The Metabolism of 3,5-Dinitro-*o*-toluamide-C^{14} (Zoalene) in Chickens

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Investigations on the metabolism of 3,5-dinitro-o-toluamide-C^{14} indicated that the compound rapidly enters the blood once the chicken is placed on medicated feed and is rapidly eliminated once the drug is withdrawn. Tissue studies indicated a preferential accumulation of radioactivity in the liver and kidneys. Solvent extraction studies revealed that part of the radioactivity was bound to the tissue components and could not be removed by solvent extraction. There appeared to be at least two radioactive compounds in the various tissues.

RECENTLY 3,5-dinitro-*o*-toluamide (Zoalene, trademark of The Dow Chemical Co. abroad) was introduced as an anticoccidial drug for the prevention and control of coccidiosis in chickens (*1–3, 5*). This drug is normally fed to broiler chickens at a concentration of 0.0125% for 8 to 10 weeks. Following use of the drug in this manner, it was necessary to determine if the drug was metabolized in the chicken and if the drug or any of its possible metabolites accumulated in the edible tissues to such an extent that it would present a residue problem. Studies were conducted to obtain information concerning the metabolic fate of zoalene in the chicken. The investigation was divided into two phases.

In the first phase, a series of short-term feeding experiments were conducted to determine how rapidly zoalene enters the blood stream and is transferred to the tissues. Tissue studies were initiated to determine if there was preferential accumulation of the radioactive compounds in specific tissues or organs. Additional studies were conducted to determine how rapidly the radioactive compounds were eliminated from the blood and tissues when the drug was removed from the diet of the bird.

In the second phase of the investigation, a long-term feeding study was conducted to determine if the level of radioactivity in the blood changed with the age of the bird and if there was a gradual accumulation of the metabolites in the tissues which would be different from the results observed in the short-term studies.

The feeding experiments were conducted with carboxyl-C^{14}-labeled zoalene having a specific activity of approximately 3.2 mc. per mmole. This would permit the detection and identification of any radioactive compounds present in the tissues at a concentration of greater than 0.1 p.p.m.

Methods

In the continuous feeding experiment with radioactive feed, it was desirable to use a feeding arrangement where the birds had free access to the feed and water but were restricted so that contamination of the cage and bird were kept at a minimum. Since chickens are coprophagous, it is necessary to remove all fecal material that is eliminated so that the birds cannot eat their droppings; otherwise the radioactivity might be circulated through the chicken's body several times.

The birds were housed in wire-floored cages which had continuous illumination and a constant temperature. Feed was provided in polyethylene bottles containing an oval opening on the side approximately 2.5 cm. wide and 4 cm. high. This allowed the bird to reach the feed and decreased the possibility of contaminating the other parts of the body.

The radioactive feed was prepared from carboxyl-labeled-C^{14} zoalene having a specific activity of 3.15 mc. per mmole (*4*). The zoalene was mixed with soybean meal to give a premix containing 25% of the drug. This premix was then mixed with a commercial broiler ration to give a feed containing 0.0125% zoalene. When a uniform mix was obtained as determined by radiochemical and chemical analysis, the feed was refrigerated until needed.

In all feeding experiments, blood samples were taken at regular intervals with hypodermic syringes wet with heparin. With birds less than 5 weeks old, blood samples were taken by heart puncture, and sampling was restricted to once a week. With birds 5 or more weeks old, blood samples were taken via the wing veins. The blood samples were plated and counted directly for radioactivity. This counting procedure, used under controlled conditions, was reproducible within ±5%.

At the termination of the medication period, the birds were placed on nonmedicated feed and sacrificed at various time intervals. When each bird was sacrificed, large blood samples were collected by heart puncture just prior to decapitation. All birds were bled out and the individual tissues quickly removed and immediately frozen. Great care was exercised to prevent cross-contamination due to bleeding or use of contaminated instruments.

The tissues and organs were analyzed for radioactivity by combustion of samples and collection of the radioactive carbon dioxide as barium carbonate. The barium carbonate was then counted as infinitely thick plates. The samples were combusted with the Van-Slyke reagent, and the carbon dioxide was trapped in sodium hydroxide (*6*). Barium chloride was added to the sodium hydroxide to precipitate the carbonate and the excess alkali titrated with hydrochloric acid using phenolphthalein as an indicator. From this titration, the amount of carbon dioxide liberated by the combustion could be calculated.

