with a slight growth response and enhanced bile-S^{35} concentration over the control while lithocholic acid was growth depressing and toxic at the same dietary level (Table V). The livers of the birds fed lithocholic acid were abnormally large and contained more S^{35} per gram than did the livers of the other dietary groups of chicks. The liver taurocholate-S^{35} 6 hours after dosing was highest when cholic and deoxycholic were fed alone and when dehydrocholic was supplemented with taurine.

Studies are in progress concerning the chick liver reactions whereby sulfate-sulfur is converted to taurine-sulfur without passing through cysteine, which is a normal reaction pathway of mammals.

Summary. Chicks utilize sulfate-S^{35} to synthesize taurine. Dietary cholic acid, taurine or both are associated with increased volumes of bile fluid. Cholic acid feeding enhances sulfate-S^{35} incorporation into the bile while taurine decreases such reactions.

1. Lowe, I. P., Roberts, E., J. Biol. Chem., 1955, v212, 477.
2. Machlin, L. J., Fed. Proc., 1954, v13, 466.
3. Virtue, R. W., Doster-Virtue, M. E., J. Biol. Chem., 1937, v119, 697.
4. ———, ibid., 1939, v127, 431.
5. Martin, W. G., 1963 Ph. D. Dissertation, West Virginia Univ., Morgantown.
6. Bremer, J., Acta Chem. Scand., 1955, v9, 683.

Received September 9, 1965. P.S.E.B.M., 1966, v121.

Cycasin Excretion in Conventional and Germfree Rats. (30793)

M. SPATZ, E. G. MCDANIEL AND G. L. LAQUEUR
Laboratories of Experimental Pathology and of Nutrition and Endocrinology, National Institute of Arthritis and Metabolic Diseases, Natinal Institutes of Health, U. S. Department of HEW, Bethesda, Md.

The glycoside cycasin, β-D-glucosyloxyazoxymethane, isolated from *Cycas circinalis* L (1,2) was previously reported to be hepatotoxic and carcinogenic in rats(3), guinea pigs (4) and mice(5). It was demonstrated furthermore that the aglycone of cycasin, methylazoxymethanol, produced acute and chronic toxic manifestations in rats similar to those observed with cycasin(6), and metabolic studies of cycasin *in vitro* had shown that a β-glucosidase was responsible for the hydrolytic cleavage of the aglycone from cycasin (7). The absence of signs of acute toxicity after intraperitoneal injections of the intact glycoside and its excretion in the urine suggested that cycasin, in order to produce acute toxic affects, required oral administration and that its cleavage most likely occurred in the alimentary tract(8,9,10). It was of some importance, therefore, to examine the question whether the enzymatic breakdown of cycasin was dependent on the presence of bacterial flora in the intestine.

The availability of germfree rats offered the opportunity to investigate this question. Preliminary observations in which germfree rats had been fed large amounts of the glycoside for 20 consecutive days indicated that such animals grew well without showing evidence of toxicity as judged from food intake, body weight gain and the microscopic appearance of the tissues. This was in striking contrast to conventional rats of the same strain in which food intake and body weight gain were reduced and in which severe liver necrosis and a high mortality commencing with the ninth day of the experiment were found(3).

The purpose of this report is: (1) to quantitate cycasin excretion in germfree animals and compare it with that in conventional animals; (2) to determine the time required for the disappearance of cycasin in urine and feces after discontinuation of feeding in these two groups of rats; and (3) to present a preliminary report on tumor development in cycasin-fed germfree rats.

Materials and methods. Thirty-five Sprague-

Dawley germfree male rats* were housed individually and fed the basal diet† until they attained a weight of 100 ± 10 g. They were then maintained for 20 days on the experimental diet consisting of 200 mg of cycasin per 100 g basal diet. Food intake was measured daily and the animals were weighed every fifth day. Five of the 35 rats were housed in metabolic cages for the collection of excreta during the 20-day experimental period. The remaining 30 rats were fed an identical diet for 20 days after which they were returned to the basal diet for long-term observation for neoplasis.

Twenty-four-hour urine outputs were collected during 3 consecutive days from the 5 germfree rats in metabolic cages while total fecal collection was done once on the third and again on the 20th day. The time necessary for disappearance of cycasin from urine and feces was determined in 2 of the 5 rats by cycasin assays for 3 additional 24-hour periods after the animals had been returned to the basal diet.

