STATUS REPORT

October 6, 1965 to April 6, 1966

NASA Grant NsG-292-63/17-001-001

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METHODS AND MATERIALS

A Bendix Time of Flight Model 14-101 instrument was utilized for the investigations described herein. The pumping system of this instrument consists of a Welch forepump, mercury diffusion pump, and liquid nitrogen trap in series. The best vacuum attainable is 2.2×10^{-8} torr as measured by a hot filament, Bayard Alpert gauge placed in the nitrogen trap region downstream from the ion source or electron gun area.

The electron beam producing ionization of sample operates in a continuous mode, while ion withdrawal from the electron beam remains pulsed. This operation mode, together with sample pressures of 8×10^{-7} torr (maximum, as measured by the Bayard Alpert gauge) gives a sensitivity detection limit of approximately 1.7×10^{-14} torr[(a)].

If the pressure in the sample inlet system (described below) does not exceed 100 microns, and the Bayard Alpert gauge pressure does not exceed 8×10^{-7} torr, then flow conditions from the sample inlet system to the electron beam are such that the partial pressure determined by relative ion intensities approximates[(b)] the partial pressure of that component in the sample inlet system as determined by calibrated gas mixtures.

Sample Inlet System

Materials, usually relatively high vapor pressure components, are introduced into the mass spectrometer ion source via ambient-temperature, glass-metal sample inlet system, consisting of pyrex glass tubing, high vacuum stopcocks, a pressure gauge (thermocouple gauge tube DV-1M), and a Hoke 2RB280 stainless steel valve (1/16" orfice 20 turns) placed immediately before the ion source inlet tube.

(a) Smallest peak seen on the recorder trace corresponds to 10^{-12} amperes (amps). The total ionization current is 3×10^{-6} amps, and sample pressure is 5×10^{-8} torr. Hence $\frac{10^{12} \times 5 \times 10^{-8}}{3 \times 10^{6}}$ gives 1.7×10^{-14} torr.

(b) For estimations to approximate ±5%, the ionization cross sectional areas for each component must also be considered.

This sample system is equipped with an auxillary pumping unit attaining vacuums of the order of 10^{-5} torr. Apiezon L stopcock grease is used for all stopcocks and ground glass joints. Glass-Kovar seals and stainless steel Swagelok fittings are utilized in the construction. The total volume of the sample inlet system is 175 ± 1 ml, and pressure in this system is measured with thermocouple gauge tube. With the system closed from the mass spectrometer ion source, sample tubes isolated by a stopcock, are mounted on the sample inlet system by means of ball and socket ground glass joint. The excess air is then removed by (1) auxillary and (2) the mass spectrometer pumping units, and a recording of ion current vs. mass to charge ratio (M/e) of the background is taken. The sample inlet system is again isolated from the ion source and the sample tube stopcock is opened to give a measureable pressure in the sample inlet system. The sample is allowed to flow into the ion source such that a recording of ion current vs. M/e (cracking pattern) is obtained without noticeable change in the sample inlet system pressure. By varying the electron energy of the ionizing beam, molecular weights of the various components can be obtained, and by comparing cracking patterns with those in the literature [1,2] the components in the mixture can be identified.

Preparative Sample System:

Respiring tissue plus medium are placed in a 500 ml round bottom flask fitted with a conditioned carbon dioxide absorber tube (Mallcosorb 30-50 mesh)(c) and attached to a glass preparative sample system via ball and socket ground glass connections. A mercury manometer, a pumping system (10^{-5} torr maximum), and a detachable collection tube (50 ml. containing a stopcock and a ground glass socket joint) comprise the glass preparative system.

(c) Mallcosorb (Mallenckrodt) CO_2 absorber contained traces of solvents which would purp away in 5 hours

The system is flushed several times through a side arm on the incubation flask and filled with 0_2 gas to a final pressure of 700 torr. The flask contents are stirred with a magnetic stirrer and incubation allowed to proceed at room temperature (25-30°C). At the end of the incubation period the collection tube is removed from the preparative sample system and attached to the mass spectrometer sample inlet system. During immersion in liquid nitrogen the excess 0_2 is pumped away from the collection tube, after which the tube is allowed to warm by removal of the liquid nitrogen flask. Thus a cryoscopic separation of certain components is effected and constitutes a critical step in the identification of the volatile metabolite mixture.

For estimation of the amount of evolved material, the collection tube is allowed to warm to room temperature and an aliquot (60-90 microns) is introduced into the sample inlet system. The partial pressure of each component is determined from the recorded ion intensity as follows: the % relative intensity of each component base peak is determined from the cracking pattern of the mixture. This % relative intensity corresponds to a % relative partial pressure of each component. Since each component peak measured is the base peak for that pure component, the % relative partial pressures are all divided by 100 and summed. The relative partial pressure divided by the summed relative partial pressures and multiplied by 100 gives the % composition of each component in the sample inlet system. By multiplying the total pressure in the sample inlet system by the determined % composition, one obtains the partial pressure of that component in the sample inlet system.[d]

Since the temperature, volume, and pressure of each component is known, the number of moles of each component can be readily calculated using the ideal gas law.

