The Isolation and Identification of the Amino-nitro-*o*-toluamide Formed by the Biological Reduction of 3,5-Dinitro-*o*-toluamide

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Biological reduction of 3,5-dinitro-o-toluamide results in the formation of amino-nitro-o-toluamide. The isomer isolated from tissue homogenates was identified by chemical and physical tests as the 3-amino-5-nitro-o-toluamide. The isomer which is generally obtained by the chemical reduction of 3,5-dinitro-o-toluamide is the 5-amino-3-nitro-o-toluamide.

In the previous papers (*7*, *9*, *11*), it has been shown that 3,5-dinitro-*o*-toluamide is metabolized in the tissues of a chicken with the formation of an amino-nitro-*o*-toluamide. This compound is bound to the tissue components and can be liberated only by enzymatic digestion. The identity of this compound was tentatively established as amino-nitro-*o*-toluamide (ANOT) by use of paper chromatographic procedures. The amino-nitro-*o*-toluamide can exist in two isomeric forms, and the paper chromatographic procedures could not adequately differentiate between the two forms. To determine the toxicity of the compound in chicken tissues, it was necessary to obtain definite proof of its structure. This meant that sufficient material must be isolated in a pure form to permit characterization by conventional physical and chemical methods.

It was realized, at the outset, that it would be necessary to isolate at least 5 mg. of the compound to permit satisfactory identification. Since the compound was present in the liver tissue at a concentration of about 3 p.p.m., it did not appear that satisfactory results could be obtained by trying to isolate the compound directly from the tissue. It had been observed that reduction of the 3,5-dinitro-*o*-toluamide could be brought about in vitro by liver homogenates. By using this observation, a biosynthesis approach was used to prepare sufficient quantities of the amino-nitro-*o*-toluamide to permit its identification.

Methods

The biosynthesis of amino-nitro-*o*-toluamide was accomplished by preparing a chicken liver homogenate by blending 100 grams of fresh liver tissue with 100 ml. of cold 0.2*M* phosphate buffer, pH 7.4, in a Waring Blendor. To this homogenate was added 30 mg. of 3,5-dinitro-*o*-toluamide and 0.57 mg. of 3,5-dinitro-*o*-toluamide-C^{14} having a specific activity of 9.46 mc. per mmole. Both samples of 3,5-dinitro-*o*-toluamide dissolved in 1 ml. of acetone were added to the homogenate. The homogenate was transferred to a constant temperature bath and incubated for 6 hours at 39° C. The temperature employed was approximately equal to the normal body temperature of a chicken. At the end of the incubation period, the homogenate was poured into 2 liters of boiling acetone (Figure 1). The sample was then placed on a steam bath and allowed to boil approximately 15 minutes. The hot acetone solution (a) was filtered through a 50-cm. Reeve-Angel No. 804 folded filter paper. The resulting residue of precipitated protein (b) was extracted with two 500-ml. portions of boiling acetone. The acetone extract and washings were combined (c) and concentrated in a flash evaporator. One milliliter of concentrated hydrochloric acid was added during this operation to prevent loss of any of the amino derivatives of 3,5-dinitro-*o*-toluamide. The extract was essentially an aqueous solution since the acetone had been removed by evaporation, and only the water from the tissue and buffer remained. The water solution was extracted by Soxhlet extraction for 8 hours with ethyl ether. This extraction removed all fatty material together with the unchanged 3,5-dinitro-*o*-toluamide. During the extraction, aliquots of the water solution were chromatographed at various time intervals using solvent system B (*8*) to establish when the extraction was complete. As soon as the 3,5-dinitro-*o*-toluamide could no longer be detected, it was assumed that the extraction was complete. The ether phase (f) was removed from the extractor, and the water phase (g) was adjusted to pH 7.5 by the addition of solid sodium bicarbonate. The water was again continuously extracted with ether for 8 hours. During this extraction, the amino - nitro - *o* - toluamide derivatives would pass into the ether layer. The water phase was checked from time to time until it was essentially free of radioactivity. After the extraction, the two phases were separated and the water phase (i) was discarded. The ether layer (h) was then evaporated to dryness and the residue taken up in 90% ethyl alcohol. A considerable amount of insoluble material remained. This was filtered off, checked for radioactivity, and discarded. The alcohol solution was evaporated to a few milliliters and made slightly alkaline by the addition of a few drops of 1*N* sodium hydroxide. The sample was transferred to a sublimator and the sample sublimed under a high vacuum. The sublimed sample was saved for analysis.