All organs were homogenized in a glass homogenizer with an equal weight of water and aliquots of the homogenate taken for combustion. The other tissues were sectioned, and various slices selected at random were combined to form a single sample for combustion.

Triplicate samples of each tissue or homogenate were combusted and duplicate plates of barium carbonate made from each combustion. The samples were counted in a Nuclear-Chicago automatic sample changer using a D-47 gas flow counter. The scale was set at a predetermined count of 4000. The background count was 17 counts per minute (c.p.m.). The net counts of the samples were always equal to or greater than the background count.

The blood was separated into red cells and plasma by centrifuging at 2500

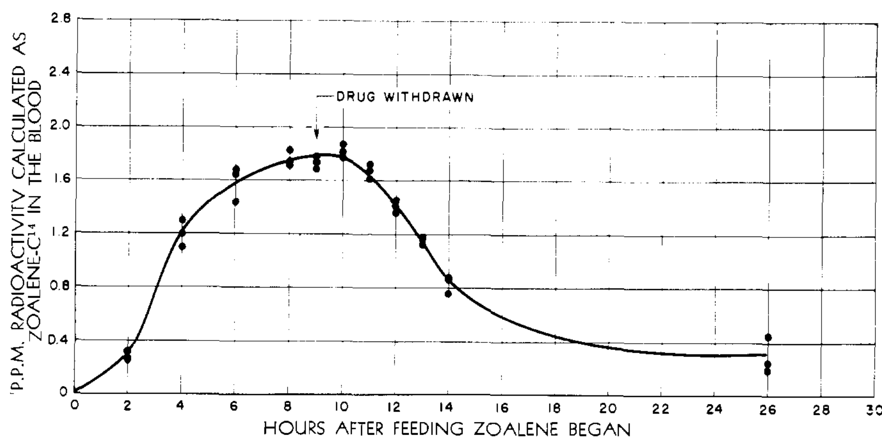

Figure 1. Changes in the level of radioactivity in the blood of chickens fed zoalene-C^{14} for 12 hours

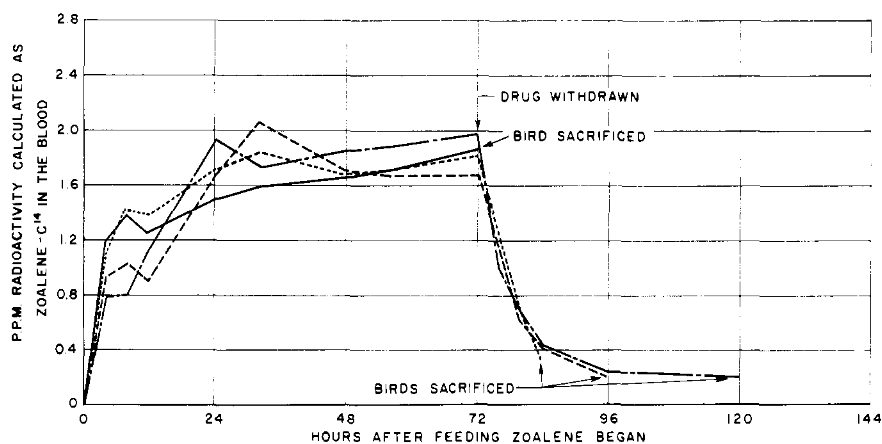

Figure 2. Changes in the level of radioactivity in the blood of chickens fed zoalene-C^{14} for 3 days

