Mass spectrometry of the phosphatidylcholines: fragmentation processes for dioleoyl and stearoyl-oleoyl glycerylphosphorylcholine

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ABSTRACT Mass spectra for the various phosphatidylcholines, together with accurate mass measurements on the more abundant fragment ions, have been described in a previous paper (Ref. 5). No detailed fragmentation sequence was proposed on the evidence available. In the case of dioleoyl glycerylphosphorylcholine, some question arose as to whether certain ions were produced by electron impact or by pyrolysis.

In this paper, results are reported which enable a more detailed fragmentation sequence to be proposed. By observing metastable transitions in the first field free region of a double-focusing mass spectrometer, it can be shown that the major ions in the spectrum are produced by electron impact processes, and not by pyrolysis; moreover, many of these ions are directly related to one another by metastable processes.

In particular, it has been demonstrated that the ions at m/e 603 for dioleoyl glycerylphosphorylcholine and at m/e 604 for stearoyl-oleoyl glycerylphosphorylcholine are derived from the appropriate molecular ions by an electron impact-induced process.

From measurements of the metastable ion intensities, as well as from the appearance potentials and ionization efficiency curves, conclusions may be drawn about many of the fragmentation mechanisms, allowing a distinction to be made between rearrangement and cleavage reactions.

SUPPLEMENTARY KEY WORDS first field free region · metastable intensities · appearance potentials · cleavage · rearrangement · electron impact

The demonstration that two ions are related by a metastable transition is important evidence for the ions being involved in an electron impact-induced process. The identification of metastable transitions between the electrostatic and magnetic analyzers of a double-focusing mass spectrometer is difficult because of low sensitivity, and also because of ambiguities that arise with complicated fragmentation sequences. Recently, metastable scanning in the 1FFR between the ion source and the electrostatic analyzer has been introduced, and this method possesses greater sensitivity than the detection of metastable transitions between the analyzers. It is thus possible to detect many more of the metastable transitions, and these may be unequivocally identified with the appropriate daughter ion by operating the mass spectrometer in the defocused mode (1–3). The presence of a metastable transition for two fragment ions usually means that these ions are related by a single-step process, although this is not always true (4).

The major fragment ions already described for dioleoyl and stearoyl-oleoyl GPC (5) may be incorporated into a fairly detailed fragmentation sequence, using the information gained from metastable scanning techniques. Information is also required about the type of reaction involved in a particular fragmentation process. Within the last few years, certain criteria have been put forward to distinguish between simple cleavage and rearrangement reactions. Most of these criteria rely on the quasi-equilibrium theory (QET) of mass spectra (6), and it has been found that, in general, rearrangement reactions have low frequency factors (7), low activation energies (8), and abundant metastable ions (9) compared with simple cleavages. In particular, Williams and Cooks (7) have shown that as the electron beam energy is reduced, those peaks associated with rearrangement reactions be-

Abbreviations: dioleoyl GPC, dioleoyl glycerylphosphorylcholine; stearoyl-oleoyl GPC, 1-stearoyl-2-oleoyl glycerylphosphorylcholine; GLC, gas–liquid chromatography; TLC, thin-layer chromatography; 1FFR, first field free region.

come relatively more abundant compared with those associated with cleavage reactions.

Brown (10) has suggested that $\Delta(AP - IP)$ should be used as a measure of the activation energy for the reaction, and he has shown that this difference is low for rearrangement reactions and high for cleavage reactions. An absolute measure of the energy of activation is complicated by inaccuracies in the measurement of the ionization and appearance potentials; this is due to a spread of electron energy in the beam, excess internal kinetic energy, and any kinetic shift during the decomposition (11).

In the case of dioleoyl GPC, the appearance potentials for the major fragments have been measured. As the molecular ion is of exceedingly low intensity (5), no molecular ionization potential could be obtained. For those ions directly related to one another by the presence of a metastable ion, the *apparent* activation energy was taken as equal to $\Delta(AP_{II} - AP_I)$, for the process $I^+ \rightarrow II^+$.

