Metabolism and Distribution of [2,3-^{14}C]Acrolein in Laying Hens

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The metabolism and distribution of [2,3-^{14}C]-acrolein were studied in 10 laying hens orally administered 1.09 mg/kg of body weight/day for 5 days. Eggs, excreta, and expired air were collected. The hens were killed 12−14 h after the last dose and edible tissues collected. The nature of radioactive residues was determined in tissues and eggs. All of the identified metabolites were the result of the incorporation of acrolein-derived radioactivity into normal natural products of intermediary metabolism in the hen except for 1,3-propanediol, which is a known degradation product of glycerol in bacteria.

Keywords: *Metabolism; acrolein; hen*

INTRODUCTION

Apart from being used extensively as a chemical intermediate, acrolein (propenal) has been utilized as an aquatic herbicide for some time (*1*−*4*). Because it is cytotoxic to plant tissue and decomposes rapidly in water (*2*, *5*, *6*), it is an effective herbicide that leaves virtually no residue that would be harmful to the environment (*7*−*9*). The objective of this study was to quantify and characterize the nature of the radioactive residues of acrolein in the eggs and edible tissues of laying hens.

Until recently, no complete study of acrolein metabolism in any species has been reported in the literature, although some partial studies in the rat have been reported. Acrolein is reported to be metabolized to glycidaldehyde, glyceraldehyde, and acrylic acid in rat liver microsomes, although the metabolites were identified only by cochromatography (*10*). No other phase I metabolites of acrolein have been reported.

Although the acrolein−glutathione adduct, 3-oxopropyl-*S*-glutathione, also has not been isolated in vivo, mercapturic acids resulting from further metabolism of glutathione adducts have been reported for acrolein (*11*). 3-Hydroxypropylmercapturic acid [*S*-(3-hydroxypropyl)-*N*-acetylcysteine] has been reported to be present in the urine of rats treated with acrolein (*12*−*14*).

Another mercapturic adduct that has been identified in the urine of acrolein-exposed rats is the carboxyethylmercapturic acid [*S*-(3-carboxyethyl)-*N*-acetylcysteine]. Draminski et al. (*15*) identified this metabolite by comparison of the gas chromatographic retention time and mass spectrum of the methyl ester to that of a synthetic standard. Linhart et al. (*14*) have recently described this metabolite in the urine of rats following both inhalation and intraperitoneal administration of acrolein.

We recently have reported an extensive study of the identification of the urinary and fecal metabolites of acrolein in Sprague−Dawley rats (*16*, *17*). To our knowledge, this paper and the accompanying paper on the metabolism of acrolein in the goat (*18*) are the first reports of the metabolism of acrolein in livestock and poultry.

Apart from the seminal work of Dorough and Ivie (*19*−*21*), there are very few reports of studies of the incorporation of xenobiotic-derived radioactivity into the products of intermediary metabolism in the open literature. Much work of this nature, however, is conducted in support of regulatory submissions of agrochemicals. Additionally, studies of the incorporation of xenobiotic-derived radioactivity into products of normal intermediary metabolism are not readily accessible by computerized literature searches as relevant techniques are scattered among studies of different compounds. We have reported in detail the techniques used in our laboratory in order that they might prove useful for others.

EXPERIMENTAL PROCEDURES

Test Materials. [2,3-^{14}C]Acrolein (specific activity = 8.9 mCi/mmol) was supplied as a solution in dimethylformamide (DMF) containing 0.25% hydroquinone as a stabilizer with a stated radiochemical purity of 93% by Sigma Chemical Co., St. Louis, MO. The non-radiolabeled acrolein (stabilized with 0.25% hydroquinone) was used as supplied by Baker Petrolite Corp. It contained 96.05% acrolein by UV analysis (the remainder was mostly water). The non-radiolabeled acrolein was dissolved in acetonitrile (ACN) containing 0.25% hydroquinone and mixed with the radiolabeled acrolein solution.

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Table 1. Total Recovery of Radioactivity in Laying Hens Administered [2,3-^{14}C]Acrolein (1.09 mg/kg of Body Weight/Day) by Gavage for Five Consecutive Days

	%
tissues and gastrointestinal tract	3.88
excreta	66.3
egg yolk	0.39
egg white	0.14
volatiles	4.6
total	75.3

Immediately before each dose, the required amounts of this solution were diluted in water to final ACN, DMF, and hydroquinone concentrations of 4, 0.5, and 0.01%, respectively.

Animal Husbandry and Sample Collection. The facilities and staff of the Colorado State University Metabolic Laboratory were used for the housing and handling of the hens. Thirty White Leghorn hens were obtained from a local supplier and were acclimated for 31 days. Fourteen of these hens were selected for the study on the basis of food and water consumption, egg production, and general health, with 10 of these randomized into the treatment group and the remainder serving as controls. The hens received 2.6−4.5 mL of the dosing solution by gavage after the morning feeding for five consecutive days. This resulted in a dose administered of 1.65 mg/hen/day (1.09 mg/kg/day) and a final specific activity of 72555 dpm/μg acrolein. The control hens received a similar volume of a solution containing ACN, DMF, and hydroquinone at the same concentrations as the dose solution.

Eggs were collected twice daily, and the whites and yolks were separated, pooled by collection interval, and stored frozen until analysis. The shells were discarded. Excreta were collected each morning and stored frozen. Six hens were monitored for expired radioactivity by means of a modified indirect calorimetry chamber system. After the third dose, six of the treated hens were placed in a respiration chamber for 10 h. The air from the chamber was pumped sequentially through a flow meter to record total airflow, and then a scrub fraction (0.864%) was pumped through a second flow meter and then through two CO_2 scrubbers, each containing R. J. Harvey carbon 14 cocktail (200 mL). All samples were stored frozen at −20 °C until analysis.

The hens were killed by cervical dislocation. Heparinized blood, liver, kidneys, and samples of breast and thigh muscle and fat were collected and pooled. The gastrointestinal tracts and their contents also were collected and pooled. All samples were immediately frozen on dry ice and were stored frozen at −20 °C until analysis.

Sample Preparation and Radioanalysis. The tissue samples, egg white, and egg yolk were partially thawed and trimmed of extraneous material, and all tissues but the gastrointestinal tract and blood were homogenized in a food processor. The gastrointestinal tracts were homogenized with dry ice in a food chopper and/or a blender. Excreta were homogenized with deionized water in a blender. Blood was homogenized with a probe-type homogenizer. The CO_2 scrubber solutions were assayed directly for total ^{14}C by liquid scintillation counting (LSC). All of the other matrices were oxidized, and the resulting CO_2 was analyzed by LSC. The values were corrected for combustion efficiency using the values obtained from control matrices fortified with a ^{14}C reference solution.

