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.	Zoalene Found 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

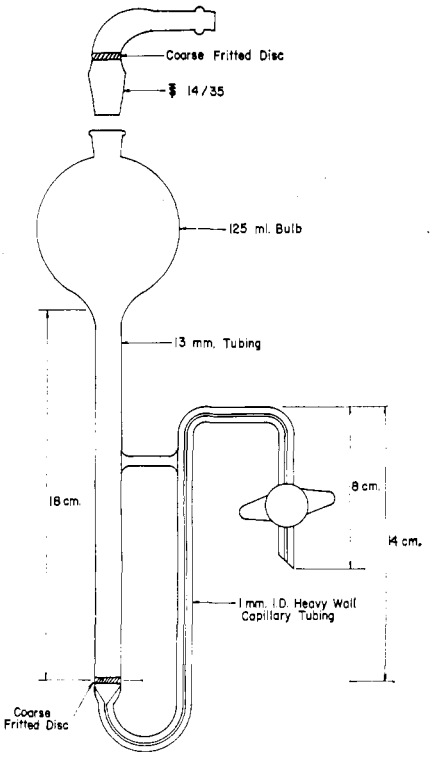

Fig. 1. Ion exchange column for resin

in a meat grinder and liver in a Waring Blendor) and place 50 grams of the tissue in a quart Mason jar. Add 125 ml. of water and 15 ml. of 1*N* HCl and mix on the Multi-Mixer for 5 minutes. Add 5 grams of ficin and continue the mixing for 2 minutes. Cap the jar loosely and incubate the sample in a water bath for 24 hours at 30° C.

Extraction of Sample. After the incubation period, place the Mason jar in a water bath and heat to 70° to 80° C. for 30 minutes. Caution should be observed in placing the jar into the hot water to prevent breakage.

Remove the jar from the water bath and allow to cool to room temperature. Slowly add 10 grams of $NaHCO_3$ to the digest with continuous stirring, being careful that the sample does not foam over the top of the jar. Add 500 ml. of acetone and stir on the Multi-Mixer for 5 minutes.

Place a Whatman No. 1 filter paper on a Büchner funnel and wet with water while applying a vacuum. Add a slurry containing 5 to 10 grams of Super-Cel in acetone. While the filter pad is still wet with acetone, decant the supernatant solution from the digest onto the filter pad, and collect the filtrate in a 1000-ml. filtering flask. Add 200 ml. of acetone to the residue in the jar and mix for another 3 minutes on the Multi-Mixer. Transfer the residue and acetone to the Büchner funnel. After filtration is complete transfer the filter pad and paper with the residue to the Mason jar. Add 500 ml. of chloroform and mix for 5 minutes. While the sample is being mixed, transfer the acetone filtrate from the filtering flask to a 2-liter separatory funnel. Reassemble the filtering flask and Büchner funnel and prepare another filter pad as described above. Transfer the chloroform and residue to the Büchner funnel. After filtration is complete wash the residue with two 250-ml. portions of chloroform. Combine the chloroform filtrate and washings with the acetone extract in the separatory funnel. Rinse the filtering flask with 50 ml. of chloroform and add it to the separatory funnel. Shake the contents of the separatory funnel well and let stand for about an hour or until the layers separate. Draw off the chloroform layer into a 2-liter beaker. Extract the aqueous phase with 200 ml. of chloroform. After the layers separate remove the chloroform layer and combine it with the first extract in the beaker.

Evaporate the chloroform extract to about 50 ml. under an infrared heat lamp using an air jet. Add 100 ml. of chloroform to the extract and again evaporate to about 50 ml. If the solution is not clear (because of the presence

