ISOLATION AND IDENTIFICATION OF TWO ISOMERIC TRIHYDROXY OCTADECENOIC ACIDS WITH PROSTAGLANDIN E-LIKE ACTIVITY FROM ONION BULBS (Allium cepa)

L. Üstünes[1,2], M. Claeys[1], G. Laekeman[1], A.G. Herman[1], A.J. Vlietinck[1] and A. Özer[2].

[1]Department of Pharmaceutical Sciences, University of Antwerp, Universiteitsplein 1, B-2610 Wilrijk, Belgium,
and
[2]Faculty of Pharmacy, Ege University, Bornova, Izmir, Turkey

ABSTRACT

Two fractions with prostaglandin E-like activity were isolated from onion (Allium cepa) by using XAD-2 adsorption, silicic acid column chromatography and thin layer chromatography. The fractions were analyzed by gas chromatography/mass spectrometry and were characterized as isomeric mixtures of 9,10,13-trihydroxy-11-octadecenoic and 9,12,13-trihydroxy-10-octadecenoic acid, which are lipoxygenase metabolites of linoleic acid. Bio-assay, for which cascade superfusion was used and the rabbit coeliac and mesenteric arteries and the rat fundus strip were employed as assay organs, was utilized to monitor the bio-active profile throughout the isolation procedures. The activity of 1 µg of the pharmacologically active fractions T1 and T2 was found to be equivalent to that of respectively 1.33 and 0.63 ng of prostaglandin E_2.

INTRODUCTION

Although the presence and functions of prostaglandins in various mammalian cells and tissues have extensively been investigated, research on prostaglandins or prostaglandin-like substances of plant origin is limited.

Send correspondance and reprint requests to M. Claeys.

Some plant species, e.g. the Allium species onion and garlic, are still used in folk medicine for the treatment of atherosclerotic disease and gastrointestinal ulcers, which are pathologies where prostanoids are involved (1,2). The beneficial effects of onion and garlic for preventing atherosclerotic disease, a predisposing factor in heart disease, stroke and high blood pressure, have been ascribed to the presence of platelet aggregation inhibitors (3-11). The latter have been characterized as sulphide containing substances, which include methylallylsulphide, diallyldisulphide, diallyltrisulphide, 2-vinyl-1,3-dithiene and allyl-1,5-hexadienyltrisulphide (10,11). Blood pressure lowering properties have also been attributed to the presence of prostaglandin A_1, which has first been suggested in yellow onion by Attrep et al. (12). Later on this finding has been confirmed by the same investigators (13) and has been supported by Pobozsny et al. (14), who also reported prostaglandin B- and F-type substances. These data and our own finding that crude ethanolic extracts of onion show prostaglandin E-like activity, which to our knowledge has not been demonstrated in a plant yet and which might explain the favourable effects of onion in the treatment of gastrointestinal ulcers, prompted us to pursue our investigation on the isolation and characterization of compounds with prostaglandin E-like activity. In the present work, two fractions with prostaglandin E-like activity were isolated from onion bulbs and were identified as mixtures of two positional isomers, i.e. of 9,10,13-trihydroxy-11-octadecenoic and 9,12,13-trihydroxy-10-octadecenoic acid, which are lipoxygenase metabolites of linoleic acid. By using a very sensitive mass spectrometric method, i.e. selected ion monitoring, prostaglandin E_1 could not be detected in the pharmacologically active fractions at the nanogram level.

EXPERIMENTAL

ONION SAMPLES

The onion samples used in this study were specially breeded Yalova onions, which were obtained from the Yalova Agricultural Research Institute in Turkey.

CRUDE EXTRACTS

In order to screen the biological activity of onion, an ethanolic crude extract was prepared starting from 3 kg of onions. The material was

homogenized in 1.5 l of distilled ethanol (80 %). After stirring at room temperature for 1hr, the macerated plant suspension was filtered and the marc was further percolated with ethanol (80 %). The filtrate and percolate were combined and concentrated under water pump pressure at a temperature not exceeding 40°C. The residue was diluted with 300 ml of 5 mM Na_2HPO_4 (*) buffer and fractionated by solvent-solvent partition according a slightly modified procedure described by Salmon et al. (15), by which proteins, neutral lipids, polar lipids and other water soluble polar compounds are separated in crude fractions. The biological activity of each of these fractions was tested qualitatively in a cascade superfusion system, in which the isolated rabbit coeliac and mesenteric artery and the rat fundus were used as assay organs, and by a blood platelet aggregation bioassay.

EXTRACTION AND PURIFICATION OF PROSTAGLANDIN-LIKE MATERIAL

Since it has been reported by several investigators that the lipid soluble fraction of onions contains prostaglandin (PG)-like material (12-14) and in view of our own preliminary observations supporting this finding, a first extraction of PG-like material was carried out using Amberlite XAD-2 resin. An amount of 3 kg of onions was homogenized for 3 min in 2.5 l of 5 mM Na_2HPO_4 buffer using a Waring blendor and fibrous material was removed by filtration and centrifugation. The volume of the aqueous supernatant was approximately 5 l. After pH adjustment to pH 4 with 2 % formic acid, the aqueous supernatant was percolated through a 3 x 50 cm column, filled with 200 g Amberlite XAD-2, at a flow rate of 5 ml/min. The column eluent was percolated a second time through the same column in order to increase the adsorption of the active principles. Subsequently, the column was washed with 4 l of bidistilled water and the PG-like material was eluted with 3.5 l of methanol. The eluent, which contained a certain amount of water, was evaporated until methanol free under water pump pressure at a temperature of 35°C. The aqueous solution was further diluted with 200 ml of bidistilled water, adjusted to pH 8 with 1N NaOH and washed 3 times with equal volumes of petroleum ether. The petroleum ether layers, which contained the neutral lipids, were discarded. The pH of the remaining aqueous solution was adjusted to 4 with 2 % citric acid and extracted 3 times with equal volumes of diethyl ether. The combined diethyl ether layers were

(*) When not specified, reagents and solvents were of analytical grade.

concentrated under water pump pressure at 35°C and the fraction thus obtained was designated as the crude onion extract. It showed PGE-like activity in the above mentioned cascade superfusion system.

