achieve maximum levels of this pool. For any substance following first-order kinetics, 90% of the steady-state level is achieved when the dosing period is 3.3 times the half-life.

Because of the apparent rapid depletion of this pool, the on-drug level is influenced by the drug consumption in the time period immediately before termination of the experiment. After 3 days the level of drug residues in this pool is probably too low to contribute significantly to the total residue.

The half-life of the persistent pool appeared to be 3.3 days based on the 4-day radioactive pulse data. This pulse is only 1.2 half-lives and hence is of insufficient duration to achieve a steady-state level for this pool. The data from the 4-day radioactive pulse experiments were used to estimate both the duration of the radioactive pulse required to achieve 95% of the steady-state level and the size of the persistent pool.

In these experiments the animals were exposed to nonradioactive drug for a prolonged period before introducing the radioactive dose. The tissues therefore would have achieved a steady residue level. During the pulse period radioactive residues are replacing the nonradioactive residues already present in the bird. Thus, during the 4-day pulse interval, the nonradioactive residue would deplete 1.2 half-lives to a level of 43% of the initial level. Since the radioactivity reflects only 57% of the total pool, the anticipated persistent residue would be about 0.53 ppm at zero withdrawal. Similarly, it can be estimated that about 14 days of radioactive pulse would be required to displace 95% of the nonradioactive residue with the radioactive residue. For this reason an experiment using a 14-day pulse was carried out. The estimated on-drug level of the persistent pool was found to be 0.55 ppm, thus corresponding closely to that predicted. The apparent half-life, however, appeared to be about 4.1 days instead of 3.3 days determined from the 4-day pulse. This suggests that the persistent residue pool present after a 4-day pulse consists of substances with half-lives both longer and shorter than 3.3 days and that a pulse period greater than 14 days could be required to attain 95% of the steady-state level. Determination of the probable residue level due to the persistent pool using a 4.1-day half-life gave an estimated on-drug level of 0.6 ppm.

When a lifetime exposure to radioactive drug was carried out, the estimated value of the persistent residue (Table VII) was found to be about 0.68 ppm. This level may be influenced by even a trace amount of the radioactivity incorporated into the endogenous metabolism of the host.

In these experiments, the half-life of the persistent pool did not change appreciably (3.3–4.1 days) as the length of the radioactive pulse was increased. Thus, any single experiment of any pulse length can be used to estimate the steady-state level of the persistent pool. The slight change in the half-life also is an indication that only a small fraction of the drug is converted to endogenous residues since such residues would have a longer half-life by virtue of reincorporation (Von Wittenau, 1967).

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Tissue Residues of Arprinocid in Chickens. 2. A Common Derivative Approach for the Analysis of Residues in Liver

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Residue analysis of tissues taken from chickens administered ^{14}C-labeled arprinocid [9-[(2-chloro-6-fluorophenyl)methyl]-9*H*-purin-6-amine] showed that liver is the edible tissue containing the highest level of radioactivity after drug withdrawal. Less than 10% of this residue was assayed as either unchanged drug or known metabolities. Solvent extractions carried out by using homogenates of liver samples taken from birds on drug through 5 days after drug withdrawal showed that most of the radioactivity could not be separated from the insoluble fraction. Strong acid hydrolysis converted 88–74% of the respective residues to a readily extractable compound identified as 2-chloro-6-fluorobenzylamine. Through analysis of this amine, both drug and unknown (but drug-related) metabolites can be quantitated—regardless of whether the residue is solvent extractable or covalently bound to cellular macromolecules. Direct assay of the free base 2-chloro-6-fluorobenzylamine has serious limitations; however, the dansyl derivative proved to be an ideal compound and serves as the basis of the analytical assay of arprinocid-derived residues.

As described in the preceding paper (Jacob et al., 1982), tissue distribution studies with ^{14}C-labeled arprinocid [9-[(2-chloro-6-fluorophenyl)methyl]-9*H*-purin-6-amine (Figure 1)], a new coccidiostat (Kilgore et al., 1978; Olson et al., 1978) showed that liver is the edible tissue containing the highest level of radioactive residue. This residual radioactivity was equivalent to 0.1–0.3 ppm, expressed as

