Isolation and identification of inhibitory factor(s) of leukotriene D4 dipeptidase in human serum

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ISOLATION AND IDENTIFICATION OF INHIBITORY FACTOR(S) OF LEUKOTRIENE D₄ DIPEPTIDASE IN HUMAN SERUM

By

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LTD₄ dipeptidase is the enzyme that converts the most active leukotriene, LTD₄, to the least active form, LTE₄. The biological activity of LTD₄ has been reported to be several times higher than that of LTE₄. Therefore, the nature and intensity of a leukotriene response could be influenced by the relative concentration of LTD₄ and LTE₄, which in turn, could be determined by the relative rate of metabolism of LTD₄ to LTE₄. In undiluted, untreated human serum, inhibition of LTD₄ dipeptidase was observed. The specific activity of LTD₄ dipeptidase increased on dilution of human serum. To isolate and identify inhibitory factor(s), size exclusion chromatographic techniques were employed. The inhibitory factor(s) was separated from the serum dipeptidase by ultrafiltration with Amicon YM-10 membranes. The inhibitory activity in the YM-10 filtrate was stable on prolonged (over 30 minutes) heating at 95° C, stable in 0.3 M NaOH, and was destroyed by acid treatment at 0° C and 30° C. Ultrafiltration with YM-10, YM-3, YM-1, and YC-05 membranes suggested that the molecular weight of the inhibitor(s) was less than 500. Based on these observations and the elution volume of the inhibitor activity from a P-2 gel filtration column, the inhibitor has been tentatively identified as carbonate (bicarbonate). The LTD₄ dipeptidase activity in a 50-75% ammonium sulfate saturated fraction of serum was inhibited by carbonate (bicarbonate) or phosphate, but not by nitrate, sulfate and chloride. A maximum of 50% inhibition of dipeptidase activity was observed at 250 mM bicarbonate. Comparable concentrations of phosphate result in 100% inhibition. The physiological inhibitor in serum appeared to be carbonate (bicarbonate). The relative carbonate (bicarbonate) concentration could be a factor in determining the rate of LTD₄ to LTE₄ conversion, and therefore important in modulating the leukotriene mediated response.
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CHAPTER 1

INTRODUCTION

Arachidonic acid (5,8,11,14-eicosatetraenoic acid) is a 20-carbon polyunsaturated fatty acid. It is commonly present in cell membrane phospholipid (phosphatidylcholine, phosphatidyl-inositol, etc.) esterified primarily to the second carbon (C-2) position of glycerol. Release of arachidonic acid from the lipid pool by the action of phospholipase A_2 is mediated by different types of stimuli (57), which can be physiological or pathological. The released arachidonic acid has a very short half life. It is either metabolized by the enzyme, cyclooxygenase, to form prostaglandins and thromboxanes, or by lipoxygenase pathways to form leukotrienes and lipoxins (57), or is reincorporated into the membrane lipids (Fig. 1).

Arachidonic acid is derived primarily from two phospholipids, phosphatidylinositol and phosphatidylcholine. Two enzymes, phospholipase C and phospholipase A_2 are mainly involved in the release of arachidonic acid (Fig. 2). When cells are activated by stimuli, it leads to the increased permeability of ions across the membrane, especially a flow of Ca^{2+} ions from the extracellular to intracellular compartment. The resulting increased intracellular Ca^{+} concentration stimulates the cytoplasmic phosphatidylinositol specific phospholipase C, which acts on phosphatidylinositol to give inositoltriphosphate (IP_3) and diacylglyceride (DAG) (57). DAG is acted upon by other lipases to yield a
Figure 1. The metabolism of arachidonic acid
Figure 2. The release of arachidonic acid
monoacylglyceride and arachidonic acid. IP$_3$ induces additional Ca$^{2+}$ mobilization from the endoplasmic reticulum to the cytoplasm, and this further activates phospholipase C. Protein kinase C (PKC) is the major enzyme activated by DAG. PKC phosphorylates a phospholipase A$_2$ inhibitory protein. This phosphorylation causes inactivation of the inhibitory protein, resulting in increased activity of phospholipase A$_2$, which cleaves arachidonic acid from phosphatidylcholine (1).

The formation of prostaglandins and thromboxanes is initiated by the enzyme prostaglandin endoperoxide synthase (PES). The cyclooxygenase activity of the prostaglandin endoperoxide synthase can transform arachidonic acid and certain other polyunsaturated fatty acids into prostaglandins. The enzyme oxidizes arachidonic acid to yield a 15-hydroperoxy-9,11-endoperoxide with a substituted cyclopentane ring (PGG$_2$). The peroxidase activity of PES then reduces PGG$_2$ to its 15-hydroxy analogue (PGH$_2$). The cyclooxygenase and peroxidase activities of PES reside in a single protein (51). PGH$_2$ can be converted into PGD$_2$, PGE$_2$, PGF$_2$, and PGI$_2$ by the enzymes glutathione-S-transferase, prostaglandin endoperoxide E isomerase, prostaglandin endoperoxide reductase and prostaglandin endoperoxide I isomerase, respectively.

The formation of thromboxane A$_2$ is catalyzed by thromboxane A synthase. The enzyme that converts prostaglandin endoperoxides to thromboxanes was first found in platelets (51). The physiological effects of
prostaglandins and thromboxanes have been studied in great detail. They affect nearly every system in the body and include inflammation, clotting, osmotic gradients and smooth muscle contraction.

The lipoxygenase pathways involve insertion of oxygen molecules at 5-, 12-, and 15- carbon positions of arachidonic acid by 5-, 12-, and 15-lipoxygenases, respectively. The platelet 12-lipoxygenase converts arachidonic acid to 12-S-hydroperoxyeicosa-5,8,10,14-tetraenoic acid (12-S-HPETE). Peroxidase activity associated with the 12-lipoxygenase hydrolyzes 12-HPETE into its hydroxy analogue 12-HETE. The biological activities of the 12-lipoxygenase products include the migration of aortic smooth muscle cells in vitro, inhibition of collagen-induced platelet aggregation, inhibition of cyclooxygenase activity at high concentration, and the amplification of the production of LTB₄ (50).