MATERIALS AND METHODS

All the measurements reported in this paper were made using an Associated Electrical Industries Ltd. MS902 double-focusing mass spectrometer, fitted with peak matching accessory and a metastable scanning device. Accurate mass measurements and low resolution mass spectra were obtained using operating conditions as described previously (5). Metastable spectra were obtained by operating the spectrometer in the defocused mode (1–3), with an accelerating potential of 4 kv. The voltage ratio necessary to observe the metastable ion was obtained by averaging the 50% values on either side of the metastable peak.

Appearance potentials were obtained using the method of Lossing, Tickner, and Bryce (12), in which the logarithm of the ionization efficiency is plotted against electron beam energy. The extrapolation was taken to 0.1% of the ion intensity at approximately 6 ev above threshold; this amounted to 17 ev for all the ions with the exception of m/e 55, for which the reference energy was 20 ev. Although a reference energy of 50 ev has been commonly used (10, 12), the curves of ionization efficiency against electron voltage for the fragments produced by dioleoyl GPC became complicated at high electron voltages, possibly due to multiple fragmentation modes. It is realized that the choice of reference energy is arbitrary, but by taking a reference energy which is a fixed number of electron volts above threshold, a satisfactory normalization procedure could be achieved.

Appearance potentials were measured with respect to the first ionization potential of argon gas, which was present in the spectrometer during all measurements; the ionization potential was estimated by extrapolation to 0.1% of the intensity at 22 ev and compared with the spectroscopic value for argon, 15.77 ev (13). Typical values obtained for argon were 15.73 and 15.80 ev; the small differences between these values and the spectroscopic value were used as correction factors for the experimentally determined appearance potentials. During the measurement of appearance potentials, the spectrometer was run under the following operating conditions: accelerating voltage, 8 kv; resolution, 1200 for 10% valley definition; trap current, 10 μa stabilized; temperature of ion source and direct insertion probe, 250°C. The repeller plate was maintained at the same potential as the ion source chamber; source pressure totalled approximately 10^{-6} mm Hg, and the analyzer pressure was $<10^{-7}$ mm Hg. The sample was applied to the direct insertion probe in chloroform solution; approximately 50 μg was found to be adequate. It has been found that analyzer pressures should be as low as possible so as to eliminate the collision-induced component of the metastable ion decompositions in the 1FFR (10, 14, 15).

Beynon (16) has discussed the factors which may affect the accuracy of ionization and appearance potential measurements, and has suggested that the accuracy to be expected under ideal conditions is of the order of ± 0.1 ev for ions which give ionization curves of shape similar to that of the calibrant gas. The measurements reported in this paper for dioleoyl GPC were found to be repeatable to within ± 0.1 ev (short term), and in the worst case ± 0.25 ev (long term).

For an appearance potential to be accurate, only one ionization process should occur just above threshold. If this is the case, one would expect the "normalized" ionization efficiency curves to be similar for the fragment ions, and that the straight line portions would be parallel to one another. This was found to be essentially correct, with the exception of ions *f*, *g*, and *h*, which are shown in Fig. 4. If more than one ionization process occurs above threshold, the apparent appearance potential will be for that process with the lowest activation energy, in all probability a rearrangement reaction.

Dioleoyl and stearoyl-oleoyl GPC were prepared synthetically (17), and they were purified by silicic acid chromatography; their purity was determined by GLC and TLC (1). No contaminants were found to be present, and the purity exceeded 99.5%.

RESULTS AND DISCUSSION

Mass spectra, recorded at 70 ev and 14 ev, for dioleoyl GPC are shown in Figs. 1 and 2 for m/e values between 50 and 700; peaks less than 1% relative abundance are not reproduced. Curves of ionization efficiency vs. electron beam voltage for the fragments m/e 603, 449, 393,

