Biochemical Pharmacology, Vol. 29, pp. 1491/1496

0006-2952/80/0601-1491 $02.00/0

ISOCARBOXAZID METABOLISM *IN VITRO* BY LIVER MICROSOMAL CARBOXYLESTERASE OF MONKEY

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(*Received* 27 *June* 1979; *accepted* 21 *November* 1979)

Abstract—An amidase that hydrolyzes isocarboxazid, a monoamine oxidase inhibitor, was studied in the monkey. High activity was observed in the liver, moderate activity in the pancreas, and low activity in the kidney, intestine and lung. No activity was observed in the brain, spleen, heart, uterus, or serum. This enzyme was thought to be located mainly in the microsomal fraction of liver, since the subcellular distribution pattern was similar to that of glucose-6-phosphatase, a marker enzyme of the microsomal fraction. This amidase was stable at low temperature (−20°) for at least 1 month, and no cofactors were required. The enzyme was not inhibited by either the substrate or its metabolites. However, significant inhibition of the enzyme was produced by organophosphorous compounds such as parathion and *O*-ethyl *p*-nitrophenyl phenylphosphorothioate (EPN). Both α-naphthylacetate and *p*-nitrophenyl acetate, substrates of carboxylesterase, competitively inhibited the activity of the isocarboxazid amidase. These results suggest that isocarboxazid amidase is also a carboxylesterase. A comparison between monkey and rat isocarboxazid amidases is also discussed in this paper.

In previous papers, we have demonstrated that an enzyme that catalyzes the hydrolysis of isocarboxazid (ISOC), a monoamine oxidase inhibitor, is found in various animal tissues and that guinea pig liver has significantly higher activity compared to tissues of the rabbit, rat and mouse [1, 2]. The enzyme appears to be located mainly in the liver microsomal fraction in rats and guinea pigs, and seems to have carboxylesterases activity [3, 4].

Microsomal carboxylesterases are known to be widely distributed in animals (rat [5, 6], pig [7, 8], horse [9], ox [10], chicken [9], sheep [9] and human [11]) and to hydrolyze various drugs possessing an ester or amide bond, i.e. acetanilide [5, 7], Hostacaine [6], procaine [6], steroid hormone esters [8] and xylocaine [10]. No report concerning monkey carboxylesterase, however, has been available.

The present studies were designed to investigate the *in vitro* metabolism of ISOC by monkey liver microsomes and the properties of this enzyme.

MATERIALS AND METHODS

Chemicals. ISOC and *O*-ethyl *p*-nitrophenyl phenylphosphonothioate (EPN) were supplied by the Nippon Roche Research Center, Kamakura, Japan, and E. I. du Pont de Nemours & Co. Inc., Wilmington, DE, respectively. Reduced glutathione and glucose-6-phosphate were obtained from the Sigma Chemical Co., St. Louis, MO. Sephacryl S-200 was purchased from Pharmacia A.B, Uppsala, Sweden. All other chemicals used were of analytical grade, and all solutions were prepared in redistilled water.

Enzyme assays. All enzyme activities were measured spectrophotometrically in a Hitachi Perkin–Elmer 139 spectrophotometer. ISOC amidase activity was determined by measuring the amount of benzylhydrazine (BZH) produced from ISOC. BZH was determined according to a method described previously [12]. Esterase activity was also assayed using procaine [4], *p*-nitrophenyl acetate (*p*-NPA) [13] and α-naphthylacetate (α-NA) [14] as substrates. For the assays of ISOC amidase and procaine (PROC) esterase activities, a mixture, consisting of 0.5 ml of substrate (2×10^{-3} M) in 0.2 M Tris–HCl buffer, pH 8.0, 0.1–0.5 ml of enzyme solution and enough redistilled water to make a final volume of 1.0 ml, was incubated at 37° for 30 min in air. Produce formation by liver amidase or esterase activity toward ISOC or PROC as substrate, respectively was linear for at least a 2-hr incubation period. Enzyme activity and specific activity were expressed as μmoles of product formed per g liver wet wt. per 30 min and nmoles of product formed per mg protein per min, respectively. Succinic dehydrogenase activity was measured by the method of Pennington [15], and incubation was carried out for 15 min. The activities of acid phosphatase and glucose-6-phosphatase (G-6-Pase) were assayed by the procedures of Alvarez [16] and Hübscher and West [17] respectively. Protein was determined by the method of Lowry *et al.* [18], using bovine serum albumin as the standard.