Five conventional male rats of the Sprague-Dawley strain were placed in metabolic cages and fed a basal diet containing the same concentration of cycasin as the germfree rats.‡ Urine and feces were collected in a comparable fashion including 2 additional days after the rats had been returned to the basal diet.

Possible daily variations in cycasin excretion were investigated in an additional group of 3 conventional Sprague-Dawley rats receiving 100 mg cycasin per 100 g of diet for 5 days during which daily urinary and fecal cycasin determinations were performed. Another group of 5 rats received 400 mg of cycasin per 100 g of diet to determine the effect of a high cycasin intake on the excretion pattern. Cycasin was assayed during the day of feeding and for 2 additional days thereafter.

Crystalline cycasin was obtained from the Dept. of Biochemistry, Kagoshima University, Japan, where it had been extracted from seeds of *Cycas circinalis* L.§ The cycasin was dissolved in sterile water and filtered through a Seitz filter into an ampoule which was transferred into the isolator after sterilization of the outside of the ampoule with peracetic acid. Mixing of the diet with cycasin was performed in the isolator.

Cycasin assay. Cycasin in urine and feces was assayed according to the method of Kobayashi and Matsumoto(10). Utilizing this method for the assay in feces, abnormally high values for fecal cycasin were obtained in trial runs. It was found that with the assay method used, fatty acids and glycerol were formed from neutral fat after exposure to the chromotrophic acid reagent(11), the glycerol having been responsible for the false high cycasin values in the trial runs. The method was revised as follows: The refrigerated watery fecal filtrate was treated with activated charcoal and washed with 250 ml of ether and 2000 ml of distilled water. This washing procedure proved to be effective for removal of the interfering fat and carbohydrate. The charcoal adsorbates were eluted with 200 ml of 20% alcohol. The quantity of cycasin was determined spectrophotometrically. The eluates were concentrated by evaporation to 0.5 ml-1 ml, facilitating the assay of cycasin by paper chromatography. Both basal diets used in these experiments were tested in this way and found to be free of cycasin.

Results and discussion. The data obtained on urinary and fecal excretion of cycasin in germfree and conventional male Sprague-Dawley rats are presented individually, together with the respective food and cycasin

* The germfree rats were obtained at weanling age from the Germfree Unit, Division of Research Services at N.I.H. and transferred to isolators in the Laboratory of Nutrition and Endocrinology, NIAMD, N.I.H.

† The basal diet of germfree rats consisted of 5% casein, 4% corn oil, 2% alfalfa, 2% liver powder, 21.4% corn meal, 10% lactalbumin, 10% whole milk powder, 21.4% cornstarch, 21.4% whole wheat flour, 3% salt mixture supplemented with all necessary vitamins.

‡ The basal diet of conventional rats consisted of Purina N.I.H. Chick Startena and contained 23.0% crude protein (min.), 4.5% crude fat (min.), 4.0% crude fiber (max.) and 67% nitrogen-free extract (min.). The exact composition is available upon request.

§ Cycasin used in this study was kindly given to us by Drs. L. Kurland and M. G. Whiting of NINDB, N.I.H.

TABLE I. Cycasin Excretion in Rats Fed 200 mg % Cycasin in Basal Diet for 3 Days.

No. of rat	Food intake, g	Cycasin intake, mg	Cycasin excretion: Urinary mg	Urinary %	Fecal mg	Fecal %	Total, mg	Cycasin, unaccounted mg	unaccounted %
			Germfree rats						
2	37.0	74.0	44.4	60.0	23.6	31.9	68.0	6.0	8.1
8	24.8	49.6	48.0	96.8	trace*		48.0	1.6	3.2
5	39.0	78.0	57.8	74.1	18.3	23.5	76.1	1.9	2.4
1	37.0	74.0	50.8	68.7	20.9	28.2	71.7	2.3	3.1
4	36.2	72.4	63.4	87.6	9.0	12.4	72.4	0.0	0.0
			Conventional rats						
1066	16.5	33.0	5.9	17.9	.0	.0	5.9	27.1	82.1
1068	19.5	39.0	12.1	31.0	1.6	4.1	13.7	25.3	64.9
1071	18.5	37.0	11.7	31.6	.0	.0	11.7	25.3	68.4
1073	25.5	51.0	12.2	23.9	.0	.0	12.2	38.8	76.1
1075	8.5	17.0	3.2	18.8	.9	5.3	4.1	12.9	75.9

