COCCIDIOSTAT IDENTIFICATION

Methods of Distinguishing Zoalene and Its Metabolite from Other Coccidiostats

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A method of distinguishing between 3,5-dinitro-o-toluamide and 3,5-dinitrobenzamide is described. The method involves the formation of a colored complex and measurement of the absorbance at several wave lengths. The ratio of the absorbance at 600 mμ to that at 540 or 560 mμ is used to identify the compound. The absorbance ratio for 3,5-dinitro-o-toluamide is between 0.5 and 0.6, while that for 3,5-dinitrobenzamide is between 0.1 and 0.2. This method can also be used to identify 3-amino-5-nitro-o-toluamide after conversion to the dinitro compound with peracetic acid.

RECENTLY, tolerances were established for the combined residues of zoalene (3,5-dinitro-*o*-toluamide), and its metabolite, ANOT (3-amino-5-nitro-*o*-toluamide), which would be permitted in edible chicken tissues (*3*). (Zoalene is a trade-mark of the Dow Chemical Co. in all countries except the United States and Canada.) One of the requirements necessary to establish these tolerances was that analytical methods be developed to identify and determine each of these compounds in the tissues.

The quantitative method developed for the determination of zoalene is based on the reaction of the compound with 1,3-diaminopropane in the presence of dimethylformamide to form a highly colored complex (*4*, *5*). This color test is specific for dinitrobenzamide-type compounds which contain two nitro groups meta to one another without substitution between the groups. Thus, this test will give a positive reaction with either 3,5-dinitrobenzamide or 3,5-dinitro-*o*-toluamide. Since these compounds are both used in commercial coccidiostats, a test was needed to distinguish between these compounds when they are present in coccidiostats, finished feeds, or tissues.

Similarly, the test developed for the determination of ANOT is based on the Bratton-Marshall procedure (*1*) for the determination of arylamines, and is therefore not specific for ANOT. Several commercial coccidiostats contain aryl nitro compounds which could be reduced by the tissue enzymes with the formation of aryl amines. Several of the reduced compounds will give a positive test with the procedure used for the determination of ANOT. A test was needed which would distinguish ANOT from other possible amino compounds.

The present paper describes a simple method for distinguishing between 3,5-dinitro-*o*-toluamide and 3,5-dinitrobenzamide in coccidiostats, feeds, and chicken tissues. This procedure is based on the differences in the spectral curves of the two colored complexes obtained when the compounds are reacted with 1,3-diaminopropane in the presence of dimethylformamide.

A test is also described which can be used to distinguish ANOT from other possible arylamines which could arise from the metabolism of other coccidiostats. This test is based on the conversion of ANOT to zoalene and the use of the test for zoalene for positive identification of the compound.

Method of Distinguishing between 3,5-Dinitro-o-toluamide and 3,5-Dinitrobenzamide

Two drugs being marketed for the control of coccidiosis contain dinitro compounds which will give a positive test with the color reaction described for zoalene. These drugs are Zoamix and Unistat coccidiostats, the composition of which is shown in Table I. The colorimetric test for 3,5-dinitro-*o*-toluamide using 1,3-diaminopropane and dimethylformamide is specific for aromatic compounds having a carboxamide group plus two nitro groups meta to one another. A purple-colored complex is produced with 3,5-dinitro-*o*-toluamide, while 3,5-dinitrobenzamide gives a pink-colored complex. The absorption maximum for the 3,5-dinitro-*o*-toluamide is at 560 mμ, while that for 3,5-dinitrobenzamide is at about 545 mμ (Figure 1). Visually these complexes appear to be similar, but an inspection of their spectral curves reveals a significant difference between the two curves. At wave lengths above 545 mμ the absorbance of the dinitrobenzamide complex decreases very rapidly so that at 600 mμ the absorbance has decreased approximately to 10% of what it was at 545 mμ. In the case of 3,5-dinitro-*o*-toluamide, the absorbance decreases only about 50%. These differences in the absorption curves can be used to distinguish the two compounds. If the absorbance of the two complexes is measured at 540, 560, and 600 mμ and the ratios of the absorbance at 600 mμ/540 mμ or 600 mμ/560 mμ are calculated, it is possible to distinguish between the two compounds, as shown in Table II. The ratios for 3,5-dinitrobenzamide are in the order of 0.10 to 0.20, while the ratios for 3,5-dinitro-*o*-toluamide are between 0.50 and 0.60. This procedure has been used to distinguish between the two compounds when present in coccidiostats, finished feed, and chicken tissue.