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[d] See sample calculations

Biological Tissue:

Rat liver tissue is used either as a sucrose homogenate or as a mitochondrial suspension. Mitochondria are prepared according to E. Weinbach[3]. Such a mitochondrial suspension consumes 8.8 μ atoms of oxygen /20 min/ mg mitochondrial N, with a P/O ratio of 1.9 for succinate as substrate at 37°C(e). Each manometer flask contained, in a final volume of 2 ml, 80 μ moles glycylglycine, 20 μ moles succinate, 20 μ moles phosphate as KH_2PO_4, 5 μ moles ADP.Na, 2 μ moles NAD^+, 0.03 μ moles cytochrome c, 50 μ moles glucose, 0.5 mg of hexokinase (Sigma Chemical Company Type III), 10 μ moles $MgCl_2.6H_2O$, and 0.5 ml. of a 10% mitochondrial preparation. Final pH=7.35.

(e) Average of 6 determinations in duplicate

Table 1

Compounds Evolved From the Incubation Flask (Note 1)

Compound	Total Moles (Note 2)	Total mg Nitrogen	Moles/mg N	Conditions
Ethylene (C_2H_4)	2×10^{-12}	20	1×10^{-13}	+A
"	6×10^{-9}	20	3×10^{-10}	+B
"	1.5×10^{-8}	--	--	*
"	3.2×10^{-9}	90	3.5×10^{-11}	*A
"	9×10^{-10}	20	4.5×10^{-11}	*A
"	1.2×10^{-8}	90	1.3×10^{-10}	*B
Acetone ($H_3C-\overset{\overset{O}{\|}}{C}-CH_3$)	6.1×10^{-10}	--	-----	* (Note 3)
"	8.1×10^{-9}	20	4.1×10^{-10}	+B
"	4.0×10^{-9}	20	2.0×10^{-10}	+A
"	2.1×10^{-8}	90	2.3×10^{-10}	*A
"	2.2×10^{-7}	90	2.4×10^{-9}	*B
2,3-dithiabutane ($H_3C-S-S-CH_3$)	4.2×10^{-12}	--	-----	*
"	2.6×10^{-8}	90	2.9×10^{-10}	*A
"	1.9×10^{-8}	90	2.1×10^{-10}	*B
"	1×10^{-9}	20	5.0×10^{-11}	*A
2-Thiapropane ($H_3C-S-CH_3$)	1×10^{-12}	--	-----	(Note 4)
"	1×10^{-10}	20	5.0×10^{-12}	*A
"	6.5×10^{-9}	90	7.2×10^{-11}	*B
"	6.9×10^{-8}	90	7.7×10^{-10}	*A
M/e 83	2.3×10^{-8}	--		* (Note 5)
"	4.5×10^{-9}	90	5.0×10^{-11}	*A
"	1.4×10^{-8}	90	1.6×10^{-10}	*B
"	2.0×10^{-8}	20	1.0×10^{-9}	*A
M/e 60	3.5×10^{-9}	90	3.9×10^{-11}	* (Note 6)

Note 1) Incubation time is 2 hr. at room temp in an oxygen atmosphere.

Note 2) Estimated values subject to accuracy described in Methods and Materials (see sample calculations)

Note 3) Acetone evolution does not depend on the presence of methionine

Note 4) This amount has been observed to evolve from 6.0 g wet weight of excised liver placed in a flask containing no medium and subjected directly to the vacuum of the sample inlet system.

Note 5) M/e 83 remains to be identified; however, the other peaks produced in the cracking patterns are 85, 47, 48, 49, and 35. This pattern is consistent with fragmentation pattern of chloroform ($CHCl_3$)

Note 6) M/e 60: acetic acid is a possibility

† Incubation medium has the following composition:

4 millimoles glycylglycine, 1 mmole succinic acid, 1 mmole KH_2PO_4, 0.25 mmole ADP.Na, 0.1 mmole NAD+, 0.0015 mmole cyctochrome c, 2.5 mmoles glucose, 0.5 mmoles $MgCl_2.6H_2O$ and 6.0 mg of hexokinase (Sigma Chemical Co. Type III). Final pH is 7.3 and total volume is 150 ml.

A Fresh mitochondrial preparation

B Aged mitochondrial preparation, frozen for 3 months

* 30 mg of L-Methionine plus 150 mg of ascorbic acid added to the basic medium

Explanation of Figure I

% relative intensity vs. mass to charge ratio
of respiring, fresh mitochondrial suspension (90 mg N total)
incubated at 30°C for 2 hours in an oxygen atmosphere.

The incubation medium contains L-methionine (30 mg) and ascorbic acid (150 mg) as well as the constituents listed under basic medium enumerated in note † Table I.

This cracking pattern is taken at 70 electron volts (ev) as read on a vacuum tube volt meter (VTVM), with a sample pressure of 31 microns in the sample inlet system.

The mercury isotopes 198 through 204 are extremely large due to the presence of a mercury manometer in the preparative sample system as a consequence, mercury vapor is trapped in the liquid N_2 cooled collection tube.

The group of peaks 129 through 136 arise from Xe isotopes which is an impurity in the O_2 gas.

Peaks 28 and 32 are not in a 4 to 1 ratio as expected for air, since a trace of O_2 remains from the oxygen incubation atmosphere.

