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EFFECT OF SPIRONOLACTONE ON BILIRUBIN METABOLISM IN RAT LIVER AND SMALL INTESTINAL MUCOSA

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Abstract—*In vitro* and *in vivo* experimental models were designed for the study of the effects of spironolactone (SP) on bilirubin metabolism in rat liver and small intestinal mucosa. *In vitro* studies included uptake of bilirubin by liver slices and intestinal sheets, determination of glucuronyltransferase activity in mucosal homogenates, and the handling of bilirubin by the isolated perfused liver after bilirubin overload. *In vivo* studies were carried out to measure the plasma disappearance rate of bilirubin and to determine the extent of bilirubin conjugation and biliary excretion of the pigment infused intravenously. The results obtained suggested that the mechanisms involved in the uptake of bilirubin by tissues were not influenced by SP pretreatment. Glucuronyltransferase activity in the small intestinal mucosa was significantly induced by SP, as previously observed in rat liver. Isolated perfused livers from SP-treated rats, as well as treated living rats, exhibited a greater than normal capacity for bilirubin excretion into bile at the expense of bilirubin diglucuronide. Conjugated bilirubin in the small intestinal mucosa of rats infused with unconjugated pigment was also increased after SP pretreatment. The results favoured the conclusion that SP is an inducer of bilirubin conjugation in the liver as well as in extrahepatic tissues, such as the small intestinal mucosa.

Spironolactone (SP), currently used as a potassium-sparing diuretic, has been reported to have pharmacological effects in addition to that of aldosterone antagonism. As far as the liver is concerned, SP treatment increases bile flow and biliary excretion of organic anions [1–3]. This steroid also is an inducer of hepatic microsomal enzyme activity and, thus, increases drug metabolism rates [4–8]. It has been reported that bilirubin UDP-glucuronyltransferase activity (GT) is also enhanced in the liver by SP treatment [2, 9]. In a preliminary communication [10], we reported that GT and glycosyltransferase activities were increased in liver homogenates of SP-treated rats. Furthermore, we observed that SP treatment resulted in a more rapid rate of bilirubin diglucuronide excretion into the bile.

In this study, we examined inducer properties of SP on several steps of bilirubin (BR) metabolism in the liver and small intestinal mucosa. The results favoured the conclusion that BR metabolism is enhanced by SP mainly due to the increase of BR conjugation.

MATERIALS AND METHODS

Chemicals

All the chemicals used were of reagent grade quality. BR, SP, bovine albumin, UDPGA and glucaro 1,4-lactone were purchased from the Sigma Chemical Co. (U.S.A.). Ethyl anthranilate was from Eastman Organic Chemicals (U.S.A.), Silica gel G (Kieselgehl 60 G) from Merck (West Germany) and pentan-2-one from British Drug Houses (England).

Animals and drug treatment

Male Wistar rats weighing 250–300 g were used. SP was injected intraperitoneally as a daily dose of 240 μmoles/kg body wt dissolved in 1 ml propylene glycol for 3 consecutive days prior to the experiment [10]. Control rats were injected with propylene glycol. The animals were allowed free access to water and saline solution during treatment and were maintained *ad lib.* on a standard laboratory pellet diet until 24 hr prior to the experiment.

In vitro *studies*

BR uptake by liver slices and intestinal sheets. Control and treated rats were killed by cervical dislocation and bled by heart puncture. The liver was perfused with cold saline through the portal vein, and then it was removed and blotted on filter paper. Liver slices were cut by hand with a razor blade. The small intestine was also perfused with cold saline. The proximal jejunum was removed and kept in Ringer–Krebs solution. Segments about 1–2 cm in length were cut open to expose the mucosa.

Each incubation flask contained usually two liver slices (total 250 mg wet wt/flask) or two intestinal sheets. The incubation medium consisted of BR in Ringer–Krebs–bicarbonate buffer (pH 7.4). BR was dissolved previously in 0.1 N NaOH (60 μl/μmole of pigment) and bovine albumin. The ratio of BR to albumin was 20:1. This ratio was enough to stabilize the BR solution and to ensure that the pigment was mostly diffusible [11].