The mass balance and tissue and egg concentrations of radioactivity are found in Tables 1−3.

High-Performance Liquid Chromatography (HPLC). HPLC analyses were performed with the following columns and solvent systems. Detection was by either ultraviolet (UV) absorbance or refractive index (RI) detection, as many of the standards used for comparison to the radioactive residues had no UV absorbance at wavelengths >210 nm. Radioactivity was detected either by using fraction collection followed by static LSC or by on-line detection using Radiomatic HPLC radioactivity detectors (Packard Instrument Co., Downers Grove, IL) and liquid scintillant.

Table 2. Total ^{14}C Residues in Tissues in Laying Hens Administered [2,3-^{14}C]Acrolein (1.09 mg/kg of Body Weight/Day) by Gavage for Five Consecutive Days

tissue	ppm[a]	% total dose
liver	0.731	0.27
kidney	0.839	0.09
breast muscle[b]	0.091	0.23
thigh muscle[b]	0.135	0.25
fat[b]	0.137	0.07
blood[b]	0.209	0.20

[a] Parts per million acrolein equivalents. [b] The percentage of dose for breast muscle, thigh muscle, fat, and blood is extrapolated using the following percentages of body weight: breast muscle, 13.3%; thigh muscle, 9.9%; fat, 2.5%; blood, 5% (personal communication from Donald E. Johnson, Colorado State University Metabolic Laboratory).

Table 3. Total ^{14}C Residues in Eggs from Laying Hens Administered [2,3-^{14}C]Acrolein (1.09 mg/kg of Body Weight/Day) by Gavage for Five Consecutive Days

collection interval	whites[a]		yolks	
	ppm	% dose	ppm	% dose
day 1	0.008	0.00	0.000	0.00
day 2	0.070	0.03	0.049	0.01
day 3	0.105	0.02	0.460	0.04
day 4	0.128	0.04	0.763	0.12
day 5	0.127	0.05	1.25	0.22

[a] Parts per million acrolein equivalents.

System A (Reversed Phase): column, Phenomenex Spherex 5 μm C-18, 4.6 × 250 mm; temperature, ambient; gradient, 100% 5 mM KH_2PO_4, pH 3.0/ACN (95:5) to 100% ACN over 20 min, followed by 15 min at 100% ACN; detection, UV at 210 nm; flow rate, 0.8 mL/min; scintillant flow rate, 3.2 mL/min.

System B (Anion Exchange): column, Supelco Supelcogel C610H-SP anion exchange, 7.8 × 300 mm; temperature, 60 °C; mobile phase, isocratic 0.1% phosphoric acid; detection, RI or UV at 210 nm; flow rate, 0.5 mL/min; scintillant flow rate, 2.0 mL/min.

System C (Ion Exclusion): column, Phenomenex ORH-801 organic acids column, 6.5 × 300 mm; precolumn Ionguard polyether ketone cartridge; temperature, 35 °C; mobile phase, isocratic 1 mM sulfuric acid; detection, RI or UV at 210 nm; flow rate, 0.8 mL/min; scintillant flow rate, 3.2 mL/min.

System D (Reversed Phase): column, Phenomenex Spherex 5 μm NH_2, 4.6 × 250 mm; precolumn 4.6 × 50 mm Spherex NH_2 5 μm; temperature, ambient; gradient, (A) 10 mM KH_2PO_4, pH 4.3; (B) ACN/water (88:12); (C) 50 mM KH_2PO_4, pH 4.3; 95:5 A/B for 5 min to 30:70 A/B at 20 min to 50:50 A/B at 27 min, followed by 23 min at 50:50 A/B to 50:50 B/C at 60 min to 10:90 B/C at 70 min, followed by 10 min at 10:90 B/C (For amino acid analysis, the gradient was ended at 50:50 A/B. For aqueous and methanol extracts of tissues, the remainder of the gradient employing solvent C was necessary to ensure complete elution of the radioactivity.); detection, UV at 210 nm; flow rate, 1.0 mL/min; scintillant flow rate, 4.0 mL/min.

System E (Reversed Phase): column, Alltech Nucleosil C_{18} 5 μm, 4.6 × 250 mm; temperature, 52 °C; gradient, (A) 50 mM ammonium acetate, pH 6.8/ACN (95:5); (B) 100 mM ammonium acetate, pH 6.8/methanol/ACN (46:10:44); 100% A to 85:15 A/B at 15 min to 50:50 A/B at 30 min to 100% B at 34 min, followed by 11 min at 100% B; detection, UV at 254 nm; flow rate, 1.0 mL/min; scintillant flow rate, 3.2 mL/min.

System F (Reversed Phase): column, Phenomenex Spherex 5 μm C-18, 4.6 × 250 mm; temperature, 45 °C; gradient, isocratic ACN/2-propanol (50:50); detection, UV at 210 nm; flow rate, 0.5 mL/min; scintillant flow rate, 2.0 mL/min.

Lipid Fractionation and Fatty Acid Analysis. Lipid-containing extracts were fractionated on an aminopropyl bonded silica gel column (30−70 μm, 60 Å, Alltech Associates, Deerfield, IL). The column was eluted sequentially with 2-propanol/$CHCl_3$ (1:2), 2% acetic acid in ether, methanol, and