Fractionation of the amidase. Female monkeys (*Macaca mulatta*), weighing 2–3 kg, were used for all experiments. The liver was removed, weighed and homogenized in 4 vol. of ice-cold 0.25 M sucrose, using a Potter-Elvehjem glass homogenizer with a loosely fitting Teflon pestle. Subcellular fractionation was carried out by differential centrifugation by the method of Sedgwick and Hübscher [19]. The homogenate was centrifuged at 600 *g* for 10 min, then at 5000 *g* for 10 min, 10,000 *g* for 20 min and finally at 100,000 *g* for 60 min, to separate nuclear, mitochondrial, lysosomal and microsomal fractions, respectively. All subfractions prepared as described above were washed once in 1.15% KCl solution,

resuspensed and recentrifuged prior to assays of each enzyme activity. When only the isolation of microsomes was necessary, the liver homogenate was first centrifuged at 10,000 *g* for 20 min. After recentrifugation of the supernatant fraction at 100,000 *g* for 60 min, the microsomal pellet was washed once in 1.15% KCl to remove adsorbed protein. The final pellet was resuspended in 1.15 per cent KCl to contain 1–10 mg protein/ml. Experiments on the determination of the characteristics of the amidase or esterase described here were done using microsomes as an enzyme source.

Polyacrylamide gel electrophoresis. Disc electrophoresis was carried out according to the method of Davis [20]. The gel concentration was 7.5 per cent (pH 9.4). Monkey microsomes were solubilized by adding Triton X-100 and were applied to polyacrylamide gels. After electrophoresis for 2 hr the gels were stained with Coomassie Blue for protein or with 2 ml of 0.1 M phosphate buffer containing Fast Blue RR (2 mg) and α-NA (10^{-3} M) dissolved in acetone. ISOC amidase activity was determined by incubating the sliced gels with ISOC as substrate, following them to estimate the BZH formation.

RESULTS

Tissue distribution. Studies were made to determine the distribution of the ISOC amidase in various tissues, i.e. liver, kidney, brain, heart, intestine, pancreas, spleen, lung, uterus, and serum, of the monkey. Table 1 shows that the liver possessed a relatively high ISOC amidase activity compared to the other tissue; a moderately high activity was found in the pancreas. The brain, spleen, heart, uterus and serum had no enzyme activities toward ISOC.

Localization of the ISOC amidase in subcellular fractions. The activities of the ISOC amidase and of known marker enzymes in three subcellular components were studied in the fractions obtained from monkey liver. The marker enzymes chosen here were succinate dehydrogenase (mitochondria), acid phosphatase (lysosomes) and G-6-Pase (microsomes).

Table 1. Tissue distribution of monkey ISOC amidase activity

Tissue	Enzyme activity*
Liver	14.20 ± 2.61 (5)†
Pancreas	7.43 ± 1.27 (4)
Kidney	1.16 ± 0.82 (4)
Small intestine	1.01 ± 0.27 (4)
Lung	0.34
Spleen	ND‡
Heart	ND
Brain (cerebrum)	ND
(cerebellum)	ND
(brain stem)	ND
Uterus	ND
Serum	ND

* Enzyme activity is expressed as μmoles BZH formed/g tissue wet wt/30 min; means ± S.E.

† Numbers in parentheses indicate the number of monkeys used.

‡ Not detectable.

