entirely compatible with the rabbit sperm as the rabbit uterus. On two occasions it was noted that if the rabbit sperm were incubated in the dog uterus for 8 to 10 hours the sperm became immobilized. In several rats immobilization of the sperm, as well as an occasional overwhelming leukocyte infiltration, was noted when sperm were incubated 8 hours. Because of this experience results are reported only for rabbit sperm incubated in the dog uterus for 6 to 7 hours and the rat uterus for 7 to 8 hours when the sperm remained motile and relatively free of leukocytes. The comparatively low fertilizing rate in the inter-species series of experiments may be due to the short incubation time of 6 to 8 hours and incomplete capacitation of the sperm. In the inter-organ series of experiments where the incubation time was increased to 9 to 11 hours there was a doubling of the fertilizing ability of sperm incubated in the rabbit uterus when compared with the inter-species series of experiments.

Summary. It has been found that washed rabbit sperm may be capacitated in the uterus of an estrous rabbit, rat, and dog, indicating that the capacitation mechanism is similar in these species. It was shown that washed rabbit sperm are capacitated only in the uterus and oviduct of the estrous rabbit and not in the bladder, colon and knee joint, indicating that the factors necessary for capacitation are limited to the female reproductive tract.

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Role of Intestinal Microorganisms in Determining Cycasin Toxicity. (31826)

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Cycasin is a compound present in plants of the family *Cycadaceae* which is hepatotoxic and carcinogenic when ingested by small laboratory animals(1). The compound is of special interest because various products from cycad plants are ingested by humans in subtropical and tropical regions of the world(2).

Cycasin is a β-glucoside with the structure methylazoxymethanol-β-*D*-glucoside. The glucose can be removed enzymatically and the purified aglycone has the same hepatotoxic and carcinogenic properties as the glucoside (3). Moreover, although cycasin is not toxic when administered intravenously, subcutaneously or intraperitoneally, the aglycone is toxic by these routes as well as after ingestion.

Previously published experiments have shown that cycasin is nontoxic when ingested by germfree animals, and that it can be recovered quantitatively and unchanged from the urine and feces of these animals(4). In conventional animals, in contrast, as much as 82% of administered cycasin is not recoverable. On the basis of these results, it was proposed that the aglycone is the proximate carcinogen and toxic substance, that

cycasin must be deglucosylated before it is toxic, and that intestinal microorganisms perform this deglucosylation. Moreover, it was suggested that individual variations in the intestinal flora could explain differences in toxicity found in animals exposed to comparable doses of cycasin, and also variations in the amount of cycasin which could be recovered in different animals(5).

The purpose of the present work was to study the role of bacteria in deglucosylating cycasin by monocontaminating germfree animals with microorganisms of known glucoside splitting ability. Monocontamination by these organisms resulted in predictable responses to cycasin depending on which organism was present.

Material and methods. a. *Animals.* Germfree male Sprague-Dawley rats weighing 100 to 180 g were obtained from the NIH animal production facility and maintained in metal isolators throughout the experimental period. Food* and water were available at all times except for a period of 18 hours prior to administration of cycasin.

The rats were housed individually within the isolator in wire mesh metabolic cages fitted with metal pans covered by wire screens for separate collections of urine and feces. Fecal cultures were prepared several times prior to the administration of cycasin and at termination of the experiments to assure the germfree state of the controls and the successful colonization by the monocontaminants of the experimental animals.

b. *Chemicals.* Crystalline cycasin prepared from the seeds of *Cycas circinalis* was obtained from Dr. H. Matsumoto. p-Nitrophenyl-β-*D*-glucoside (PNPG), adenosine triphosphate (ATP) and salicin were purchased from the Sigma Chemical Co. Yeast hexokinase, glucose-6-phosphate dehydrogenase and triphosphopyridine nucleotide (TPN) were purchased from the Boehringer Chemical Co.

