other BP metabolites (BP-phenols and BP-diols) were purchased from the National Cancer Institute (NCI), Chemical Carcinogen Reference Standard Repository at the Midwest Research Institute (Kansas, MO). [1,3-³H]BPDE-I (439 mCi/mmol) and [³H]BP (66 Ci/mmol) were obtained from the NCI Radiochemical Carcinogen Reference Standard Repository at Chemsyn Science Laboratories (Lenexa, Kansas).

Preparation of total lipid from animals and humans

The total lipid was prepared from hepatocytes or sera of Sprague-Dawley rats, and human sera. After a male rat (250 \pm 20 g) was anesthetized, the abdomen was cut open and perfusion was initiated through the portal vein. Ca²⁺-free buffer and collagenase buffer were successively pumped from a reservoir through the portal vein at 37°C (17). The released hepatocytes were cultured in a

CO₂ incubator for 24 h and sound hepatocytes were separated. Whole blood (10 ml) was taken from the animals or human volunteers (mean age: 24±2, N=10) and the serum was separated promptly after centrifugation at 3,000 rpm for 20 min. Folch solution (chloroform : methanol, 2:1 by vol.) was added to hepatocytes or serum and agitated for 3 h. After centrifugation at 3,000 rpm for 10 min, the lower layer was separated and the concentration of total lipid was determined by UV absorption spectroscopy at 590 nm as described in the dichromate oxidation method (18). The total lipid was dissolved in tetrahydrofuran (THF) at a concentration of 1 mg/ml. The male rats (250±20 g) were orally treated with BP (25 mg/rat) in 2 ml of corn oil to investigate BP-lipid adducts *in vivo*. After 6 h, the rats were anesthetized and 10 ml of whole blood was taken from the abdominal aorta. Other procedures were as above.

In vitro detection of lipid adducts

Standard lipids (cholesterol, triglyceride, triolein, tripalmitin, tristearin, palmitic acid, oleic acid, linoleic acid, stearic acid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and sphingomyelin) (50 µg each in 50 µl THF or chloroform) or total lipids from animals and humans were incubated with BP or BP metabolites (50 µl, 1 mg/ml THF or chloroform) for 2 h at 37°C. After incubation, each sample was applied as a 1 cm streak to TLC plates (silica gel 60; Merck, Darmstadt, Germany) and the solvent front was

allowed to migrate to a predetermined boundary mark. The cholesterol and triglyceride were separated using a THF/hexane two component system: THF/hexane/formic acid (20:80:2, by vol.) to 18 cm above the origin and THF/hexane/formic acid (100:20:2, by vol.) to 10 cm (19,20). Total lipids, phospholipids and free fatty acids were separated using a three-development system: diethylether/hexane/ammonia (50:50:0.25, by vol.) to 18 cm above the origin, chloroform/methanol/water/ammonia (70:30:3:2, by vol.) to 10 cm and ethylacetate/acetone/water/glacial acetic acid (40:40:2:1, by vol.) to 16 cm (21). After TLC the plates were dried in a desiccator, and the spots were located under UV light (254 and 365 nm) or observed after spraying with 10% sulfuric acid. The R_f values from the reaction of triglyceride, tripalmitin, triolein, and tristearin with BPDE-I were all 0.88.

Analysis of lipid adducts and modification levels by HPLC

Each standard lipid (50 μg) was mixed with [1,3- ^3H]BPDE-I (3.5 nmol) in THF for 2 h at 37°C and was applied to a TLC plate. Solvent systems were as described above. All spots were scraped off into a screw-cap scintillation vial and the radioactivity was measured using a liquid scintillation counter (LSC, Pharmacia-LKB 1209 Rackbeta, Uppsala, Sweden). The modification level of the [1,3- ^3H]BPDE-I-TG adduct was determined on the basis of the molar ratio of [1,3- ^3H]BPDE-I to TG. For HPLC analysis, the reaction mixture was

centrifuged and the supernatant was filtered through a 0.45 μm membrane prior to injection into an Econosil silica column (Alltech; 250 \times 10 mm inner diameter; 10- μm particle size, Deerfield, Illinois) attached to a HPLC system (Gilson 805; Gambetta, France) using a THF/hexane solvent system (22,23).

