vigorous stirring. After 1.5-hr stirring, the aq. layer was extracted with ether (25 ml) which was washed with sat. NaCl and dried over anhyd. Na_2SO_4. After evaporation of ether, the residual oil was purified by column chromatography (SiO_2, hexane: AcOEt=7: 3 for elution) to give benzoylpiperidine (440 mg, 23% from acetanilide) which was identified by comparison of spectral data with the authentic sample and by gas chromatographic analysis.

Acknowledgement We thank Prof. S. Yamada, University of Tokyo and Dr. Z. Horii, Dean of Josai University, for their encouragement.

[Chem. Pharm. Bull. 24(8)1943—1947(1976)]

UDC 547.541.04.09 : 615.31.076.9

Hydrolysis of Bis(*p*-hydroxyphenyl)pyridyl-2-methane Disulfate. I. Presence of Arylsulfatase and Laxative Activity

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(Received March 22, 1976)

The laxative action of bis (*p*-hydroxyphenyl)pyridyl-2-methane disulfate is considered to be dependent on the formation of the free phenolic compound, bis(*p*-hydroxyphenyl)-pyridyl-2-methane by hydrolysis. Therefore, the activity of the parent compound as a laxative must be governed by arylsulfatase activity in the human intestine. When examinations of human feces were made, arylsulfatase activity in the intestine was detected, and about 98% of the samples were shown to be positive.

Validity of the fecal arylsulfatase test in the test population might be supported by the liquefaction test of rat feces with the injection of the parent compound into duodenum.

It is well known that many naturally occurring and synthetic phenolic compounds are used in medicinals for their laxative properties. In addition, it has been hypothesized that the free phenolic group is essential for the laxative action.[2-5] Pala, *et al.*[6] described bis(*p*-hydroxyphenyl)pyridyl-2-methane disulfate (I) as a new laxative but asserted that no phenolic groups were released when it was administered to rats. The study did not mention arylsulfatases which have been detected in intestines, microorganisms of intestinal flora and other tissues.

According to Ferlemann, *et al.*,[5] bisacodyl (III) (Fig. 1), whose structure is similar to that of I, is decomposed by either fecal homogenates or freshly collected fluid from the small intestine of the rat. It was found that the hydrolytic activity of the homogenates and fluids could be abolished by heating.

In contrast to the manner in which bisacodyl is hydrolyzed, the hydrolysis of the disulfate compound (I) can be attributed to microorganisms of the intestinal flora.[7] Scheline[8] described

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the presence of arylsulfatase in these microorganisms. Findings obtained by Jauch, *et al.*[9] support this mechanism of hydrolysis: (a) Fecal homogenates of germfree rats hydrolyzed bisacodyl in the same manner as fecal homogenates from normal rats, and prior autoclaving of the homogenates abolished the hydrolytic activity; (b) After oral administration of I, germfree rats failed to excrete bis(*p*-hydroxyphenyl)pyridyl-2-methane (II) in their feces. In addition, fecal homogenates from these rats were unable to hydrolyze I; (c) When rats that normally excreted free II in their feces after oral administration of I were given neomycin for several days, they failed to excrete free II in their feces. Fecal homogenates from these rats were also unable to hydrolyze I during the neomycin treatment. Discontinuance of the antibiotic was followed by a resumption of hydrolytic activity in the gut as well as in the fecal homogenates.

They concluded that the hydrolysis of I and III resulted in the same active products (see Figure 1), but that the hydrolysis was accomplished by esterases of a different origin. Bisacodyl can be hydrolyzed by enzymes in the small intestine.[5] However, I appears to be hydrolyzed only by arylsulfatases of microorganisms found in colonic flora. So far, these microorganisms have neither been identified nor cultured.

