Table V. Recovery[a] of Sevin from Main-Stream Smoke of Treated Cigarettes

Cigarette, γ/Cigarette	Smoke, γ/Smoked Cigarette[b]	Recovery, %
Sevin		
50	0.50[c,d]	1.0
100	1.06[c,d]	1.0
150	1.81[c,d]	1.2

[a] Values shown are means of three determinations.
[b] Corrected for: Av. background of apparent Sevin for check smoke of 0.49 γ/smoked cigarette.
[c] Positive absorbance value, positive Sevin peak (blue) at 575 mμ.
[d] Based on smoke from 90 cigarettes.

drying for 48 hours. Analysis of the cigarette tobacco indicated that Sevin was not dissipated during this presmoking period.

Sevin was added to a series of cigarettes to produce levels of approximately 50, 100, and 150 γ of Sevin per cigarette. These sets of treated cigarettes were smoked as previously described (*2*) and the acetone-trapped smoke was analyzed for Sevin content. Table V shows approximately 1% recovery of Sevin from main-stream smoke of Sevin-treated cigarettes. It is highly probable that much of the Sevin is converted to a volatile material with loss of the detecting functional group. Future studies in this area are contemplated.

Conclusions

Residue studies with Sevin on tobacco indicate that of the total residue on green tobacco at priming time, only 12% was detectable after flue-curing. Residue recovered from main-stream cigarette smoke was approximately 1%.

To place these findings on practical terms in relation to Sevin usage in the field, as an insecticide, for the control of tobacco insects and the amount of Sevin residue reaching the smoker, the following conclusions have been made:

The residue load on tobacco sprayed at the recommended level (1 pound per acre) of Sevin, primed the same day as treated and flue-cured, approximated 15.2 p.p.m. of Sevin. Residual Sevin levels four times greater than these would be required to produce any measurable contamination of main-stream cigarette smoke. Priming tobacco 1 week after treatment would increase this margin by a factor of 10.

Thus, it is felt that Sevin can be used for insect control on flue-cured tobacco and produce cigarettes with essentially no detectable Sevin in main-stream smoke.

Acknowledgment

The authors thank Eileen Lecce and Irene Haithcock for their assistance in processing the many tobacco and cigarette smoke samples used in this study.

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FEED ADDITIVE RESIDUES

Determination of 3,5-Dinitro-*o*-toluamide (Zoalene) in Chicken Tissues

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An analytical procedure for the determination of zoalene in chicken tissues is described. Zoalene is extracted from the tissue with acetone and benzene, and chromatographed on an alumina column. The compound is determined colorimetrically by the addition of 1,3-diaminopropane in the presence of dimethylformamide. The average recovery from muscle tissue over the range of 0.1 to 4.0 p.p.m. was 77 ± 4%, and from liver tissue over the range of 0.2 to 4.0 p.p.m. it was 86 ± 5%.

Zoalene (3,5-dinitro-*o*-toluamide) is a new drug which is effective in preventing caecal and intestinal coccidiosis of chickens. The metabolism of this compound in chickens was investigated in a feeding experiment (*3*) using C^{14}-labeled zoalene ($-C^*ONH_2-C^{14}$). When chickens were sacrificed while on feed containing the radioactive zoalene, two compounds were found in the tissues. The compounds were identified as 3,5-dinitro-*o*-toluamide and the metabolite, 3-amino-5-nitro-*o*-toluamide (ANOT).

Methods for the determination of zoalene and for the metabolite, ANOT (*4*), were developed to measure the residue of each compound in chicken tissues. This paper describes the method for the determination of zoalene in chicken tissue.