Results

Coccidiostats. Zoamix (25% 3,5-dinitro-*o*-toluamide) and Unistat (25% 3,5-dinitrobenzamide) coccidiostats were extracted by the method of Smith (*4*), using a 1-gram sample of each coccidiostat. Each extract was made to 1000 ml. with dimethylformamide and a 10-ml. aliquot was transferred to a 100-ml. volumetric flask and diluted to 100 ml. with dimethylformamide. A 5-ml. aliquot of the diluted extract was mixed with 5 ml. of 1,3-diaminopropane. The absorbance of each solution was measured at 540, 560, and 600 mμ 10 minutes after the addition of the diamine. The absorbance values obtained and the calculated ratios are given in Table III. The differences in the ratios obtained with Zoamix compared with those obtained with Unistat easily permit identification of each material.

Since the other nitro compounds in Unistat do not give a positive reaction with this color test, they do not inter-

Table I. Composition of Several Commercial Coccidiostats

Coccidiostat	Composition	Per Cent
Zoamix, The Dow Chemical Co.	3,5-Dinitro-*o*-toluamide	25.00
	Inert material	75.00
Unistat, Dr. Salsbury's Laboratories	3,5-Dinitrobenzamide	25.00
	Acetyl(*p*-nitrophenyl)sulfanilamide	30.00
	3-Nitro-4-hydroxyphenylarsonic acid	5.00
	Inactive ingredient: bentonite	40.00
Polystat, Dr. Salsbury's Laboratories	Acetyl(*p*-nitrophenyl)sulfanilamide	15.00
	Dibutyltin dilaurate	10.00
	Dinitrodiphenylsulfonyl ethylenediamine	10.00
	3-Nitro-4-hydroxyphenylarsonic acid	3.75
	Inactive ingredients	61.25
Nicarbazin mixture, Merck and Co.	4,4′-Dinitrocarbanilide. 2-hydroxy-4,6-dimethylpyrimidine	25.00
	Corn distillers dried grains, wheat standard middlings, and dried extracted vitamin B_{12} fermentation solubles	75.00

Table II. Absorption Characteristics of Colored Complexes of 3,5-Dinitro-o-toluamide and 3,5-Dinitrobenzamide

		Absorbance	
Wave Length, Mμ		3,5-Dinitro-o-toluamide	3,5-Dinitrobenzamide
540		0.690	1.040
560		0.770	0.950
600		0.400	0.125
Ratio:	600 mμ/540 mμ	0.58	0.12
	600 mμ/560 mμ	0.52	0.13

Table III. Absorbance Values and Ratios for Compounds Present in Coccidiostats and Finished Feeds

		Coccidiostat		Finished Feed	
Wave Length, Mμ		Zoamix	Unistat	0.0125% 3,5-dinitro-o-toluamide	0.025% 3,5-dinitrobenzamide
540		0.440	0.650	0.275	0.980
560		0.495	0.605	0.307	0.905
600		0.275	0.090	0.165	0.134
Ratio:	600 mμ/540 mμ	0.62	0.14	0.60	0.14
	600 mμ/560 mμ	0.56	0.15	0.54	0.15

Table IV. Absorbance Values and Ratios for Compounds Added to Muscle Tissue

(0.5 p.p.m. of each compound added)

		Absorbance					
Wave Length, Mμ		No compd. added		3,5-Dinitro-o-toluamide		3,5-Dinitrobenzamide	
540		0.034	0.040	0.155	0.168	0.202	0.200
560		0.028	0.033	0.157	0.165	0.174	0.180
600		0.014	0.018	0.080	0.084	0.037	0.037
Ratio:	600 mμ/540 mμ	...	...	0.52	0.50	0.18	0.18
	600 mμ/560 mμ	...	...	0.51	0.51	0.21	0.21

fere with the reaction. Polystat and Nicarbazin coccidiostats (Table I) also gave a negative test and thus, Zoamix and Unistat coccidiostats can be distinguished from them.

