Metabolic Fate and Pharmacokinetics of Tissue Residues of the Anticoccidial Drug Robenidine in the Rabbit. Incidence of Coprophagy on Its Bioavailability

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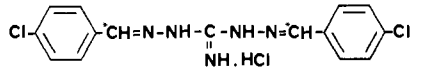
When rabbits were given a single oral dose of [^{14}C]robenidine hydrochloride, tissue retention was low and accounted for less than 0.5% after 7 days. Unchanged robenidine was excreted in the feces (80% of the dose) while the remaining radioactivity was recovered in the urine as a major metabolite, namely, *p*-chlorohippuric acid. Polar metabolites and traces of *p*-chlorobenzoic acid were also observed. The kinetics study of tissue residues showed that the total radioactivity leveled off after 6 days of continuous administration in most samples; during the depletion phase a fast elimination generally occurred but a longer persistence of the residues was noticed in the liver. A comparison between normal rabbits and rabbits bearing collars led to the observation that coprophagy was involved in the bioavailability of robenidine and resulted in the recycling of 8.5% of the ingested drug.

Anticoccidial drugs such as nitrofurans derivatives and metichlorpindol are commonly used as prophylactic agents in rabbit production in the european countries. Robenidine, 1,3-bis[(*p*-chlorobenzylidene)amino]guanidine hydrochloride, or Cycostat is a coccidiostat in use for a number of years as a feed additive for chickens. Recently (Coudert, 1978; Peeters et al., 1979; Peeters and Halen, 1980; Licois and Coudert, 1980) the efficacy of this substance against rabbit Eimeria, when incorporated at 100- and 66-ppm levels in the diet, has been demonstrated. The toxicological evaluation for the human consumer of robenidine residues in chickens has been carried out, based on metabolic studies performed in the rat (Zulalian and Gatterdam, 1973) and the chicken (Zulalian et al., 1975). Robenidine was metabolized mainly to *p*-chlorobenzoic acid and *p*-chlorohippuric acid for the former or (*p*-chlorobenzoyl)ornithine for the latter species. Extension of the use of robenidine to the rabbit involves a reexamination of the pharmacological and toxicological data if we keep in mind that the biotransformation pathways of xenobiotics are highly species dependent. As far as lagomorpha are concerned, coprophagy is a very physiological peculiarity (Thacker and Brandt, 1955). Its role on feeding behavior and nutritional status of the rabbit has been highlighted [see the review of Laplace (1978)], but little is known about its incidence on xenobiotic behavior throughout recycling of part of the caecal contents.

The aim of this study was to establish the metabolic pathways of robenidine in the rabbit, and especially the pharmacokinetics of residues in edible tissues, in order to fix a withdrawal time and bring additional data to the toxicological evaluation for the human consumer. At the same time this study gave the opportunity to investigate the incidence of coprophagy on robenidine bioavailability.

MATERIALS AND METHODS

Labeled Robenidine. [α-(*p-chlorobenzylidene*)-^{14}*C*]-Robenidine hydrochloride was obtained from American



Cyanamid Co. (Princeton, NJ). Its specific activity was 3.52 mCi/mM. Radiochemical purity, checked by thin-layer chromatography (TLC) in benzene–dioxane–acetic acid (45:12:2), using a thin-layer scanner II (Berthold, Karlsruhe, West Germany), was better than 99%.

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Spontaneous degradation of [^{14}C]robenidine, when kept in ethanol and in subdued light, has been mentioned (J. Zulalian, unpublished data). A test has been performed in our normal laboratory storage conditions, i.e., in the cold (+4 °C) and in the dark. A dilute ethanolic solution (0.5 mg/mL 70% ethanol) exhibited a perfect stability over a 15-day period.

Treatment of Rabbits. *Administration of* [^{14}*C*]*Robenidine.* Labeled robenidine was incorporated in the feed under the following conditions: with a syringe, 1 mL of a 70% ethanolic solution was spread out on the surface of about 30-g pellets, allowing quick impregnation. The rabbits were fasted for 12 h prior to the administration of this small quantity of feed, which was eaten completely in a few minutes.

