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portion of the radioactivity appears to be chemically bound to the tissues and is slowly eliminated once the drug is withdrawn.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.

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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

Table I. Color Tests to Detect 3,5-Dinitro-o-toluamide and Possible Related Metabolites on Paper Strips

	Detection Method[a]								
								8	
Compound	1	2	3	4	5	6	7	Long 366 mμ	Short 254 mμ
3,5-Dinitro-o-toluamide	..	S	..	Y	G	Pu	G	..	D
3,5-Dinitro-o-toluic acid	..	S	..	Y	G	..	G	..	D
3-Amino-5-nitro-o-toluamide	S	S	Y	Y	..	..	..	D	D
3-Amino-5-nitro-o-toluic acid	S	S	Y	Y	..	..	..	D	D
5-Amino-3-nitro-o-toluamide	Pi	Pi	Y	Y	..	..	..	G	D
5-Amino-3-nitro-o-toluic acid	Pi	Pi	Y	Y	..	..	..	D	D
3,5-Diamino-o-toluamide	Pu	Pu	Y	Y	..	..	..	F	F
3,5-Diamino-o-toluic acid	Pu	Pu	Y	Y	..	..	..	F	F
3-Acetyl-amino-5-nitro-o-toluamide	..	S	..	Y	..	..	..	..	D
4,6-Dinitrophthalide	..	S	..	Y	R	R	R	..	D
3,5-Dinitrophthalic acid	..	S	..	Y	Pu	Pu	Y	..	D
N-(3,5-Dinitro-o-toluyl)-glycine	..	S	..	Y	G	Pu	G	G	D

[a] D, Dark colored spot; F, fluorescent spot; G, green spot; Pi, pink spot; Pu, purple spot; R, red spot; S, salmon colored spot; Y, yellow spot.

Table II. R_f Values of 3,5-Dinitro-o-toluamide and Possible Related Metabolites

	Solvent System				
Compound	A	B	C	D	E
3,5-Dinitro-o-toluamide	0.80	0.88	0.81	0.86	0.82
3,5-Dinitro-o-toluic acid	0.48	0.10	0.73	0.61	0.20
3-Amino-5-nitro-o-toluamide	0.68	0.40	0.69	0.78	0.52
3-Amino-5-nitro-o-toluic acid	0.23	0.00	0.60	0.45	0.02
5-Amino-3-nitro-o-toluamide	0.64	0.33	0.73	0.82	0.56
5-Amino-3-nitro-o-toluic acid	0.31	0.00	0.57	0.48	0.03
3,5-Diamino-o-toluamide	0.22	0.02	0.46	0.38	0.03
3,5-Diamino-o-toluic acid	0.05	0.00	0.36	0.14	0.02
3-Acetyl-amino-5-nitro-o-toluamide	0.74	0.34	0.80	0.78	0.66
4,6-Dinitrophthalide	0.04	0.00	0.62	0.60	0.00
3,5-Dinitrophthalic acid	0.70	0.00	0.28	0.03	0.00
N-(3,5-Dinitro-o-toluyl) glycine	0.30	0.04	0.56	0.40	0.05

dried at room temperature, and the position of the individual compounds was determined by the methods described below.

Reference standards were run with the unknown compounds in each of the solvent systems, and their location was compared with the location of the unknown compounds. In this way, it would be possible to identify the unknown compounds. A positive identification was assumed if the results obtained with two or more solvent systems were in agreement.

The following methods were used to detect the compounds on the strips:

Method 1. The chromatograms were sprayed with a 0.2% sodium nitrite solution in 0.1*N* hydrochloric acid (*4*). After 1 minute, the strips were sprayed while still moist with a solution of 0.1 gram of *N*-(1-naphthyl) ethylenediamine dihydrochloride in 95 ml. of *n*-butyl-alcohol and 5 ml. of 2*N* hydrochloric acid (*1*, *5*). Amino compounds appeared as intense pink, purple, or salmon colored spots.

