HISTOCHEMICAL LOCALIZATION OF CHOLINESTERASE IN THE AMPHIBIAN SPINAL CORD AND ALTERATIONS FOLLOWING VENTRAL ROOT SECTION*

By L. W. CHACKO† and JEAN A. CERF‡
Department of Anatomy, School of Medicine, University of California, Los Angeles, Los Angeles 24, California

The distribution of cholinesterases has been investigated in the spinal cord of various mammalian species, and the grey matter generally found to yield a positive histochemical reaction for the 'specific' enzyme (acetylcholinesterase, AChE), sometimes confined to motor nuclei in the ventral and lateral horns (Koelle & Friedenwald, 1949; Koelle, 1950, 1951, 1954, 1955; Hard & Peterson, 1950; Gerebtzoff, 1953; Ravin, Zacks & Seligman, 1953; Giacobini & Holmstedt, 1958). Sites of maximum AChE activity were detected in the cytoplasm or surface membrane of motor nerve cells and neurites, or in their immediate vicinities in the neuropil. In contrast to the 'neural' localization of AChE in the mammalian spinal cord, 'non-specific' cholinesterase (ChE) was found in non-neural structures, with the exception of some motor neurons exhibiting both enzyme activities (Koelle, 1954).

Besides the purpose of obtaining comparative histochemical data on the amphibian spinal cord, the present study appeared to be of interest in view of a feature described originally by Lavdowsky (1891), Sala (1892) and Cajal (1911), and recently reinvestigated with other techniques (Cerf & Chacko, 1957, 1958). In lower vertebrates, numerous branching expansions extend peripherally from the polar dendrites of the motoneurons (and from some interneurons), infiltrating the ventral and lateral columns and terminating in a dense marginal net under the pia. This 'perimedullary plexus', or 'lateral neuropil' receives axonal contributions constituted mainly by collaterals from the white matter and axons of some commissural cells, although the presence of motor axon and primary afferent collaterals has also been reported (Cajal, 1911; Sala, 1892; Kappers, Huber & Crosby, 1936; Silver, 1942*a*). Particular attention was given to this and other neuropil areas of the spinal cord, in view of the fact that a relatively high concentration of AChE has been noted histochemically in parts of the central nervous system where synaptic fields are well developed. In frog brain, for example, Shen, Greenfield & Boell (1955) reported the localization of

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† Visiting Professor, Fellow of the Rockefeller Foundation. Present address: Department of Anatomy, All-India Institute of Medical Sciences, New Delhi, India.

‡ 'Chercheur qualifié' of the Belgian National Foundation for Scientific Research. Fulbright Research Scholar of the United States Educational Foundation in Belgium. Present address: Laboratoire de Pathologie Générale, Faculté de Médecine, Brussels University, Belgium.

the enzyme in centres rich in interneuronal connexions and poor in cellular elements, except for the motor nuclei of cranial nerves.

Further, alterations in the distribution of cholinesterase in the spinal cord after ventral root section were investigated. This procedure has been shown to initiate a retrograde reaction in motoneurons, which extends as far as the dendritic terminations in the perimedullary plexus (Cerf & Chacko, 1958).

MATERIAL AND METHODS

The bullfrog (*Rana catesbeiana*) and common toad (*Bufo boreas halophilus*) were used in this study. The spinal cord was removed from the decerebrated animal after perfusion of the vascular system with saline. Since the cholinesterase of amphibian tissues seems easily destroyed by ordinary fixation procedures (Couteaux & Taxi, 1952; Chessick, 1954; Shen *et al.* 1955), the lumbar enlargement was rapidly sliced into pieces 3–5 mm. thick and immediately lowered in liquid nitrogen. Fresh-frozen transverse sections (20 μ) were cut with the microtome placed in a cryostat kept at -10 to $-15°$ C., and allowed to thaw on slides just before further processing. The histochemical method for detection of cholinesterases, using acetylthiocholine (AcThCh) or butyrylthiocholine (BuThCh) as substrates, was essentially similar to Koelle's improved technique (1951). Incubation in the presence of the following inhibitors was used to confirm the identity of the esterase at the site of enzymic reaction: (*a*) DFP (di-*iso*propyl-fluorophosphate), known as a potent inhibitor chiefly of cholinesterases other than AChE (Hawkins & Mendel, 1947; Koelle, 1950); (*b*) Burroughs and Wellcome B.W. 284 ^{51}C [(1-5-bis-4-allyl dimethylammonium phenyl) = pentan-3-one dibromide; Austin & Berry, 1953] found to be a highly selective inhibitor of AChE (Koelle, 1955). Appropriate concentrations were prepared from stock solutions of 10^{-1}M DFP in propylene glycol stored in the desiccator, and 10^{-2}M B.W. 284 kept in the refrigerator. The sections were divided in four groups, and incubated for 2 hr. at 37° C. in the following media, respectively: group I: AcThCh substrate solution; group II: same solution with incorporation of 3×10^{-5}M B.W. 284; group III: same solution with incorporation of 3×10^{-10}M DFP; group IV: BuThCh substrate solution. After incubation, the slides were rinsed, toned by ammonium sulphide, rinsed again, fixed in formalin 10 %, dehydrated in alcohol, cleared in xylol, and mounted with permount. Stained and unstained sections were observed under both ordinary light and phase-contrast microscopes.