The ether phase (f) from the first extraction contained radioactive material which was presumed to be 3,5-dinitro-*o*-toluamide. In order to prove that the radioactive material was 3,5-dinitro-*o*-toluamide, it was necessary to separate the material from the liver components which were also present in the ether solution.

A procedure previously developed for the isolation of 3,5-dinitro-*o*-toluamide from feed was employed. The procedure is based on the absorption of the 3,5-dinitro-*o*-toluamide on to aluminum oxide from an acetone-heptane solution. The aluminum oxide is washed with heptane to remove interfering substances. The 3,5-dinitro-*o*-toluamide is then eluted with 80% ethyl alcohol.

The original ether solution (f) was evaporated to dryness and the residue taken up in boiling acetone. The acetone solution was cooled and diluted to 50 ml. To this was added 80 ml. of heptane. This solution was then introduced into an 18 × 60 cm. column containing 50 grams of aluminum oxide which had been previously saturated with heptane. After

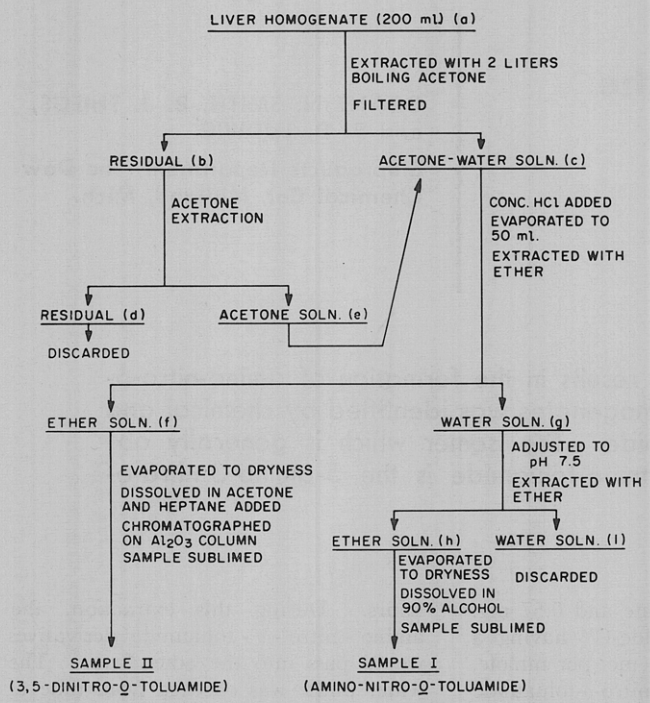

Figure 1. Isolation of amino-nitro-o-toluamide formed by biological reduction of 3,5-dinitro-o-toluamide

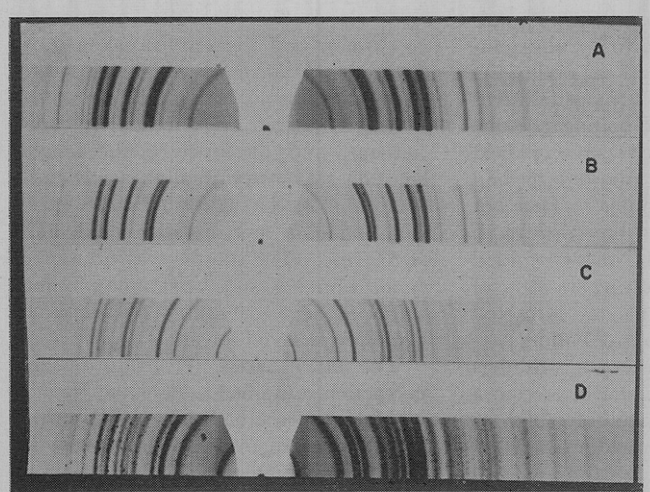

Figure 3. X-ray patterns of various crystalline forms of 3-amino-5-nitro-o-toluamide