FRACTIONATION OF THE CRUDE ONION EXTRACT BY SILICIC ACID COLUMN CHROMATOGRAPHY

For column chromatography, silicic acid (Sil-A-200; Sigma Chem. Co., St. Louis, Mo.; 60-200 mesh) was used. The solvent systems described by Samuelsson (16) for the purification of prostaglandins from human seminal plasma were employed for gradient elution. A column of 71 cm height and 1 cm internal diameter was prepared with 20 g of silicic acid, and the crude onion extract was applied onto the column after preadsorption onto a small amount (500 mg) of silicic acid. A benzene/ethyl acetate gradient at a flow rate of 1 ml/min was used and the column was finally eluted with methanol. A total of 26 fractions of 250 ml were collected and evaporated to dryness under water pump pressure at 35°C. The residues were dissolved in 1 ml of methanol and kept at -20°C until bioassay and chemical analysis.

THIN LAYER CHROMATOGRAPHY ANALYSES

1. Analysis of silicic acid column chromatography fractions

The fractions obtained after silicic acid column chromatography were monitored by thin layer chromatography (TLC). Commercial silicagel 60 F_{254} TLC plates of 20 x 20 cm size and 0.25 mm thickness were used (Merck, Darmstadt G.F.R.). A sample of 3 µl of each fraction and PGE_2, PGA_2 and $PGF_{2\alpha}$ (10 µg) were applied on the same plate. A mixture of chloroform/diethyl ether/methanol/acetic acid (45/45/5/2; by volume) which has been described by Granström (17) for the separation of prostaglandins, was used as developing system. PG-like substances were visualized by spraying with 10 % phosphomolybdic acid in ethanol and heating at 100°C.

2. Further purification of the pharmacologically active fractions

The pharmacologically active fractions were combined and further separated by preparative TLC, for which silicagel 60 F_{254} plates with a thickness of 0.5 mm were used. PGE_2 and $PGF_{2\alpha}$ standards (10 µg) and a small aliquot of the fraction to be separated, approximately 1/100, were applied to the same plate as markers. The same solvent system as for analytical TLC was employed. After development, the plate was cut and the standards visualized

as previously described. Three zones, the products corresponding to the PGE_2 and $PGF_{2\alpha}$ markers and another zone with a Rf-value close to that of PGE_2 were scraped off and extracted from the silica with 2 ml of chloroform/methanol (1/1; by volume). After centrifugation, the supernatants were evaporated to dryness and the residues taken up in 2 ml of distilled water. The pH of the solutions was adjusted to 4 with 2 % formic acid and extracted 3 times with equal volumes of diethyl ether in order to remove the silicagel, which was co-extracted in the previous step. The diethyl ether extracts from each zone were combined and evaporated with a N_2 stream. These fractions, designated as T_1, T_2 and F and corresponding with the TLC zones with a Rf-value of 0.28, 0.22 and 0.11 respectively, were dissolved in 2 ml of methanol and further subjected to the biological activity tests and to gas chromatography/mass spectrometry (GC/MS) analysis.

CONTINUOUS CASCADE SUPERFUSION SYSTEM

To detect the biological activity of extracts, column fractions and the separated substances T_1, T_2 and F, a cascade of isolated organs was used (18). Rabbits of either sex (2.5 - 3 kg, Dendermondse Witte) were killed by a blow on the head, bled and the coeliac and mesenteric arteries removed . These arteries were cut spirally, whereas the rat fundus strip (Wistar rats of either sex, 200-300 g) was prepared by zigzag cutting. The tissues were fixed in a cascade system and a load of 3 g was applied. The isolated organs were superfused by means of a Gilson, Minipuls II roller pump at a rate of 5 ml/min. The superfusion fluid was Krebs with the following composition in g/l (mM) : NaCl, 6.9 (118); KCl, 0.35 (4.7); $CaCl_2.2H_2O$, 0.55 (2.5); K_2HPO_4, 0.16 (1.2); $MgSO_4.7H_2O$, 0.29 (1.17); glucose, 2.0 (11.1); $NaHCO_3$, 2.1 (25.0). The following antagonists were added to the Krebs solution (final concentrations) : methysergide maleate (Sandoz; 2 x 10^{-7} g/ml), mepyramine maleate (Baker; 1 x 10^{-7}g/ml), phenoxybenzamine (Smith, Kline and French; 1 x 10^{-7}g/ml),propranolol hydrochloride (Imperial Chemical Incorporation; 2 x 10^{-6} g/ml). Indomethacin (Merck, Sharp and Dohme; 1 x 10^{-6} g/ml) was infused by means of a Braun pump to enhance the sensitivity of the organs for exogenous prostaglandins (19) (Upjohn Co.). The contractions of the organs were auxotonically detected by Harvard smooth muscle transducers and recorded with a Watanabe 6-channel recorder. All fractions from silicic acid column chromatography and the final fractions T_1, T_2 and F were tested on the cascade. For this purpose, 0.15 ml samples were employed, the methanol evaporated under a stream of N_2 and the residue dissolved in 10 µl of 50 mM Tris-HCl buffer (pH 8.5) and 90 µl

Krebs. Exogenous PGE_2, $PGF_{2\alpha}$ and PGA_2 standards were applied to evaluate the sensitivity of the isolated organs and to compare the biological responses of the samples under trial.

RABBIT PLATELET AGGREGATION

For platelet aggregation assays, rabbit platelet rich plasma was used and the methodology, as described by Born (20) and modified by Laekeman et al. (21) was followed. Another series of experiments was performed with rabbit whole blood according to Bult et al. (22).

GAS CHROMATOGRAPHY/MASS SPECTROMETRY ANALYSES

Samples

The substances T_1 and T_2 isolated by preparative TLC, which showed PG-like bio-activity at the end of the bio-assay guided isolation were analyzed by GC/MS.