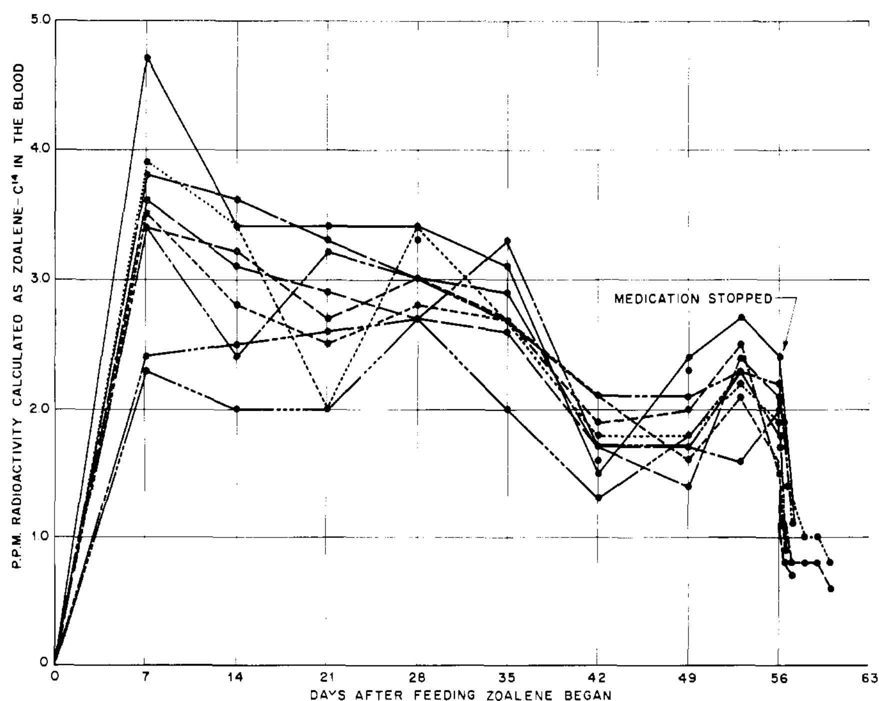

Figure 3. Changes in the level of radioactivity in the blood of chickens fed zoalene-C^{14} for 7 days

r.p.m. for approximately 30 minutes. Aliquots of the whole blood, red cells, and plasma were analyzed for total radioactivity.

It was observed in the course of the investigation that only a portion of the radioactivity in the tissues could be removed by an acetone extraction. The radioactivity removed by the solvent extraction appeared to be different from that which remained in the tissues. The acetone extraction was therefore used to give an indication of the bound and free radioactive material in the tissues. Each tissue was homogenized with an equal weight of water and then mixed with 10 times its volume of acetone. The sample was then transferred to a Soxhlet extractor and extracted for 4 hours. For convenience, the radioactivity remaining with the tissue residue was designated as bound radioactivity and that which was extracted was designated as free radioactivity.

Results and Discussion

In the first series of short-term feeding experiments, 8-week-old White Leghorn chickens were fed gelatin capsules containing radioactive zoalene at hourly intervals. Each capsule contained 0.57 mg. of radioactive zoalene mixed with soybean meal. This was equal to 0.013% in the feed based on a consumption rate of 100 grams of feed per day. Between forced feedings, the birds were allowed to feed normally on nonmedicated feed.

Each bird was given a total of 10 capsules and then placed on nonmedicated feed for an additional 17 hours. Blood samples were taken via the wing veins just prior to each forced feeding and at various time intervals after medication was discontinued. The blood samples were immediately analyzed for radioactivity. The results of a typical experiment are shown in Figure 1. The data were plotted in terms of p.p.m. of radioactive zoalene originally administered. However, this does not mean that all the radioactivity was present as zoalene.

The data in Figure 1 show that the radioactivity in the blood rapidly increased when the birds were placed on medicated feed. After 8 hours, the level of radioactivity appeared to be plateauing, and forced feeding was discontinued after the 10th treatment at 9 hours. Within a few hours, there was a significant decrease of radioactive compound in the blood. This rapid decrease continued from the 10th to the 14th hour. After this, the rate of elimination of the compounds decreased with time. This change in slope of the curve suggests the possibility that several radioactive compounds might be present in the blood. The compound (or compounds) present in the largest quantities appears

to have a very short biological half-life on the order of 4 to 6 hours, while the other compound (or compounds) appears to have a half-life on the order of days.

Two additional series of short-term feeding studies were conducted in which birds were allowed to feed continuously in a normal manner on medicated feed containing 0.0125% radioactive zoalene.

In one series, the birds were on medicated feed 3 days and then on nonmedicated feed for 2 days. Blood samples were taken at various time intervals throughout the feeding period (Figure 2). After medication was discontinued, birds were sacrificed at various time intervals and their tissues analyzed for radioactivity. The results indicate that approximately 24 hours were required for the radioactivity in the blood to reach a more or less constant level. There was considerable variation in the amount of radioactivity found in the birds. Visual observation made while the birds were on medicated feed suggested that these variations were primarily due to the differences in feeding patterns of the individual birds. They did not appear to be eating at a constant rate, and any slight change in the amount of feed consumed per unit of time was reflected in a change in the blood picture.

