Studies on the Tissue Clearance and Metabolism of 2-Acetamido-5-nitrothiazole-C^{14} (Enheptin-A) in Turkeys

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Studies on the tissue clearance and in vitro metabolism of carbon-14–labeled 2-acetamido-5-nitrothiazole in turkeys show that following a 14-day dosing period this compound and/or its metabolites remain in turkey tissue with a biological half life of from 3.5 to over 12 days for the tissues examined. In vitro metabolism studies using turkey liver and kidney slices show that the 5-nitro group of 2-acetamido-5-nitrothiazole is susceptible to reduction, and that this pathway apparently represents a major route of metabolism for this compound. Paper chromatography shows that, under the in vitro conditions employed, six metabolites of 2-acetamido-5-nitrothiazole are produced by the liver and three by the kidney including unchanged compound. It is not implied that a similar spectrum of metabolites is formed under in vivo conditions.

ENHEPTIN-A (American Cyanamid Co.) is a widely used agent for the prevention and treatment of infectious enterohepatitis in turkeys (*5*). Studies on its chemotherapeutic effects, toxicity, and extratherapeutic effects have been reported (*2*, *6*). Previous studies, using a colorimetric method as the analytical procedure, on the clearance of this compound following withdrawal from treated birds indicated essentially complete removal within 48 hours (*3*).

The purpose of the work reported here was to determine clearance of the compound from tissues of birds to which had been administered carbon-14–labeled 2-acetamido-5-nitrothiazole for two weeks. As the determination of carbon-14 in tissue would not differentiate between unchanged compound and metabolites, information concerning the possibility that metabolites are formed and stored could be obtained by comparing these results with data previously referenced.

The metabolism of 2-acetamido-5-nitrothiazole in turkeys was studied using in vitro techniques.

Materials and Methods

Drugs. Both carbon-14–labeled 2-acetamido - 5 - nitrothiazole (thiazole-2-C^{14}) and the unlabeled compound were supplied by the American Cyanamid Co. In clearance studies, the labeled compound was mixed with lactose diluent and administered by capsule. In in vitro metabolism studies, labeled material was added to turkey liver or kidney slices. Before use in clearance studies, the labeled compound was examined for radiochemical purity by paper chromatography. The compound was dissolved in acetone and spotted on Whatman No. 1 paper. Development was carried out in a system of 2-propanol–ammonia–water (160:8:16). Chromatograms were dried and fumed with ammonia to visualize the compound. They were then examined using a Forro Radiochromatograph (Forro Scientific Co., Evanston, Ill.). By use of these procedures, the labeled compound was found to contain a small amount (5% or less) of a radiochemical contaminant which did not migrate in the solvent system employed.

Turkeys. In those experiments involved with tissue clearance of carbon-14 2-acetamido-5-nitrothiazole and/or its metabolites, 4-week-old, broad breasted, white tom turkey poults were used. They were housed in a wire-floored battery and maintained on a starter ration during the course of the study. In vitro metabolism studies were undertaken using tissue preparations obtained from broad breasted Bronze hen turkeys from 5 to 13 weeks old. They were maintained on a starter ration and were not fasted prior to sacrifice.

Clearance Studies. Carbon-14–labeled drug was administered by capsule as a single daily dose to each of 20 birds over a 14-day period. The daily dose was calculated (based upon an average feed consumption of 50 grams per day at the start of the experiment) at 0.05% of feed level (25 mg. per day) and contained 19.1 μc. of radioactivity. Birds were allowed food and water *ad libitum* during the experimental period. At termination of the dosing period, the birds were taken randomly in groups of four and sacrificed for tissue analysis at 1, 2, 4, 7, and 10 days following drug withdrawal.