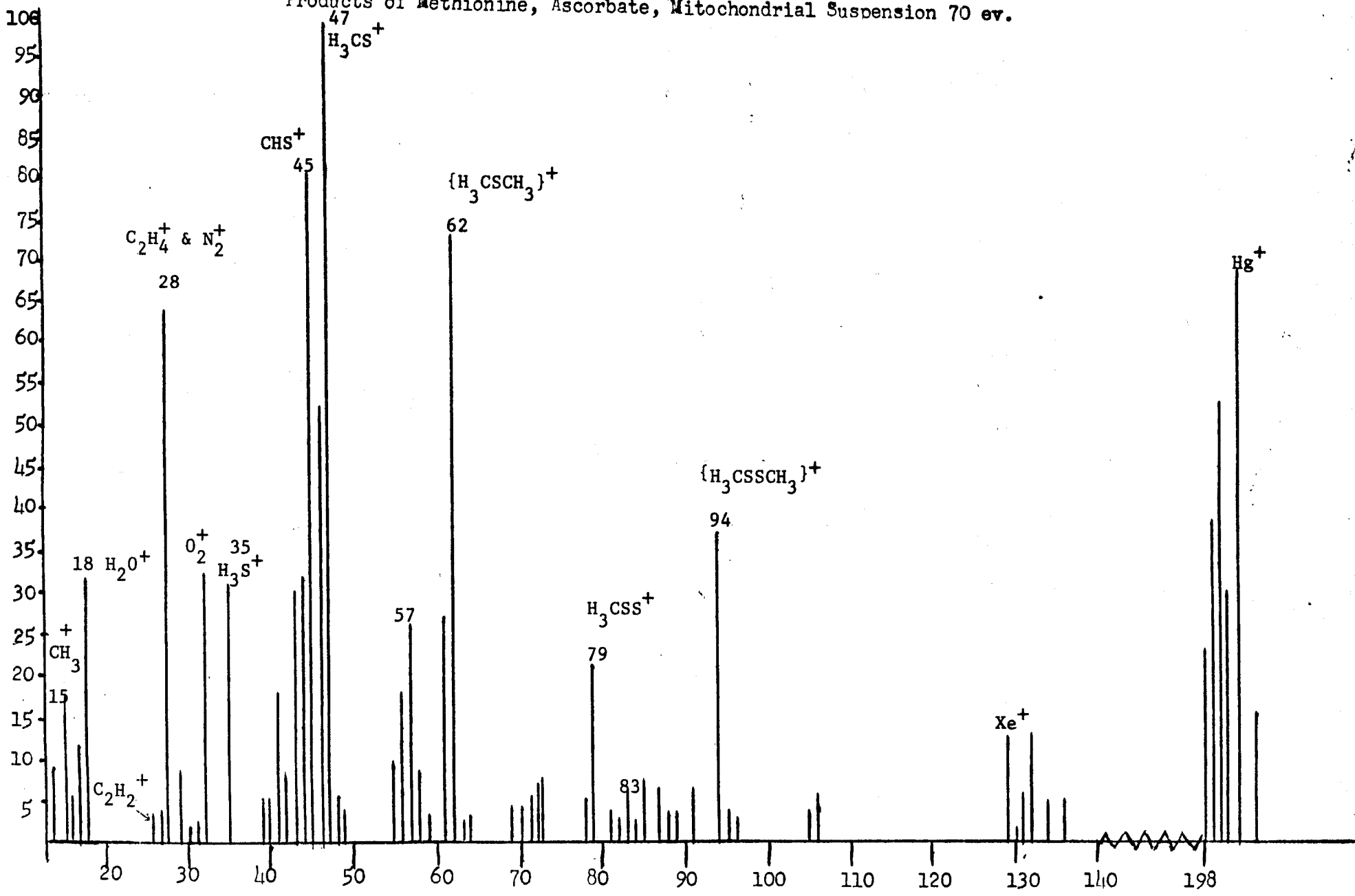
All M/e values less than 3% were excluded as well as those of Hg^{++}.

The collection tube was allowed to warm to room temperature before sample is introduced into sample inlet system.

FIGURE I

% RELATIVE INTENSITY VS. MASS TO CHARGE RATIO (M/e)

Products of Methionine, Ascorbate, Mitochondrial Suspension 70 ev.