When the birds were withdrawn from radioactive feeds, there was immediate and rapid decrease in the radioactivity in the blood so that within 24 hours 90% of the radioactive compounds had been eliminated. After the drug had been withdrawn for 24 hours, there was the equivalent of 0.2 p.p.m. radioactive zoalene in the blood. This level of radioactivity decreased only slightly in the next 24-hour period. These results again indicated the presence of at least two major radioactive components.

In the third series of short-term feeding studies, the birds were on medicated feed 7 days and on nonmedicated feed from 0 to 5 days. Blood samples were collected each morning and analyzed immediately. The results are shown in Figure 3. Here again, there was considerable variation in the amount of radioactivity found in the blood, but essentially the same blood profile was obtained. There was a rapid increase of activity in the blood when the birds were placed on medicated feed and a rapid loss when the drug was withdrawn. The longer withdrawal period showed that the residual radioactivity was slowly being eliminated from the blood. At 8 days, there was 0.5 p.p.m. present, while at 12 days this had decreased to about 0.3 p p.m.

In the long-term studies, 2-week old White Leghorn cockerels were selected for uniformity, and nine birds were placed on feed containing radioactive zoalene. The average weight at the start of the feeding period was 111±2 grams per bird. At the end of the 8-week feeding period, the average weight was 1124±85 grams per bird with a net gain of 1012±79 grams. During this period, the average feed consumption calculated from the total feed consumption for all the birds was approximately 3000 grams of feed per bird. This would amount to an average intake of approximately 384 mg. of radioactive zoalene per bird during the feeding period.

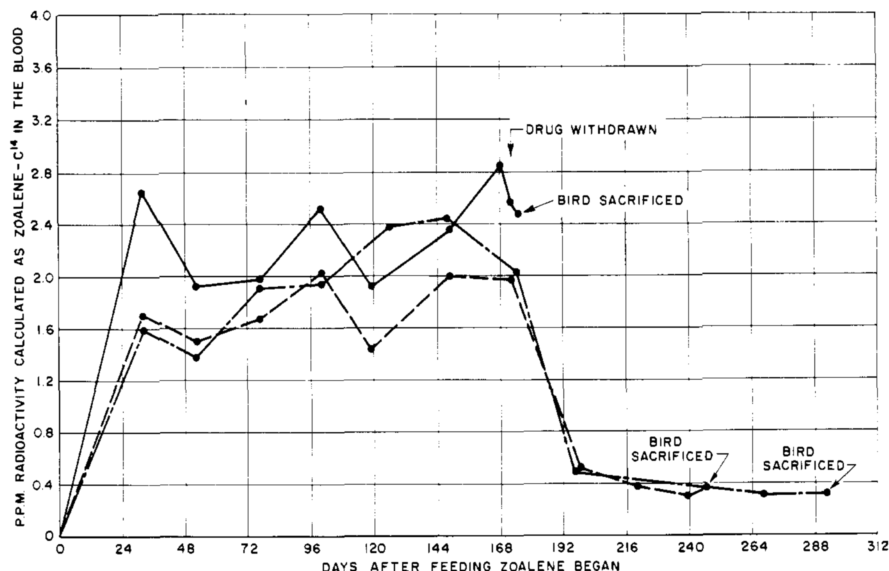

Figure 4. Changes in the level of radioactivity in the blood of chickens fed zoalene-C^{14} for 8 weeks

Blood samples were taken via the wing veins at weekly intervals and analyzed for radioactivity (Figure 4). As the feeding period continued, there was a gradual decrease in the level of radioactivity in the blood. It is known that as a chicken grows older, the amount of food consumed per unit of body weight decreases. This decrease in food consumption results in a decreased drug intake which in turn is reflected in a decrease in the level of radioactivity in the blood. This pattern was observed in the feeding experiments with radioactive zoalene. As the birds become older, the food intake per day decreased and the level of radioactivity in the blood decreased.

When the birds were taken off medicated feed, the activity dropped very rapidly so that within 24 hours the majority of the radioactive compounds had disappeared. Traces of radioactive material (0.6 to 0.8 p.p.m.) remained in the blood during the remaining portion of the observation period.