* Less than .5 mg.

intakes in Table I. The most significant difference between the two groups of rats is shown in the last column headed "cycasin unaccounted," being small in germfree and large in conventional rats. Since collection procedures and assay techniques were identical in all, and the magnitude of the differences was considerable between the two groups, the findings indicate that germfree rats nearly quantitatively excreted the ingested cycasin while the recovery rate of cycasin in conventional rats varied from 18 to 35% of the intake, the remainder presumably having been metabolized to compounds not detected by the assay procedures for cycasin.

A further difference between the two groups is apparent when food intakes are compared. The germfree rats ingested nearly twice as much cycasin as the conventional animals. The reduction in food intake of the conventional rats can be readily related to the progressive liver injury which was absent in germfree rats. Since in previous experiments a good correlation between the levels of dietary cycasin and degrees of hepatic injury were found, the striking decrease in recoverable cycasin is indicative of its predominant metabolic (enzymatic) breakdown to the highly active hepatotoxic aglycone in conventional rats.

Although it was originally thought that fecal excretion of cycasin might represent the major excretory route in germfree rats, this was not the case. The data in Table I show that the unmetabolized cycasin was predominantly excreted through the kidneys in both germfree and conventional rats. Therefore, it appears that cycasin is absorbed by the intestinal mucosa as the intact glycoside and excreted as such by the kidneys without producing toxic effects. It thus behaved as an inert substance. The observations in germfree rats are well supported by the finding that intraperitoneal injections of cycasin into conventional rats failed to produce toxicity with a high urinary excretion of the unaltered compound(9,10).

The fecal excretion of cycasin between the two groups was found to be considerably higher in germfree than in conventional rats. Whether this is, in some way, due to the greatly enlarged cecum, typical of germfree rats, or to continuous breakdown of the glycoside along the length of the intestine in conventional rats is unknown. It would seem possible that both factors play a role, the former in germfree, the latter in conventional rats. There was good evidence that the percentile fecal excretion remained substantially unchanged with time in those 3 germfree rats from which feces had been collected over a 20-day period. The pertinent data are summarized in Table II. For example, rat no. 8 which excreted the smallest amount of cycasin during the first 3 days (Table I) continued to do so during the remaining 16 days of observation.

The individual variations in the total amount of cycasin excreted by conventional rats raised the question whether different ex-

cretion patterns might exist in similar animals. Since such differences could indirectly reflect variations in rate of metabolic breakdown, daily urinary and fecal excretions of cycasin were studied over a 5-day period in a separate group of 3 rats. The data are summarized in Table III and indicate that not only were there considerable variations among rats, but also daily variations for the individual rat. The amount of cycasin consumed apparently had little influence since it was nearly identical, as with rats 1059 and 1061. Yet, rat 1059 excreted a total of 9.6 mg while 27.5 mg of cycasin was recovered from rat 1061. Similar variations in cycasin excretion have been previously reported(10). Further investigations of this type appear necessary to explore the possibility that the future development of neoplasia may depend predominantly on the percentage of metabolized cycasin in the intestinal tract.

The possibility existed that excessive amounts of cycasin might alter the excretion pattern. In earlier experiments microscopically visible liver cell injury was found within 24 hours or less under such conditions. A level of cycasin concentration of 400 mg per 100 g diet was chosen which, while excessive, was known to permit survival of the majority of the rats for many months provided exposure was no longer than 2 days. The findings on cycasin excretion in such rats are summarized in Table IV. Assays were continued for one additional day at which time cycasin could no longer be demonstrated in either feces or urine. Of special interest again is the great variability in cycasin excretion. It is strikingly apparent in the last 2 columns in which that part of cycasin intake is recorded both as amount and percentage which

TABLE II. Fecal Excretion of Cycasin in Germfree Rats* Fed 200 mg % Cycasin in Basal Diet.

No. of rat	Duration of exp, days	Food intake, g	Cycasin intake, mg	Cycasin excretion	
				mg	%
2	20	316.0	632.0	218.8	34.6
4	20	211.5	423.0	61.1	14.4
8	19	99.8	198.6	11.3	5.7

* The 3 rats are the same included in Table I. Fecal collection was continued for 16-17 more days. Values for cycasin intake and excretion were combined from the 2 collection periods.