In a series of experiments, unilateral section of three ventral roots (8th, 9th and 10th) was performed under barbiturate anaesthesia as described previously (Cerf & Chacko, 1958), and the animals were sacrificed after a 2–9 days' survival period. The spinal cord of the corresponding segments was excised and treated as above.

RESULTS

The sections belonging to groups I (AcThCh) and III (AcThCh and DFP) showed a positive enzymic reaction (Pl. 1, fig. 1), while sections of groups II (AcThCh and B.W. 284) and IV (BuThCh) remained blank (Pl. 1, fig. 2). From these results, it appears that the cholinesterase present in amphibian spinal cord belongs to the so-called specific type, or acetylcholinesterase.

The topographical distribution of the cholinesterase is illustrated in Pl. 1, fig. 1,

showing a section prepared with group I solution. The pattern was essentially similar in sections of group III, although there was an indication of slight but generalized reduction in activity.

The motor nuclei exhibited a high degree of enzymic activity, often obscuring the details of individual motoneurons in the ventro-lateral part of the ventral horn. No exact intracellular localization of the enzymic activity was attempted; however, cellular characteristics were better defined when DFP was used in slightly stronger concentration, although the interneuronal spaces in the motor nuclei still remained somewhat diffusely stained. The perikaryon of motoneurons took a granular type of staining throughout, extending to varying distances into the cell processes, but the method proved inadequate to verify an eventual concentration of the enzyme at the surface membrane of neurocytes, as described by Gerebtzoff (1953), Ravin *et al.* (1953) and Koelle (1954) (Pl. 1, fig. 3). The nuclear membrane was sharply defined, but the nucleus appeared clearer in those neurons in which the details were not obscured by intense diffuse staining (Pl. 1, fig. 3). The results are in general agreement with the intraneuronal localizations reported in the microchemical study of Giacobini (1957). No other cell-bodies in the spinal cord showed such a marked cholinesterase activity: the dorsal horn cells, interneurons, and neuroglial cells were not brought out at all by the method.

The medial (grey matter) and lateral (marginal zone) neuropil areas revealed a moderate reaction best marked in a region of the dorsal horn ('dorsal neuropil') bordering the dorsal column of white matter (Pl. 1, figs. 1, 4). The staining of the lateral neuropil is evident in figs. 1, 4, 6 and 7 of Pl. 1. The possibility of its being an artifact of diffusion from the adjacent pia into the marginal zone can safely be ruled out, as no such diffusion occurred into the dorsal column from the pia bordering it (Pl. 1, figs. 1, 4). The ventro-lateral column was relatively clear and showed, if at all, only a very mild enzymic activity whereas the dorsal column appeared completely blank (Pl. 1, figs. 1, 4). The leptomeninges showed a fair degree of cholinesterase activity (Pl. 1, figs. 1, 4, 6, 7).

Blood vessels of the spinal cord were intensely stained with the AcThCh method, as seen on sections from groups I and III (Pl. 1, figs. 1 and 4–7). These vessels, of calibre ranging between 10 and 20 μ, were distributed throughout the sections. The anterior spinal artery also showed consistently a marked reaction. Thorough perfusion of the animal with saline to wash out the blood vessels revealed the localization of activity in the capillary walls.

Some of the slides yielded a transverse section through spinal roots. The ventral root revealed a cholinesterase reaction both in the axonal fibres and in the Schwann cells, but the myelin sheaths were devoid of enzymic activity (Pl. 1, fig. 6). In posterior root sections, only a very small proportion of the axons were even faintly stained histochemically (Pl. 1, fig. 7).

After unilateral ventral root section, the motoneurons showed the usual histological signs of retrograde reaction. Additionally, the motor nuclei displayed a marked diminution of the histochemical reaction on the operated side (Pl. 1, figs. 4, 5). This decrease in cholinesterase activity was not very marked 2 days after ventral root section, but became increasingly recognizable from 4-days' survival period onwards. In the ventro-lateral white column and in the lateral neuropil, no consistent alteration

in depth or pattern of staining was observed on the affected side of the spinal cord; however, a reduction in staining was noticed occasionally (Pl. 1, fig. 4).