A study of the distribution of radioactivity in the blood revealed that the red cells contained about twice the concentration of radioactive compounds as did the plasma when calculated as p.p.m. of zoalene-C^{14} per gram of sample (Table I). On a volume basis, whole blood is made up of approximately 65% plasma and 35% red cells. On this basis, there is approximately uniform distribution of the radioactivity in the whole blood between the plasma and red cells.

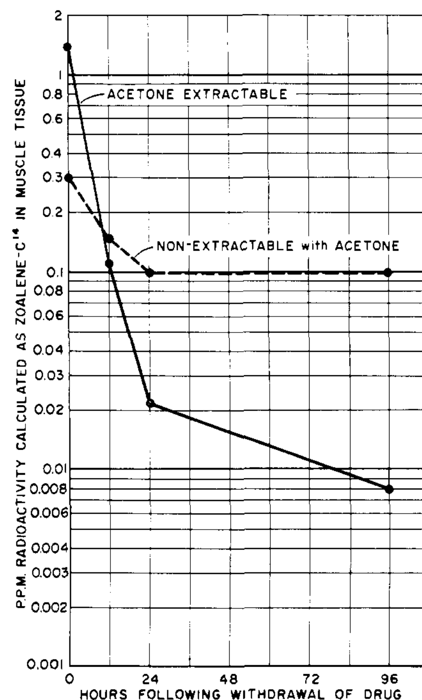

Figure 5. Elimination of radioactive compound from muscle tissues

When the drug was withdrawn from the birds, the radioactivity in the plasma rapidly disappeared so that within 24 hours only a trace of radioactivity could be detected. The radioactivity in the red cells decreased from 2.5 to 1.7 p.p.m within the first 12 hours then appeared

Table I. Concentration of Radioactive Compounds in Various Blood Fractions Obtained from Chickens Fed Zoalene C^{14} for 8 Weeks[a]

Time Off Medicated Feed, Hours		Whole Blood		Plasma		Red Cells	
		Total	Bound	Total	Bound	Total	Bound
0		1.7	0.4	1.2	0.05	2.4	1.2
		1.7	0.4	1.2	0.05	2.4	1.2
		1.8	0.5	0.9	0.05	2.6	1.3
		1.8	0.4	1.0	...	2.6	1.3
	Av.	1.8	0.4	1.1	0.05	2.5	1.2
12		0.7	0.5	0.1	0.05	1.9	1.2
		0.8	0.6	0.1	0.04	1.6	1.3
		0.8	0.5	0.1	0.05	1.6	1.2
		0.7	0.6	...	...	..	..
	Av.	0.8	0.6	0.1	0.05	1.7	1.2
24		0.7	0.7	0.07	0.05	1.6	1.7
		0.5	0.4	0.05	0.06	1.9	1.6
		1.1	0.4	0.06	0.03	1.4	1.6
		0.8	0.7	...	0.03	..	1.1
		0.7	0.7	...	0.06	..	1.6
		1.1	0.7	...	0.06	..	1.0
	Av.	0.8	0.6	0.06	0.05	1.6	1.4
96		0.6	0.7	0.06	0.04	1.1	1.0
		0.6	0.6	0.08	0.04	1.4	1.1
		0.5	0.5	0.07	0.05	0.9	1.3
		0.5	0.5	0.08	0.05	1.0	1.3
	Av.	0.6	0.6	0.07	0.04	1.1	1.2

[a] Results are expressed in terms of p.p.m. of the radioactive Zoalene.

Table II. Concentration of Radioactivity in P.P.M. in Tissue of Chickens Fed Radioactive Zoalene-C^{14}

	Days on Medicated Feed						
	3				7		
	Hours Off Medicated Feed				Hours Off Medicated Feed		
Tissue	0	12	24	48	0	72	96
Liver	5.3	1.8	1.0	0.7	6.3	0.6	0.4
Kidney	4.9	1.3	0.7	0.7	6.5	0.5	0.3
Heart	1.7	0.4	0.1	0.1	2.5	0.1	<0.1
Gizzard	1.8	0.2	0.1	0.1	2.3	0.1	0.1
Thigh muscle	1.8	0.2	0.1	<0.1	2.3	0.1	<0.1
Breast muscle	1.8	0.2	0.1	<0.1	2.1	0.1	0.1
Fat	0.9	0.1	<0.1	<0.1	1.2	0.1	<0.1
Blood	1.9	0.4	0.2	0.2	2.5	0.2	0.2
Skin	1.6	0.3	0.1	0.1	1.5	0.1	0.1
Lung	2.1	0.6	0.1	0.1	3.9	0.2	0.1
Spleen	1.8	0.3	0.1	0.1	2.8	0.1	0.1
Testes	2.3	0.3	0.1	0.1	2.5	0.1	<0.1
Feathers	..	..	..	..	4.9	2.6	3.2
Bones	..	..	..	..	0.7	<0.1	<0.1

