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COMBINED GAS CHROMATOGRAPHY-MASS SPECTROMETRY OF BRAIN POLYPHOSPHOINOSITIDES

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The quantitative assay of tissue polyphosphoinositides has generally been accomplished by first deacylating the extracted lipids (Dittmer and Douglas, 1969) and then separating the products on an ion-exchange column. With this method, relatively large tissue samples are required, the procedure is cumbersome and the assay method, phosphorus determination, is not specific to the phosphoinositides. Thus, there has been a need for a more reliable and sensitive assay for tissue polyphosphoinositides. In this paper, we report a method for measuring and identifying MPI[1], DPI and TPI in rat brain extracts by combined gas chromatography-mass spectrometry.

METHODS

LIPID EXTRACTION: Four month old rats were decapitated, the brains rapidly removed (always in less than 45 seconds) and frozen in liquid nitrogen. The brains were then weighed while frozen and homogenized in 10 vols of $CHCl_3$:MeOH (1:1, v/v). The homogenate was centrifuged, the supernatant fluids were saved and the precipitate was washed twice with $CHCl_3$:MeOH (2:1, v/v) and then extracted at 37° C for 20 min with $CHCl_3$:MeOH (2:1, v/v) containing 0.25% concentrated HCl. The extract was filtered through a plug of glass wool and the filtrate collected. The latter extraction was repeated three times. The combined extracts (i.e., $CHCl_3$: MeOH [1:1] and $CHCl_3$:MeOH [2:1] with 0.25% HCl) were then washed with 0.2 volumes of 1N HCl. The lower phase was collected, washed twice with 0.5 vols $CHCl_3$:MeOH: 1N HCl (3:48:47) and neutralized with cyclohexylamine.

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[1] Abbreviations used MPI, DPI and TPI: mono-, di- and triphosphoinositide respectively; DAMPI, DADPI and DATPI, the deacylated lipids; TMS, trimethylsilyl.

DEACYLATION: The extracted lipids were taken to dryness and then dissolved in $CHCl_3$:MeOH (1:4, v/v) and enough 1.2 N NaOH in MeOH:H_2O (1:1) was added to give a final concentration of 0.2 N. Following a 10 min. incubation period at 37° C, the methanolysis was terminated by first cooling the reaction mixture in ice and then neutralizing with 1N acetic acid. Two ml of $CHCl_3$:MeOH (9:1), two ml of H_2O and one ml of isobutyl alcohol were then added and the entire mixture was centrifuged at 2,500 rpm for 20 min. The water phase was collected and the deacylated lipids were converted to the free acid form on a Dowex-50 W column and then lyophilized. Standard solutions of MPI were prepared by deacylating commercially available MPI (Pierce Chemical Co.) as described above, whereas DPI and TPI were first extracted from rat brain and then deacylated. Phosphorus content in the standards was determined by the procedure of Bartlett (1958).

GAS CHROMATOGRAPHY (GC): The lyophilized samples were then taken up in N,O-bis (trimethylsilyl)trifluoroacetamide (+1% trimethylchlorosilane)-dry pyridine (2:1, v/v). The samples were allowed to stand overnight at room temperature prior to GC. Although chromatography was achieved on a variety of column packings, optimal separation was obtained on a 2 ft. x 1/4 in. glass U-tube packed with 1% SE-30 on Gas Chrom Q (Applied Science Laboratories). Two µl aliquots were injected into the gas chromatograph for analysis. Helium was used as carrier gas at a flow rate of 75 cc/min. Mass spectra of the TMS derivatives of the deacylated phosphoinositides were obtained with a LKB-9000 mass spectrometer according to procedures fully described elsewhere (Zinbo and Sherman, 1970).