TABLE III. Daily Cycasin Excretion in Conventional Rats Fed 100 mg % Cycasin in Basal Diet.

No. of rat	Experimental day	Food intake, g	Cycasin intake, mg	Cycasin excretion					Cycasin, unaccounted	
				Urinary		Fecal		Total, mg		
				mg	%	mg	%		mg	%
1058	1	17.6	17.6	3.8	21.5	1.0	5.6	4.8	12.8	72.7
	2	14.3	14.3	5.5	38.4	1.0	6.9	6.5	7.8	54.5
	3	12.6	12.6	3.2	25.4	.9	7.1	4.1	8.5	67.4
	4	16.8	16.8	1.5	8.9	.8	4.7	2.3	14.5	86.3
	5	13.9	13.9	5.6	40.2	.8	5.7	6.4	7.5	53.9
	Total		75.2	19.6		4.5		24.1	51.1	
1059	1	17.2	17.2	2.4	13.9	.0	.0	2.4	14.8	86.0
	2	20.0	20.0	1.2	6.0	1.0	5.0	2.2	17.8	89.0
	3	18.0	18.0	1.1	6.1	1.1	6.1	2.2	15.8	87.7
	4	20.0	20.0	.5	2.5	1.7	8.5	2.2	17.8	89.0
	5	18.3	18.3	.6	3.2	.0	.0	.6	17.7	96.7
	Total		93.5	5.8		3.8		9.6	83.9	
1061	1	19.0	19.0	2.7	14.2	.0	.0	2.7	16.3	85.7
	2	19.8	19.8	6.0	30.3	.7	3.5	6.7	13.1	66.1
	3	17.3	17.3	3.2	18.5	1.6	9.2	4.8	12.5	72.2
	4	20.0	20.0	4.7	23.5	.0	.0	4.7	15.3	76.5
	5	19.4	19.4	8.6	44.3	.0	.0	8.6	10.8	55.6
	Total		95.5	25.2		2.3		27.5	68.0	

TABLE IV. Cycasin Excretion in Conventional Rats Fed 400 mg % Cycasin in Basal Diet for 24 Hours.

No. of rat	Food intake	Cycasin intake, mg	First day				Second day				Grand total excreted as % of intake	Cycasin, unaccounted	
			Cycasin excretion, mg			Total excreted as % of intake	Cycasin excretion, mg			Total excreted as % of intake			
			Urinary	Fecal	Total		Urinary	Fecal	Total			mg	%
1053	12.4	49.6	11.82	.68	12.50	25.20	1.9	.00	1.9	3.83	29.03	35.20	70.97
1057	13.4	53.6	35.80	1.52	37.32	69.62	5.3	"	5.3	9.88	79.51	10.98	20.49
1062	12.4	49.6	27.20	.00	27.20	54.83	4.5	"	4.5	9.07	63.91	17.90	36.09
1063	14.3	57.2	29.80	1.68	31.48	55.04	4.6	"	4.6	8.04	63.08	21.12	36.92
1064	11.7	46.8	4.80	.00	4.80	10.26	1.1	"	1.1	2.35	12.61	40.90	87.39

was absent from the excreta and, therefore was presumably metabolized by the experimental animals, *e.g.,* rat 1057 metabolized only 11 of the 53.6 mg cycasin it had consumed, while rat 1064 metabolized 41 to 46.8 mg. The magnitude of variations in cycasin breakdown from animal to animal may well explain observations recently made by us in which rats of the same strain and on an identical cycasin concentration in the diet produced neoplastic disease in only 50% of the animals. Future studies with cycasin should take into consideration the evidence that the intestinal flora is the source of β-glucosidase and, therefore, the major factor which determines the enzymatic breakdown of the inert cycasin into the active aglycone.

When conventional or germfree rats were returned to the basal diet, cycasin excretion rapidly decreased in both feces and urine and was no longer demonstrable in specimens collected 48 hours after discontinuation of cycasin feeding. This would suggest that cycasin is not stored in a tissue site to be subsequently released at a slow rate over prolonged periods.