to level off so that the decrease in the next 84 hours was from 1.7 to 1.1 p.p.m.

Acetone extraction was used to determine the amount of bound radioactivity in the whole blood, plasma, and red cells. This was accomplished by pouring the fresh blood fraction into 50 times its volume of boiling acetone. The solution was allowed to cool and the precipitated protein, etc., removed by filtration through a Soxhlet thimble. The residue was then extracted for 4 hours. The residue and the extract were both analyzed for radioactivity. The material remaining with the residue was designated as bound material and that extracted as free material. The use of these designations does not imply that all the radioactivity remaining in the tissues was present as a single radioactive compound. There would be a distribution of all the radioactive compounds between the extract and the residue. Those which are chemically bound to the tissue will remain primarily with the tissue residue.

The average weights of the dried acetonized residue obtained from the three blood fractions were 0.150 gram per ml. of fresh blood, 0.050 gram per ml. of plasma, and 0.308 gram per ml. of red cells. On a dry-weight basis, the whole blood contained approximately 2.6 p.p.m. bound radioactivity compared to 1.0 p.p.m. for plasma and 3.9 p.p.m. for red cells at zero time withdrawal.

The data presented in Table I indicate that the rapid loss of radioactivity from the blood is primarily associated with the loss of the free radioactive compounds. The bound radioactivity appeared to remain more or less constant during this period and is associated primarily with the red cell fraction. This is probably due to the fact that the bound compounds are associated with the protein fraction of the blood which is largely confined to the red cells.

Table III. Concentration of Radioactivity in Tissues of Chickens Fed Radioactive Zoalene-C^{14} for 8 Weeks

(Radioactivity calculated as p.p.m. of Zoalene-C^{14})

	Hours Off Medicated Feed			
Tissues	0	12	24	96
Liver	5.1	3.6	3.0	1.4
Kidney	5.0	1.3	1.2	1.2
Heart	1.5	0.2	0.2	0.1
Gizzard	1.4	0.2	0.2	0.2
Breast muscle	1.6	0.2	0.1	0.1
Thigh muscle	1.5	0.2	0.1	0.1
Fat	0.9	0.1	0.1	0.1
Blood	1.8	0.8	0.8	0.6
Skin	1.4	0.4	0.3	0.2
Spleen	1.5	0.2	0.2	0.2

When the birds were sacrificed, samples of each tissue were collected for analysis. The samples were immediately prepared for combustion and aliquots weighed out in glass dishes. The aliquots were then stored in a frozen condition until combusted. Duplicate combustions were made of each tissue. If the results checked, no further analyses were made. The results obtained in the short-term feeding experiments are summarized in Table II and the long-term feeding experiments in Table III. The results reported are the averages obtained from the analyses of tissues from the various birds sacrificed at the same time. The methods used to measure the radioactivity were sensitive enough to detect 0.1 ± 0.05 p.p.m. of radioactivity expressed as zoalene-C^{14}. The data presented in Tables II and III indicate that the radioactivity appears to be accumulating preferentially in the liver and kidneys compared with the other tissues. Furthermore, there is a large percentage of bound radioactivity in these tissues as evidenced by the slow decrease in radioactivity when the birds were placed on nonmedicated feed. In most tissues, there was a decrease of approximately 80 to 90% in the level of radioactivity within the first 12 hours the birds were on nonmedicated feed.

In the short-term feeding experiments, there was a decrease of approximately 65% in the liver and approximately 70% in the kidney in the first 12 hours after withdrawal. This was even more apparent in the data from the long-term feeding studies shown in Table III. Extraction of the liver tissue with acetone also confirmed these observations since at zero time withdrawal, the liver contained a total of 5.1 p.p.m. of radioactivity of which about 3.4 p.p.m. was nonextractable (bound). At 12 hours, there was a total of 3.6 p.p.m. of which

approximately 3.3 p.p.m. was bound. After a 24-hour withdrawal period, the radioactivity remaining in the liver tissue was apparently all bound compound.