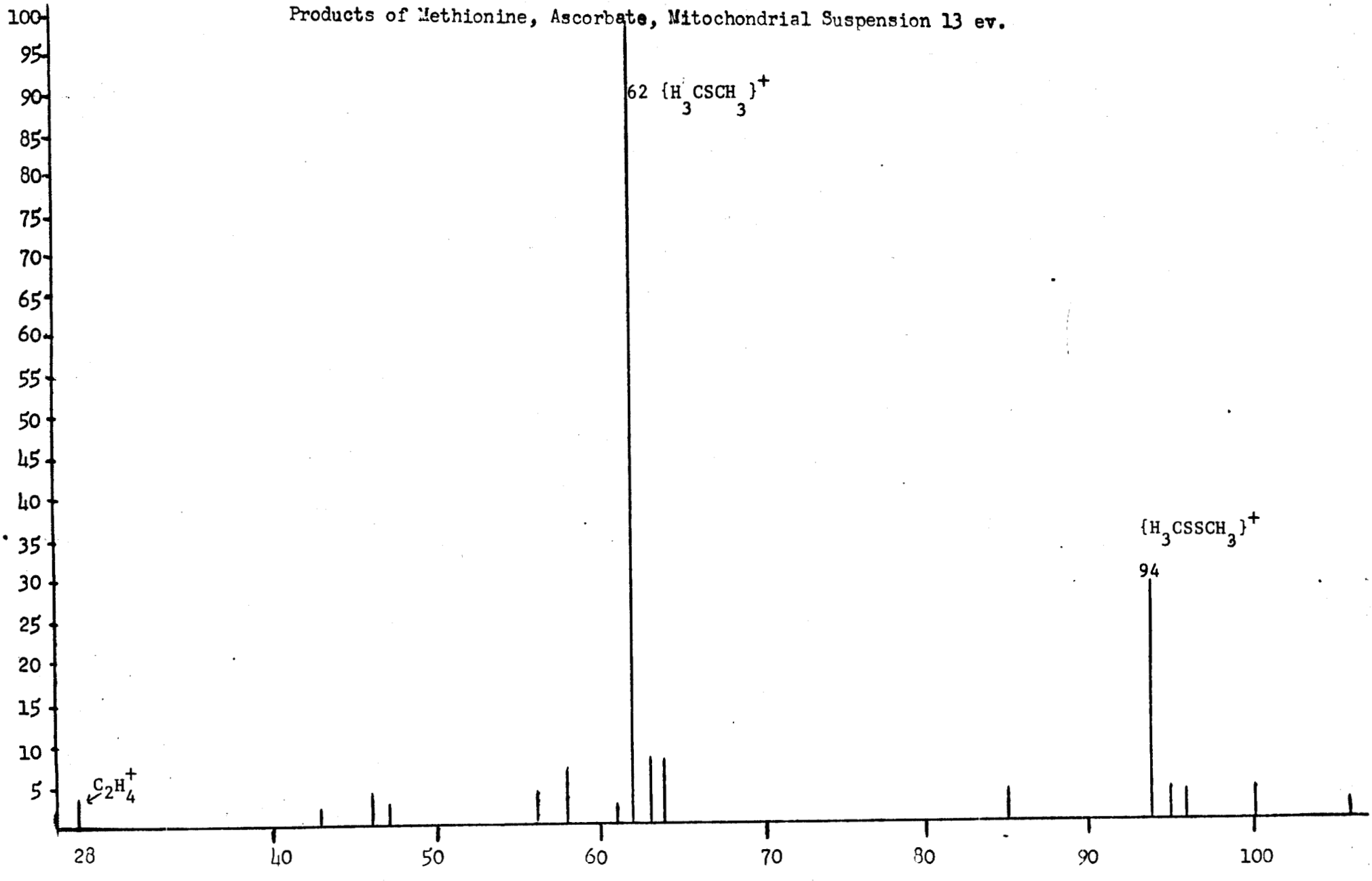
Explanation of Figure II

This is the same sample as in Figure I, with the following conditions:

A) Electron energy is 13 ev (VTVM)

B) M/e's are scanned to 204; however, Hg isotopes are not shown.

FIGURE II


% RELATIVE INTENSITY VS. MASS TO CHARGE RATIO (M/e)
Products of Methionine, Ascorbate, Mitochondrial Suspension 13 ev.
62 {H3CSCH3}+
{H3CSSCH3}+
94
C2H4+
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
28
40
50
60
70
80
90
100