The 15-lipoxygenase converts arachidonic acid to 15-hydroperoxide eicosatetraenoic acid (15-HPETE) and this product inactivates the enzyme by oxidizing a single methionine residue (44). At least two trihydroxylated compounds, lipoxin A (5,6,15(L)-trihydroxy-eicosa-7,9,11,13-tetraenoic acid) and lipoxin B (5(O),14,15 (L)-trihydroxy-eicosa-6,8,10,12-tetraenoic acid), are formed when human neutrophils are incubated with 15-HPETE and a calcium ionophore. These lipoxins are another group of biologically active derivatives which can cause contraction of lung strips, dilation of microvasculature, and inhibition of natural killer cell cytotoxicity. They
may also play an important role in inflammation and immunity (49).

The leukotriene family is a group of biologically active molecules, formed by leukocytes, mast cells, macrophages, and other tissues and cells in response to immunological and nonimmunological stimuli. They were first reported in 1938 as a smooth muscle-contracting factor in lung perfusates and referred to as "slow reacting substance of anaphylaxis" (SRS-A). It was not until 1979 that their structures were identified and their physiological functions elucidated.

The enzyme 5-lipoxygenase catalyzes the lipoxygenation of arachidonic acid at C-5 to initiate the synthesis of leukotrienes (23) (Fig. 3). Leukotriene A₄ (LTA₄) is a key intermediate in leukotriene biosynthesis. It is derived from arachidonic acid by two consecutive reactions, oxygenation at C-5 followed by dehydration, both catalyzed by 5-lipoxygenase. The highly unstable allylic epoxide LTA₄ can be metabolized by two alternative pathways. Hydrolysis of LTA₄, catalyzed by LTA₄ hydrolase leads to the formation of leukotriene B₄ (LTB₄) (50). LTB₄ can be further metabolized by ω-hydroxylation to 20-hydroxy and 20-carboxy LTB₄ (7). Alternatively, the addition of glutathione by a glutathione transferase leads to the peptidyl leukotriene, leukotriene C₄ (LTC₄) (3, 43). LTC₄ can be cleaved successively by peptidase enzymes to yield leukotrienes D₄ (LTD₄) and LTE₄.

The cysteinyl-containing leukotrienes (LTC₄, LTD₄, and LTE₄) are potent bronchoconstrictors. They increase permeability in postcapillary venules

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Figure 3. 5-lipoxygenase pathway
and stimulate mucus secretion (14, 43, 49). The dihydroxy derivative, LTB₄, which is a calcium ionophore, causes adhesion and chemotactic movement of leukocytes and stimulates aggregation, enzyme release, and generation of superoxide in neutrophils (43). LTC₄, LTD₄, and LTE₄, which are released from lung tissues of asthmatic subjects on exposure to specific allergens, seem to play a pathophysiological role in immediate hypersensitivity reactions (54). All of the leukotrienes have proinflammatory effects (47).

Leukotrienes are also formed in the central nervous system (50). The highest production of LTC₄ is found in hypothalamus and the median eminence. Immunohistochemical studies revealed the coexistence of LTC₄ and luteinizing hormone-releasing hormone (LHRH) in neurons in the median eminence. Furthermore, LTC₄ caused release of luteinizing hormone (LH) from rat anterior pituitary cells suggesting a neuroendocrine role of LTC₄.

Several other functions of LTs have been identified recently. Investigations by Quinn et al. showed LTC₄, LTD₄ and LTE₄ played a role as mediators of delayed onset acute lung injury after smoke inhalation (42). It was also reported that leukotrienes contributed to the persistence of a hydrochloric acid induced gastric lesion (39). Leikaut and his colleagues found that cysteiny1 leukotrienes enhanced growth of airway epithelial cells which suggested a new physiological role for the leukotrienes in the lung that links inflammation with epithelial cell proliferation (32). It was also
reported that LTC₄ decreased the activity of respiratory cilia \textit{in vitro} (61).

LTC₄ is converted to LTD₄ by cleavage of the glutamic acid residue by a γ-transpeptidase (Fig. 4). This enzyme has been found in rat basophil leukemia cells (25), in the supernatant of PMNs (6, 45), in human plasma (28) and lung (29), and in guinea pig lung tissue. The study of the transpeptidase in human serum in our lab and other laboratories showed that the metabolism of LTC₄ into LTD₄ was dependent on time, amount of enzyme present and on the substrate concentration (60). Several forms of the serum γ-glutamyl transpeptidase have been reported and have been described as isoenzymes or modifications of a single gene product.

Cleavage of a glycine residue from LTD₄ by dipeptidase activity results in the formation of LTE₄. The relative activity of the γ-transpeptidase and the LTD₄ dipeptidase might have important physiological consequences. The dipeptidase catalyzes the conversion of the most active peptidyl leukotriene, LTD₄, to the least active peptidyl leukotriene, LTE₄. The pioneering work on dipeptidase was done by Campbell who first purified a porcine renal dipeptidase (9). Dipeptidase enzymes have been subsequently isolated from many different tissues and cells including: rat, hog (9), human (11), and monkey kidneys, sheep (10), rat (22), human (29), and guinea-pig lungs, rat liver (24), rat basophil leukemia cells (25), human PMNs (36, 45, 46), and human serum (28, 60). Time course studies showed a relatively slow conversion of LTD₄ to LTE₄,
Figure 4. Peptidyl leukotriene metabolism

- **γ-glutamyl-transpeptidase** catalyzes the conversion of **LEUKOTRIENE C₄** to **GLUTAMATE**.
- **DIPEPTIDASE** catalyzes the conversion of **LEUKOTRIENE D₄** to **GLYCINE**.
- **DIPEPTIDASE** catalyzes the conversion of **GLYCINE** to **LEUKOTRIENE E₄**.
compared to the rate of conversion of LTC₄ to LTD₄ (28). Dipeptidase is usually found as a membrane bound enzyme. Recently, Campbell and his coworkers (10) showed that the protein is anchored to the lipid bilayer by a covalent attachment to phosphatidylinositol via a C-terminal glycolipid extension. The dipeptidase was released by the treatment of phosphatidylinositol-specific phospholipase C from Bacillus thuringiensis. Size exclusion chromatography (SEC)-HPLC and electrophoresis analysis showed the molecular weight of the enzyme is between 105,000-140,000 daltons. The enzyme is composed by two monomers of molecular weight 50,000 to 70,000 daltons (10). The optimal reaction condition for the dipeptidase activity was obtained with 1 mM of Mn²⁺, Co²⁺, or Zn²⁺ (44, 36), and was completely inhibited by Cu²⁺ (44), bestatin (11, 40) and a renal dipeptidase inhibitor, cilastatin (MK0791) (27). The presence of a natural inhibitory effect of LTD₄ conversion has been mentioned briefly by Raulf and coworkers (45, 46). Studies in our lab have suggested some type of inhibitor(s) is present in human serum (60). The LTD₄ dipeptidase activity in human serum appeared to be regulated by the amount of the inhibitor(s) rather than the amount of enzyme. This indicates that the inhibitor(s) in human serum may play a very important role in regulating the leukotriene levels in human circulation under different physiological and pathological conditions (60).