(A, ANOT–alcohol; B, ANOT–water; C, ANOT–glacial acetic acid; D, ANOT–acetone)

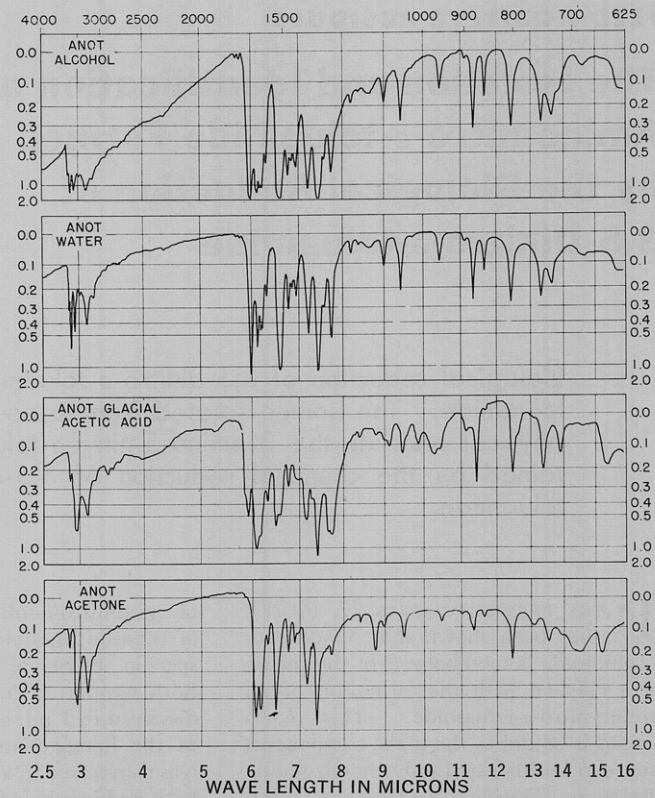

Figure 2. Infrared scans of various crystalline forms of 3-amino-5-nitro-o-toluamide

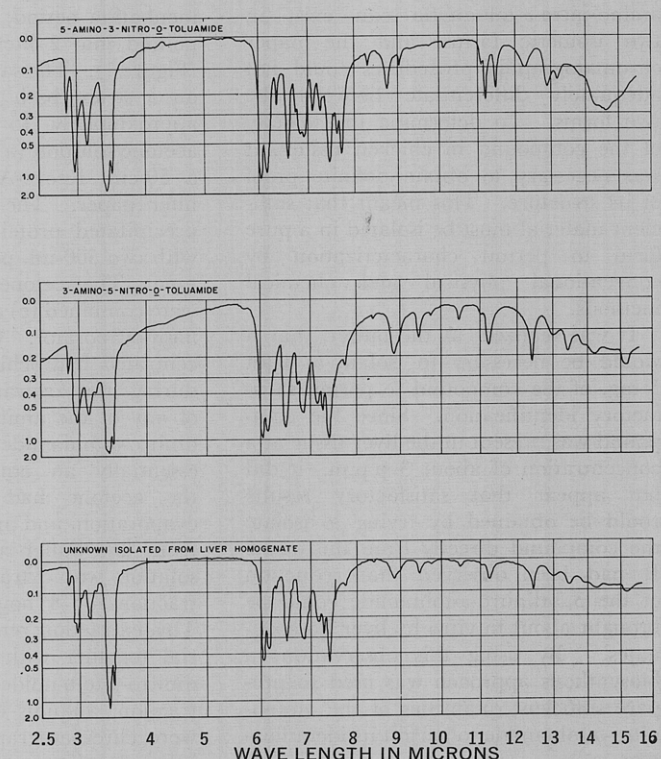

Figure 4. Infrared scans of 5-amino-3-nitro-o-toluamide, 3-amino-5-nitro-o-toluamide, and unknown compounds isolated from liver homogenate

the solution had entered the aluminum oxide, the column was washed with 75 ml. of heptane. The heptane washing contained no significant amount of radioactivity and was discarded. The radioactivity was then eluted from the column with 100 ml. of 80% ethyl alcohol. The effluent was collected in 10-ml. fractions, and each fraction was analyzed for radioactivity. Those fractions containing radioactive material were combined and the remaining fractions discarded.