c. *Bacterial monocontaminants.* Bacterial strains were chosen on the basis of their ability to ferment the β-glucoside salicin. The utilization of this compound as a carbon source requires a β-glucosidase which can release free glucose from it. A strain of *Streptococcus fecalis* which is a known salicin fermenter was purchased from the American Type Culture Collection (No. 12755). *Lactobacillus salivarius salicinius,* a known fermenter of salicin and *Lactobacillus salivarius salivarius* which is known not to ferment salicin were obtained from Mr. Morrison Rogosa, National Dental Institute.† These organisms were maintained on a modified Rogosa's cysteine broth‡ to which either 1% salicin or 0.5% glucose was added, depending on the organism being grown. The medium containing salicin was selective for the salicin fermenting organisms and was useful in maintaining the cultures free of contamination. In addition, the cultures were checked frequently for contamination by Gram staining and for growth in fermentation tubes containing salicin in Rogosa's medium, pH 6.8, with crystal violet as an indicator of acid production. These tests were also used to check cultures from the feces of monocontaminated rats for the presence of the appropriate microorganisms.

d. *Assays for β-glucosidase.* Bacteria for assay were harvested in late log phase and washed 3 times by centrifugation. Approxi-

* The diet was a modification of the Lobund breeder diet 462 and consisted of: vitamin-free casein 5%, lactalbumin 10%, whole milk powder 10%, salt mixture (Wesson) 3%, alfalfa meal 2%, liver powder 2%, corn oil 4%, and 21.4% each of yellow corn meal, whole wheat flour and cornstarch. To each 100 g of diet were added 5500 USP units of vit. A and 1100 USP units of vit. D (as Natola), 75 mg α-tocopherol acetate, 1 mg menadione, 6 mg thiamine hydrochloride, 2 mg pyridoxine hydrochloride, 15 mg calcium pantothenate, 10 mg niacinamide, 100 mg ascorbic acid, 0.1 mg biotin, 0.01 mg vitamin B_{12}, 100 mg choline chloride, 5 mg p-aminobenzoic acid, 1 mg folic acid and 3 mg riboflavin.

† These same strains are also obtainable from the American Type Culture Collection as No. 11742 and 11741 respectively.

‡ The medium, which was prepared by the Media Unit at the NIH, contained the following per liter: Tripticase (Baltimore Biological Laboratory), 10 g; yeast extract (Difco), 5 g; K_2HPO_4, 3 g; KH_2PO_4, 3 g; $MgSO_4 \cdot 7H_2O$, 0.575 g; $FeSO_4 \cdot 7H_2O$, 0.034 g; $MnSO_4 \cdot 7H_2O$, 0.120 g; ammonium citrate, 2 g; Tween 80, 1 g; sodium acetate, 1 g; and L-cysteine HCl, 0.2 g.

mately 0.5 to 1.0 g of bacteria were disrupted in 2.0 ml of 0.01 M tris, pH 7.5, by means of four 15-second periods of exposure to the probe of a Branson sonifier Model LS-75 at setting 4. The procedure was performed at 4°, and at least one minute for cooling was provided after each exposure. The sonified bacteria were centrifuged for 20 minutes at 48,000 × *g* and the crude extract was assayed on the day of its preparation.

Two different assays, both performed at room temperature (25°), for β-glucosidase were used, depending upon the substrate(6,7). When PNPG was used, the following were combined in a total volume of 0.3 ml: triethanolamine buffer, pH 7.0, 30 μmoles; PNPG, 1.6 μmoles; and the enzyme to be assayed. The rate of increase in absorbancy at 395 mμ was determined using a Gilford Model 2000 recording spectrophotometer. Since p-nitrophenol has a pK in the region of pH 7.0, careful control of the pH was essential. The release of 0.01 μmole of p-nitrophenol under the conditions described above results in an increase of 0.310 absorbancy unit (1 cm light path length).