Finished Feed. In finished feeds, Zoamix was mixed with the feed constituents to give a final concentration of 0.0125% 3,5-dinitro-*o*-toluamide, and Unistat was mixed to give a concentration of 0.025% 3,5-dinitrobenzamide. Finished feeds containing these two coccidiostats were prepared and extracted by a feed assay method for zoalene (*2*). A 10-gram sample of feed was extracted with 85% acetonitrile and the extract was diluted to 100 ml. A 4-ml. aliquot of this solution was transferred to a 50-ml. beaker and evaporated to dryness, using moderate heat and an air jet. The residue was dissolved in 5 ml. of 20% ethyl alcohol–80% dimethylformamide, and 5 ml. of 1,3-diaminopropane were added to produce the color. The absorbance of each solution was measured at 540, 560, and 600 mμ after 10 minutes. The absorbance values and the ratios obtained with the finished feeds are given in Table III. The results indicate that the method can be used to identify either 3,5-dinitro-*o*-toluamide or 3,5-dinitrobenzamide in finished feeds.

Dinitro Compounds Added to Tissue. A series of 50-gram samples of untreated chicken muscle tissue to which 25 μg. of 3,5-dinitro-*o*-toluamide had been added was analyzed for the compound by the method of Smith *et al.* (*6*).

The absorbance of the final solution was measured at 540, 560, and 600 mμ rather than only at 560 mμ as called for in the method. Untreated samples and muscle samples containing 25 μg. of 3,5-dinitrobenzamide were analyzed by the same procedure with absorbance readings being taken at the three wave lengths.

The readings at each wave length and the absorbance ratios are given in Table IV. The ratios for 0.5 p.p.m. of 3,5-dinitro-*o*-toluamide in the tissues were approximately 0.5, while those for 0.5 p.p.m. of 3,5-dinitrobenzamide were in the range of 0.2. The low absorbance readings for untreated tissue samples are representative of the values obtained when neither compound is present. When a significant absorbance reading is obtained, the calculation of the ratios will indicate the compound present.

Tissues from Treated Chickens. Samples of muscle tissue were obtained from White Rock chickens fed continuously for 8 weeks on feed containing Zoamix (1 lb. per ton) and on feed containing Unistat (2 lb. per ton). The chickens were sacrificed while still on medicated feed and 50-gram tissue samples were analyzed for zoalene by the method of Smith *et al.* (*6*).

Absorbance readings were made at 540, 560, and 600 mμ and the ratios were calculated. Typical results obtained are shown in Table V.

The absorbance ratios for tissues from birds fed Zoamix are close to 0.5, indicating that the color is due to 3,5-dinitro-*o*-toluamide. The absorbance readings for tissue from birds fed Unistat are low, but the ratios indicate the color is due to 3,5-dinitrobenzamide.

The absorbance values given for the treated tissues were not corrected for the tissue blank. In the case of benzamide the tissue is contributing a significant amount of color to the benzamide sample at the 600-mμ reading. If the tissue blanks are subtracted and the ratios calculated, ratios of 0.51 and 0.48 will be obtained with the toluamide and 0.14 and 0.17 with the benzamide.

The absorbance values were not corrected for the blanks in order to show that the compounds can be distinguished in the tissues when tissue blanks are not available.

The very low absorbance values for muscle tissue from untreated birds again indicate that neither compound is present. In this experiment the birds were sacrificed while still on medicated feed so that there would be a residue of both compounds in the tissues. In prac-