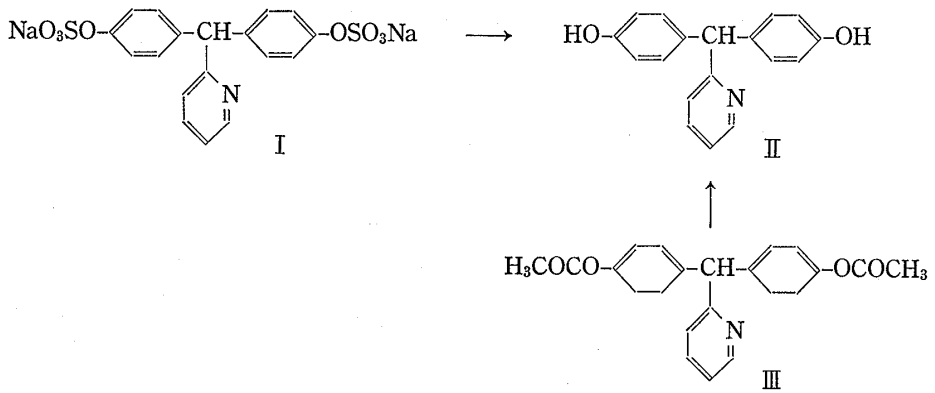

Fig. 1. Hydrolysis of Bis(*p*-hydroxyphenyl)pyridyl-2-methane Disulfate (I) and Bisacodyl (III) to Bis(*p*-hydroxyphenyl)pyridyl-2-methane (II)

In the present study, human feces were tested for the presence of arylsulfatase to (A) Support the conclusion of Jauch, *et al.*, and (B) Evaluate I as a possibility for a new laxative.

The term "arylsulfatase" was taken from Jauch, *et al.* and is used to describe the enzyme which hydrolyzes I to II. As mentioned before, this enzyme and the microorganisms from which it is derived have not yet been identified.

Materials and Methods

Peptone Culture Medium——Eight grams of Bacto-nutrient broth dehydrated (Difco Laboratories, Detroit, U.S.A.) were dissolved in 1000 ml of distilled water and the solution was sterilized by autoclaving for 15 min at 15 lb/sq. in. pressure at 121°.

Fecal Sample Solution——A mixture of 300 mg of fresh feces and 5 ml of peptone culture medium was incubated at 37±1° for 48 hr. To this mixture, 2.5 ml of 0.3% aqueous solution of I was added and further incubated at 37±1° for another 48 hr. After centrifugation, 50 μl of the supernatant was subjected to Thin-layer chromatography (TLC).

TLC for the Detection of Bis(*p*-hydroxyphenyl)pyridyl-2-methane (II)——For TLC, the solvent system selected was benzene-isopropanol (9:1). The adsorbent was diatomite (Kieselgel G). The distance developed was 10 cm at 0.5 mm thickness, and the color developer was iodine tincture JP. The Rf value of II was 0.58 (blue) at room temperature, and the identification limit of II by iodine tincture was 0.02 μg per spot.

Liquefaction of Feces in Rat Intestine——Each group consisted of 12 female rats (Wistar strain, average weight 300 g). The rats are anesthetized with ether. Through a midline incision the duodenum is exposed.

9) R. Jauch, R. Hankwitz, K. Beschki and H. Pelzer (Biochemical Department, Dr. Karl Thomai GmbH, Germany), unpublished.

One-tenth milliliter of a solution containing 30 mg of I per kg of body weight was injected into the duodenum at a site about 1.5 cm distance from pylorus and the incision is closed. Whenever needed after the injection, through a midline indision apparent changes such as softening and liquefaction of feces could be observed by looking through a gut wall.

Results and Discussion

I did not give any spot from TLC, and the components of the peptone culture medium gave only one spot (*Rf* 0.68, pale yellow). Therefore, it was very easy to identify II, the product from the hydrolysis of I by arylsulfatase.

Phosphate buffer is often used in hydrolytic reaction with arylsulfatase, and thus a mixture of I and 1/15 M phosphate buffer (pH 6.2) was tried and incubated at 37±1° for 24 hr. However, the use of phosphate buffer was abandoned, since a spot of II was detected from TLC without any feces or arylsulfatase present. When a mixture of peptone culture medium and I was incubated at 37±1° for 48 hr (no feces) and examined for II, no spot of II was observed on the TLC plate.

