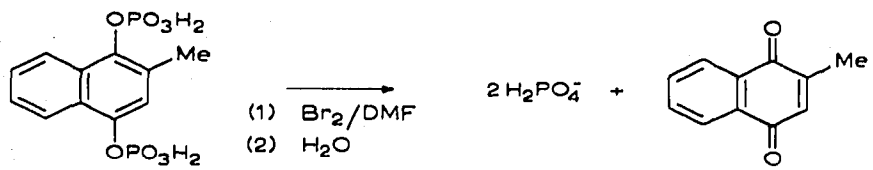
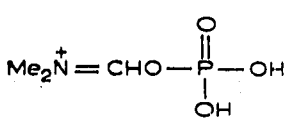
CHROM. 5383

Combined gas–liquid chromatography/mass spectrometry study of the bromine oxidation of Synkavit (2-methylnaphthalene-1,4-diol diphosphate) in ^{18}O-enriched dimethylformamide

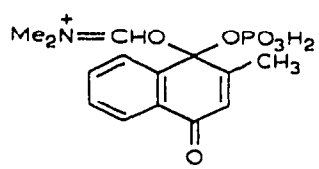
The combination of gas–liquid chromatography (GLC) and mass spectrometry (MS) has proved to be applicable to a wide variety of analytical problems[1–12]. The success of this technique has prompted us to employ it in a re-examination of the bromine oxidation of 2-methylnaphthalene-1,4-diol diphosphate (Synkavit, I) in ^{18}O-enriched dimethylformamide as solvent. Both the 2-methyl-1,4-naphthoquinone



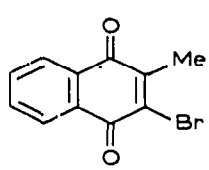
I II



III



IV



V

(Menadione, II) and the inorganic phosphate (P_i) products of this reaction were found in the initial study[13] to be isotopically labelled by conversion of each to carbon dioxide and determination of the ^{18}O to ^{16}O ratio by MS. With the combination technique one uses the GLC column to present a few micrograms of sample to the mass spectrometer for determination of the excess ^{18}O content of the molecule itself (molecular ion) or fragment ions thereof. We have recently reported on the determination of the ^{13}C content of amino acids from algae grown on ^{13}C-enriched CO_2 via combined GLC–MS of their TMSi derivatives[11]. It was felt that a comparison of results for the direct (GLC–MS) and the indirect (conversion to CO_2 followed by MS) methods for the determination of ^{18}O content of Synkavit and P_i would be a test of their relative merits and consistency. This was considered particularly worthwhile in view of the conclusions drawn from observing the presence of label in the two products—*i.e.*, that the bromine oxidation proceeded via the intermediates III and

IV to the extent of 26% (P–O bond cleavage) and 11%* (C–O bond cleavage), respectively[13].

Experimental

GLC–MS experiments with 1–2 μg samples were carried out with an LKB Model 9000 instrument. The spiral glass GLC column was 4 ft. × 3 mm I.D.; 3% OV-17 on acid-washed and silanized[4] Gas-Chrom P; 30 ml/min helium carrier gas. Mass spectra were obtained with a 70 eV ionizing potential, a 3.5 kV accelerating potential, a 50 μA filament current, and a 270° ion source temperature.

Phosphoric acid (Merck) and the ^{18}O-enriched acid (obtained from P_i—magnesium phosphate—by ion-exchange) were taken up in bis-trimethylsilyltrifluoroacetamide and allowed to react for 30 min at 45° to form the tri-trimethylsilyl (TMSi) derivative[14]. This compound exhibited a retention time of 6 min (temperature-programmed analysis: initial temperature 80°, 5°/min). MS peak intensity measurements were made on both the molecular ion M (*m/e* 314) and M-15 (*m/e* 299) fragment and for the corresponding isotope signals (*m/e* 316 and 301, respectively). Multiple GLC–MS runs were made and mean values determined. Calculation of the percent excess ^{18}O for the M-15 fragment is given below; the same value was obtained from the molecular ion.

	M-15	
	m/e 299	*m/e 301*
Intensity ^{18}O-enriched $PO(OTMSi)_3$	100	19.0
Intensity reference $PO(OTMSi)_3$	100	13.5
Intensity difference		5.5

$$\frac{5.5}{5.5 + 100} \times 100 = 5.2\% \text{ excess } ^{18}O$$

Intensity measurements and calculations were also made on the M (*m/e* 172) and the M+2 (*m/e* 174) signals of the ^{18}O-enriched 2-methyl-1,4-naphthoquinone and a reference sample. A value of 2.9% excess ^{18}O was obtained.

