are not obtained in chemical ionization. Hence the MS/MS analysis must be performed on a fragment ion and molecular structure specificity is decreased. Clearly, the combination of SIMS ionization with MS/MS analysis would maximize structural information and minimize complexity and time of analysis.

The liquid chromatography experiments suggest several important conclusions. First, conventional ionization methods are unsuitable for the quaternary compounds studied here so that the interfaced liquid chromatograph/mass spectrometer system does not yield spectra which adequately characterize the analyte. The identification is obscured by the abundance of background species in the chemical ionization source. Such species are better resolved using MS/MS, allowing positive identification from a more impure (mushroom tissue) material. On the other hand, liquid chromatography followed by SIMS analysis yields structure specific spectra. This is clearly the method of choice if LC is selected over TLC although development of an interface is clearly needed. At least one group is working toward this end (*18*).

The ion beam technique used here has considerably higher spatial resolution than has ben utilized in this experiment. As a minimum, some use of the two-dimensional resolution capability in conjunction with two-dimensional chromatography could easily be implemented. It is also worth noting that the use of a highly selective (SIMS) detector for chromatography should lead to an increase in effective chromatographic resolution. This procedure also addresses a common difficulty in liquid chromatography of natural products, viz, assignment of a peak to a particular constituent, even when this is known to be present in the sample.

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Permeable Membrane–Mass Spectrometric Measurement of Reaction Kinetics

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The compound to be analyzed is introduced into the source of a mass spectrometer after selective permeation through a thin membrane which permits passage of apolar molecules. The in situ measurement of ethyl 2-furoate produced in an α-chymotrypsin catalyzed transesterification is described. Under present experimental conditions, and in a selective ion mode operation, ethyl furoate production can be detected with a sensitivity of 0.1 μM and a response time of approximately 13 s at 25 °C.

The mass spectrometer (MS) with its wide applicability, sensitivity, and specificity is a useful detector for monitoring reactions in which reactants and products are dilute components of a liquid phase—when a suitable procedure is available for sample introduction into the MS. However, sample preparation has usually been required prior to analysis, which precludes a primary advantage of in situ measurements, collection of data as the reaction proceeds. This paper presents our current refinement of permselective membrane/mass spectrometric (PM/MS) analysis, which is an in situ method.

Westover and co-workers (*1*) were the first to couple permselective membranes with a mass spectrometer in order to measure apolar solutes dissolved in a polar liquid. Hollow fibers of dimethylsilicone polymer were fabricated into probes such that outside contact was with the solution to be analyzed, while the interior was connected to the evacuated inlet of a mass spectrometer. Dimethylsilicone membranes are particularly suitable for measuring apolar solutes in aqueous solutions, since water is almost totally excluded while the solute can permeate into the MS. These authors were able to measure directly the transient appearance of methoxyflurane ($CH_3OCF_2CHCl_2$) in the venous blood of a rat briefly exposed to that anaesthetic and to detect various organic

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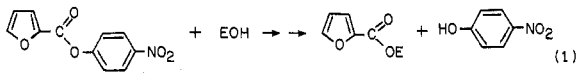
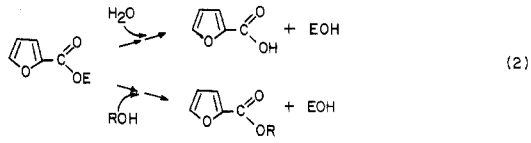
solutes at 100 ppm in water within <1 to 14 s. In a subsequent publication, Tou and Kallos (2) described the measurement of chloromethyl methyl ether and bis(chloromethyl) ether hydrolysis in humid air. As an indication of the potential sensitivity of the PM/MS method, with no prior workup of the aqueous solutions, bis(chloromethyl) ether was detected at 10 ppb (3), and benzene and methyl salicylate were detected at 0.5 and 2 ppb, respectively (4). Polar compounds with little permeability through dimethylsilicone have also been measured (5, 6). In one approach, solute is pumped across the face of a flat membrane, against which is immobilized an enzyme to catalyze conversion into a permeable product. Thus, urea concentrations were measured from the CO_2 released in a urease catalyzed hydrolysis and NADH via the ethanol produced in the alcohol dehydrogenase catalyzed reduction of acetaldehyde. The cyclohexene produced from the electrochemical reduction of dibromocyclohexane was detected through a membrane mounted on a grid electrode (7); volatile metabolites in organ perfusates and whole cell suspensions have been measured by the PM/MS method (8, 9); the use of semipermeable membranes as gas separators in gas chromatography/mass spectrometry (GC/MS) is well-known (e.g., ref 10), and there has been a similar application in liquid chromatography/mass spectrometry (LC/MS) (11).