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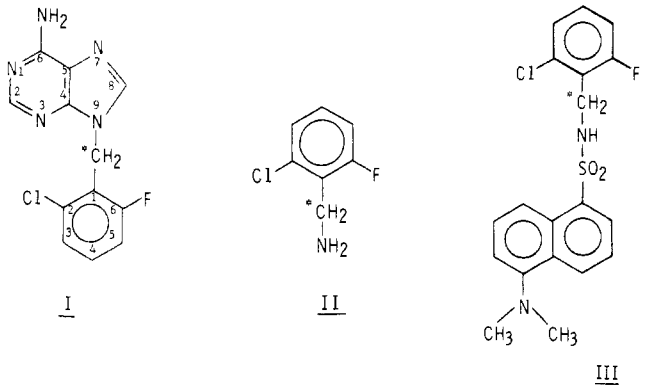


I II III

Figure 1. Structure of arpronocid (I), 2-chloro-6-fluorobenzylamine (II), and the dansyl derivative of 2-chloro-6-fluorobenzylamine (III). The site of the radioactivity in the ^{14}C-labeled form of each compound is noted (*). The analogous compounds I and II are not radioactively labeled when used as carriers.

arprinocid, at 3–5 days after drug withdrawal. Using a modification of the reverse isotope dilution assay technique (Jacob et al., 1975), we found that arprinocid accounted for less than 4% of these residues and known metabolites for about 2%; thus, an "intractable" residue of greater than 90% was apparent. Fractionation of the radioactive residues in these livers into solvent-soluble, water-soluble, and insoluble components showed that over 50% of the radioactivity could not be separated from the insoluble fraction. Release of additional radioactivity from these residues required chemical treatment capable of degrading the macromolecular cellular constituents. As the major radioactive substance liberated by strong acid hydrolysis of liver residues is 2-chloro-6-fluorobenzylamine, methods of analyzing for this substance were investigated. The dansyl derivative of this amine was found to be suitable for this purpose and was used to determine the fraction of the total radioactive residue which yields this drug-related moiety upon acid hydrolysis. Further studies employing the dansyl derivative of 2-chloro-6-fluorobenzylamine for the practical analysis of tissue residues have been reported elsewhere (Cala-Tway et al., 1979).

MATERIALS AND METHODS

Chemicals. *Dansyl chloride [5-(dimethylamino)-1-naphthalenesulfonyl chloride]* was obtained from Sigma Chemical Co. and recrystallized from isooctane before use.

Arprinocid, obtained from the Merck Sharp & Dohme Research Laboratores sample collection, was synthesized by reaction of 2-chloro-6-fluorobenzyl chloride with adenine. The product was of greater than 98% purity and contained about 1.0-1.2% of the 3 isomer.

The ^{14}C-labeled arprinocid, prepared by Dr. R. L. Ellsworth of our laboratoreis, was >99% pure and contained less than 0.5% of any single impurity.

2-Chloro-6-fluorobenzylamine (II) was prepared by treating 2-chloro-6-fluorobenzyl alcohol (Aldrich Chemical Co.) with 48% HBr to produce 2-chloro-6-fluorobenzyl bromide. This intermediate was reacted with methanolic ammonia to yield the benzylamine. The hydrochloride was recrystallized from 2-propanol.

(2-Chloro-6-fluorobenzyl)urea was prepared by heating 2-chloro-6-fluorobenzylamine with nitrourea in aqueous 2-propanol at 90–95 °C for 20 min. The solution was concentrated and water added, whereupon the urea crystallized.

9-(2-Chloro-6-fluorobenzyl)-6-oxopurine was prepared by the nitrous acid hydrodeamination of arprinocid.

9-(2-Chloro-6-fluorobenzyl)-6-aminopurine 1-oxide was prepared by oxidizing arprinocid with 3-chloroperbenzoic acid in methanol at reflux. The *N*-oxide was recrystallized from 2-propanol–water–acetic acid (5:5:1).

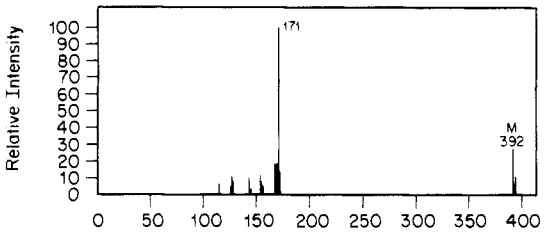


Figure 2. Electron impact mass spectrum of the dansyl derivative of 2-chloro-6-fluorobenzylamine. Conditions are given under Materials and Methods.