In vivo dose-response relationships for BP adducts to DNA, protein, lipid and triglyceride in different mouse tissues

ICR mice (32 ± 2 g) (3 animals/dose) were injected i.p. with 0.33, 1 or 3 mg [^3H]BP (66 Ci/mmol) in 0.5 ml corn oil. After 24 h, the mice were sacrificed, and the liver, heart, lung, stomach, kidney, spleen and brain were collected. The organs were washed 3 times with 0.9% saline and, after homogenization, were incubated (37°C , 1h) in 4 vol of 10% SDS buffer solution. The total lipid and protein were separated using a Folch solution, and DNA was isolated from the tissues using a standard phenol, chloroform/isoamyl alcohol extraction, and RNase treatment (24). The concentration of triglyceride was measured in the total lipid using the TG assay kit. All samples were counted on an LSC at a concentration of 1 mg/ml.

Results

To investigate the formation of carcinogen-lipid adducts, total lipids were prepared from rat serum or hepatocyte membrane and human serum, and were reacted with BP and BPDE-I. Thin layer chromatography showed that BPDE-I, but not BP, covalently bound to a certain lipid regardless of the lipid source (Fig. 1). On the TLC plates, a new spot (spot F) was formed with a greenish fluorescence, whereas BPDE-I fluoresces blue under the UV light (365 nm). To further investigate BPDE-I-lipid damage, standard lipids (cholesterol, triglycerides, phospholipids and free fatty acids) were reacted with BPDE-I and the R_f value of each spot produced by TLC was estimated. Only TG, tripalmitin, triolein and tristearin reacted with BPDE-I and produced a BPDE-I-TG adduct spot on the TLC plates, similar to the BPDE-TG adduct spot observed in Fig. 1.

The formation of a BPDE-I-TG adduct was reconfirmed using [1,3- ^3H]BPDE-I. A two dimensional TLC was developed for a reaction mixture of TG (50 μg) and [1,3- ^3H]BPDE-I (1.5×10^6 CPM). Four different spots were detected and the CPM values of each spot were measured by LSC (Fig. 2). Spots A and B were BPDE-I (two separate regions in this solvent system), spot C was BPDE-I-TG adduct, and spot D was TG, which was confirmed by separate TLC application of BPDE-I and TG. The radioactivities of the spots were as follows : A-423232, B-44862, C-253215 and D-151 CPM.

In a dose-response experiment, [1,3-³H]BPDE-I-TG was increasingly formed *in vitro* as a function of [1,3-³H]BPDE-I dose (Fig. 3). In a time-response experiment, the [1,3-³H]BPDE-I-TG adduct reached a peak level 24 h after incubation at 37°C and remained at this level for 48 h (data not shown). The formation of the [1,3-³H]BPDE-I-TG adduct was further detected by HPLC analysis and chromatograms of the separations are shown in Fig. 4.

In animal experiments, a BP-metabolite-lipid adduct was formed in the serum of rats orally treated with BP (25 mg/rat). The fluorescent hue under UV light and the R_f value of the BP-metabolite-lipid adduct so formed, were identical to those of [1,3-³H]BPDE-I-TG formed *in vitro* (spot F in Fig. 1). The correlation coefficients of the BP-DNA/BP-TG adducts or the BP-protein/BP-TG adducts were 0.99 for all tissues (Table I).

Discussion

Biomolecular damage is widely believed to be a critical event during mutagenesis and carcinogenesis. Carcinogen-macromolecule adducts (DNA, RNA or protein adducts) have been largely studied with known chemical carcinogens (24). However, the analysis of DNA or protein-adducts in human tissues still has limitations in terms of sampling, sensitivity and specificity. DNA or protein adducts have been measured in human tissues for the etiological investigation of carcinogen exposure and qualitative risk assessment. Lipids are abundant in human tissues and may be susceptible to the exposure of carcinogens. If carcinogen-derived lipid damage occurs, it could be useful as an alternative to DNA or protein adduct.

In this study, BPDE-lipid adduct formation was detected *in vitro* and *in vivo* by the exposure of BPDE-I or BP. The formation of the BPDE-lipid adduct *in vitro* indicated that the TGs, including tripalmitin, triolein and tristearin, are likely target lipids for BPDE-I. Triglycerides (also called *fats*, *triglycerols* and *depot lipids*) constitute 90% of dietary lipids and are the major form of metabolic energy storage in humans (40-150 mg/100 ml blood in adults). These nonpolar, water-insoluble substances consist of glycerol triesters of fatty acids such as palmitic, oleic and stearic acids (25).