We have used the α-chymotrypsin catalyzed transesterification of p-nitrophenyl 2-furoate in developing the PM/MS method for the specific purpose of kinetic measurements. Reactions of this enzyme proceed through a multistep path (e.g., ref 12). In the first stage (eq 1), the



(1)



(2)

hydroxyl group of the active site (EOH) serine is esterified, with concomitant release of p-nitrophenol. In the second stage the enzyme is deacylated yielding free enzyme and acid. With an alcohol present there is competing transesterification, and the ratio of product ester to acid increases linearly with increasing alcohol concentration (13, 14). Since formation of the acyl enzyme is much faster than the subsequent deacylation, initiating the reaction with excess substrate results in a first-order burst followed by a zero-order, steady-state formation of p-nitrophenol. Ethyl 2-furoate, the ester product with ethanol as nucleophile, is permeable through dimethylsilicone membranes and is conveniently detected from either the parent (m/e = 140) or base (m/e = 112: loss of ethylene) ion. The PM/MS monitoring of this transesterification reaction is the subject of our report.

EXPERIMENTAL SECTION

Apparatus and Material. The reaction cell we used is represented diagrammatically in Figure 1. A dimethylsilicone polymer membrane with a nominal thickness of 20 or 25 μm (General Electric, Schenectady, NY) is supported on a Spectro/Por PC filter (Spectrum Medical Industries, New York, NY), backed in turn by a stainless steel screen. The membrane and its two supports are clamped between two stainless steel blocks using two O-rings: one for a liquid, the other for a vacuum seal. The completed assembly forms a reaction cell, with the membrane forming the bottom (area = 1 cm^2). Water from a constant-temperature bath is pumped through a channel in the upper block in order to maintain the cell temperature. The cell is connected to the MS source via a 9 in. long, $^1/_{16}$ in. i.d. stainless steel tube, a $^1/_4$ in. i.d. tee joint, a $^1/_4$ in. bellows valve (Nupro Corp., Willoughby, OH), and a second 12 in. long, $^1/_{16}$ in. i.d. stainless steel tube. The side arm of the tee joint is connected through a second bellows valve to an oil diffusion pump (20 L/s), which is closed off during an analysis. The tubing and valves are heated to approximately 100 °C to minimize solute adsorption to the walls. The normal operating parameters of the MS, a DuPont Model 21-490 single focusing, magnetic sector machine, were an ionization voltage of 20 eV at 100 μA and the source temperature and pressure at 210 °C and 10^{-4} torr when open to the reaction cell. The ion current was measured with a 16-stage electron multiplier (ITT, Fort Wayne, IN, Model F4074) connected through an integrator of our own design to a Bell & Howell Datagraph Model 5-134 (band-pass, 10 Hz). The MS was operated in a selected-ion monitoring mode. Two sets of up to four contiguous mass units each, separated by 30–40 mass units could be scanned with each peak integrated. Stopped flow experiments were performed with a Durrum Model D110. (Dionex, Sunnyvale, CA). All other absorbance measurements were made with a Cary 16 spectrophotometer (Varian Corp., Palo Alto, CA).

