Phosphorus Residues in Wheat Due to Phosphine Fumigation[1]

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ABSTRACT

Treatment of wheat with phosphine-^{32}P ($^{32}PH_3$) under practical fumigation conditions results in the formation of non-PH_3 residues. The residues cannot be removed from the wheat by aeration or application of a vacuum. The distribution of residues is 85, 12, and 4% in the bran, endosperm, and germ fractions. A portion (64%) of the residues in the bran can be extracted with water and appears to be essentially hypophosphite (88%) and pyrophosphate (12%). For wheat, residues of approximately 3.3 p.p.m. form at the recommended fumigation level of 6.6 p.p.m. PH_3; somewhat higher amounts of residues form with flax and rapeseed.

Fumigation with phosphine (PH_3) involves treatment of materials with tablets composed of aluminum phosphide (AlP) and ammonium carbamate. On exposure to moist air, the AlP slowly evolves PH_3; and the ammonium carbamate, a mixture of carbon dioxide (CO_2) and ammonia (NH_3). The presence of CO_2 is desirable in that it deters the spontaneous ignition of PH_3 in air.

As the practice of PH_3 fumigation is increasing in the cereal industry, it was decided to examine the amount of PH_3 which reacts with wheat to form any nonvolatile residues. Knowledge concerning the effects of fumigation on residue formation is especially necessary for a food material such as wheat, which is one of man's most basic foods. Additional research on PH_3 fumigation is particularly necessary since there is controversy in the literature regarding whether PH_3 fumigation results in residue formation (1).

In the present study, PH_3, labeled with radioactive phosphorus (^{32}P), was used to indicate the extent of reaction between $^{32}PH_3$ and wheat. With $^{32}PH_3$, any reaction forming nonvolatile residues is conveniently indicated by the amount of radioactivity remaining on the wheat.

MATERIALS AND METHODS

The samples used in the study are described in Table I. The oilseeds were composite samples of 1969 western Canadian samples; the wheat was a bulk sample of a hard red spring wheat variety, Thatcher, grown in northern Saskatchewan during 1969; and the flour was a straight-grade sample of approximately 72% extraction milled from the above wheat.

Two lots of PH_3 labeled with ^{32}P with specific activities of 10.8 and 15 mc. per mmoles were purchased from The Radiochemical Centre. The $^{32}PH_3$ was stored in a gas buret over mercury. The volume of $^{32}PH_3$ was measured with a cathetometer.

Treatment with $^{32}PH_3$ was carried out by weighing 70 or 25 g. of wheat into 75-ml. pear-shaped bottles (The Macbick Co.) and then injecting $^{32}PH_3$ with a gastight syringe into the bottle through a rubber septum. In the first series of

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TABLE I. MATERIALS USED FOR PHOSPHINE-^{32}P TREATMENT

	Grade	Protein %	Moisture %	Oil Content %
Wheat	No. 2	13.7[a]	9.3	n.d.
Flour	...	12.7[b]	14.1	n.d.
Rapeseed	No. 1	39.4[c]	n.d.	44.3[d]
Flax	No. 1	39.1[c]	n.d.	42.4[d]

[a]13.5% moisture basis.

[b]14% moisture basis.

[c]Moisture-free basis, oil-free meal.

[d]Moisture-free basis.

experiments, the $^{32}PH_3$ was diluted by 50% with CO_2; just prior to the injection of $^{32}PH_3$, the air in the bottles was flushed out with CO_2; and an exposure time of 4 days was used. In the second series, no diluent was used; the air was not flushed away from the fumigation bottles; and a 5-day exposure time was used. On the day before the experiments were begun, the wheat moisture was adjusted to 14%. After exposure to $^{32}PH_3$, the samples were poured into shallow trays and air was drawn at 400 linear ft. per min. over the samples for 2 days to remove all of the excess $^{32}PH_3$. Samples were then counted in a proportional counter to determine the amount of radioactivity remaining on the wheat. The samples were counted a number of times for at least 4 weeks to ensure that only nonvolatile radioactive materials were being detected, and also to check that the $t_{1/2}$ of the radioactivity corresponded to ^{32}P.

Solutions made of glutathione (Schwarz); ribonuclease (Mann); bovine pancreas, 5X cryst. (Mann); insulin, bovine pancreas, recryst. (Mann); and hemoglobin, bovine, 2X cryst. (Mann), were dissolved in water or buffer and treated with $^{32}PH_3$ as described for wheat.