Samples for radioactivity measurements were taken of liver, kidney, abdominal fat, skin, white muscle, and dark muscle. All radioassays were made using a liquid scintillation spectrometer. Tissue samples varied from 100 to 370 mg. in weight and were dissolved in 3.0 ml. of a 3 to 1 mixture of M/1 Hyamine hydroxide (Rohm and Haas Co.) in methanol and 30% potassium hydroxide in a glass counting vial. The tissue samples were allowed to stand in this mixture at room temperature for 12 hours and were then heated at 80° C. with intermittent shaking over 30 minutes to effect complete dissolution. To the dissolved tissues were then added 1.0 ml. of glacial acetic acid and 10 ml. of a liquid scintillator {composed of *p*-xylene, 500 ml.; *p*-dioxane, 500 ml.; 2-ethoxyethanol, 300 ml.; naphthalene, 102 grams; 2,5-diphenyloxazole, 5 grams; and 1,4-bis[2-(5-phenyloxazoyl)]-benzene, 0.13 gram}. Correction of samples for counting efficiency was made using an internal standard technique. Data from the radioassays representing both unchanged 2-acetamido-5-nitrothiazole and/or its metabolites were calculated on the basis of disintegrations per minute (d.p.m.) of carbon-14 per milligram of tissue.

Metabolism Studies. Turkey liver or kidney slices (100 to 300 mg.) were added to 25-ml. Erlenmeyer flasks containing 5.0 ml. of Krebs-Ringer phosphate buffer (pH 7.4, gassed with nitrogen) and 2.14×10^{-4} mole (200 μg.) of 2-acetamido-5-nitrothiazole. Flasks were incubated in a Dubnoff metabolic shaking incubator at 37° C. for 2 hours under nitrogen. Reduction of the nitro group was followed. A polarographic method was employed. After the incubation period, tissue was removed, an equal volume (5.0 ml.) of 2*M* KCl was added, and the solution saturated with borax. Polarograms were then run (Model XXI recording polarograph, E. H. Sargent Co.). The calibration curve for this assay was linear over the 20- to 200-μg. range of 2-acetamido-5-nitrothiazole per 5.0 ml. of incubation mixture.

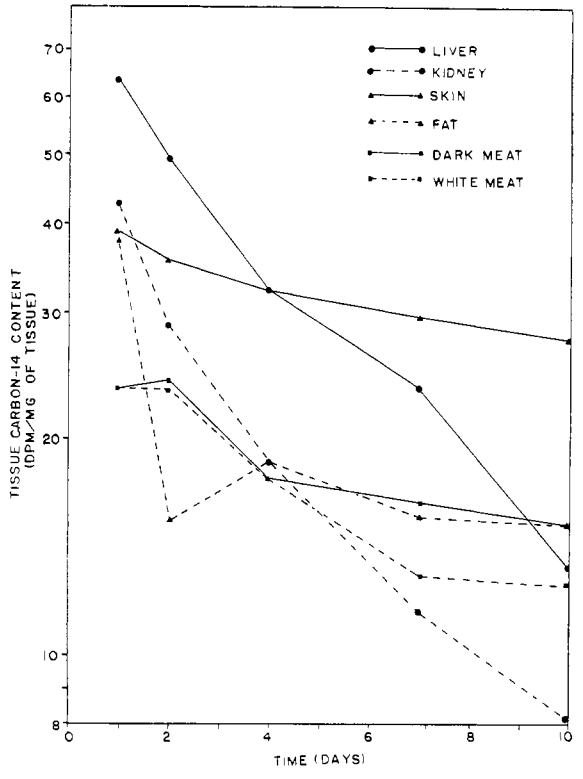

Figure 1. Tissue clearance of carbon-14 2-acetamido-5-nitrothiazole and/or its metabolites in the turkey