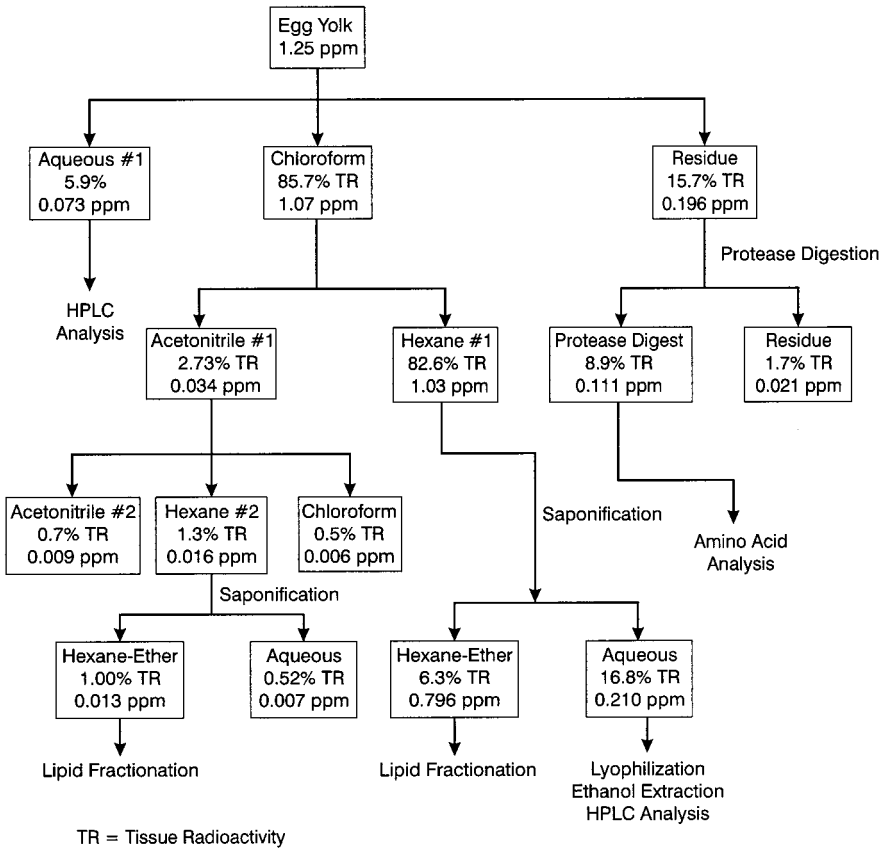

Figure 1. Fractionation scheme for egg yolk.

$CHCl_3$/methanol/0.8 M sodium acetate (60:30:45) to deliver neutral lipid, fatty acid, other lipid, and phospolipid fractions, respectively (*22*).

Free fatty acid fractions were derivatized with phenacyl bromide and analyzed by HPLC using a modification of the method of Hanis (*23*).

Amino Acid Analysis. Amino acids were derivatized with phenyl isothiocyanate using the method of Heinrickson and Meredith (*24*). The phenyl thiocarbamoyl amino acids were resolved on HPLC system E.

Protease Digestion. Unextractable residues were digested with protease (type XIV, Sigma Chemical Co., St. Louis, MO). The residues were incubated in a shaking water bath at 37 °C overnight with a 20 mg/mL solution of protease in Tris-HCl buffer, pH 7.0, containing $CaCl_2$ (13.9 mM).

Thin-Layer Chromatography (TLC). The extracts were applied to either a 0.5- or 0.25-mm thickness 20-cm × 20-cm Merck Kieselgel 60 F254 glass plate. Plates were developed to a height of 16 cm in a preequilibrated tank using *n*-butanol/acetic acid/water (4:2:1). Following development, the plates were analyzed for radioactivity on an Ambis Radioanalytical Imaging System.

Egg and Tissue Extraction and Fractionation Procedures for Metabolite Identification. Homogenized pooled egg yolk (day 5 p.m. collection, 5.28 g) was extracted with $CHCl_3$/methanol/water using the method of Bligh and Dyer (*25*) to give $CHCl_3$/methanol (85.7% of yolk radioactivity), aqueous (5.9% of yolk radioactivity), and a residue (15.7% of yolk radioactivity). Figure 1 contains the fractionation scheme for egg yolk. The aqueous fraction was analyzed in HPLC system E. The ^{14}C residues had the same retention times as allantoin, proline, glycine, arginine, and glutamate at 0.1, 1.7, 1.1, 0.5, and 0.6% of the yolk radioactivity, respectively.

The $CHCl_3$ extract (85.7% of the yolk radioactivity) was evaporated under reduced pressure, and the residue was partitioned between hexane and ACN. The hexane 1 fraction (82.6% of yolk radioactivity) was evaporated to dryness under reduced pressure, and the residue saponified in 3 M KOH at reflux for 48 h. The mixture was acidified to pH 2 with concentrated HCl and extracted three times with hexane/ether (1:1) to give a hexane/ether fraction and an acidic aqueous fraction. The hexane/ether fraction (63.7% of yolk radioactivity) was evaporated to dryness, dissolved in $CHCl_3$/2-propanol (1:2, 5 mL), and fractionated as described under Lipid Fractionation to obtain a free fatty acid fraction and another nonpolar lipid fraction (58.6 and 4.1% of yolk radioactivity, respectively). The free fatty acid fraction obtained was derivatized and analyzed as described under HPLC. The HPLC chromatograms indicated the presence of radioactivity in myristic, oleic/palmitic, and stearic acids at 3.1, 41.3, and 6.9% of the yolk radioactivity, respectively. The other lipid fraction was analyzed in HPLC system F and shown to be cholesterol.

The acidic aqueous fraction (16.8% of yolk radioactivity) was lyophilized, and the resulting solids, which were mostly KCl, were extracted with absolute ethanol (2 × 50 mL). The ethanol (14.0% of yolk radioactivity) was evaporated to dryness, and the residue was reconstituted in deionized water and extracted with hexane (no. 3, 2 mL). The solids remaining contained 2.14% of the yolk radioactivity. The aqueous fraction was analyzed in HPLC systems B and C. The HPLC chromatograms indicated the presence of glycerol (3.8% of yolk radioactivity). Hexane 3 was fractionated as previously described under Lipid Fractionation into a fatty acid fraction and another lipid fraction (0.6 and 7.5% of the yolk radioactivity, respectively). The other lipid fraction was analyzed in HPLC system F; radioactivity had the same retention times as cholesterol and fatty acids (0.6 and 5.7% of yolk radioactivity, respectively).

The ACN fraction (2.7% of yolk radioactivity) was back extracted with hexane to give a hexane 2 (1.27%) and an ACN 2 fraction (0.73% of yolk radioactivity) as well as an insoluble fraction that was $CHCl_3$-soluble (0.52% of yolk radioactivity). The hexane 2 was saponified and extracted with hexane/ether to give an aqueous (0.52% of yolk radioactivity) and a fatty acid fraction (1.00% of yolk radioactivity), indicating that this fraction also contained triglycerides.

The solid residue from the Bligh and Dyer procedure was digested with a protease solution (7.58 mL) as described above.