Further metabolism of LTE₄ has been reported in several tissues.
Bernstrom and his colleagues (5) reported that rat liver homogenates can covert LTE\(_4\) to acetyl-LTE\(_4\), with the acetyl group from acetyl coenzyme A. Acetyl-LTE\(_4\) was the major metabolite recovered in the bile (21). It was also found that the human and rat kidneys convert the LTE\(_4\) to acetyl-LTE\(_4\) which was then found in the urine. LTA\(_4\) can also undergo nonenzymatic hydrolysis into the C-12 epimers of, 12-dihydroxy-6,8,10-trans-14-cis-eicosatetraenoic acid and two isomers of 5,6-dihydroxy-7,9,11,14-eicosatetraenoic acid (20).

Although the biological effects of LTE\(_4\) and acetyl-LTE\(_4\) are 100-fold lower than that of LTD\(_4\), they still have biological activity (48). So, the further metabolism of LTE\(_4\) and acetyl-LTE\(_4\) resulting in complete termination of the biological activity has been extensively examined (4). More recently, Shamhoun and his coworkers showed another route of degradation of LTE\(_4\) and acetyl-LTE\(_4\) via \(\omega\)-oxidation and \(\beta\)-oxidation which alters the fatty acid (16, 48). The end products formed here were 20-COOH-LTE\(_4\), 20-COOH-N-acetyl LTE\(_4\), 18-COOH-19,20-dinor-LTE\(_4\) (dinor-LTE\(_4\)), and 16-COOH-17,18,19,20-tetranor-LTE\(_4\) (tetranor-LTE\(_4\)). These results demonstrate that the metabolism of leukotrienes may be a detoxification rather than an inactivation of cysteinyI-containing leukotrienes.
THESIS OBJECTIVE:

The major objectives of this study were to isolate, characterize, and hopefully identify the factor(s) which inhibits LTD₄ dipeptidase activity in human serum.
Materials:

HPLC Grade methanol and acetonitrile were purchased from Fisher Chemical, Fisher Scientific (Fair Lawn, NJ). Leukotrienes were purchased from Cayman Chemical (Ann Arbor, MI), and the tritium labelled leukotrienes were purchased from New England Nuclear (Boston, MA). The resin, nucleosil 5 C18 100A, for reverse phase HPLC was obtained from Phenomenex (Torrance, CA), and the columns were generously packed by ChromatoChem, Inc. (Missoula, MT). YM membranes were purchased from Amicon Div., W.R. Grace & Co. (Danvers, MA). The Bio-Gel P-2 was from Bio-Rad Laboratories (Richmond, CA). The bicinchoninic acid protein assay reagents were purchased from Sigma Chemicals (St. Louis, Mo). All other chemicals were of reagent grade and purchased from standard sources.

Methods:

1. Collection of Human Blood and Sera:

Human blood from healthy volunteers was drawn from the cubital vein into heparinized or heparin-free vacutainers. The blood was set in the 30° C water bath for 30 min until the clotting was completed, and was centrifuged at 2,000 rpm, at 4° C, for 30 min. The serum was collected using a Ulti-Sep
Serum Separator, and pooled. The pooled sera was divided into 1 ml aliquots and stored at -70° C.

2. Separation of LTD₄ Dipeptidase from the Inhibitory Factor(s)

Serum was mixed with 20 mM Tris-HCl buffer (pH 7.4) in a 1:50 ratio and filtered with an Amicon Micro-ultrafiltration system using a YM-10 membrane. The flow rate was 10 ml per hour under 40 psi nitrogen at 5° C. The filtered serum was divided into 1 ml aliquots and stored at -70° C.

3. Ammonium Sulfate Fractionation of LTD₄ Dipeptidase:

The method developed by B. J. Campbell (9) was modified and used. In a typical preparation, 3 grams solid ammonium sulfate were added to 10 ml of pooled sera, mixed and allowed to stand at 4° C for 12 hours. The 50% ammonium sulfate saturated sera was then centrifuged at 4,500xg for 1 hour. The pellet was resuspended in 5 ml 20 mM Tris-HCl Buffer at pH 7.4 and dialyzed against the same buffer for 18 hours with four changes of buffer. Solid ammonium sulfate was then added to the supernatant to result in 75% saturation. After standing at 4° C over night, the 75% ammonia sulfate saturated suspension was centrifuged at 10,000xg for 1 hour. The sediment was resuspended in 5 ml 20 mM Tris-HCl buffer at pH 7.4 and dialyzed as previously described. The two different fractions will be designated as 50% precipitate and 75% precipitate.
4. Isolation and Concentration of Inhibitor by Ultrafiltration:

In a typical experiment, 50 ml pooled sera was added to an Amicon ultrafiltration system model 52 with a YM-10 membrane, under 40 psi nitrogen at 4° C. The filtrates from several runs (about 40 ml) were pooled, divided to 20 ml aliquots and lyophilized. The lyophilized filtrate was resuspended in 1 ml triple distilled water and was designated as 20X concentrated inhibitor solution.