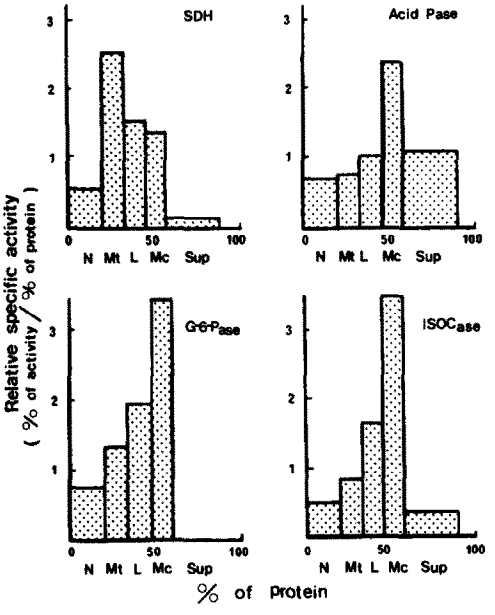

Fig. 1. Subcellular distribution patterns of monkey liver enzymes. Abbreviations: SDH, succinate dehydrogenase; Acid Pase, acid phosphatase; G-6-Pase, glucose-6-phosphatase; and ISOCase, isocarboxazid amidase. Abscissa: fractions are represented by their relative protein content, in the order in which they are isolated, i.e. from left to right, nuclear, mitochondrial, lysosomal, microsomal and supernatant fraction. Ordinate: relative specific activity.

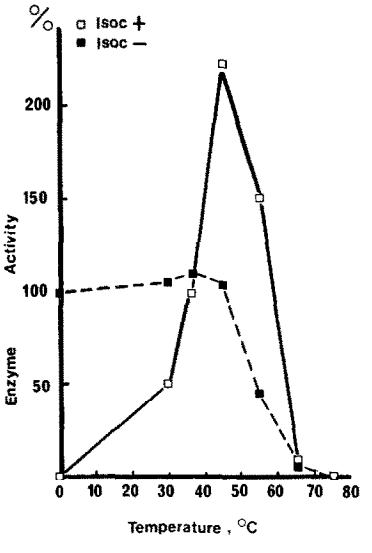

Fig. 2. Temperature optimum and temperature sensitivity of the liver microsomal ISOC amidase. Enzyme activities were measured after 30 min of incubation at different temperatures with ISOC (—□—). After a 10-min preincubation without added substrate at the different temperatures, incubation was carried out for the next 30 min at 37° with added ISOC (---■---).

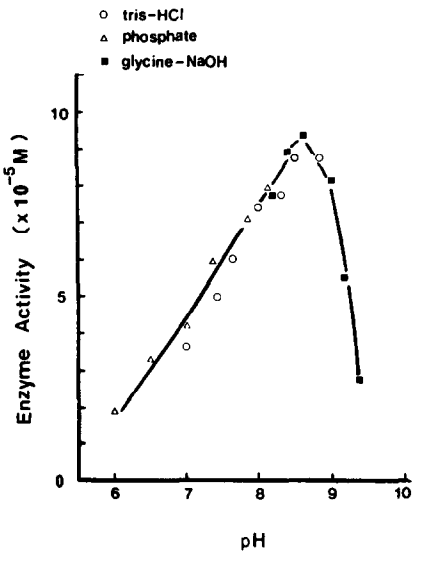

Fig. 3. pH optimum. The following three buffer systems were used: (1) 0.2 M phosphate buffer prepared from KH_2PO_4 and NaOH (pH 6.0 – 8.1); (2) 0.2 M Tris–HCl buffer (pH 7.0 – 8.8); and (3) 0.1 M glycine–NaOH buffer prepared from glycine–NaCl and NaOH (pH 8.2 – 9.6).

Figure 1 shows the distribution of the ISOC amidase and of the marker enzymes in the various fractions, expressed as a percentage of the recovered activities. The distribution pattern of the ISOC amidase was roughly similar to that of G-6-Pase; the highest activities were in fractions consisting of membranes, with a little in the cell sap. This ISOC amidase was localized in the microsomes, as reported for rat [5, 6] and pig [7, 8]. Therefore, various enzymatic properties were assayed, using microsomes as the enzyme source. The reaction velocity was linear with enzyme concentrations up to 7 mg of microsomal protein in the presence of 1 mM ISOC, and with time up to 120 min.

Stability of the ISOC amidase. The microsomal pellet, suspended in 1.15 per cent KCl solution, was maintained at 4° or −20°, and enzyme activity was measured every 5 days. Enzyme activity toward ISOC gradually decreased to 50 per cent of the initial activity in about 20 days at low temperature (4°), while no loss of enzyme activity was observed at −20° for 30 days.