Explanation of Figure III

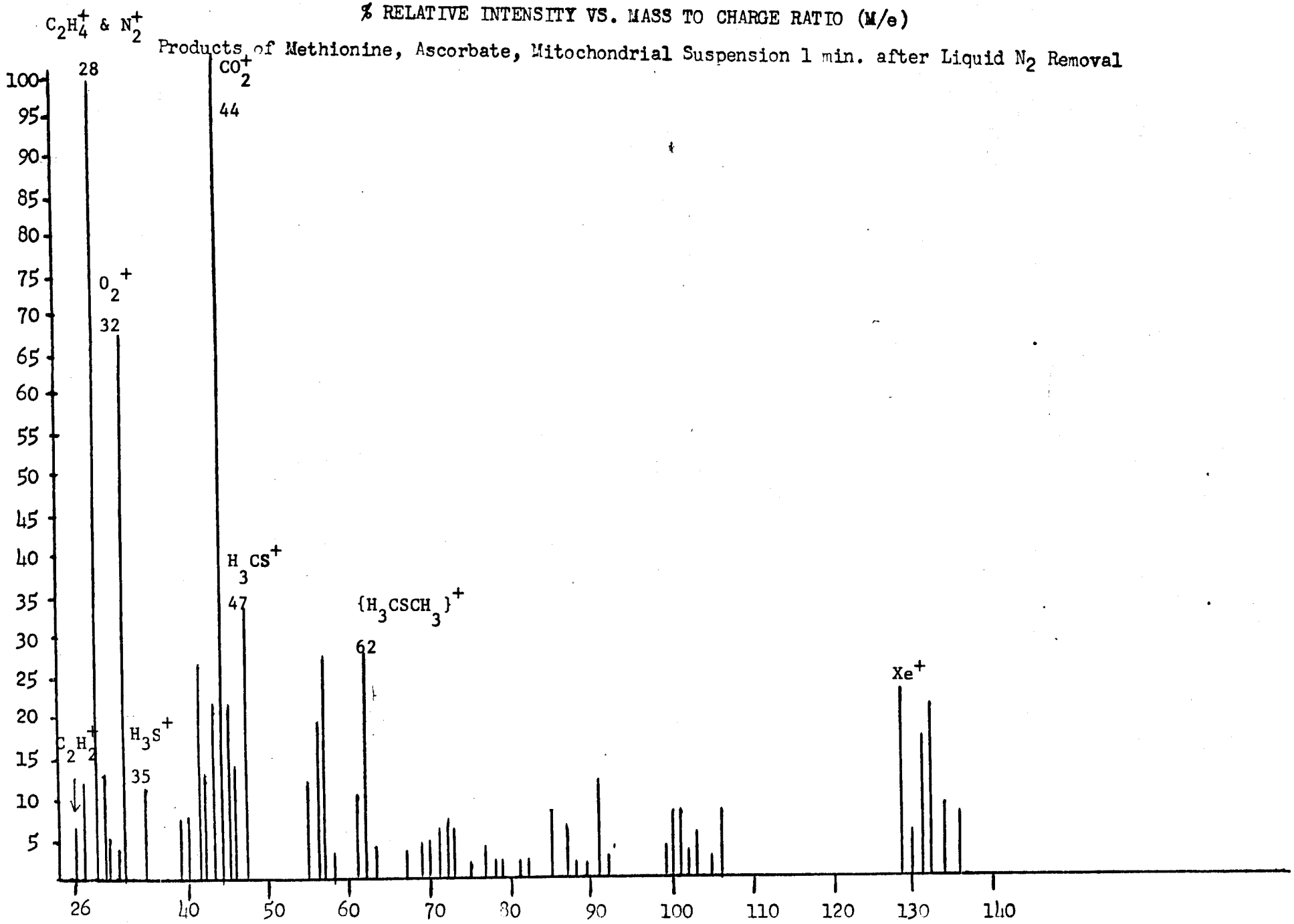
This is the same sample as in Figure I, with the following conditions:

A) Electron energy 70 ev (VTVM)

B) Cracking pattern taken 1 minute after removing liquid N_2 flask from collection tube attached to sample inlet system.

This figure shows that the sample is warm enough to allow certain M/e values to appear, but cold enough to keep M/e 94, and 79 in the solid state as evidenced by their disappearance from the cracking pattern.

FIGURE III


% RELATIVE INTENSITY VS. MASS TO CHARGE RATIO (M/e)
Products of Methionine, Ascorbate, Mitochondrial Suspension 1 min. after Liquid N_2 Removal
$C_2H_4^+$ & N_2^+
28
CO_2^+
44
O_2^+
32
H_3CS^+
47
H_3S^+
35
$C_2H_2^+$
$\{H_3CSCH_3\}^+$
62
Xe^+
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
26
40
50
60
70
80
90
100
110
120
130
140

Explanation of Figure IV

This is the same sample as in Figure I, with the following exceptions.

A) The N_2 peak (M/e 28) was subtracted out

B) Cracking pattern is taken 10 sec. after the liquid N_2 flask is removed from the collection tube.

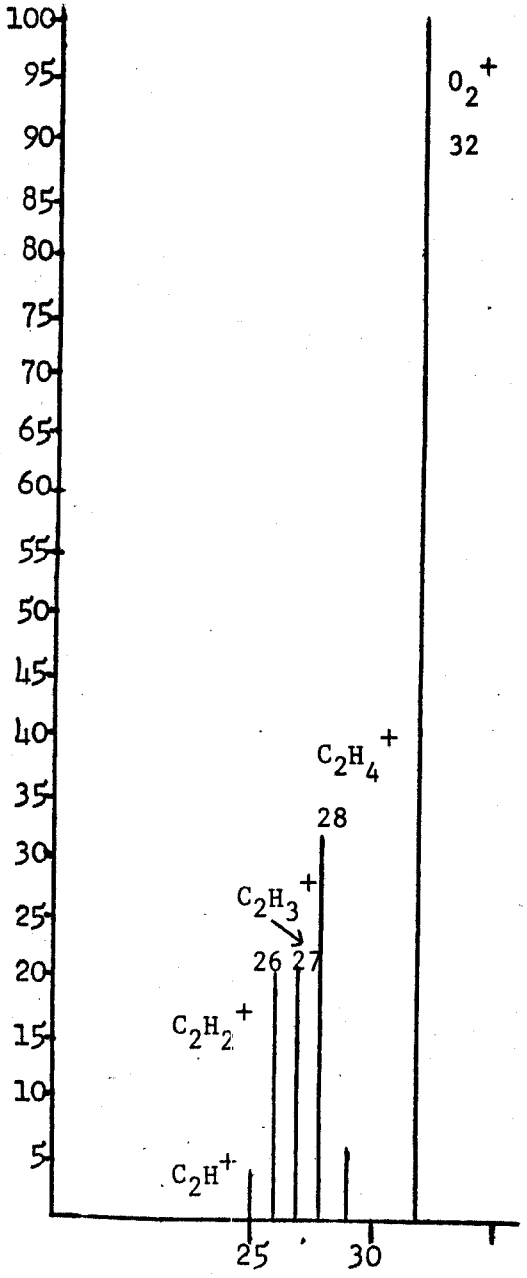
C) Sample inlet pressure reads zero and Bayard Alpert (B.A.) gauge reads 4×10^{-8} torr.