Logically, hydrolysis reactions are very pH sensitive, and since the fecal samples came from different sources, there might be different pH's. Therefore, pH's of the fecal sample solution (a mixture of 300 mg of fresh feces and 5 ml of peptone culture medium) were monitored before and after incubation at 37±1° for 48 hr, and as a result, no changes of pH's were detected by showing pH 6.8 which was the pH of peptone culture medium itself.

Since it was already known through TLC that I can be hydrolyzed by arylsulfatase (in the presence of 5 ml of peptone culture medium and 0.1 ml of β-glucuronidase/arylsulfatase [Boehringer Manheim GmbH, Germany] (1: 5) incubated at 37±1° for 48 hr), it was concluded that hydrolysis of I by feces may be due to arylsulfatase from intestinal bacterial flora.

Since the assumption that the laxative action of I is dependent on individuals having arylsulfatase, it is important to determine what percentage of the population has no arylsulfatase in their intestine. Normal, healthy human test subjects were divided into groups by region, age and sex as seen in Table I, Section 1. The number of subjects selected was limited and insufficient for a thorough statistical treatment of the data obtained; however, results were deemed adequate to warrant some general conclusions. Relative concentrations of fecal arylsulfatase from the test population were graded from (—) to (卌), as shown in Table I. There did not appear to be any correlation between the amount of arylsulfatase present and the sex or age of the subject nor the region in which they lived. Out of the test population, only two of the subjects (2.2%) had no arylsulfatase in their feces. The other corresponding percentages obtained were: 卌, 5.6%; 艹, 12.3%; +, 74.3%; and ±, 5.6%.

If the assumption that arylsulfatase comes from intestinal bacterial flora is valid, it should be possible to detect lowered levels in the feces after the administration of oral antibacterial drugs. Accordingly, several antibacterial drugs were selected, and each one of them was given to a different test subject. Ingestion of chloramphenicol, berberine preparation, creosot pills and a sulfa drug resulted in a lowering of arylsulfatase levels, but not as distinctly nor as prolonged as tetracycline. Administration of tetracycline lowered arylsulfatase levels down to zero for at least seven days after the last dose. However, the detection of arylsulfatase may have been hampered because of a delay in the rate of passage of fecal matter through the gut. A more complete and detailed study will be made in the future of the effect of antibacterial drugs on arylsulfatase levels in human feces.

In order to ascertain laxative activity of II produced by hydrolysis of I in a gut, a solution of I was injected into rat duodenum under ether anesthesia. Doses to each group used for the injection, were 5, 10, 15, 20, 30, and 35 mg of I per kg of body weight. Below the doses of 15 mg/kg showed through 4 hr observation every 30 min neither softening nor liquefaction of feces in the region of about 30 cm length of a small intestine measured from the pylorus. When 20 mg/kg of I was given to rats, softening of feces was observed in the same region of a gut

TABLE I. Hydrolysis of Bis(p-hydroxyphenyl)pyridyl-2-methane Disulfate to Bis(*p*-hydroxyphenyl)pyridyl-2-methane (II) by Arylsulfatase in Human Feces

Section 1. Normal, Healthy Human Test Subjects

Region	Age in years	Sex	Arylsulfatase concentration[a] (Number of test subjects) ⧺	⧺	+	±	−	Total
Tokyo	—15	M			6			6
		F			5			5
	16—35	M	1		3	1		5
		F		1	3			4
	36—50	M			6			6
		F			5			5
Mihara (near Hiroshima)	—15	M			5		1	6
		F			4	1	1	6
	16—35	M			3			3
		F		1	7			8
	36—50	M	1	1	3	1		6
		F	2	1	3			6
Osaka	—15	M						0
		F			1	2		3
	16—35	M		2	5			7
		F	1	2	3			6
	36—50	M		1	2			3
		F		2	2			4
Total (% of Total)			5 (5.6)	11 (12.3)	66 (74.3)	5 (5.6)	2 (2.2)	89 (100)