Effects of temperature on the ISOC amidase activity. As shown in Fig. 2, exposure of the enzyme to temperatures below 45° without the substrate caused no alteration. However, exposure to temperatures higher than 50° resulted in a gradual decrease, with almost complete inactivation of enzyme activity at 70°. On the other hand, the enzyme activity was gradually increased with increasing temperatures, up to 45°; above this temperature the activity rapidly decreased.

pH optimum. Microsomal ISOC amidase has a pH optimum at 8.5–9.0 for enzymatic hydrolysis of ISOC. There was no significant difference in the enzyme activity with the three buffer systems used (Fig. 3).

Inhibition studies. A number of substances commonly used in enzyme characterization were tested, with preincubation of enzyme for 10 min in the presence of inhibitors. As shown in Table 2, remarkable inhibition was observed with EPN and parathion, moderate inhibition was also noted in the presence of high concentrations of sodium fluoride, eserine and α-NA.

Table 3. Effects of various divalent cations on liver microsomal ISOC amidase activity

Metal ion	Concentration (M) 10^{-3}	10^{-4}	10^{-5}
None	100.0*	100.0	100.0
Hg^{2+}	3.5	80.4	100.1
Cu^{2+}	57.0	96.6	93.9
Zn^{2+}	88.6	99.7	99.5
Co^{2+}	61.9	89.8	102.8

* Numbers in this table show the mean values from three experiments and represent per cent activity of control.

Table 2. Effects of inhibitors on liver microsomal ISOC amidase activity

Inhibitor	Inhibitor concentration (M) 10^{-3}	10^{-4}	10^{-5}	10^{-6}
Parathion		9.5*	60.1	93.0
EPN†	0.0	75.4	94.6	
Bis *p*-nitrophenylphosphate	13.4	36.9	93.6	
Dithiobis nitrobenzoate	98.6	107.3	115.3	
p-Chloromercuribenzoate	99.9	106.2	106.7	
N-Ethylmaleimide	91.0	104.0	108.5	
Sodium fluoride	16.4	56.1	91.9	
Eserine	82.2	101.3	100.9	
EDTA	95.8	91.8	99.2	
p-Nitrophenylacetate	44.5	95.3	104.5	
α-Naphthylacetate	12.6	35.5	72.1	
Acetanilide	89.1	101.4	95.9	
Procaine		105.6	102.6	

* Numbers in this table show the mean values from three experiments and represent percent of control activity.

† *O*-Ethyl *p*-nitrophenyl phenylphosphonothioate.

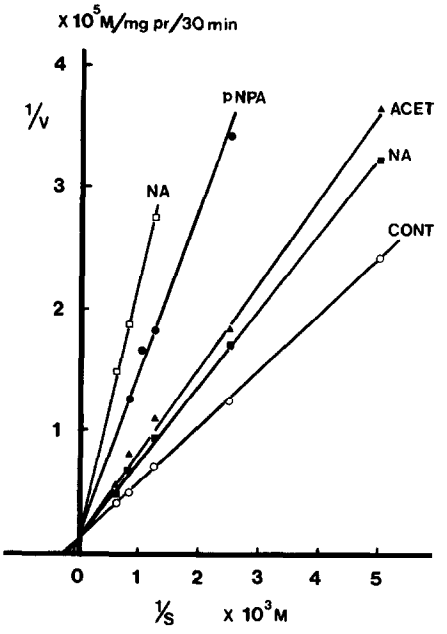

Fig. 4. Double reciprocal plots of the hydrolysis of ISOC in the presence of other possible substrates: a 10^{-4} M (□, NA) and a 10^{-5} M (■, NA) concentration of α-naphthylacetate, a 10^{-4} M concentration of *p*-nitrophenylacetate (●, *p*-NPA) and a 10^{-2} M concentration of acetanilide (▲, ACET). Each point is the mean of three experiments with the same microsomes.

Little or no inhibition of the enzyme was observed with EDTA, *p*-chloromercuribenzoate, *N*-ethylamaleimide or dithiobis nitrobenzoate, even at a concentration of 1 mM. Reductants, i.e. reduced glutathione, dithiothreitol, cysteine and mercaptoethanol, did not affect the enzyme activity at a concentration of 1 mM. The enzyme was not inhibited by either the substrate or its metabolites.

Effects of metal ions. Among the divalent metal ions tested, Hg^{2+}, Cu^{2+}, Co^{2+} and Zn^{2+} had inhibitory effects on ISOC amidase, while Mg^{2+}, Fe^{2+} and Ca^{2+} had no effects. Moreover, none of the metal ions used activated the ISOC amidase (Table 3).