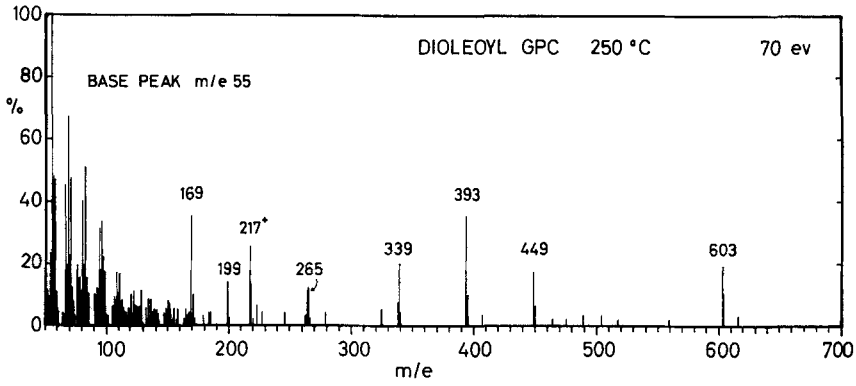


FIG. 1. Mass spectrum of dioleoyl GPC recorded at 70 ev. The percentage relative abundance, compared with the base peak, is plotted against m/e values between 50 and 700. Only peaks greater than 1% relative abundance are shown.

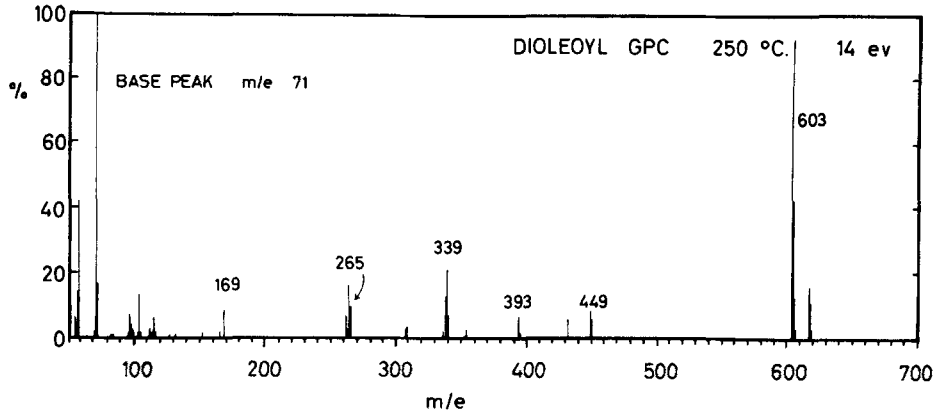


FIG. 2. Mass spectrum of dioleoyl GPC recorded at 14 ev. The percentage relative abundance, compared with the base peak, is plotted against m/e values between 50 and 700. Only peaks greater than 1% relative abundance are shown.

339, 265, 169, 58, and 55, in the spectrum of dioleoyl GPC, are shown in Figs. 3 and 4, together with the curve for argon. Appearance potentials derived from the semi-logarithmic extrapolation in Figs. 3 and 4 are shown schematically against fragment mass in Fig. 5. This figure is intended to represent visually the breakdown of high mass fragments of low appearance potential to low mass fragments of relatively high appearance potential. Table 1 shows the data obtained for dioleoyl GPC by metastable scanning in the 1FFR. The data obtained for stearoyl-oleoyl GPC are essentially similar, differing only by the expected two mass units, and are therefore not shown; the same fragmentation steps were observed for both compounds. The apparent parent ion associated with each daughter ion is calculated from the accelerating voltage ratio. The ion in the spectrum of dioleoyl GPC most likely to correspond with this calculated ion is shown in the last column of the table. The resolution of the metastable scanning device is such that the error involved at 700 amu is of the order of ±3 amu. This should be compared with the data quoted by Jennings (2) or Shadoff (18).

The intensities for the metastable ions are compared with the intensity of the daughter ion. For the fragmentation $(M^+) \rightarrow (A^+)$, the intensity ratio corresponds to $(m^*)/(A^+)$, where (m^*) is the metastable ion intensity. The appearance potentials for each fragment are also shown in Table 1.

The effects of activation energy and frequency factor on the mass spectrum are described by the quasi-equilibrium theory (QET) (6) in which the rate constant k for the decomposition process is related to the frequency factor v and the activation energy for the reaction E_a^a by the expression

$$k = v \frac{(E^i - E^a)^{s-1}}{E^i}$$

where E^i is the internal energy of the molecule and s is the number of harmonic oscillators. The internal energy of the decomposing species is not usually in excess of 5 ev, once the electron beam energy exceeds 20 ev. The frequency factor, v, has a magnitude of the same order as the bond vibrational frequency, approximately 10^{14}/sec (19).

