Saccharin Metabolism and Tumorigenicity

Abstract. *Exposure of male Charles River CD1 rats to a 5 percent saccharin diet in utero and throughout weaning, conditions associated with tumor induction, did not induce detectable metabolism (< 0.4 percent of the oral dose) of tritiated saccharin in vivo. No metabolites (< 0.06 microgram per kilogram per 24 hours) were detected in the urine of normal rats given a tracer dose. Pretreatment with 3-methylcholanthrene did not induce saccharin metabolism.*

A recent report on the risks and benefits of saccharin (*1*) concluded that saccharin is a weak bladder carcinogen in male rats but that consistent effects were observed only in rats that had received saccharin-containing diets during two consecutive generations. However, saccharin differs from most chemical carcinogens in that it is eliminated from the body largely unchanged. Previous studies on saccharin metabolism have yielded equivocal results with some workers detecting 2-sulfamoylbenzoic acid (*2-4*), 2-sulfobenzoic acid (*2, 3*), and carbonate (*4*) as urinary metabolites in the rat ($\leq$1 percent of the dose), and others (*5-7*), using similar techniques, failing to confirm these findings. None of the studies determined the fate of saccharin in rats receiving saccharin in the diet (5 percent by weight) for two generations. Such long-term administration during development might result in the metabolism of the compound being induced, which has been shown to occur for cyclamate (*8*), especially during the weaning period (*9*). Furthermore, a minor saturable metabolic pathway may have remained undetected by previous workers because of the relatively large doses used. The possibility of induction of saccharin metabolism by polycyclic aromatic hydrocarbons has not been investigated, although it has been shown that prior treatment with phenobarbital does not induce saccharin metabolism (*5*). However, phenobarbital induction would not expose a metabolic route involving cytochrome P_1-450 (cytochrome P-448), which would be of greater relevance to the metabolic activation of an aromatic compound. The recent report (*1*) reviewed these conflicting data and concluded that "either saccharin is unusual in that its carcinogenic effects are due to the unmetabolized parent compound or the effects are due to small quantities of undetected metabolites," and that "because of the limits of experimental detection it is possible that a small percentage of saccharin may be modified enzymatically."

In an attempt to resolve these conflicting reports and doubts over the possibility of a minor metabolic route being involved in the reported tumorigenicity of saccharin we have studied the fate of [5-^{3}H]saccharin under conditions likely to maximize any metabolism. We used male Charles River CD1 rats, the same strain as used in the most recent positive Canadian two-generation carcinogenicity study (*1*).

The [5-^{3}H]saccharin (synthesized from 5-chlorosaccharin by the Radiochemical Centre, Amersham, U.K.) was purified by repeated preparative thin-layer chromatography (TLC) until it contained > 99.8 percent of the activity as saccharin and < 0.02 percent as 2-sulfamoylbenzoic acid by TLC and reverse isotope dilution (*7, 10*). The high-specific-activity product (approximately 50 mCi/mg) was incorporated into a diet containing 5 percent saccharin by weight to give an activity of 6.1 μCi/g. Analysis of the food by TLC showed that 98.3 percent of the ^{3}H was saccharin. This result was lower because of the poorer quality of the chromatography rather than because of the presence of other ^{3}H constituents.

Adult male rats that had been exposed to saccharin in utero and maintained on a 5 percent saccharin diet, conditions associated with an increased incidence of bladder tumors (*1, 11*), were given free access to the labeled diet for a period of 24 hours and then returned to the unlabeled 5 percent saccharin diet (pulsed-dose experiment). The urine and feces were collected at 24-hour intervals and analyzed for total ^{3}H and metabolites as described previously (*7, 10*). In a separate experiment the metabolism and excretion of a single oral dose of [^{3}H]saccharin was studied (i) in normal rats given 60 μg/kg and (ii) in rats first treated with 3-methylcholanthrene and then given the single dose of saccharin (400 μg/kg). Both groups were maintained on saccharin-free food and water.