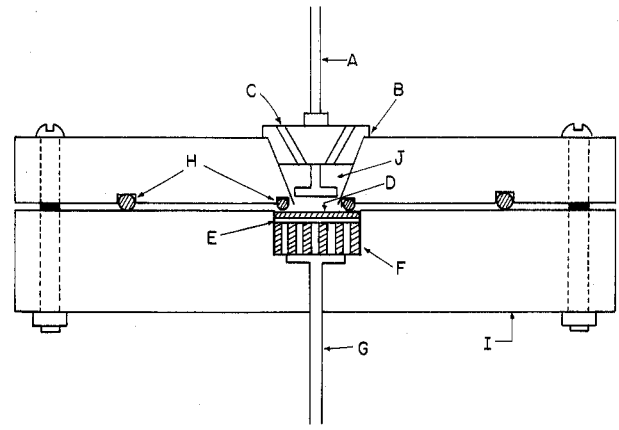


Figure 1. Reaction cell diagram: (A) mechanical stirrer; (B) Teflon plug; (C) sample injection ports; (D) dimethylsilicone rubber membrane supported on a polycarbonate filter; (E) stainless steel screen; (F) porous brass support; (G) tubing to the mass spectrometer; (H) Viton O-rings; (J) reaction compartment with a volume of approximately 0.5 mL.

The synthesis of p-nitrophenyl 2-furoate has been described previously (15). Ethyl 2-furoate was synthesized from furoic acid which had been crystallized twice from toluene. A solution of 15 g of acid dissolved in 100 mL of 95% ethanol and 3 mL of concentrated H_2SO_4 was refluxed for 24 h and then neutralized with Na_2CO_3. Rotary evaporation removed the excess ethanol, and the pale yellow ester was crystallized repeatedly from 30% (v/v) methanol in water (mp 34 °C). All other reagents were obtained commercially and used with no further purification.

Procedures. To obtain absolute rate constants requires an initial machine calibration. In a typical experiment we add, under constant stirring, a known amount of ethyl 2-furoate into the reaction cell already containing 450 μL of buffer, 13 μL of 95% ethanol, and 20 μL of dimethyl formamide. The base ion (m/e = 112) is scanned and integrated for 1.2 s with each scan integration beginning at 2.6-s intervals. The resultant signal rises after ester addition and reaches a plateau (Figure 2). The time response is then fit (the three constants unconstrained) to

$$I = a_1(1 - a_2e^{-a_3t}) \tag{3}$$

with the nonlinear least-squares algorithm GRIDLS (16). Within experimental uncertainty, the best fit values of a_1 were identical with the magnitudes of the signal plateau measured directly.

In a typical kinetic experiment the reaction is initiated by adding, under constant stirring, 20 μL of 12.5 mM p-nitrophenyl 2-furoate in dimethyl formamide to 420 μL of buffer, 50 μL of chymotrypsin solution (20 mg/mL in 10^{-3} M HCl; Worthington, Freehold, NJ; lot no. CDI-39H745), and 13 μL of 95% ethanol. After an initial lag, the signal intensity rises linearly with time (Figure 3). The time response is fit to

$$I = b_1\left[t - \frac{(1 - e^{-b_2t})}{b_2} - \frac{a_2(1 - e^{-a_3t})}{a_3} + \frac{a_2(e^{-b_2t} - e^{-a_3t})}{a_3 - b_2}\right] \tag{4}$$

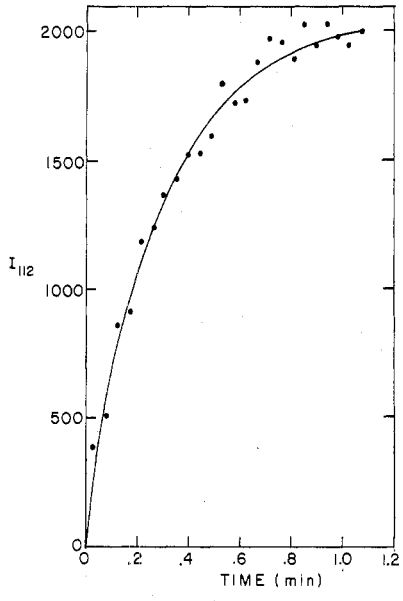


Figure 2. Mass spectrometer signal response after a single addition of ethyl 2-furoate. The signal magnitude obtained in the base peak (m/e = 112) is given in arbitrary units. The line through the points was calculated from the best fit of eq 3 to the data. Ester concentration was 15 μm and temperature was 25 °C.