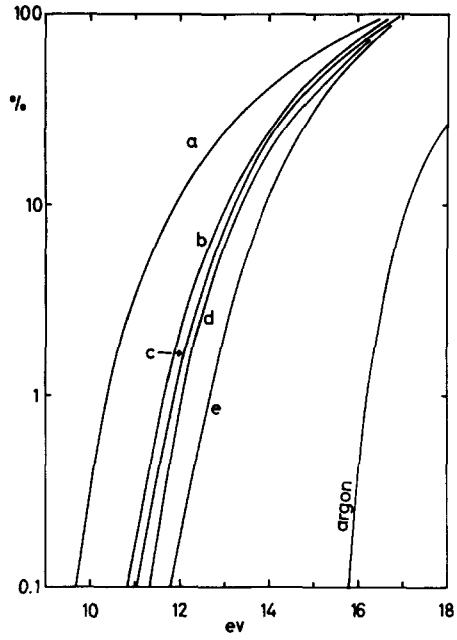


FIG. 3. Ionization efficiency curves for various ions in the mass spectrum of dioleoyl GPC. Ionic abundance, shown on a logarithmic scale, is plotted against electron voltage. Ions shown: *a*, m/e 603; *b*, m/e 265; *c*, m/e 339; *d*, m/e 393; and *e*, m/e 169.

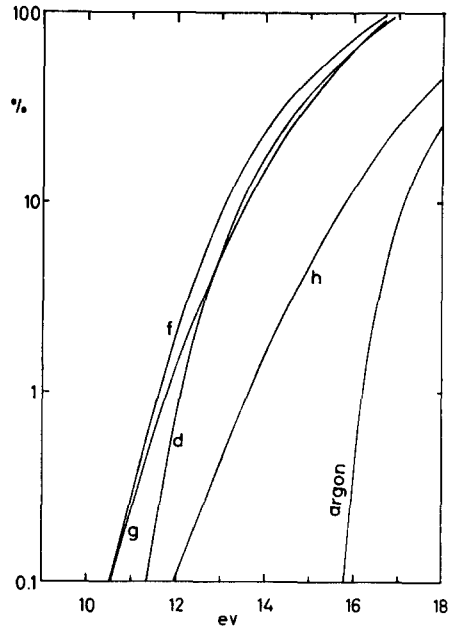


FIG. 4. Ionization efficiency curves for various ions in the mass spectrum of dioleoyl GPC. Ionic abundance, shown on a logarithmic scale, is plotted against electron voltage. Ions shown: *d*, m/e 393; *f*, m/e 449; *g*, m/e 58; and *h*, m/e 55.

When k is greater than 10^6 sec^{-1} most of the fragmentations take place in the source, and when k is less than 10^5 sec^{-1} most of the ions reach the collector without fragmenting. Metastable ions are usually observed when k is approximately 10^5 sec^{-1} (20).

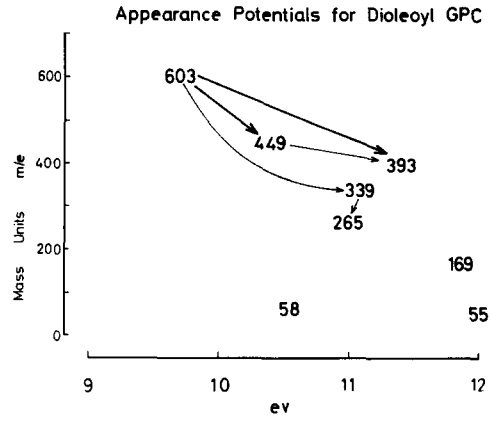


FIG .5. A schematic representation of the appearance potential for various ions plotted against the mass of the ion. The arrows indicate decomposition processes for which a metastable transition has been observed. The arrows shown in heavy type are for those processes exhibiting particularly abundant metastable ions.

TABLE 1 METASTABLE DECOMPOSITIONS OBSERVED FOR DIOLEOYL GPC IN THE FIRST FIELD FREE REGION. APPEARANCE POTENTIALS ARE ALSO SHOWN.