The determination of zoalene is based on the highly colored complex which the dinitro compound forms with 1,3-diaminopropane in the presence of dimethylformamide. The tissue is extracted with acetone and benzene and the extracts are combined in a separatory funnel and shaken. After the solution has separated into two phases, the aqueous phase is discarded. The acetone-benzene phase is concentrated and taken up in chloroform. The chloroform solution is passed through an alumina column which retains the zoalene but allows the fat and some other extraneous matter to pass through. The compound is eluted from the column with 80% ethyl alcohol which is subsequently evaporated to dryness. The residue is dissolved in a mixture of ethyl alcohol and dimethylformamide

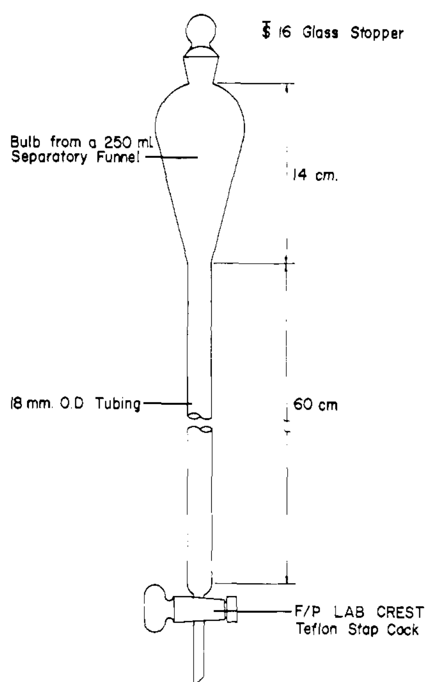

Figure 1. Chromatographic column for alumina

and the colored complex formed by the addition of 1,3-diaminopropane. This reaction is specific for compounds having a carboxamide group plus two nitro groups meta to one another with no substitution between them (*1, 2*).

Experimental

Apparatus. Chromatographic columns (Figure 1).

Heat lamps, Fisher Infra-Rediators.

Multi-Mixer, Lourdes Model MM.

Spectrophotometer, Beckman Model DU.

Waring Blendor or suitable substitute for macerating samples.

Reagents. Acetone-Benzene Solution. Mix 35 parts of acetone with 65 parts of benzene (v./v.).

Alumina. Alcoa grade F-20, 80- to 200-mesh.

1,3-Diaminopropane. Union Carbide Chemicals Co. Should be clear and colorless. Store in refrigerator except when in use. Caution must be observed as this is a hazardous liquid rapidly absorbed through the skin, causing skin burns and eye injury.

Dimethylformamide – Ethyl Alcohol Solution. Mix 1 part of 100% ethyl alcohol with 4 parts of C.P. grade dimethylformamide (v./v.).

3,5 - Dinitro - *o* - toluamide (Zoalene). Recrystallized reference standard, The Dow Chemical Co.

Extraction of Sample. Collect and freeze samples immediately and store in frozen state until analyzed to prevent enzymatic breakdown of the compound.

Grind the frozen samples (muscle is ground in a meat grinder and liver in a Waring Blendor) and weigh 50 grams into a quart Mason jar. Add 250 ml. of acetone and blend with the Multi-Mixer for about 5 minutes. Filter through Whatman No. 1 paper with 5 grams of Hyflo Super-Cel on a Büchner funnel into a 1000-ml. filtering flask. Wash the residue with 100 ml. of benzene. Transfer the residue and filter paper back to the Mason jar, add 250 ml. of benzene and blend again in the Multi-Mixer for 3 minutes. Filter as before using the same flask and wash the residue with 100 ml. of benzene. Transfer the combined filtrates to a 1000-ml. separatory funnel, using an additional 50 ml. of benzene to rinse the filtering flask.

Shake the contents of the separatory funnel well and let stand for 30 minutes. Swirl the funnel and let stand another 30 minutes. Swirl again and let stand several hours or until the layers are well separated. Draw the water layer off into a 250-ml. centrifuge bottle, removing any interfacial debris with the water. Transfer the organic layer to a 1000-ml. beaker. Rinse the separatory funnel with 100 ml. of the acetone-benzene solution and add the washings to the aqueous phase in the centrifuge bottle. Shake this mixture and centrifuge for about 20 minutes at approximately 1700 r.p.m. Remove the lower aqueous phase with a suction tube. Decant the organic layer into the beaker containing the first extract.

