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~~Jeffrey Kempter~~
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OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Follow-up to Methyl Bromide Registration Standard. Interim Reports on the Metabolism of MeBr Following Post Harvest Fumigation. Report of 11/17/88. (DEB No. 4680) No MRID No.

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Background

The MeBr Registration Standard had cited the need for metabolism studies reflecting postharvest fumigation. At the very least, data were required on representative members of the root and tuber vegetables, tree nuts, cereal grains (corn and a small grain), citrus fruits, pome or stone fruits, and nongrass animal feeds.

In a telecon with the registrant on 2/11/88, DEB (W. Hazel) told Dr. White (Methyl Bromide Industry Panel or MBIP) that MBIP

would need to send in a plant metabolism protocol--even if the metabolism study was already underway. In its review of 7/14/88, DEB reminded the registrant that a protocol for the metabolism had not yet been received. At a meeting with the MBIP (among others), Dr. White informed the Agency that two copies of a "study plan" had been submitted. However, neither DEB nor RD had a copy on file.

Present Submission

The registrant has included a third copy of the "study plan" with the current submission. Because of the transmission delays, DEB did not have a chance to review the study plan until the metabolism studies were quite far along. That metabolism study plan is discussed under the "Detailed Considerations" section of this review.

The registrant has also submitted arguments against a requirement for metabolism studies using radioactive bromine studies. These arguments had been submitted earlier with the MBIP response of 9/22/88 and reviewed (memo of C. Deyrup, 11/3/88). In this review, DEB agreed that radiolabeled Br studies are not feasible. The need for using other means to determine whether 5-bromouracil may be present is discussed on page 14 of this review.

The present submission includes six progress reports/articles covering the time period 5/86-10/88, and future study plans. A previously submitted progress report was dated 4/88.

Recommendations

The registrant has submitted future study plans which are detailed below. DEB's recommendations concerning the study plans and the currently completed work follow the outline of the "Future Study Plans."

Future Study Plans concerning Plant Metabolism Studies

The authors list the following tentative plans for the metabolism studies:

1. The identity of the 1-methyl and 3-methyl histidines will be confirmed.
2. The authors will attempt to identify the methylated amino acids contained in Peak 3B. Possible candidates are 1,3-dimethylhistidine, epsilon-methyl lysine, and methyl arginine.
3. The authors will attempt to identify the major methylated amino acids in peak 1.
4. The authors will determine if S-methyl cysteine is the source of methyl mercaptan when fumigated commodities are

treated with alkali.

5. The sites of methylation in the DNA of apples and potatoes will be investigated.
6. Possibly the apparent increase in chemically bound residues as a function of storage time will be investigated.

DEB's Recommendations Concerning the Metabolism Studies

The above future study plans appear to be appropriate. However, the plans should be modified to include the following:

1. The submitted study plan and the work completed thus far are aimed at characterizing chemically bound residues only. The contribution that MeBr makes to the total radioactive residue (TRR) will need to be taken into account.
2. DEB concludes that it is highly unlikely that volatile metabolites of concern would arise from the proposed use, as the methylation of polysaccharides, proteins, DNA, etc., should not yield volatile products. Therefore, the only volatile residue which needs to be determined is MeBr per se. The level of MeBr should be determined after a 1-2 aeration period.
3. Since the only volatile residue which needs to be quantitated is MeBr itself, it may be unnecessary to repeat all the radiolabeled metabolism studies. DEB suggests that the registrant fumigate the various commodities with MeBr (unlabeled), exactly as in the previously conducted metabolism studies and determine MeBr levels from replicate fumigations. If there is little variation in residue levels from replicate fumigations, the ppm MeBr could be added to the ppm of chemically bound MeBr equivalents (determined from radiolabeled studies) to yield an estimate of the TRR and the MeBr contribution to the TRR. The ppm in both cases should be based on the weight of the commodities before lyophilization and/or extraction.
4. If there is significant variation between replicate fumigations, the metabolism studies will need to be repeated in order to determine the contribution of the parent to the TRR.
5. Whenever ether extraction is used to defat the commodities before characterizing the chemically bound residues, the radioactivity in the ether extract should be measured. DEB could not distinguish whether this had been done in each case.
6. The decline studies do not enable DEB to estimate the

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initial MeBr levels in the metabolism studies, because MeBr per se was not determined, the treatment rates in the storage studies differed from that used in the metabolism studies, and it is not possible to determine how much of the activity remaining after 10 days was due to physically bound MeBr.