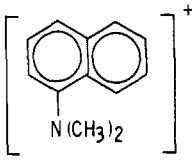
9-(2-Chloro-6-fluorobenzyl)-2,6-dioxopurine was prepared from the *N*-oxide. The latter was hydrolyzed in 1 N HCl at reflux for 4 h and then allowed to stand at 25 °C for 3 h and filtered. The residue obtained by evaporating the filtrate was dissolved in 6 N HCl and the solution stirred at 25 °C whereupon 5-amino-1-(2-chloro-6-fluorobenzyl)-4-imidazolecarboxamide crystallized. After recrystallization from ethanol, the amide was heated with urea at 175 °C for 2 h. The solid product was washed with water and then extracted with 0.5 N NaOH. The xanthine crystallized upon acidification of the basic solution.

1-[(2-Chloro-6-fluorobenzyl)sulfonamido]-5-(dimethylamino)naphthalene (III) was synthesized by reacting dansyl chloride (200 mg) dissolved in 60 mL of acetone with 2-chloro-6-fluorobenzylamine hydrochloride (160 mg) dissolved in 50 mL of 0.2 M $NaHCO_3$ (pH 8.5). The mixture was heated for 30 min at 50–55 °C and the reaction mixture was then evaporated to 40 mL. The concentrate was diluted with 300 mL of water and extracted twice with equal volumes of ethyl acetate. The combined ethyl acetate extracts were evaporated to dryness. The residue was dissolved in 10–15 mL of warm 2-propanol, and the product was crystallized by adding water dropwise to the propanol solution. The crystalline product was isolated by filtration, washed sequentially with water and isooctane, and then air-dried. The yield was 181 mg, 85% of theory. Product evaluation: elemental analysis was as follows. Calcd: C, 58.10; H, 4.60; N, 7.13; Cl, 9.02. Found: C, 57.99; H, 4.56; N, 7.00; Cl, 9.39. mp 145 °C; no weight loss was observed by differential thermal analysis; UV_{MeOH} (Cary Model 15) λ_{max} 216 nm (ϵ_{mol} 42.4 × 10^{-3}), 252 (ϵ_{mol} 13.3 × 10^{-3}), 336 (ϵ_{mol} 4.1 × 10^{-3}).

Methods. *High-Performance Liquid Chromatography (HPLC).* A Du Pont 841 liquid chromatograph was used with a Waters C_{18} μBondapak column (30.5 cm × 4 mm). An acetonitrile–water system (45:15) was used for column development at a flow rate of 1 mL/min at 1000 psi. The product was detected as a single UV peak (254 nm) at 15 min.

Gas–Liquid Chromatography (GLC). A Hewlett-Packard 5730A gas chromatograph was used with a nitrogen bead detector and an 8 m × 0.3 mm i.d. SE-30 glass capillary column. The column oven, detector, and injector temperatures were 265, 300, and 300 °C, respectively. The single product peak appeared at 3.8 min with a helium carrier gas linear velocity of 40 cm/s.

Mass Spectrometry. The LKB 9000 mass spectrometer (direct inlet sample introduction) was operated at 70-eV ionization potential, 3.5-kV accelerating potential, 50-μA trap current, 250° source temperature. The computer (Varian 620 i) output spectrum (only ions greater than 5% relative intensity) of the dansyl derivative III is presented in Figure 2. The monochloro molecular ion is found at *m/e* 392, as required, with the base peak at *m/e* 171.



m/e 171

Solvents and Chromatographic Materials. All solvents were glass distilled. Silica GF plates were purchased from Analtech, Inc. The plates were developed with methanol before use to remove traces of fluorescent material. The following thin-layer chromatographic systems were used:

	R_f of dansyl derivative III
system 1: *n*-hexane-2-propanol-ammonium hydroxide (28%), 90:10:0.5	0.60
system 2: isooctane-2-propanol, 80:20	0.50
system 3: isooctane-diethyl ether, 10:90	0.54
system 4: benzene-dioxane-ammonium hydroxide (28%), 10:80:10	0.70

Radioactivity Measurements. A Packard Tri-Carb (Model 3320) liquid scintillation spectrometer was used for the radioactivity measurements. Samples were counted either directly in a toluene–ethanol (70:30) solution of Omnifluor phosphor (4 g/L) or combusted in a Packard oxidizer (Model 306) in the presence of oxygen. The generated $^{14}CO_2$ was trapped in a Carbo-Sorb/Permafluor phosphor and the solution counted as above. Counting efficiencies for each sample were determined by the external standard method and all results expressed as dpm.