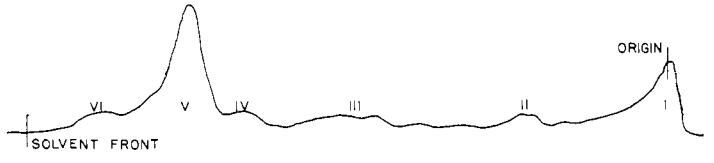

Figure 2. Radiochromatogram of liver incubation extract

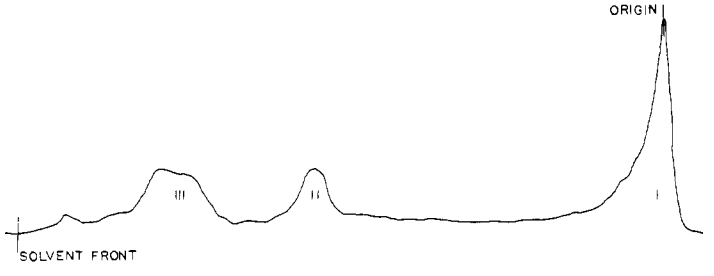

Figure 3. Radiochromatogram of kidney incubation extract

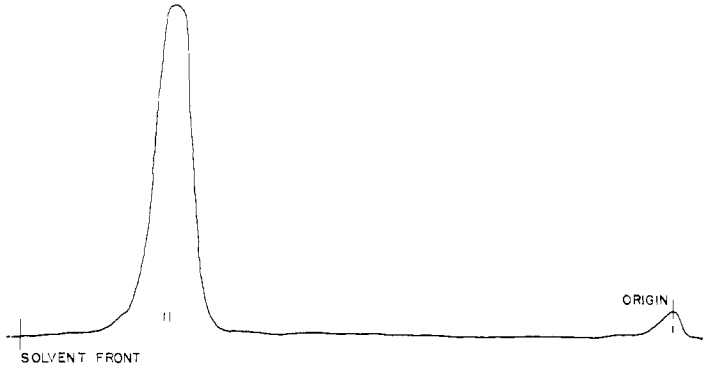

Figure 4. Radiochromatogram of carbon-14 2-acetamido-5-nitrothiazole incubation extract

Table I. In Vitro Reduction of 2-Acetamido-5-nitrothiazole by Turkey Liver and Kidney Slices[a]

Experiment	Number of Animals	2-Acetamido-5-nitrothiazole Metabolized, μG./Gram Tissue/2 Hr.[b]		Ratio: Liver/Kidney
		Liver	Kidney	
1	3	626	837	0.75
2	5	774	1015	0.76
3	2	701	928	0.77
4	2	592	1066	0.56

[a] Approximately 200-mg. tissue slices incubated in 5.0 ml. Krebs-Ringer phosphate buffer containing 200 μg. 2-acetamido-5-nitrothiazole at 37° C. under N_2 for 2 hours. Flask contents assayed polarographically for disappearance of substrate.

[b] Each value represents over-all mean of duplicate determinations on tissues of each animal used. Appropriate tissue blanks and 2-acetamido-5-nitrothiazole blanks were included in each experiment.

In other experiments, the incubation mixture was examined using paper chromatography to determine the number and approximate amounts of metabolites formed. In these experiments, labeled compound was incubated as described above and the incubation mixture subsequently treated with 2,2-dimethoxypropane (*7*) to remove water prior to chromatographic examination. The residue was taken up in dry acetone and chromatographed as described earlier. Radioactive spots were identified through the use of the chromatogram scanner. Relative areas under each peak were determined by use of a planimeter.

Results and Discussion

Data obtained in experiments measuring the disappearance of drug from tissues (Figure 1) suggest that 2-acetamido - 5 - nitrothiazole and/or its metabolites have a relatively long storage time in turkey tissue following drug withdrawal. When compared with results obtained by other techniques specific for measuring unchanged 2-acetamido - 5 - nitrothiazole in similar studies (*3*), the data obtained using labeled material suggest that metabolites are formed and stored. Biological half-lives (time in days required for tissue radioactivity concentrations to decrease by 50%) of the radioactive material in the tissues examined ranged from 3.5 days in kidney to over 12 days (extrapolated) in the skin.

In vitro metabolism studies in which reduction of the 5-nitro group was measured show that this route of metabolism of 2-acetamido-5-nitrothiazole is common to both liver and kidney of the turkey. Kidney preparations were consistently more active than those for liver in effecting this reduction as shown in Table I. It is likely that other tissues

would also be able to reduce the nitro group of this compound, since Fouts and Brodie (*4*) have shown that nitro reductase activity is a property of several tissues in other species. Further, it is probable that such a reduction could be effected by bacteria of the intestinal flora. The high level of nitro reductase activity shown by both turkey liver and kidney suggests that this pathway may represent a major route of metabolism of this compound.

