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

Table I. Identification of Free and Bound Radioactive Material Isolated from Liver and Reference Compounds by Chemical Test

Compound	Test[a] Bratton-Marshall Test	Diamino-propane Test	NaMe Test	TMAOH Test	DMAB Test	KCN Test	KCN-DMF Test
3,5-Dinitro-*o*-toluamide	−	P	G	G	−	+	B
3,5-Dinitro-*o*-toluic acid	−	−	G	G	−	+	C
3-Amino-5-nitro-*o*-toluamide	+	−	−	−	+	−	C
3-Amino-5-nitro-*o*-toluic acid	+	−	−	−	+	−	C
5-Amino-3-nitro-*o*-toluamide	+	−	−	−	+	−	C
5-Amino-3-nitro-*o*-toluic acid	+	−	−	−	+	−	C
3,5-Diamino-*o*-toluamide	+	−	−	−	+	−	C
3,5-Diamino-*o*-toluic acid	+	−	−	−	+	−	C
3-Acetamido-5-nitro-*o*-toluamide	−	−	−	−	−	−	C
4,6-Dinitrophthalide	−	−	R	R	−	+	R
3,5-Dinitrophthalic acid	−	−	P	B	−	+	C
N-(3,5-Dinitro-*o*-toluyl) glycine	−	P	G	G	−	+	C
Free radioactivity from liver	?	P	G	G	−	+	B
Bound radioactivity from liver	+	−	−	−	+	−	C

[a] B, Blue; C, colorless; G, green; P, purple; R, red.

was an oily solution. This was mixed with 100 ml. of chloroform, and 75 ml. of the chloroform solution was distilled off to remove any traces of water which might still be present.

The chloroform solution was chromatographed on an alumina column using the procedure previously described (*10*). The effluent from the column was collected in 10-ml. fractions, and each fraction was analyzed for radioactivity. The fractions containing activity were combined and evaporated to about 5 ml. Aliquots of this solution were spotted on Whatman No. 1 filter paper strips and developed with the five solvent systems previously described (*9*). The unknowns were run with reference standards of the possible metabolites of zoalene.

The radioactive compounds were located on the strips by scanning each strip in a Nuclear-Chicago Actograph II using a D-47 flow counter with a Micro-mil window. The reference compounds were located by the colorimetric procedures previously described (*9*). A quantitative estimation of each radioactive spot was obtained by sectioning the paper and counting each section in a Tri-Carb scintillation counter.

The unused portion of the original extract was subjected to a series of spot tests described below to further characterize the radioactive compound.

The Bratton-Marshall test for aryl amines (*1*, *12*) was conducted by mixing 0.1 ml. of the extract with 10 ml. of 0.25*N* HCl. To this was added 0.5 ml. of 0.1% solution of sodium nitrite. After 5 minutes, 0.5 ml. of 0.5% solution of ammonium sulfamate was added and the solution allowed to stand 3 minutes. Finally 0.5 ml. of a 0.1% solution of *N*-(1-naphthyl)-ethylenediamine dihydrochloride was added. A pink color indicates aryl amines (Table I).

In the diaminopropane test (*5*, *7*), 0.1 ml. of the extract was mixed with 5 ml. of dimethylformamide and 5 ml. of 1,3-diaminopropane. 3,5-Dinitro-*o*-toluamide and *N*-(3,5-dinitro-*o*-toluyl)glycine give distinct purple solutions while 4,6-dinitrophthalide gives an orange-red colored solution (Table I). In the sodium methylate test (NaMe Test), the 0.1 ml. of the extract was mixed with 10 ml. of dimethylformamide and 5 drops of 5*N* sodium methylate solution added. Certain of the dinitro-*o*-toluamide derivatives give distinct colors (Table I).

In the tetramethylammonium hydroxide test (TMAOH Test), 10 ml. of the dimethylformamide solution containing 0.1 ml. of the extract was treated with five drops of a 20% aqueous solution of TMAOH. Certain of the toluamide derivatives give specific tests (Table I).

The *p*-dimethylaminobenzaldehyde test (DMAB Test) (*11*) was used to detect aryl amines (Table I).