Acid Hydrolysis Procedures. Samples were dissolved in 6 N HCl and hydrolyzed by either of two procedures. In the first, the sample in HCl was sealed in a glass tube and heated for about 20 h in an oil bath maintained at 120 or 150 °C. In the second, a loosely stoppered flask containing the sample in 6 N HCl was autoclaved for 18 h at about 135–140 °C.

Liver tissue samples were homogenized for 1 min in a high-speed Virtis homogenizer by using a ratio of 1 g of wet tissue to 10 mL of 6 N HCl.

Reverse Isotope Dilution Analysis (RIDA) of Tissue Samples. Most determinations were carried out in duplicate. The nonradioactive species of either aprinocid (I) or 2-chloro-6-fluorobenzylamine (II) was added and served as the carrier. After hydrolysis the sample was cooled and the pH adjusted to 10–11 with 10 N sodium hydroxide. The aqueous layer was extracted 3 times with an equal volume of toluene, and the combined extracts were washed with about $^1/_3$ volume of 1 N HCl. The acid extract was evaporated to dryness, the residue was dissolved in 2 mL of 0.2 M sodium bicarbonate, and 1.5 mL of a 1% solution of dansyl chloride in acetone was added. The tube was loosely capped and heated at 50 °C for 30 min. The acetone was removed under a stream of nitrogen. After the aqueous residue was diluted with water, the pH was adjusted to 10 with sodium hydroxide and the solution extracted twice with 4 mL of hexane.

The hexane extract was evaporated to dryness and the residue purified by a thin-layer chromatographic procedure. The residue was dissolved in a small quantity of methanol and the solution applied as a streak across a 2 × 8 in. Analtech GF plate. After development with solvent system 1, the product zone, located by visual inspection under UV light, was carefully removed and the product eluted with methanol. The methanol eluate was rechromatographed by using solvent system 2.

A specific activity determination was carried out on the methanol eluate of the product zone from solvent system 2 by determining the mass concentration of product (based on absorbance measurements at 336 nm) and radioactivity content (based on scintillation counting) of an aliquot of the methanol solution (Jacob et al., 1975). The remaining methanol eluate was rechromatographed in system 3 and in system 4 if necessary. The analysis was considered satisfactory when the specific activity of successive chromatographic eluates did not vary significantly.

Verification of Assay Procedure for Arprinocid in Liver. Two methanol solutions were prepared each containing about 1.0 μg of ^{14}C-labeled arprinocid and 600 μg of unlabeled arprinocid. The specific activity of the drug in each solution was determined by measuring (as described above) the radioactivity and total arprinocid. The methanol was evaporated from each solution under a stream of nitrogen. The first methanol residue (A) was hydrolyzed in 6 N HCl at 150 °C for 18 h. The second methanol residue (B) was hydrolyzed at 150 °C for 18 h following the addition of a homogenate of 2 g of chicken liver in 20 mL of 6 N HCl. A reverse isotope dilution assay was carried out on each sample by using the standard dansylation procedure.

Animal Handling. Chickens (Hubbard–Hubbard strain) were grown on a standard broiler mash containing 0.007–0.008% arprinocid. Following administration of the ^{14}C-labeled arprinocid for 4 or 14 days, the labeled drug was removed from the diet, and three males and three females were slaughtered at each of several withdrawal periods. Samples of edible tissues from each bird were taken, blended in a grinder, and stored frozen until assayed.

Tissue Analysis. Samples of frozen ground tissues were carefully thawed and weighed into an appropriate Virtis homogenizer cup. After a weighed quantity of water or 6 N HCl was added, the samples were homogenized at high speed to produce a uniform tissue homogenate. The homogenate was sampled for radioactivity determinations or solvent extraction studies.

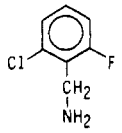
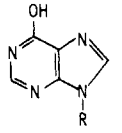
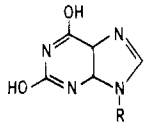
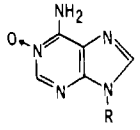
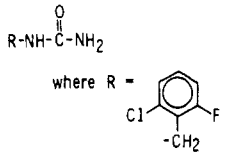
RESULTS AND DISCUSSION

Preliminary studies showed that arprinocid and closely related metabolites can be extracted from aqueous solution by use of ethyl acetate. Thus solvent extraction was employed to characterize the nature of the radioactive residue found in aliquots of liver tissue taken from birds 1 and 5 days after drug withdrawal. The ethyl acetate extracts were found to contain 25% and 15% of the total tissue radioactivity, respectively, whereas the corresponding solid residues contained 53% and 70% of the total tissue radioactivity.