Effect of substrate concentration on reaction velocity and substrate specificity. The effect of substrate concentration on the rate of hydrolysis was determined with concentrations of ISOC from 5×10^{-5} to 1.6×10^{-3} M. The apparent K_m and V_{max} values were 3.2 mM and 3.0 nmoles BZH/mg protein/min, respectively. The monkey microsomes were also observed to have high esterase activity toward α-NA and *p*-NPA as substrates, and low activity toward PROC and acetanilide (Table 4).

To determine the relation between the enzyme that hydrolyzed the ISOC and those that hydrolyzed the other substrates α-NA, *p*-NPA or acetanilide, the hydrolysis of different concentrations of ISOC in the presence and absence of α-NA, *p*-NPA or acetanilide was investigated. As shown in Fig. 4, the double reciprocal plots show competitive inhibition

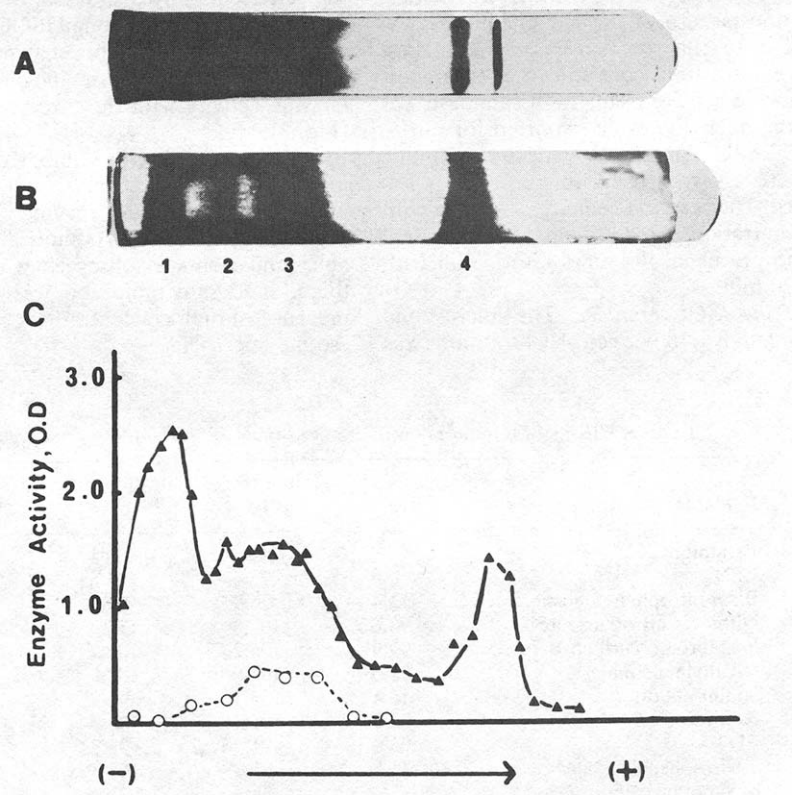

Fig. 5. Polyacrylamide gel electrophoresis of monkey microsomes after solubilization with Triton X-100. (A) Protein bands stained with Coomassie Blue. (B) Enzyme activity bands stained with α-naphthylacetate. The bands of activity were numbered from the cathodic end of the gel. (C) Esterase and ISOC amidase activities after gel electrophoresis. The gels were sliced and incubated with α-naphthylacetate (—▲—) for 5 min or ISOC (---○---) for 3 hr. The enzyme activities were estimated by the methods described in Materials and Methods.

Table 4. Substrate specificity of liver microsomal ISOC amidase activity and carboxylesterase activity*

Substrate	Enzyme activity	K_m	V_{max}
Isocarboxazid	0.6	3.20×10^{-3}	3.0
Procaine	0.5	0.56×10^{-3}	0.7
Acetanilide	0.02	5.00×10^{-3}	0.4
p-Nitrophenylacetate	675	0.71×10^{-3}	3.85×10^{3}
α-Naphthylacetate	1010	0.08×10^{-3}	4.0×10^{3}

* Enzyme activities and V_{max} values are expressed as nmoles product formed/mg microsomal protein/min, and K_m as M of each substrate.

of ISOC-hydrolyzing activity in the presence of 10^{-4} M concentration of α-NA *p*-NPA or a 10^{-2} M concentration of acetanilide. The effect of PROC was not determined in this experiment because a concentration of more than 10^{-4} M PROC developed color with the *p*-dimethylaminobenzaldehyde reagent which was used for measuring BZH amounts and inhibited the color development of BZH.