Standards were prepared by injecting $^{32}PH_3$ into bottles containing Br_2-water. Evaporation of aliquots of the $^{32}PH_3$-Br_2 solution provided counting standards.

Milling of the $^{32}PH_3$-treated wheat was carried out in a Brabender Quadruplex mill, followed by rebolting through a 60-grit gauze sieve.

Thin-layer chromatography of wheat extracts was carried out on silica-gel plates using a methanol/conc. NH_4OH/10% trichloroacetic acid/water (50:15:5:30 v./v.) solvent (2). The thin-layer plates were scanned for radioactivity by a Nuclear-Chicago Actigraph III scanner.

Amino acid analysis of 6N acid hydrolysates (3) of bran and proteins was carried out on a Model 120 Beckman amino acid analyzer.

Gel chromatography of bran extracts and protein solution was carried out on 2 X 160-cm. columns of Sephadex G-10, with 1N acetic acid as an elution solvent.

The sulfhydryl (SH) and disulfide (SS) contents of flour were determined as described by Kolthoff et al. (4) by potentiometric titrations with 0.001M silver nitrate in a cell equipped with a rotating platinum electrode and a Hg-HgO-$Ba(OH)_2$ reference electrode (5). The titrations were carried out in 0.40M Tris-0.30M KCl-0.003M EDTA buffer, pH adjusted to 7.4 with nitric acid. Separate analyses were carried out to determine the SH, and the SH plus SS contents. The SH plus SS content was determined by analyzing for SH content of samples which had been

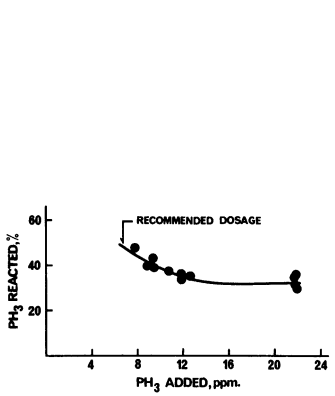
reduced with sodium sulfite. Values for SS content were obtained by difference. Reactive and total SH and SS contents refer to analyses carried out in the absence and presence of 8M urea. The technique used to analyze insulin and glutathione for SH content was to react them with iodoacetic acid-2-^{14}C, followed by hydrolysis with 6N HCl, and then analysis by ion-exchange chromatography with simultaneous radioactivity monitoring. The amount of S-carboxymethyl-2-^{14}C-cysteine found is a measure of the cysteinyl-SH present (6,7).

RESULTS

Treatment of wheat with 8 to 23 p.p.m. $^{32}PH_3$ (w./w.) for 4 and 5 days resulted in 3.8 to 7.4 p.p.m. of $^{32}PH_3$ reacting with wheat to form nonvolatile residues (Fig. 1). The extent of residue formation varied with the initial dose of PH_3, with larger relative amounts of PH_3 reacting at the smaller dosages. No apparent difference in amount of residue formation was observed when the experiments were carried out in CO_2 or air atmospheres. Larger amounts of residues (~45%) formed with rapeseed and flax, and smaller amounts (~15%) with flour (Table II).

The distribution of residues in wheat was determined by milling $^{32}PH_3$-treated wheat and examining the fractions for radioactivity. The results (Table III) showed that 85, 12, and 4% of the residues were in the bran, flour, and germ.

To determine the identity of residues in wheat, a portion of the bran was extracted with water. The extraction removed 64% of the radioactivity. Gel chromatography of the extract on a Sephadex G-10 column showed that all of the



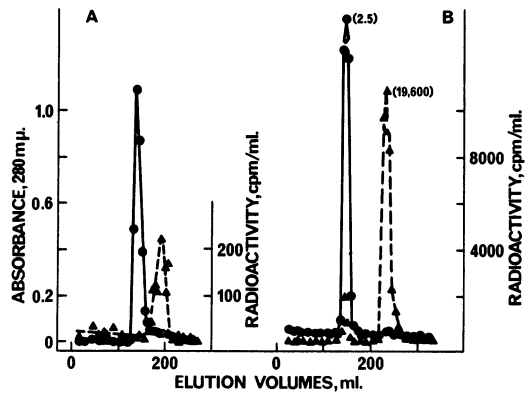


Fig. 1 (left). Percentage of PH_3 reacted with wheat at various dosages, based on nonvolatile residue remaining in the nearly filled fumigation vessels containing 70 g. wheat.