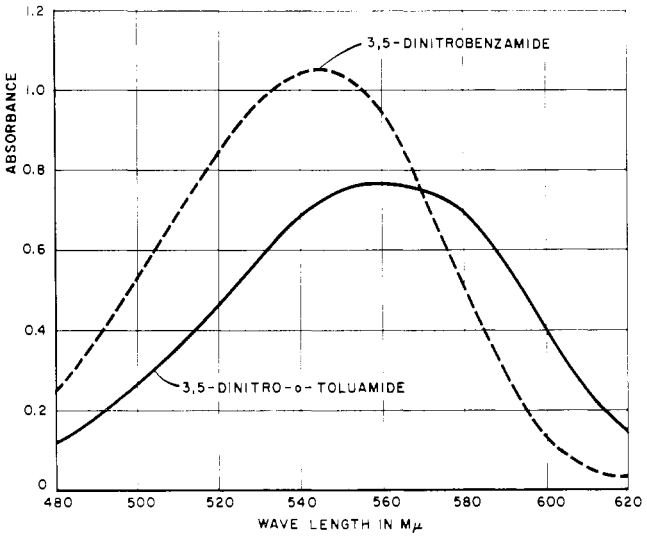

Figure 1. Spectral curves for 3,5-dinitro-o-toluamide and 3,5-dinitrobenzamide with 1,3-diaminopropane

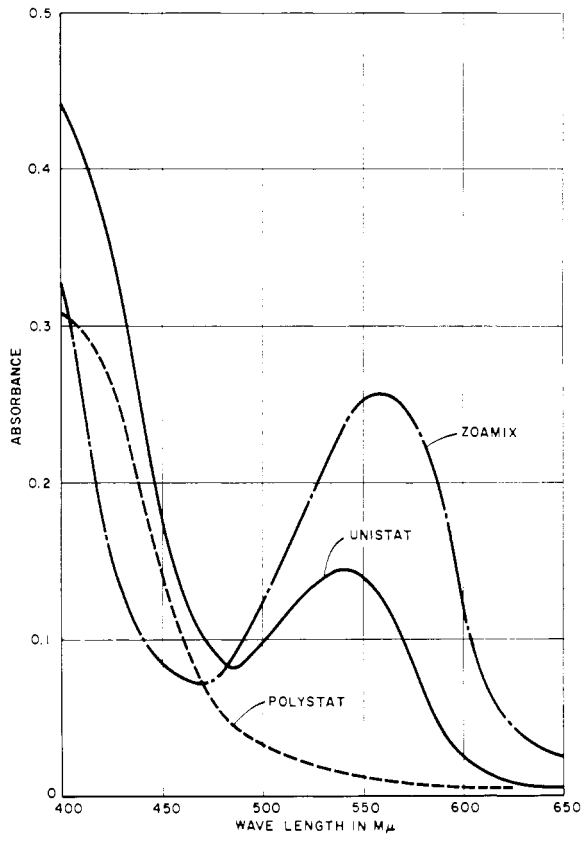

Figure 2. Spectral curves for residues from chickens fed Zoamix, Unistat, and Polystat after treatment with peracetic acid ▶

Table V. Absorbance Values and Ratios for Compounds in Muscle Tissue from Treated and Untreated Chickens

	Absorbance		
Wave Length, Mμ	*Untreated*	*Zoamix at 1 lb. per ton*	*Unistat at 2 lb. per ton*
540	0.028	0.320	0.080
560	0.023	0.333	0.065
600	0.010	0.158	0.017
Ratio: 600 mμ/540 mμ	...	0.49	0.21
600 mμ/560 mμ	...	0.47	0.26

tice, a residue tolerance for zoalene has been established so that the birds can be sacrificed while on medicated feed; with Unistat, however, a 4-day withdrawal period is recommended.

Identification of Residues Giving Positive ANOT Test

Tissues from chickens fed Zoamix, Unistat, or Polystat contain residues of arylamino compounds which can be detected by the procedure used to determine ANOT (3-amino-5-nitro-*o*-toluamide) (*7*). Thus, a positive test would not indicate which of the three coccidiostats had been fed to the chickens. It was, therefore, necessary to develop a qualitative test to determine if the residue was due to zoalene or to one of the other coccidiostats.

The qualitative test used involves converting the reduced compound (such as ANOT) back to the original dinitro compound and then identifying the dinitro compound by the colorimetric procedure described above. Thus, a residue of ANOT is converted to dinitro-*o*-toluamide while a residue of reduced benzamide is converted back to the dinitrobenzamide. A positive color test with 1,3-diaminopropane and dimethylformamide will indicate that the original compound fed the chickens was either dinitro-*o*-toluamide or dinitrobenzamide and the spectral curves will show which compound was fed. If no color is produced with the diaminopropane and dimethylformamide after the conversion, the residue is not due to either the toluamide or benzamide but may be due to Polystat, since this coccidiostat gives a negative color test.