Results and discussion

GLC of the tri-TMSi phosphate derivative of P_i indicated the sample to be homogeneous, and the compound was identified by its retention behavior and mass spectrum. The value of methods for readily converting inorganic anions to volatile derivatives[14] amenable to GLC and MS is evident. Analysis of the 2-methyl-1,4-naphthoquinone** isolated from the Synkavit oxidation, on the other hand, disclosed the presence of two components (see Fig. 1). The earlier eluted component was

* A factor of 2 should be included in the calculation of this value in ref. 13 to take account of both phosphate sites in I.

** A GLC method for the determination of Menadione has been reported[15].

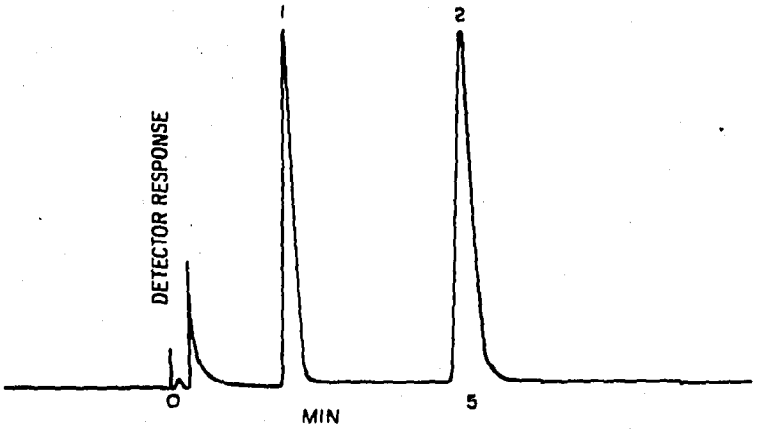


Fig. 1. GLC of the quinone fraction resulting from bromine oxidation of Synkavit in ^{18}O-enriched dimethylformamide. Column conditions are given in *Experimental*; temperature-programmed analysis: initial temperature 145°, 5°/min.

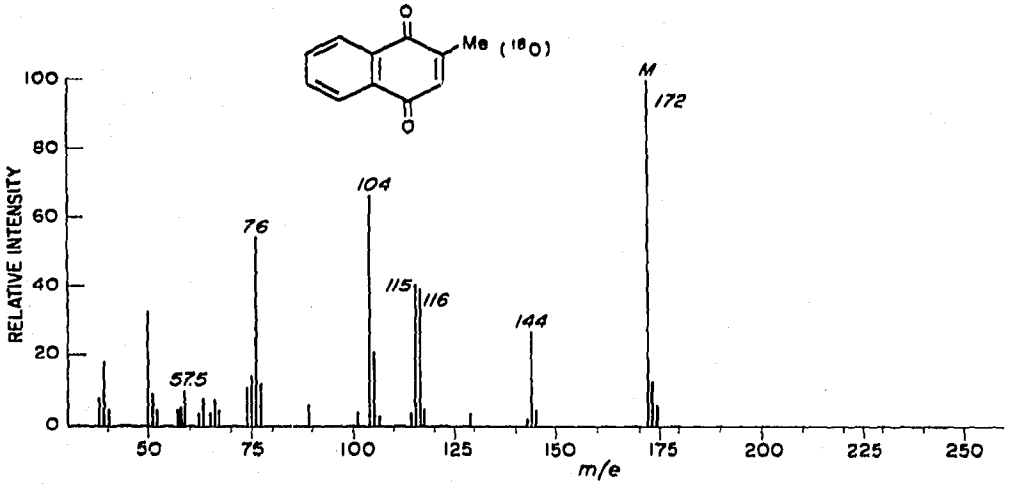


Fig. 2. Mass spectrum of the 2-methyl-1,4-naphthoquinone resulting from bromine oxidation of Synkavit in ^{18}O-enriched dimethylformamide.