7. The decline studies did not specify the temperature used in conducting the investigation. For the sake of completeness, the authors should include the storage temperatures in the final report.
8. In the studies aimed at determining the extent of O-, S-, or N- methylation, the specific activity of the MeBr was not given so that DEB could not calculate the ppm MeBr equivalents. DEB needs this information in order to judge whether the nature of the residue has been adequately delineated.
9. Although DEB can conclude that methylmethionylsulfonium derivatives are the source of some of the dimethyl sulfide, it is not clear if they are the only source. As planned, the authors should determine whether S-methyl cysteine is the source of methyl mercaptan. They should also consider whether there are other sources of methyl mercaptan and dimethyl sulfide besides S-methyl cysteine and methylmethionyl-sulfonium derivatives.
10. DEB has discussed with TOX (D. Ritter, 1/24/88) the degradative approach taken by the authors in determining the extent of O- and S-methylation. TOX said that as much information as possible should be generated on the original site of methylation. This approach was taken for studying the extent of methylation of DNA and for the determination of the methylated histidines. The authors should provide any information available on the original sites of methylation (polysaccharides, phenols, etc.).
11. TOX is especially concerned with the extent of methylation that occurs in proteins and whether methylated glutathione is formed (D. Ritter, 1/24/89). Do the authors have any information on the presence of methylated glutathione?
12. It may be necessary to study the volatiles which are derived from the hydrolysis of protein fractions instead of from the whole commodities. At the very least, DEB needs to know what proportion of the TRR is contained in the protein fractions, as was done with corn.
13. If a significant portion of the TRR is contained in non-protein fractions, attempts should be made to identify the sites of reaction in these fractions as well.
14. At the meeting of 11/10/88, Dr. Starrat said that it might be possible to look for 5-BrU with HPLC with electrochemical

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detection. DEB recommends that this route be pursued.

15. The authors reported the presence of 3-methylguanine in wheat DNA hydrolysates. The authors should indicate on the chromatograms where this residue would elute, as they did with the other methylated DNA bases.

The present submission only concerned metabolism studies aimed at delineating the nature of the residue in plants. The data gaps cited in the Methyl Bromide Registration Standard remain outstanding.

Detailed Considerations

For the purposes of discussion, the present submission was broken down into the following categories: 1) Proposed plan of study; 2) Decline of residues upon storage; 3) O-, S-, and N-methylation; 4) The presence of brominated metabolites; 5) The methylation of DNA; and 6) Future study plans. The registrant's submission pertaining to each of these categories will be presented, followed by DEB's Comments/Conclusions.

1) Registrant's Submission--Proposed Metabolism Study Plan

The proposed study plan, dated 10/26/87, consists of a brief one page outline. The investigation will include metabolism studies on corn, wheat, almonds, oranges, apples, alfalfa, and potatoes. After fumigation, the crop samples will be ground and defatted or lyophilized and ground, if the water content is high. The samples will be heated with 1N NaOH at 100°C, and the volatiles will be trapped and quantitated for radioactivity. The non-volatile residue will be neutralized, dried, and hydrolyzed with 6N HCl. The hydrolysate will be subjected to ion exchange chromatography in order to identify N-methylated amino acids.

DNA will be isolated from several of the above crops, in addition to corn and wheat, which have already been investigated. The methylation pattern of DNA will be determined.

The storage stability of the methylated components will be determined for corn and one other commodity. The samples will be stored 6 months at room temperature. All tests will be done in duplicate.

DEB's Comments/Conclusions, re: Proposed Metabolism Study Plan

In its review of the interim report of 4/88, DEB said that the study appeared to be aimed at characterizing bound radioactive residues, since levels of the parent had not been reported, and apparently samples were extracted with ether or were lyophilized before counting (memo of C. Deyrup, 7/14/88). The study plan makes it clear that samples were not radioassayed before lyophilization and/or extraction. If DEB had had the opportunity to review the protocol before the work had actually begun, we

would have stipulated that the registrant would have to account for the contribution that MeBr and its volatile metabolites make to the total radioactive residue (TRR), as required in DEB's memo of 7/14/88. DEB agrees with the registrant's argument that volatile radioactive residues would consist of the parent and, perhaps, methanol, from the reaction with water. The methylation of polysaccharides, proteins, DNA, etc., would not produce residues which would be removed by lyophilization.