5. Purification of Inhibitor by Gel Filtration:

Size Exclusion Chromatography (SEC) was used to attempt to further isolate the inhibitor. The Bio-Rad polyacrylamide gel resin P-2 was used. Best resolution was obtained with a 1.3 X 45 cm column using an eluent buffer containing 10 mM Mops and 10 mM NaCl, pH 7.4. The flow rate was controlled between 12-15 ml per hour, and ferritin, vitamin B₁₂, and dinitrophenylalanine (DNP-ala) were used as the molecular weight markers. One ml fractions of eluate were collected with a ISCO model 1850 Fraction Collector and UV-absorbance was measured with a model UA-5 Absorbance/Fluorescence Monitor (ISCO, INC.).

6. Bicarbonate Determination:

BaCl₂, at a final concentration of 20 mM, was added into all 1 ml-fractions from the P-2 column. The white precipitation of Ba(HCO₃)₂ was
monitored by absorbance reading at a wavelength of 600 nm with a Perkin-
Elmer Lambda 3A UV/VIS spectrophotometer.

7. Assay of Leukotriene D₄ Dipeptidase Activity:

The reaction mixture consisted of a mixture of 100 ng synthetic unlabeled
LTD₄, tritium labeled LTD₄ 0.8 μl (0.1 ng LTD₄, specific activity: 1.8x10⁴
dpm), and 100 μl enzyme (protein concentration: 27.1-60.6 μg/μl) in a 100
mM NaCl, 5 mM CaCl₂, 0.1% triton, and 100 mM Mops buffer at a pH of
7.4. The mixture was incubated in 37° C water bath for 15 to 35 min
depending on which enzymatic activity was being measured. The reaction
was stopped by adding 0.5 ml cold methanol (-20° C). The mixture was then
stored at -70° C for at least 1 hour prior the lipid extraction.

8. Lipid Extraction:

Cold methanol was used to stop the reaction and extract the lipid in the
mixture. The mixture was centrifuged at 2,000xg for 30 min at 4° C. The
supernatant was transferred to a conical centrifuge tube and the residue
was extracted with another 0.5 ml methanol. After centrifugation the
supernatants were pooled and evaporated to dryness under vacuum at 40°
C. The samples were stored at -70° C until HPLC analysis. All glassware
used for the assays was silicone-treated to prevent the adherence of lipids to
the surface, and to minimize loss of leukotreines.
9. RP-HPLC Analysis:

A C18 5 µm 100 Å nucleosil HPLC column, 4.6 X 250 mm, was used for reverse-phase HPLC analysis. The solvent used for isocratic eluting contained acetonitrile, methanol, acetate, and distilled water (320:200:1:479). Disodium EDAT (0.5%) was added and pH was adjusted to 5.5 with ammonium hydroxide. The leukotriene extract was dissolved in 0.15 ml eluting solvent and eluted from the column at a flow rate of 1.0-1.5 ml/min. The products of [³H] LTD₄ metabolism were quantified by the distribution of tritium counts in the different fractions using an in-line radioactivity flow detector.
CHAPTER 3
RESULTS

1. LTD₄ dipeptidase inhibitor activity in human serum:

Previous experiments in our laboratory showed that dilution of serum did not result in a corresponding dilution of LTD₄ dipeptidase activity. A typical experiment using a series of two-fold serum dilutions is shown in Fig. 5. The highest specific activity was usually observed at a 1:32 dilution. This indicates the presence of some type of inhibitory factor(s) in the serum.

2. Serum fractionation:

The inhibitory factor(s) was successfully separated from the LTD₄ dipeptidase by using an Amicon ultrafiltration system with a YM-10 membrane. As shown in Fig. 6, the LTD₄ dipeptidase activity was increased after removing the inhibitory factor(s) by filtration, and showed an almost linear decrease in dipeptidase activity with serial dilution. The products of LTD₄ formed in the presence of a 25-50% ammonium sulfate fraction and a 50-75% ammonium sulfate serum fraction are shown in Fig. 7. Based on the lower amount of the unidentified metabolite produced by the 50-75% fraction, this preparation was used for the separated studies. Using the synthetic LTE₄ as a substrate, it was shown that the unknown metabolite was a product of LTE₄ metabolism (Fig. 8). There was no dipeptidase activity in either 0-25% ammonium sulfate fraction or in the 75-100%
Figure 5. Untreated serum was 2-fold series diluted with 100 mM Mops buffer, pH 7.4. The enzyme assay was carried out as described in Materials and Methods (M&M) part 7. HPLC method described in M&M part 8 and 9 was used to quantify the conversion of leukotrienes. Solid line shows the LTD₄ conversions with the dilution of the untreated serum. Dotted line shows the specific activities of the untreated serum with the dilution. The specific activity is expressed as pmol leukotriene converted per min per mg protein.
Figure 6. Human serum LTD₄ dipeptidase activity was separated from the inhibitory factor(s) by an Amicon Micro-ultrafiltration system using a YM-10 membrane as described in the part 2 of M&M. Then dilution and enzyme assay were carried as described in Fig. 5. Solid line shows the filtered dipeptidase activity. Dotted line shows the untreated serum.
Figure 7. Ammonium sulfate fractionation of serum was carried out as described in the part 3 of M&M. Different fractions were tested for the dipeptidase activity. The distribution of radioactivity counts on chromatograph was used to monitor the conversion of LTD₄. (a) LTD₄ control. (b) LTD₄ was mainly converted to LTE₄ by a dipeptidase activity in 50-75% ammonium sulfate precipitation. (c) LTD₄ was converted to an unknown metabolite by an enzymatic activity in the 25-50% ammonium sulfate precipitation fraction.
Figure 8. 100 ng LTE₄ was used as the substrate incubating with the 25-50% saturated ammonium sulfate precipitation fraction. Standard LTE₄ and metabolized LTE₄ were measured at a wavelength of 280 nm. (a) LTE₄ control. (b) Unknown metabolite with a later retention time after LTE₄ was incubated with 25-50% saturated ammonium sulfate precipitation fraction.
ammonia sulfate supernatant.

3. Optimal conditions for LTD₄ dipeptidase activity:

Fig. 9 shows the time course study of the LTD₄ dipeptidase activity in the 50-75% saturated ammonium sulfate fraction. Under this assay condition, over 90% of the synthetic LTD₄ was converted after 50 min incubation. The influence of different cations is shown in Fig. 10. The enzyme was about 30% inhibited by 20 mM EDTA and 10 mM EGTA, and was activated by 10 mM Ca²⁺ and 2.9 mM Mn²⁺. Fig. 11, 12, and 13 show that optimal activity was obtained with 100 mM NaCl and 5 mM CaCl₂, which both are close to the physiological concentration in human serum.