Fig. 2 (right). Gel-chromatography elution profiles of (A) aqueous extract of bran, obtained by milling PH_3-treated wheat,, and (B) of a solution of the residue obtained by freeze-drying a PH_3-treated hemoglobin solution. The absorbance (circles) and radioactivity (triangles) values show that the protein (first peak) and small-molecular-weight components (second peak) are completely separated.

TABLE II. REACTION OF PHOSPHINE-^{32}P WITH WHEAT AND OILSEEDS

Material	Wheat Treated g.	PH_3 Added p.p.m.	PH_3 Reacted %
Wheat	70	14.3	37[a]
Wheat	25	11.8	7[b]
Wheat flour	50	10.2	14
Wheat flour	50	9.9	17
Flax	25	9.5	28
Flax	59	13.4	45
Flax	58	12.0	47
Rapeseed	25	14.2	27
Rapeseed	57	12.8	34
Rapeseed	58	10.3	54

[a]Average of 12 experiments.

[b]Average of two experiments.

TABLE III. DISTRIBUTION OF PHOSPHINE-^{32}P RESIDUES IN WHEAT

	Specific Radioactivity cpm./g.	Constituent %	Distribution of Radioactivity %
Wheat	5,520	100	...
Bran	27,400	17	84.7
Flour	838	80.5	12.2
Germ	9,570	2.5	4.3

extractable radioactivity was due to small-molecular-weight components, since all of the radioactivity was eluted out at a position completely separated from the soluble bran proteins (Fig. 2). Thin-layer chromatography of the radioactive fraction from gel chromatography of the bran extract, followed by radioscanning, showed that two components were present. Of the total radioactivity present on the thin-layer plate, 88% corresponded to hypophosphite (R_f, 0.96) and 12% to pyrophosphate (R_f, 0.05) as shown in Fig. 3. The radioactive spots could not be located colorimetrically with molybdate spray.

Additional information on the PH_3 reaction was obtained by examining the reaction of PH_3 with solutions of hemoglobin, insulin, ribonuclease, and oxidized glutathione. After the excess $^{32}PH_3$ was removed with a stream of nitrogen, the protein solutions were freeze-dried. Gel-chromatography fractionation of the freeze-dried material followed by radioactivity measurements of the fractions showed that PH_3 had formed mostly small-molecular-weight products and some radioactive protein derivatives (Fig. 2 and Table IV). Relatively small amounts of PH_3 reacted with the proteins, except with hemoglobin, where the reaction corresponded to 12 μmoles PH_3 per μmole hemoglobin.

Thin-layer chromatography of the small-molecular-weight fraction from the gel-chromatography fractionation of the hemoglobin experiment (Fig. 3) showed that hypophosphite and orthophosphate had formed as with wheat but in different proportions, and in addition some other unknown products were present. Approximately 55, 27, and 18% of the radioactivity was due to hypophosphite, pyrophosphate, and unknown components (Fig. 3).

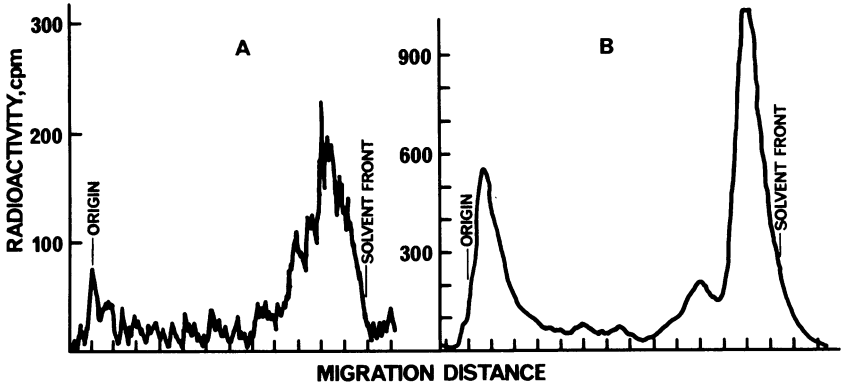


Fig. 3. Radioactivity scans of thin-layer chromatograms of the small-molecular-weight radioactive fractions obtained during gel chromatography (Fig. 2) of (A) bran and (B) hemoglobin. The peaks by the origin and solvent fronts correspond to elution positions of pyrophosphate and hypophosphite.