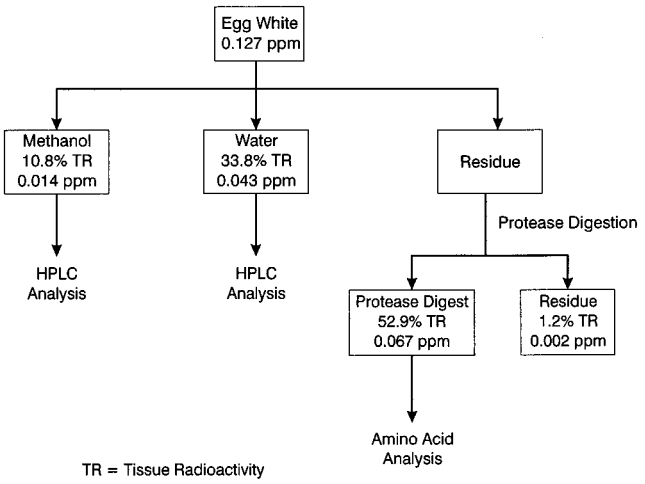

Figure 2. Fractionation scheme for egg white.

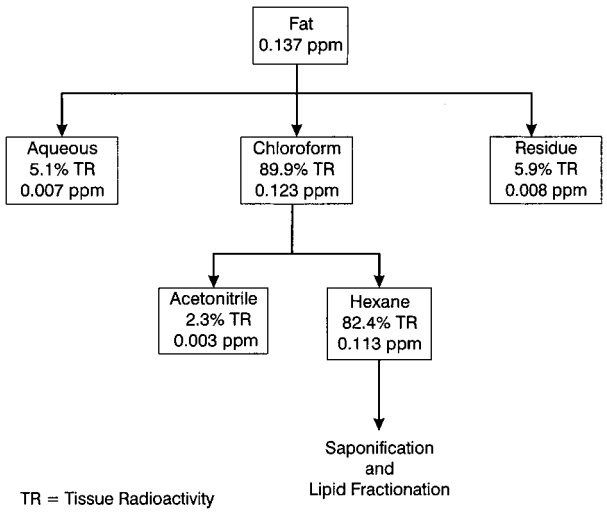

Figure 3. Fractionation scheme for fat.

The digest was centrifuged, and the pellet was washed with water and recentrifuged. The pellet was again digested with protease, and the supernatants of the two digestions (8.9% of yolk radioactivity) were combined, lyophilized, derivatized, and analyzed for amino acids using HPLC system E. Major radiolabeled amino acids were glycine and glutamate (2.7 and 2.2% of the yolk radioactivity, respectively) with lesser amounts of threonine and alanine (both 0.5% of the yolk radioactivity).

Egg White. Figure 2 summarizes the fractionation scheme for egg white. The homogenized pooled egg white (day 5 p.m., 10.33 g) was extracted sequentially with methanol and deionized water (3 × 50 mL) using a probe-type tissue homogenizer for ~1 min, followed by centrifugation. The extracts for each solvent were combined. The methanol and aqueous fractions contained 10.8 and 33.8% of the egg white radioactivity, respectively. The residue after extraction was digested with protease solution (59 mL) as described above. The digest contained 52.9% of the egg white radioactivity, and the remaining undigested solids contained 1.2% of the egg white radioactivity. The protease digest was lyophilized and analyzed for amino acids as described above. Radioactivity was incorporated into glutamate, glycine, and alanine (7.9, 7.8, and 5.1% of egg white radioactivity, respectively).

The methanol extract was analyzed in HPLC systems A−D. These indicated the presence of glyceric acid, lactic acid, and 1,3-propanediol (2.0, 2.3, and 2.2% of the egg white radioactivity, respectively, quantitated in HPLC system C), as well as a number of unidentified peaks.

The water extract was lyophilized and reconstituted in deionized water (2 mL). The sample was filtered using a 0.2-μm filter and stored in a freezer. Additional insoluble material precipitated during storage, so the sample was filtered again through a 0.45-μm filter before being analyzed in HPLC system E. Radioactivity was incorporated into glycine, glutamate, and aspartate (3.2, 1.2, and 1.4% of egg white radioactivity, respectively).

Fat. Fat homogenate (6.14 g) was extracted with $CHCl_3$/methanol/water using the method of Bligh and Dyer (*25*). Figure 3 contains the fractionation scheme for fat. The $CHCl_3$/methanol, aqueous, and insoluble fractions contained 89.9, 5.1, and 5.9% of the fat radioactivity, respectively. The $CHCl_3$ was evaporated to dryness under reduced pressure and dissolved in hexane, and the hexane was partitioned with ACN. The resultant hexane and ACN extracts contained 82.4 and 2.3% of the fat radioactivity, respectively. The hexane fraction was evaporated to dryness under reduced pressure, and the residue saponified with 3 M KOH (45 mL). The saponified sample was acidified to pH 2 with concentrated HCl and extracted with hexane/ether (1:1, 4 × 40 mL). The extract was evaporated to dryness and fractionated as outlined under Lipid Fractionation. This procedure had to be repeated twice more due to apparent incomplete saponification. The final combined hexane/ether extracts contained 70.0%, and the acidic aqueous fraction contained 25.2% of the fat radioactivity. Lipid fractionation separated the hexane/ether extract into fatty acids (62.5% of fat radioactivity) and other lipids (2.0% of fat radioactivity). The fatty acid fraction was derivatized and analyzed by radiochemical HPLC as described above. Radioactivity was incorporated into myristic, oleic/palmitic, and stearic acids (3.5, 33.5, and 5.2% of fat radioactivity, respectively).

The acidic aqueous fraction was lyophilized, and the residue was extracted with absolute ethanol. The residue and the ethanol contained 7.2 and 14.3% of the fat radioactivity, respectively. The ethanol fraction was dissolved in water and analyzed in HPLC systems B and C. Glycerol (9.7% of fat radioactivity) was the predominant radioactive peak in the chromatograms.

Liver. Liver homogenate (~9−15 g) was extracted sequentially with hexane, methanol, and water (3 × 50 mL) using a probe-type homogenizer, and the supernatants for each solvent extraction were combined. Figure 4 contains the fractionation scheme for the liver. The hexane 1, methanol 1, water 1, and insoluble fractions contained 6.1, 62.0, 7.2, and 23.1% of the radioactivity in liver, respectively. Water extract 1 was lyophilized and extracted sequentially with methanol (2 × 12.5 mL) and water (2 × 10 mL). Water extract 2 was lyophilized, reconstituted in a small amount of deionized water, filtered through a 0.45-μm filter, and analyzed in HPLC system D. The retention times of radioactivity indicated the presence of ornithine, glycine, arginine, glutamate, and aspartate (0.08, 0.09, 0.41, 0.13, and 0.21% of the radioactivity in liver, respectively). The methanol extract of water 1 was added to methanol 1 to form methanol 2. Methanol 2 was concentrated under reduced pressure to ~15−20 mL and centrifuged. The pellet was washed with methanol (10 mL) and centrifuged. The wash was added to the concentrated methanol 2 and then back extracted with hexane to give hexane 2. Hexane 2 contained 18.8% of the radioactivity in liver, whereas MeOH 2 contained 40.4% of the radioactivity in liver. Hexane 2 was combined with hexane 1 to give hexane 3 (24.8% of the radioactivity in liver).