RESULTS AND DISCUSSION

Fig. 1 presents a representative chromatogram of TMS-DAMPI (peak I), TMS-DADPI (III) and TMS-DATPI (IV) extracted from whole brain and treated as described above. A fourth peak (II) is also seen in Fig. 1 and is, at present, an unknown. The tissue concentrations of the phosphoinositides were determined by a comparison of their chromatographic peak heights with standards run under identical conditions. The calculated values, based on tissue wet weight, were 2.42 µmoles/g for MPI, 0.30 µmoles/g for DPI and 0.19 µmoles/g for TPI. These tis-

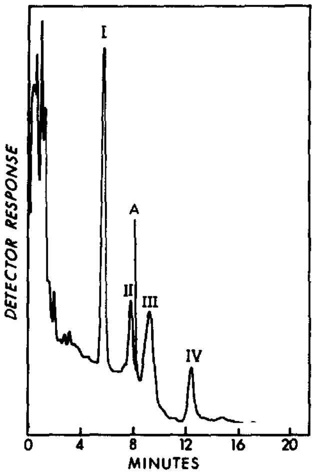
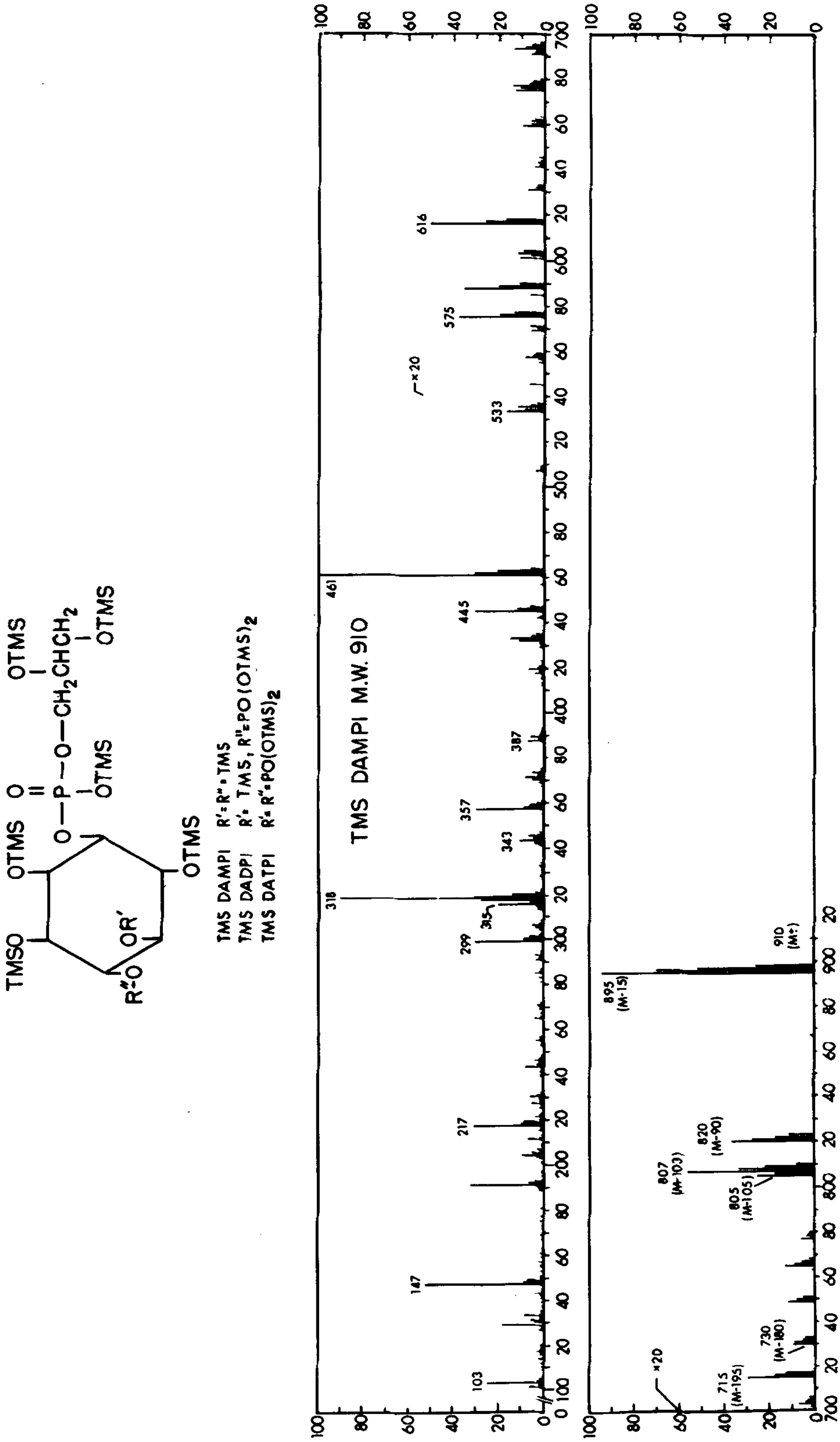