Experimental. Four separate experiments were conducted by using New Zealander rabbits 2.5 months old, each weighing about 2 kg. In experiment I, which was designed to establish a metabolic balance, six (three males and three females) rabbits were accustomed for 15 days in individual metal wire-mesh metabolic cages suitable for a quantitative and separate collection of urines and feces. A pelleted commercial feed containing 50 ppm of robenidine was supplied ad libitum during this period of time, and the access to water was free. At day 0 of the experiment, following a 12-h fasting period, 5 μCi (0.37 mg) of [^{14}C]robenidine was administered to each animal. After complete consumption of the impregnated feed, free access to the supplemented feed was given again to the animals. Urines and feces were collected separately and daily for 8 consecutive days. Sodium azide was added to the urines in order to avoid microorganism development.

In experiment II, carried out to identify robenidine metabolites in excreta and tissues, two rabbits received a low pigmented diet, i.e., alfalfa free, during the 15-days preexperimental period, and then 20 μCi of [^{14}C]robenidine was administered to each animal, and the urines and feces were collected during 0–24- and 24–96-h periods. Another rabbit placed under the same experimental conditions received 100 μCi, the slaughter occurring 24 h later, and different tissues (muscle, fat) and organs (liver, kidneys) were sampled.

Experiment III was conducted to investigate the pharmacokinetics of robenidine residues. Twelve male rabbits were accustomed for 15 days to separate metabolic cages as described in experiment I but were fed a pelleted commercial diet free from drugs or additives. At day 0 of the experiment the same feed supplemented with 50 ppm of robenidine was substituted, and 5 μCi of [^{14}C]robenidine (0.37 mg, i.e., about 5–7% of the nonradioactive robenidine administered) was delivered to each animal and daily, as

0021-8561/82/1430-1071$01.25/0

Table I. Mean Cumulative Excretion of Radioactivity by Rabbit after One Oral Dose of [^{14}C]Robenidine

	excretion, % of dose			
	males		females	
day	urine	feces	urine	feces
1	12.5	47.51	13.70	49.36
2	3.99	28.75	5.12	28.22
3	0.92	3.20	0.85	2.53
4	0.29	0.30	0.26	0.43
5	0.12	<0.15	0.13	<0.15
6	0.11		0.13	
7	0.07		0.08	
8	<0.05		<0.05	
total	18.00 ± 2.98	79.76 ± 4.36	20.27 ± 2.11	80.59 ± 2.81
	97.76 ± 1.38[a]		100.86 ± 1.08[a]	

[a] Average of three animals.

already described. One rabbit was slaughtered after 1, 3, 6, 9, and 15 days. On the 16th day and hereafter, administration of labeled robenidine was stopped and the remaining rabbits received the control diet ad libitum. Thus, one animal was slaughtered on the following 1, 2, 3, 5, 8, and 12 days. Blood was collected from the carotid artery at slaughter and kept on heparin. The following tissues and organs were sampled: liver, kidneys, heart, lungs, brain, muscle (fillet), and perirenal fat. The remaining carcass and digestive tract were ground separately. Depending on their size, the samples were lyophilized directly or ground and then homogenized (Ultra-Turrax homogenizer), and about a 10-g aliquot was lyophilized. After lyophilization, samples were ground again.

In the experiment IV, the incidence of coprophagy on robenidine bioavailability was investigated; twelve rabbits were accustomed for 15 days in separate metabolic cages as previously described. At day 0 of the experiment they each received 5 μCi of [^{14}C]robenidine. The animals were fasted for 12 h so that the administration occurred at 6 p.m. Immediately after, the fortified pellets were eaten, and collars designed to prevent coprophagy were fixed around the neck of six of the rabbits. Urines were collected every 6 h during 72 h.

Radioactivity Measurements. Aliquots of urine samples were counted directly in a Intertechnique SL 32 liquid scintillation spectrometer by using an Aqualuma (Lumac Systems A.G.) fluors mixture and external standardization for quenching corrections.

Feces were dry ground by using a domestic coffee grinder, and then four aliquots 100–200 mg were weighed in polycarbonate capsules. The capsules were combusted under an oxygen flow in an automatic apparatus (Oxymat, Intertechnique, Kontron) that ensures trapping of $^{14}CO_2$ in a scintillation mixture containing phenylethylamine (Carbomax, Lumac Systems A.G.).