D) M/e from 32 up are left out since these are only background M/e.

FIGURE IV

% RELATIVE INTENSITY VS. MASS TO CHARGE RATIO (M/e)

Cracking Pattern of Ethylene (C_2H_4)



Explanation of Figure V

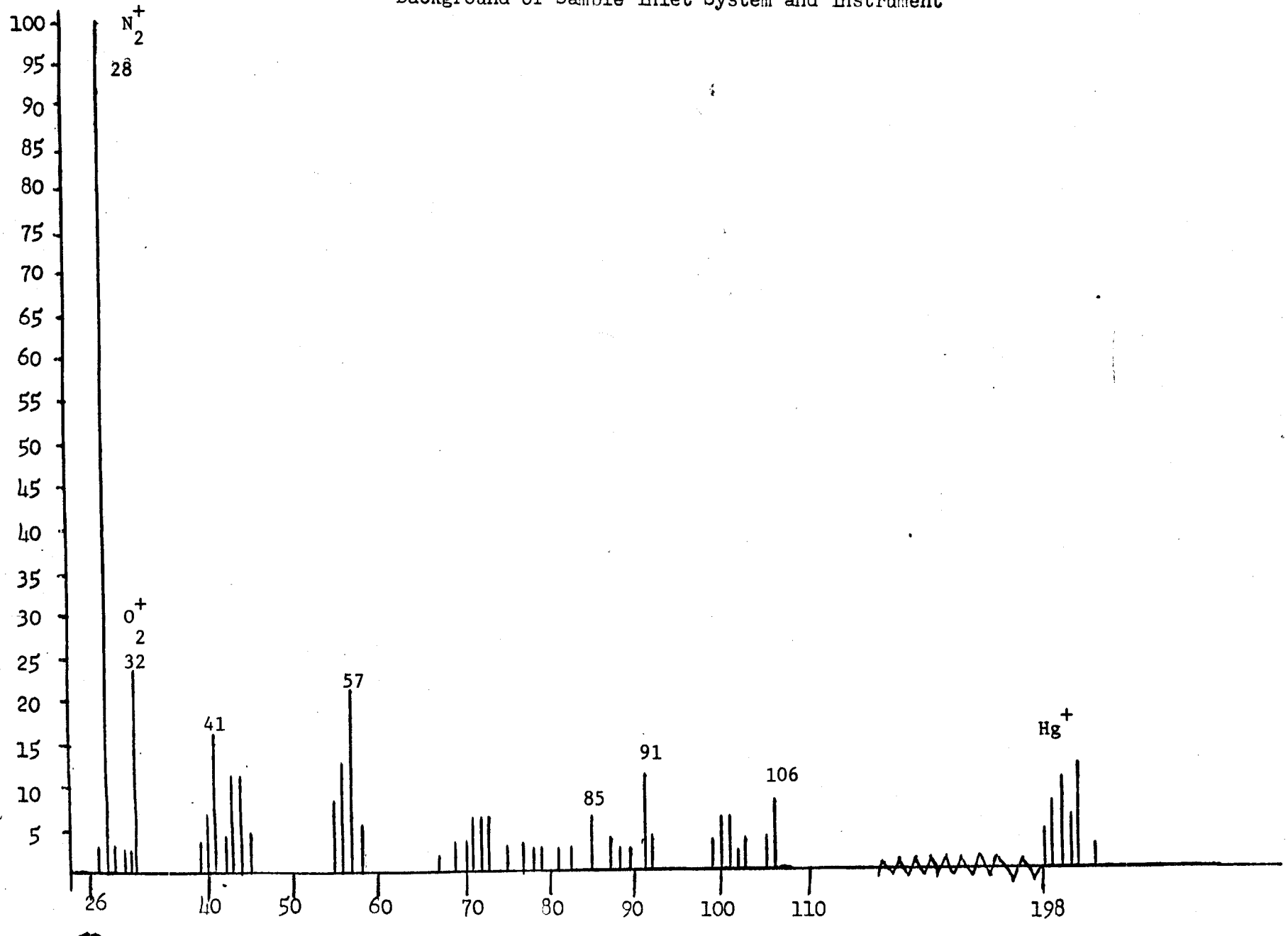
This figure represents the constant background level of the sample inlet system and instrument at a pressure of 3×10^{-8} torr B.A. gauge reading. At low electron energies of 10-11 ev (VTVM), M/e values of 106, 100, 85, 58, and 56 are prevalent. These have been shown to come from the stopcock grease (Apiezon L) used in the sample inlet system.

The air leak of the system is such that the speed of the mass spectrometer pumping system can maintain the pressure at 3×10^{-8} torr.