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The final concentration of BR in the medium was 0.3 mM. Four ml of the incubation medium was added to each flask. Control flasks without BR were kept on ice. Incubation was carried out in dim light at 37° in a Dubnoff water bath shaker and for different time intervals (1, 5, 15 and 30 min). After incubation, the slices and intestinal sheets were briefly washed in 1% (w/v) albumin solution, at 0° to remove adherent BR, and the mucosa was scraped from the inner wall.

Liver slices and mucosa were then homogenized (Omni-Mixer, I, Sorvall, Inc., U.S.A.) [12] to obtain a tissue concentration of 100 mg/ml. The homogenates were chilled on ice until used for determination of total BR concentration.

Total tissue water and extracellular space were measured in liver slices as well as in mucosal fractions, in duplicate incubations without BR.

Determination of GT in the small intestinal mucosa. The proximal small intestine (10 cm) was opened lengthwise and the mucosa was obtained as stated above. Homogenates for enzyme assay were prepared as previously described for the liver [13, 14], but tissue concentration in the homogenate was 300 mg/ml. The optimal amount of UDPGA in the incubation medium was investigated. The results of such experiments are shown in Fig. 1, where it can be seen that the optimal concentration was 22.5 mM. Since the reaction was found to obey first order kinetics for up to 30 min, this time was used for all the incubations. It was also demonstrated previously that enzyme activity was not increased by the addition of glucaro 1,4-lactone (a specific inhibitor of beta-glucuronidase activity) to the incubation medium (the final concentration was 1.0 mM) [15].

GT activity was expressed as nmoles of bilirubin conjugated/10 min per mg of protein, and as nmoles of bilirubin conjugated/10 min per g of wet tissue.

Studies in isolated perfused rat liver. To avoid problems inherent to the intact organism, we examined the liver capacity for BR excretion by using the isolated rat liver preparation described by Brauer *et al.* [16] with some modifications [17]. The perfusion medium consisted of heparinized rat blood in Ringer–Krebs–Henseleit buffer (pH 7.4) with an hematocrit value of 10–11%. The liver was placed in a thermostatically controlled chamber at 37°. A gas mixture (95% O_2 and 5% CO_2) was continuously bubbled. The medium was recirculated by means of a peristaltic action pump (American Instrument Co., Cat. No. 5-8954, U.S.A.). A constant pressure of 14 cm H_2O was maintained at the portal vein level. Flow rate through the liver (Q), determined by a direct measurement, was 44.5 ± 1.5 ml/min (mean ± S.E.M. N = 13). A single dose of BR (16.5 μmoles ± 1.0) was incorporated into the system. BR was dissolved in isotonic carbonate solution (0.5 g, Na_2CO_3, 0.52 g NaCl, 100 ml H_2O) [18], and centrifuged prior to use. Six ml of the supernatant fraction was added to the system. An aliquot of the supernatant fraction was used for BR determination. Bile samples were collected through a polyethylene catheter inserted into the bile duct (PE-50, Intramedic, U.S.A.) every 10 min for 60 min. One sample of the perfusate was taken from the reservoir 5 min after the injection, to test percent dose retained at that time. Biliary excretion of BR was expressed as percent dose excreted/10 min and as nmoles/10 min per g of liver weight. Bile flow was expressed as μl/10 min per g of liver weight.

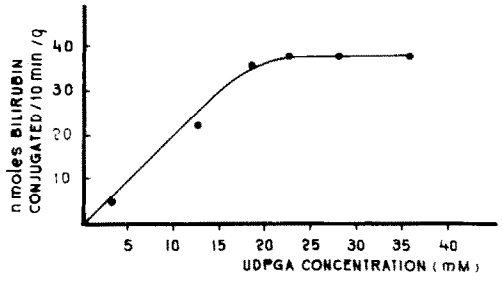

Fig. 1. Effect of varying the concentration of UDPGA in the incubation mixture on glucuronyltransferase activity. Each data point is the mean of two separate experiments.

In vivo *studies*

BR uptake. Polyethylene catheters (PE-50, Intramedic, U.S.A.) were inserted into a femoral vein and into a femoral artery. The arterial catheter was heparinized.

The rats were maintained under ether anaesthesia. After the injection of a single dose of BR (3500 nmoles/100 g body wt) through the venous catheter, arterial blood samples (0.2 to 0.3 ml) were collected at 30 sec, 1, 2, 3 and 4 min after the injection. The samples were centrifuged for plasma separation.