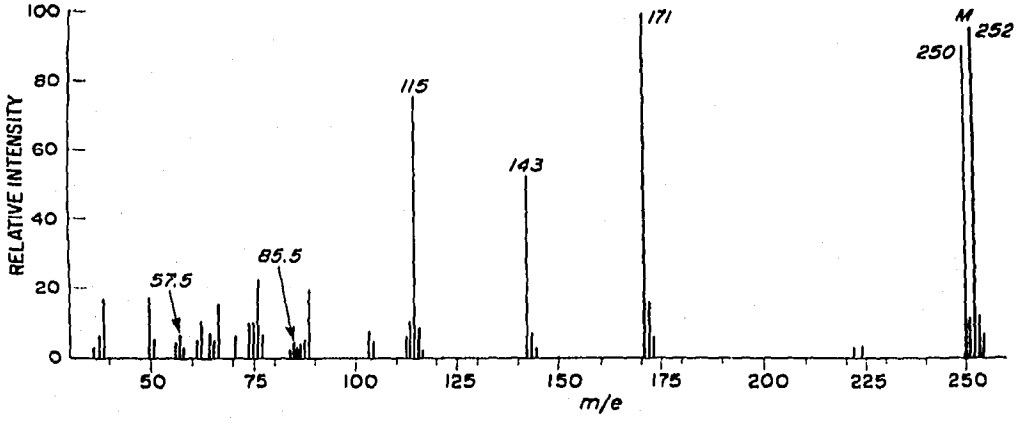


Fig. 3. Mass spectrum of the 3-bromo-2-methyl-1,4-naphthoquinone resulting from bromine oxidation of Synkavit in ^{18}O-enriched dimethylformamide.

identified by retention time and mass spectrum (see Fig. 2) as the expected Menadione (II). The mass spectrum of the later eluted component is presented in Fig. 3. This compound is clearly mono-bromo-Menadione—note the characteristic bromine isotope cluster for the molecular ion (*m/e* 250/252). NMR data on the mixture strongly suggest that the bromine atom is at the 3-position. The key NMR features leading to the structural assignment for the contaminant were the low areas of the $CH_3C = CH$ protons relative to the aromatic resonances and the appearance of a new methyl singlet at 7.62 τ. The two methyl peaks had a combined relative area of three protons. The absence of splitting in the 7.62 τ signal plus the downfield shift compared to that in Menadione are both diagnostic for a substituent at C-3. The significance of the production of this compound during the bromine oxidation of Synkavit is presently unclear*, although participation of such a species has previously been intimated[16].

The data obtained from the mass spectra indicated that II and P_i contain 2.9 and 5.2% excess ^{18}O, respectively (see *Experimental*). This would suggest that the oxidation in 17% enriched ^{18}O-dimethylformamide proceeded via III and IV to the extent of 31% and 17%, respectively. The former value (from P_i, as the tri-TMSi derivative) is seen to be consistent with, although greater than that noted previously[13]. The presently determined value (17%) for the second route (value obtained from 2-methyl-1,4-naphthoquinone uncontaminated with the bromination product) is also greater than the original value of 11% (ref. 13), perhaps because of the large amount of 3-bromo-2-methyl-1,4-naphthoquinone (V) present in the sample when it was investigated by the classical MS (conversion to CO_2) technique.

A disadvantage of the indirect (CO_2) approach for determination of ^{18}O (or ^{13}C) isotope content is that it does not allow differentiation between CO_2 from the compound of interest and that from possible sample contaminants. There is also the possibility of the introduction of impurities during combustion, or of incomplete combustion[17]. Although this method is capable of yielding isotope ratios with great accuracy[18], MS on the actual compound of interest, while producing less accurate data[18], does not suffer from the above disadvantages. The latter approach also allows the determination of isotope content on the molecular ion or appropriate fragment ion[19], providing a contaminant does not produce interfering ions. Of course, fractional sublimation of the components of a mixture may occur during sample evaporation into the ion source even with closely related compounds[20], and thus a contaminant will not necessarily volatilize with the compound of interest. However, the great separating power of GLC, resulting from the judicious choice of stationary phase, when combined with MS results in a superior technique for indicating non-homogeneity of sample and yielding isotope data on each component of a mixture on the microgram scale[11,12]. The potential of the method in chemical and biological studies employing limited amounts of partially purified compounds is obvious.

We wish to thank Mrs. VIRGINIA GRUBER for preparation of the ^{18}O-enriched phosphoric acid from P_i, Mr. JACK L. SMITH for assistance with the mass spectro-

* Calculations based on the signals at *m/e* 171 and 173 (M–Br) in the mass spectrum of bromo-Menadione suggest that this compound contains almost the same percent excess ^{18}O as the ^{18}O-enriched Menadione.

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Merck Sharp & Dohme Research Laboratories, Rahway, N.J. 07065 (U.S.A.)
W. J. A. VANDENHEUVEL
B. H. ARISON

Division of Computer Research and Technology, National Institutes of Health, Bethesda, Md. 20014 (U.S.A.)
J. S. COHEN

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