Two-hundred-milligram aliquots of blood and tissues previously lyophilized and homogenized were processed the same as feces.

Excretion of radioactivity as $^{14}CO_2$ was measured from a trapping system connected to a small respiratory chamber, housing one rabbit administered per os a single 5-μCi dose of [^{14}C]robenidine. $^{14}CO_2$ was reacted with phenylethylamine by bubbling directly into 500 mL of the Carbomax scintillation mixture. The measurements were operated in a cumulative mode by sampling every 6 h for 48 h. The $^{14}CO_2$ absorption capacity of the Carbomax was saturated in 12 h; thus, the trapping mixture was changed. The experiment was conducted in duplicate.

Identification of the Metabolites. *Extraction and Cleanup Procedures.* Due to the physical and chemical characteristics of rabbits' urines and feces, extraction and cleanup procedures had to be perfected. Very cloudy urines with high mineral salts contents were acidified by using 1 N hydrochloric acid before extraction with ethyl acetate. Soxhlet's extraction of feces using methanol gave only 60% recovery of the total radioactivity; the use of a chloroform–methanol (2:1) mixture according to the method of Folch et al. (1957) resulted in a very improved recovery (95%) with much less interfering material like oxygenated pigments that originate from the alfalfa of the diet.

Where tissue metabolites were concerned, various techniques were used: extraction with the chloroform–methanol (2:1) mixture, acid hydrolysis (6 N hydrochloric acid) under reflux followed by ethyl acetate extraction, and chemical fractionation according to Shibko et al. (1967) that leads to the isolation of the proteins in bulk.

Chromatographic Procedures. Robenidine, *p*-chlorobenzoic acid, *p*-chlorohippuric acid, and more polar metabolites were resolved with a one-dimensional thin-layer chromatography on silica (silicagel G, Merck) using benzene–dioxane–acetic acid (45:12:2) as the developing solvent mixture. *p*-Chloro[^{14}C]benzoic acid was determined as a methyl ester by radio–gas chromatography (RGC), using a $^1/_8$ in. o.d. × 15 m stainless steel column packed with 3% diethylene glycol succinate (DEGS) on Chromosorb W AW DMCS operated at 150 °C under a nitrogen flow; nine-tenth of the sample went through a proportional counter after oxidation to $^{14}CO_2$ over hot copper oxide. *p*-Chloro[^{14}C]hippuric acid was determined as a methyl ester in the same way but with a nonpolar silicone type, 3% SE 30 column at 220 °C.

RESULTS AND DISCUSSION

Balance Study. Table I summarizes the results of the urinary and fecal excretion of radioactivity following a single oral administration of [^{14}C]robenidine to rabbits fed a diet containing 50 ppm of robenidine. It shows that the radioactivity was excreted fairly rapidly and mainly in the feces. However, if more than 92% of the dose administered was excreted during the first 48 h, radioactivity was still measurable in urines at day 7. A more detailed analysis of the kinetics of the depletion of urinary radioactivity indicates a biphasic-type elimination (Figure 1) that corresponds to a major metabolite with a 13-h half-life and quantitatively minor metabolite(s) with a 3–5 day half-life. No difference was noticed in the excretion pattern in males and females. Only 0.2% radioactivity was detected in expired air during 48 h after the dosage of the animals, indicating that the radioactivity was located in a metabolically stable position.

Table II. Pharmacokinetics of [^{14}C]Robenidine in the Blood and Tissues of the Rabbit[a]

	incorporation[b]						depletion[c]					
days	1	3	6	9	12	15	1	2	3	5	8	12
blood	0.28	0.14	0.20	0.21	0.14	0.23	0.14	0.14	0.15	0.10	0.08	0.08
liver	2.95	3.00	1.56	1.60	1.34	1.77	1.32	1.14	0.99	0.70	0.37	0.23
kidneys	1.70	1.80	0.99	0.82	0.80	1.18	0.14	0.22	0.30	0.26	0.13	0.09
heart	0.36	0.19	0.23	0.17	0.26	0.35	0.50	0.13	0.11	0.39	0.08	0.07
lungs	0.74	0.44	0.69	0.61	0.65	1.31	0.24	0.24	0.37	0.37	0.11	0.10
brain	0.15	0.19	0.15	0.09	0.14	0.14	0.06	0.04	0.04	0.03	0.09	0.06
muscle	0.21	0.14	0.15	0.14	0.17	0.19	0.06	0.05	0.05	0.06	ND[d]	ND
fat	0.37	0.29	0.25	0.43	0.29	0.29	0.42	ND	ND	ND	ND	ND
remaining carcass	0.32	0.19	0.16	0.20	0.11	0.14	0.08	ND	ND	ND	ND	ND