The rate of glucose formation from cycasin and salicin could be measured by coupling glucosidase activity to TPN reduction by means of hexokinase and glucose-6-phosphate dehydrogenase. The following were combined in a total volume of 0.3 ml: triethanolamine, pH 7.0, 30 μmoles; substrate (cycasin or salicin), 1 μmole; $MgCl_2$ 5 μmoles; TPN, 0.14 μmole; ATP, 2 μmoles; glucose-6-phosphate dehydrogenase, 0.7 unit; hexokinase, 1.4 units; and the enzyme to be assayed. The rate of TPNH formation was determined from the increase in absorbancy at 340 mμ. The activity of the coupling enzymes was tested each day using a known quantity of glucose, and it was determined that the rate of TPN reduction by the coupling system was well in excess of any rates which were observed when a glucosidase was being assayed. The reduction of 0.01 μmole of TPN causes an increase in absorbancy of 0.186.

The conditions described for these assays provided optimal pH. Extracts from each organism used were tested for stimulation by magnesium in the assay for PNPG glucosidase. With the exception of one case to be described below, the substrate concentrations as indicated were well above the K_m and were essentially nonlimiting.

The protein concentration of the extracts was determined by the method of Lowry *et al*(8).

e. *Experimental procedures.* The respective bacterial strains were introduced into the isolators in sealed containers through a peracetic acid lock. Monocontamination of the previously germfree rats was accomplished by swabbing the oral cavity, front paws, and head of each rat with a broth culture of the living organisms. The suspension was also applied to the food pellets. This procedure was repeated once the following day. Monocontamination was considered successful after pure cultures of the respective monocontaminants were obtained from the feces on two occasions, usually between the fourth and seventh day. Prior to administration of cycasin, food was withheld for 18 hours. Cycasin in aqueous solution (50 mg/ml) was sterilized by filtration through a Millipore® filter and introduced in a sealed container through the peracetic acid lock. It was given by stomach tube to each rat in a dose of 750 mg/kg body weight, which is substantially larger than the LD 50 for this strain of rats. Collections of urine and feces on a 24-hour schedule were started after the rats had received the cycasin. Determination of cycasin in urine and feces was done for 3 consecutive days according to the method described previously(4). All rats which had not already died were sacrificed on the seventh day after cycasin had been given and the livers were examined for pathologic changes. On the day of autopsy, samples of the remaining cycasin solutions which had been administered to the rats were removed from the isolators for assay to assure that no degradation had taken place during filtration or entry into the isolator.

Results. The results of assaying β-glucosidase activity *in vitro* in extracts of the bacteria under the conditions described above are shown in Table I.

The *Streptococcus* has a glucosidase with

TABLE I. Specific Activity of β-Glucosidase in Extracts of Bacteria Used as Monocontaminants.

Organism	Carbon source used for growth of bacteria	Substrate used in assay	Specific activity (mμmoles of substrate split/5 min /mg of protein)
Streptococcus fecalis (known salicin fermenter)	salicin	PNPG	21
	"	salicin	312
	"	cycasin	54
	glucose	"	19
Lactobacillus salivarius salicinius (known salicin fermenter)	glucose	PNPG	NA*
	"	salicin	NA
	salicin	PNPG	9
	"	salicin	13
	"	cycasin	NA†
Lactobacillus salivarius salivarius (known not to ferment salicin)	glucose	PNPG	NA
	"	salicin	NA
	"	cycasin	NA

* NA means no β-glucosidase activity was detectable under conditions of the assay.