Derivatization

For the GC/MS characterization two types of volatile derivatives were made. The most simple derivatives prepared were the methyl ester, trimethylsilyl (TMS)-ether derivatives. In order to obtain information about the positional isomerism (vide infra), the hydrogenated methyl esters, n-butyl boronate, TMS-ether derivatives were made. Methyl esters were prepared with an ethereal diazomethane solution. Catalytic hydrogenation was performed with the esterified samples dissolved in 0.5 ml of methanol with 1 mg PtO_2 as catalyst and by bubbling hydrogen gas through the suspension for 1 min at room temperature. Subsequently, the suspension was filtered through a a small silicic acid column (5 x 10 mm), the column washed with 0.5 ml of methanol and the methanol eluant evaporated under N_2. n-Butyl boronates were prepared with 30 µl of a n-butyl boronic acid solution (5 mg/ml in acetone/benzene, 2/1, by volume) and by reacting the mixture for 20 min at 60°C, as described by Kelly (23). Trimethylsilylation was carried out with 30 µl of a mixture of bis(trimethylsilyl)trifluoroacetamide (BSTFA)/pyridine (2/1; by volume) for at least 1hr at room temperature. Immediately before GC/MS analysis, samples were taken to dryness under N_2 and redissolved in an appropriate volume of n-hexane. The samples were analyzed in the absence and presence of methyl esters of saturated fatty acids in order to determine the equivalent chain lenghts (C) of compounds of interest.

Instrument conditions

GC/MS analyses were carried out on a Finnigan 4000 quadrupole instrument, connected to an Incos 2000 data system, using the jet separator as GC/MS interface and with the mass spectrometer operated in the electron impact ionization mode. GC was performed on a 2 m x 2 mm i.d. glass column, packed with 1 % OV-1 coated on Chromosorb WHP 100-120 mesh, which was programmed from 180°C to 230°C at a rate of 5°C/min. Helium was used as carrier gas at a flow rate of 25 ml/min. Injector and GC/MS interface temperatures were 250°C and 230°C respectively. The MS conditions were : electron energy, 70 eV, emission current, 0.3 mA and ion source temperature 230°C. Mass spectra were obtained by averaging over the entire GC peaks.

Quantification of active fractions T_1 and T_2

The quantity of substances T_1 and T_2 was estimated by means of GC/MS using $PGF_{2\alpha}$, which is structurally related as it also contains three hydroxyl functions, as an internal standard. To a sample of 20 µl of the methanol solutions of T_1 and T_2 (2 ml), 5 µg of $PGF_{2\alpha}$ was added and after conversion into the methyl ester, TMS-ether derivatives, the GC/MS analyses were carried out. The quantity of the substances T_1 and T_2 was determined by comparing the peak areas in the reconstructed ion chromatograms of the compounds to be estimated to those of $PGF_{2\alpha}$ and by assuming that the mass spectral response for the derivatives of T_1 and T_2 are the same as for the derivative of $PGF_{2\alpha}$. By proceeding in this manner, the quantities of T_1 and T_2 could be estimated as 150 and 410 µg respectively.

Detection of prostaglandin E_1

For the detection of PGE_1, the methoximated methyl ester, TMS-ether derivatives were prepared for a sample (1/10) of the combined pharmacologically active fractions, which were obtained after silicic acid column chromatography. Methoximation was carried out according to the method described by Grêen (24). Following methoximation, the sample was esterified using ethereal diazomethane, trimethylsilylated with BSTFA as described above and subjected to GC/MS under the same GC/MS conditions as for the analysis of T_1 and T_2, except that the mass spectrometer was operated in the selected ion monitoring mode. The ions selected for the detection of the two (syn- and anti-) PGE_1-derivatives were at m/z 470, 380 and 199 for the first eluting isomer and at m/z 368 and 297 for the second eluting isomer (24).

Using this sensitive mass spectrometric technique, no PGE_1 could be detected in this sample.

Free fatty acid analysis

A small part of the crude onion extract (1/20) was subjected to analytical TLC under the conditions given above and the compounds present in the zone corresponding to arachidonic acid, which was applied separately on the plate, were eluted from the silica, derivatized into the methyl esters using ethereal diazomethane, and analyzed by GC/MS. The GC/MS conditions were the same as for the analysis of T_1 and T_2. Based on the GC/MS results, the following fatty acid composition could be estimated : palmitic acid (C16:0), 12.6 %; linoleic acid (C18:2), 70.5 %; stearic acid (C18:0), 7.0 %; oleic acid (C18:1), 6.1 % and α-linolenic acid (C18:3), 2.6 %. Other minor fatty acids (C14:0 and C15:0) only represented 1.2 % of the total free fatty acid fraction.

BIOSYNTHESIS OF TRIHYDROXY LINOLEATES

Lyophilized onion juice (30 mg) was incubated in 1 ml of 0.2M Tris-HCl buffer (pH 8) for 20 min at 37°C with [1-^{14}C]-linoleic acid (1 µCi; 2.7 µg). In incubations carried out in the presence of the lipoxygenase inhibitor nordihydroguaiaretic acid (5×10^{-4}M), a preincubation with the inhibitor present in Tris-HCl buffer containing 1 % ethanol was done at room temperature for 20 min and the enzymic reactions were started by addition of [1-^{14}C]-linoleic acid. Control incubations were also performed with enzyme suspensions which were heat-deactivated by boiling for 1, 2, 4 and 8 min. After incubation, acidifying to pH 3 with 0.2M aqueous citric acid solution, the lipids were extracted twice with 3 ml of diethyl ether. The diethyl ether extracts were dried with a stream of nitrogen and subjected to TLC analysis on 0.25 mm silicagel plates (Silicagel 60 F254, Merck). A standard of 13-hydroxy-9,11-octadienoic acid (13h-18:2; 4 µg) and samples of fractions T1 and T2 (2 µg) were applied separately on the plate. The TLC plates were developed with the organic phase of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (100:60:20:100, by vol.). Detection was carried out by reaction with phosphomolybdic acid by spraying the plates with a 10 % ethanolic solution and heating at 110°C. Radioactive metabolites were also localized by autoradiography and the radioactivity present in the radioactive zones was determined by liquid scintillation counting.