[a] Concentrations are expressed as microgram equivalents of [^{14}C]robenidine per gram of fresh blood or tissue. [b] Days after starting the administration of 50 ppm of Cycostat plus a daily oral dose of 5 μCi of labeled robenidine in the diet. [c] Days after the simultaneous withdrawal of robenidine and labeled drug from the diet of rabbits at the end of the incorporation phase. [d] ND = concentration below the detection limit in the corresponding tissue (ppm): fat, 0.2; liver, 0.07; muscle and carcass, 0.05; blood, kidneys, lung, and heart, 0.04; brain, 0.03.

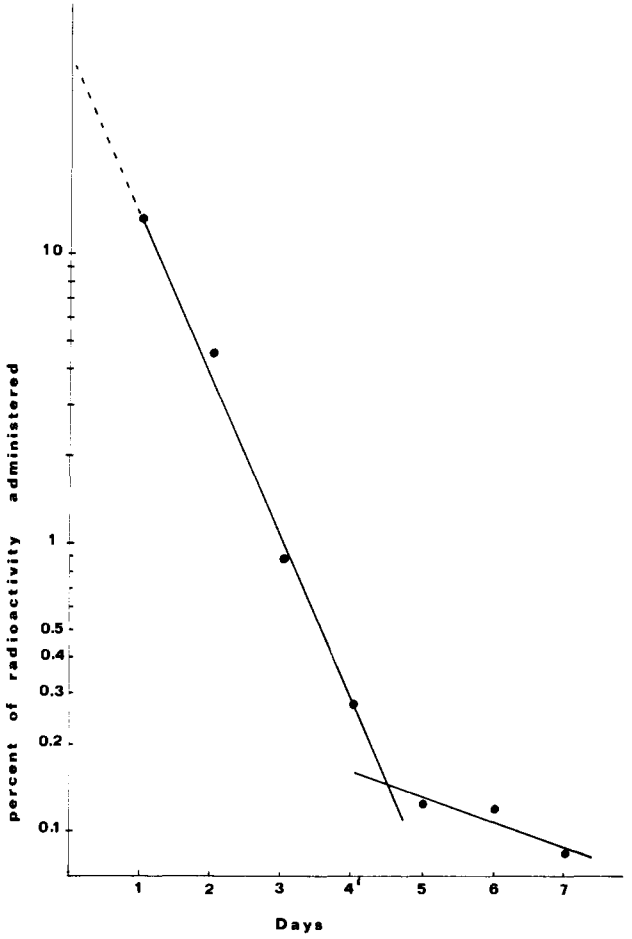
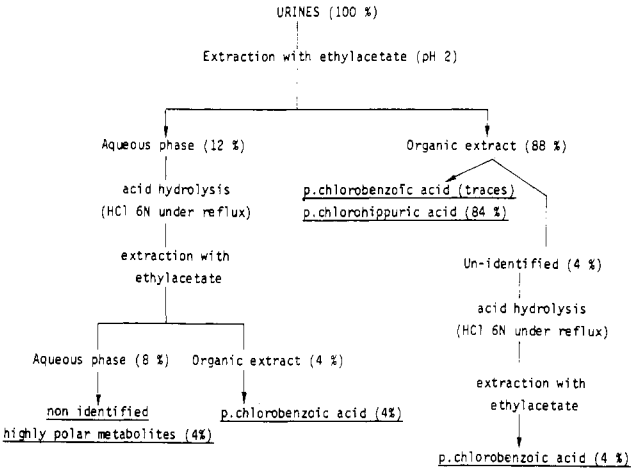


Figure 1. Kinetics of the radioactivity in rabbit urine after administration of a single dose of [^{14}C]robenidine.

When the ^{14}C distribution between urines and feces is compared, these results are very similar to those obtained by Zulalian and Gatterdam (1973) in the rat.