DEB concludes that it is highly unlikely that volatile metabolites of concern would arise from the proposed use. Therefore, the only volatile residue which needs to be quantitated is MeBr itself.

Whenever ether extraction is used, the radioactivity in the ether extract should be measured. Although the registrant has reported the activity in ether extracts from almonds, corn, alfalfa, and orange peels, it is not clear if the activity was measured whenever ether extraction was used.

DEB understands that the samples which were used in the metabolism studies described in the progress reports (4/88 and 10/88) cannot be used to generate MeBr residue data, because the volatile MeBr may have dissipated. Since DEB has concluded that the only volatile radioactive residue that needs to be accounted for is MeBr itself, it may not be necessary to repeat all the radio-labeled metabolism studies. DEB suggests that the registrant fumigate the various commodities with MeBr (unlabeled); the fumigations should exactly mimic the conditions used in the metabolism studies. As recommended at the 11/10/88 meeting, the commodities should be aerated for 1-2 hours, since similar aeration periods are encountered in commercial practice. The levels of MeBr could then be determined by the King headspace method. It would be necessary to determine MeBr levels from several fumigations so that a coefficient of variation could be determined. If there is little variation in residue levels from replicate fumigations, the ppm MeBr could be added to the ppm of chemically bound MeBr equivalents to yield an estimate of the TRR and the MeBr contribution to the TRR. The ppm in both cases should be based on the weight of the commodities before lyophilization and/or extraction.

If there is significant variation between replicate fumigations, the metabolism studies will need to be repeated in order to determine the contribution of the parent to the TRR.

2) Registrant's Submission--Decline of Residues upon Storage

The following study was carried out for MBIP by A.N. Starrat and E.J. Bond (Agriculture Canada).

The distribution of activity after storage was examined in Granny Smith apples. Six apples were fumigated at a rate of 32 mg/l for 2.5 hours at 25°C with ¹⁴C-MeBr. After 14 days

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of storage at some unspecified temperature, the total radioactive residue (TRR) was distributed as follows:

% TRR (PPM MeBr Equiv.)		
Skin	Pulp	Seeds
12.8-19.5	2.9-4.2	77.6-84.0
(30.1-57.5)	(5.7-8.7)	(150-229)

The decline of radioactive residues with time and the distribution of the TRR as a function of fumigation temperature were also investigated. The dosages were different for the two temperatures, but the higher fumigation temperature led to higher residue levels even though the treatment rate was lower. The results are tabulated below.

Days after treatment	Temperature [dose]	PPM (MeBr equiv)		
		Pulp	Skin	Seed
0	10°C [48 mg/l]	3.8	7.3	0
	25°C [32 mg/l]	14.6	33.7	22.4
7	10°C [48 mg/l]	2.9	11.9	58
	25°C [32 mg/l]	2.7	18	109
14	10°C [48 mg/l]	1.9	9.7	59
	25°C [32 mg/l]	2.3	8.1	83.5

The absorption and retention of ¹⁴C-MeBr in other commodities were also investigated. The commodities were treated for 24 hours at a rate of 24 mg/L. The commodities were stored at an unspecified temperature, and analyzed on Day 0, 1, 3, and 10. Only the results for Day 0 and Day 10 are given in the table below.

Commodity	Days after treatment	PPM		Avg % Retained after 10 Days
		¹⁴ C-MeBr	Equiv	
Maize	0	47.2-56.0		57.5
	10	21.3-35.1		
Maize Meal	0	63.0-67.7		72.6
	10	44.6-50.4		
Wheat	0	78.6-88.3		96.1
	10	78.1-82.3		
Whole wheat flour	0	82.3-91.4		87.6
	10	74.1-77.8		
White flour	0	148.3-148.8		62.3
	10	90.2-95.0		

Commodity	Days after treatment	PPM ¹⁴ C-MeBr Equiv	Avg % Retained after 10 Days
Oatmeal	0	129.6-206.8	80.5
	10	109.2-151.5	
Peanuts	0	116.2-154.9	50.2
	10	66.9-69.2	
Walnuts	0	119.3-163.5	78.1
	10	107.5-113.4	
Almonds	0	100.3	74.9
	10	80.0	
Alfalfa	0	103.2-187.1	69.0
	10	71.2-135.3	

DEB's Comments/Conclusions, re: Decline of Residues upon Storage

In its 7/14/88 review, DEB cited the need for determining the composition of the total radioactive residue, including the parent. At the meeting of 11/10/88, the registrant suggested that it may be possible to estimate initial MeBr levels from the available storage data.