Evaporate the extract to a volume of approximately 10 ml. under an infrared heat lamp using an air jet. Add 100 ml. of chloroform and evaporate to 50 ml. If the solution is clear it is ready for application to the alumina column. If it is not clear (because of the presence of water), evaporate to 10 ml. again, add an additional 100 ml. of chloroform, and evaporate to 50 ml. This procedure may be repeated several times, if necessary, to get a clear solution.

Chromatography. Add the chloroform solution to the alumina column (see above) which has been previously washed with chloroform. When the solution has drained to the top of the column, wash it with 50 ml. of chloroform. Use three additional 50-ml. portions of chloroform to rinse the beaker and wash the column. Allow the liquid level to drain to the top of the alumina each time before adding the next wash. Discard the effluent collected up to this point.

Elute the zoalene by adding 90 ml. of 80% ethyl alcohol to the column after the last chloroform wash has drained to the level of the alumina. Discard the first 30 ml. of this effluent and collect a second fraction of 50 to 60 ml., which contains the zoalene, in a 100-ml. beaker. Evaporate under a heat lamp using an air jet just until the residue no longer flows. Care should be taken not to heat the residue too long, and it should not be taken to absolute dryness. This is the most critical step in the procedure since continuous heating of the residue after the liquid has evaporated will give low results.

Development of Color. Add 5 ml. of the dimethylformamide–ethyl alcohol solution to the residue and stir for several minutes. Warming the contents of the beaker for a short time in a water bath at 45° to 50° C. will aid in dissolving the residue. Generally the residue is completely soluble but if a trace of insoluble matter remains it may dissolve in the following step. Add 5 ml. of 1,3-diaminopropane to the solution to produce the colored complex. If the solution is not clear, filter after 5 minutes through a small fluted filter paper (Eaton-Dikeman No. 509) to remove suspended matter. Measure the absorbance at 560 mμ exactly 10 minutes after the addition of the diaminopropane. If the color is too intense to measure, dilute the solution with the above reagents in the same proportion. The intensity of the color may vary with different lots of diaminopropane.

Preparation of Alumina Column. Insert a plug of glass wool in the bottom of the chromatographic tube (Figure 1). Just prior to use, add 60 grams of alumina while gently shaking or tapping the tube to pack the column as uniformly as possible. The height of the alumina in the column should be approximately 30 cm. With the stopcock open, wash the column with 100 ml. of chloroform and drain it to just above the level of the alumina.

Preparation of Standard Curve. Dissolve 100 mg. of zoalene in 50 ml. of acetone and make up to 1000 ml. with water. Dilute an aliquot of this solution, which contains 100 μg. per ml., with water to give a solution containing 10 μg. per ml. Transfer appropriate aliquots of the latter solution containing from 10 to 100 μg. of zoalene to 100-ml. beakers and evaporate just to dryness under a heat lamp, being careful to avoid overheating.

Add 5 ml. of the dimethylformamide–ethyl alcohol solution to the residue and stir for several minutes. To produce the colored complex, add 5 ml. of 1,3-diaminopropane. Measure the absorbance at 560 mμ exactly 10 minutes after the addition of the diaminopropane. Construct a standard curve by plotting absorbance readings against the concentration of zoalene.

Results and Discussion

Recovery Experiments. Known amounts of zoalene were added to muscle and liver tissue samples obtained from 8- to 10-week-old White Rock chickens. The samples were analyzed for zoalene and the results are given in Table I.