Fig. 1. Gas chromatogram of the TMS derivatives of deacylated MPI (I), DPI (III) and TPI (IV) obtained from rat brain. Peak II is an unknown. A marks an electrometer gain change to an eight-fold greater sensitivity.

sue concentrations are in good agreement with previously reported values (Dittmer and Douglas, 1969). The reasons for the somewhat lower levels of TPI found in the present study are currently under investigation (i.e., post-mortem change, non-specificity of phosphorus determination, etc.). On the basis of the determinations made in these experiments, it appears that phosphoinositide concentrations in one mg or less of tissue can readily be measured. Hence, the sensitivity of our method appears to be at least 100 times greater than previously reported methods.

The mass spectra of TMS-DAMPI, TMS-DADPI and TMS-DATPI are presented in Figures 2 and 3. As can be seen the molecular ions ($\overset{+}{M\cdot}$) are found in only trace amounts for each of these compounds. In addition, each compound undergoes the loss of a number of neutral fragments typical of TMS derivatives which were confirmed by labelling the parent compound with trimethylsilyl-d_9 groups (McCloskey _et al._, 1968). These losses include M-15 (CH_3), M-90 (TMSOH), M-103 ($TMSOCH_2$), M-105 ($TMSOH+CH_3$), M-180, (2TMSOH) and M-195 ($2TMSOH+CH_3$). Below _m/e_ 400 the mass spectra of these compounds are similar to the spectra of a number of TMS sugar phosphates (Zinbo and Sherman, 1970).


TMSO
OTMS
O
O—P—O—CH2CHCH2
OTMS
OTMS
OTMS
OR'
R''O
OTMS
TMS DAMPI R'=R''=TMS
TMS DADPI R'= TMS, R''=PO(OTMS)2
TMS DATPI R'=R''=PO(OTMS)2
TMS DAMPI M.W. 910
103
147
217
299
315
318
343
357
387
445
461
533
575
616
×20
715 (M-195)
730 (M-180)
805 (M-105)
807 (M-103)
820 (M-90)
895 (M-15)
910 (M+)
100
80
60
40
20
0
100
200
300
400
500
600
700
800
900