where the constants a_2 and a_3 are those defined by eq 3 and b_1 and b_2 are unconstrained best fit constants. The justifications for eq 3 and 4 are given in the following section (see eq 7 and 10). The rate-limiting transesterification constant k_{ROH} can be calculated from

$$k_{ROH} = b_1/K(CH_3CH_2OH)E_0 \qquad (5)$$

where b_1 is the best fit constant obtained with eq 4 and K is the slope of the linear a_1 vs. ethyl furoate concentration dependence; E_0 the enzyme active site concentration is obtained by burst titration (*17*) with *p*-nitrophenyl acetate as substrate. The rate constant k_{ROH} was also determined by an independent procedure in which *p*-nitrophenol release was monitored spectrophotometrically (*14*).

RESULTS AND DISCUSSION

With the addition of ethyl 2-furoate to the PM/MS cell there is a rapid appearance of that ester's mass spectrum. An example of the time course of this appearance, monitored at the m/e = 112 base peak is shown in Figure 2. Since solute flux through the membrane is analogous to heat flux through a flat conductor of uniform and finite thickness, the solution of the heat problem (*18*) should apply directly to membrane permeation

$$I(t) = c_sK[1 + 2\sum_{n=1}^{\infty}(-1)^n \exp(-D\pi^2n^2t/l^2)] \qquad (6)$$

where c_s is the solute concentration in the aqueous phase after a single, extremely rapid addition and D the diffusion coefficient of the solute within the membrane of uniform thickness l. The constant K, which may be considered as instrumental, includes D, l, the membrane's effective area, the solution/membrane partitioning coefficient for the solute, and the proportionality constant between solute flux in the MS and the resultant signal intensity. As a first approximation we have omitted terms with $n > 1$, eq 7. Assigning $D\pi^2/l^2$ to

$$I(t) = c_sK[1 - \exp(-D\pi^2t/l^2)] \qquad (7)$$

a_3 and c_sK to a_1, and introducing a_2 as an empirical correction for the error due to the approximation, we arrive at eq 3, which was used for data analysis. (Note that the coefficient of 2, omitted in eq 7, is required in eq 6 because the series $\sum_{n=1}^{\infty}(-1)^n = -1/2$.)

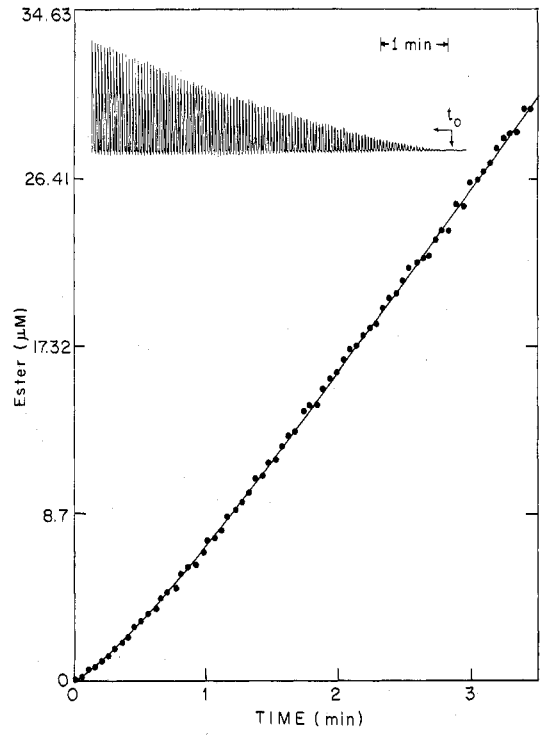


Figure 3. Mass spectrometer signal response during the enzymatic production of ethyl 2-furoate. Experimental conditions have been given in the text. The curve on the top is a tracing of the chart recording with the MS scanning the base peak (m/e = 112). The solid curve was calculated from the best fit of eq 4 to the points.