The potassium cyanide test (KCN Test) was used to indicate the presence of a *meta*-dinitro compound in which there is no substitution between the nitro groups. The test is conducted by mixing 0.1 ml. of the tissue extract with 5 ml. of water, 10 drops of a 10% solution of potassium cyanide, and 2.5 ml. of *N*-propyl alcohol. The sample was heated in boiling water for 10 minutes. A yellow color indicates a positive test.

In the potassium cyanide–dimethylformamide test (KCN-DMF Test), 0.1 ml. of the extract was mixed with 10 ml. of dimethylformamide, and five drops of 10% potassium cyanide solution was added. Certain of the toluamide derivatives give distinct color reactions (Table I).

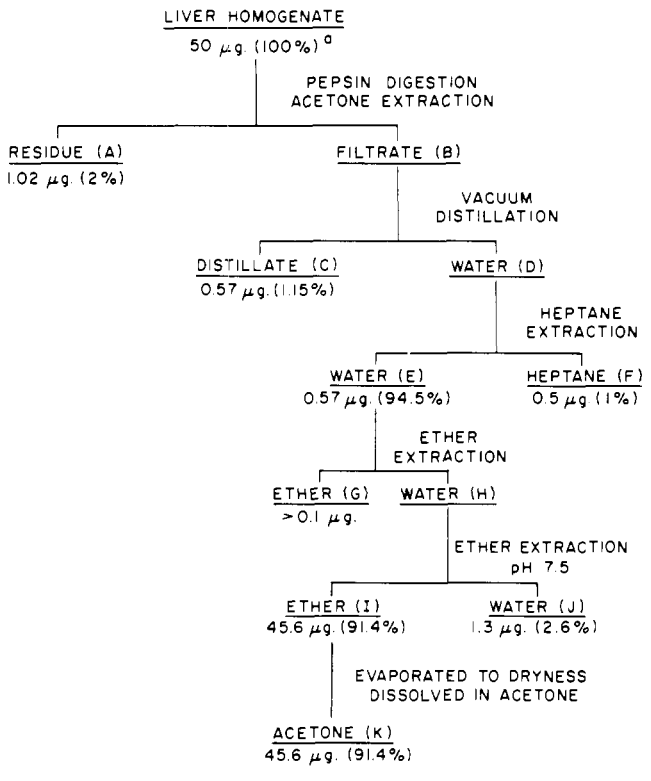
The procedures described above were satisfactory to establish the chemical structure of the free or easily extractable radioactivity in the liver tissue.

To characterize the bound radioactivity in the liver, it was first necessary to develop a method to liberate and extract it. The procedure which was developed is outlined in Figure 1. In this procedure, the fresh frozen livers were homogenized with an equal volume of ice water in a Waring Blendor. As soon as possible, 100 ml. of cold 0.6% hydrochloric acid was added for each 20 ml. of homogenate. To each 100 ml. of the hydrochloric acid suspension was added 25 mg. of 1 to 10,000 pepsin. The sample was then incubated at 40° C. for 48 hours. To the digest was then added eight times its volume of boiling acetone. After allowing the acetone solution to stand 4 hours, it was filtered through a 43 × 123 mm. Soxhlet extraction thimble. The residue was then extracted 4 hours with acetone. The acetone extract was then added to the original filtrate. The acetone-water phase (B, Figure 1) was reduced in volume by flash evaporation. A small quantity of concentrated hydrochloric acid was added to prevent any loss of amino compounds. The volume of the acetone-water phase was reduced to about 25 ml. At this point, the solution was essentially a water phase. The residue (A) and the distillate (C) were saved and analyzed for radioactivity.

The water solution (D) was transferred to a continuous extractor (*6*) and extracted for 8 hours with heptane. The heptane (F) was then removed and saved for analysis. The water layer (E) was extracted for 8 hours with ether. The ether solution (G) was removed and analyzed for radioactivity. The water solution was adjusted to pH 7.5 by the addition of solid sodium bicarbonate. The basic solution (H) was then extracted for 8 hours with ether. The water phase (J) was analyzed for radioactivity. The final ether solution (I) was found to contain the majority of the radioactivity. This solution was taken to dryness and the residue taken up in acetone (K). This solution was analyzed by the spot tests described above and chromatographed (*9*).