Conversion Test for Reduced Compounds. To run this qualitative test, the reduced compounds must first be isolated from the tissue. This is accomplished by the analytical method used for ANOT (*7*). The compound is liberated from the tissue by enzymatic digestion and extracted with acetone and chloroform. The compound is isolated on an alumina column, eluted with 80% ethyl alcohol, and isolated further by chromatographing on a Dowex 50 ion exchange resin column and eluting with 4*N* HCl. The HCl eluate is evaporated to 0.5 to 1.0 ml. under an infrared lamp using an air jet. Three milliliters of distilled water are added and the solution is neutralized with 2*N* NaOH. The pH of the solution is then adjusted carefully to 8.0 to 8.5 with 0.1*N* NaOH solution. The final volume of the solution should be from 5 to 10 ml.

The solution is tranferred to a 125-ml. separatory funnel and 25 ml. of acetone and 50 ml. of chloroform are added. The separatory funnel is shaken vigorously for several minutes and then set aside to allow the layers to separate. The organic layer is drawn off into a 150-ml. beaker and the solution is evaporated down to 5 to 10 ml. using an infrared lamp. While this solution is being evaporated, the water layer is extracted again with 25 ml. of acetone and 50 ml. of chloroform. The organic layer is transferred to the same beaker containing the first extract and the combined extracts are evaporated to 1 to 2 ml. This solution is transferred to a 50-ml. round-bottomed flask using 5 ml. of chloroform to wash the beaker. The beaker is rinsed with 5 ml. of 40% peracetic acid and this is also transferred to the flask. The flask is fitted with a condenser and the contents of the flask are refluxed for 30 minutes.

Table VI. Absorbance Values and Ratios for Compounds Obtained after Peracetic Acid Treatment of Amino Compounds Added to Chicken Tissue

(1 p.p.m. of each compound added)

		Absorbance					
		No compd. added		3-Amino-5-nitro-o-toluamide		3-Amino-5-nitrobenzamide	
Wave Length, Mμ							
	540	0.059	0.062	0.362	0.315	0.213	0.225
	560	0.046	0.048	0.357	0.325	0.186	0.199
	600	0.026	0.027	0.205	0.172	0.060	0.062
Ratio:	600 mμ/540 mμ	...	...	0.57	0.55	0.28	0.32
	600 mμ/560 mμ	...	...	0.57	0.53	0.32	0.31

Table VII. Absorbance Values Obtained When Liver Tissues from Chickens on Medicated Feeds Are Analyzed for ANOT

(50-gram samples of tissue used)

Coccidiostat Added to Feed	Pounds per Ton of Feed	Absorbance,[a] 540 Mμ
None	0	0.025
Zoamix	1	0.275
Unistat	2	0.548
Polystat	4	0.430
Nicarbazin	1	0.042

[a] Absorbance values have not been corrected for liver tissue blank of 0.025.

Table VIII. Absorbance Values and Ratios for Compounds Obtained after Peracetic Treatment of Residues Giving Positive ANOT Test

		Absorbance		
Wave Length, Mμ		Zoamix, 1 lb. per ton	Unistat, 2 lb. per ton	Polystat, 4 lb. per ton
	540	0.237	0.145	0.018
	560	0.258	0.130	0.014
	600	0.135	0.023	0.008
Ratio:	600 mμ/540 mμ	0.57	0.16	...
	600 mμ/560 mμ	0.52	0.18	...
Visual color		Purple	Pink	Yellow

After the flask has cooled, the contents are transferred back to the 150-ml. beaker used previously. The flask is rinsed with 5 ml. of chloroform and this solution is added to the beaker. The solution is evaporated almost to dryness under an infrared lamp. It is recommended that heating be stopped when a trace of peracetic acid still remains in the beaker. Prolonged heating of the sample to remove the last trace of the acid tends to cause darkening of the sample. The residual peracetic acid in the beaker is neutralized with 0.5 ml. of concentrated ammonium hydroxide. The excess ammonium hydroxide is evaporated off under an infrared lamp. The beaker should be removed from the heat as soon as it becomes dry. The color test is run by adding 5 ml. of 20% ethyl alcohol–80% dimethylformamide to the beaker and stirring for several minutes to dissolve as much of the residue as possible. Color is developed by the addition of 5 ml. of 1,3-diaminopropane. The solution is filtered and the absorbance of the solution is measured at 540, 560, and 600 mμ. The ratios of the absorbances can then be calculated in the manner described previously to determine the compound giving the color.