These storage studies do not address this issue, because MeBr per se was not determined; the levels of activity, given in terms of MeBr equivalents, apparently reflect radioanalysis. In any case, the treatment rates in the storage studies differed from that used in the metabolism studies. Furthermore, it is not possible to determine how much of the activity remaining after 10 days was due to physically bound MeBr.

The submitted storage data do demonstrate that radioactive residue levels decline during storage at unspecified temperatures. For the sake of completeness, the authors should include the storage temperatures in the final report.

3) Registrant's Submission--S-, O-, and N-Methylation

The present study (authored by A.N. Starrat E.J. Bond of Agriculture Canada) involved peanuts, almonds, corn, oatmeal, alfalfa, oranges, potatoes, wheat, and apples. Peanuts and oatmeal represent additions to the original study plan.

The authors explain that their studies were aimed at the characterization of chemically bound residues, as opposed to physically bound residues; therefore the samples were ground and lyophilized (if the water content was high) and/or ground and extracted with ether to remove oil. The oil was shown to contain less than 1% of the chemically bound residues in almonds, corn, alfalfa, and orange peels.

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The authors cite several studies in the literature which indicate that the amount of methylation is related to the protein content; O-, N-, and S-methylation of proteins have also been reported in the literature. A radiolabeled study in the '50's demonstrated that the gluten (a plant protein) fraction of wheat accounted for 80% of the activity.

The present studies, which used MeBr treatments at a rate of 48 mg/L for 3 days, showed that the corn germ, which comprises less than 12% of the kernel, contained almost half of the activity; as in earlier studies, the activity concentrated in the protein fraction. After removal of fats with ether, the corn protein was fractionated by successive solvent extractions into albumins, globulins, Zein-1, Zein-2, glutelin, and amino acids. Activity was distributed throughout these fractions.

To a large extent, the characterization of O- and S- methylation sites relied upon degradative techniques, because it was already known that MeBr concentrates in the protein fraction. The hydrolytic conditions which free the methylated amino acids also hydrolyze the sites of O- and S- methylation.

After lyophilization and/or ether extraction, the whole commodity (not a protein fraction) and methyl methionine sulfonium bromide were heated with 1N NaOH for 5 hours. Under these conditions, it had been shown that methylmethionylsulfonium bromide decomposes to dimethyl sulfide. O-Methylated compounds give methanol under these conditions. The radiolabeled methyl mercaptan may derive from S-methylcysteine. Since the whole commodity, rather than protein fractions, was hydrolyzed, the authors suspect that some of the methyl mercaptan may come from enzyme-catalyzed conjugation with glutathione.

Five traps were connected in series to the system; the traps contained:

1. Water (ice-cooled)
- 2 and 3. Saturated Mercuric cyanide
- 4 and 5. Saturated Mercuric chloride.

A slow stream of nitrogen was passed through the system. Dimethyl sulfide was treated with sodium borohydride to produce methyl mercaptan, which was carried to Trap 2 with a stream of nitrogen.

Methanol would be trapped in Trap 1, dimethyl sulfide would collect in Traps 4 and 5, and Traps 2 and 3 were included to collect products which are less volatile than dimethyl sulfide, such as methyl mercaptan.

After the NaOH treatment, the residues were hydrolyzed with 6N HCL to completely hydrolyze proteins, and the hydrolysate was examined by ion exchange chromatography. At the end of the 4/88 report, radioactive peaks corresponding to histidine (major

peak) and arginine and lysine had been reported. In its 7/14/88 review (memo of C. Deyrup), DEB had asked whether the authors had meant methylated histidine, arginine, and lysine. From the present submission, it is apparent that the chromatographic conditions only separated the amino acids into acidic, basic, and neutral species and did not differentiate between methylated and non-methylated species. Most of the activity was contained in Peak 3, which would contain the basic amino acids histidine, lysine, arginine, in addition to methylmethionyl sulfonium chloride.