Daughter Ion	Appearance Potential*	Voltage Ratio†	Intensity $\times 10^{-3}$‡	Calculated Parent Ion	Observed Parent Ion§
603.53	9.6[7]	1.0310	<0.1	622.2	?621
		1.2077	6	728.9	?726
		1.3041	<0.1	787.1	785 (M^+)
449.36	10.6[1]	1.0940	4	491.6	491
		1.1245	4	505.3	505
		1.1565	2	519.7	519
		1.3381	12	601.3	603
393.30	11.4[1]	1.1075	2	435.6	435
		1.1436	<0.1	449.8	449
		1.2099	2	475.8	475
		1.2485	2	491.0	491
		1.2827	3	504.5	505
		1.5295	20	601.6	603
339.29	11.1[3]	1.1300	0.8	383.4	381
		1.2890	4	437.3	435
		1.789‖	≪0.1	607.0	(603)‖
265.25	10.9[2]	1.0657	4	282.7	281
		1.2232	6	324.5	323
		1.2750	2	338.2	339
		1.3432	2	356.3	353
169.02	11.8[2]	1.1067	4	187.1	
		1.1740	<0.1	198.4	199
		1.2555	<0.1	212.2	
		1.4930	<0.1	252.3	
		1.6990	<0.1	287.2	
58.06	10.6[9]	1.0230	2.5	59.4	59
		1.6315	0.5	94.7	95
55.06	11.9[9]	none measured			

* Obtained by semilogarithmic extrapolation; referred to argon (15.77 ev).

† Voltage ratio necessary to bring metastable ions to a focus.

‡ Ratio of metastable ion intensity to daughter ion intensity.

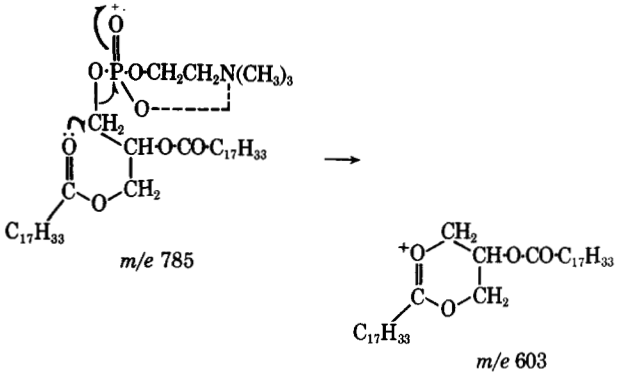
§ The ion observed in the mass spectrum that accounts most satisfactorily for the calculated parent ion. Underlining of the observed parent ion (281 and 353) indicates that the identification was not unequivocal.

‖ Indicates loss of accuracy due to low intensity.

At high electron beam energy, the frequency factor, v, determines which rate constant, whether for the cleavage or for the rearrangement reaction, is the greater. At low electron beam energies, the activation energy is the determining factor, particularly at levels just above threshold, thus accentuating peaks produced by rearrangement reactions (7).

Both the ion at m/e 603 in the spectrum of dioleoyl GPC and that at m/e 604 for stearoyl-oleoyl GPC are related to the appropriate molecular ion by a metastable ion, indicating that the fragmentation is induced by electron impact. The two ions must, however, possess different structures. Also, the metastable ion intensities for precursor fragmentation reactions suggest a difference in the mechanism by which these ions are formed. Dioleoyl GPC shows an intense metastable ion corresponding to the transition 729 → 603 and a weak one for the transition 622 → 603, whereas stearoyl-oleoyl GPC shows the reverse order for the metastable ion intensities for the analogous fragmentations.

