Table I. Recoveries of Ethoxyquin following Addition of the Antioxidant to Broiler Meat, Fish Meat, Fish Meal, and Water

Sample, 10 g	No.	Amount added, mg	Recovery ($\bar{x}$), %	Std dev (S_x), %
Broiler meat	10	1	36	1.6
Fish meat	10	1	34	1.4
Fish meal	8	1	28	1.1
Water	6	1	72	1.2

Only EMQ (mol wt 217) is determined in the GLC analysis. Therefore, the choice of solvent is important for the loss of GLC measurable antioxidant caused by oxidation in the analytical procedure to be kept at a minimum.

For quantitization of the antioxidant, a suitable internal standard is required. Quinoline (QI) is found acceptable in these systems. Figure 2 shows the ratio of the area of EMQ and QI as plotted against different concentrations of EMQ. The linearity is observed in the range from 0.1 to 1.0 mg/ml corresponding to 0.2 to 2.0 μg of EMQ injected. The lower limit of detection is 10 ng.

Table I gives the results of the recovery studies. Only about one-third of the antioxidant added to the three different biological materials is found as GLC measurable EMQ following the homogenization and clean-up procedure. Recoveries of approximately 70% of the ethoxyquin added to water are found, which indicates a loss in the analysis of only 25–30%.

Even though the experimental conditions varied with respect to time (2–20 days) due to the practical limitations of the laboratory capacity, it is seen in Table I that the standard deviations of the recoveries are all acceptable. The observed difference of the recoveries from water and biological systems is probably explained by EMQ working as an antioxidant in the biological redox system. Earlier experiments carried out by Monsanto Chemical Co. (Gordon and Maddy, 1958) showed that the primary oxidation products of EMQ, in addition to EMQ itself, have antioxidative effects. Therefore, the recovery of 30% observed, regardless of the biological system to which the antioxidant is added and the variations of time between homogenization and analyses of the homogenate, may indicate that a spontaneous consumption of the GLC measurable antioxidant is taking place until an intermediate equilibrium state is reached. The general oxidation inhibition is then proceeding.

As can be seen antioxidants pose a special analytical problem since a static image has to be established of a dynamic system. This study presents a method of determination of unchanged or unreacted antioxidant 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline (EMQ) in feed and food products.

LITERATURE CITED

Bickoff, E. M., Guggolz, I., Livingston, A. L., Thomson, C., *Anal. Chem.* **28**(3), 376 (1956).
Fish. News Int. **9**(12), 37 (1970).
Gordon, R. S., Conkin, R. A., Machlin, L. J., *J. Assoc. Off. Anal. Chem.* **47**(3), 512 (1964).
Gordon, R. S., Maddy, K. H., Ed., "Use of Santoquin in Dehydrated Forage Crops", Monsanto Development Department, Organic Chemicals Division, St. Louis, Mo., 1958, pp 30–88.
Skaare, J. U., Dahle, H. K., *J. Agric. Food Chem.*, preceding paper in this issue (1975).
Weilmann, H. R., Hurter, I., Stoll, K., Temperli, A., *Lebensm.-Wiss. Technol.* **5**, 106 (1972).
Witt, S. C., Bickoff, E. M., Kohler, G. O., *J. Assoc. Off. Anal. Chem.* **56**(1), 167 (1973).

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Characterization of Bound Residues of Nitrofen in Cereal Grains

Joseph P. Wargo,[1] Richard C. Honeycutt,* and Irving L. Adler

Starch isolated from the grain of mature rice and wheat which contained radioactive residues resulting from the preemergence use of ^{14}C-labeled nitrofen was found to be radioactive. The starch was hydrolyzed to glucose and derivatized to the osazone with phenylhydrazine. The osazone was isolated and recrystallized several times. From 64 to 90% of the radioactivity in the rice and wheat grain was found to be in the starch, leading to the conclusion that the ^{14}C from the nitrofen had been reincorporated into glucose and subsequently into starch.