Three metabolites of linoleic acid (Rf = 0.85) were formed : X_1 (Rf = 0.65), X_2 (Rf = 0.27), X_3 (Rf = 0.23) and X_4 (Rf = 0.05). The standard, 13h-18:2, showed a Rf-value of 0.65, indicating that X_1 contains monohydroxy linoleates. Under the same TLC conditions fractions T1 and T2 co-chromatographed with X_2 and X_3 respectively. Peak X_4 co-chromatographed with phospholipids. The conversion of [1-^{14}C]-linoleic acid into radioactive metabolites was 5.4 %; of these radioactive metabolites the monohydroxy linoleates present in zone X_1 represented 23.5 %, whereas the trihydroxy linoleates present in zones X_2 and X_3 represented 8.0 and 8.7 % respectively. The lipoxygenase inhibitor nordihydroguaiaretic acid resulted in inhibition (80 %) of the formation of the above mentioned metabolites. Compared to control incubations, only 10 % of the radioactivity associated with the trihydroxy linoleates remained in the case incubations were carried after boiling the enzyme suspension for 1 min. Boiling the enzyme suspension for a longer time period (2, 4 and 8 min) did not result in a further decrease of the radioactivity present in zones X_2 and X_3.

RESULTS AND DISCUSSION

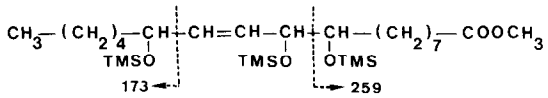
A crude ethanolic extract of onion was biologically active in the cascade system and inhibited tne arachidonic acid induced platelet aggregation. Since the lipid soluble fraction of onion showed the same profile in the cascade superfusion system or more specifically, displayed PGE-like activity, we decided to examine the PGE-like compounds of onion into more detail.

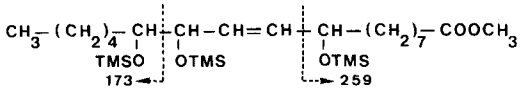
Bio-assay was used as a basic tool for the detection and characterization of the biological activity of extracts, column fractions and isolated substances. PGE-like bio-activity was detected as an in vitro vascular smooth muscle relaxing and non-vascular smooth muscle stimulating activity in the cascade superfusion system. By monitoring the bio-activity profile throughout the extraction and separation procedures, the biologically active fractions T_1 and T_2 were obtained and their PGE-like activity was characterized.

Conventional solvent extraction procedures which have previously been used by other investigators to isolate PG's from onion were unsuccesful in our hands (13, 14) due to severe emulsion problems. In a first step, acidic lipids with PGE-like activity were isolated by XAD-2 adsorption according to a slightly modified method reported by Keirse and Turnbull (25). Subsequently, this crude acidic lipid extract was fractionated by silicic acid

column chromatography using stepwise elution with benzene/ethyl acetate mixtures of increasing polarity following procedures described by Samuelsson (16). TLC analysis of the silicic acid column chromatography fraction showed compounds with Rf-values similar to those of PGE_2, $PGF_{2\alpha}$ and PGA_2. Only the fractions containing compounds with Rf-values similar to the former two PG's were active in our pharmacological test systems and were consequently further investigated. Therefore, a final separation was achieved by preparative TLC, which resulted in the isolation of three different fractions. Fraction I, designated as T_1, contained material with a Rf-value similar to that of PGE_2 (Rf = 0.28), and showed PGE-like bio-activity. Fraction II, designated as T_2, contained material with a Rf-value of 0.22 and also resulted in PGE-like bio-activity. Fraction III, designated as F, contained a compound with a Rf-value very close to that of $PGF_{2\alpha}$ (Rf = 0.11), but without any bio-activity, and was designated as F. The Rf-values of PGE_2 and $PGF_{2\alpha}$, obtained in the same experimental conditions, were found to be 0.29 and 0.14 respectively.

As the TLC behavior of fractions T_1 and T_2 suggested polar lipids with at least three hydroxyl groups, T_1 and T_2 were analyzed by GC/MS after conversion into methyl ester, TMS-ether derivatives. The mass spectra obtained for T_1 and T_2 were identical, except for small differences in relative ion abundances, indicating that T_1 and T_2 are stereoisomeric (Fig. 1). The mass spectra were similar to the mass spectrum obtained by Graveland (26) for a mixture of two isomeric trihydroxylated octadecenoic acids, which were isolated from a wheat flour incubate of linoleic acid, i.e. of the two positional isomers, 9,10,13-trihydroxy-11-octadecenoic acid (9,10,13-th-18:1) and 9,12,13-trihydroxy-10-octadecenoic acid (9,12,13-th-18:1). The mass spectra showed a small ion at m/z 545, corresponding to the loss of a CH_3-radical and diagnostic ions at m/z 173 and m/z 259, which correspond with the fragments represented in the formulae below and at m/z 460, which corresponds with the loss of hexaldehyde and is indicative for a TMSO-group at the C13-position (27).