4. Separation of the inhibitory factor(s) from serum by ultrafiltration:

The Amicon ultrafiltration system with the YM-10 membrane was used to separate the LTD₄ dipeptidase inhibitory factor(s) from the serum as described in materials and methods. After lyophilization, the filtrate was resuspended in a volume of 20 times concentrated (20X). The addition of the concentrated inhibitor(s) to the dipeptidase assay mixture resulted in the inhibition of LTD₄ dipeptidase action (Table 1).

5. Characterization of the inhibitor:

The concentrated YM-10 filtrate was incubated at 95°C for 30 min and assayed for inhibitory activity. The inhibitory activity was not destroyed. The concentrated YM-10 filtrate (20X) was stable in 0.3 N NaOH (20 μl 2 N NaOH in 100 μl concentrated filtrate, 20X), whereas the
Figure 9. LTD₄ Dipeptidase of human serum incubated with LTD₄ for various incubation period. Same assay conditions were used as described in Fig. 5.
Figure 10. Different cations and chelators were added to observe their effects on the dipeptidase. Standard enzyme assays as described in Fig. 5. was used. Different cation concentrations were added based on their physiological concentrations. 1. Buffer control. 2. Enzyme control. 3. EDTA (20 mM). 4. EGTA (10 mM). 5. Ca$^{2+}$ (10 mM). 6. Mg$^{2+}$ (4 mM). 7. Fe$^{3+}$ (0.084 mM). 8. Mn$^{2+}$ (2.9 mM). 9. Zn$^{2+}$ (0.44 mM). 10. Cu$^{2+}$ (0.071 mM).
Figure 11. The influence of NaCl concentration on LTD₄ dipeptidas activity was studied by adding different NaCl concentrations into the standard enzyme assays.
Figure 12. The influence of CaCl$_2$ concentration on LTD$_4$ dipeptidase activity was studied by adding different concentration of Calcium into the standard enzyme assays.
Figure 13. Optimal condition for dipeptidase activity was achieved by adding sodium chloride and Calcium chloride into the reaction mixture. 1. Buffer control. 2. Enzyme control. 3. 100 mM NaCl 4. 5 mM CaCl$_2$. 5. 100 mM NaCl + 5 mM CaCl$_2$. 

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### TABLE 1. THE DIPEPTIDASE INHIBITION PROPERTIES OF YM-10 FILTRATE

<table>
<thead>
<tr>
<th>SAMPLES</th>
<th>W/ YM-10 FILTRATE % OF LTD. CONVERSION</th>
<th>W/O YM-10 FILTRATE % OF LTD. CONVERSION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17.47</td>
<td>79.90</td>
</tr>
<tr>
<td>2</td>
<td>13.91</td>
<td>83.84</td>
</tr>
<tr>
<td>3</td>
<td>18.34</td>
<td>83.50</td>
</tr>
<tr>
<td>4</td>
<td>21.04</td>
<td>86.20</td>
</tr>
<tr>
<td>5</td>
<td>17.71</td>
<td>84.86</td>
</tr>
<tr>
<td>6</td>
<td>17.47</td>
<td>86.36</td>
</tr>
</tbody>
</table>

\[X \pm SD, n=6\] \hspace{1cm} 17.66\pm2.08\% \hspace{1cm} 84.11\pm2.17\%

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Figure 14. The separation of LTD₄ Dipeptidase inhibitor by Bio-Rad gel P-2 Column was described in part 5 of M&N. (a) Molecular weight markers control: peak 1, ferritin; peak 2, VitB₁₂; peak 3, DNP-ala. (b) Concentrated inhibitor from YM-10 filtrate (20X). The inhibition was recovered from 49 to 60 ml with a pH range from 8 to 8.5. (c) Bicarbonate with the MW markers.
Figure 15. Comparison of retention time of synthetic bicarbonate and LTD$_4$ Dipeptidase Inhibitor from Serum. As described in part 6 of M&M, two indicators were mainly measured: pH and carbonate (bicarbonate). Solid line: pH of concentrated YM-10 filtrate eluted from P-2 column. Dotted line: Absorbance of Ba(HCO$_3$)$_2$ precipitation formed by synthetic bicarbonate eluted from P-2 column and BaCl$_2$ added.
inhibitory activity was destroyed in 0.3 N HCl (20 µl 2N HCl in 100 µl concentrated filtrate, 20X). The inhibitory effect was eliminated by treating with 0.3 N HCl at either 0°C or at 65°C for 30 min (Fig. 17).

Concentrated filtrate from the YM-10 membranes was filtered with YM-3, YM-1, and YC-05 membranes. Inhibitory activity was recovered in the filtrate from each of these membranes which suggested a molecular weight of less than 500 (Fig. 16). Elution from the P-2 gel filtration column, however, suggested a molecular weight of about 800-1000 (Fig. 14B).

6. Purification of inhibitory factor(s) by gel filtration:

The inhibitor(s) was eluted from a Bio-Rad gel P-2 column with a 10 mM NaCl, 10 mM Mops buffer pH at 7.4. As shown in Fig. 14B, the inhibitory factor(s) was recovered from the P-2 column in fractions from 49 ml to 60 ml. The retention time of the inhibitory factor(s) from the P-2 column corresponds to a molecular weight around 800-1,000 based on internal molecular weight markers vitamin B₁₂ (MW 1,355) and DNP-ala (MW 255) (Fig. 14A). The pH of the 49th ml-60th ml fractions was 8.0-8.4 compared to 7.0-7.4 for all other fractions (Fig. 15). The pH of all concentrated fractions was adjusted to 7.4 for the enzyme inhibitor assay.

7. Identification of inhibitor(s):

The inhibitor(s) was heat stable, acid labile, and resulted in an increase in pH when concentrated. Based on these observations we suspected that the inhibitor(s) might be bicarbonate. This did not
correspond to the expected elution volume from the P-2 column. The apparent contradiction was solved when analytical grade sodium bicarbonate was run through the P-2 column and the same retention time was obtained as that of the inhibit activity and high pH of the concentrated filtrate, 20X (Fig. 14, 15). The bicarbonate elution was qualitatively monitored by adding BaCl$_2$ resulting in a production of white precipitate.