The initial disappearance rate from plasma of BR, expressed as the first order rate constant K_1, was determined [19]. This was considered an indirect measure of BR uptake by tissues, mainly due to net transfer from plasma into the liver.

BR conjugation and biliary excretion of conjugated BR. In other group of rats, the bile duct and a femoral vein were catheterized as described above. The rats were placed in restraining cages and remained conscious throughout the experiment. After recovery from anaesthesia (3 hr after surgery) [15], bile was collected for 30 min to determine the endogenous bilirubin output (nmoles/hr per 100 g body wt) and bile flow (μl/min per 100 g body wt). Then, a priming dose of BR (3500 nmoles/100 g body wt) was injected i.v. followed by the i.v. administration of pigment for 40 min (170 nmoles/min per 100 g body wt) [18] (Unita Braun Melsungen, West Germany). Bile was collected in the dark every 10 min during the infusion, and body temperature during bile collection was maintained by placing the rats in a warming, thermostatically controlled, chamber. The apparent maximum rate of BR excretion during the infusion was estimated. At the end of the infusion, the animals were bled by heart puncture, the livers were removed and weighted, and small intestinal mucosa was obtained as described above. Liver homogenates (200 mg/ml) and mucosal homogenates (400 mg/ml) were prepared in Ringer–Krebs solution for determination of conjugated BR. The relative amounts of monoglucuronide (MG) and diglucuronide (DG) were estimated in bile samples and tissue homogenates.

Analytical methods

Total BR in plasma, bile samples, isolated liver perfusates, injected solutions and incubation media was determined by the diazo reaction [20].

Total BR in liver and mucosal homogenates after the incubation was also determined [12, 21].

Total tissue water of liver slices and mucosal fractions was calculated after the incubation by the difference between wet and dry weight (24 hr at 100°). The hepatic and mucosal extracellular space was determined with inulin [21].

Conjugated BR in hepatic and mucosal homogenates obtained in the *in vivo* studies was estimated after treatment of samples with diazotized ethyl anthranilate at pH 6.0 [15]. The azo derivatives were extracted in pentan-2-one (2 ml), and their concentrations were measured by spectrophotometry. Bile samples (diluted 1:50) were diazotized following the same procedure. The extracts containing the azo derivatives were subjected to thin-layer chromatography [22]. The relative amounts of MG and DG [23, 24] were estimated by densitometry.

Bile flow in living rats and in isolated livers was measured by gravimetry. The ratio of liver wt to body wt was also calculated in the *in vivo* studies.

Protein estimation in mucosal homogenates used for GT determination was performed by the method of Lowry *et al.* [25].

Statistics

Student's *t*-test was used in the comparison of data. $P < 0.05$ was considered to be significant. The slope of the BR disappearance curve in plasma was calculated by least squares regression.

RESULTS

BR uptake in vitro

Determination of BR in tissue homogenates from SP-treated rats did not differ from the values obtained in controls, at any time of incubation. It was also found that time course of BR uptake was similar for both tissues up to 5 min of incubation.

Then it was observed that liver slices exhibited a greater capacity than mucosal fractions to accumulate BR, independently of treatment. A difference was detectable after 15 min and was statistically significant after 30 min of incubation ($P < 0.02$) (Fig. 2).

Total tissue water was found to be constant for both tissues during the entire period of incubation. The hepatic and mucosal extracellular space gave a value of 0.25 ml/g wet tissue ± 0.01 (mean value ± S.E.M.), which is similar to that observed previously [21].

The final tissue:medium ratio after 15 min of incubation was less than 1 for mucosa (0.86 ± 0.05) and greater than 1 for liver (1.6 ± 0.11).

GT in small intestinal mucosa

SP treatment resulted in a greater concentration of total proteins in mucosal homogenates. GT was also increased per mg of protein ($P < 0.05$) as well as per g of wet tissue ($P < 0.01$). Data of enzyme activities and protein concentrations are presented in Table 1.