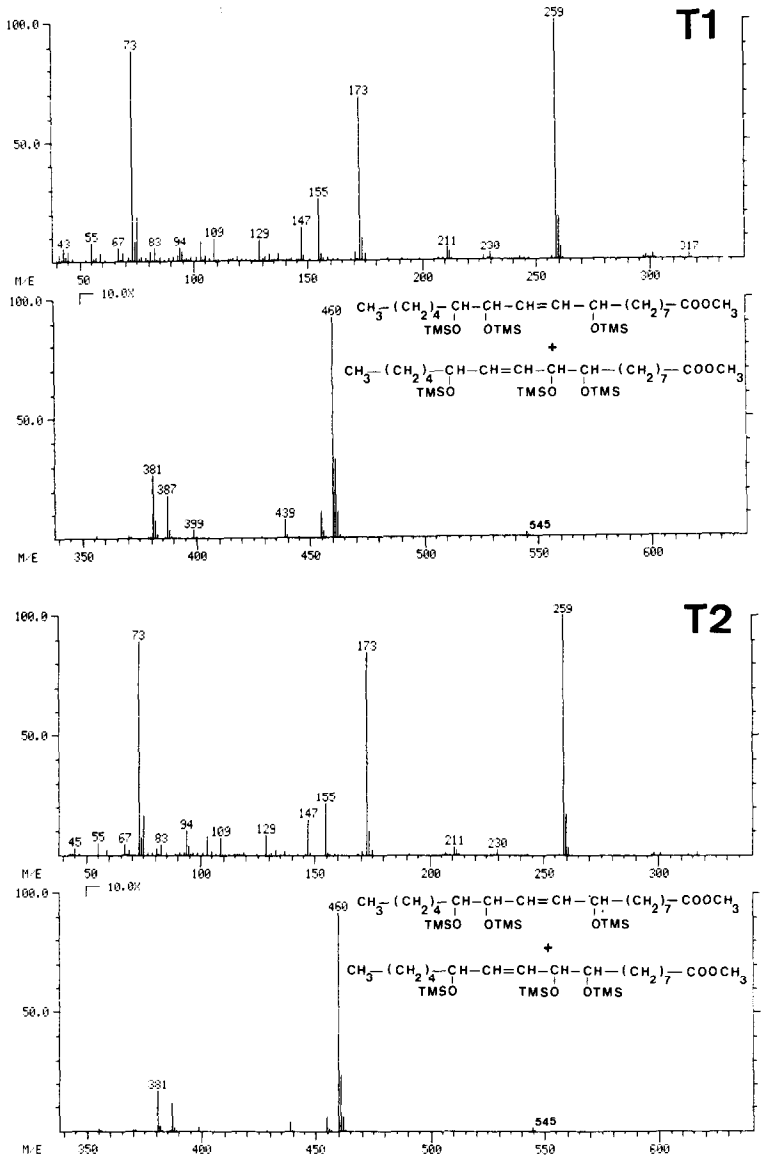
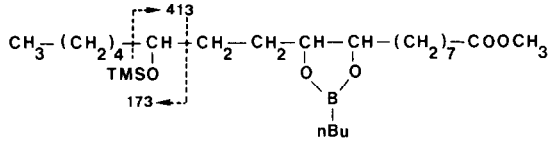


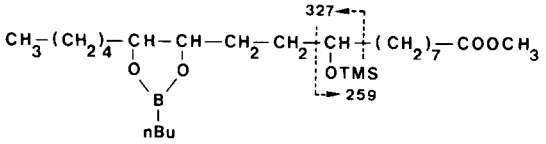
Fig. 1. Mass spectra of the methyl ester, TMS-ether derivatives of fractions T1 and T2. For each fraction, GC peaks with two maxima were obtained : fraction T1 showed maxima with corresponding C-values of 22.5 and 22.7, whereas for fraction T2, maxima with C-values of 22.5 and 22.8 were obtained.

In order to determine the relative amounts of the trihydroxy octadecenoic acid isomers, we prepared n-butyl boronate derivatives as described by Funk and Powell (28). The compounds of each fraction were esterified with diazomethane, hydrogenated using PtO_2 as catalyst, converted to the n-butyl boronate derivatives in order to derivatize the vicinal diol function and trimethylsilylated. The two fractions T_1 and T_2 resulted in virtually identical mass spectra (Fig. 2), which contain ions at m/z 469 ($M^{+\cdot} - CH_3^{\cdot}$), m/z 453 ($M^{+\cdot} - CH_3O^{\cdot}$), m/z 394 ($M^{+\cdot}$ - TMSOH), m/z 369 (394 - $CH_3^{\cdot}$), m/z 129 ($TMSO^{+}=CH-CH=CH_2$) and ions characteristic for each of the positional isomers, which are explained in the formulae below. Ions characteristic for the 9,10,13-th-18:1 derivative are at m/z 413, 384, 323 and 173, whereas ions characteristic for the 9,12,13-th-18:1 derivative are at m/z 327, 237 and 259.



323 : 413 - TMSOH

384 : $M^{+\cdot}$(484) - C_5H_{11}CH(=O)



237 : 327 - TMSOH

From the sum of the ions characteristic for the positional isomers, the relative amounts of each of the isomers was estimated. Fraction T_1 was found to contain 56.7 % of 9,10,13-th-18:1 and 43.3 % of 9,12,13-th-18:1, whereas fraction T_2 was shown to contain 46.2 % of 9,10,13-th-18:1 and 53.8 % of 9,12,13-th-18:1. These data indicate that fraction T_1, which is the most active one, differs in composition from fraction T_2 and is the most enriched in 9,10,13-th-18:1.

Despite the fact that PGA_1 has been characterized in onion (13), PGE_1 could not be detected in the combined pharmacologically active fractions, which were obtained after silicic acid column chromatography. This result is in line with the finding that dihomo- γ-linolenic acid, the direct precursor