Table I. Recovery of Zoalene Added to Chicken Tissues

Zoalene Added, P.P.M.	Tissue Wt., Grams	Absorbance[a]	Zoalene Recovered	
			P.p.m.	%
		MUSCLE TISSUE		
0.1	50	0.029	0.07	70
	50	0.027	0.07	70
	50	0.029	0.07	70
	100	0.058	0.08	80
	100	0.060	0.08	80
	100	0.056	0.08	80
0.2	50	0.061	0.15	75
	50	0.055	0.14	70
	50	0.061	0.15	75
0.5	50	0.147	0.38	76
	50	0.158	0.40	80
	50	0.158	0.40	80
	100	0.276	0.39	78
	100	0.262	0.37	74
	100	0.259	0.36	72
1.0	50	0.325	0.83	83
	50	0.320	0.82	82
	50	0.321	0.82	82
	100	0.545	0.76	76
	100	0.525	0.73	73
	100	0.540	0.76	76
	100	0.514	0.72	72
	100	0.514	0.72	72
	100	0.559	0.78	78
2.0	50	0.629	1.61	81
	50	0.625	1.60	80
	50	0.592	1.52	76
	100[c]	0.577	1.61	81
	100[c]	0.577	1.61	81
	100[c]	0.567	1.59	79
4.0	100[c]	0.544	3.04	76
	100[c]	0.554	3.10	78
	100[c]	0.604	3.38	84
		Av. recov.		77 ± 4[b]
0	50	0.013		
	50	0.013		
	50	0.014		
	50	0.012		
	50	0.013		
	Av.	0.013		
0	100	0.015		
	100	0.020		
	100	0.012		
	100	0.017		
	100	0.018		
	100	0.017		
	Av.	0.016		
		LIVER TISSUE		
0.2	50	0.074	0.19	95
	50	0.069	0.18	90
	50	0.069	0.18	90
	50	0.070	0.18	90
	50	0.066	0.17	85
0.5	50	0.176	0.45	90
	50	0.186	0.47	94
	50	0.161	0.41	82
	50	0.164	0.42	84
	50	0.156	0.40	80
0.8	50	0.285	0.73	91
	50	0.276	0.70	88
	50	0.266	0.68	85
	50	0.251	0.64	80
	50	0.246	0.63	79
1.0	50	0.321	0.82	82
	50	0.326	0.83	83
	50	0.316	0.81	81
	50	0.321	0.82	82
4.0	50	1.372	3.51	88
	50	1.382	3.52	88
	50	1.328	3.38	85
			Av. recov.	86 ± 5[b]
0	50	0.025		
	50	0.025		
	50	0.035		
	50	0.035		
	50	0.042		
	50	0.042		
	50	0.033		
	50	0.035		
	50	0.030		
	Av.	0.034		

[a] For 50- and 100-gram muscle samples, values were corrected for blank values of 0.013 and 0.016, respectively; for liver samples, values were corrected for a blank of 0.034. Different lots of diaminopropane were used for 50- and 100-gram samples.
[b] Standard deviation.
[c] Samples diluted before reading.

Table II. Distribution of Radioactive Zoalene in Various Fractions in Chemical Determination of Zoalene

Fraction	Activity in Fraction, C.P.M.[a]	Distribution of Radioactivity, %	
Zoalene added to liver tissues	4,340,000	100	
Extraction		94.8	
Remaining in tissue	224,000		5.2
Extracted in water phase	24,000		0.6
Extracted in acetone-benzene phase	4,091,000	94.2	
Chromatography acetone-benzene phase	4,091,000	94.2	
Remaining on alumina column	163,000		4.0
In chloroform washings	25,000		0.6
In 80% alcohol effluent	3,751,000		86.4
Total activity accounted for			96.8

[a] Counts per minute corrected for background, self-absorption, etc., and rounded off to the nearest 1000 c.p.m.

Although these recoveries were considered satisfactory, radiochemical studies were conducted to determine where the zoalene was being lost. For this investigation radioactive zoalene (amide-labeled C^{14}) was added to liver tissue and the samples were analyzed. The amount of radioactivity in each fraction was determined using a liquid scintillation spectrometer and calculated in counts per minute. Table II shows that 94.8% of the added zoalene was extracted, the majority of which was concentrated in the organic phase (94.2%). When the zoalene was chromatographed on the alumina, 0.6% of the zoalene was eluted with the chloroform washings. Approximately 86% of the radioactivity was found in the alcohol effluent from the column. The results indicate that 4% remained on the column. This figure is probably low because of the difficulties involved in counting the radioactive materials adsorbed on the alumina.

A similar experiment with radioactive zoalene was made in which the effluent from the alumina column was collected in 5-ml. fractions. The activity in an 0.5-ml. aliquot of each fraction was determined using a liquid scintillation spectrometer. The activity in each fraction was calculated after correcting for absorption due to color in the solution. The absorbance of each fraction at 400 mμ was also measured to determine the distribution of the yellow color. The activity in counts per minute × 1000 and the absorbance × 100 of each fraction are shown in Figure 2. The zoalene and the yellow pigments are both eluted rather sharply, with the pigments preceding the zoalene slightly. The yellow color may be used to indicate the location of the zoalene.