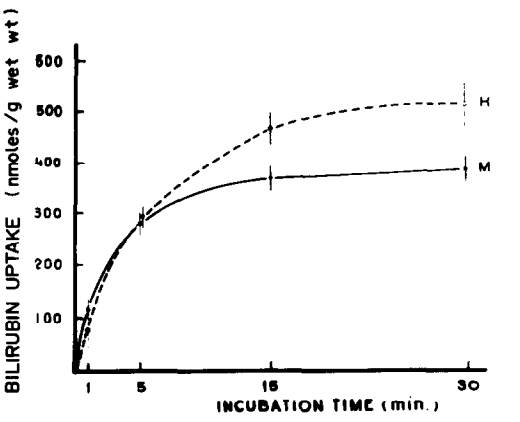

Fig. 2. Time course of bilirubin accumulation by intestinal mucosa and liver slices. Results of four to six sets of experiments with controls and of five to eight experiments with SP-treated rats have been averaged (± S.E.M.). Key: (H) hepatic uptake (dotted line); and (M) mucosal uptake (solid line).

BR excretion in isolated liver

As shown in Fig. 3, BR excretion was increased in isolated livers of SP-treated rats, as compared to the excretion observed in controls. The increase (expressed as nmoles/10 min per g of liver) was statistically significant after 10 and 20 min of BR injection. When data were represented as percent dose excreted/10 min by the whole liver, the differences were even greater due to the increase of liver weight produced by SP. Furthermore, percent dose of BR excreted by livers of SP-treated rats after the entire experimental period (60 min) was also significantly greater than that of normal livers. On the other hand, after 5 min of the incorporation of pigment in the perfusion medium, percent dose retained was similar in control and SP preparations.

Bile flow (found to be constant in both groups during perfusion) was increased significantly by SP

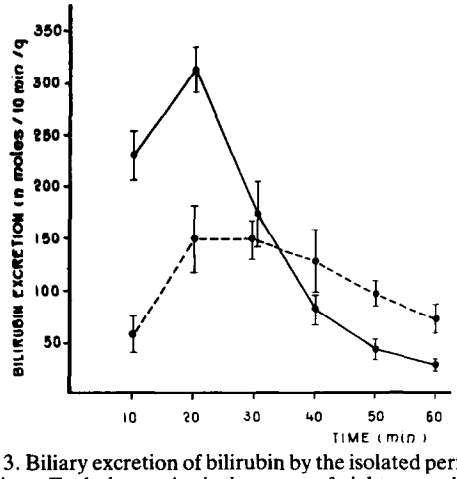

Fig. 3. Biliary excretion of bilirubin by the isolated perfused rat liver. Each data point is the mean of eight normal liver perfusions (dotted line) and of five SP-treated liver perfusions (solid line), ± S.E.M.; the differences at 10 and 20 min of perfusion were statistically significant ($P < 0.001$). Chromatographic studies showed that bilirubin excretion in both groups was mainly at the expense of bilirubin diglucuronide (68%).

Table 1. Effect of spironolactone on bilirubin UDP-glucuronyltransferase activity in the small intestinal mucosa*

	Control rats	Spironolactone-treated rats	P
Protein concentration (mg/ml)	15.2 ± 0.4	19.1 ± 1.1	<0.02
Glucuronyltransferase activity [nmoles bilirubin conjugated · 10 min^{-1} · (mg protein)$^{-1}$]	0.71 ± 0.05	0.89 ± 0.04	<0.05
Glucuronyltransferase activity [nmoles bilirubin conjugated · 10 min^{-1} · (g wet tissue)$^{-1}$]	35.6 ± 1.5	55.6 ± 3.9	<0.01

* Values are means ± S.E.M. for groups of five control rats and eight SP-treated animals.

treatment. The results obtained are presented in Table 2.

BR uptake in vivo

The initial disappearance rate from plasma of injected BR did not show differences between treated and control rats. The value of K_1 for SP-treated rats was 0.120 ± 0.040 (N = 6) and 0.160 ± 0.024 (N = 6) for control animals.

Bile flow, biliary excretion of BR, and BR conjugation in vivo

Bile flow, liver weight and the ratio of liver wt to body wt were considered indicative of the steroid inducer effect. The three variables were increased significantly in SP-treated rats.