Some samples were hydrolyzed with HCl without a prior NaOH hydrolysis. These samples consistently gave higher yields of radioactive basic amino acids. The authors attribute the higher yields to:

- 1) methylmethionylsulfonium chloride, which elutes with the basic amino acids and is only partially destroyed by the acid hydrolysis. This moiety would be absent in samples treated with alkali because it is converted to dimethyl sulfide, or
- 2) a methylated basic amino acid, such as arginine, which is wholly or partially destroyed by alkali:

The peak containing the basic amino acids and methylmethionylsulfonium chloride, Peak 3, was rechromatographed on Dowex 50W in the NH_4^+ form. Peak 3 was resolved into two peaks, Peak 3A, corresponding to the elution time of histidine, and Peak 3B, corresponding to the elution times of arginine, lysine, and methylmethionylsulfonium chloride. Peak 3B was consistently greater in samples which had not been treated with NaOH. It was suspected the greater yield of Peak 3B reflected a contribution from undegraded methylmethionylsulfonium chloride. Therefore, Peak 3B from treated corn was isolated and treated with 1N NaOH in the system equipped with the series of traps. The mercuric chloride trap collected 52-60% of the Peak 3B radioactivity as dimethyl sulfide; no activity was collected in the other traps. Therefore, acid hydrolysis of the sample leads to a partial recovery of methylmethionylsulfonium chloride.

According to the authors, Peak 3B could include contributions of arginine and lysine after base hydrolysis, although the presence of methylated arginine and lysine has not yet been confirmed. Peak 3B comprises a significant portion of the chemically bound residue in oatmeal (16-17%), and accounts for 5.3% of the chemically bound residue in wheat; however, Peak 3B accounts for less than 5% of the chemically bound residue in peanuts, almonds, corn, alfalfa, oranges, potatoes, and apples.

^{14}C -Methyl histidines were synthesized in order to confirm their presence in the fumigated commodities. The methyl histidines were shown to be stable to the alkaline and acidic conditions used to hydrolyze the protein in the samples. The presence of 1-methylhistidine and 3-methylhistidine was established by ion exchange

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chromatography and reversed phase HPLC. The material from almonds and corn was treated with dansyl chloride; a product was isolated with the same reversed phase HPLC retention time as dansyl 1-methylhistidine. The following table delineates the composition of the chemically bound radioactivity, with the exception of the methylated DNA bases which were identified in another study. [It was reported that only 0.3-0.4% of the chemically bound activity was contained in the DNA fraction; the same dosage, 48 mg/L for 72 hours, was followed in the DNA study. See the "Methylation of DNA" section of this review.]

The author explains that the entries for the contribution of activity liberated as methanol, methyl mercaptan, and dimethyl sulfide are slightly different from the values previously submitted because of differences in the counting procedures. The results presented below reflect counting after the precipitation step for the sulfur compounds; also, counts in the trap following the methanol trap are no longer attributed to carry over of methanol but to unprecipitated methyl mercaptan. The differences between the present submission and the previous submission for the trapped volatiles amount to 1-2% or less.

Commodity	% Chemically Bound Residues*					% Bound Residues Characterized**
	Trap 1 MeOH	Trap 2 CH ₃ SH	Trap 4 (Me) ₂ S	1-Me His	3-Me His	
Wheat	21.2	3.3	32.3			75.1
	21.5	5.4	25.7	9.9	7.0	70.7
Oatmeal	17.4	5.2	18.5	8.5	4.5	54.7
	19.2	5.6	20.2	8.1	6.4	60.3
Peanuts	20.7	11.5	23.3	18.1	7.8	82.1
	22.4	9.9	24.9	19.8	6.9	84.6
Almonds	16.3	3.5	11.2	54.5	11.2	96.8
	16.2	6.2	11.8	46.2	14.2	95.2
	14.8	5.0	11.9	53.7	11.5	97.5
Corn	29.3	4.4	21.7	9.4	7.8	72.9
	29.3	3.6	23.5		7.6	74.5
Alfalfa	50.2	2.2	5.9	4.6	3.3	66.5
	52.3	4.3	6.4	5.2	4.9	73.8
Apples	26.5	22.7	31.5	4.6	2.7	90.0
	22.9	18.0	30.6		2.7	79.7
Orange pulp	24.2	24.5	25.4	1.0	1.2	77.7
	22.2	26.1	23.5		1.0	74.7
Orange peels	14.6	14.6	18.1	1.4	1.2	77.7
	15.0	15.0	33.2		1.6	74.7

Commodity	% Chemically Bound Residues*					% Bound Residues Characterized**
	Trap 1 MeOH	Trap 2 CH ₃ SH	Trap 4 (Me) ₂ S	1-Me His	3-Me His	
Potato skins	11.1 12.5	8.8 8.1	28.8 27.9	1.1	1.7 1.6	52.6 52.4
Potato pulp	7.4 8.8	22.7 27.5	25.4 28.1	0.8	0.9 0.6	59.1 67.6
Beef serum albumin	41.0 38.3	2.1 2.0	3.4 4.9			

* Physically bound residues of MeBr were first removed by lyophilization and/or ether extraction.