TABLE IV. REACTION OF PHOSPHINE-^{32}P WITH PROTEIN SOLUTIONS

Protein	Protein Concentration mg./ml.	Total PH_3 Reacted	PH_3 Bound to Protein
		μmoles PH_3 /μmole protein	
Insulin, pH 2.4	7.7	0.064	0.014
Insulin, pH 8.0	5.4	0.073	0.020
Ribonuclease	10	0.025	0.0042
Hemoglobin	9.5	12	0.85
Glutathione	8.1	0.0096	0.0011

An attempt was made to obtain some information on the nature of the radioactive protein derivatives by examining their amino acid compositions. After acid hydrolysis of the freeze-dried gel-chromatography protein fractions, amino acid analysis with simultaneous radioactivity was carried out. A single radioactive ninhydrin-negative peak was obtained, as well as amino acid compositions which corresponded to the published values for the proteins. The identity of the radioactive peak was not determined, but its elution position corresponded to the position where negatively charged inorganic ions would elute out, such as orthophosphate. These results indicate that the phosphorus-protein derivative formed by PH_3 in flour is not stable to 6N HCl hydrolysis at 110°C. for 24 hr.

The results obtained on analyzing flour for SS and SH content, and glutathione and insulin for SH content after treatment with PH_3, are given in Table V. The results indicate that some reduction of SS to SH occurs.

DISCUSSION

The present work demonstrates directly that, when wheat is treated with $^{32}PH_3$ under conditions closely resembling those used during commercial fumigation, approximately 23 to 44% of the added PH_3 reacts with the wheat to form residues.

TABLE V. SULFHYDRYL AND DISULFIDE CONTENTS OF PH_3-TREATED FLOUR

Flour	Reactive		Total	
	SH	SS	SH	SS
	μmoles/g. flour[a]			
PH_3-treated	0.75	5.0	0.90	15.2
Control	0.55	5.7	0.80	15.1

[a]Anhydrous.

Treatment of flax and rapeseed at 12-p.p.m. PH_3 levels resulted in approximately 45% of the PH_3 reacting to form residues. The moisture of the wheat was adjusted to 14% before treatment, since it is cereals with a higher moisture content which usually require fumigation.

Two different types of residues appear to form in wheat. The first type, representing about 36% of the total residues, are those which cannot be extracted with water and therefore are probably attached covalently to a wheat constituent. The constituent is probably not a protein, since gel chromatography of bran extracts showed the protein fraction to contain no radioactivity (Fig. 2). Although control experiments with pure protein solutions showed that $^{32}PH_3$ can react to form radioactive protein derivatives, perhaps the reason that $^{32}PH_3$ did not react with wheat protein is due to the existence of gaseous-solid reaction conditions with wheat, whereas gaseous-solution conditions were present during the control protein solution experiments. The second type of residues are those which can be extracted with water, and form approximately 64% of the total residues. Approximately 88% of the water-soluble residues appear to be hypophosphite ($H_2PO_3^-$) and 12% to be pyrophosphate ($H_2P_2O_7^{--}$). The results show also that PH_3 can penetrate through the bran layers to react with the endosperm, since 12% of the total residues were found in the flour milled from the PH_3-treated wheat.

The reactions in wheat caused by the reaction of PH_3 are not known. As PH_3 is a strong reductant it was expected that some SS may have been reduced to SH bonds. However, analysis of flour before and after PH_3 treatment (Table V) showed that there was only a slight increase in SH and decrease in SS contents. Analysis of PH_3-treated glutathione and insulin solutions showed that no reduction of SS to SH had occurred. Taken together, these results suggest that PH_3 does not readily reduce SS bonds – a finding contrary to that of Robinson and Bond, who found that PH_3 readily reduced cystine to cysteine (8).

The present results support the finding of Berck, who first noted that all of the PH_3 added to wheat could not be recovered (1), and with the recent report by Robinson and Bond (8), who also demonstrated directly that $^{32}PH_3$ forms nonvolatile residues with wheat. Although Robinson and Bond did not try to duplicate field fumigation conditions, or to calculate the amount of residues formed, they found, as in the present study, that 65 to 75% of the residues could be extracted with water and that approximately 70% of the extractable residues are due to hypophosphite. Robinson and Bond reported that the minor residue occurring in the largest amount is orthophosphate (8). Conclusions perhaps should be reserved as to whether the minor residue is actually pyrophosphate in wheat, as found in the present work, or orthophosphate in flour, as found by Robinson and

Bond. While pure orthophosphate and pyrophosphate have reasonably different R_f values [0.64 - 0.68 and 0.46 - 0.5 (9), and 0.2 - 0.4 and 0 - 0.2 (2)] in the different thin-layer chromatography systems used, the combination of component trailing and peak spreading due to radioscanning and chromatography of wheat extracts containing numerous components made accurate identification more difficult. Unfortunately it was not possible to carry out studies to resolve the discrepancy in the identification of the minor residue, as our studies with $^{32}PH_3$ were completed before Robinson and Bond's work was published.