8. Inhibitory activities of bicarbonate and other anions:

The effect of different anions is shown in Fig. 18. The inhibitory activity was found for bicarbonate and phosphate, whereas no inhibition was observed with sulfate or nitrate. Fig. 19 and 20 show that the inhibitory activity of carbonate and phosphate was dose dependent. The maximum inhibition caused by bicarbonate (250 mM) was about 50%, while that of the phosphate (400 mM) was 100%.
Figure 16. Amicon membranes were used to determine the Molecular Weight of LTD₄ dipeptidase inhibitor. 100 µl of 20X inhibitor solution passed different membranes were added to standard enzyme assays. Inhibition was observed in all size of membranes used. 1. Buffer control. 2. Enzyme control. 3. YM-10. 4. YM-3. 5. YM-1. 6. YC-05.
Figure 17. 20X Inhibitor solution was pretreated with 0.4 N HCl under heating and non-heating conditions. The inhibitory activity was eliminated by the acid. 1. Buffer control. 2. Enzyme control. 3. Inhibitor control. 4. Inhibitor with 0.4 M HCl on ice. 5. Inhibitor is heated at 65° C for 30 min with presence of 0.4 M HCl. 6. NaCl control. 7. Salt+inhibitor control.
Figure 18. Inhibition of anions on LTD₄ dipeptidase activity was assayed by adding different anions concentration into standard enzyme assay. 1. Buffer control. 2. Enzyme control. 3. Bicarbonate (100mM). 4. Phosphate (100mM). 5. Sulfate (100mM). 6. Nitrate (200mM).
Figure 19. Different amount of bicarbonate was incubated with 50-75% ammonium sulfate saturated precipitation fractionation of human serum. A 50% inhibition was observed when 250 mM bicarbonate was added.
INHIBITION OF DIPEPTIDASE
THE EFFECT OF PHOSPHATE

Figure 20. Different amount of phosphate was incubated with 50-75% ammonium sulfate saturated precipitation fractionation. A 100% inhibition was observed when 400 mm phosphate was added.
The peptidyl leukotrienes of the 5-lipoxygenase pathway of arachidonic acid metabolism have been identified as the active ingredients of the substances collectively known as slow-reacting substance of anaphylaxis (SRS-A). The leukotrienes rapidly gained substantial attention when the potency of their biological activities on certain cells and organs were found to exceed that of many traditional autocoids such as histamine, catecholamines, kinins, and prostaglandins (8). Of particular importance is the action of the leukotrienes on the pathological processes of allergic reactions, endotoxemias, tissue injury, and inflammatory responses. Identification and characterization of the receptors of leukotrienes and their natural and synthetic inhibitors have been extensively studied (18, 26, 35, 45, 55, 60, 63). Such studies were undertaken to try to develop specific pharmaceutical agents which may prevent, moderate, or reverse leukotriene-dependent reactions, and to use such therapeutic reagents to treat leukotriene related diseases.

LTD$_4$ dipeptidase is the enzyme which converts the most active leukotriene, LTD$_4$, to the least active form, LTE$_4$. The biological activity of LTD$_4$ has been reported to be 25 to 100 times higher than that of LTE$_4$ (19, 48, 58). It is probable, therefore, that the nature and intensity of the
response would be influenced by the relative concentrations of LTC₄, LTD₄, and LTE₄ which, in turn, could be determined by the relative rates of metabolism of LTC₄ to LTD₄ and LTD₄ to LTE₄. The marked increase in the specific activity of the LTD₄ dipeptidase in the serum dilution experiments (Fig. 5) suggested that the enzyme activity is determined by inhibitor concentration rather than enzyme concentration. Since the first dipeptidase was isolated from porcine kidney in 1969 (9), enzymes from several different species and tissues have been isolated and characterized (10, 11, 17, 22, 27-29). Likewise, several dipeptidase inhibitors have been also studied. Generally, bestatin (10, 11, 40) and D-penicillamine (24) cause noncompetitive inhibition of dipeptidase, whereas cilastatin (27), dithiothreitol, and chloroacetyldehydrophenylalanine are competitive inhibitors of the enzyme (10, 11). Inorganic phosphate, ATP, ADP, and AMP have also been reported to inhibit the dipeptidase (9). Some divalent metal ions may also play important roles in dipeptidase metabolism, since the metal chelator, o-phenanthroline, can inhibit the dipeptidase activity, and the inhibition can be reversed by adding divalent metal ions such as Co²⁺, Mn²⁺, and Zn²⁺. This also suggests that the dipeptidase from certain sources may be a metalloenzyme (36). None of these dipeptidase inhibitors seems a likely candidate for the natural inhibitor in serum (Table. 2). The inhibition of the dipeptidase in undiluted human serum was suggested by the observations of Koller et al (28). They assayed the γ-transpeptidase
TABLE 2. COMPARISON OF THE LTD₂ DIPEPTIDASE
SENSITIVITY TO BESTATIN AND CILASTATIN

<table>
<thead>
<tr>
<th>Dipeptidases</th>
<th>Inhibitor</th>
<th>Concentration (mM)</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Serum</td>
<td>Bestatin</td>
<td>0.001</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.005</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.05</td>
<td>50%</td>
</tr>
<tr>
<td></td>
<td>Cilastatin</td>
<td>0.001</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.005</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.05</td>
<td>50%</td>
</tr>
<tr>
<td>Sheep Lung</td>
<td>Bestatin</td>
<td>0.001</td>
<td>6%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.005</td>
<td>41%</td>
</tr>
<tr>
<td></td>
<td>Cilastatin</td>
<td>0.001</td>
<td>48%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.005</td>
<td>100%</td>
</tr>
</tbody>
</table>