Hexane 3 was concentrated to dryness under reduced pressure and the residue partitioned between ACN and hexane (35 mL each). Hexane 4 contained 21.6% and ACN 1 contained 2.4% of the radioactivity in liver. Hexane 4 was concentrated under reduced pressure and saponified for 7 h with 3 M KOH (30 mL). The sample was acidified to pH 2 with concentrated HCl and extracted with (1:1) hexane/ether (3 × 50 mL). The organic layer was washed three times with water (20 mL), and the washings were added to the aqueous layer to give acidic aqueous 1 (4.0% of the radioactivity in liver). The organic layer was designated hexane/ether 1 (16.8% of the radioactivity in liver) and was fractionated as described under Lipid Fractionation into fatty acids and other lipids (11.8 and 3.9% of the

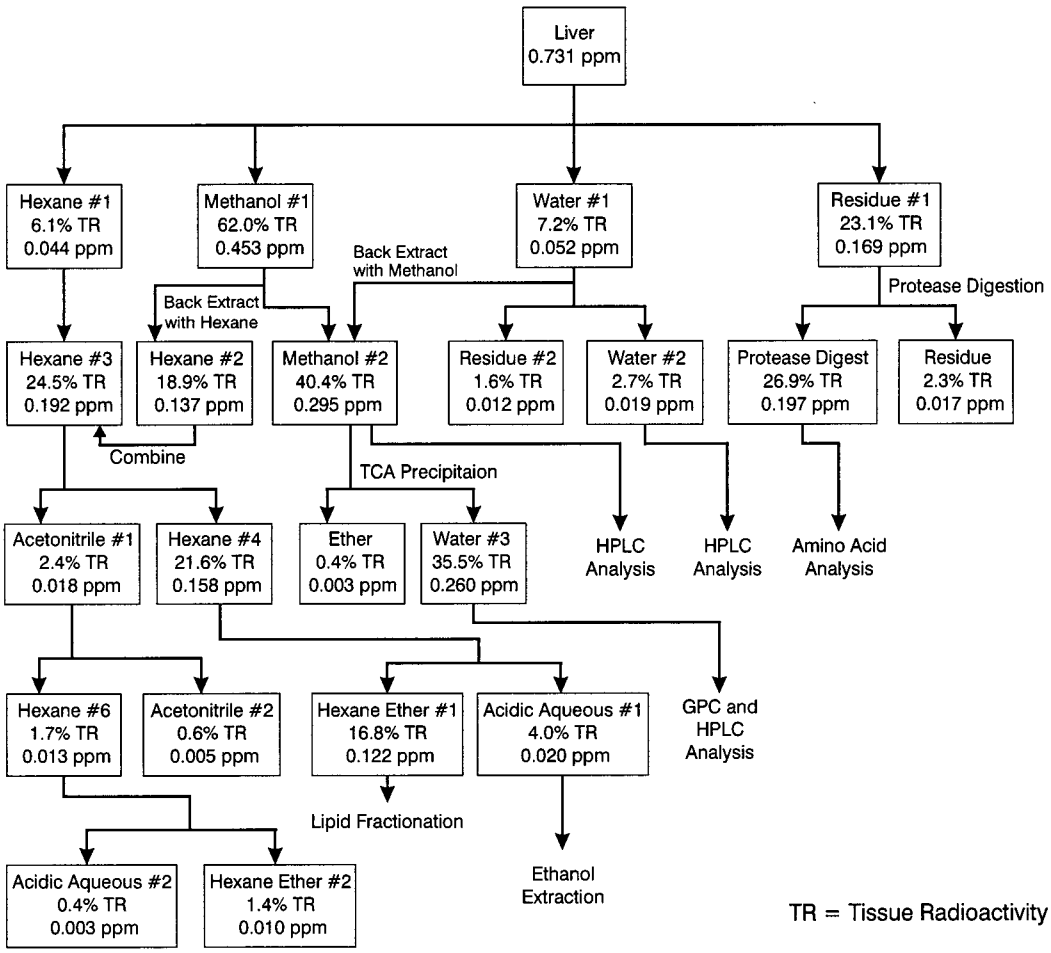

Figure 4. Fractionation scheme for liver.

radioactivity in liver, respectively). Derivatization and HPLC analysis of the fatty acids fraction indicated incorporation of radioactivity into linoleic, palmitic, oleic, and stearic acids (0.5, 2.7, 3.6, and 3.0% of liver radioactivity, respectively). The other lipid fractions were found to be cholesterol (3.1% of liver radioactivity). The acidic aqueous extract 1 was lyophilized and repeatedly extracted with absolute ethanol, cooled, and filtered. The residue contained 0.9% and the ethanol 2.6% of the liver radioactivity. The filtrates were combined, lyophilized, reconstituted in absolute ethanol (2 mL), and filtered. Approximately half of the sample was dried under N_2 and reconstituted in deionized water. The sample contained insoluble materials and was extracted with hexane (2 × 1 mL), which was designated hexane 5 (1.2% of liver radioactivity), and the water layer was filtered. It contained 0.9% of the liver radioactivity and was not analyzed further, although it presumably contains glycerol.

ACN 1 was evaporated, reconstituted in 2-propanol/ACN (2 mL), and analyzed in HPLC system F to give cholesterol (0.1% of liver radioactivity) and free fatty acids (1.6% of liver radioactivity). The remainder was dried under a stream of N_2 and partitioned with hexane to give hexane 6 (1.7% of the liver radioactivity) and ACN 2 (0.6% of the liver radioactivity). Hexane 6 was concentrated under reduced pressure, saponified with 3 M KOH (10 mL) for ~20 h, and worked up the same way as hexane 4. The acidic aqueous extract 2 contained 0.4% and the hexane/ether 2 extract 1.4% of the liver radioactivity.