Since it was not practical to separate the zoalene from the yellow tissue pigments, spectral curves of the zoalene complex and muscle and liver tissue blanks were prepared to ascertain if the pigments would interfere with the determination of zoalene (Figure 3). Curves were also prepared from the zoalene isolated from muscle and liver samples obtained from birds on medicated feed. The spectral curves shown in Figure 3 indicate that the absorption of the yellow pigments is insignificant at 560 mμ, which is the wave length at which the zoalene complex exhibits an absorption peak.

Preliminary observations indicated that another factor which could contribute to the loss of zoalene was the enzymatic destruction of the compound. If the tissues were allowed to stand at room temperature or above for a period of time, the zoalene would rapidly disappear. In order to ascertain the magnitude of this loss, 100-gram samples of muscle tissue were homogenized with 100 μg. of zoalene. Samples were analyzed for zoalene immediately and after incubation periods of 4, 8, and 24 hours at 30° C. The results, as shown in Table III, indicate a rapid disappearance of the zoalene during incubation. In order to minimize this loss by enzy-

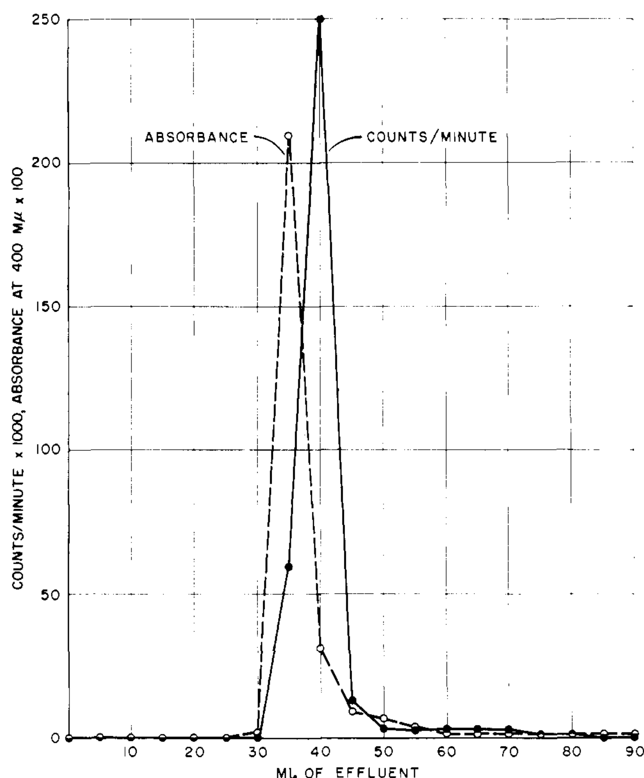

Figure 2. Elution of radioactive zoalene and yellow pigments from alumina column

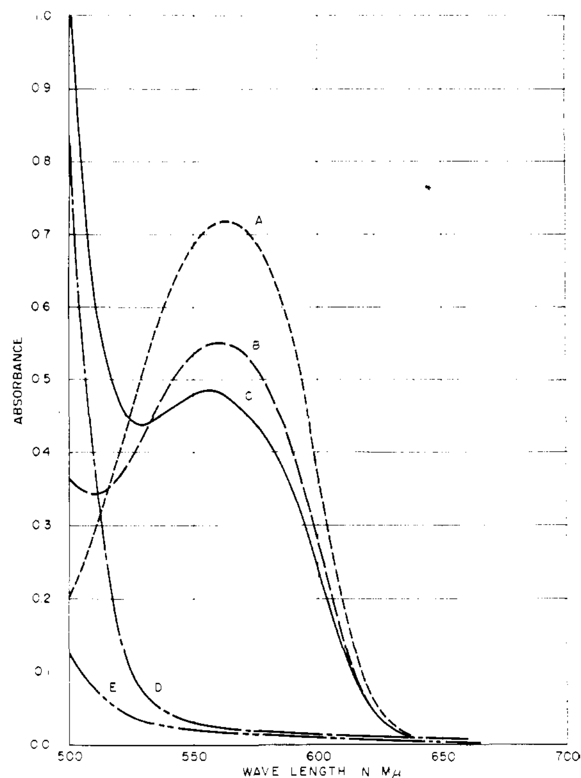

Figure 3. Spectral curves of colored complexes obtained with zoalene, zoalene extracted from tissues, and tissue blanks