Results and Discussion

To identify unknown compounds extracted from tissues by paper chromatographic techniques and spot tests, it is necessary to have some idea of the nature of the compounds which might be en-



[a] Radioactivity reported in μg. of 3,5-dinitro-o-toluamide-C^{14} and per cent of radio radioactivity in original liver homogenate.

Figure 1. Extraction and isolation of radioactivity found in chicken liver tissues

countered. From a review of the structure of 3,5-dinitro-*o*-toluamide, it appeared likely that the compound could undergo several enzymatic reactions with the formation of a wide variety of compounds. It is known that tissue enzymes can reduce nitro groups (*13*), hydrolyze amide groups (*2*), and possibly oxidize the methyl group (*2, 3*). In addition, it has been established that the *o*-toluamide can be converted to a phthalide by enzymatic reactions (*2*). Many of these compounds have characteristic reactions which can be used to distinguish them.

In the present investigations, the tissue extract containing the free or easily extractable radioactivity was first subjected to a series of color tests shown in Table I. The Bratton-Marshall test and the DMAB test were both negative, indicating that the compound was not an aryl amine. The diaminopropane, NaMe, and TMAOH tests were positive. These results suggested that the compound was either 3,5-dinitro-*o*-toluamide or *N*-(3,5-dinitro-*o*-toluyl) glycine. The KCN test supported this assumption, and the KCN-DMF test suggested the compound was probably the 3,5-dinitro-*o*-toluamide.

The chromatographic scans of the free extract were compared with reference strips. Typical scan obtained is shown in Figure 2. Of the various systems employed, it appeared that solvent system B (chloroform saturated with aqueous methylamine) (*9*) gave the best separation of 3,5-dinitro-*o*-toluamide from the compounds. The R_f value for 3,5-dinitro-*o*-toluamide was approximately 0.88 while for *N*-(3,5-dinitro-*o*-toluyl)glycine it was 0.04. The R_f value for the free radioactive compound was 0.88. This substantiated the fact that the free radioactive compound isolated from the liver was 3,5-dinitro-*o*-toluamide.

Similar results were obtained with the free radioactive material isolated from muscle, kidney, heart, and other tissues.

The identification of the bound radioactivity from the tissues was complicated by the problem of liberating the material from the tissues. The liberation must be carried out under conditions which would not destroy the radioactive compounds present.

Preliminary studies indicated that the radioactivity was bound to the proteins and it would be necessary to hydrolyze them to liberate the compound (or com-

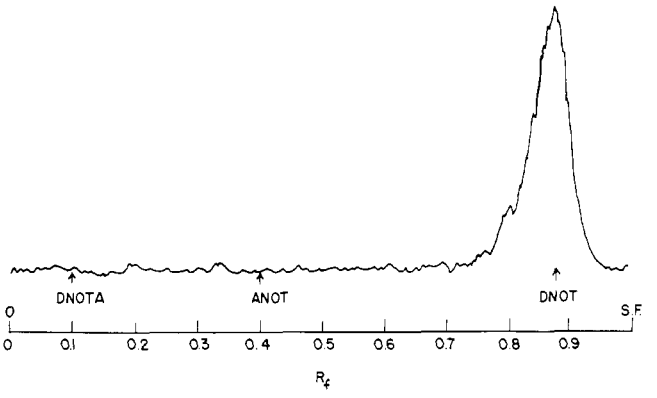


Figure 2. Chromatographic scan of free radioactive material isolated from liver

Abbreviations used in Figures 2 and 3: (ANOT) amino-nitro-o-toluamide; (ANOTA) amino-nitro-o-toluic acid; (DAOT) 3,5-diamino-o-toluamide; (DAOTA) 3,5-diamino-o-toluic acid; (DNOT) 3,5-dinitro-o-toluamide; (DNOTA) 3,5-dinitro-o-toluic acid; (DNP) 4,6-dinitrophthalide; (S.F.) solvent front; (S.S.A.) solvent system A; (S.S.B.) solvent system B; (S.S.C.) solvent system C; (S.S.D.) solvent system D