Amino Compounds Added to Tissues. Fifty-gram samples of chicken liver tissue to which 50 μg. of 3-amino-5-nitro-*o*-toluamide had been added were carried through the ANOT procedure and the conversion back to the dinitro compound. The color was developed with 1,3-diaminopropane and the absorbance was read at 540, 560, and 600 mμ following the method described above. Fifty-gram samples of liver tissue containing 50 μg. of 3-amino-5-nitrobenzamide were carried through the same procedure. The absorbance readings (uncorrected for tissue blanks) and the absorbance ratios obtained are shown in Table VI. The ratios for dinitro-*o*-toluamide are 0.5 or greater, while those for dinitrobenzamide are in the neighborhood of 0.3. The higher ratios obtained with dinitrobenzamide (0.3) than observed previously (0.1 to 0.2) can be explained on the basis of the high readings obtained with the tissue blanks at 600 mμ. If the tissue blanks are subtracted, the ratios obtained with the dinitrobenzamide would be 0.16 and 0.17.

Tissue from Treated Birds. Liver samples were obtained from birds fed continuously for 8 weeks on feed containing Zoamix (1 lb. per ton) by sacrificing the birds while still on medicated feed. These samples were then analyzed for ANOT according to the standard procedure. Similar liver samples were obtained from birds fed on feed containing Unistat (2 lb. per ton), Polystat (4 lb. per ton), and Nicarbazin (1 lb. per ton) and analyzed by the ANOT method. All the treatments except Nicarbazin gave positive tests for ANOT, as shown in Table VII. From these results it would have been concluded that all tissues giving positive results contained ANOT.

A similar series of liver tissue samples which gave a positive ANOT test was carried through the conversion procedure described above. The spectral curve of each colored complex was determined over the range from 400 to 650 mμ (Figure 2). The absorbance values at 540, 560, and 600 mμ together with the calculated ratios are shown in Table VIII.

The results indicate that only the tissues obtained from birds fed Zoamix or Unistat contain a residue which could be converted to a dinitro compound and which could give a positive test with the diaminopropane-dimethylformamide reaction. The residue in liver tissues from birds on Zoamix was converted to a dinitro compound which gave a purple color complex whose absorbance ratios were greater than 0.5. This indicates the compound to be 3,5-dinitro-*o*-toluamide. The residue in liver tissue from birds on Unistat, after conversion to the dinitro compound, gave a pink-colored complex whose absorbance ratios were less than 0.2. This indicates the compound to be 3,5-dinitrobenzamide.

With liver samples obtained from birds fed Polystat, a positive test was obtained with the ANOT procedure and a negative test was obtained with conversion procedure. Thus, the residue which might result from feeding Polystat can easily be distinguished from the residue which will result from feeding Zoamix.

The conversion test can then be used to distinguish ANOT from other possible arylamino compounds which may result from the metabolism of other nitro coccidiostats.

Discussion

Of the four coccidiostats listed in Table I only two, Zoamix and Unistat, respond to the test with 1,3-diaminopropane. This test can be used to distinguish between 3,5-dinitro-*o*-toluamide and 3,5-dinitrobenzamide by measuring the absorbance at several wave lengths and by calculating the ratio of absorbances as described. The ratio of absorbances (600 mμ/540 mμ or 600 mμ/560 mμ) for 3,5-dinitro-*o*-toluamide is in the range of 0.50 to 0.60, while that for 3,5-dinitrobenzamide is much lower, being in the range of 0.1 to 0.2.

The reduced derivatives of Zoamix,

Unistat, and Polystat formed in the chicken tissues all give positive tests with the method for the determination of 3-amino-5-nitro-*o*-toluamide described by Thiegs, Smith, and Bevirt (*7*). These residues cannot, therefore, be distinguished by this method. If the derivatives of Zoamix and Unistat are converted back to their corresponding dinitro compounds by treatment with peracetic acid, they can then be distinguished by the 1,3-diaminopropane test.