Nitrofen (I), 2,4-dichloro-1-(4-nitrophenoxy)benzene, is a selective herbicide which has been used under the trade name of TOK to control annual grasses and many broadleaved weeds. This herbicide is currently registered for use on many crops in the United States.

Nitrofen can be metabolized in vivo to the corresponding amine (II) and to the acetamide (III) [Gutenman and Lisk, 1967; Adler et al., 1971]. It has also been shown that ^{14}C from nitrofen-^{14}C can be found in the lignin from ^{14}C-treated rice and wheat straw (Honeycutt and Adler, 1975). Even though diphenyl ether cleavage of this herbicide has not been reported in plants, Frear and Swanson (1973) have shown that the diphenyl ether herbicide fluorodifen is readily metabolized in peas at the diphenyl ether linkage.

In the course of work with the use of nitrofen-^{14}C as a preemergence herbicide in rice and wheat plots, we obtained grain from these two crops which contained ^{14}C. Very little of the radioactive residue, however, could be removed by conventional solvent extraction techniques. These residues are commonly referred to as bound residues.

A high percentage of grain is composed of starch ("Food and Life", 1959). The studies reported here were undertaken to determine if starch isolated from grain of wheat and rice crops treated with nitrofen-^{14}C contained radioactivity and whether the radioactivity was incorporated into the natural metabolic pool.

Research Laboratories, Rohm and Haas Company, Bristol, Pennsylvania 19007.
[1] Present address: P.O. Box 744, Monument, Colo. 80132.

Table I. Radioactivity in Glucosazone Samples from Wheat Grain[a]

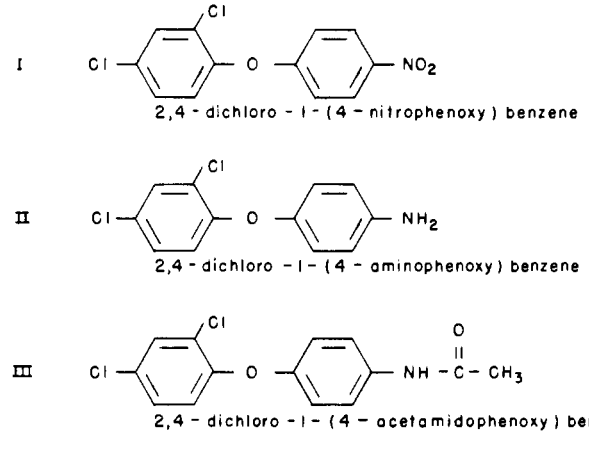
Experiment no.	dpm/g for variety of wheat at label site			
	Era, NO_2 ring	Anza, NO_2 ring	Era, Cl_2 ring	Anza, Cl_2 ring
1, 1st recrystallization	168	127	293	190
2, 1st recrystallization	122	80	116	
2, 2nd recrystallization	147	118	150	197
2, 3rd recrystallization	133	83	126	166
3, 1st recrystallization	126	104	171	110
3, 2nd recrystallization	103	137	219	138
3, 3rd recrystallization	126	204	242	145
Average	132 ± 20	122 ± 42	188 ± 42	158 ± 33

[a] Values presented in this table are averages of duplicate combustion samples of glucosazones.

Table II. Radioactivity in Starch Isolated from Grain of Rice and Wheat Plants Treated with Nitrofen-^{14}C

Crop	Site of label	Specific radioact. of grain, dpm/g	Specific radioact.[a] in osazone calcd as starch, dpm/g	% of ^{14}C at[b] harvest in starch
Rice	Cl_2 ring	395	358	64
Wheat (Era)	NO_2 ring	303	300 ± 45	69
Wheat (Era)	Cl_2 ring	422	427 ± 150	70
Wheat (Anza)	NO_2 ring	260	277 ± 95	74
Wheat (Anza)	Cl_2 ring	268	359 ± 75	94

[a] These values are calculated from the average specific radioactivities of the osazones appearing in Table I. A factor of 2.27 was used to convert the specific radioactivity of the glucosazones to the specific radioactivity of starch. [b] Percent values were calculated by dividing the specific radioactivity of the starch by that of the original grain and multiplying by 0.70, since rice and wheat grain are about 70% starch ("Food and Life", 1959).