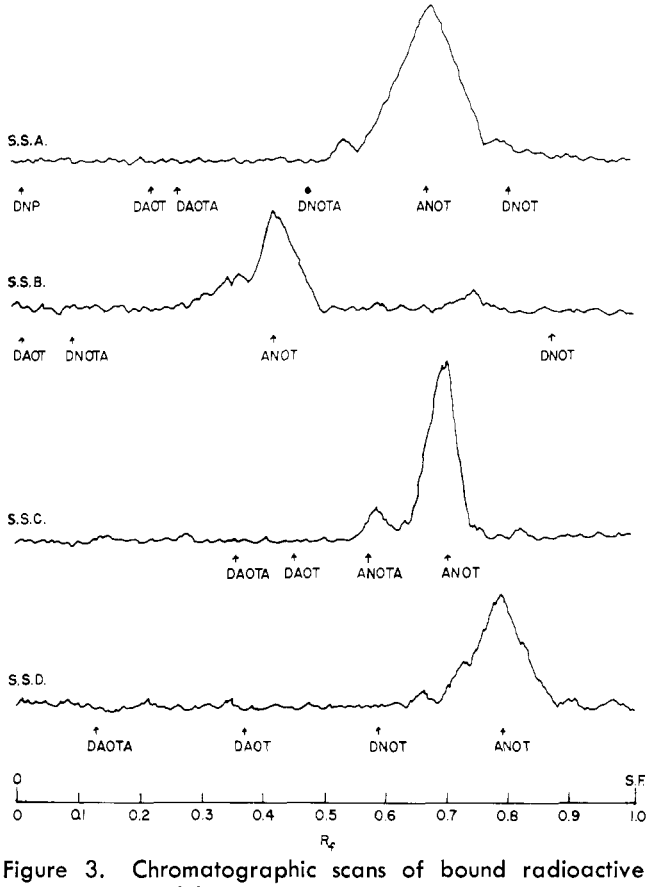


Figure 3. Chromatographic scans of bound radioactive material isolated from liver

pounds). Therefore, various standard methods for hydrolyzing proteins were investigated (*4*). To ascertain if a procedure was satisfactory, reference compounds were added to liver homogenates, and the homogenates were hydrolyzed. Attempts were then made to isolate and identify the reference compounds in the hydrolyzates. It was observed that acid or base hydrolysis gave poor results due to the action of the acids or bases on the toluamide compounds. Increasing the temperature during the hydrolysis also gave low recovery of the reference compounds.

During these investigations it was observed that when the tissues were allowed to stand at room temperature for a period of time self-autolysis occurred which resulted in the liberation of the radioactive compound.

This suggested the possibility of using an enzymatic hyrolysis of the tissues. It was decided that the hydrolysis of the tissue should be carried out under conditions which would minimize enzymatic destruction of the radioactive compounds. It is known that liver tissue can rapidly reduce nitro groups (*13*) and hydrolyze amide groups (*2*). Both of these reactions can be minimized by carrying out the hydrolysis reaction in a strongly acid medium. By using a pepsin digestion at pH 1.5, satisfactory results were obtained and most of the radioactivity could be isolated (Figure 1).

The extract containing the bound radioactivity was analyzed by the various color reactions described above. Positive Bratton-Marshall and DMAB tests were obtained while negative NaMe, TMAOH, KCN, and KCN-DMF tests were obtained. These results indicated that the compound was an aryl amine.

To be sure that the radioactivity was associated with the colored complex obtained in the Bratton-Marshall test, the complex was isolated from the reaction medium by adjusting the solution to pH 6.5 and extracting with butanol. The majority of the colored complex and the radioactivity was in the butanol layer. The butanol layer could be washed with 0.1*N* sodium hydroxide solution without losing any significant quantities of the radioactivity or color.

Under the conditions of this test, the amino acids from the proteins would be converted to keto acids, which would remain in the water layer. Since the water layer was essentially free from radioactivity after the butanol extraction, it is unlikely that any significant quantity of the 3,5-dinitro-*o*-toluamide-C^{14} originally fed the birds was metabolized to carbon dioxide and water and the carbon dioxide assimilated during the biosynthesis of the proteins.