When the derivative of Polystat is treated with peracetic acid and tested with 1,3-diaminopropane, a negative test is obtained. This will, therefore, distinguish it from the derivatives of Zoamix and Unistat.

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NATURALLY OCCURRING INSECTICIDES

Identification of 2-Phenylethylisothiocyanate as an Insecticide Occurring Naturally in the Edible Part of Turnips

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A chemical having insecticidal properties was found in the edible part of turnips and was identified as 2-phenylethylisothiocyanate. In the turnip variety investigated (Purple Top Strap Leaf), the concentration amounted to 63 p.p.m., as determined by bioassay. The toxic action of 2-phenylethylisothiocyanate against various insects has been established. It has a definite knock-down effect which is not as great as that of Lethane 384; however, its killing effect is superior to that of Lethane 384. 2-Phenylethylisothiocyanate is an insecticide which occurs naturally in plant tissue that has been consumed for centuries by humans without causing any obvious harm.

CHEMICALS having pesticidal activity are synthesized yearly and marketed in relatively large amounts for agricultural purposes. These chemicals, which are applied to either crops or soils, are in many cases of residual nature. If proper precaution is not exercised, these residues may contaminate animal and human food supplies. Therefore, all pesticides, unless they are of low persistence, necessarily have to have a low mammalian toxicity as determined by extensive animal tests. From such toxicity data, conclusions are then drawn relative to humans.

In tests conducted at the University of Wisconsin, a chemical of insecticidal action was found in the edible part of turnips. Since this crop has been consumed for centuries by humans and no harmful effects have been attributed to it, the toxicity of the insecticidal compound to humans cannot be high.

Preliminary Experiments with Turnips

Turnips (Purple Top Strap Leaf) were grown in a soil (Carrington silt loam), which did not contain any insecticidal residues. After harvest, the edible part of the crops was washed with water and then macerated in a food grinder to a puree-like consistency. Three grams of this material were placed on filter paper in each of eight small test jars (bioassay jars—$2^3/_4$ inches in diameter and 3 inches deep). Fifty vinegar flies (*Drosophila melanogastor* Meig.) were then introduced into each of the test jars (*2*). Shortly after exposure, the flies were affected by paralysis; 50% of the test insects were dead within 2 to $2\ ^1/_2$ hours. Total mortality was registered 3 hours after exposure. It was evident in these early tests that the action of the insecticidal principle resembled a relatively fast knock-down effect.

During the next step, the edible part of the turnips was extracted and purified. For this purpose, 350 grams of the macerated crop material was mixed with 750 grams of anhydrous sodium sulfate; the mixture was kept overnight in a refrigerator and then placed in a 2-quart wide-mouthed Mason jar. A mixture of 900 ml. of commercial grade *n*-pentane (purified by passing through Florex and redistilling) and acetone (4 to 1 by volume) was then added. After 1 hour of head-to-end tumbling, the jars and their contents were chilled to minimize evaporation of solvents during filtering. The supernatant liquid was then decanted through glass wool, and the recovery volume was recorded.

The acetone was removed from the extracts by washing once with water and three times with a 2% solution of sodium sulfate. The pentane solution was then dried over anhydrous sodium sulfate and concentrated to about 25 ml. One gram of Nuchar activated carbon (C-190 N, pH 6) was added; the mixture was swirled gently for 1 minute, and then filtered through a $^1/_2$-inch layer of asbestos with some glass wool on top, held in a glass tube ($7 \times {}^3/_4$ inch). After several washings with a total of 140 ml. of pentane, the clear filtrate was concentrated in a 50° C. water bath and then added to a 10-gram Florisil (60/100 mesh) column (20 mm. diameter). To elute, 150 ml. of 6% ether in pentane was used. Finally, the extract was adjusted to volume with pentane.

The insecticidal activity of the purified extract was tested by pipetting aliquots representing 34.88 grams of turnip material into bioassay jars. After the solvent had been evaporated at room temperature at the opening of a fume hood, the jars were covered with a fine screen. Fifty vinegar flies were intro-