A. Zoalene *B.* Zoalene extracted from muscle *C.* Zoalene extracted from liver *D.* Liver blank *E.* Muscle blank

Table III. Effect of Incubation on Disappearance of Zoalene from Muscle Tissue Homogenates

Hours of Incubation at 31° C.	Absorbance[a]	Zoalene Found,[b] μg.
0	0.494	107
	0.459	102
4	0.131	29
	0.113	25
8	0.055	12
	0.043	10
24	0.006	1
	0.002	0

[a] Corrected for a muscle tissue blank of 0.016.
[b] Corrected for 77% recovery from muscle tissue.

Table IV. Zoalene Residues in Chickens Sacrificed While on Medicated Feed

Tissue	Weight, Grams	Date Sacrificed	Absorbance, Corrected	Zoalene Found μg.	Zoalene Found P.p.m.[a]
Muscle					
White Leghorn	100	9–59	0.544	76	1.0
	100	9–59	0.399	56	0.7
White Rock	100	9–59	0.412	58	0.8
	100	9–59	0.426	60	0.8
	50	1–60	0.389	49	1.3
	50	1–60	0.367	47	1.2
Liver					
White Leghorn	50	9–59	0.148	21	0.4
	50	9–59	0.160	23	0.5
White Rock	50	9–59	0.220	31	0.7
	50	9–59	0.185	26	0.6
	50	1–60	0.274	35	0.8
	50	1–60	0.281	36	0.8

[a] Corrected for 77% recovery from muscle tissue and 86% recovery from liver tissue.

matic decomposition, it is necessary to freeze tissue samples immediately in dry ice and analyze them as soon as possible.

Residues. Muscle and liver tissues were obtained from several flocks of 8- to 10-week-old chickens which had been started as day-old chicks on a broiler ration containing 0.0125% zoalene. The tissues were frozen with dry ice as soon as they were removed from the birds and analyzed for zoalene within a few hours after the birds were sacrificed. The results obtained from these analyses are given in Table IV. The residue of zoalene in muscle tissue was 0.7 to 1.3 p.p.m. and in liver 0.4 to 0.8 p.p.m.

Disappearance of Zoalene from Tissues. Previous investigations with radioactive zoalene indicated that the drug rapidly disappeared from the tissues when the chickens were taken off medicated feed (*3*). The analytical method was used to follow the disappearance of zoalene from muscle and liver tissues when chickens were taken off medicated feed. Nine-week-old White Rock chickens, which had been raised on feed containing 0.0125% zoalene, were used. One group of birds was sacrificed while on the medicated feed, and similar groups were sacrificed 4, 8, 12, and 24 hours after being taken off the medicated feed and put on nonmedicated feed.

The muscle and liver samples were collected as soon as the birds were killed and immediately frozen with dry ice. Muscle samples were taken from individual birds while the livers from each group were combined. All analyses

Table V. Effect of Time Off Medicated Feed on Zoalene Residues in Chicken Tissue

(50-gram sample used for each determination)