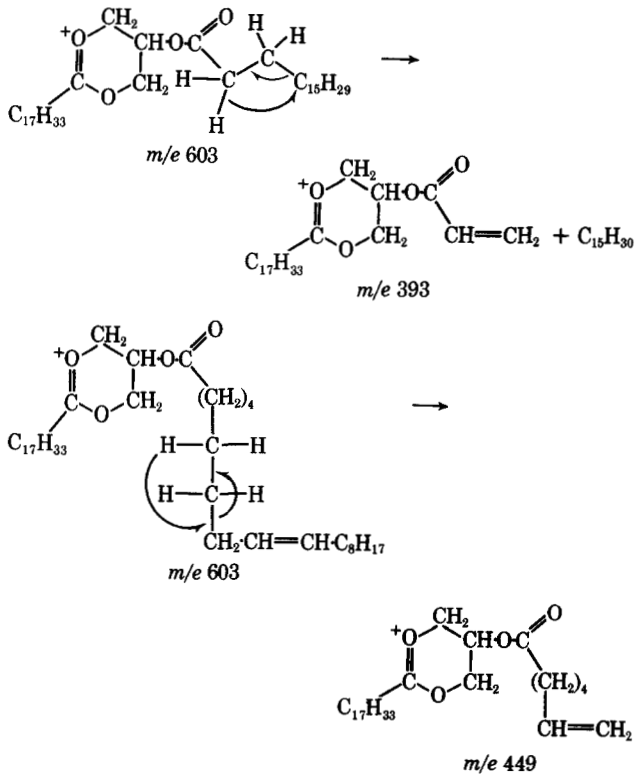
The fragmentation of the molecular ions to yield m/e 603 or m/e 604 gives rise to weak metastable ions for both compounds. Low intensity metastable ions are normally associated with cleavage reactions (9). The fragmentation of dioleoyl GPC to yield the ion at m/e 603 is likely to be brought about by a phosphoryl-assisted cleavage; the resonance-stabilized product would account for the considerable intensity of this peak, particularly at low electron voltages.



In their paper on the mass spectrometry of the deuterated 1,3-distearins, Morrison, Barratt, and Aneja (21) suggest two plausible fragmentation pathways for the dehydration of the distearin to yield a fragment at m/e 606. In the case of stearoyl-oleoyl GPC, it is probable that such mechanisms operate, producing the ion at m/e 604 by removal of the phosphorylcholine group in a manner analogous to the removal of water from the distearin molecule. In the 1,3-distearins, one of the protons that is removed is thought to originate from the hydrocarbon chains (21), and a similar origin has been suggested for one of the protons removed during the formation of the m/e 604 ion in the spectrum of stearoyl-oleoyl GPC (5). Although no accurate mass measurements are available to support such a contention, it is possible that the ions present (on the basis of a metastable transition) at approximately m/e 621–2 and 726–9 in both spectra are produced by cleavage of the molecular ion α to the phosphorus atom, and by removal of the trimethylnitrogen group (M^+-59).

The fragmentation sequence will be discussed with reference to dioleoyl GPC, although similar conclusions may be drawn from the data available for stearoyl-oleoyl GPC. Many of the metastable transitions observed for both phosphatidylcholines involve the successive loss of hydrocarbon fragments, the fragment ions being spaced at multiples of 14 mass units.

The major pathways for the fragmentation of the m/e 603 ion are shown in Fig. 5. Pronounced metastable ions are observed for the fragmentations 603 → 393 and 603 → 449, suggesting rearrangement reactions. Possible reaction mechanisms for these two reactions are outlined below. The production of the ion at m/e 449 may occur by specific cleavage at the carbon atom that is β to the double bond. This would need verification using deuterium labeling of the hydrocarbon chain; however, work in this laboratory with 9,10-deuterated phosphatidylcholines would suggest that this approach might be fraught with difficulties, as considerable D–H scrambling is observed in the mass spectrum (22, 23).

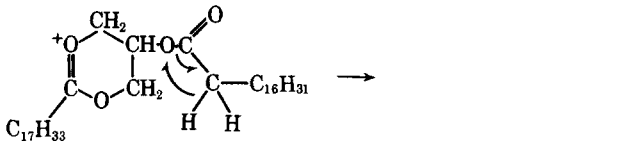


Although the reaction mechanisms show the fragmentation of only one of the hydrocarbon chains, it is

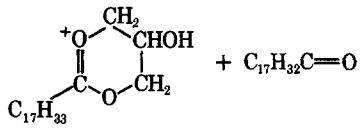
known that both chains fragment as judged from the spectrum of stearoyl-oleoyl GPC (5). The fragment at m/e 449 may then yield the ion at m/e 393 by loss of four more methylene units.

The metastable ion intensities suggest that these reactions are of the rearrangement type (9), and the apparent activation energies are also in keeping with this conclusion. The values of 1.74 ev for ($AP_{393} - AP_{603}$) and 0.94 ev for ($AP_{449} - AP_{603}$) compare well with the range of values found by Brown (10) (range of values 0.11–2.91 ev for simple rearrangements, as compared with 1.46–6.31 ev for cleavages).