The results (Table 1) show that the ^{3}H label was eliminated, largely in the urine, within 48 hours, despite the widely differing conditions. The extent of fecal excretion in the pulsed-dose study (14 percent) is in agreement with previous results with F_0 female Wistar rats that were fed on a 5 percent saccharin diet but were returned to normal food during studies of metabolism (*7*). The lower fecal excretion in rats given 60 μg of saccharin per kilogram suggests that absorption may be reduced at the higher doses used in previous metabolism studies. Analysis of urine samples obtained 0 to 24 hours after labeled saccharin administration in the pulsed-dose experiment showed that saccharin was the only ^{3}H-labeled component (98.5 percent of urinary ^{3}H by TLC); < 0.05 percent of the ^{3}H was detected by reverse isotope dilution as 2-sulfamoylbenzoic acid, the reported metabolite (*2-4*). Similarly, in untreated rats given 60 μg/kg, 99.9 percent (0 to 24 hours) and 99.1 percent (24 to 48 hours) of the urinary ^{3}H was unchanged saccharin, while < 0.01 percent of the dose was excreted as 2-sulfamoylbenzoic acid. This result was confirmed by

Table 1. The excretion of ^{3}H by rats given [5-^{3}H]saccharin. Results are expressed as averages (± standard deviation) of three male Charles River CD1 rats.

Time after dose (days)	Percent of dose in		
	Urine	Feces	Total
*Pulsed-dose experiment**			
1	67.9 ± 3.5	8.6 ± 2.8	82.0 ± 3.6
2	79.3 ± 2.4	13.3 ± 4.3	99.1 ± 0.7
3	80.1 ± 2.3	13.6 ± 4.6	100.0
Low-dose experiment 1—(single dose of saccharin)†			
1	83.5 ± 1.7	2.5 ± 1.9	88.4 ± 3.5
2	84.8 ± 2.1	2.7 ± 1.7	90.0 ± 3.8
6	85.0 ± 2.1	2.9 ± 1.5	90.7 ± 3.6
Low-dose experiment 2—(methylcholanthrene and single dose of saccharin)‡			
1	91.3 ± 8.2	6.7 ± 3.0	102.9 ± 9.9
2	93.3 ± 7.0	7.1 ± 3.3	105.7 ± 8.1
3	94.0 ± 7.5	7.8 ± 2.4	106.7 ± 7.4

*Rats (290 g of body weight) exposed to saccharin in utero and maintained on 5 percent saccharin diet were given a diet containing 5 percent [^{3}H]saccharin for 24 hours (day 1) and then returned to the unlabeled diet. Because the diet took up moisture in the cages the recovery of ^{3}H was > 100 percent of that apparently consumed. The results given are, therefore, the percentage of the total recovered in 3 days. †Normal rats (260 g of body weight) were given a single dose of [^{3}H]saccharin (60 μg/kg; 750 μCi per rat) by intragastric intubation. The results given are the percentage of the dose. ‡Rats (330 g of body weight) were given 3-methylcholanthrene (20 mg/kg, orally) 3 and 2 days before the single oral dose of [^{3}H]saccharin (400 μg/kg; 5 mCi per rat). Animals treated similarly showed a 193 percent increase in hepatic aryl hydrocarbon hydroxylase activity and a 48 percent increase in total hepatic cytochrome P-450 compared with controls. The results given are the percentage of the dose.

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reverse phase high-pressure liquid chromatography which showed the absence of other ^3H-labeled components including ^3H_2O. Saccharin was the only ^3H compound detected (99.7 percent of activity) in urine samples (0 to 24 hours) from the rats that received 3-methylcholanthrene before saccharin.

These results demonstrate that significant metabolism is not induced by long-term administration of saccharin during the neonatal and weaning stages of two-generation studies. Because of the poorer quality of the chromatography of the pulsed-dose urine samples the limit of detection for any metabolite separated from saccharin by TLC was approximately 0.4 percent. In the low-dose studies the limits of detection were 0.2 percent by TLC in 2-methylcholanthrene-treated rats and 0.1 percent by TLC, 0.05 percent by high-pressure liquid chromatography and 0.01 percent of the dose as 2-sulfamoylbenzoic acid by reverse isotope dilution in normal rats. This last study sets the limit of detection of a saturable metabolic process at 0.06 μg per kilogram of body weight per 24 hours. Thus in animals fed a 5 percent saccharin diet this would represent < 0.015 μg of metabolite per gram of saccharin consumed (< 0.015 part per million), assuming each rat (250 g) consumes 20 g of diet per day. The total exposure of the bladder epithelium during long-term administration to any metabolite would be a maximum of 0.015 μg per day or approximately 5 μg per year. This is considerably below the concentration of organic impurities in commercial saccharin (up to 20 parts per million) that the recent report (*1*) concluded were present at such low levels that they would have to show "exquisite potency" to be responsible for the development of bladder tumors in rats fed diets high in saccharin.