As seen in Figure 2, there is good correlation between the empirical eq 3 and the experimental results; moreover, plots of a_1 ($=c_sK$) vs. ethyl 2-furoate concentration are linear. For example, in a set of five determinations (to which the results of Figure 2 belong), c_s was varied from 0.5 to 14.6 μM; regression analysis yielded a linear correlation coefficient of 0.9998, an intercept of -15 ± 13 in arbitrary units, and a slope of 138.6 $\pm$ 1.75 μM^{-1}. The estimated sensitivity was 0.1 μM at S/N = 2. In other experiments, linearity was observed up to 0.1 mM. The measurement of a_1 as a function of c_s constitutes a machine calibration with the value of K given by the slope. If eq 7 is a reasonable approximation of eq 6, then the empirical constant a_2 in eq 3 should be near 1. In the experiment just cited $a_2 = 0.95 \pm 0.075$. Assuming the validity of eq 7, we define the instrumental response time as $(\ln 2)/a_3$, which is 12.9 $\pm$ 0.39 s using the best fit value of a_3. The response time is thus the effective half time to attain maximal signal intensity.

When ethyl 2-furoate is formed in the α-chymotrypsin catalyzed transesterification reaction (eq 1 and 2), c_s increases with time. Experimentally, we observe a brief delay followed by a linearly increasing signal intensity (Figure 3). The delay must arise from a combination of instrumental response and the reaction presteady state. The zero-order portion of the curve corresponds to steady-state enzyme turnover. Applying the Laplace–Carson transform (*19*) to the differential equations for the minimal scheme

$$E + S \underset{}{\overset{P_1}{\rightleftharpoons}} X \xrightarrow{EtOH} E + P_2 \qquad (8)$$

yields the function $P_2(t)$ when substrate and alcohol are in excess

$$P_2(t) = k_0E_0[t - (1 - e^{-k_1t})/k_1] \qquad (9)$$

While this reaction scheme is too simple to describe the chymotrypsin mechanism, the function derived from it for the time dependence of P_2 production is a good representation

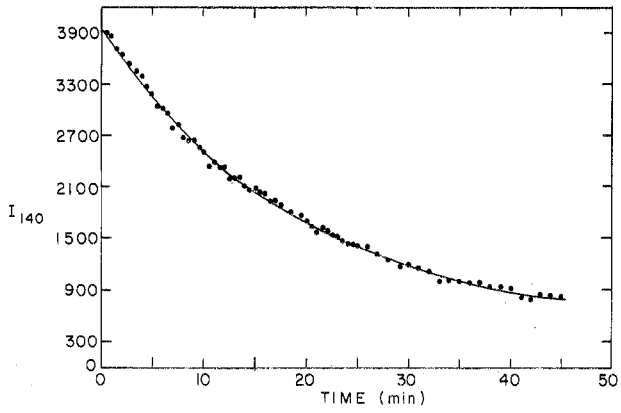


Figure 4. Permeation loss of ethyl 2-furoate through the membrane. The conditions have been given in the text; initial ester concentration was 20 μM. The signal obtained from the molecular ion (m/e = 140) is given in arbitrary units. The solid curve represents the best fit of a first-order decay curve through the experimental points.

of experimental results. Thus, we consider eq 9 to be phenomenologically appropriate; the construction of the apparent constants k_0 and k_1 from the true elementary rate constants depends on the detailed reaction mechanism.

The time dependence of the MS signal intensity can be derived from eq 3 and 9 (see Appendix)

$$I(t) = a_1 k_0 E_0 \left[t - \frac{1 - e^{-k_1 t}}{k_1} - \frac{a_2(1 - e^{-a_3 t})}{a_3} + \frac{a_2(e^{-k_1 t} - e^{-a_3 t})}{a_3 - k_1} \right] \quad (10)$$

With a nomenclature change eq 10 and 4 become identical. As shown in Figure 3, the derived function fits the experimental results well. The value of k_0 obtained from this fit yields a transesterification rate constant k_{ROH} (eq 5) which is the same as that obtained by an independent spectrophotometric method (*14*). For example, at 25.2 °C the PM/MS method results in k_{ROH} = 0.47 ± 0.067 M^{-1} min^{-1}; at 25.4 °C the spectrophotometric method results in k_{ROH} = 0.51 ± 0.020 M^{-1} min^{-1}. (The active site titration required to measure E_0 in the PM/MS procedure makes the largest contribution to the uncertainty.) The presteady-state portion of the enzymatic reaction has a half-life of 2 s at 25 °C, measured by the stopped flow spectrophotometric detection of *p*-nitrophenol release. The present instrument response time of 13 s is too slow to allow for the PM/MS measurement of the presteady state. For example, the best fit value of k_1 obtained from the data of Figure 3 was (9 ± 20) × 10^8 s^{-1}.