Pharmacokinetics in Blood and Tissues (Table II). *Incorporation Phase.* Starting the administration of [^{14}C]robenidine to the rabbits resulted in a general labeling of the tissues and organs. The radioactivity reached the highest values in the liver and the kidneys and the lowest in the blood and other tissues and organs. At day 6 of the experiment the radioactivity leveled off. It is noteworthy that the equilibrium was obtained after a general decrease of higher concentrations reached during the first 24 h, except in the brain and fat where the differences were nonsignificant. This phenomenon may be explained as a result of an induction of the enzymatic systems involved in the metabolism of robenidine.

Scheme I. Identification of Urinary Metabolites of [^{14}C]Robenidine in the Rabbit (All Members Expressed as Percent Total Radioactivity in Urine)



Depletion Phase. When labeled and nonlabeled robenidine were simultaneously withdrawn from the rabbits diet, a decrease of radioactivity in all the tissues and organs occurred. This phenomenon was fast in the fat and remaining carcass but slower in the muscle, heart, lungs, kidneys, and brain. A longer remanence of the radioactivity was observed in the liver where the depletion was of the exponential type (Figure 2), with a half-life of 4.5 days. This result is relevant with the above-mentioned slow release of minor metabolite(s) in the urine.

Metabolic Pathways. Separation, identification, and quantitation of urinary metabolites were carried out according to the procedures summarized on Scheme I. They emphasize that robenidine was metabolized by the rabbit to *p*-chlorobenzoic acid and *p*-chlorohippuric acid, the latter being by far the major metabolite (84%). A small proportion of the urine radioactivity was uncompletely identified as *p*-chlorobenzoic-containing metabolites and highly polar metabolites.

Thus, the metabolic pathways of robenidine are the same in the rabbit and the rat, even if polar metabolites seem to occur in higher proportions in the urine of the former (12% vs. 6%). It is well-known that the rabbit is able to conjugate *p*-chlorobenzoic acid to glycine (Bray et al., 1952), and this ultimate metabolic step is generally not considered as a limiting factor in most species. However, there is a lack of data concerning the breakdown of the semicarbazide function. Zulalian and Gatterdam (1973)

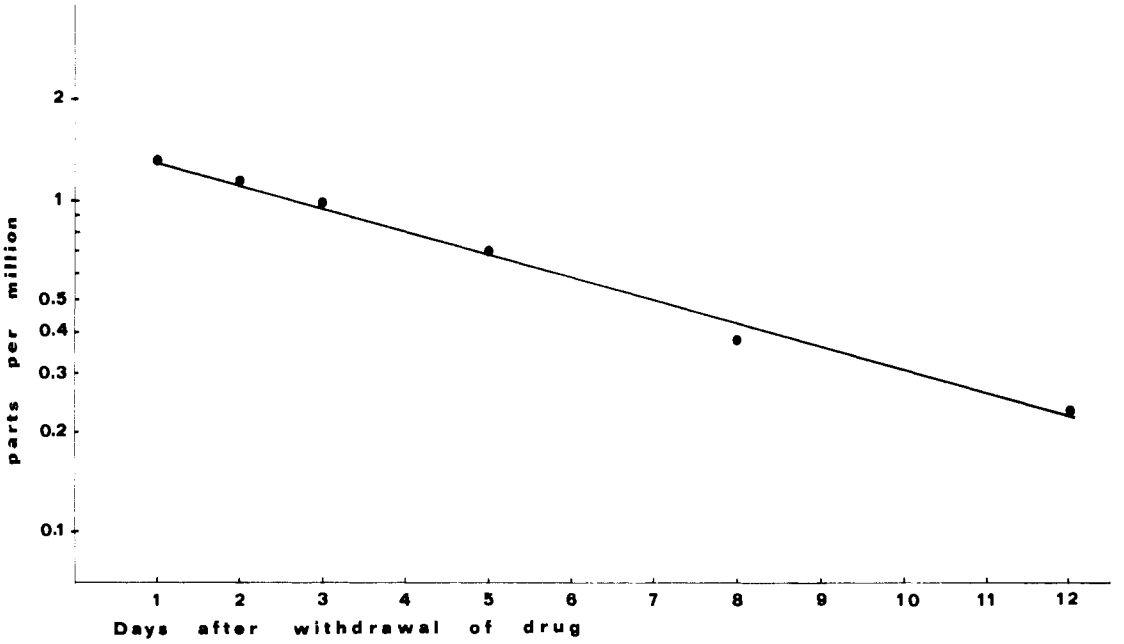


Figure 2. Depletion kinetics of the residues in the liver.