Method 2. The chromatograms were sprayed with a solution of 10 grams of stannous chloride in 25 ml. of concentrated hydrochloric acid and 100 ml. of water (*8*). After the strips stood for 5 minutes, they were exposed to bromine vapors for 10 seconds (*5*). The strips were aerated until the bromine color had disappeared and then were sprayed with the solutions described in detection method 1. Nitro and amino compounds gave intense pink, purple, or salmon colored spots with this method.

Method 3. The chromatograms were sprayed with a solution of 1 gram of *p*-dimethylaminobenzaldehyde in 30 ml. of ethyl alcohol, 30 ml. concentrated hydrochloric acid, and 180 ml. of *n*-butyl alcohol (*1*). Amino compounds gave intense yellow colored spots.

Method 4. The chromatograms were sprayed with a solution of 10 grams of stannous chloride in 25 ml. of concentrated hydrochloric acid and 100 ml. of water. After air-drying, the strips were sprayed with detection method 3. Nitro and amino compounds gave intense yellow colored spots.

Method 5. The chromatograms were sprayed with a solution of 10 ml. of 2*N* sodium methylate in methyl alcohol and 90 ml. of dimethylformamide. The solution must be made fresh each time it is used. Certain of the nitro compounds give specific color reactions with this method (Table I.).

Method 6. The chromatograms were sprayed with a 50% solution of 1,3-diaminopropane in dimethylformamide. The solution must be made fresh each time it is used (*6*). Certain of the nitro compounds give specific color reactions with this test (Table I).

Method 7. The chromatograms were sprayed with a solution of 10 ml. of tetramethylammonium hydroxide (10% in water) and 90 ml. of dimethylformamide. The solution must be made fresh each time it is used. Certain of the nitro compounds give specific color reactions with this test (Table I).

Method 8. The chromatograms were viewed under ultraviolet light at 366 and 254 mμ wave lengths. Diamino-o-toluamide and diamino-o toluic acid gave fluorescent spots. Other compounds appear as dark purple spots.

The solvent systems employed were as follows:

System A. *n*-Butyl alcohol–concentrated ammonium hydroxide = 100:25 (v./v.).

System B. Chloroform–40% aqueous solution of methylamine = 230:30 (v./v.).

System C. Ethyl alcohol–water–concentrated ammonium hydroxide = 80:16:4 (v./v.).

System D. *n*-Butyl alcohol–ethyl alcohol–concentrated ammonium hydroxide–water = 40:10:15:45 (v./v.).

System E. Isoamyl alcohol–concentrated ammonium hydroxide = 90:10 (v./v.).

Results and Discussion

Table I lists some of the various degradation products of 3,5-dinitro-o-toluamide which have been studied by the methods described.

The color tests which have been used can be grouped into three general classes. These are tests which detect aryl amino compounds (methods 1 and 3); tests in which nitro compounds are first converted to aryl amino compounds and then detected by using the procedures for aryl amino compounds (methods 2 and 4); and in certain cases there are specific tests for individual compounds (methods 5, 6, 7, and 8).

Detection method 1 was found to give salmon colored spots with 3-amino-compounds, while the 5-amino-compounds gave pink spots, and the 3,5-diamino compounds gave purple spots. These various amino compounds could therefore be distinguished on the basis of the color obtained.

Detection method 3 also is used to detect aryl amino compounds. Some differences in the rate of color formation were noticed with the various amino compounds listed in Table I. These differences were, however, not distinct enough to be used as a means of distinguishing the compounds.

Detection methods 2 and 4 are used to detect nitro or amino compounds. The nitro compounds are reduced with stannous chloride and the resulting amino compound is detected by methods 1 and 3. If method 1 is to be used, the stannous chloride must be destroyed

before diazotizing and coupling. With method 3, this is not necessary.

Detection methods 5, 6, 7, and 8 are used to locate specific compounds on the basis of the color produced (Table I).