The combined fractions were extracted twice with an equal volume of heptane. The alcohol solution was made slightly alkaline with a few drops of 1*N* sodium hydroxide solution and then evaporated to dryness. The residue was then sublimed. The sublimed sample (II) was saved for analysis.

A series of chemical and physical determinations was made on both samples to determine the structure of each compound. The carbon and hydrogen content was determined by a modification of the Pregel method (*3*), and nitrogen content was determined by the Dumas method (*4*). Molecular

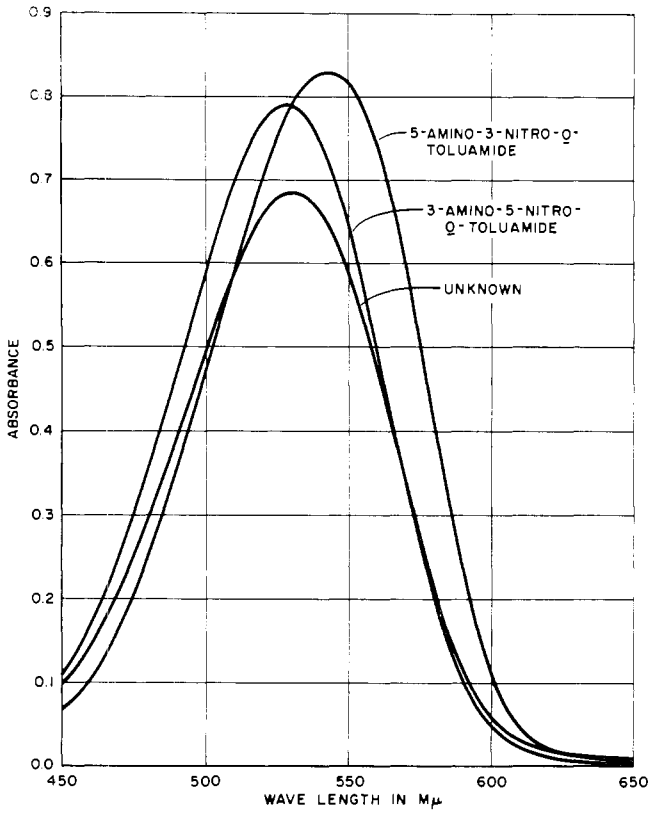

Figure 5. Spectral curves of dyes formed from 3-amino-5-nitro-o-toluamide and 5-amino-3-nitro-o-toluamide by coupling with *N*-(1-naphthyl)ethylenediamine dihydrochloride

weight was determined by mass spectroscopy. The purity of each sample was determined by chemical analysis using a modified Bratton-Marshall procedure for the aryl amino and the diaminopropane procedure for zoalene. In addition, paper chromatographic procedures were used to check purity (*8*). The samples were recrystallized from absolute ethyl alcohol and infrared scans obtained using infrared equipment which would give satisfactory resolution (*1, 2*). Since the 3,5-dinitro-*o*-toluamide and amino-nitro-*o*-toluamide are not very soluble in the solvents normally used for infrared analysis, it was necessary to use the Nujol Mull technique, which requires that the compound be run in a crystalline state. The crystalline form of each compound was ascertained by x-ray diffraction.