Preliminary data on the 30 germfree rats, fed for 20 consecutive days a diet containing 200 mg cycasin per 100 g feed, show 23 survivors without evidence of neoplastic disease, 11 to 13 months after initial exposure to the carcinogen. The 7 rats which were sacrificed, had been on the basal diet for 4 to 12 months after discontinuation of cycasin feeding and were free of tumors on careful gross and microscopic examination. The survivors are now under careful observation until their natural death. The noteworthy aspect of the experiment thus far has been that germfree rats survived for one year after having been exposed to a high concentration of cycasin which conventional rats cannot tolerate. In an earlier study, not included here, in which conventional rats received one-half of the cycasin concentration used in the present experiment for only 13 days, a mortality of 80% was obtained within the first 2 months. The prolonged survival time of germfree rats after exposure to large amounts of cycasin is in keeping with the inability of such rats to hydrolyze the glycoside and

with the nearly quantitative recovery of the unaltered glycoside from urine and feces.

Summary. Urinary and fecal excretion of the naturally occurring toxic and carcinogenic glycoside, cycasin, β-D-glycosyloxyazoxymethane, was determined in germfree and conventional Sprague-Dawley male rats. Excretion in germfree rats was nearly quantitative while only 18 to 35% was recovered in conventional rats. Since cycasin is inert until acted upon by a β-glucosidase, the lack of enzymatic cleavage in germfree rats suggests the bacterial flora as the most likely source for the enzyme in conventional rats. Considerable variation was encountered in the amount of cycasin which was not recovered and was presumably metabolized in conventional rats. The possible significance of this variability is discussed in relation to toxicity and carcinogenicity.

1. Nishida, K., Kobayashi, A., Nagahama, T., Bull. Agr. Chem. for Japan, 1955, v19, 77.
2. Riggs, N. V., Chem. and Fed., 1956, 926.
3. Laqueur, G. L., Fed. Proc., 1964, v23, 1386.
4. Spatz, M., ibid., 1964, v23, 1384.
5. O'Gara, R. W., Brown, J. M., Whiting, M. G., ibid., 1964, v23, 1383.
6. Matsumoto, H., Strong, F. M., Arch. Biochem., Biophys., 1963, v101, 299.
7. Kobayashi, A. Agr. Biol. Chem. (Tokyo), 1962, v26, 203.
8. Nishida, K., Kobayashi, A., Nagahama, T., Kojima, K., Jamane, M., Seikagaku, 1956, v28, 218 (in Japanese, translation available at Library of Medicine).
9. Kobayashi, A., Matsumoto, H., Fed. Proc., 1964, v23, 1354.
10. ———, Arch. Biochem. Biophys., 1965, v110, 373.
11. Burton, R. M., in Methods of Enzymology, 1957, vIII, 246.

Received September 9, 1965. P.S.E.B.M., 1966, v121.

Formation Constants of Certain Zinc-Complexes by Ion-Exchange Method.* (30794)

PRAN VOHRA, ELLEN KRANTZ AND F. H. KRATZER
Department of Poultry Husbandry, University of California, Davis

The effectiveness of disodium ethylenediaminetetraacetic acid (EDTA) in improving the availability of zinc has been shown for poults(1) and chicks(2,3) fed diets containing isolated soybean protein. The optimum stability constant for a chelating agent to be effective was found to be between 13 and 17, using maximum values from the literature (4). To explain the mechanism of the biological action of chelating agents it is desirable to know the formation constants for additional compounds and to have these values for a physiological pH.

Let us consider a general reaction between zinc ion and any complexing agent *A* with a valency of x which can be described by the following equation:

$$Zn^{++} + nA^{-x} = Zn(A)_n^{2-xn} \qquad (1)$$

The formation constant for this reaction is:

$$K_{f\ (absolute)} = \frac{[Zn\ (A)_n^{2-xn}]}{[Zn^{++}]\ [A^{-x}]^n} \qquad (2)$$

The log value of K_f is called the stability or the formation constant for the reaction. The values for formation constants are usually determined under well-defined conditions of ionic strength, optimal pH at which the complexing agent is present in its completely dissociated form A^{-x}, and a temperature of 20°C. Such conditions are seldom attainable in studies involving biological systems in intact animals.

As the biologists are more interested in the relative value of the formation constants at about the physiological pH of 7.4, the current investigation was undertaken following the procedures outlined by Schubert(5). The values so determined are termed as apparent formation constants and are related to the absolute formation constants as follows:

* Supported by Grant No. A5334 from the Nat. Institutes of Health, U.S.P.H.S.