** Includes contributions from Traps 1-5

The authors conclude that the variation in metabolic profile reflects the difference in the composition of the proteins in the various commodities. For example, in beef serum albumin, methanol accounted for a large proportion of the trapped radioactivity, relative to that trapped as dimethyl sulfide, and this is in accordance with the known ratio of free carboxyl groups to methionine in this matrix.

The effect of storage time at room temperature on the levels of chemically bound residues was also examined. The level of chemically bound residues in corn and almonds appeared to increase after 6 months of storage, although there was little difference in the observed distribution of methylation sites (i.e., O-, N-, and S- methylation).

The authors concede that experimental error cannot be ruled out, especially since the radioactive recovery from almonds exceeded 100%; however an alternative explanation is further reaction of MeBr with natural plant constituents during the storage period.

The authors cite literature references which report that S-methyl methionylsulfonium salts occur widely in plants, methylated histidines are present in biological fluids, S-methyl cysteine occurs in legume seed and may be found in the hemoglobin of many species, and that methylated lysine and arginine are present in every organism examined. The methylated lysine, arginine, and 3-methyl histidine are not reused by the organism.

DEB's Comments/Conclusions, re: Sites of S-, O-, and N-Methylation

In the studies aimed at determining the extent of O-, S-, or N- methylation, the specific activity of the MeBr was not given. The data in the metabolism studies are represented in

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terms of dpm only; the percent of the bound activity was also provided. However, the ppm MeBr equivalents cannot be derived without the specific activity of the MeBr that was used in the experiments. DEB needs this information in order to judge whether the nature of the residue has been adequately delineated.

Since degradative techniques were used to examine the whole commodities (minus the fat), the original sites of O-methylation and S-methylation cannot be specifically delineated. Since it is known that MeBr reacts with proteins, it is logical to conclude that some, if not most of the methanol, dimethyl sulfide, and methyl mercaptan are derived from methylated amino acids.

Although DEB can conclude that methylmethionylsulfonium derivatives are the source of some of the dimethyl sulfide, it is not clear whether these are the only source. As planned, the authors should determine whether S-methyl cysteine is the source of methyl mercaptan. They should also consider whether there are other sources of methyl mercaptan and dimethyl sulfide besides S-methyl cysteine and methylmethionylsulfonium derivatives.

DEB has discussed with TOX (D. Ritter, 1/24/88) the degradative approach taken by the authors. TOX said that as much information as possible should be generated on the original site of methylation. This approach was taken for studying the extent of methylation of DNA and for the determination of the methylated histidines. The authors should provide any information available on the original sites of methylation (polysaccharides, phenols, etc.).

TOX was especially concerned with the extent of methylation that occurs in proteins and whether methylated glutathione is formed. Do the authors have any information on the presence of methylated glutathione?

It may be necessary to study the volatiles which are derived from the hydrolysis of protein fractions instead of from the whole commodities. At the very least, DEB needs to know what proportion of the TRR is contained in the protein fractions, as was done with corn.

If a significant portion of the TRR is contained in non-protein fractions, attempts should be made to identify the sites of reaction in these fractions as well.

4) Registrant's Submission--Possibility of Bromination

The authors state that brominations in the lab are effected by positive bromine, and MeBr, unlike N-bromosuccinimide, is not a source of bromonium ions. Bromometabolites are well-known in nature, where they originate from the attack of positive bromine produced by an enzymatic reaction with bromide ion. However, the bromide ion produced from fumigation would be

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indistinguishable from endogenous bromide.