Polyacrylamide gel electrophoresis and ISOC amidase activity. After the monkey microsomes were solubilized with 2.5 per cent Triton X-100 to a final concentration of 0.25 per cent Triton X-100, the extracts containing 200 μg of microsomal protein were subjected to disc polyacrylamide gels and electrophoresed for 2 hr. Figure 5 shows the distribution of protein bands stained with Coomassie Blue (A) and of α-NA esterase activity (B). This figure demonstrates that microsomes have at least four esterase activities toward α-NA. To investigate the relation between the ISOC amidase and the α-NA esterases, the gels were sliced 1.0 mm thin and three pieces of them per tube were incubated for 3 hr with ISOC. Esterase activity was also measured by incubating the sliced gel with α-NA for 5 min. As shown in Fig. 5 (C), the ISOC amidase activity migrated the same distance as the third band of esterase activity. This indicates that one of the esterases may have ISOC-hydrolyzing activity.

DISCUSSION

These experiments show that monkey tissues contain an ISOC hydrolyzing amidase, mainly in liver, and that the enzyme activity of monkey liver is lower than that of rabbit liver (51.6 μmoles BZH/g liver wet wt/hr) but higher that that of rat liver (9.6 μmoles BZH/g liver wet wt/hr) [1]. This enzyme was found to be located in the microsomal fraction of liver. Figure 4, showing competitive inhibition of the ISOC amidase in the presence of α-NA, *p*-NPA and acetanilide, suggests that these four substrates (two amides and two esters) can be hydrolyzed by the same enzyme. In addition, the sensitivities to certain esterase inhibitors such as organophosphates, the optimum pH, the temperature stability, and the effect on SH-inhibitors and reductants suggest that this enzyme may be one of the carboxylesterases. This suggestion seems to be confirmed by the result of disc electrophoresis; the third band among four bands having esterase activity also had ISOC amidase activity. This enzyme, moreover, was found to be similar in the properties summarized in Table 5, to the ISOC amidase from rat liver microsomes that was demonstrated previously, [4], and to the microsomal carboxylesterases derived from various mammals [5–11]. The microsomal ISOC amidase from monkey, as well as rat, was effectively solubilized

Table 5. Comparison between monkey and rat ISOC amidase

	Monkey	Rat
Tissue distribution	Liver	Liver
Subcellular distribution	Microsomes	Microsomes
Optimum pH	8.5 – 9.0	8.5 – 9.0
Optimum incubation temperature	45°	60°
Stability	Stable (−20°)	Stable (−20°)
Inhibitor	Organophosphates	Organophosphates
	Heavy metals	Heavy metals
	Sodium fluoride	SKF 525-A
Activator	None	None
Substrate specificity	Isocarboxazid	Isocarboxazid
	α-Naphthylacetate	α-Naphthylacetate*
	p-Nitrophenylacetate	*p*-Nitrophenylacetate*
	Acetanilide	Procaine
K_m	3.20×10^{-3} M	0.55×10^{-4} M
V_{max}	3.0 nmoles/mg protein/min	2.0 nmoles/mg protein/min
Solubilization	Cholic acid*	Cholic acid*
	Triton X-100*	Triton X-100*

* Unpublished data.

with low concentrations of cholic acid and Triton X-100, but not of trypsin, and the molecular weight of the solubilized enzyme was estimated by the Sephacryl S-200 gel chromatography method to be about 180,000 (unpublished data). This value is similar to the molecular weight of rat liver microsomal carboxylesterase [6]. Further investigations are necessary for the comparison of monkey ISOC amidase with the other ISOC-hydrolyzing enzymes or the other carboxylesterases.

Acknowledgements—The authors wish to express appreciation to Dr. Tetsuo Satoh for his valuable discussions and comments on the manuscript. Chemicals used in this study were donated by the following companies: Nippon Roche Research Center (isocarboxazid) and E. I. du Pont de Nemours & Co. Inc. (EPN).

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