In the triglycerides of human lipids, palmitate tends to be concentrated at

position 1 and oleate at position 2 of the base glycerol structure (26). Considering the structure of the TG species, a possible explanation for the formation of the BPDE-I-TG adduct might be the covalent binding of position 10 of BPDE-I to the ester bonds of TG between the glycerol portion and free fatty acids of TG, though this must be investigated further. One important role of TG is transporting free fatty acids, either to various tissues or during the biosynthesis of several lipids in the body (27,28). Therefore, the adduct formation between BPDE-I and TG may be of biological significance and it may also serve as an index of any internal damage caused by carcinogens. *In vivo* correlations between either the biomolecular adducts (DNA, protein) or damage (lipid, in this case as BP, BP metabolites, and BPDE-I-TG adducts are expected to be incorporated into the lipid) and TG adduct formation are obvious such that the lipid damage or TG adducts could serve as a surrogate biomarker for DNA or protein adducts.

These preliminary data suggest that the formation of the BPDE-I-TG adduct may be an important piece of evidence that lipids (especially esterified forms) can be directly damaged by reactive carcinogens. The carcinogen-lipid adduct or -lipid damage may be useful biomarkers for carcinogen exposure, although carcinogen-lipid adduct is limited to a TG adduct in this study. It is of interest whether the formation of a carcinogen-lipid adduct plays a role in the process of chemical carcinogenesis. In the future, further investigation is needed on the chemical structure of BPDE-I-TG, its biological role and any correlation

with carcinogen-DNA or carcinogen-protein adduct formation in human tissues.

In addition, the development of better technology to detect TG adducts could facilitate their use for cancer research as well as risk assessment.

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Table I. Dose-response relationships for [³H]BP adducts to DNA, protein, lipid and triglyceride in various tissues of mice i.p. treated with [³H]BP

		BPDE-I-adduct (pmol/mg)						
		Organ						
Per 1mg	BP dose (mg)	Heart	Lung	Spleen	Kidney	Stomach	Brain	Liver
DNA	0.33	3.12±1.34*	4.42±0.94	1.28±0.44	7.98±1.07	11.63±6.78	0.96±0.66	19.16±1.99
	1	7.68±2.21	11.19±2.96	3.52±0.38	22.98±3.59	31.06±7.02	2.28±1.19	52.21±9.31
	3	20.95±4.68	26.18±8.23	8.46±1.46	54.64±12.74	76.43±11.27	5.87±3.17	126.08±17.35
Protein	0.33	7.57±1.17	32.95±3.48	4.36±0.62	23.49±7.83	46.53±3.44	1.72±0.61	53.32±1.37
	1	19.07±2.61	87.33±5.78	11.52±2.32	68.58±6.92	132.13±21.42	4.58±1.44	141.50±4.65
	3	54.53±14.72	249.76±18.37	27.41±3.97	183.12±19.46	358.07±26.74	12.33±5.24	393.37±13.22
Lipid	0.33	43.80±4.52	193.65±13.79	48.57±2.77	127.44±11.57	335.39±17.29	14.31±4.77	257.91±11.49
	1	117.82±10.85	532.55±29.83	118.50±9.38	369.75±22.55	784.80±41.84	40.65±10.83	684.44±31.94
	3	301.61±29.42	1230.19±35.72	311.65±42.68	965.04±84.92	2048.42±121.65	96.34±20.03	1902.71±60.28
TG	0.33	2.33±1.01	20.95±4.11	3.52±1.07	16.90±4.61	22.69±10.19	1.29±0.23	38.80±6.31
	1	5.42±1.78	50.49±12.52	8.40±2.23	39.53±10.23	52.42±17.82	3.33±1.19	88.86±7.62
	3	13.24±2.91	112.07±20.49	20.50±4.97	93.31±16.71	126.33±18.67	8.09±2.31	206.15±13.55

* Mean ± SD

Figure Legend

Fig. 1. TLC analysis of total lipid treated in vitro with BP and BPDE-I. A : BP, B : BPDE-I, C : lipid of rat serum, D : BP mixed with C, E : BPDE-I mixed with C, F : BPDE-I-lipid adducts spot, G : Triglyceride.

Fig. 2. Two-dimensional TLC analysis of TG was reacted with [1,3-³H]BPDE-I. A and B : [1,3-³H]BPDE-I isomers, C : [1,3-³H]BPDE-I-TG adducts, D : TG ; samples were two-dimensionally (arrows 1 and 2) developed from the origin.

