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Duodenal Glucuronide Synthesis

III. Identification of Phenolphthalein Glucuronide as a Conjugation Product of Phenolphthalein in the Intestinal Tract in Rat

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Incubation of phenolphthalein in the presence of surviving duodenal slices resulted in the partial disappearance of the free phenolphthalein from the incubation mixture. The treatment with β-glucuronidase of the ethylacetate extract made from the mixture revealed that some 66 % of the original phenolphthalein appeared as a glucuronide hydrolysable by this enzyme.

Recent studies have indicated that the mucous membrane of the gastrointestinal tract posesses its own defence mechanism against harmful chemical substances. It has been found that the glucuronide conjugation is *in vitro* carried out not only by the liver and the kidney, but also by the gastrointestinal mucous membrane. These observations were made by incubation of the tissue specimens in the presence of *o*-aminophenol [1,2]. This phenomenon has been verified by others using *o*-aminobenzoic acid and *p*-aminobenzoic acid [3]. Most recently we have been able to demonstrate that estradiol glucuronide is formed upon incubation of estradiol with duodenal mucosa in rat [4] and also that the feeding of a glucurogenic substance, cinchophen, leads to increased glucuronide contents in the portal blood in dog [6].

In the present study another glucurogenic substance has been used to confirm these earlier observations. Phenolphthalein was considered to be suitable for this purpose. It is known to be converted into a glucuronide in the organisms, in which form it also is excreted in urine [7]. Phenolphthalein glucuronide is widely used in connection with quantitative determination of β-glucuronidase. This enzyme hydrolyzes the conjugate readily in a linear course to phenolphthalein and glucuronic acid. The quantitative measurement of free phenolphthalein is a simple procedure [8].

MATERIAL AND METHODS

Albino rats (Wistar breed) were used in the experiments.

Incubation. The incubation of free phenolphthalein with duodenal mucosa slices was performed in the Warburg apparatus at 37°C for 90 min. The experimental procedure has been described in detail in previous papers [9,10].

Phenolphthalein concentrations. A stock solution of phenolphthalein was made by dissolving 200 mg of phenolphthalein (Fluka, pro anal.) in 1 ml 10 % NaOH. From this solution 0.02 ml was taken and transferred to 50 ml of the Ringer solution. Thus, this contained 4 mg/50 ml of phenolphthalein or 0.72 mg/9 ml. This 9 ml sample was made by putting the contents of 3 Warburg flasks together, each containing 3 ml of the incubation mixture. The tissue slices from these 3 flasks were removed after the incubation, washed with 1 ml of pure Ringer solution and dried on glass plates for later weighing. The washing solution was added to the combined 9 ml incubation mixture.

Deproteinization. The incubation mixture (10 ml) was made 2 N with hydrochloric acid. By using 4.44 N HCl, the amount used for this was 8.2 ml. This mixture was then heated at 60°C for 2 min and the protein precipitate was separated by centrifugation. The clear supernatant was transferred to an extraction funnel and shaken with 13 ml ethylacetate (2 × 15 min). The precipitant left after the centrifugation was washed with ethanol (1 ml), centrifuged and the alcohol supernatant added to the ethylacetate solution. The ethylacetate was removed in vacuum. The residue was dissolved in 2 ml of an acetate buffer (pH 4.5).

Hydrolysis of phenolphthalein conjugate. 2 mg of β-glucuronidase (Worthington Co) was added to the buffered solution of the residue and this was then incubated together with the separate control samples without β-glucuronidase at 37°C for 4 h. The proteins were removed by heating the mixture at 60°C for 2 min and centrifuging thereafter. The clear supernatant was diluted with 3 ml of distilled water and made alkaline with 3 ml glycine-sodium hydroxide buffer (pH 10.45–10.7). By this means the optimum pH

Table 1. Duodenal glucuronide synthesis. I, II, and III different Warburg runs. C control analysis made from the mixture incubated without the tissue slice. + β, hydrolysed sample, − β, unhydrolysed sample. BF: bound phenolphthalein calculated from the difference of the control and nonhydrolysed values. FG, phenolphthalein present as a glucuronide. Calculated from the difference of the hydrolysed and nonhydrolysed values. Δ "lost amount", difference of BF and FG.