DEB's Comments/Conclusions, re: Possibility of Bromination

DEB understands that the formation of 5-bromouracil (5-BrU) would entail attack by positive bromine, since nucleophilic attack on uracil leads to 6-substituted compounds. Although the iBr from fumigation may be indistinguishable from endogenous iBr, fumigation with MeBr does lead to elevated iBr residues. DEB consulted with Dr. Edward Thomas (St. Jude's Hospital), who has worked extensively with plant peroxidases. According to Dr. Thomas, MeBr itself would probably not be a substrate for peroxidases. Bromide ion, though unreactive toward the well-known horseradish peroxidase, is quite a good substrate for some other plant peroxidases. The product is an active form of bromine. Dr. Thomas could not predict whether a significant amount of 5-BrU would arise. He pointed out that the plasma concentration of iBr in human plasma was about 0.1 mM or about 23 mg in the body, assuming a plasma content of 6 pints. Although he hadn't seen any published work on the subject, it seemed likely to him that small amounts of 5-BrU could arise in humans from the reaction of peroxidases with plasma iBr. According to S. Stanton (SAOS/SACB) the top 10 foods in the diet could bear up to 16 mg iBr (assuming tolerance levels) which is not an insignificant amount, relative to endogenous iBr (23 mg). DEB has already agreed with the authors that studies using radioactive Br would not be feasible. (memo of C. Deyrup, 11/3/88).

At the meeting of 11/10/88, Dr. Starrat said that it might be possible to look for 5-BrU with HPLC with electrochemical detection. DEB recommends that this route be pursued.

5) Registrant's Response--Methylation of DNA

Corn and wheat were treated at a rate of 48 mg/L for 1-3 days. After aeration overnight, the corn and wheat were ground and defatted by extracting with ether. The DNA was isolated by standard procedures and hydrolyzed with 98% formic acid or 0.1 N HCl. The hydrolyzed DNA was examined by HPLC. The major methylated DNA base was 7-methylguanine. The levels of activity in the DNA fractions are given below.

Commodity	Exposure time (h)	PPM MeBr in Commodity	% Bound Activity in DNA	% Guanines 7-Methylated
Corn	24	43	0.17	0.21
	72	77-83	0.29-0.38	0.61
Wheat	72	94	0.6	0.76-0.92

The methylated DNA bases were identified by their chromatographic behavior using HPLC. Both cationic exchange columns and reversed phase columns were used so that the authors were able to confirm identities of the methylated bases.

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The following methylated bases were identified:

- 7-Methylguanine
- 3-Methylcytosine
- 1-Methyladenine
- 3-Methyladenine

The relative proportions of these bases is given below.

Commodity	Exposure time (hr)	Ratio				PPM (7-MeG)
		*7-MeG	1-MeA	3-MeC	3-MeA	
Corn	24	9.6	5.3	1.4	1/--	
	72	8.1	4.5	1.1	1/--	0.1
Wheat	72	6.7	4.2	1.2	1/1	0.3
		5.9	4	1.5	1/1.1	

* 7-Methylguanine:1-Methyladenine:3-Methylcytosine:3-Methyladenine:3-Methylguanine
DEB's Comments/Conclusions, re: DNA Methylation Studies

Although 3-methylguanine was identified in wheat DNA hydrolysates, DEB could not find any standards that corresponded to this compound in the submitted chromatograms. The authors should indicate on the chromatograms where this residue would elute, as they did with the other methylated DNA bases.

6) Registrant's Response--Future Plans

The authors list the following tentative plans for the metabolism studies:

1. The identity of the 1-methyl and 3-methyl histidines will be confirmed.
2. The authors will attempt to identify the methylated amino acids contained in Peak 3B. Possible candidates are 1,3-dimethylhistidine, epsilon-methyl lysine, and methyl arginine.
3. The authors will attempt to identify the major methylated amino acids in peak 1.
4. The authors will determine if S-methyl cysteine is the source of methyl mercaptan when fumigated commodities are treated with alkali.
5. The sites of methylation in the DNA of apples and potatoes will be investigated.
6. Possibly the apparent increase in chemically bound residues as a function of storage time will be investigated.

DEB's Comments/Conclusions, re: Future Study Plans

The future study plans appear to be appropriate. However, the authors should consider whether there are other sources of methyl mercaptan besides S-methyl cysteine.

In addition, the future study plans should include the modifications which have been delineated in DEB's Comments/ Conclusions in the preceding text of this review.

cc: Amy Rispin (EFED/SACS), PMSD/ISB, SF, RF, Reg. Std.
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