Similar results were obtained with muscle tissue using a scintillation counting technique which permitted detection of lower concentrations of radioactivity (5). The majority of the extractable material appeared to disappear very rapidly so that within 24 hours the majority of it had disappeared from the tissue (1.4 to 0.2 p.p.m.). The nonextractable activity decreased slightly (0.3 to 0.1 p.p.m.) during the first 24 hours and then remained more or less constant during the remaining portion of the observation period.

Although the acetone extraction does not clearly distinguish between free and bound radioactive material, it is apparent from the shape of the curves presented in Figure 5 that at least two major components are present in the tissues.

The change in the slope of the acetone extractable curve also suggests a two-component system. The first component (1.4 to 0.02 p.p.m.) represents approximately 98% of the extractable activity while the second component (0.02 to 0.008 p.p.m.) represents about 2% of the extractable activity. The change in slope of the nonextractable curve may be explained partly by the lack of complete extraction of the free radioactivity by the acetone extraction. This change in slope could be explained if about 15% of the free radioactivity was not removed by the simple acetone extraction.

From the results obtained, it appears that the majority of the radioactivity found in tissues, other than liver and kidney, of chickens being fed C^{14}-labeled 3,5-dinitro-*o*-toluamide exists in a free form which can be easily removed by a simple acetone extraction. When the birds are taken off medicated feed, the material is rapidly dissipated from the tissues. A very small portion of the radioactivity appears to be chemically bound to the tissues and is slowly eliminated once the drug is withdrawn.

From the results obtained in these feeding studies, it is apparent that the level of bound radioactivity has not changed significantly when the feeding period was extended from 3 or 7 days to 8 weeks; therefore, the length of the feeding period apparently doesn't affect the magnitude of the bound compound.

The identification of these two components in the tissues will be the subject of a later paper in this series.

Acknowledgment

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FEED ADDITIVES DETECTION

The Identification of 3,5-Dinitro-*o*-toluamide (Zoalene) and Possible Metabolites by Paper Chromatography

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Paper chromatographic techniques have been adopted for the separation and identification of 3,5-dinitro-o-toluamide and possible metabolic products. By employing various solvent systems in combination with a series of color tests, it has been possible to distinguish the various compounds that may be formed by the enzymatic reduction, hydrolysis, and oxidation of 3,5-dinitro-o-toluamide.

To INVESTIGATE the metabolism of 3,5-dinitro-*o*-toluamide (Zoalene, trademark of The Dow Chemical Co. abroad) in chickens, it was necessary to develop methods for the separation and identification of the parent compound and metabolites which were formed. A review of the literature suggested that zoalene might be degraded by various pathways. Bray *et al.* (2) have demonstrated that *o*-toluamide can undergo hydrolysis of the amide, oxidation of the methyl group, and ring closure with the formation of a phthalide derivative. In addition, it has been demonstrated that chicken liver tissue can readily reduce dinitro compounds (9). These observations suggest that 3,5-dinitro-*o*-toluamide may be metabolized with the formation of a wide variety of products (7).

A possible approach to the separation and isolation of these compounds would be the use of paper chromatographic techniques. It was realized at the outset that the possibility of finding a single solvent system or a detection method which would permit separation and identification of all possible compounds was very unlikely. However, by using several solvent systems and detection methods for specific functional groups, it might be possible to identify each compound. This technique has proven successful with other nitro compounds (8).

Methods

Chromatograms of the reference standards and unknown solutions were run by the descending method (3) using Whatman No. 1 filter paper strips 2.5 × 45 cm. Approximately 10 μl. of the solution containing from 10 to 25 μg. of the compound was applied 10 cm. from one end of the filter paper in such a manner that the area covered by the solvent did not exceed 0.5 cm. in diameter. The reference standards were generally applied as acetone solutions while the unknown samples were generally dissolved in 50% acetone.

After the strips were spotted, they were air dried to remove the solvent and then developed at 25° C. in a chamber equilibrated with the vapors of the solvent system. After the solvent had run the desired distance (30 cm.), the strips were removed, and the solvent front was marked. The strips were air