MATERIALS AND METHODS

Chemicals. Nitrofen-^{14}C was prepared at Rohm and Haas Company, Bristol, Pa. Nitrofen-^{14}C labeled uniformly about the nitrophenyl ring had a specific radioactivity of 0.8 mCi/g and was applied to wheat at 3.2 lb/acre. Dichlorophenyl ring labeled nitrofen-^{14}C had a specific radioactivity of 0.99 mCi/g and was applied at 4 lb/acre to wheat. The specific radioactivity of the dichlorophenyl ring labeled nitrofen was 1.37 mCi/g for the rice experiments and was applied at 3 lb/acre.

Rice and Wheat Samples. Rice grain samples were from one small rice plot (4 ft × 4 ft) which had been sprayed preemergence with nitrofen-^{14}C as described above. Grain samples were taken 147 days after treatment and contained a radioactive residue of 0.13 ppm calculated as parent compound.

Wheat grain samples were from two small wheat plots which had been sprayed preemergence as described above. Two varieties of spring wheat were planted for each type of label. Grain samples were taken 110 days after planting and contained radioactive residues of 0.1 to 0.2 ppm calculated as nitrofen.

Starch Isolation. The procedure followed was adapted from that of Wolf et al. for the determination of amylose in corn (1970). Five grams of rice or wheat grain was weighed into a Waring Blendor jar. The grain was disintegrated by blending at high speed for several minutes. Ninety milliliters of dimethyl sulfoxide (Me_2SO) and 10 ml of water were added, and the mixture was blended at high speed for 5 min. The blended sample was transferred to a centrifuge flask and allowed to stand overnight at room temperature with occasional stirring. The next morning, the flask was centrifuged for 30 min at 600*g* and the supernatant was decanted into another centrifuge flask. Sufficient anhydrous ethanol was added to cloud the supernatant and precipitate the starch. The flask was centrifuged for 30 min at 600*g* and the supernatant was discarded. The precipitated starch was redispersed in anhydrous ethanol and centrifuged again. The supernatant was discarded and the precipitated starch was removed by filtration and washed several times with anhydrous alcohol. The washed starch was dried in an oven at 60–70° for 48 hr.

Samples of the dried starch were assayed for radioactivity by combustion and liquid scintillation counting on a Packard Model 3320 liquid scintillation spectrometer.

Hydrolysis of Starch and Derivatization of the Glucose. The starch isolated above was hydrolyzed to glucose by heating 10 g plus 500 ml of 0.05 *N* HCl in a 1-l. erlenmeyer flask on a steam bath for 4 hr. After the 4-hr hydrolysis, the acid was neutralized to pH 7 with 40% KOH, and a

mixture of 20 g of phenylhydrazine hydrochloride and 30 g of sodium acetate was added. The solution was heated for an additional 3 hr on a steam bath.

The mixture was allowed to cool overnight in a refrigerator at 3–4°. The precipitated glucosazone was removed by filtration, washed several times with water, and recrystallized three times from a methanol–water mixture. Melting points were determined for each sample, and samples of the dried glucosazone from each recrystallization were combusted for assay of radioactivity.

Samples of glucosazone were prepared for infrared spectroscopy by pressing the sample into a potassium bromide pellet. A Perkin-Elmer RE infrared 137 spectrophotometer was used to determine the infrared spectra.

RESULTS AND DISCUSSION

Starch was isolated from wheat and rice grain and radioassayed by combustion followed by liquid scintillation counting. Preliminary experiments revealed that crude starch from nitrofen-^{14}C treated wheat did indeed contain radioactivity. To demonstrate that the radioactivity in the starch fraction was a part of the glucose unit, the starch was hydrolyzed; the resulting glucose was derivatized to the osazone and recrystallized several times. An infrared spectrum of the osazone was taken and appeared identical with the infrared spectrum of a standard of glucosazone.