		Phenolphthalein recovered				Tissue dry weight, mg	Phenolphthalein, mg/100 mg tissue dry wt		
		mg	BF mg	FG mg	Δ		BF	FG	Δ
I.		0.78							
	1+β	0.58		0.39	0.20	24.9		1.5 (6)	0.8 (0)
	2+β	0.62		0.43	0.16	26.2		1.8 (5)	0.6 (1)
	3−β	0.19	0.59			23.5	2.5 (4)		
II.		−0.76							
	1+β	0.56		0.39	0.20	25.0		1.5 (6)	0.8 (0)
	2+β	0.54		0.37	0.22	24.8		1.5 (0)	0.8 (9)
	3−β	0.17	0.59			23.5	2.5 (4)		
III.		−0.79							
	1+β	0.58		0.40	0.21	26.0		1.5 (6)	0.8 (2)
	2+β	0.60		0.42	0.19	28.0		1.5 (1)	0.6 (8)
	3+β	0.59		0.41	0.20	21.4		1.8 (8)	0.9 (2)
	4−β	0.18	0.61			20.1	3.0 (5)		
	Mean		0.60	0.40	0.20				

for the stability of the colour given by phenolphthalein was maintained. After this the mixture was centrifuged. The colour intensity of the clear solutions was measured with a Beckman DU spectrophotometer (540 mμ). This method is a modified Fishman method, originally designed for the determination of β-glucuronidase activity [11]. In our procedure the unknown factor was the amount of free phenolphthalein produced from the conjugate during the incubation with β-glucuronidase.

Calibration curve. This was made by preparing a dilution 0.005 ml/2 ml in 10 % NaOH from the phenolphthalein stock solution and making a dilution series (in Ringer solution) from this. The colour intensity was then measured after mixing the samples with the glycine-sodium hydroxide buffer (3 ml buffer + 3 ml H_2O).

RESULTS AND DISCUSSION

The results are listed in Table 1. First the methodical accuracy can be checked from the values. The measured amount of phenolphthalein in the original incubation mixture was 0.72 mg. The recovery in the triplicate control samples was 0.78, 0.76 and 0.79, mean 0.78. No loss of the original phenolphthalein is indicated. By studying the results it is apparent that a great part of the phenolphthalein present in the mixture is not found in free form after incubation with surviving duodenal tissue. It is also evident that a major portion of this bound glucuronide can be liberated by β-glucuronidase. The calculations indicate that 66 % of the ethylacetate extractable bound phenolphthalein was converted into the glucuronide. The nature of the remaining part (Δ) has not been identified in these studies. There are several possibilities. It might include material conjugated to sulphate and it might also be bound to the protein present in the original mixture.

These studies add to the previous evidence that the duodenal mucosa is able to carry out active glucuronide synthesis. The physiological meaning of this intestinal function is still obscure. In the liver this conjugation serves the purpose of active detoxication. In the intestine it might also serve as a part of the selective absorption mechanism. Our studies now in progress aim at elucidation of some of these points.

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REFERENCES

1. Hartiala, K. J. W. *Acta Physiol. Scand.* **31** (1954) suppl. 114, 20.
2. Hartiala, K. J. W. *Ann. Med. Exptl. Fenniæ Helsinki* **33** (1955) 239.
3. Shiray, Y. and Ohkubo, T. *J. Biochem. (Tokyo)* **4** (1954 b) 341.
4. Lehtinen, A., Nurmikko, V. and Hartiala, K. *Acta Chem. Scand.* **12** (1958) 1585.
5. Lehtinen, A., Hartiala, K. and Nurmikko, V. *Acta Chem. Scand.* **12** (1958) 1589.
6. Hartiala, K. J. W., Leikkola, P. and Savola, P. *Acta Physiol. Scand.* **42** (1958) 36.
7. Fleig, C. *J. Pharm. Chem.* **29** (1909) 55.
8. Talalay, P., Fishman, W. H. and Huggins, C. *J. Biol. Chem.* **166** (1946) 757.
9. Levvy, G. A. and Storey, I. D. E. *Biochem. J.* **44** (1949) 295.
10. Hartiala, K. J. W. and Sirkku Rontu *Ann. Med. Exptl. Fenniæ Helsinki* **33** (1955) 213.
11. Fishman, W. H., Springer, N. and Brunetti, R. *J. Biol. Chem.* **173** (1948) 449.

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