Since most of the radioactivity was associated with the solid fraction, the liver tissue was digested with ficin (Glazer and Smith, 1971) to determine whether extractable substances were produced. This treatment resulted in only a slight increase in ethyl acetate extractable substances. Thus it is unlikely that the radioactivity associated with the solid residue was bound to the cellular constituents by a simple adsorptive process.

Vigorous acid hydrolysis of a liver sample taken from a chicken slaughtered 5 days after drug withdrawal increased the ethyl acetate extractable radioactivity to more than 50% of the total radioactivity in the sample. After purification by thin-layer chromatography, the radioactive hydrolysis product was identified as 2-chloro-6-fluorobenzylamine (II). Formation of radiolabeled macromolecular liver components could result from oxidative cleavage of the labeled benzyl moiety from the adenine ring. In this case the labeled macromolecule would be

Table I. Yield of 2-Chloro-6-fluorobenzylamine after Hydrolysis with 6 N HCl for 20 Hours at 150 °C

compound added (nonradioact)	% yield of II[a]
	98
	87
	71
	92
	72

[a] Analyses based on the determination of the specific activity of the dansyl derivative when ^{14}C-labeled arprinocid was added to the sample before hydrolysis. Percentage values are compared to the arprinocid assay taken as the 100% reference.

associated with incorporated 2-chloro-6-fluorobenzyl alcohol (or an oxidized product) lacking the nitrogen atom from the adenine. A second possibility involves the formation of a covalent bond between a macromolecular compound and the adenine moiety. In this case the nitrogen atom of the adenine ring would remain attached to the benzyl residue and acid hydrolysis would yield 2-chloro-6-fluorobenzylamine. As the covalent bonding leading to macromolecularly bound residues involves the purine rather than the benzyl moiety of the arprinocid, the generation of II could thus be used as a measure of the drug-related residues present in tissue—both the solvent-extractable residue and that associated with the macromolecular constituents.

As II is a liquid at room temperature, is volatile as the free base, and has low UV absorbance, it was considered important to convert this amine to a more manageable compound to allow its assay. We initially tried derivatizing II with *o*-phthalaldehyde in the presence of 2-mercaptoethanol (Roth, 1971). The derivative was stable in aqueous solution but was not produced in acceptable yield. An almost quantitative yield of derivatized II was obtained by using fluorescamine (Udenfriend et al., 1972; Nakumura and Pisano, 1972); however, the derivative rapidly hydrolyzed in water to form nonfluorescent products and underivatized amine. Dansyl chloride was found to be a very useful reagent for converting primary amino group containing compounds to appropriate derivatives for analytical purposes (Seiler and Wiechmann, 1970), and dansyl derivatives have been employed in pesticide residue analysis (Lawrence et al., 1976). The dansyl derivative of II was found to be an ideal product for assay purposes. This derivative has strong fluorescence, a unique UV absorbance, is easily synthesized, and is stable in aqueous solution. Chromatographic properties were generally excellent and a variety of solvent systems were found which gave excellent thin-layer chromatographic separations; suitable conditions for its HPLC and GLC were also established. In addition, the relatively nonpolar character of the derivative allowed its ready separation from most of the other dansyl products present in extracts of the tissue hydrolysates.

Table II. Comparison of Assay Results from Hydrolyses Carried Out either in Sealed Tubes or in an Autoclave in Open Flasks

sample	hydrolysis conditions	% of total radioact converted to dansyl-(chlorofluoro-benzyl)amine[b]
liver, 5 days postdose[a]	sealed tube; 150 °C; 20 h	72.4, 71.2, 76.0, 75.1
liver, 5 days postdose[a]	autoclave; 138 °C; 18 h	71.2, 70.2

[a] Liver from chickens dosed with ^{14}C-labeled arprinocid for 14 days. [b] Determined by RIDA with the nonradioactive form of I as the carrier and isolation of III.

Table III. Liberation of 2-Chloro-6-fluorobenzylamine (II) from Fractions of Chicken Liver Taken One Day after Drug Withdrawal

fraction	% of total radioact in fraction	% of total radioact recovered as II[a]	% of radioact in fraction recovered as II[a]
total homogenate	100	65	
ethyl acetate extract	25	24	96
water-soluble fraction	23	7	30
insoluble fraction	53	26	49

[a] Based on dansyl RIDA with the nonradioactive form of I added as a carrier before hydrolysis with 6 N HCl at 120 °C for 20 h.