† As described below in the text, the extract of *Lactobacillus salivarius salicinius* can deglucosylate cycasin in high concentrations. In the presence of 0.1 M cycasin the specific activity is 74 mμmoles/5 min/mg of protein.

substantial activity toward cycasin, even though this substrate is not hydrolyzed as rapidly as salicin. Comparison of specific activity of the enzyme in organisms grown on salicin with that of organisms grown on glucose indicates that glucose acts as a modest repressor of the enzyme, but that significant activity is present in bacteria grown on glucose. Growing organisms in an equimolar mixture of salicin and glucose resulted in specific activities similar to those found in organisms grown in glucose alone. *Lactobacillus salivarius salicinius* contained a glucosidase that hydrolyzed PNPG and salicin but not cycasin under the conditions of the assay. This enzyme is induced when the organisms are grown on a β-glucoside and is not detectable if the medium contains glucose. *Lactobacillus salivarius salivarius* has no detectable glucosidase activity, although, of course, the bacteria could not be grown on a glucoside.

Since the results based on the assay conditions described above do not explain the mild toxicity and decreased excretion of cycasin in animals contaminated with the salicin fermenting *Lactobacillus*, experiments were done to determine the effect of substrate concentration on glucosidase activity. At sufficiently high concentrations, cycasin was a suitable substrate for the glucosidase of this *Lactobacillus*, however, the K_m for cycasin was 0.83 M compared to a K_m for salicin of 3.5×10^{-5} M.§ At a cycasin concentration of 0.10 M the specific activity was 74 mμmoles of substrate split/5 min/mg of protein. In the case of the Streptococcal glucosidase, the K_m for cycasin is 3.1×10^{-5} M compared to a K_m for salicin of 7.7×10^{-5} M.

The excretion studies of cycasin are presented in Table II. All detectable cycasin is excreted during the first 2 days following administration, the determinations being negative on the third day. The figures for total percent excretion in the germfree rats and for those monocontaminated with *Lactobacillus salivarius salivarius* probably represent quantitative excretion with the limits of experimental error. Microscopic examination showed the livers of these rats to be uniformly normal. Monocontamination of germfree rats with *Streptococcus fecalis* resulted in a striking reduction in cycasin excretion, with values comparable to those seen previously in conventional rats. Microscopic study of livers from these animals showed severe centrolobular hemorrhagic necrosis, such as has been reported previously in cycad toxicity(10).

A definite though less impressive reduction in cycasin excretion was noted in the rats monocontaminated with *Lactobacillus salivarius salicinius*. Sections of the liver from

§ K_m values were determined from plots made by the method of Lineweaver and Burk(9).

TABLE II. Cycasin Excretion in Germfree and Monocontaminated Rats.

Monocontaminant	No. of rat	Intake in mg	Excretion in mg in 48 hr		Total excretion		Unrecovered cycasin	
			Urinary	Fecal	mg	%	mg	%
None	1	85.0	80.8	.0	80.8	95.0	4.2	5.0
	2	90.0	79.0	3.1	82.1	91.2	7.9	8.8
	3	85.0	75.5	1.4	76.9	90.5	8.1	9.5
	4	120.0	118.0	.0	118.0	98.3	2.0	1.7
	5	110.0	96.2	2.3	98.5	89.5	11.5	10.5
	6	85.0	78.2	.7	78.9	92.8	6.1	7.2
	7	95.0	84.2	1.7	85.9	90.4	9.1	9.6
Streptococcus fecalis	1	125.0	33.4	.0	33.4	26.7	91.6	73.3
	2	112.0	30.0	"	30.0	26.7	82.0	73.3
	3	118.0	36.8	"	36.8	31.2	81.2	68.8
	4	108.0	36.0	"	36.0	33.3	72.0	66.7
Lactobacillus salivarius salicinius	1	126.5	81.6	.0	81.6	64.5	44.9	35.5
	2	126.5	77.6	"	77.6	61.3	48.9	38.7
	3	126.5	95.2	"	95.2	75.3	31.3	24.7
	4	132.3	120.0	"	120.0	90.7	12.3	9.3
Lactobacillus salivarius salivarius	1	125.0	121.5	2.2	123.7	99.0	1.3	1.0
	2	110.0	106.0	1.3	107.3	97.5	2.7	2.5
	3	75.0	69.0	1.0	70.0	93.3	5.0	6.7
	4	80.0	73.3	1.4	74.7	93.4	5.3	6.6

TABLE III. Influence of Duration of Streptococcal Monocontamination of Germfree Rats on Cycasin Excretion.