Table I. Recovery of ANOT Added to Chicken Tissues

ANOT Added, P.P.M.	Tissue Wt., Grams	Absorbance[a]	ANOT Recovered P.p.m.	ANOT Recovered %
		Muscle Tissue		
0.1	50	0.016	0.08	80
	50	0.020	0.10	100
	50	0.016	0.08	80
	50	0.015	0.08	80
0.3	50	0.053	0.27	90
	50	0.054	0.28	93
	50	0.051	0.26	87
	50	0.050	0.26	87
0.5	50	0.080	0.41	82
	50	0.083	0.43	86
	50	0.085	0.44	88
0.8	50	0.129	0.66	82
	50	0.135	0.72	90
	50	0.136	0.70	87
1.0	50	0.166	0.85	85
	50	0.170	0.87	87
	50	0.160	0.83	83
			Av. recov.	86 ± 5[b]
0	50	0.006		
	50	0.005		
	50	0.006		
		Av. 0.006		
		Liver Tissue		
0.25	50	0.037	0.19	76
	50	0.041	0.21	84
0.50	50	0.081	0.42	86
	50	0.090	0.46	92
	50	0.098	0.50	100
1.00	100	0.329	0.85	85
	100	0.334	0.86	86
	100	0.324	0.84	84
2.00	50	0.329	1.70	85
	50	0.342	1.76	88
	50	0.330	1.70	85
2.50	50	0.434	2.24	90
	50	0.444	2.28	91
	50	0.432	2.22	89
4.00	50	0.678	3.48	87
	50	0.684	3.52	88
	50	0.696	3.58	89
			Av. recov.	87 ± 5[b]
0	50	0.007		
	50	0.007		
	50	0.010		
	50	0.012		
	50	0.018		
	50	0.022		
		Av. 0.013		

[a] For muscle samples, values were corrected for a blank of 0.006: for 50- and 100-gram liver samples, values were corrected for blank values of 0.013 and 0.026, respectively.
[b] Standard deviation.

Table II. The Conversion of Zoalene to ANOT during the Determination of ANOT in Chicken Tissues

(50-gram sample used for each determination)

Zoalene Added, P.P.M.	Absorbance[a]	ANOT, P.P.M. Found[b]	ANOT, P.P.M. Calcd. as zoalene	Conversion of Zoalene, %
		Muscle Tissue		
0.5	0.001	0.00	0.00	0
	0.004	0.02	0.02	4
				Av. 2
1	0.019	0.11	0.13	13
	0.017	0.10	0.12	12
	0.019	0.11	0.13	13
	0.015	0.10	0.12	12
				Av. 12
2	0.038	0.23	0.26	13
	0.039	0.23	0.26	13
	0.041	0.24	0.28	14
	0.041	0.24	0.28	14
				Av 14
		Liver Tissue		
0.5	0.011	0.07	0.08	16
	0.016	0.09	0.10	20
	0.022	0.13	0.15	30
				Av. 22
1.0	0.036	0.22	0.25	25
	0.037	0.22	0.25	25
	0.035	0.20	0.23	23
				Av. 24
2.0	0.056	0.34	0.40	20
	0.062	0.36	0.42	21
	0.058	0.34	0.40	20
				Av. 20

[a] Corrected for a muscle blank of 0.006 and a liver blank of 0.013.
[b] Corrected for 86% recovery of ANOT from muscle tissue and 87% from liver tissue.

Table III. ANOT Residues in Chickens Sacrificed While on Medicated Feed

(50-gram sample used for each determination)

Tissue	Date Sacrificed	Absorbance, Corrected	ANOT Found, μg.	ANOT Found, P.p.m.[a]
Muscle				
White Leghorn	9–59	0.081	21.0	0.5
	9–59	0.093	24.0	0.6
White Rock	8–59	0.120	31.0	0.7
	8–59	0.099	25.5	0.6
	1–60	0.111	28.5	0.7
	1–60	0.111	28.5	0.7
Liver				
White Leghorn	9–59	0.293	75.5	1.7
	9–59	0.295	76.0	1.7
	10–59	0.291	75.0	1.7
	10–59	0.300	77.0	1.8
White Rock	8–59	0.230	59.0	1.4
	8–59	0.225	58.0	1.3
	8–59	0.257	66.0	1.5
	8–59	0.229	59.0	1.4
	1–60	0.284	73.0	1.7
	1–60	0.245	63.0	1.5

[a] Corrected for 86% recovery from muscle tissue and 87% recovery from liver tissue.

of water), the addition and evaporation of chloroform may be repeated several times.

Chromatography. The procedure is the same as for zoalene (*4*) up to where the compound is eluted and a second fraction of 50 to 60 ml. is collected.