Fig. 3. Dose-related formation of [1,3-³H]BPDE-I-TG in triglyceride (50 µg) treated with [1,3-³H]BPDE-I. The sensitivity of TLC to detect BPDE-I-TG was 20 pmol BPDE-I/µg total lipid.

Fig. 4. HPLC analysis of BPDE-I-TG adduct in TG treated with BPDE-I. A : triglyceride, B : BPDE-I, C : BPDE-I-TG adduct. Samples were eluted at a flow rate of 3 ml/min and detected at 280 nm with a sensitivity of 1.0 mA. Solvent A (hexane, 1% methanol, 0.5% tetraethylammonium (TEA) and B (THF, 1% methanol, 0.5% TEA) were used as a gradient mobile phase system : 40% B was used as the eluent for the first 25 min, and then increased to 100% B during the next 15 min, and remained at 100% B for 30 min.

Fig. 1.

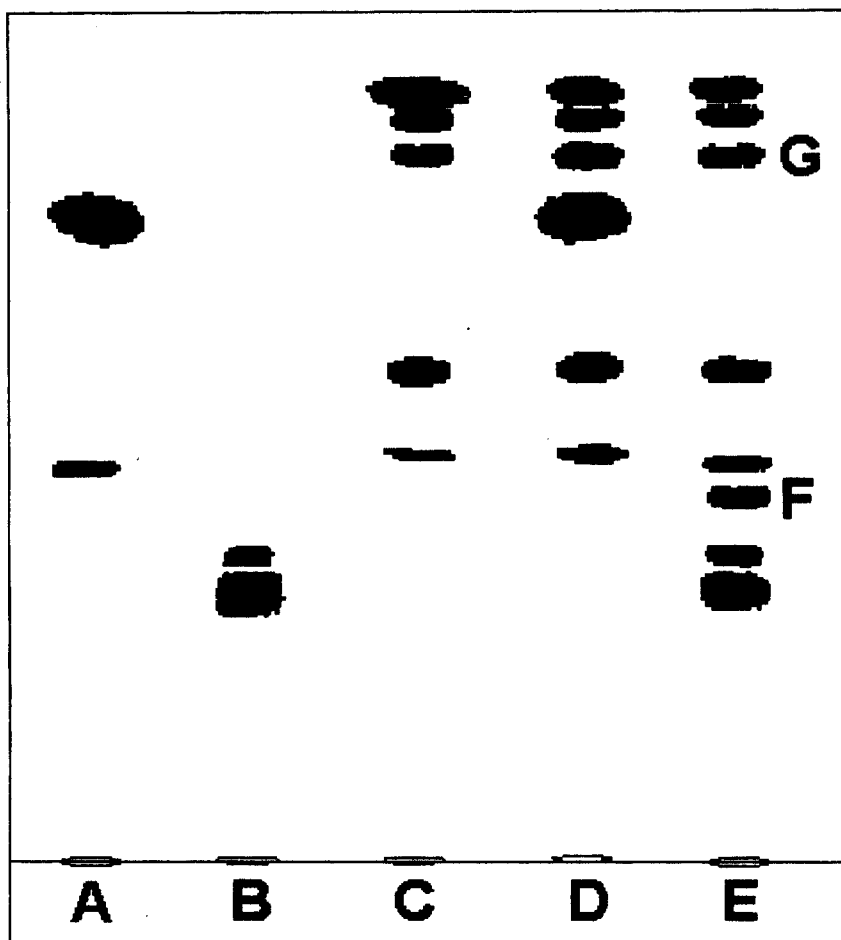


Fig. 2.