Table I shows the results of typical experiments in which starch was isolated from wheat grain and hydrolyzed and the glucose derivatized to the glucosazone. The glucosazone was then recrystallized three times and radioassayed each time. The data in Table I indicate that a constant specific radioactivity was obtained for the wheat grain osazones. Similar experiments with rice gave radioactive starch, which after hydrolysis and derivatization yielded an osazone of constant specific radioactivity upon recrystallization. The radioactivity present in the purified glucosazones from both rice and wheat was significantly high enough to conclude that carbon atoms from nitrofen had entered the metabolic pool and had been channeled into glucose and subsequently into starch.

Table II shows that when the specific radioactivities of these glucosazones are used to calculate the percent of total radioactivity present as starch in wheat or rice grain, a large portion of the residue is accounted for. About 64–94% of the radioactive residues in nitrofen treated wheat or rice grain are in the starch 110 and 147 days, respectively, after planting and treatment. The question of which nitrofen ring is labeled or the species or variety of grain does not appear to make a difference in the extent to which ^{14}C residues appear as starch within the scope and experimental error of this work.

These data lead to the conclusion that the carbon atoms of nitrofen are channeled through at least three structural reorganizations during the metabolism of nitrofen to glucose. First, there must be a cleavage of the diphenyl ether bond during this process. As of now, no intermediate which would demonstrate conclusively that there has been such a cleavage has been isolated. However, Frear and coworkers have shown that the herbicide fluorodifen can be cleaved by extracts of *Pisum sativum* which documents the existence of such an enzymatic diphenyl ether cleaving system in plants (Frear and Swanson, 1973).

A second major metabolic occurrence appears to be ring opening eventually leading to glucose or simpler, presumably aliphatic moieties. Finally, a third anabolic process must then take place to convert these ^{14}C-containing materials to glucose and finally to starch.

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LITERATURE CITED

Adler, I. L., Roser, R. L., Wargo, J. P., Rohm and Haas Company, unpublished data, 1971.
"Food and Life", The Yearbook of Agriculture, U.S. Government Printing Office, Washington, D.C., 1959, p 90.
Frear, D. S., Swanson, H. P., *Pestic. Biochem. Physiol.* **3**, 473 (1973).
Gutenman, W. H., Lisk, D. J., *J. Dairy Sci.* **50**, 1516 (1967).
Honeycutt, R. C., Adler, I. L., *J. Agric. Food Chem.*, following paper in this issue (1975).
Wolf, M. J., Melvin, E. H., Garcia, W. J., Dimler, R. J., Kwolek, W. F., *Cereal Chem.* **47**, 437 (1970).

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Characterization of Bound Residues of Nitrofen in Rice and Wheat Straw

Richard C. Honeycutt* and Irving L. Adler

Crude lignin and cellulose isolated from rice and wheat straw after postemergence and preemergence treatment with nitrofen-^{14}C were found to contain radioactivity. The lignin was purified to a constant specific radioactivity. About 30% of the radioactive residues in rice and wheat straw was in lignin. The cellulose from rice and wheat straw was hydrolyzed to glucose and derivatized to the osazone with phenylhydrazine. The osazone was recrystallized to constant specific radioactivity. Very little of the radioactivity in the rice and wheat straw was found to be in cellulose.

The selective herbicide 2,4-dichloro-1-(4-nitrophenoxy)-benzene, sometimes referred to as nitrofen (I) has been used to control annual grasses and broadleafed weeds in many crops in the United States. Investigations into the metabolism of this herbicide have shown that the amine (II) as well as the acetamide (III) may be produced in vivo (Gutenmann and Lisk, 1967; Adler et al., 1971). Even though no direct evidence for cleavage of the diphenyl ether bond has been reported for nitrofen in plants, a similar herbicide, fluorodifen, has been shown to be cleaved at the diphenyl ether in peas (Frear and Swanson, 1973).

In the course of investigations using nitrofen as a preemergence herbicide for wheat and rice, we obtained straw from these crops which contained radioactive residues. These residues could not be quantitatively removed by conventional organic solvent extraction techniques. Such

Research Laboratories, Rohm and Haas Company, Bristol, Pennsylvania 19007.