The extraction procedure used in isolating the bound compound also indicates that the compound possesses an amino group. Amino compounds form hydrochloride salts which would remain in the water solution when they were extracted with an organic solvent. It is difficult to ascertain if the compound contained a free carboxyl group from the extraction procedure. Most carboxyl compounds would form salts in the alkaline solution and would not be extracted with ether. A study of the amino derivatives of *o*-toluic acid revealed that some of these compounds form internal salts so that the amino or carboxyl groups do not act as would be predicted. For example, the amino-nitro-*o*-toluic acid can be extracted from a weak alkaline solution (pH 7.5) by continuous extraction with ether.

The possibility that trace quantities of 3,5-dinitro-*o*-toluamide and/or 3,5-dinitro-*o*-toluic acid might be present in the bound fraction was indicated by the very weak reactions obtained with the diaminopropane, NaMe, and KCN tests. These tests were so weak that it was impossible to arrive at any conclusions as to the amount of these compounds which might be present.

From the results obtained in the chemical tests, it appeared that the most likely metabolite in the tissues would be an amino-nitro-*o*-toluamide.

The extract was then chromatographed to determine if the compound was amino-nitro-*o*-toluamide (ANOT). The solvent systems employed (*9*) had previously been shown to separate many of the metabolites, but none of them are by themselves sufficient to give complete separation and identification of the various components. By combining solvent systems with detection methods, it was possible to limit or separate all the various components for which reference standards were available for comparison.

The results of the chromatographic test are shown in Figure 3. In this study, reference compounds were run with the unknown solutions and were detected by chemical means. The positions of the reference compounds are indicated with each scan.

With solvent system A, the amino-nitro-*o*-toluamide could be separated from 4,6-dinitrophthalide; 3,5-diamino-*o*-toluamide; *N*-(3,5-dinitro-*o*-toluyl)glycine; 3,5-dinitro-*o*-toluic acid; and 3,5-dinitro-*o*-toluamide. The scan obtained with this solvent system (Figure 3) indicates that the majority of the radioactivity isolated from the liver is related to amino-nitro-*o*-toluamide.

With solvent system B, amino-nitro-*o*-toluamide can easily be separated from the 3,5-dinitro-*o*-toluamide; 3,5-dinitro-*o*-toluic acid; and 3,5-diamino-*o*-toluamide. In this system, 3,5-dinitro-*o*-toluamide and *N*-acetyl-3,5-dinitro-*o*-toluamide migrate at about the same rate. Similarly, the amino-nitro-*o*-toluic acids; 3,5-dinitrophthalic acid; 4,6-dinitrophthalide; and *N*-(3,5-dinitro-*o*-toluyl)glycine all remain at the origin. The scan obtained with solvent system B shows that the radioactive compound has migrated part way down the paper, and its position corresponds to that of the amino-nitro-*o*-toluamide.

The extract was also chromatographed with solvent system C. In this system, amino-nitro-*o*-toluamide can be separated from amino-nitro-*o*-toluic acid, diamino-*o*-toluamide, and diamino-*o*-toluic acid. Here again the position of the radioactive compound corresponds to the amino-nitro-*o*-toluamide with possible traces of amino-nitro-*o*-toluic acid.

Solvent system D was used to further identify the radioactive compound. In this scan (Figure 3), the peak of radioactivity again corresponds to amino-nitro-*o*-toluamide and thus distinguishes it from the other amino compounds which might be present.

The results obtained in the chromatographic studies agree with those obtained in the chemical studies and identify the bound compound as amino-nitro-*o*-toluamide.

There are two possible isomers of amino-nitro-*o*-toluamide. They are 3-amino-5-nitro-*o*-toluamide and 5-amino-3-nitro-*o*-toluamide. Both compounds will give similar reactions in all the tests employed. Positive identification of which isomer of amino-nitro-*o*-toluamide is present in the tissues can be obtained only by isolating sufficient quantities of the material to permit infrared analysis. This will be the subject of a later paper in this series.

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