Data obtained in experiments relative to separation of metabolites resulting from incubation of carbon-14–labeled 2-acetamido-5-nitrothiazole with turkey liver or kidney slices are shown in Figures 2 and 3. Figure 4 represents a blank containing labeled compound carried through the incubation and separation procedure. The small amount of radioactive material remaining at the origin in this chromatogram was also found in freshly prepared solutions of the compound in acetone, and thus was not produced during incubation and subsequent separation steps. In the liver incubation mixture, six radioactive spots are evident with spot 5 corresponding to unchanged 2-acetamido-5-nitrothiazole. In the kidney incubation mixture, three spots are apparent with spot 3 corresponding to unchanged compound. Values obtained for the analysis of incubation media for unchanged compound by polarography correspond well with the concentrations of unchanged compound present on chromatograms. This suggests that the majority of the metabolites produced under these in vitro conditions no longer have the nitro group intact. Further work is required to substantiate this point.

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FAT COMPONENTS

Unsaponifiable Fraction of Pork Fat as Related to Boar Odor

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The unsaponifiable matter obtained from pork fat was fractionated and found to contain carbonyls, cholesterol, squalene, vitamin A, and four saturated hydrocarbons. In addition, cholesterol esters, a 7-ketosterol, a triterpene alcohol, and two oxidation products of squalene were indicated. No evidence of primary or secondary alcohols or of sulfur- or nitrogen-containing compounds could be found. No important differences were observed in the components from fat containing boar odor as compared to fat free from boar odor.

Although meat from the uncastrated male pig (boar) has been known for many years to produce an undesirable permeating odor on heating, attempts to establish the frequency and to identify the responsible components have been relatively recent (*5, 17*). Craig *et al.* (*5*) reported that the component(s) responsible for boar odor was (were) located in the unsaponifiable fraction of pork fat. The presence of cholesterol in lard and whole pig fat was noted by Lange (*12*). Fitelson (*8*) reported the presence of squalene in pork lard and beef tallow. The presence of cholesterol and squalene in the unsaponifiable material from both boar and barrow fat was also reported by Craig *et al.* (*5*). However, these authors were unable to associate the presence of either cholesterol or squalene with boar odor in pork. The presence of vitamin A in pork lard was indicated by others (*2, 10*).

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The present study was undertaken to ascertain the chemical composition of the unsaponifiable fraction from pork fat and to determine relationship to boar odor.

Experimental Methods

Saponification. Both boar fat containing boar odor and barrow (castrated male pig) fat free from boar odor were saponified in the cold as suggested by Deuel (*6*). The cold saponification method has been described in some detail by Craig *et al.* (*5*). In brief, the procedure consisted of saponifying the fat dissolved in diethyl ether at room temperature by adding a concentrated solution of sodium ethylate. The unsaponifiable material was isolated and purified by filtering off the soap and washing repeatedly with water. The solution was then dried with anhydrous sodium sulfate, filtered, and reduced to a few milliliters under vacuum. The unsaponifiable residue was stored in the refrigerator under nitrogen.

Qualitative Tests. The nitrochromic acid test for alcohols, the 2,4-dinitrophenylhydrazine test for carbonyls, and the LeRosen test for aromatic nucleus and aliphatic unsaturation were carried out utilizing the reagent solutions described by Walsh and Merritt (*16*). The Lieberman-Burchard test for cholesterol as described by Litwack (*13*) and the hydroxylamine test for esters as described by Hall and Shaefer (*9*) were employed. Sulfur-containing compounds were determined by the Feigl method (*7*), and the micro-Kjeldahl procedure was used to measure the nitrogen content of the unsaponifiable material (*1*).

Removal of Free Fatty Acids. The free fatty acids remaining in the unsaponifiable material were removed by the basic copper carbonate method described by Capellas *et al.* (*4*).

Column Chromatography. A 100-mg. sample of unsaponifiable material was subjected to silicic acid chromatography (*4*). The unsaponifiable matter was dissolved in a minimum of hexane and added to the top of a chromatographic column 11 mm. in diameter fitted with a stopcock and containing 3 grams of Mallinckrodt's 100-mesh silicic acid. The type and