Hours Off Medicated Feed	Absorbance[a]	Zoalene Found	
		µg.	P.p.m.[b]
	Muscle Tissue		
0	0.255	32.5	0.8
	0.225	29.0	0.8
	0.382	49.0	1.3
	0.395	50.5	1.3
	0.320	41.0	1.1
	0.334	42.5	1.1
	0.497	63.5	1.7
	0.447	57.0	1.5
	0.232	30.0	0.8
	0.234	30.0	0.8
4	0.183	23.5	0.6
	0.168	21.5	0.6
	0.312	39.5	1.0
8	0.055	7.0	0.2
	0.039	5.0	0.1
	0.016	2.0	0.1
12	0.012	1.5	<0.1
	0.030	4.0	0.1
	0.019	2.5	0.1
24	0.000	0.0	0.0
	0.000	0.0	0.0
	0.000	0.0	0.0
	Liver Tissue		
0	0.499	63.5	1.5
	0.494	63.0	1.5
4	0.207	26.5	0.6
	0.198	25.5	0.6
	0.199	25.5	0.6
8	0.051	6.5	0.2
	0.044	5.5	0.1
	0.047	6.0	0.1
12	0.021	2.5	<0.1
	0.018	2.5	<0.1
	0.018	2.5	<0.1
24	0.000	0.0	0.0
	0.000	0.0	0.0
	0.000	0.0	0.0

[a] Corrected for a muscle tissue blank of 0.015 and a liver tissue blank of 0.031. [b] Corrected for 77% recovery from muscle tissue and 86% recovery from liver tissue.

were started within a few hours after the birds were sacrificed. The results, given in Table V, show essentially no zoalene is present in either muscle or liver tissues 12 hours after the birds are taken off the medicated feed. Analyses of duplicate muscle samples taken from each bird sacrificed with no withdrawal time indicate good reproducibility of the method. The analyses at zero withdrawal time also indicate considerable variation in the zoalene content of muscle from individual birds.

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FEED ADDITIVE RESIDUES

Determination of 3-Amino-5-nitro-*o*-toluamide (ANOT) in Chicken Tissues

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A method for the determination of ANOT in chicken tissues is described. ANOT is liberated from the tissue by enzymatic digestion, extracted, and chromatographed on alumina and ion exchange columns. It is determined colorimetrically by diazotization and coupling with *N*-1-naphthylethylenediamine dihydrochloride. The average recovery from muscle tissue, over the range of 0.1 to 1.0 p.p.m., was 86 ±5% and from liver tissue, over the range of 0.25 to 4.0 p.p.m., was 87 ±5%.

When zoalene (3,5-dinitro-*o*-toluamide) is fed to chickens, both the original compound and a metabolite, 3 - amino - 5 - nitro - *o* - toluamide (ANOT), are found in the tissues (*3, 4*). This paper describes the method for determining ANOT in chicken tissue.

An investigation of the metabolism of radioactive zoalene ($-C^{*}ONH_2-C^{14}$) indicated that residues of ANOT were tissue-bound and could not be removed by the usual extraction procedures (*3*). The compound is liberated by enzymatic hydrolysis of the tissue with ficin and extracted from the tissue digest with acetone and chloroform. It is isolated from fats, pigments, and other extraneous material by chromatographing on an alumina column. The compound is further separated from interfering materials by chromatographing on a Dowex 50W ion exchange column. The ANOT is determined quantitatively by diazotization and coupling with *N*-1-naphthylethylenediamine dihydrochloride (*1*).

Materials and Procedures

Apparatus. Same as described in the zoalene method (*4*), with the addition of ion exchange columns for Dowex resin (Figure 1) and water baths, 30° and 70° C.

Reagents. Alumina, Alcoa grade F-20, 80- to 200-mesh.

3-Amino-5-nitro-*o*-toluamide, recrystallized (The Dow Chemical Co.).

Ammonium sulfamate, reagent grade (Fisher Scientific Co.), 1.25% solution in water prepared fresh weekly.

Dowex 50W-X8, H^+ form, 200- to 400-mesh (J. T. Baker Chemical Co.).

Ficin (Nutritional Biochemicals Corp.).

Hyflo Super-Cel (Johns-Manville Co.).

N - 1 - Naphthylethylenediamine dihydrochloride, Eastman No. 4835, 0.25% solution in water prepared fresh weekly.

Sodium nitrite, reagent grade, 0.25% solution in water prepared fresh daily.

Digestion of Sample. Collect the tissue, immediately freeze in dry ice, and keep frozen until analyzed to prevent enzymatic breakdown of the compound. Grind the frozen tissue (muscle is ground