The biliary excretion of endogenous BR was also increased in treated animals. The apparent maximum rate of BR excreted into bile of SP-treated rats was higher than that of controls, and it was reached earlier, in coincidence with previous data [10]. As expected, due to the higher rate of BR glucuronide formation and of DG biliary excretion produced by SP, conjugated BR in liver homogenates was similar in both groups, whereas the concentration of conjugated BR in the small intestinal mucosa was increased in SP-treated rats. Conjugated BR in bile samples of both groups infused with BR was mainly due to BR-DG, as observed previously [10]. The predominance of BR-DG in tissue homogenates was less marked. There were no differences between groups of the relative amounts of MG and DG estimated in bile samples or tissues. The results are presented in Table 3.

DISCUSSION

In a preliminary communication [10], we observed that the activities of enzyme systems involved in BR conjugation were increased in liver homogenates of rats pretreated with SP, and that steroid pretreatment resulted in a more rapid excretion rate of BR-DG into bile. In the present study, we examined the effects of SP on BR metabolism in the rat liver in more detail, and in comparison to those observed in the rat small intestinal mucosa.

The initial step in the transfer of BR from the plasma into the hepatocyte is a bidirectional process but a major portion of BR is removed by cells in each circulation by a mechanism not completely elucidated [26–28]. The uptake of BR by the rat small intestinal mucosa has been also reported pre-

Table 2. Effect of spironolactone on bile flow and bilirubin excretion by the isolated perfused rat liver*

	Control livers	Spironolactone-treated livers	P
Bile flow [μl · 10 min^{-1} · (g liver)$^{-1}$]	5.5 ± 1.0	11.4 ± 1.0	<0.01
Percent dose of bilirubin in perfusate 5 min after injection	63.1 ± 4.7	61.1 ± 4.1	NS†
Percent dose of bilirubin excreted into bile 60 min after injection	40.5 ± 1.3	67.6 ± 2.8	<0.001

* Values are means ± S.E.M. for groups of eight control livers and five SP-treated livers. A single dose of bilirubin of 16.5 μmoles ± 1.0 was injected into the system after 30 min of equilibration.

† Not significant.

Table 3. Liver weight, bile flow, endogenous bilirubin output, and bilirubin conjugation in control and spironolactone-treated rats*

	Control rats	Spironolactone-treated rats	P
(Liver wt/body wt) × 100	3.18 ± 0.09 (8)	3.68 ± 0.15 (5)	<0.01
Bile flow [$\mu l \cdot min^{-1}(100\ g\ body\ wt)^{-1}$]	4.6 ± 0.3 (10)	7.9 ± 0.6 (9)	<0.001
Endogenous bilirubin output [$nmoles \cdot hr^{-1} \cdot (100\ g\ body\ wt)^{-1}$]	26.0 ± 0.8 (8)	46.5 ± 4.9 (4)	<0.01
Maximum rate of bilirubin excretion‡ [$nmoles \cdot min^{-1} \cdot (100\ g\ body\ wt)^{-1}$]	99.8 ± 6.1 (3)	135.7 ± 2.1 (3)	<0.01
Conjugated bilirubin in hepatic homogenates (nmoles/g wet tissue)	13.6 ± 2.2 (11)	11.6 ± 3.4 (5)	NS†
Conjugated bilirubin in mucosal homogenates (nmoles/g wet tissue)	2.5 ± 0.3 (11)	4.2 ± 0.8 (5)	<0.05
% Diglucuronide in bile	71.3 ± 4.1 (9)	71.4 ± 2.8 (8)	NS
% Diglucuronide in hepatic homogenates	61.5 ± 1.6 (4)	55.4 ± 12.5 (5)	NS
% Diglucuronide in mucosal homogenates	53.7 ± 4.3 (7)	57.4 ± 8.1 (5)	NS

* After a priming dose of unconjugated bilirubin, a continuous infusion of pigment was maintained for 40 min (see text). Endogenous bilirubin output and bile flow were measured prior to bilirubin overload. Data are mean values ± S.E.M. The number of animals is given in parentheses.

† Not significant from controls.

‡ Maximum rates were observed 30 min after the injection in controls and 20 min after the injection in treated rats.

viously, involving a sodium-dependent mechanism not entirely explained by simple passive diffusion [21, 29].