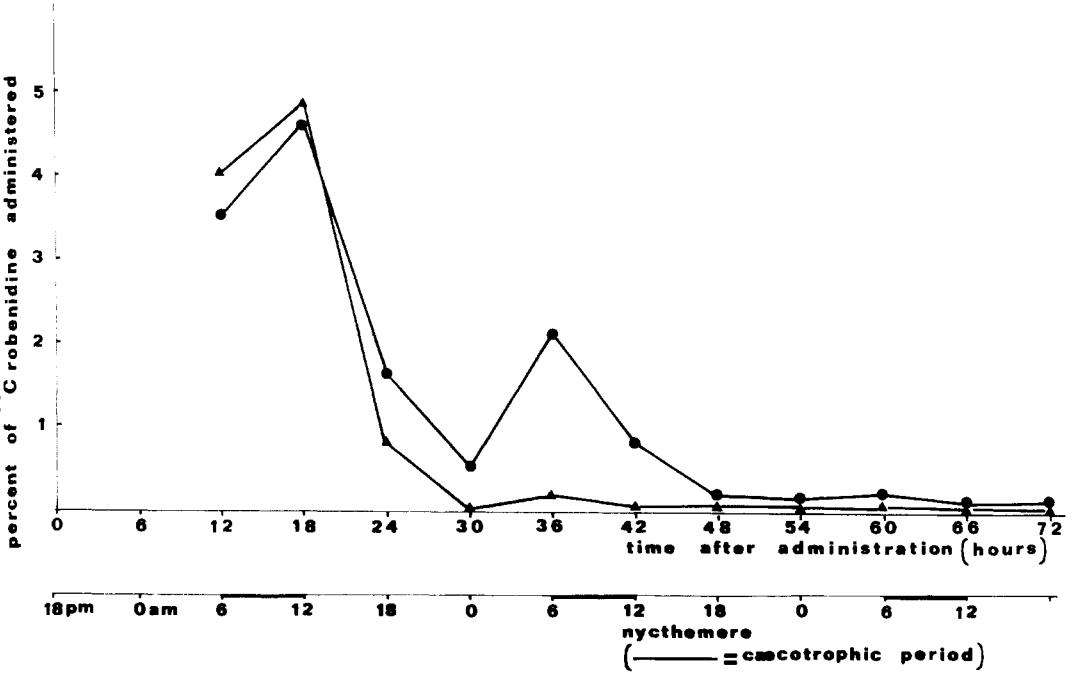


Figure 3. Effects of prohibiting coprophagy on the urinary excretion of radioactivity in the rabbit after administration of a single oral dose of [^{14}C]robenidine (average of six animals in each group). (●) Control rabbit; (▲) rabbit with a collar.

established that the rat realizes that enzymatic hydrolysis, and this study indicates that the rabbit also is able to do it.

Most of the fecal radioactivity was associated with unchanged robenidine. It must be noted that the biliary excretion of radioactivity was very low, which states that most of the robenidine excreted in the feces corresponds to the nonabsorbed fraction.

Identification of Tissue Residues. In order to establish the relevance of the metabolites that were identified in excreta to those occurring in the tissues, we analyzed the radioactivity in selected edible tissues (experiment II). Twenty-four hours after administration of a 100-μCi dose, residue levels in fat and muscle were very low (<0.05%); however, 1.3% of the administered dose was recovered in the liver and the analysis of that radioactivity led to reliable results. Chloroform–methanol extracts containing 85% of the total radioactivity revealed the presence of *p*-chlorohippuric acid and robenidine as a liver residue. After acid hydrolysis a total removal of ^{14}C in the *p*-chlorobenzoic acid form was obtained. Chemical fractionation of cellular components showed that 15% of the liver radioactivity was strongly bound to the proteins; this must be related to the above-mentioned observation that ^{14}C depletion in the liver is slower than in other tissues. However, a complete recovery as *p*-chlorobenzoic acid was achieved after acid hydrolysis.