The demonstration in the present work that in wheat PH_3 can penetrate to form residues with the endosperm agrees with two other recent related studies by Matthews et al. (10,11). The latter studies showed that PH_3 fumigation of wheat affects the properties of flour milled from it, as the flour resulted in a dough with a higher viscosity and mixing tolerance (10) and in bread with a firmer crumb (11). Taken together, the present work and the studies of Berck (1), Robinson and Bond (8), and Matthews (10,11) all show that PH_3 fumigation of wheat results in the formation of residues and also affects the properties of flour milled from the PH_3-treated wheat. The five studies quoted above, however, do not agree with reports sponsored by the manufacturers of PH_3 for fumigation purposes, which claim that PH_3 does not form any residues. For example, Bruce et al. (12) report somewhat contradictorily that no PH_3 residues are to be expected in bread even though they obtain PH_3 recoveries ranging from 67 to 101%; Dieterich et al. (13), in their summary of largely unpublished reports, state that there is no evidence of absorption of PH_3 by or adsorption onto any of the foods studied and that negligible residues of approximately 0.01 p.p.m. or less occur; Rauscher and Mayr, in an oral report (14), rebut the evidence published by Berck and again state that "there is obviously no reaction between the commodities tested and the fumigant phosphine"; and the manufacturers of PH_3 for fumigation purposes also claim that PH_3 does not contaminate the grain being fumigated (15).

It is interesting to note that examination of the study by Bruce et al. (12) reveals that it contains evidence for the opposite view – that PH_3 fumigation results in significant formation of PH_3 residues. These workers, using approximately 450 times the recommended dosage of PH_3, examine the P content of wheat as an indication of whether non-PH_3 residues are present. They found that the treated wheat had a P content 0.03% higher than the untreated wheat, and apparently dismissed the finding as insignificant as no comment was made on the increase. A calculation shows that an increase of 0.03% in P is equivalent to 1.1 p.p.m. P – a very significant increase in terms of a fumigant reaction, and an increase equivalent to that found in the present study with $^{32}PH_3$.

[Wheat contains approximately 0.38% P (13,14); thus a 0.03% increase ≡

$$\left(\frac{0.03}{100} \times \frac{0.38}{100} \times 1\right) = 1.14 \times 10^{-6} \text{ g. P/g. wheat} \equiv 1.1 \text{ p.p.m. P}]^{2}$$

Studies claiming that there is no reaction between PH_3 and the substance being fumigated may have reached their conclusions because of the manner of defining "residues", interpreting results, and the selection of experimental conditions.

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[2] A reviewer has suggested that an alternative interpretation is more probable, that the authors meant a P increase from 0.38 to 0.41%. This interpretation is equivalent to a 300-p.p.m. increase in P content, which is possible, as a 450-fold excess of PH_3 was used.

It is not satisfactory to define "an absence of PH_3" to mean "an absence of residues" (12,13,14), since such a definition neglects the possible occurrence of non-PH_3 residues. In addition, in experiments carried out with PH_3 gas as the source of fumigant, the finding of "an absence of PH_3 after aeration" cannot be construed to mean that "residues are absent", since in field fumigation AlP is used as the source of PH_3 and under field conditions AlP can still be present after aeration. The presence of AlP (AlP is a nonvolatile solid, whereas PH_3 is a volatile gas) means that a potential source of PH_3 is still present in the substance being fumigated.