BR within the cells is conjugated mainly with glucuronic acid, and a microsomal UDP-glucuronyltransferase system catalyzes transfer of a glucuronyl residue from UDPGA for BR-MG formation or to BR-MG for DG synthesis [30]. BR-DG is the predominant BR conjugate in rat and human bile [23] and some investigators have suggested that BR-DG may be indeed formed at the canalicular membrane [31, 32]. The formation of glucuronides has been demonstrated in the gastrointestinal mucosa of several species [33–36], and GT was detected in biopsy specimens of human mucosa [37].

The results presented in this paper indicate that liver slices have a greater capacity than mucosal fractions to accumulate BR taken up from a medium with a high molar ratio of BR to albumin. The uptake by both tissues with time was initially very rapid but then the amount of BR accumulated tended to reach a plateau, as observed previously [21, 27]. The tendency to reach a constant value with the incubation time could be explained as being due to saturation of BR binding sites in both tissues or as an expression of a net flux [21]. The ratio of equilibrium between mucosal cells and medium after 15 min of incubation was similar to that described previously for BR dissolved in sodium taurocholate [21]. The ratio was found to be greater than 1 for liver, denoting a greater capacity for BR accumulation that might have been due to a higher concentration of ligandin within the hepatic cells [26]. Although the fraction taken up by the extracellular space cannot fully account for the total uptake observed in both tissues, it was impossible to distinguish between uptake into intracellular compartments and binding to some component of the membrane [27, 38]. The uptake of BR *in vitro* was not influenced by SP, in coincidence with data on the disappearance of BR from plasma seen in the experiments *in vivo* and of BR retained in the perfusion medium of isolated livers after 5 min of pigment incorporation into the system. These results are in agreement with those reported by other authors, but with the use of hepatocytes isolated from phenobarbital-pretreated rats [27], despite the fact that phenobarbital pretreatment has been shown to induce ligandin [26].

Conversely, glucuronidation of BR was clearly enhanced by SP in both tissues. Just as GT was enhanced by SP in rat liver homogenates [10], a similar effect in the small intestinal mucosa has been described in this paper, but this was achieved by using a high concentration of UDPGA in the incubation medium due to a rapid breakdown of the nucleotide in adult rat gut [33]. Enzyme activity determined in mucosal homogenates of untreated rats, when expressed per mg of protein, was similar to that observed previously in normal rat liver [10]. When GT was expressed per g of wet tissue, the activity estimated in the mucosa was about one-fifth of that observed previously in the liver and about one-tenth after SP pretreatment. Since the concentration of total proteins in mucosal homogenates was also increased by SP, we can assume that the steroid produced induction of the enzyme, although it is difficult to determine how far increased concentration of GT catalytic units is responsible for the increase of GT activity seen *in vitro* [39]. It was reported that the activity of UDPGA-dependent enzyme system is clearly enhanced in the liver by phenobarbital [30, 40], but conjugation of several

compounds with glucuronic acid in the rat small intestinal mucosa is not affected in a similar way by that treatment [35, 41].

The results of *in vivo* studies support the conclusions of the experiments *in vitro* since BR-MG and DG were both present in the small intestinal mucosa after unconjugated BR overload, and conjugated BR was increased in the mucosa of SP-treated animals. On the other hand, the experiments with isolated rat liver were designed to determine the effect of SP on BR hepatic transport after unconjugated BR overload without the participation of other tissues. The dose of BR was chosen to obtain in the perfusion circulating medium a concentration of pigment high enough, but below that obtained under conditions of saturation of the hepatic excretory mechanism [42]. The results clearly demonstrate that SP increases the rate of BR-DG excretion into bile, as proved in living rats [10]. Furthermore, the greater endogenous bile BR output observed in SP-treated rats reflects increased BR metabolism and biliary excretion produced by SP.

Glucuronidation of exogenous and endogenous compounds is probably the most important truly detoxicatory process for, unlike hydroxylation, it does not produce transient toxic intermediates [39]; thus, the effect produced by SP on BR conjugation seems of great interest. On the other hand, SP was found to be less effective for hepatic removal of BR from plasma, which may be related to a poor induction of ligandin produced by SP, in comparison to the effect of other inducers [43].

The results described in this paper may help in understanding several aspects of BR metabolism in more detail, particularly those related to the mechanism of BR-DG synthesis [30, 44].

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