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in undiluted human serum but used a 1:50 dilution for the dipeptidase assay. A similar phenomenon was observed by Foster and his coworkers (17) in their studies of LTC₄ metabolism in the anesthetized rat. Intravenously injected LTC₄ was rapidly converted to LTD₄, while the conversion of LTD₄ to LTE₄ was considerably slower. The results of our studies suggested to us that carbonate (bicarbonate) is responsible for the LTD₄ dipeptidase inhibitory activity in normal serum. The dipeptidase activity did not decrease until the serum was diluted 32 fold. After filtration with a YM-10 membrane, the bicarbonate and phosphate were removed in the filtrate, and the retained enzyme activity decreased with dilution as expected. Inhibitions caused by bicarbonate and phosphate are dose dependent. Bicarbonate is a more likely the main inhibitor in serum than phosphate because of its higher physiological concentration (25 mM, for bicarbonate, vs. 2 mM for phosphate). The finding that inorganic phosphate inhibits human serum LTD₄ dipeptidase is consistent with the results of Campbell and his coworkers who identified inorganic phosphate as an inhibitor for porcine renal dipeptidase (9). Our studies represent the first time that bicarbonate was identified as a natural inhibitor of LTD₄ dipeptidase in human serum. The experiments in our lab also showed that LTD₄ dipeptidase activity in human serum is less sensitive than previously reported inhibitors, cilastatin and bestatin (Table 2). Additionally, the dipeptidase activity in human serum is not dependent on divalent metal
ions, and was not specifically activated by Co$^{2+}$, Mn$^{2+}$, and Zn$^{2+}$.

Furthermore it was interesting to find that higher concentrations of divalent ions (5mM), which can precipitate bicarbonate, would increase the dipeptidase activity. Addition of the chelator, EDTA, restores the inhibitory effect. These results suggest that it was the depletion of bicarbonate by the divalent ions which increased the dipeptidase activity in human serum rather than a stimulation of enzymatic activity.

Asthma is a disease characterized by episodic bronchoconstriction, hypersecretion of mucus, and inflammation of airways. It was estimated that approximately five percent of adults and seven to ten percent of children in the United States have the disorder (56). Evidence suggests that the 5-lipoxygenase pathway metabolites of arachidonic acid, especially the peptidyl leukotrienes (LTD$_4$, LTC$_4$, LTE$_4$), have a very important role in mediating the physiologic events in asthma. Schwartzberg and his colleagues observed a 50-fold elevation of LTC$_4$ levels in children with an acute asthma attacks (52-54). An increased level of leukotrienes was found in bronchoalveolar lavage fluid from atopic asthmatics (62). Radeau and his coworkers reported an enhanced arachidonic acid metabolism in asthma patients (43). The elevated level of leukotrienes may be a result of either increased release from the inflammatory cells (eosinophiles, macrophage etc.) or inhibition of leukotriene degradation by the leukotriene metabolizing enzymes or both. Our experimental data showed that bicarbonate is one of
the inhibitory factors in human serum. Because it is inhibitory at physiological concentrations, it may play a major regulatory role in regulating the LTD₄ dipeptidase activity in human serum.

Bicarbonate is the major component of the carbonic/bicarbonate buffering system in human circulation. The system is defined by the Henderson-Hasselbach equation: pH = 6.1 + log ([HCO₃⁻]/[H₂CO₃]). Mainly two organs and one enzyme are involved in regulating the carbonic acid/bicarbonate buffer system. The carbon dioxide produced by aerobic metabolism of different tissues is transported to the lung and exhaled. The rate of respiration and CO₂/O₂ exchange capacity of the lung is one method contributing to the regulation this system. The kidneys are the second contributor in regulating the buffer system. The bicarbonate is reabsorbed when its serum concentration is less than 26 mEq/L, and it is excreted in the urine if its serum concentration is greater than 26 mEq/L. Bicarbonate is reabsorbed in the proximal and distal tubule, with 85 to 90 percent reabsorbed proximally, and 10 to 15 percent distally. The enzyme involved in regulating this buffer system is carbonic anhydrase. This enzyme exists at a high concentration in red blood cells, and on the luminal surface of proximal tubule cells in kidneys. It catalyzes the reversible reaction: CO₂ + H₂O = H₂CO₃ = H⁺ + HCO₃⁻. The first reaction rate, catalyzed by carbonic anhydrase, is several hundred times higher than that without the enzyme. The second reaction occurs rapidly without an enzyme. The CO₂ produced
by the aerobic metabolic process diffuses from cells into tissue capillaries and enters red blood cells. The carbonic anhydrase in the red blood cells converts the CO_2 into HCO_3^- . The newly produced bicarbonate diffuses into plasma because cell membranes are much more permeable to negatively charged anions (e.g. HCO_3^-, Cl^-) than to positively charged cations (e.g. K^+, H^+). The electrostatic difference across the cell membrane created by the movement of HCO_3^- can be neutralized by the movement of Cl^- into the red blood cells, which is called the "chloride shift". This is the major source of bicarbonate for the carbonic acid/bicarbonate buffer system. The reverse reaction is carried out when bicarbonate is reabsorbed in the kidney by the carbonic anhydrase on the surface of proximal tubule cells. Under normal physiological conditions, when a fixed acid is produced in extracellular circulation, it can be buffered by bicarbonate. The CO_2 produced is exhaled from lung. When an airway obstruction happens, the O_2/CO_2 exchange is partially or completely dysfunctional, the CO_2 produced by the human body will be retained in the tissue capillaries. The retained CO_2 may accelerate the enzymatic conversion to bicarbonate, which is catalyzed by carbonic anhydrase. This study suggests that increased bicarbonate levels might inhibit the LTD_4 dipeptidase activity, causing a higher concentration of the more biologically active leukotrienes. The elevated LTD_4 may enhance the inflammatory process, mediate the immediate hypersensitivity reaction, and cause more severe clinical symptoms and manifestations. Previous
researchers have also shown that there are LTC₄ and LTD₄ receptors on the surface of human red blood cells (18). No physiological functions of these receptors have been identified, and it has been confirmed that there are no leukotriene metabolizing enzymes in the red blood cells. It would be interesting to determine the effect of LTD₄ on carbonate metabolism in erythrocytes. Under asthmatic conditions, bicarbonate levels in the blood should increase because of oxygen deprivation. This may result in increased CO₂ and HCO₃, and consequently decreased detoxification of LTD₄ by serum dipeptidase. Modulating the effects of leukotriene may be proposed as follows. At elevated levels, leukotrienes may bind to the receptors on red blood cells. This binding may result in the generation of a second messenger, such as cAMP, which would modulate and ultimately inhibit the activity of carbonic anhydrase. Decreased carbonic anhydrase activity would result in decreased bicarbonate levels, leading to increased dipeptidase activity. As leukotriene levels fall on detoxification by dipeptidase, decreased generation of the second messenger would occur, carbonic anhydrase activity would increase and serum bicarbonate levels would return to normal. These processes would be initiated by the obstruction and relief of the obstruction of the airways. The discovery of a possible role for bicarbonate as the inhibitor of leukotriene metabolism may lead to the development of new therapeutic agents in treating leukotriene associated diseases, especially anti-asthma drugs.
The major phosphate stores in humans reside intracellularly in cell cytoplasm. The organic phosphate is incorporated into lipid as phospholipids which compose the cell membranes. Phosphorous is mainly found as a bone component and exists at relatively low levels as phosphate in the extracellular circulation. A phosphate buffering system also plays a modest role in regulating the pH of blood. Three buffer pairs, $\text{H}_2\text{PO}_4^-$ /$\text{H}_3\text{PO}_4$, $\text{HPO}_4^{2-}$/$\text{H}_2\text{PO}_4^-$, and $\text{PO}_4^{3-}$/$\text{HPO}_4^{2-}$, could potentially be formed. Under the physiological conditions at pH 7.4, the $\text{HPO}_4^{2-}$/$\text{H}_2\text{PO}_4^-$ system with a pK of 6.8 is most important. At pH 7.4, there will be four molecules of $\text{HPO}_4^{2-}$ for every molecule of $\text{H}_2\text{PO}_4^-$. Experimental data indicated that inhibition by phosphate occurs at a concentration almost 20 times higher than normal physiological levels (2 mM). This suggests that the phosphate inhibition of LTD$_4$ dipeptidase may play a minor part in regulating the leukotriene levels in serum, except under extreme conditions. High concentrations of intracellular phosphate can be released into a lesion, and could cause a limited and localized inhibitory effects. As demonstrated by Campbell and his coworkers (9), ATP, ADP, and AMP showed some inhibition in hog renal dipeptidase. The phosphate groups on the nucleotide may explain the inhibition, and the adenosine with most phosphate residues causes the stronger inhibition, such that, ATP is more inhibitory than ADP (ATP>ADP>AMP). Further research may be carried out to reveal the physiologic functions of intracellular phosphate, organic phosphate of
phospholipids, and phosphorous in bone, in the regulation of leukotriene metabolism.