FIGURE V

% RELATIVE INTENSITY VS. MASS TO CHARGE RATIO (M/e)

Background of Sample Inlet System and Instrument



DISCUSSION

Figures I through IV represent information that has been used to identify the compounds listed in Table I. These cracking patterns show that a complex gas mixture (Fig. I) can be identified with the aid of low energy cracking patterns (Fig. II) and short warm up periods of liquid nitrogen cooled sample (Fig. III). Moreover, by recording the sample inlet pressure and knowing the components of the mixture, it is possible to estimate the amount of each component present (see sample calculation). Figure II demonstrates that M/e volumes 94,62,58, and 28 could be molecular ions, since fragmentation is slight at this low energy (13 ev)[f] and that M/e 62 is not a fragment of M/e 94 (Fig III). Moreover, upon a stepwise increase in electron energy, M/e values of 35,45,46,47 and 61 all increase in intensity as M/e 62 decreases. The ions responsible for these fragments are: H_3S^+ (35), $HC\text{-}S^+$ (45), $H_2C\text{-}S^+$ (46), $H_3C\text{-}S^+$ (47)[g], $C_2H_5\text{-}S^+$ (61). At 70 ev the relative intensities of the above ions match those of 2-thiapropane fragmentation. 2-thiapropane has a molecular weight of 62.13 and an appearance potential of 8.70 ± 0.20 ev[4]. The major fragments at 70 ev of M/e 94 are 15,45,46,47 and 79. M/e 79 corresponds to the $H_3C\text{-}S\text{-}S^+$, and M/e 15 represents CH_3^+. The other fragments give rise to ion species mentioned above. Cracking patterns of $H_3C\text{-}S\text{-}S\text{-}CH_3$ and $H_3C\text{-}S\text{-}CH_3$, obtained at 70 ev show no significant differences from those found in the literature[2].

The molecular ion at M/e 28 (Fig. III) and cracking pattern illustrated in Figure IV, show that ethylene is present in the mixture. Moreover, M/e 26 in Figure I arises from ethylene fragmentation only and represents 62% of the total

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(f) Low energy (13 ev) electron impact of background reveals M/e values of 106, 100, 85, and 56.

(g) Sulfur compounds exhibit a 4% ^{34}S isotope peak found 2 mass units higher than ^{32}S containing fragment.

ethylene content (ratio of M/e 26/28 in Fig. IV is 62%)[h]. Acetone is identified by M/e 58, 43, and 15.

Evolution of ethylene (C_2H_4) from mitochondria appears to be well established[5,6] Ageing increases ethylene production as seen in Table I, confirming results of Chandra and Spencer[5]. Methionine and ascorbate addition stimulate ethylene production by mitochondria. Ethylene evolution by respiring mitochondria is less than that produced by a methionine: ascorbate-containing medium[i]. This suggests that methionine stimulated C_2H_4 production from mitochondria predominates over the C_2H_4 produced by the methionine: ascorbate medium alone.

Ageing increases acetone evolution compared to that of normal respiring mitochondria.

2,3-dithiabutane and 2-thiapropane are both produced by normal, respiring mitochondria using L-methionine as substrate. The amount of 2,3-dithiabutane evolved from either an aged or fresh mitochondrial suspension is about the same, while maximal production of 2-thiapropane occurs with fresh, respiring mitochondria. Small amounts of 2,3-dithiabutane are produced by the medium alone, while no detectable amount of 2-thiapropane is seen. Moreover, 2-thiapropane is produced from whole liver containing no added methionine, indicating that the appearance of this compound may be due to tissue decomposition. The breakdown of methionine by mitochondria, producing 2-thiapropane and 2,3-dithiabutane, appears to be uncommon. Future investigation of these metabolites may provide interesting and significant additions to established pathways of methionine metabolism.

M/e 83 and 85, appearing in the cracking pattern of L-methionine: ascorbate medium alone, disappear as mitochondrial nitrogen increases. Although the fragmentation pattern fits that of $CHCl_3$, the origin of $CHCl_3$, either by contamination

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(h) Acetone gives rise to <1% M/e 26.

(i) Lieberman, M. et al[7] reported a Cu^+ catalyzed breakdown of methionine in phosphate buffer + ascorbate at 30°C in air with ethylene as the major product.

or as background constituent, can not be accounted for.

M/e 60 comes from aged mitochondrial preparations only and has not yet been identified, though acetic acid (CH_3COOH) may be a possibility.

FUTURE PLANS

A) Possible bacterial contribution will be evaluated as follows:

1) Identical runs (as described above) will be preformed under sterile conditions.

2) An agar smear of an aliquot from a non-sterile run may be made, and the agar medium allowed to incubate at 37°C for 24 hr. Isolated bacterial colonies will be placed in the methionine-containing medium with no mitochondria. After incubating 2 hours, a sample is collected and volatile compounds analyzed mass spectrometically.

B) The nature of the process producing the volatile components will be further elucidated by the addition of well-characterized inhibitors of electron transport and oxidative phosphorylation such as ethylene diaminetetracetic acid (EDTA), CN^-, azide, etc. Moreover, use of isolated fractions of disrupted mitochondria would help localize the process involved.

C) The contribution of other volatile materials from isolated cell fractions (cytoplasm, cell membrane, nuclei,,and microsomal) from a variety of tissues (liver, kidney, spleen, bacteria, etc.), incubated with a variety of common substrates (pyruvate, glucose, fatty acids, etc.) may be investigated.

SUMMARY

The foregoing data clearly demonstrates that the mass spectrometer is a useful tool for detecting and characterizing small amounts of material evolved by a normal, respiring mitochondrial suspension.