A portion of methanol 2 was evaporated under a stream of N_2 and reconstituted in deionized water. A 10% trichloroacetic acid solution (1.25 mL) was added dropwise, and the sample was mixed by vortexing and centrifuged. The supernatant was extracted with ether (4 × 5 mL). The aqueous layer was concentrated under a stream of N_2, filtered, and applied to a Sephadex G-10 column (~10 g of gel, slurry-packed in water). The column was eluted with deionized water, and 3-mL fractions were collected. The radioactivity eluted into fractions 3−10, which were combined and lyophilized, reconstituted in deionized water (2 mL), and applied to a Sephadex G-50 column, which was also prepared and eluted with deionized water, and fractions were collected as with the G-10 column. Fractions 9−13 were combined, lyophilized, reconstituted in deionized water, and analyzed on HPLC system D. Radioactivity coeluted with allantoin, creatine, arginine, glutamate, and aspartate (0.7, 5.7, 1.7, 2.3, and 2.3% of liver radioactivity, respectively). This extract was injected multiple times into HPLC (system E), and fractions were collected. The major peaks were analyzed by TLC, which confirmed the presence of creatine, aspartate, and glutamate. The amino acid spots were ninhydrin positive as were a number of spots that did not correspond to the amino acid standards, indicating that they were peptides or small proteins.

The residue after extraction was digested with a protease solution (25 mL) as described above. The digest contained 26.9% and the residue 2.3% of the liver radioactivity. The digest was purified by cation exchange chromatography and derivatized and analyzed for amino acids as described above. Radioactivity was incorporated into aspartate, glutamate, serine, threonine, alanine, and methionine (0.5, 5.3, 2.5, 0.9, 1.1, and 0.9% of liver radioactivity, respectively).

Muscle. Breast or thigh muscle homogenate (~20 g) was extracted sequentially with hexane, methanol, and water in the same manner as liver. The fractionation schemes for breast and thigh muscle are in Figures 5 and 6, respectively. As with liver, the methanol was back extracted with hexane, and the hexane was combined with the original hexane extract. The combined hexane, methanol, and water fractions contained 1.4, 56.7, and 8.3% of the breast muscle radioactivity, respectively. The corresponding values for thigh muscle were 4.1, 61.8, and

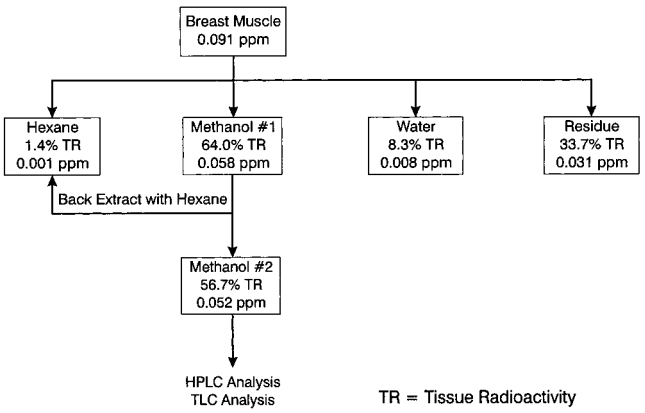

Figure 5. Fractionation scheme for breast muscle.

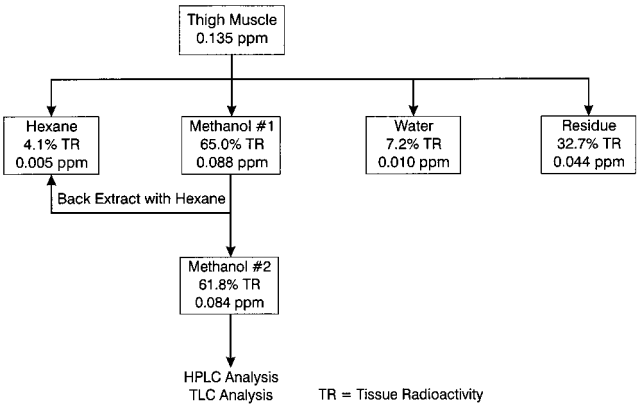

Figure 6. Fractionation scheme for thigh muscle.

7.2%, respectively. The residue contained 33.7 and 32.7% of the breast and thigh muscle radioactivity, respectively. The methanol extract was concentrated under reduced pressure and analyzed by HPLC in systems B and D and by TLC. Radioactivity was incorporated into lactate, glycine/serine, and ornithine/arginine (16.0, 5.5, and 5.7% of breast muscle radioactivity and 11.6, 7.7, and 3.1% of thigh muscle radioactivity, respectively). The amino acid TLC spots were ninhydrin positive. Because of the low levels of radioactivity (0.091 and 0.135 ppm, respectively) in the breast and thigh muscle, no analysis was performed on the hexane or water extracts or the residue.

Kidney. Kidney homogenate was extracted with hexane, methanol, and water in the same manner as liver and muscle. A flowchart of the fractionation scheme for kidney is shown in Figure 7. As with liver and muscle, the methanol was back extracted with hexane, and the hexane was combined with the original hexane extract. The combined hexane, methanol, and water fractions contained 7.7, 63.3, and 6.0% of the radioactivity in kidney, respectively. The residue contained 22.0% of the radioactivity in kidney.

The hexane extract was concentrated under reduced pressure and partitioned between hexane and ACN. The hexane 3 and ACN 1 extracts contained 7.7 and 1.7% of the radioactivity in kidney, respectively. Hexane 3 was concentrated under reduced pressure and the residue saponified with 3 M KOH. The solution was acidified with concentrated HCl and extracted with hexane/ether (1:1). The organic layer was washed with water, and the water was added to the acidic aqueous layers. The water layer contained 1% of the radioactivity in kidney and was not analyzed further, although it presumably contained glycerol. Hexane/ether 1 (7.1% of the radioactivity in kidney) was fractionated as described under Lipid Fractionation into fatty acids (3.9% of radioactivity in kidney) and other lipids (2.6% of radioactivity in kidney). The fatty acid fraction was derivatized as described above. Radioactivity was incorporated into palmitic, oleic, and stearic acids (1.1, 1.0, and 1.2% of the radioactivity in kidney). The other lipid component was shown to coelute with cholesterol (2.1% of the radioactivity in kidney) in HPLC system F. AC was concentrated under reduced pressure and partitioned with hexane (no. 4, 1.5% of the radioactivity in kidney) and ACN (no. 2, 0.3% of the radioactivity in kidney). Hexane 4 was saponified, acidified, and extracted with hexane/ether (1:1). The hexane/ether contained 1.1% and the acidic aqueous 0.3% of the radioactivity in kidney.