Transfer the second fraction to the Dowex 50W-X8 column (see below). Slight air pressure may be used to increase the flow of the liquid through the resin. After the solution has passed through the resin, wash the column with 50 ml. of 80% ethyl alcohol. Wash again with 50 ml. of water. Discard all washings since the ANOT remains on the resin. Elute the ANOT with 45 ml. of 4*N* HCl and collect the effluent in a 50-ml. volumetric flask.

Color Development. Add 1 ml. of 0.25% sodium nitrite to the volumetric flask containing the ANOT, mix, and allow to stand for 5 minutes. Then add 1 ml. of 1.25% ammonium sulfamate, mix again, and allow to stand another 5 minutes. Add 1 ml. of 0.25% *N*-1-naphthylethylenediamine dihydrochloride and bring the volume up to 50 ml. with 4*N* HCl. Mix well, let stand 15 minutes, then measure the absorbance at 540 mμ using a Beckman spectrophotometer and a 1-cm. cuvette. Reagent blanks should be run periodically.

Preparation of Dowex Column. Heat 100 grams of Dowex 50W-X8 (200 to 400-mesh) on a steam bath with 400 ml. of 6*N* HCl for several hours. Filter the resin on a Büchner funnel and wash with water until the washings are free of acid. Wash the resin once with 100 ml. of 80% ethyl alcohol and suspend it in 250 ml. of 80% ethyl alcohol. Pour sufficient resin in the ion exchange column (Figure 1) to give a bed height of 4 to 5 cm. after settling. Wash the column with 25 ml. of 80% ethyl alcohol. It is ready for use when the resin has settled and the liquid level has drained to just above the resin. Slight air pressure can be used to speed the flow of liquid through the resin. However, care should be taken that the liquid level does not go below that of the resin.

Preparation of Standard Curve. Dissolve 100 mg. of recrystallized ANOT in 50 ml. of acetone and make up to 1000 ml. with water. Dilute an aliquot of this solution, which contains 100 μg. of ANOT per ml., to give a solution containing 10 μg. per ml. Transfer appropriate aliquots of the latter solution containing from 10 to 100 μg. of ANOT to 50-ml. volumetric flasks and add 4*N* HCl until the volume is about 40 to 45 ml. Follow the color development procedure described above. Construct a standard curve by plotting the absorbance readings against the concentration of ANOT.

Results and Discussion

Previous experiments (*3*) using radioactive zoalene indicated that the ANOT formed in the tissues was tissue-bound and was not removed by the usual extraction procedures. To measure residues of ANOT it was first necessary to liberate the compound from the tissues so it could be extracted. Chemical hydrolysis with acids and bases (*2*) was unsuccessful, as low recoveries of added ANOT were obtained. Self-autolysis of the tissues under slightly acid conditions gave results which suggested that an enzymatic digestion might release the compound. Of the enzymes tested

Table IV. Effect of Time Off Medicated Feed on ANOT Residues in Chicken Tissues

(50-gram sample used for each determination)

Hours Off Medicated Feed	Absorbance[a]	ANOT Found μg.	ANOT Found P.p.m.[b]
	Muscle Tissue		
0	0.081	21.0	0.5
	0.093	24.0	0.6
4	0.048	12.5	0.3
	0.053	13.5	0.3
	0.050	13.0	0.3
8	0.067	17.5	0.4
	0.028	7.5	0.2
	0.044	11.5	0.3
12	0.021	5.5	0.1
	0.010	2.5	<0.1
24	0.012	3.0	<0.1
	0.013	3.0	<0.1
	0.006	1.5	<0.1
	Liver Tissue		
0	0.291	75.0	1.7
	0.300	77.0	1.8
	0.293	75.5	1.7
	0.295	76.0	1.7
4	0.187	48.0	1.1
	0.162	42.0	1.0
8	0.144	37.0	0.9
	0.136	35.0	0.8
	0.125	32.5	0.7
12	0.053	13.5	0.3
	0.051	13.0	0.3
24	0.032	8.0	0.2
	0.036	9.0	0.2
48	0.038	10.0	0.2
	0.028	7.5	0.2

[a] Corrected for a muscle tissue blank of 0.006 and a liver tissue blank of 0.004.
[b] Corrected for 86% recovery from muscle tissue and 87% from liver tissue.