Analytical studies, using the dansyl derivative for quantitation, were carried out to establish satisfactory conditions for the generation of II from model compounds and from various radioactive tissue samples and fractions. The model compounds all gave high yields of II on acid hydrolysis (Table I) with 6 N HCl at 150 °C for 20 h.

The validity of the assay for unaltered arprinocid in liver was demonstrated by assaying mixtures of nonradioactive and ^{14}C-labeled arprinocid in the presence and absence of liver (see Methods for details). In the presence of liver, the assay value was 96.5% of theory, and in the absence of liver it was 98.2% of theory. It was concluded that liver had no significant effect on the assay results.

The two hydrolysis procedures described (see Methods) were compared by assaying liver from a chicken sacrificed 5 days following drug withdrawal. The assay results in Table II show no significant differences between the two procedures.

In order to characterize the types of drug-related metabolites present in liver, a sample taken from a chicken slaughtered 1 day after drug withdrawal was fractionated into ethyl acetate soluble, water-soluble, and water-insoluble fractions. Each fraction was then subjected to acid hydrolysis and analyzed for II by the dansyl RIDA procedure. The results in Table III show that of the total fractions analyzed, the percentage of the radioactivity in each fraction which was hydrolyzed to II ranged from 30% of the water-soluble fraction to 96% of the ethyl acetate

Table IV. Liberation of 2-Chloro-6-fluorobenzylamine (II) from Liver Tissue[a] from Chickens Dosed with ^{14}C-Labeled Arprinocid

hydrolysis conditions		% of the total radioact residue in liver	
time, h	oil bath temp, °C	arprinocid (I)	2-chloro-6-fluoro-benzyl-amine (II)
4	100	30	24
4	150	98	60
24	150	76	70
48	150	78	77

[a] Chickens slaughtered 4 days after withdrawal from radioactive drug.

Table V. Assay of Liver from Chickens after Dosing with ^{14}C-Labeled Arprinocid for 14 Days

time of withdrawal, days	total radioact, ppm[a] (expressed as I)	fraction recovered as II[b]	
		%	ppm (expressed as I)
on drug	2.99	88.0	2.63
3	0.338	78.2	0.264
4	0.274	75.0	0.206
5	0.237	73.5	0.174

[a] Determined by combustion analysis. [b] Determined by RIDA using the nonradioactive form of I as the carrier and III for isolation. Samples hydrolyzed at 150 °C for 20 h.

extract. These data demonstrate the complexity and varied nature of the compounds which comprise the residue.

Further studies were designed to indicate the possibility of using the acid hydrolysis as a basis for a practical analysis of drug-related residues in tissues. These studies included hydrolyses run under a variety of conditions and a measure of the stability of II under these conditions.

The results of assays carried out on liver tissue with either I or II as the nonradioactive carrier and under conditions of incomplete (4 h; 100–150 °C) or complete (48 h; 150 °C) hydrolysis are given in Table IV.

The results in Table IV suggest a significant fraction of drug-related metabolites is more readily hydrolyzed than is arprinocid. This conclusion is based on the following observations. (1) The use of II as a carrier will give the true assay value or else a value that is too low because of the incomplete conversion of I or metabolite to yield ^{14}C-labeled II. (2) The use of I as a carrier will give, under conditions of incomplete hydrolysis, values that are too high if the metabolite(s) hydrolyzes (hydrolyze) more completely than does the carrier. Hydrolysis for 4 h at 100 °C was insufficient to hydrolyze the carrier completely, and I was recovered from the reaction mixture. (3) The hydrolysis carried out for 48 h at 150 °C gave essentially the same assay value for each carrier (78% vs. 77%), suggesting that the hydrolysis of the carrier and metabolites was complete and that the yield to II was very high.

Finally, the fraction of the total radioactivity which can be recovered as II was compared for livers taken from chickens slaughtered at different withdrawal times. The data in Table V show that although the fraction of radioactivity recovered as II gradually depletes, greater than 70% of the total radioactivity in the tissue is liberated by acid hydrolysis.

These data thus conclusively prove that most of the radioactive substances present in chicken liver are not the result of metabolic changes involving the benzyl moiety of I. It is evident that although the exact chemical nature of the drug residues is unknown and regardless of whether the residues are solvent extractable or are covalently bound to cellular macromolecules, hydrolysis with acid liberates 2-chloro-6-fluorobenzylamine. This acid hydrolysis product of the drug and metabolites represents most of the drug-related species present in liver tissue, and its quantitation via the dansyl derivative offers a microchemical approach for assay arprinocid-derived residues.

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