The methanol extract was analyzed in HPLC system E and TLC system A. Radioactivity was incorporated into proline, glycine, citrulline, aspartate, and glutamate (0.5, 0.4, 0.3, 5.3, and 0.5% of the radioactivity in kidney, respectively). The TLC spots assigned to amino acids were ninhydrin positive, as were a number of spots that did not correspond to any of the standard amino acids, indicating that these spots were either small peptides or proteins. The water extract was lyophilized to dryness and analyzed in HPLC system E. Radioactivity was incorporated into glycine, arginine, glutamate, and aspartate (0.1, 0.2, 0.1, and 0.1% of the radioactivity in kidney, respectively).

The residue from a similar extraction was digested with protease (25 mL) as described above. The digest contained 22.8% of the radioactivity in kidney, whereas the insoluble residue contained 1.7% of the radioactivity in kidney. The supernatant was purified by cation exchange chromatography and analyzed for amino acids as described above. Radioactivity was incorporated into aspartate, glutamate, serine, and glycine (0.2, 3.4, 1.2, and 2.1% of radioactivity in kidney, respectively).

RESULTS AND DISCUSSION

A summary of the radioactive metabolites identified in the hen is found in Table 4.

Egg Yolk. Fatty acids were the primary radioactive component in egg yolk, representing 58.6% of the radioactivity in yolk. Incorporation of radioactivity into amino acids, cholesterol, allantoin, and glycerol represented 9.5, 3.8, 0.1, and 3.8% of the radioactivity in yolk, respectively. The total accountability of radioactivity from all extracts, residues, and chromatograms was 96.3%. In contrast to the metabolism of acrolein in the goat, incorporation of radioactivity into glycerol was comparable to the incorporation into fatty acids on a per-carbon basis, indicating that direct incorporation of the three-carbon unit of acrolein into glycerol is not occurring in the hen. It appears that acrolein was metabolized to CO_2, which can be incorporated by biosynthesis into fatty acids and glycerol.

Egg White. Amino acids were the primary radioactive component in egg white, representing 26.6% of the egg white radioactivity. Minor components were glyceric acid, lactic acid, and 1,3-propanediol, which represented 2.0, 2.3, and 2.2% of the egg white radioactivity, respectively. 1,3-Propanediol was the only metabolite in the hen that is not a normal product of intermediary metabolism. It has been reported as a bacterial metabolite of glycerol (*26*). The total accountability of radioactivity from all extracts, residues, and chromatograms was 97.5%.

Fat. Radioactivity in fat was found after saponification as glycerol and fatty acids, which represented 9.7 and 54.9% of the fat radioactivity, respectively. This contrasts with the results of the analysis of goat fat (*18*) in which the majority of the radioactivity was found in the glycerol fraction. This suggests that very little of the acrolein is incorporated as an intact three-carbon moiety. The total accountability of radioactivity from all extracts, residues, and chromatograms was 103%.

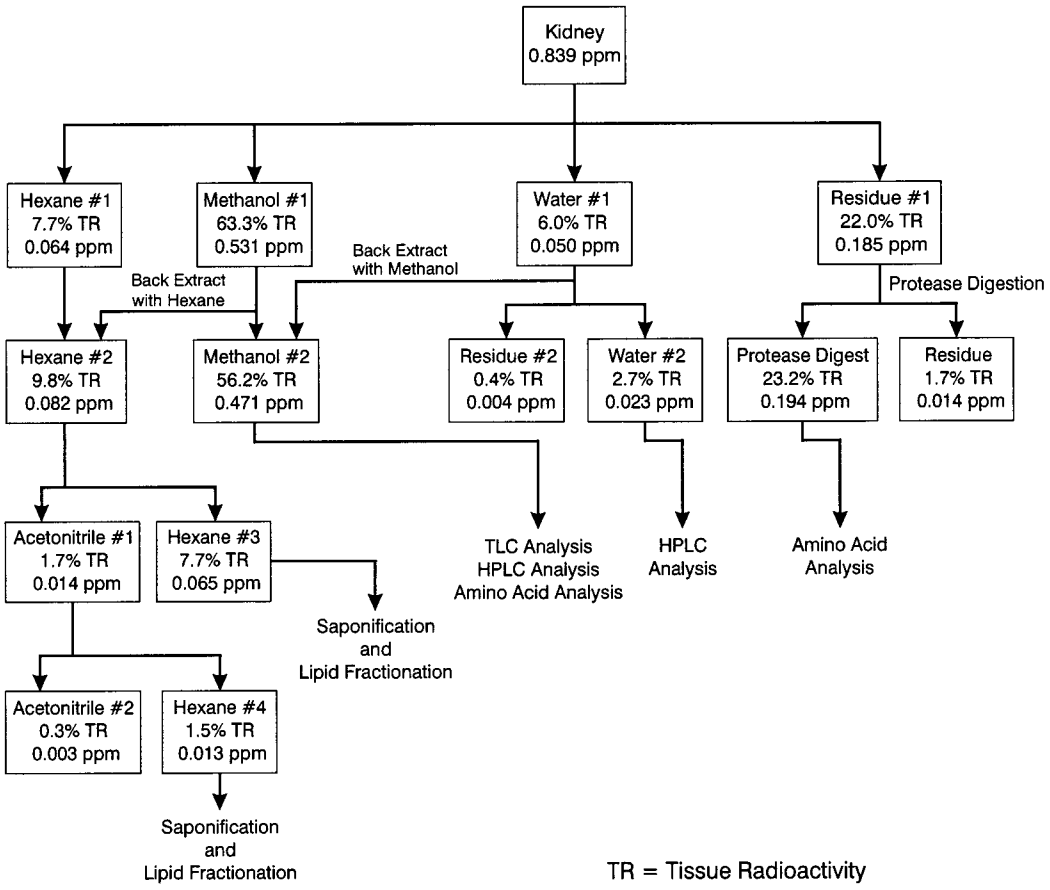

Figure 7. Fractionation scheme for kidney.