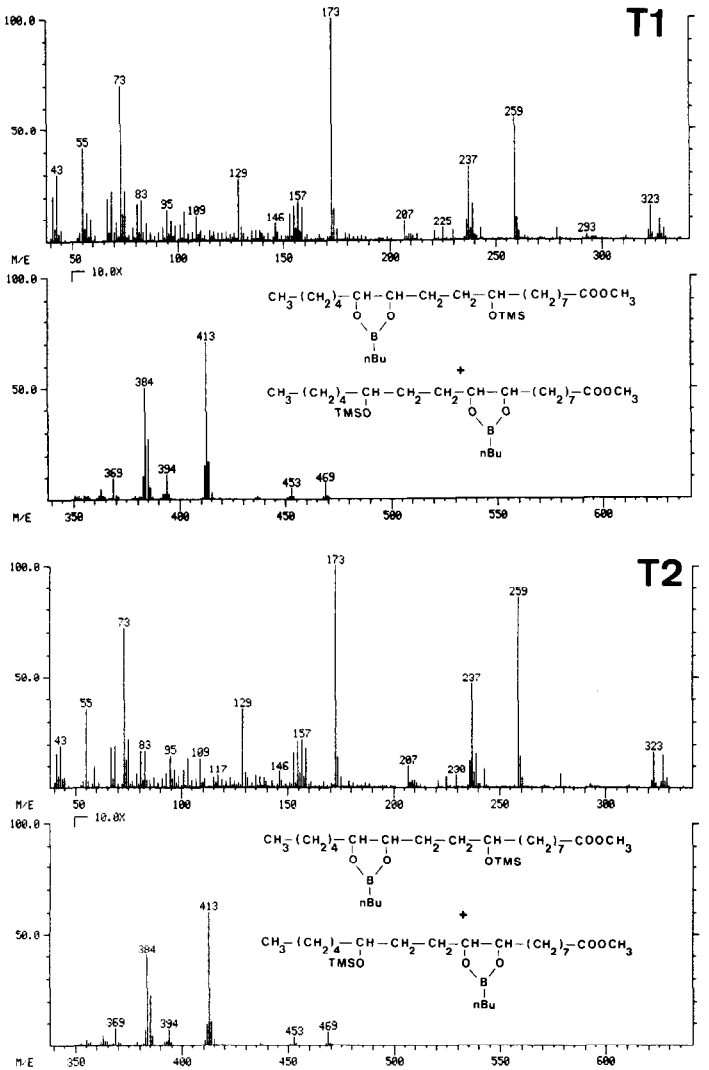


Fig. 2. Mass spectra of the hydrogenated, methyl ester, n-butyl boronate, TMS-ether derivatives of fractions T1 and T2. Mass chromatographic analysis of fraction T1 indicated that the derivatives of 9,12,13-th-18:1 and 9,10,13-th-18:1 showed corresponding C-values of 24.0 and 24.2 respectively. For the derivatives of 9,12,13-th-18:1 and 9,10,13-th-18:1 present in fraction T2, C-values were obtained of 24.1 and 24.3 respectively.

for prostaglandins of the 1 series, which is present in certain plant materials (29), could not be found in our onion samples.

After their structure determination, a quantification of the identified trihydroxylated octadecenoic acids was made by GC/MS and their activity compared with authentic PGE_2. Using simple bracketing assay dose-responses of the test and standard PGE_2 solutions, the activity of 1 µg of T_1 and T_2 was found to be equivalent to that of respectively 1.33 and 0.63 ng of PGE_2 (Fig. 3). No inhibitory activity could be demonstrated for T_1 or T_2 in final concentrations up to 10 µg/ml in blood platelet aggregation experiments in which rabbit platelet rich plasma or whole blood were employed and in which arachidonic acid was used as aggregating agent.

The presence of 9,10,13-th-18:1 and 9,12,13-th-18:1 in onion, suggests that onion bulbs contain lipoxygenase activity. The positional isomers 9,10,13-th-18:1 and 9,12,13-th-18:1 are most probably formed from linoleic acid, which was found to be a major fatty acid in onion, by the biosynthetic pathways given in Scheme 1. Initial oxygenation at C-9 and C-13 results in hydroperoxy derivatives of linoleic acid, which rearrange into unstable allylic epoxy hydroxy compounds and which, upon hydrolysis, give rise to trihydroxy-octadecenoic acids. Evidence for lipoxygenation at C-9 and at C-13 in onion was also obtained by GC/MS detection of 9-hydroxy-10,12-octadecadienoic acid and 13-hydroxy-9,11-octadecadienoic acid, which could be demonstrated in trace amounts (results not shown). Lipoxygenation at C-13 finally results in the formation of 9,12,13-th-18:1 and may also result in 9,10,13-th-18:1 (30), whereas lipoxygenation at C-9 leads to 9,10,13-th-18:1 and most likely to some extent also to 9,12,13-th-18:1. The biosynthetic pathways, given in Scheme 1, have been investigated in detail in cereals (26, 31) and recently, in porcine leukocytes (32), where the lipoxygenation products of linoleic acid also include the positional isomers 9,10,13-th-18:1 and 9,12,13-th-18:1. Proof for the enzymatic formation of the trihydroxy linoleates by lipoxygenase-mediated pathways was obtained by carrying out biosynthesis experiments with radiolabeled linoleic acid and with lyophilized onion juice as enzyme source in the absence and presence of the lipoxygenase inhibitor nordihydroguaiaretic acid. Experiments with boiled enzyme suspensions show that the formation of the trihydroxy linoleates already disappears for 90 % after boiling for 1 min, giving further evidence for the involvement of lipoxygenase, which is known to be rapidly heat-deactivated (33). The possibility how-

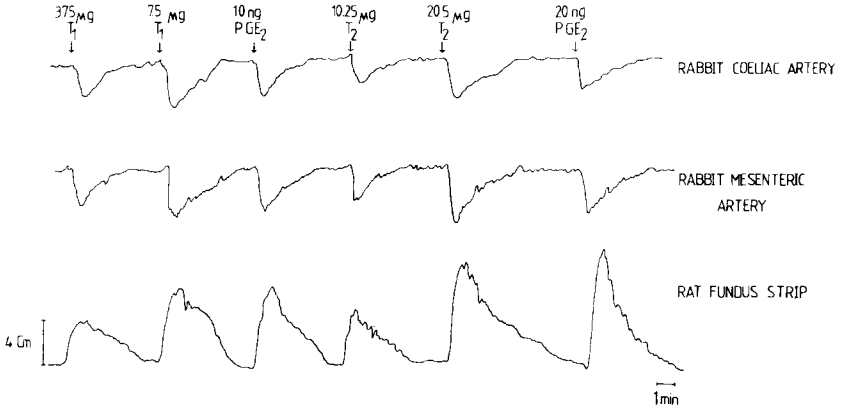
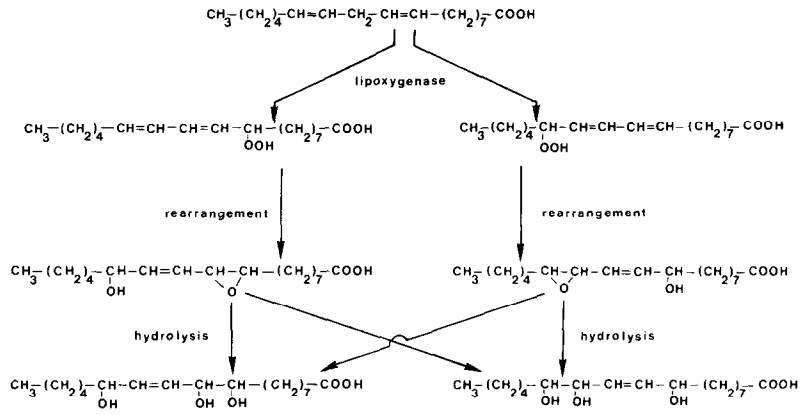


Fig. 3. Original tracings of a bioassay experiment. The products were injected after dilution in 100 µl of Krebs buffer.