Results and Discussion

The previous studies on the metabolism of 3,5-dinitro-*o*-toluamide (*7, 9, 11*) showed that the compound could undergo reduction with the formation of an amino-nitro-*o*-toluamide. It was not possible to definitely ascertain from the chromatographic studies whether the compound produced was the 3-amino-5-nitro-*o*-toluamide or the 5-amino-3-nitro-*o*-toluamide. It was, therefore, necessary to obtain a sufficient quantity of the reduced compound to determine its identity by physical and chemical methods. The compound was obtained by enzymatic reduction of the 3,5-dinitro-*o*-toluamide-C^{14} using liver homogenates. The extraction procedure outlined in Figure 1 was then used to separate the various radioactive compounds. The resulting compounds were then sublimed under vacuum to remove any traces of impurities which might still be present. The two samples obtained by these procedures were recrystallized from ethyl alcohol. Both were light yellow in color.

Sample I, assumed to be the amino-nitro-*o*-toluamide, gave a positive Bratton-Marshall test and negative sodium methylate, tetramethylammonium hydroxide, diaminopropane, and potassium cyanide tests (*5, 6, 8, 10*).

These results indicated that the compound was an aryl amino compound. The material was chromatographed in solvent systems B and C (*8*), and the R_f values obtained corresponded to those obtained with the 3-amino-5-nitro-*o*-toluamide. Elemental analysis indicated an empirical formula of $C_8H_9N_3O_3$ which corresponds to the formula for amino-nitro-*o*-toluamide. Molecular weight was found to be 195 which again corresponds to the amino-nitro compound.

In preparing infrared scans, the Nujol Mull technique must be used since the compound was not sufficiently soluble in carbon tetrachloride or carbon disulfide to obtain satisfactory scans. Since the material was scanned in the solid state, the spectrum obtained was influenced by the crystalline state of the compound. Authentic samples of the 3-amino-5-nitro-*o*-toluamide and 5-amino-3-nitro-*o*-toluamide were synthesized and crystallized from various solvents. Each compound was found to exist in at least four crystalline forms. The crystalline form obtained depended on the conditions and the solvent used for the crystallization.

In many cases, several crystalline forms were obtained at the same time. These results meant that the scan of the unknown amino-nitro compound would have to be compared with four scans of the 3-amino-5-nitro-*o*-toluamide and four scans of the 5-amino-3-nitro-*o*-toluamide. Typical scans obtained with the 3-amino-5-nitro-*o*-toluamide compound are shown in Figure 2. If the unknown compound was not in one of these four crystalline forms, its infrared scan could not be compared with any of the reference standards. It was therefore necessary to find a method to determine if the unknown compound was crystalline and if so, what the crystalline form was. Since less than 5 mg. of the unknown compound was available, it was impossible to ascertain the crystalline form by visual observation. X-ray diffraction was therefore resorted to as a means of distinguishing the various crystalline forms. Typical patterns obtained are shown in Figure 3. By matching the x-ray patterns (Figure 3) with the infrared scans (Figure 2), crystalline form could be related to infrared spectrum. By using these techniques, it was possible to ascertain the crystalline form of the unknown compound and obtain a satisfactory infrared scan.

The structure of the 5-amino-3-nitro-*o*-toluamide reference standard was established by using two different synthesis procedures to prepare the compound. The purity of the compound was determined by mass spectrometry, infrared analysis, elemental analysis, and paper chromatography. As far as could be ascertained, this sample was at least 99% pure. The 3-amino-5-nitro-*o*-toluamide reference standard was of similar purity.

The two reference standards and the unknown compound isolated from the liver were first recrystallized from ethyl alcohol, then sublimed, and then dissolved in alcohol. The alcohol solution was then evaporated in a hood at room temperature. If the solutions were evaporated to dryness at room temperature, the same crystalline form of each compound was always obtained. This technique was therefore used to prepare all samples for infrared analysis.

The crystalline patterns of the two reference standards and the unknown

compound were obtained by x-ray diffractions. Nujol Mulls were then prepared and infrared scans made. The scans obtained are shown in Figure 4. A review of the scans shows that the material isolated from the liver homogenate corresponds closely to the 3-amino-5-nitro-*o*-toluamide.