Table 4. Summary of the Metabolites Identified in the Eggs and Tissues of Laying Hens Administered [^{14}C]Acrolein (1.09 mg/kg of Body Weight/Day) by Gavage for Five Consecutive days

	egg yolk	egg white	fat	kidney	liver	thigh muscle	breast muscle
fatty acids	58.6% 0.733 ppm		54.9% 0.075 ppm	4.3% 0.036 ppm	12.4% 0.092 ppm		
amino acids	9.5% 0.119 ppm	26.6% 0.033 ppm		14.3% 0.120 ppm	15.2% 0.111 ppm	10.8% 0.014 ppm	11.1% 0.010 ppm
cholesterol	3.8% 0.048 ppm			2.1% 0.018 ppm	3.2% 0.023 ppm		
glycerol	3.8% 0.048 ppm		9.7% 0.013 ppm				
lactate		2.3% 0.003 ppm				11.6% 0.016 ppm	16.0% 0.014 ppm
1,3-propanediol		2.2% 0.003 ppm					
glyceric acid		2.0% 0.003 ppm					
creatine					2.6% 0.019 ppm		
allantoin	0.1% 0.002 ppm						

[a] Percentages in the table represent the percentage of egg or tissue radioactivity.

Liver. Liver radioactivity was distributed into amino acids, fatty acids, cholesterol, and creatine. They represented 15.2, 12.4, 3.2, and 2.6% of the liver radioactivity, respectively. Again, in contrast to the metabolism of acrolein in the goat, there was no evidence for the direct incorporation of the three-carbon unit of acrolein into natural products. Total accountability of radioactivity from all extracts, residues, and chromatograms was 83.7%.

Muscle. The major metabolites in muscle were lactic acid, which represented 16.0 and 11.6% of the radioactivity in breast and thigh muscle, respectively, and amino acids, which represented 11.1 and 10.8% of the radioactivity in breast and thigh muscle, respectively. The presence of lactate is probably due to anaerobic metabolism during tissue collection. The total accountabilities of radioactivity from all extracts, residues, and chromatograms were 86.3 and 84.9% for breast and thigh muscle, respectively.

Kidney. The largest percentage of radioactivity in the kidney was incorporated into amino acids; they represented 14.3% of the kidney radioactivity. Other metabolites identified were fatty acids and cholesterol. They represented 4.3 and 2.1% of the kidney radioactivity,

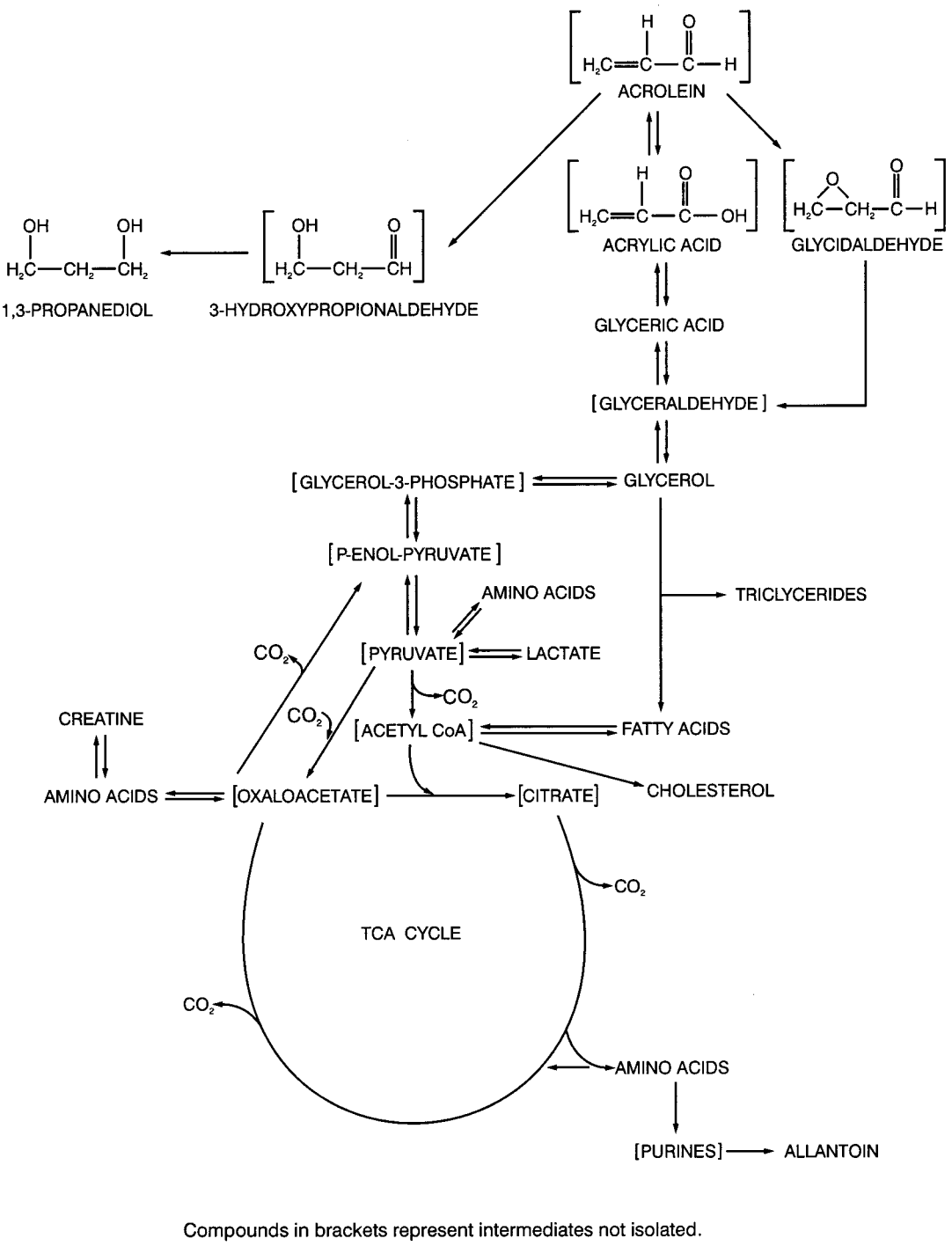

Figure 8. Proposed metabolic pathway for acrolein in the hen.

respectively. The total accountability of radioactivity from all extracts, residues, and chromatograms was 83.5%.

Summary. A proposed metabolic pathway for acrolein in the hen is shown in Figure 8. With the exception of a small amount of 1,3-propanediol in egg white, all of the identified metabolites found in laying hens after administration of acrolein were the result of the incorporation of acrolein into the normal natural products of intermediary metabolism. In contrast to the metabolism of acrolein in the goat, there was no evidence that the three-carbon unit of acrolein was incorporated intact into natural products; instead, the incorporation of radioactivity appeared to result from the metabolism of acrolein to smaller molecules followed by incorporation of these metabolites into the normal biosynthetic pathways.

SAFETY

Acrolein is toxic and a powerful irritant, and neat acrolein should be handled in an efficient hood. The usual precautions for handling radioactive materials and disposal of radioactive waste were followed.

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