Of approximately 100 solvent systems tested, only those listed in Table II appeared to give satisfactory separation of the various compounds studied.

Solvent systems A, C, and D give satisfactory separation of the dinitro compounds by using color tests 5, 6, or 7. Of these three systems, system A appears to be the most satisfactory.

Solvent system B is used specifically for the identification of traces of 3,5-dinitro-*o*-toluamide. To separate and identify the amino-nitro-*o*-toluamides from the amino-nitro-*o*-toluic acids, systems B and E are employed.

The R_f values listed in Table II are the average of six or more determinations using authentic samples. The variations in the R_f values obtained were less than $\pm 5\%$. The factors responsible for these variations have been described in detail by Consden (*3*). Because of the many factors influencing the R_f value, great significance should not be placed upon its absolute magnitude. The more important consideration lies in the relative movement of the various compounds and their characteristic reaction with the detection reagents. When chromatographed side by side in equal quantities, this relative movement is constant. In practice, the unknown compound is run individually with the reference standards and cochromatographed with each reference standard.

In the various metabolism studies, satisfactory identification of a compound was considered to be established when it was possible to identify the compound using three solvent systems and employing the various detection methods.

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FEED ADDITIVE RESIDUES

Identification of the Metabolites of 3,5-Dinitro-*o*-toluamide-C^{14} (Zoalene) in Chicken Tissues

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When 3,5-dinitro-o-toluamide-C^{14} (zoalene) was fed to chickens, the tissues were found to contain a free radioactive compound which could be extracted with acetone and a bound compound which could not be removed by any standard extraction procedure. The free compound was identified as 3,5-dinitro-o-toluamide. The bound compound was liberated by pepsin digestion of the tissue and identified as amino-nitro-o-toluamide.

IN A PREVIOUS PAPER of this series (*9*), it was shown that when Leghorn chickens were continuously fed 3,5-dinitro-*o*-toluamide-C^{14}, (DNOT) (Zoalene, trademark of The Dow Chemical Co., abroad), the tissues contained a significant amount of radioactive compounds in the liver. A portion of this radioactivity could easily be removed from the liver tissue by a simple acetone extraction, while the majority of the radioactivity appeared to be chemically bound to the tissue.

It was necessary to establish the structure of the radioactive compounds in the tissue in order to ascertain if the 3,5-dinitro-*o*-toluamide or any of its metabolites were accumulating in the tissues and if the amount of each compound present would be a problem as far as a health hazard was concerned if the tissues were eaten.

The present investigations were therefore undertaken in the hopes of identifying all the radioactive compounds in the tissues which had arisen from the metabolism of 3,5-dinitro-*o*-toluamide-C^{14}.

Previous studies had demonstrated that radioactivity in the tissues could easily be divided into bound and free fractions based on ease of extraction with acetone. This appeared to be a good approach to the problem so the present investigations were divided into three phases: the first phase consisted of identifying the easily extractable (free) radioactive material; the second phase involved an extensive study of methods for liberating the bound radioactive material from the tissues; and the third phase involved the identification of the bound radioactive material.

Methods

White Leghorn chickens were continuously fed 3,5-dinitro-*o*-toluamide-C^{14} (carboxyl labeled) at the recommended rate of 0.0125% in the feed. The 3,5-dinitro-*o*-toluamide had a specific activity of 3.15 mc. per mmole. At the end of 8 weeks, the birds were sacrificed and the livers removed and pooled. The livers were then homogenized in a Waring Blendor using an equal weight of water. The homogenate was then mixed with 10 times its weight of boiling acetone. After the protein had precipitated, the suspension was filtered through a 43 × 123 mm. Soxhlet thimble. The residue in the thimble was then extracted for 4 hours with acetone. The acetone extract was combined with the original filtrate and the sample evaporated under an infrared lamp until most of the acetone had been removed. Air jets were employed to facilitate the evaporation of the acetone. The sample was transferred to a round-bottomed flask and further concentrated by lyophilization. The resulting sample