The absence of detectable metabolism of saccharin at such low concentrations is important evidence that this anionic species cannot act as a classic electrophilic carcinogen. Thus we must conclude (*1*) that "saccharin is unusual in that its carcinogenic effects are due to the unmetabolized parent compound," and other possibilities, such as promotor effect or changes in physiological function or urine composition, must be investigated before the apparent mechanism of tumorigenicity and its specificity are understood.

T. W. SWEATMAN
A. G. RENWICK
Clinical Pharmacology Group, University of Southampton, Medical and Biological Sciences Building, Southampton SO9 3TU, England

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Tidal Vertical Migration: An Endogenous Rhythm in Estuarine Crab Larvae

Abstract. *Field-caught larvae of the estuarine crab* Rhithropanopeus harrisii *have a tidal rhythm of vertical migration when maintained in constant conditions. Laboratory-reared larvae do not show this rhythm. Endogenous tidal vertical migrations aid the retention of these planktonic larvae in estuaries near the parent populations.*

Tidal fluctuations dramatically affect the lives of benthic intertidal organisms. It is not surprising that a number of these organisms have endogenous circatidal rhythms of behavior, physiology, and reproduction [see (*1–3*)]. Phasing of their internal clocks with respect to the tidal cycle is accomplished through synchronizers such as changes in hydrostatic pressure, wave agitation, and inundation, all of which occur at the land-water interface (*2*).

Because planktonic organisms remain in the water column, the above-mentioned synchronizers have little influence on them. However, in estuarine environments the tidal cycle is often accompanied by changes within the water column itself, brought about by flow-induced turbulence (*4*). Water quality factors likely to be thus affected are salinity, dissolved organic substances, and inorganic nutrients; temperature and turbidity cycles often occur as well. These changes could act to synchronize endogenous tidal clocks in some estuarine plankton. We now describe a tidal rhythm, presumably entrained by these water column synchronizers, in the planktonic larvae of the estuarine crab *Rhithropanopeus harrisii* (Gould).

We monitored vertical distributions of laboratory-reared or field-caught crab larvae under constant conditions for up to 8 days by means of an automated television system. To obtain laboratory-reared larvae, we collected ovigerous female crabs and maintained them at 25°C and 20 per mil salinity in a cycle of 12 hours of light alternating with 12 hours of darkness. Standard rearing techniques were used for the larvae (*5*), which were initially kept in the same conditions as the parent crabs, then changed to constant dark at the start of the experiment (*6*). Field-caught larvae were taken by towing a plankton net in water of 15 per mil salinity in the Newport River estuary near Beaufort, North Carolina. These samples were immediately returned to the laboratory for sorting of crab larvae into species and stage; no more than 5 hours elapsed between collection and the start of an experiment. Although tidal height records for the area of collection are not available, tides in the Beaufort area are of the semidiurnal type, with approximately equal amplitudes. The tidal cycle at the collection site has a period length of approximately 12.4 hours.

The general procedure was as follows. Between 60 and 100 larvae were added to the top of a Lucite column (1.9 m) (*7*) containing water of the same salinity as that in which the larvae had been living (*8*). Freshly hatched *Artemia salina* nauplii were added for food at the beginning of each experiment and again at random times every second day. Introduction of fresh food had no apparent effect on larval vertical distribution. After a brief period in which the larvae were allowed to distribute themselves, the monitoring system was automatically activated each half hour (*9*), so that an elevator could raise the television camera and scan the column against a dim red backlight in a period of 2 minutes. The scanned image was stored on videotape. Events were identical on the following cycle, but the camera was lowered to the initial position. Except for the time that the backlight was on, the larvae were in total

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