Interval between bacterial inoculation & cycasin administration in days	No. of rat	Intake in mg	48 hr excretion in mg		Total excretion		Unrecovered cycasin	
			Urinary	Fecal	mg	%	mg	%
5	1	76.0	66.5	1.5	68.0	89.5	8.0	10.5
	2	94.0	65.0	.5	65.5	69.6	28.5	30.4
	3	68.0	58.5	.0	58.5	86.0	9.5	14.0
	4	64.0	46.3	.5	46.8	73.1	17.2	26.9
7	1	125.0	33.4	.0	33.4	26.7	91.6	73.3
	2	112.0	30.0	"	30.0	26.7	82.0	73.3
	3	118.0	36.8	"	36.8	31.2	81.2	68.8
	4	108.0	36.0	"	36.0	33.3	72.0	66.7

these animals showed mild evidence of injury consisting mainly of loss of cytoplasmic basophilia and occasional necrotic parenchymal and Kupffer cells. This *Lactobacillus* is the organism described above which requires a higher cycasin concentration for glucosidase activity compared with *Streptococcus fecalis*.

The importance of permitting sufficient time for colonization of the intestines of germfree rats by a monocontaminant is shown in Table III. The rats monocontaminated with the *Streptococcus fecalis* which received cycasin on the fifth day after inoculation excreted more than twice as much cycasin as the rats monocontaminated for 7 days. Although fecal cultures indicated the presence of the organisms, insufficient time had apparently been allowed for their establishment to assure a level of enzymatic activity comparable to that found after 7 days.

Table IV summarizes the principal results of the various experiments.

Discussion. These experiments confirm the role of intestinal bacteria in deglucosylating cycasin to yield the aglycone. The bacterial species, of which strains were selected on the basis of their known glucoside-splitting ability, are known to be a part of the intestinal flora of the normal rat(11). No attempt was made to survey the entire intestinal flora in this respect. It is sufficient here to recognize that bacteria vary in their ability to deglucosylate cycasin, and that this variation represents one biologic variable which de-

TABLE IV. Summary of Effects of Bacteria on Cycasin in Monocontaminated Germfree Rats.

Monocontaminant	*In vitro* test for β-glucosidase in bacterial extracts	No. of rats	Avg cycasin intake (mg)	Cycasin excreted in 48 hr		Degree of liver injury (microsc.)	Alive 7 days after cycasin administration
				mg	%		
None		7	95.7	88.7	92.7	None	7/7
Streptococcus fecalis	+++	4	115.8	34.1	29.4	Severe	0/4
Lactobacillus salivarius salicinius	+	4	128.0	93.6	73.1	Mild	4/4
Lactobacillus salivarius salivarius	—	4	97.5	93.9	96.3	None	4/4

termines the toxicity and probably the ultimate carcinogenicity of cycasin.

Experiments done previously in which germfree animals failed to deglucosylate cycasin left open the possibility that some endogenous glucosidase capable of deglucosylating cycasin was formed or released by the intestine in the presence of microorganisms. Experiments with the *Lactobacillus* which does not ferment salicin show that in animals monocontaminated with this organism cycasin is not deglucosylated and is therefore recoverable from urine and feces. Moreover, the compound is not toxic in these animals. This strongly suggests that there is no endogenous glucosidase formed by the intestine which is capable of deglucosylating cycasin, even in the presence of bacteria.

Other authors have shown, however, that mammalian intestinal mucosa contains endogenous β-glucosidases which are capable of hydrolyzing both naturally occurring disaccharides and β-glucosides with synthetic aglycones(12). These intestinal glucosidases are not able to deglucosylate cycasin as shown by its quantitative excretion and absence of toxicity.