The importance of ethylene evolution from respiring tissue has not yet been established. However, Kakanov[8] suggests that C_2H_4 evolution is a general property of life and UV radiation may convert C_2H_4 into ethylene oxide or other mutogenic derivatives in vivo.

The breakdown of methionine by mitochondria, producing $H_3C\text{-}S\text{-}CH_3$ and $H_3C\text{-}S\text{-}S\text{-}CH_3$, appears to be uncommon. Future investigation of these metabolites may provide interesting and significant additions to established pathways of methionine metabolism. The amount of M/e 83 evolved from the medium alone varies inversly with added mitochondrial nitrogen. This apparent utilization of M/e 83 by mitochondrial tissue needs further clarification.

Aged mitochondria produce M/e 60, possibly acetic acid, as well as Me 58 (acetone) in greater amounts than that produced by normal fresh mitochondrial

ADDENDUM

SAMPLE CALCULATIONS

The % relative intensities are obtained from Figure I. Compounds and M/e values used to estimate their amounts are:

H_2O,18; $N_2 + C_2H_4$, 28[j]; O_2, 32; $H_3C\text{-}\overset{\overset{O}{\|}}{C}\text{-}CH_3$, 43; CO_2, 44; $H_3C\text{-}S\text{-}CH_3$, 62[k]

$H_3C\text{-}S\text{-}S\text{-}CH_3$, 94; Xe, 132; 83[l]; Apiezon L grease, 106; Hg, 202.

(j) both N_2 and C_2H_4 contribute to M/e 28, however, M/e 26 comes from C_2H_4 only and represents 62% of the total amount present.

(k) represents 81% of the total amount present.

(l) remains unidentified.

P_i x % relative intensity of M/e in pure (i) = % relative intensity of i in the mixture

Where P_i = Partial pressure of component i.

$P_{18} \times 100 = 32.3$ or $P_{18} = 0.323$

$P_{28} \times 62 = 2.8$ or $P_{28} = 0.046$

$P_{28} \times 100 = 59.9$ or $P_{28} = 0.599$

$P_{32} \times 100 = 32.9$ or $P_{43} = 0.305$

$P_{44} \times 100 = 31.6$ or $P_{44} = 0.316$

$P_{62} \times 81 + 74.0$ or $P_{62} = 0.914$

$P_{83} \times 100 = 6.5$ or $P_{83} = 0.065$

$P_{94} \times 100 = 37.8$ or $P_{94} = 0.378$

$P_{100} \times 100 = 5.2$ or $P_{106} = 0.052$

$P_{132} \times 100 = 13.8$ or $P_{132} = 0.138$

$P_{202} \times 100 = 69.9$ or $P_{202} = 0.699$

$\Sigma P_i = 4.164$

% P_i of Total

$$\% P_{C_2H_4} = \frac{.046}{4.164} \times 100 = 1.10$$

$$\% P_{H_3C-\overset{O}{\overset{\|}{C}}-CH_3} = \frac{.305}{4.164} \times 100 = 7.32$$

$$\% P_{H_3C-S-CH_3} = \frac{.914}{4.164} \times 100 = 24.02$$

$$\% P_{83} \quad \frac{.065}{4.164} \times 100 = 1.56$$

$$\% P_{H_3C-S-S-CH_3} = \frac{.378}{4.164} \times 100 = 9.08$$

Total sample inlet pressure = 31×10^{-3} torr at 30°C in a volume of 175 ml.

$$\% P_i \times \text{Total pressure} = P_i \text{ in sample inlet system}$$

$$1.10 \times 31 \times 10^{-3} = P_{C_2H_4} = 0.341 \times 10^{-3} \text{ torr}$$

$$7.32 \times 31 \times 10^{-3} = P_{H_3C-\overset{\overset{O}{\|}}{C}-CH_3} = 2.27 \times 10^{-3} \text{ torr}$$

$$24.02 \times 31 \times 10^{-3} = P_{H_3C-S-CH_3} = 7.45 \times 10^{-3} \text{ torr}$$

$$1.56 \times 31 \times 10^{-3} = P_{83} = 0.484 \times 10^{-3} \text{ torr}$$

$$9.08 \times 31 \times 10^{-3} = P_{H_3C-S-S-CH_3} = 2.81 \times 10^{-3} \text{ torr}$$

$$PV = nRT$$

$P = P_i$, $V = 0.1751$, $R = 0.0820$ 1 atm./mole deg, n = number of moles, $T = 303°K$

P_i (in microns $\times 9.26 \times 10^{-9}$ = n (number of moles)

$0.341 \times 9.26 \times 10^{-9} = 3.16 \times 10^{-9}$ moles of C_2H_4

$7.45 \times 9.26 \times 10^{-9} = 6.9 \times 10^{-8}$ moles of $H_3C-S-CH_3$

$2.27 \times 9.26 \times 10^{-9} = 2.1 \times 10^{-8}$ moles of $H_3C-\overset{\overset{O}{\|}}{C}-CH_3$

$0.484 \times 9.26 \times 10^{-9} = 4.48 \times 10^{-9}$ moles of M/e 83

$2.81 \times 9.26 \times 10^{-9} = 2.6 \times 10^{-8}$ moles of $H_3C-S-S-CH_3$

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