The correlation between theory and experiment is surprisingly good, since a number of factors were not explicitly considered; for example, the presence of an unstirred layer adjacent to the membrane, the time required for the solute to reach partitioning equilibrium between the liquid and the membrane, etc. Until we have accounted for these presently hidden factors, the use of eq 7 and 9 is only justified empirically. One assumption for the use of eq 7 is that c_s remains constant after the one time addition of solute. However, solute is lost into the MS through the membrane, as seen by an apparent first-order decay of signal intensity over longer time intervals (Figure 4). The measured half-life for this process at 25 °C is 13.3 ± 0.44 min. Hence, over the time course of our kinetic measurements the assumption of no solute loss is approximately correct. Were longer measurement times required, then permeation loss could be incorporated into the analysis, either by altering the derivation leading to eq 7 (*18*) or by introducing a first-order decay of P_2 into the equations for the derivation of the function $P_2(t)$.

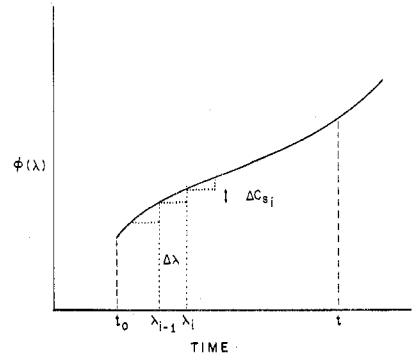


Figure 5. Approximating $\phi(\lambda)$ as a step function.

The PM/MS method should prove valuable as an analytical tool for the measurement of many time-dependent processes in addition to the reaction discussed here. We have, for example, measured the nonenzymatic, base catalyzed hydrolysis of esters and have investigated a time-dependent $^{16}O/^{18}O$ kinetic isotope effect associated with the α-chymotrypsin catalyzed transesterification (*20*). The technique has high sensitivity and a reasonably fast response time. Sensitivity and response are dependent upon a number of factors, among which are the vapor pressure and polarity of the compound to be measured, the nature and thickness of the membrane, the cell and source inlet geometries, and the S/N characteristics of the MS. By judicious changes in one or more of the methodological variables, it should be possible to enhance sensitivity and to decrease response time. Thus, the most serious constraint facing the PM/MS method may be the membrane-imposed limitation to apolar compounds. Until this difficulty is obviated, the large number of available apolar compounds should permit wide usage of this tool in kinetic measurements.

ACKNOWLEDGMENT

We are indebted to Richard Schmidt for his technical assistance with the mass spectrometer, Bob Fagan for his advice in designing the reaction cell, and Daniel Leussing for arranging the use of a stopped flow spectrophotometer. Time on an Amdahl 470 was provided by The Instruction and Research Computer Center of The Ohio State University.

APPENDIX

Assume the time-dependent change in c_s due to any physical or chemical process is given by the function $\phi(\lambda)$ where λ is the time over which the change has occurred. We define $J(t)$ by $I(t)/c_s$ for the case when there is an instantaneous step in c_s (see eq 6 and 7) with t the time at which the signal is measured. We can approximate $\phi(\lambda)$ with a staircase function as shown in Figure 5, in which $\Delta\lambda$ is a constant small time interval, and $\lambda_i = i\Delta\lambda$. The signal intensity measured at time t due to a small step in c_s during the interval $\lambda_i - \lambda_{i-1} = \Delta\lambda$ is given by

$$I_i(t) = \Delta c_{s_i} J(t - \lambda_i) \quad (A1)$$

provided $\lambda_i \leq t$. The approximate value for Δc_{s_i} is obtained from the slope of the function $\phi(\lambda)$

$$\Delta c_{s_i} \approx \Delta\lambda \phi'(\lambda_i) \quad (A2)$$

and by substitution

$$I_i(t) \approx \phi'(\lambda_i) J(t - \lambda_i) \Delta\lambda \quad (A3)$$

The total intensity at time t due to all the small concentration steps is the sum of the individual contributions

$$I(t) = \sum_{i=1}^{n} I_i(t) = \sum_{i=1}^{n} \phi'(\lambda_i) J(t - \lambda_i) \Delta\lambda \quad (A4)$$

Passing to the limit of $\Delta\lambda \rightarrow 0$ yields

$$I(t) = \int_0^t \phi'(\lambda) J(t - \lambda)\, d\lambda \qquad \text{(A5)}$$

Substituting eq 3 and 9 into eq A5 yields eq 10 (or 4).