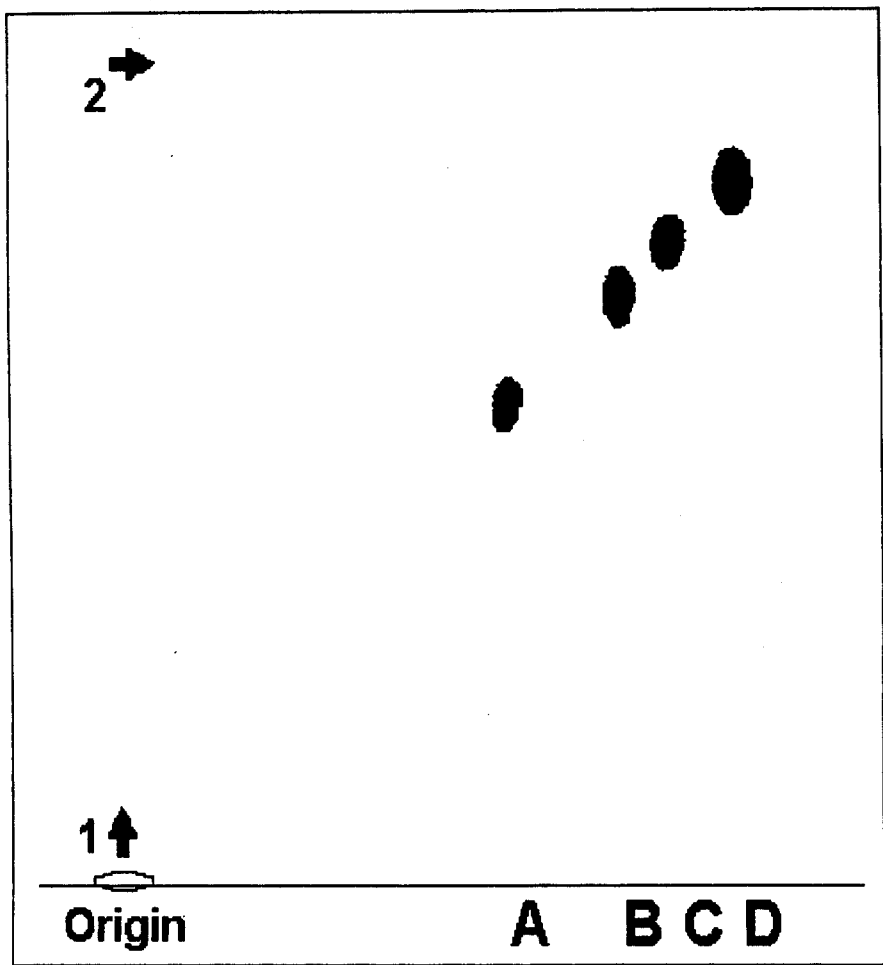


Fig. 3.

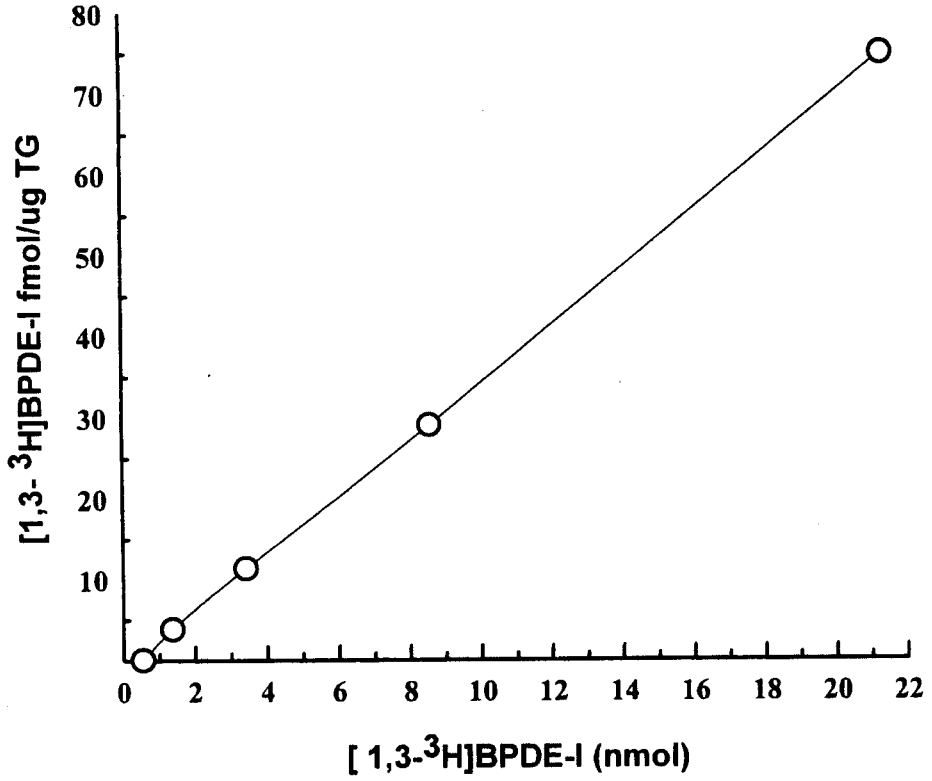


Fig. 4.