β-glucosidase activity has been investigated in other microorganisms. Although *E. coli* generally do not ferment salicin, Schaefler and Maas(13) have selected mutants able to grow on salicin which contain a β-glucosidase inducible by a variety of natural and synthetic glucosides. β-glucosidases have also been demonstrated in yeasts(14) and molds (15). The dependence of cycasin toxicity on the presence of such glucosidase containing microorganisms would make toxicity highly variable in a population of rats.

Even among experimental animals given glucosidase containing microorganisms, there is variability in the toxicity of cycasin and in the amount of unchanged cycasin which is excreted. There are several factors which might be expected to have a role in this variability. One is the success of the monocontaminant in colonizing the intestine. Unless cycasin is deglucosylated in the intestine by microorganisms, it is absorbed and excreted unchanged in the urine. The importance of successful establishment of the monocontaminant is demonstrated by the experiment in which animals given either the *Streptococci* or the salicin fermenting *Lactobacilli* four days prior to being given cycasin excreted considerably more unaltered cycasin and manifested less toxicity than the groups in which 7 days were allowed for establishment of the contaminant. The successful isolation of an organism from the feces of monocontaminated animals is not a sufficient indication of the establishment of the organism.

The inducibility of β-glucosidase by growth on a glucoside in the absence of glucose, especially in the case of the salicin fermenting *Lactobacillus* adds another variable that would be expected to influence the response of different animals to cycasin. The role of various dietary disaccharides and polysaccharides in inducing glucosidases capable of hydrolyzing cycasin in the *Lactobacilli* has not been investigated.

The relationship of cycasin concentration to glucosidase activity in the salicin fermenting *Lactobacillus* suggests that factors which would favor high concentrations of cycasin in the intestine would be important in determining the extent of its deglucosylation and therefore its toxicity. Results obtained with this organism were originally contra-

dictory because the excretion studies, the evidence of mild cycasin toxicity in animals contaminated by it, and the ability of the bacteria to ferment salicin, all indicated that it contained at least moderate amounts of a glucosidase capable of splitting cycasin. Enzymatic studies, on the other hand, failed to reveal a detectable glucosidase. A 30- to 100-fold increase in substrate concentration, however, permitted the assay of glucosidase activity in the extracts. A determination of the K_m for cycasin and a calculation of the approximate concentration of cycasin in the alimentary canal of the experimental rats revealed that adequate concentrations of cycasin were probably present initially for some deglucosylation to occur.

Summary. The role of intestinal bacteria in converting the naturally occurring glucoside cycasin to its hepatotoxic and carcinogenic aglycone (methylazoxymethanol) has been studied. Germfree rats, in which cycasin is nontoxic, were monocontaminated with several strains of bacteria prior to being given cycasin. Levels of glucosidase activity in the bacteria were determined by the assay of cell free extracts, using cycasin as a substrate. The toxicity of cycasin in rats given the various bacteria and the amounts of unchanged cycasin excreted were consistent with the glucosidase assays. Intestinal microorganisms therefore convert cycasin to the toxic aglycone, and variations in the intestinal flora probably have a role in determining the toxicity of ingested cycasin.

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A Leukemogenic Filtrable Agent from Chemically-Induced Lymphoid Leukemia in C57BL Mice. (31827)

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Mice of the C57BL strain are refractory to spontaneous lymphatic leukemia, and highly susceptible to its induction by irradiation. There are no reports of successful attempts to induce lymphatic leukemia in adult C57BL mice by chemical carcinogens.

Although the problem of whether chemicals induce leukemia by activation of a latent virus, similarly to the induction mechanism thought to be involved in radiation-induced leukemias(1), has been investigated in avian leukosis(2) and murine leukemias(3), no definite conclusions have been reached.

The aim of the present work was to demonstrate the leukemogenic activity of 7,12-dimethylbenz(a)anthracene (DMBA) in C57BL adult mice, and to isolate a leukemogenic agent from these carcinogen-induced lymphomas.

Materials and methods. The carcinogen