Incidence of Coprophagy. Figure 3 shows the urinary excretion pattern in the normal and noncoprophagous rabbit. A first peak of excretion occurred in both types of animals about 18 h after [^{14}C]robenidine was administered, but a second and smaller peak appeared in the normal rabbit 18 h later, followed by a third and just perceptible increase after a new 24-h period of time. A

slight increase of the urine radioactivity must be noticed for the rabbits unable to attend coprophagy. It is concomitant of the second peak in normal rabbit and may be explained by the fact that part of the soft feces can stick on the wiring of the cages and then be eaten by the rabbits in spite of their collars.

It is noteworthy that the periodicity of this damped phenomenon was in step with the nycthemeral rhythm of coprophagy in the rabbit fed ad libitum, as established by Corpet and Laplace (1976). Total radioactivity excreted by the noncoprophagous rabbit was 44.8% less than for the normal one. Based on a comparative balance study carried out over a 4-day period in both type animals, it was established that the difference observed in the urines (19.1% vs. 10.6%) was in the same range of that measured in the feces (80.2% vs. 88.7%) but opposite. If one considers these complementary observations and data, it may be stated that coprophagy contributes definitely to the recycling of [^{14}C]robenidine and that this phenomenon accounts for about 8.5% of the radioactivity ingested.

Such an evaluation must be compared with other values obtained during studies specially designed to measure the nutritional impact of coprophagy in the rabbit. By use of various tracers or collecting and then weighing separately hard and soft feces, the extent of the recycling was established to between 8% (Battaglini, 1968) and 13% (C. Dehalle, personal communication) of the dry matter ingested. The fact that robenidine is poorly absorbed can explain this very similar behavior.

CONCLUSION

From the comparative study of robenidine metabolism in rat, chicken, and rabbit, it appears that patterns are very similar qualitatively and quantitatively for birds and mammals. After breakdown of the semicarbazide function, elimination occurs in urine as a glycine conjugate of the *p*-chlorobenzylidene moiety of the molecule. However, our study did not give any data on the fate of the aminoguanidine moiety.

The pharmacokinetics of the tissue residues give the basic data necessary to fix a withdrawal period before slaughtering. Due to the longer persistence of residues in the liver, this organ may be considered as the target one for residue control.

Coprophagy was shown to result in the recycling of part of the unchanged robenidine, thus contributing to the longer persistence of liver residues when compared to those measured elsewhere in the rat.

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Received for review December 29, 1981. Accepted July 29, 1982.

Pyrimidines. 23. A Structure–Activity Relationship Study of 4-Chloro-2,6-(substituted amino)pyrimidines as Pre- and Postemergence Herbicidal Agents

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Two pyrimidine analogues of the herbicide atrazine were synthesized, and their herbicidal activity was evaluated. One of these deazaatrazines, 4-chloro-2-(ethylamino)-6-(2-propylamino)pyrimidine, was found to be a highly specific preemergence herbicidal agent. It inhibits the growth of Johnson grass but is noninjurious to corn, whereas atrazine has the opposite effect in each case. In the postemergence tests, a high order of tolerance by cotton plants was noted with these pyrimidine derivatives, whereas atrazine in these tests was quite injurious to this valuable crop. Some structure–activity relationships were correlated among many of the pyrimidine analogues in both the pre- and the postemergence tests.

For centuries agricultural workers have been engaged in a battle with undesired plants in their fields. During the past 40 years, the use of herbicides has become a major tool in controlling the growth of vegetation. An examination of the present-day trend in the use of herbicides indicates that the older, inorganic herbicides and defoliants are declining in usage as more selective, more effective, and less hazardous organic agents have become available. It also reveals that among the organic agents, many heterocyclic compounds are of special value. This is illustrated by the wide use of herbicides such as atrazine [2-chloro-4-(ethylamino)-6-(2-propylamino)-*s*-triazine, 1] (Gysin and Knüsli, 1955), amitrole [amizol, 3-amino-1,2,4-triazole, 2] (Tafuro et al., 1955), and bromacil [5-bromo-3-(2-bu-

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0021-8561/82/1430-1075$01.25/0