The choice of PH_3 concentrations used in many of the studies requires comment. The concentration of PH_3 used in experimental studies should be reasonably similar to those used in field practices. Field fumigation with PH_3 involves the insertion of AlP tablets directly into the grain, where the AlP slowly hydrolyzes to PH_3. The recommended dosage in reasonably airtight containers is approximately 180 AlP tablets (equivalent to 180 g. PH_3) per 1,000 bushels of grain for a minimum period of 3 to 5 days, depending on the temperature (18). For wheat, the recommended dosage is equivalent to 6.61 γ PH_3 per g. wheat; or, since bulk and kernel densities of wheat are approximately 0.748 and 1.37 g. per cc., to 10.9 γ PH_3 per cc. air space in the wheat, assuming that the PH_3 diffuses evenly throughout the grain (actually, the concentration of PH_3 will be higher in the vicinity of the AlP tablets). However, in studies reporting no residues, dosages were used that usually were significantly lower or higher than the recommended dosages; and in addition, experiments were often carried out in partially filled fumigation vessels (13). Fumigation experiments carried out under conditions significantly different from actual field conditions cannot be expected to lead to an understanding of field PH_3 fumigation.

An example of using a higher dosage than the recommended level is given by Dieterich et al. (13), who maintain that PH_3 fumigation does not lead to residue formation, and give as evidence for their view the unpublished work of Mayr and Hild. Examination of Mayr and Hild's evidence shows that 0.36 to 3.6 g. "Phostoxin", equivalent to 0.12 to 1.2 g. PH_3, was used to treat 200-g. mixtures of wheat, wheat flour, and oat flakes in a 20-liter flask. The validity of using Mayr and Hild's evidence to apply to normal PH_3-fumigation conditions may be questioned, since they used partially filled fumigation vessels and high concentrations of PH_3. The use of partially filled vessels (200 g. wheat in a 20-liter flask would indicate that a void volume is present of approximately 19.85 liters) means that PH_3 uptake would be severely reduced. Evidence that the use of partially filled fumigation vessels results in significantly reducing PH_3 uptake is presented in Table II for wheat, flax, and rapeseed; for example, when only 25 instead of 70 g. wheat was treated in 75-cc. containers, the amount of residues was only 7%, as compared to 37%. Regarding concentration, normal fumigation of 200 g. of material requires approximately 0.00044 g. PH_3; accordingly, Mayr and Hild used on the average a 15,000-fold excess of PH_3.

An example of dosages lower than the recommended levels is given by Rauscher (18), who used 1.4 to 5.0 γ PH_3 per g. wheat product in partially filled containers (0.31 to 1.1 γ PH_3 per cc. void). In this instance PH_3 uptake would be reduced

because only approximately 29% of the recommended dosage of PH_3 is present in a partially filled fumigation container.

The finding that normal commercial PH_3 fumigation of wheat results in significant residues in both the wheat (2 to 3 p.p.m.) and flour (0.4 to 0.8 p.p.m.) indicates that the practice of PH_3 fumigation of cereals is at variance with both current Canadian and U.S. Government regulations (19,20,21). Both countries stipulate a tolerance of only 0.1 p.p.m. of PH_3 residues in wheat; and in addition, the original registration of "Phostoxin" as a PH_3 fumigant was permitted on the assumption that no PH_3 residues were to be present in the commodity treated (21,22).

It must be mentioned that "residues" are defined in the present work to mean "any nonvolatile form of PH_3, or PH_3-reaction product" which remains behind in a fumigated product after prolonged aeration. A clear definition of "residue" in PH_3 fumigation does not seem to have been made, but it can be deduced in the literature that "absence of residues" has usually meant "an absence of PH_3 gas". The latter definition is not satisfactory, as PH_3 would not be expected to be present in a fumigated product after sufficient aeration (since PH_3 is a volatile gas with a b.p. of -87.7°C.); and also, such a definition ignores the possible formation of nonvolatile PH_3 reaction products.

The amount of residues found in flour (0.4 to 0.8 p.p.m.) also exceeds the FAO-WHO recommendation that the maximum PH_3, or PH_3 reaction product residues, should not exceed 0.01 p.p.m. (22).

At the present time it is not known whether fumigation of foods with PH_3 which results in 2 to 3 p.p.m. of residues is harmful to human health. The portions of the residues which occur as hypophosphite (56%), and pyrophosphate or orthophosphate (11%), are not in themselves harmful, since these are innocuous chemicals. What is not known is whether the 37% portion of the residues which are water-insoluble, and the chemical changes caused by the reactions of PH_3 in the food being fumigated, are harmful or not. Statements have been made that PH_3-fumigated foods are perfectly safe for human consumption (13,18). However, as these statements also included claims that no residues form during fumigation, it seems that the question of safety remains to be convincingly resolved.

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