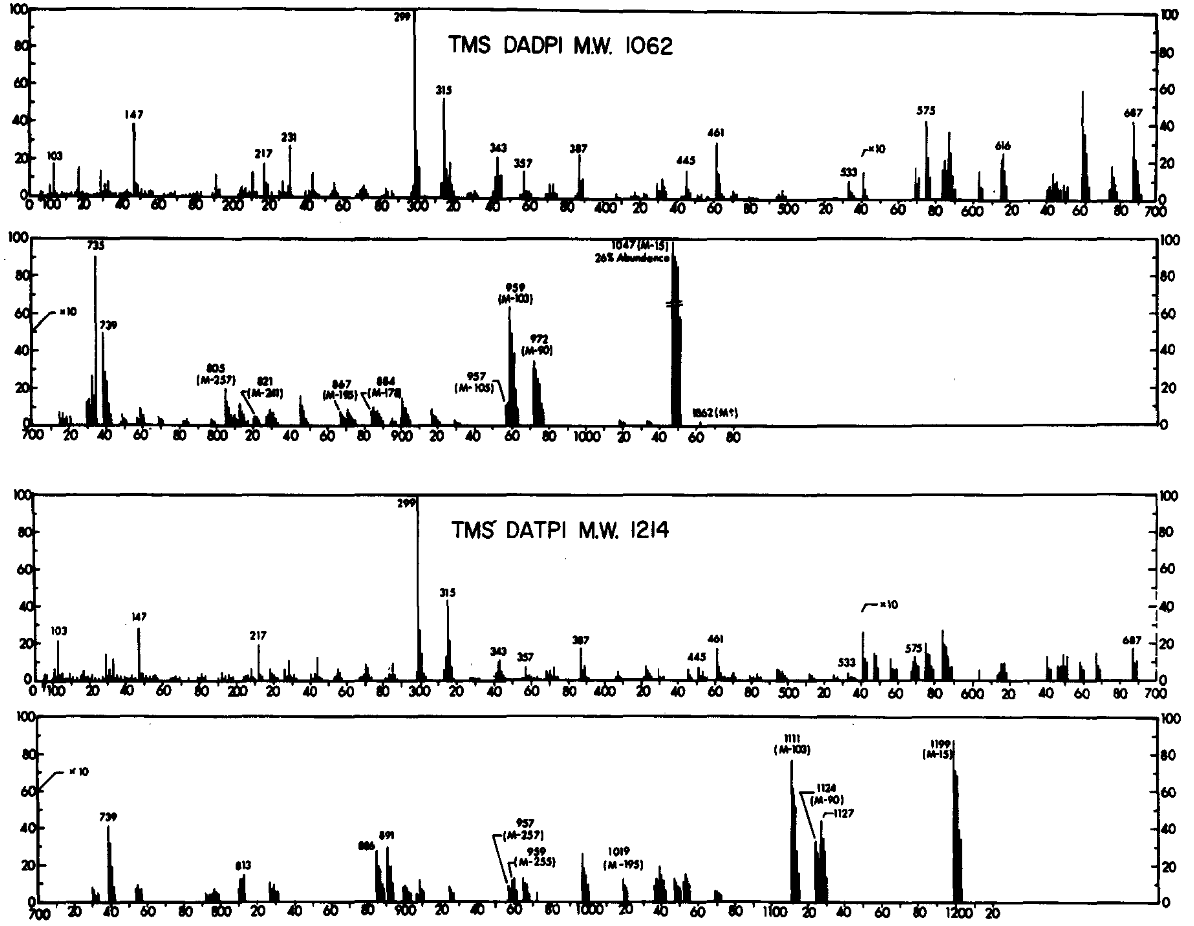


Fig. 3. The 70 electron volt mass spectrum of deacylated and trimethylsilylated (top) DPI, and (bottom) TPI.

As shown in Figs. 2 and 3, the base peak was m/e 461 for TMS-DAMPI while this ion is second in abundance in the spectra of TMS-DADPI and TMS-DATPI. TMS-d_9 labelling confirmed the structure $[TMSOCH_2CH(OTMS)CH_2-OP(OH)(OTMS)_2]^+$ for m/e 461, as suggested by Duncan *et al.* (1971), by a mass shift to m/e 497. In addition, the fairly abundant ion at m/e 533 has the structure $[TMSOCH_2CH(OTMS)CH_2OP(OTMS)_3]^+$ also confirmed by the mass shift to m/e 578 on TMS-d_9 labelling. The base peak m/e 299, in the case of TMS-DADPI and TMS-DATPI, has the structure $[(TMSO)_2PO_2-SiMe_2]^+$ as reported previously (Zinbo and Sherman, 1970).

The GC method described in this paper appears to offer a considerably more sensitive and reliable means of quantitatively assaying tissue content of the phosphoinositides than previously reported methods. As such it should have considerable application to the simultaneous determination of the phosphoinositides in tissue samples at the milligram level.

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