Scheme 1. Biosynthesis of the positional isomers, 9,10,13-trihydroxy-11-octadecenoic acid and 9,12,13-trihydroxy-10-octadecenoic acid from linoleic acid.

ever that part of the trihydroxy linoleates detected in onion, are generated through an initial non-enzymatic reaction, i.e. auto-oxidation or unspecific catalysis of linoleic acid by heme compounds (33, 34) to hydroperoxy derivatives, cannot be excluded. Stereochemical analyses could allow to determine the relative participation of the enzymatic versus the non-enzymatic oxidation of linoleic acid in the formation of the trihydroxy linoleates. These stereochemical analyses could most conveniently be done on the monohydroxy linoleates but are unfortunately quite complicated to carry out experimentally as they involve isolation and purification of the monohydroxy linoleates from onion, methylation, formation of the (-)menthoxycarbonyl derivatives, oxidative ozonolysis, followed by a second methylation step and GC/MS analysis of the resulting diastereoisomeric methyl 2-hydroxyheptanoates and dimethyl 2-hydroxysebacates (35), and have therefore not been included in the present study.

The finding that trihydroxy octadecenoic acids show PGE-like activity in *in vitro* bioassay tests is in agreement with recent results obtained by Panossian et al. (36). These investigators isolated a series of isomeric trihydroxy octadecadienoic acids from the roots of *Bryonia alba*, which are used for similar medicinal purposes as onion and could also demonstrate that the isolated trihydroxy octadecadienoic acids have PG-like activity but are about a factor of 10^3 less potent than authentic $PGF_{2\alpha}$, which is in line with our findings. A compound of non-prostanoid nature and with PGE-like activity has also been found in *Propionibacterium acnes* and has been characterized as pentadecanoic-1-monoglyceride (37). Furthermore, from these results it appears that the presence of a five-membered ring structure is not an absolute prerequisite for PG-like activity, as was also pointed out recently in investigations on the biological activity of synthetic seco-prostaglandins (38-41).

ACKNOWLEDGEMENTS

Work at the University of Antwerp was supported by grants (nrs. 3.0031.80 and 3.9001.79) from the Belgian National Fund for Scientific Medical Research.
L. Ustünes acknowledges the Flemisch Federal Government for a predoctoral exchange fellowship. M. Claeys is a research associate of the Belgian National Fund for Scientific Research. The authors are grateful to L. Van den Eynde for typing the manuscript.

REFERENCES

1. Bolton, S., G. Null and W.M. Troetel. The medical uses of garlic. Fact and fiction. American Pharmacy 22, 40 (1982).
2. Lau, B.H.S., M.A. Adetumbi and A. Sanchez. Allium sativum (garlic) and atherosclerosis : a review. Nutrition Research 3, 119 (1983).
3. Baghurst, K.I., M.J. Ray and A.S. Truswell. Onions and platelet aggregation. Lancet 101 (1977).
4. Phillips, C. and N.L. Poyser. Inhibition of platelet aggregation by onion extracts. Lancet 1051 (1978).
5. Makheja, A.N., J.Y. Vanderhoek and J.M. Bailey. Inhibition of platelet aggregation and thromboxane synthesis by onion and garlic. Lancet 781 (1979).
6. Vanderhoek, J.Y., Makheja, A.N. and J.M. Bailey. Inhibition of fatty acid oxygenases by onion and garlic oils. Evidence for the mechanism by which these oils inhibit platelet aggregation. Biochem. Pharmacol. 29, 3169 (1980).
7. Makheja, A.N., J.Y. Vanderhoek, R.N. Bryant and J.M. Bailey. Altered arachidonic acid metabolism in platelets inhibited by onion and garlic extracts. In : Advances in Prostaglandin and Thromboxane Research (B. Samuelsson, P.N. Ramwell and R. Paoletti, Editors) vol. 6, Raven Press, New York, 1980, p. 309 (1980).
8. Srivastava, K.C. Effects of aqueous extracts of onion, garlic and ginger on platelet aggregation and metabolism of arachidonic acid in the blood vascular system : in vitro study. Prostaglandins, Leukotrienes and Medicine, 13, 227 (1984).
9. Makheja, A.N., J.Y. Vanderhoek and J.M. Bailey. Effects of onion (Allium cepa) extract on platelet aggregation and thromboxane synthesis. Prostaglandins and Medicine 2, 413 (1979).
10. Ariga, T., S. Oshiba and T. Tamada. Platelet aggregation inhibitor in garlic. Lancet 150 (1981).
11. Apitz-Castro, R., S. Cabrera, M.R. Cruz, E. Lederma and M.K. Jain. Effects of garlic extract and of three pure components isolated from it on human platelet aggregation, arachidonate metabolism, release reaction and platelet ulstrastructure. Thrombosis Res. 32, 155 (1983).
12. Attrep, K.A., J.M. Mariani Jr. and M. Attrep Jr. Search for prostaglandin A_1 in onion. Lipids 8, 484 (1973).
13. Attrep, K.A., W.P. Bellman, Sr., M. Attrep, Jr., J.B. Lee and W.E. Braselton, Jr. Separation and identification of prostaglandin A_1 in onion. Lipids 15, 292 (1980).
14. Pobozsny, K., P. Tétényi, I. Héthelyi, L. Kocsar and V. Mann. Biologically active substances : investigations into the prostaglandin content of Allium species (in Hungarian). Herba Hungarica 18, 71 (1979).
15. Salmon, J.A. and R.J. Flower. Prostaglandins and related compounds. In : Hormones in Blood (C.H. Gray and V.H.T. James, Editors) vol. 2, Academic Press, p. 237 (1979).
16. Samuelsson, B. Isolation and identification of prostaglandins from human seminal plasma. J. Biol. Chem. 238, 3229 (1963).
17. Granström, E. Two-dimensional thin-layer chromatography of prostaglandins and related compounds. In : Methods in Enzymology (W.E.M. Lands and W.L. Smith, Editors), vol. 86, Academic Press, p. 493 (1982).
18. Vane, J.R. The use of isolated organs for detecting active substances in the circulating blood. Br. J. Pharmacol. 23, 360 (1964).
19. Ferreira, S.H., A.G. Herman and J.R. Vane. Prostaglandin production by rabbit isolated jejunum and its relationship to the inherent tone of the preparation. Br. J. Pharmacol. 56, 469 (1976).
20. Born, G.V.R. Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature 194, 927 (1962).