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Identification of Double Bond Positions in Polyunsaturated Fatty Acids by Chemical Ionization Mass Spectrometry

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Permethoxylated derivatives of polyunsaturated fatty acids were analyzed by combined gas–liquid chromatography–chemical ionization mass spectrometry (GLC–CIMS). According to this method, the molecular weights could be determined easily by quasi-molecular ions and also by characteristic ions derived from successive losses of methanol from quasi-molecular ions. Furthermore, all positions of the original double bond could be deduced from the recognizable ions which were formed by the cleavage of carbon–carbon between the two methoxy functions. The mode of fragmentation was confirmed by the mass spectra of deuteriomethyl 5,6,8,9,11,12,14,15-octamethoxyeicosanoate, derived from arachidonic acid.

Chemical ionization mass spectrometry (CIMS) of unsaturated fatty acids has shown advantages over conventional electron impact ionization mass spectrometry (EIMS), giving much information on molecular weight and positional and/or geometrical isomerization (*1–3*). In our initial investigation, trimethylsilyloxy derivatives were found to be useful for the determination of unsaturated fatty acid structure by CIMS (*2*). However, the drawback of this method is that increasing the number of trimethylsilyloxy moieties adds substantially to molecular weight of the compound, prohibitively lowering the yield of parent molecular ions for derivatives of those fatty acids with three or more double bonds (*3*). In the present study, permethoxylated derivatives of polyunsaturated fatty acids were analyzed by gas–liquid chromatography–chemical ionization mass spectrometry (GLC–CIMS), in order to determine whether those compounds may obviate such difficulty encountered in trimethylsilyloxy derivatives.

EXPERIMENTAL SECTION

Reagents. Methyl esters of eicosadienoic (11,14-eicosadienoic; $C_{20:2}$), arachidonic (5,8,11,14-eicosatetraenoic; $C_{20:4}$), and docosahexaenoic (4,7,10,13,16,19-docosahexaenoic; $C_{22:6}$) acids were obtained from Sigma Chemical Co., St. Louis, MO. Osmium tetraoxide, deuteriomethyl alcohol, and deuterium chloride were purchased from E. Merck Co., Darmstadt. Sodium hydride, dimethyl sulfoxide, petroleum ether, bp 30–60 °C, and methyl iodide were obtained from Wako Pure Chemicals Co., Tokyo. All of the solvents were redistilled before use.

Procedures. Stereospecific oxidation of polyunsaturated fatty acids was carried out by the method of Wolff et al. (*4*) with some modifications. A 100–200-μg sample of polyunsaturated fatty acids was dissolved in 100 μL of dioxane–pyridine (8:1, v/v) and 1 mg of osmium tetraoxide (1% solution in dioxane, freshly prepared) was added. After 2 h at room temperature, a mixture of 5 mL of methanol and 1.5 mL of 16% Na_2SO_3 solution was added, and the mixture was shaken for 1 h. After centrifugation of the mixture to remove the salts, the supernatant was evaporated to dryness. This residue was suspended in ether and evaporated to dryness (two times). The final residue was dissolved in a small volume of methanol–chloroform (1:1, v/v). It was then transferred into the screw-capped tube (13 × 100 mm), and the residue was completely dried over P_2O_5 in a desiccator.

Permethylation of polyhydroxy fatty acids was carried out with methylsulfinyl-carbanion and methyl iodide in dimethyl sulfoxide (*5*), and permethylated products were purified by silicic acid column chromatography with 5% methanol in chloroform as the eluting solvent. The permethylated fatty acids were converted

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