Additional proof of the structure of the unknown compound was obtained with the Bratton-Marshall test. If the Bratton-Marshall procedure for the determination of aryl amines were carried out in butyl alcohol, distinct color curves were obtained for the 3-amino-5-nitro-*o*-toluamide and the 5-amino-3-nitro-*o*-toluamide. This reaction was carried out by adding 1 ml. of a 0.25% solution sodium nitrite to the compound in 5 ml. of 4*N* HCl. After 5 minutes, 1 ml. of 1.25% ammonium sulfamate was added. After allowing the solution to stand another 5 minutes, 85 ml. of butyl alcohol was added followed by 1 ml. of 0.25% solution of *N*-(1-naphthyl) ethylenediamine dihydrochloride. Typical spectral curves obtained are shown in Figure 5. The spectral curve for the unknown compound corresponds to the 3-amino-5-nitro-*o*-toluamide indicating that the unknown compound was probably the 3-amino-5-nitro-*o*-toluamide.

Sample II (Figure 1) was analyzed by the Bratton-Marshall, sodium methylate, tetramethylammonium hydroxide, diaminopropane, and potassium cyanide methods (*5*, *6*, *8*, *10*). The Bratton-Marshall test was negative while all the rest were positive (*8*). The colors obtained in these tests suggested that the compound was probably 3,5-dinitro-*o*-toluamide. Elemental analysis indicated an empirical formula of $C_8H_7N_3O_5$, and molecular weight determination gave 225. These values correspond to those obtained with 3,5-dinitro-*o*-toluamide.

Infrared scans of sample II were compared with the reference standard of 3,5-dinitro-*o*-toluamide and were found to be identical. From the results obtained, it was concluded that sample II was 3,5-dinitro-*o*-toluamide.

Apparently incubation of 3,5-dinitro-*o*-toluamide with liver homogenates results in the reduction of one of the nitro groups of 3,5-dinitro-*o*-toluamide with the formation of the 3-amino-5-nitro-*o*-toluamide. This latter compound apparently binds to the tissues when it is formed in the normal metabolism of the 3,5-dinitro-*o*-toluamide in the chicken. Other compounds are also produced by the metabolism of 3,5-dinitro-*o*-toluamide, but they do not appear to be bound to the tissue to any great extent.

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FRUIT PRESERVATIVES ANALYSIS

Determination of Calcium in Cherry Brines by Versenate Titration: Elimination of Anthocyanin Interference by Means of Carbonyl Reagents

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Carbonyl reagents were used to decolorize anthocyanin pigments in cherry brines which could then be titrated with disodium dihydrogen versenate (EDTA) solution. On brines ranging in calcium content from 500 to 9000 p.p.m., the EDTA method gave values that averaged 46 p.p.m. higher than the A.O.A.C. permanganate titration method. The precision of the EDTA method was significantly better. Recovery of calcium added to brines was 97.5 to 100%. Addition of 200 p.p.m. of ferric or phosphate ions, or 500 p.p.m. of aluminum, cupric, lead, or magnesium ions to brines did not interfere with the endpoint of murexide indicator.

The preservation of sweet cherries in calcium bisulfite brine is the initial step in processing maraschino and glacé cherries, as well as cherries for fruit cocktail and fruits for salad. Control of the amount of calcium in the brine is important in achieving the desired firm texture in the product. In the search for a simple, rapid method for determining calcium in the brine, attention was focused on complexometric titration techniques to circumvent precipitation and filtration steps that are employed in the conventional oxalate precipitation method (*1*).

Numerous reports on the rapid EDTA titration method for calcium are to be found, differing chiefly as to the indicator used. Schwarzenbach (7) described the complexometric titration of calcium using murexide as the indicator. Lewis and Melnick (*3*) reported studies on the accuracy of determinations of calcium and magnesium in which calcon and cal-red indicators were used. The accuracies they reported indicated that the EDTA titration method for calcium would be satisfactory for use in determining calcium in cherry brines. Moss (*4*) studied the limits of interference by various ions in the EDTA determination of calcium using cal-red indicator. He noted the use of hydroxylamine to reduce manganic ions and thus eliminate that source of interference. Schwarzenbach (*6*), likewise, mentions the use of hydroxylamine as a reducing agent for interfering ions, but its function as a carbonyl agent in removing interfering