21. Laekeman, G.M., J. Mertens, J. Totté, H. Bult, A.J. Vlietinck and A.G. Herman. Isolation and pharmacological characterization of vernolepin. J. Natural Products 46, 161 (1983).
22. Bult, H., E. Wechsung, A. Houvenaghel and A.G. Herman. Prostanoids and hemostasis in chickens : anti-aggregating activity of prostaglandins E_1 and E_2, but not of prostacyclin and prostaglandin D_2. Prostaglandins 21, 1045 (1981).
23. Kelly, R.W. Method for the measurement of prostaglandin $F_{2\alpha}$ in biological fluids by gas chromatography-mass spectrometry. Anal. Chem. 45, 2079 (1973).
24. Greên, K. Gas chromatography-mass spectrometry of O-methyloxime derivatives of prostaglandins. Chem. Phys. Lipids 3, 254 (1969).
25. Keirse, M.J.N.C. and A.C. Turnbull. Extraction of prostaglandins from human blood. Prostaglandins 4, 607 (1973).
26. Graveland, A. Enzymatic oxidations of linoleic acid and glycerol-1-monolinoleate in doughs and flour-water suspensions. J. Am. Oil Chem. Soc. 47, 352 (1970).
27. Capella, P. and C.M. Zorzut. Determination of double bond position in monounsaturated fatty acid esters by mass spectrometry of their trimethylsiloxy derivatives. Anal. Chem. 40, 1458 (1968).
28. Funk, C.D. and W.S. Powell. Metabolism of linoleic acid by prostaglandin endoperoxide system from adult and fetal blood vessels. Biochim. Biophys. Acta 754, 57 (1983).
29. Hitchcock, C. Structure and distribution of plant acyl lipids. In : Recent advances in the chemistry and biochemistry of plant lipids (T. Galliard and E.I. Mercer, Editors), Academic Press, p. 1 (1975).
30. Gardner, H.W., E.C. Nelson, L.W. Tjarks and R.E. England. Acid-catalyzed transformation of 13(S)-hydroperoxylinoleic acid into epoxyhydroxyoctadecenoic acid and trihydroxyoctadecenoic acids. Chem. Phys. Lipids 35, 87 (1984).
31. Heimann, W. and P. Dresen. Uber den enzymatischen hydroperoxidabbau in cerealien. Enzymcharakterisierung und Reaktionsprodukte. Helv. Chim. Acta 56, 463 (1973).
32. Claeys, M., G.A.A. Kivits, E. Christ-Hazelhof and D.H. Nugteren. GC/MS characterization of lipoxygenase metabolites of linoleic acid formed by porcine leukocytes. Arch. int. Biochim., 92, 17 (1984).
33. Dupont, J., P. Rustin and C. Lance. Interaction between mitochondrial cytochromes and linoleic acid hydroperoxide. Possible confusion with lipoxygenase and alternative pathway. Plant Physiol. 69, 1308 (1982).
34. Tappel, A.L. Unsaturated lipid oxidation catalyzed by hematin compounds. J. Biol. Chem. 217, 721 (1955).
35. Hamberg, M. Steric analysis of hydroperoxides formed by lipoxygenase oxygenation of linoleic acid. Anal. Biochem. 43, 515 (1971).
36. Panossian, A.G., G.M. Avetissian, V.A. Mnatsakanian, S.G. Batrakow, J. A. Vartanian, E.J. Gabrielian and E.A. Amroyan. Unsaturated polyhydroxy acids having prostaglandin-like activity from Bryonia alba II. Major components. J. Med. Plant Research 47, 17 (1983).
37. Abrahamsson, S., L. Hellgren, J.G.A.M. Raaijmakers and J. Vincent. Prostaglandin-like substances in Propionibacterium acnes. VI. Characterization of the lipid fraction by gas chromatography in conjunction with mass spectrometry. Experientia 37, 1277 (1981).
38. Bicking, J.B., C.M. Robb, R.L. Smith, E.J. Cragoe, Jr., F.A. Kuehl, Jr., and L.R. Mendel. 11,12-Secoprostaglandins. 1. Acylhydroxy-alkanoic acids and related compounds. J. Med. Chem. 20, 35 (1977).
39. Bicking, J.B., J.H. Jones, W.J. Holtz, C.M. Robb, F.A. Kuehl, Jr., D.H. Minster and E.J. Cragoe. 11,12-Secoprostaglandins. 5. 8-Acetyl- or 8-(1-hydroxyethyl)-12-hydroxy-13-aryloxytridecanoic acids and sulfonamide iso-esters as inhibitors of platelet aggregation. J. Med. Chem. 21, 1011 (1978).

40. Carrol F.I., F.M. Hauser, R.C. Huffman and M.C. Coleman. Synthesis and biological evaluation of 10-oxa-11-deoxyprostaglandin E_1 and 10-Nor-9, 11-secoprostaglandin F_1 and their derivatives. J. Med. Chem. 21, 321 (1978).
41. Zoretic, P.A., P. Soja and T. Shiah. Synthesis and gastric antisecretory properties of an 8-aza- and a 10-oxa-8,12-secoprostaglandin. J. Med. Chem. 21, 1330 (1978).

Editor: J.E. Pike Received: 10-30-84 Accepted: 3-26-84