Section 2. Effect of Antibacterial Drugs on Arylsulfatase Activity

Drug	Days[b]	Age of male test subject (yrs.)	Arylsulfatase concentration[a] ⧺	⧺	+	±	−
Tetracycline (Lederle Co., Ltd., 250 mg four times daily for 2 days)	0	28			*		
	1					*	
	3						*
	7						*
Chloramphenicol (Sankyo Co., Ltd., 250 mg four times daily for 3 days)	0	49		*			
	1					*	
	3				*		
	7			*			
Berberine preparation ("Wakamatsu", Nakataki Seiyaku Co., Ltd., 2 Tablets three times daily for 3 days)	0	30			*		
	1					*	
	3				*		
	7					*	
Creosot pill (Daiko Yakuhin Kogyo Co., Ltd., 3 pills three times daily for 3 days)	0	27		*			
	1				*		
	4		*				
	7		*				
Sulfa drug ("Sinomin", Shionogi Seiyaku Co., Ltd., 2 tablets twice daily for 3 days)	0	26			*		
	1					*	
	3				*		
	7				*		

a) Arylsulfatase concentrations from TLC were based on color intensity of II with iodine tincture. ⧺ corresponds to 5 μg of II; ⧺ to 2—3 μg; + to 0.5—1.0 μg; ± to 0.2—0.5 μg; and − to no spot observed.

b) Day 0 is the day before administration of the antibacterial agent, and days 1, 3, 4, and 7 are the number of days after the last dose administered.

after 3.5 hr of the injection; 30 mg/kg, liquefaction of feces after 2 hr; and 35 mg/kg, liquefaction of feces after 1 hr. In the case of 0.2 ml of water injection without I to one group, no apparent changes were observed in the same region of a gut through 4 hr after the injection. On the other hand, fecal samples of the treated group (12 rats) of which 0.2 ml of tetracycline syrup (equivalent to 5 mg of tetracycline, Achromycin V Syrup, Lederle) was given orally three times daily for continuous 5 days, were taken every day for the fecal arylsulfatase test (see "TLC for the detection of II" in the Materials and Methods). Ten rats out of 12 rats in the treated group showed — (the same grade as shown in Table I) from the 3rd day after the first tetracycline administration, whereas the remainder provided ± even the 5th day after the first administration. When 30 mg/kg of I was injected into duodenum to each rat of the treated group (described above) on the next day after the end of the tetracycline administration, on the contrary to untreated normal group, no softening and liquefaction of feces within the limits of 0—40 cm distance from the pylorus were observed for 2 hr after the injection of I, except two rats which showed ± in the fecal arylsulfatase test. These two rats gave softening and partial liquefaction of feces in the same gastric region 2 hr after the injection of I.

The facts mentioned above support that II due to hydrolysis of I by arylsulfatase in a gut or feces causes laxative activity, and I itself cannot liquefy feces. In this connection, pH's (measured by the pH-Meter PT-1, Electrode 301 B, Toyo Kagaku Sangyo Co., Ltd., Tokyo) at internal mucus membrane of a gut about 20 cm distance from the pylorus in both normal and the treated groups were scattered from 5.5 to 7.5, and their mean pH with standard error was 6.35 ± 0.23, accompanying with no significant difference between normal and the treated groups. Where, pH's in the treated group were measured on the next day after the final tetracycline administration.

From the results obtained above, detection of arylsulfatase in human feces might be valid in relation with laxative effect of I. Correlation of laxative action of I with the grades ##, #, or + in the fecal arylsulfatase test shown in Table I, and a more complete and detailed studies will be made in near future.

Acknowledgements The authors are grateful to Dr. Kazuo Matsumoto, the Teijin Fher Medical Co., Ltd., for his suggestion and generous donation of samples. The authors are also much indebted to Mr. Akira Nishikawa and Mrs. Fusako Hashimoto of the same company for their technical assistance.