(ficin, pepsin, trypsin, chymotrysin, proteinase, and protease), ficin gave the best liberation of the bound metabolite. Maximum liberation of the compound occurred at a pH of approximately 4.0.

After the enzymatic digestion, heating the autolyzate at 70° to 80° C. for 30 minutes and neutralizing the solution prior to extraction increased the recovery of the ANOT. The ANOT is extracted from the autolyzate with acetone. The addition of sufficient acetone to give a solution containing at least 75% acetone causes some tissue components to precipitate so they can be removed by filtration. The extraction with chloroform and subsequent combination of the acetone and chloroform extracts permit separation into aqueous and organic phases. The ANOT goes into the organic phase while many tissue components remain in the aqueous phase. The organic phase contains, in addition to the ANOT, the fats, pigments, and other extraneous material.

The organic phase is concentrated and taken up in chloroform and evaporated again to ensure the removal of any water. The concentrated chloroform solution is added to an alumina column. The fats and some other material are washed through the column with chloroform. The ANOT is eluted from the column with 80% ethyl alcohol. The ethyl alcohol solution contains, in addition to the ANOT, some yellow pigments and other extraneous matter which were removed by the alcohol.

The ANOT is separated from these extraneous materials by chromatographing on a Dowex 50W ion exchange column. The yellow pigments pass through the column while the ANOT and some interfering materials are retained. The ANOT is then eluted with 4*N* hydrochloric acid while the interfering materials remain on the column. The compound is measured quantitatively by diazotization and coupling with *N* - 1 - naphthylethylenediamine dihydrochloride (*1*).

Recovery Experiments. Muscle and liver tissues were obtained from 8- to 10-week-old White Rock chickens which had been fed continuously on a commercial broiler ration. Known amounts of ANOT were added to tissues which were then analyzed. Results are shown in Table I.

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%. These recoveries are considered to be satisfactory for residue analysis of this nature.

Conversion of Zoalene to ANOT during Ficin Digestion. In vitro experiments with zoalene showed that it is rapidly converted to ANOT by tissue enzymes. Since the determination of ANOT requires a digestion period with ficin to hydrolyze the tissue, it appeared that part of the zoalene present might be reduced to ANOT before the tissue enzymes were inactivated.

To determine if some zoalene was reduced, muscle and liver tissues to which 0.5, 1.0, and 2.0 p.p.m. of zoalene was added were analyzed for ANOT. The results, given in Table II, show that a small amount of zoalene is converted to ANOT. This conversion of zoalene to ANOT makes the ANOT residue slightly higher than it would be if no zoalene were present. No correction has been made for the small amount of ANOT which is formed from the zoalene present in the tissue. It is desirable, however, when ANOT is being determined, to add the hydrochloric acid and ficin to tissue samples as rapidly as possible in order to inactivate the enzymes which convert zoalene to ANOT.

Residues. Muscle and liver tissue samples were obtained from several flocks of 8- to 10-week-old chickens which had been raised on a commercial broiler ration containing 0.0125% zoalene. Muscle tissue samples were taken from individual birds, while the livers from 10 birds were combined to obtain sufficient sample.

The samples were collected as the birds were sacrificed, immediately frozen in dry ice, and maintained in the frozen state until analyzed. All analyses were started within a few hours after the birds were sacrificed.

The ANOT residues found in muscle and liver tissue samples taken from several flocks of chickens are given in Table III. The ANOT content of muscle tissue ranged from 0.5 to 0.7 p.p.m., while that in liver ranged from 1.3 to 1.8 p.p.m.

Disappearance of ANOT When Chickens Are Taken Off Medicated Feed. This experiment was conducted to determine the ANOT content of muscle and liver tissue after the chickens had been off medicated feed for different periods of time. The 10- to 12-week-old White Leghorns used had been raised on a commercial broiler ration containing 0.0125% zoalene. One group of 6 birds was sacrificed while on medicated feed. Similar groups were sacrificed 4, 8, 12, 24, and 48 hours after being taken off the medicated feed and put on nonmedicated feed. The samples were collected as soon as the birds were sacrificed and immediately frozen in dry ice. Muscle samples were taken from individual birds, while the livers in each group were combined. The results given in Table IV show that most of the ANOT disappeared from both the muscle and liver within 12 hours after the birds are taken off the treated feed.

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