An unknown leukotriene metabolite was recovered when the 25-50% ammonium sulfate precipitated human serum was incubated with LTD$_4$ or LTE$_4$ (Fig. 7, 8). Our experimental data demonstrated this unknown metabolite to be enzymatically produced from LTE$_4$. Reverse phase HPLC analysis showed the retention time of the unknown metabolite is about 3-4 min later than that of LTE$_4$. This separation using a reverse phase is based on both of the polarity and size of the compound. This indicates that the unknown metabolite is less polar and perhaps has a relatively lower molecular weight than that of LTE$_4$. Previous studies showed LTE$_4$ can be converted into several different metabolites.

Acetylation of LTE$_4$ by acetylase in liver and kidney converts LTE$_4$ to N-acetyl-LTE$_4$ (5). This compound is mainly found in the bile and urine. HPLC analysis using different solvent systems showed this metabolite had an earlier elution time, which indicates the compound has a higher polarity than that of LTE$_4$. LTF$_4$ is derived from LTE$_4$ by conjugating LTE$_4$ and $\gamma$-glutamyl amino acids (2). The reaction is achieved by incubating LTE$_4$ with $\gamma$-glutamyl transpeptidase and glutathione. HPLC analysis showed the compound has a very high polarity and a very early retention time, compared to that of LTE$_4$.

LTG$_4$ is the derivative of a transamination reaction catalyzed by
cysteine conjugate aminotransferase (59). The enzyme is found in kidney and liver, but not in the lung or leukocytes. HPLC analysis showed a much later retention time of the compound compared to that of LTE₄.

Other LTE₄ metabolites include ω-hydroxy LTE₄ (4), LTE₄ sulfoxide and 6-trans LTB₄ (31). All these compounds have higher polarity than that of LTE₄ and can be excluded. Determination of the chemical structure and physiological functions of the unknown metabolite could be very crucial in studying the metabolism of peptidyl leukotrienes. This metabolite could be a completely inactivated form of the biologically active peptidyl leukotrienes. Further characterization of the unknown metabolite is currently in progress.
Peptidyl leukotrienes are a group of biological active metabolites from the 5-lipoxygenase pathway of arachidonic acid. The rate of degradation of the leukotrienes is controlled by their specific metabolic enzymes as well as the enzyme inhibitors. Isolation, identification and characterization of natural and synthetic inhibitors may provide a novel direction in preventing and treating some leukotriene-associated disease. Towards this goal, several results achieved in this study are as follow:

1. Bicarbonate was isolated and identified as the major inhibitory factor of LTD₄ dipeptidase in human serum. LTD₄ dipeptidase activity from human serum was 50% inhibited in 250 mM bicarbonate.

2. Phosphate was the other leukotriene D₄ dipeptidase inhibitor which was found in serum. It may play a modest part of the inhibition as phosphate levels needed for inhibition are almost 20 times higher than that of the physiological condition.

3. The serum LTD₄ dipeptidase activity was fractionated with ammonium sulfate. It was found that the dipeptidase activity was in the 50-75% ammonium sulfate precipitation fraction. The optimal enzymatic activity was obtained at pH 7.4 with 5 mM CaCl₂, 100 mM NaCl in the assay mixture.
4. An unknown metabolite of LTE$_4$ was formed when either LTD$_4$ or LTE$_4$ was incubated with the 25-50% ammonia sulfate precipitated fraction of serum. Reverse phase HPLC analysis indicates the unknown leukotriene metabolite may have a lower molecular weight and polarity than that of LTE$_4$. 

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34. McIntyre, T. M., G. A. Zimmerman, and S. M. Prescott. 1986

Identification and isolation of medicarpin and a substituted benzofuran as potent leukotriene inhibitors in an anti-inflammatory Chinese herb. Prostaglandins Leukotrienes and Essential Fatty Acids 38:137-143.


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