The mechanism for the elimination of the ketene group, $C_{17}H_{32}CO$, from the ion at m/e 603 may be considered to take place as follows:



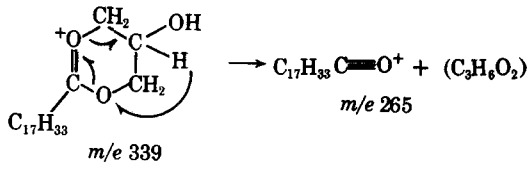
m/e 603



m/e 339

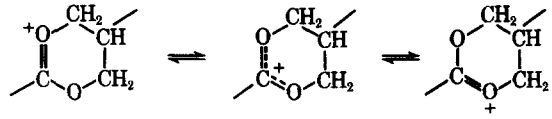
The product is equivalent to $(RCO + 74)^+$ as reported for triglycerides by Barber, Merren, and Kelly (24), and the elimination of a substituted ketene has analogies in the spectra of other esters (25). Although the metastable ion intensity is low, suggesting a simple cleavage, the reaction requires the rearrangement of one hydrogen atom, and the apparent activation energy ($AP_{339} - AP_{603}$) of 1.46 ev is more appropriate to a rearrangement (10).

The ion at m/e 339 gives rise to the acylium ion at m/e 265 and probably to an ion at m/e 264 by transfer of an extra hydrogen atom. The very small difference in the appearance potentials for m/e 265 and 339 is not significant when compared with the error involved in each appearance potential measurement of ±0.1 ev.



m/e 339 *m/e* 265

When the low and high voltage spectra are compared (Figs. 1 and 2), the abundance of the ions discussed above at low electron voltage becomes apparent. This provides confirmatory evidence that these ions arise through rearrangement reactions (7). In the reaction mechanisms outlined above, the resonance-stabilized cyclic structures put forward for many of these ions are in keeping with the stability of these ions, as judged by their ionic abundances. For example, the m/e 603 ion may be stabilized as follows:



Also it may be noted that the curves of ionization efficiency against electron voltage are similar for all these ions.

Of the three remaining ions for which the appearance potential was measured, the one at m/e 55 shows the greatest deviation in the shape of its ionization efficiency curve. The large number of possible fragmentation processes giving rise to this ion, which is a small hydrocarbon fragment, is likely to account for this deviation.

The ions at m/e 169 and 55 have particularly high appearance potentials, suggesting that these ions are, perhaps, formed by cleavage reactions. The ion at m/e 58 (ionic formula C_3H_8N [5]) shows an appearance potential that is considerably lower, however, suggesting that this ion may be produced by a reaction involving a rearrangement. The ions at m/e 169 and 58 cannot have arisen from precursor ions associated with m/e 603, or the ion m/e 603 itself, as the ion at m/e 169 contains phosphorus, and that at m/e 58 nitrogen.

The evidence presented in this paper provides a more detailed description of the various fragmentation modes for the phosphatidylcholines under electron impact in the mass spectrometer. Using 1FFR metastable scanning and appearance potential data, it is possible to postulate a fragmentation scheme involving the major ions. The theories of McLafferty and Fairweather (9) and Williams and Cooks (7) provide confirmatory evidence that rearrangement processes are of great importance in the spectra of the phosphatidylcholines studied.

In particular, it has been demonstrated that the ion at m/e 603 for dioleoyl GPC and at m/e 604 for stearoyl-oleoyl GPC are produced from the molecular ion by an electron impact-induced process, even though the molecular ion is of very low intensity. Shadoff (18) has used 1FFR metastable scanning to demonstrate the presence of "nonexistent" molecular ions, and he has pointed out that for these transitions to be observed at all, the molecular ion must have a lifetime of about 10^{-6} sec, or long enough to leave the ion source.

Very recently, work has been reported (26, 27) on the mass spectrometry of various triglycerides; this work involved an investigation of the effects of structure and also included the fragmentation of specifically deuterated compounds. These findings confirm much of the data presented for 1,3-distearins (21) and indirectly support

many of the conclusions reached for the fragmentation processes involved in the mass spectra of the phosphatidylcholines, using metastable ion and appearance potential techniques as described in the present work.

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