DISCUSSION

The histochemical identification of the cholinesterase present in frog and toad spinal cord with selective substrates and inhibitors shows that the enzyme belongs to the so-called specific type (AChE). This finding confirms the observations of Shen *et al.* (1955) on the frog brain. Thus, the amphibian central nervous system appears to show histochemically detectable AChE only, even in blood vessels or other non-neuronal structures which are well known to contain non-specific ChE in mammals. The phylogenetic trend in the appearance of non-specific ChE in non-nervous tissues, clearly brought out by Sippel (1955) on the heart of various vertebrates, seems to apply to the evolution of the nervous system also.

Since a histochemical method for choline-acetylase is lacking, the mapping of AChE activity has been frequently used for attempting a tentative identification of cholinergic neurons in the central nervous system. Indeed, a reasonable correlation has been observed between the distribution of acetylcholine-synthesizing regions (as evidenced by biochemical determinations of acetylcholine (ACh) content and choline-acetylase activity; see Feldberg, 1957) and that of AChE in nervous tissue. Hence, it has been suggested that cholinergic synaptic activity is exerted by neurons rich in all three components of the ACh system (Feldberg & Vogt, 1948; Burgen & Chipman, 1951; Koelle, 1951, 1954), although a few exceptions to this postulate have been reported in mammals (cerebellum, adrenergic nerve fibres). In the amphibian spinal cord, the nervous elements most obviously rich in AChE are found to be the anterior horn motor nerve cells and their processes. This observation is in agreement with the results of histochemical investigations on mammalian cord (Koelle & Friedenwald, 1949; Koelle, 1950, 1951, 1954, 1955; Gerebtzoff, 1953; Ravin *et al.* 1953; Giacobini & Holmstedt, 1958), and can be correlated with the cholinergic nature of motoneurons.

The depletion of the cellular enzyme in motoneurons after ventral root section is confirmative of observations by Hard & Peterson (1950) on the spinal cord of the dog, and can be compared to the retrograde reduction of AChE activity resulting from axonal severance in other nerve cell-bodies (Sawyer & Hollinshead, 1945; McLennan, 1954; Gerebtzoff & Vandersmissen, 1956; Brown, 1958). The mechanism of the decrease in AChE activity is not as yet properly understood since different factors could be operative. For instance, there is good evidence that other enzymic systems can be notably altered during retrograde reaction of nerve cells, and the relation of various chemical disturbances to chromatolysis has already been discussed (e.g. see Bodian, 1947). But whereas a generalized increase in activity, as observed regularly for acid phosphatase (references in Cerf & Chacko, 1958), is suggestive of enhanced enzymic function related to processes of neuronal repair, on the contrary, it seems reasonable to interpret the decreased cellular AChE activity as either a sign of motoneuron regression or lysis during intense retrograde reaction, or else as resulting from mobilization of the cellular enzyme towards a different site. Indeed, a few observations would seem to give credit to the latter view; first, an accumulation of AChE in the proximal, sprouting and regenerating segment of sectioned peripheral

nerves has actually been demonstrated (Sawyer, 1946); moreover, the loss of ACh in frog's sciatic nerve following prolonged stimulation is less if the central connexions of the fibres are intact (Krause, 1955); finally, an initial increase of choline-acetylase activity was found in the central part of a sectioned sympathetic nerve (Hebb & Waites, 1956). In all these observations (see also Cavanagh, Thompson & Webster, 1954), the decrease or disappearance of ACh and its enzymes in nerve fibres severed from their cell-bodies suggests the constant passing down of the enzymes from the cells in which they originate, a view expressed by Dale (1955). Accumulation in the central end of the sectioned nerve would represent a passive piling-up by damming of the enzymes that can no longer be passed along nor be utilized. To correlate these various findings with the decreased AChE activity observed in motoneuron cell-bodies following ventral root severance, as in Hard & Peterson's (1950) and in the present study, it seems necessary to postulate further that the cellular production of the enzyme is discontinued or that an enhanced centripetal flow exists in fibres during axon reaction. No suggestions as to the possible relation of these phenomena to the reparative efforts of the neuron can be offered as yet.

No other perikarya in the spinal cord revealed demonstrable AChE activity, although electrophysiological experiments have shown that there are probably cholinergic synapses on interneurons lying in the polysynaptic reflex pathways of the cat (Feldberg, Gray & Perry, 1953). However, it is difficult to dismiss the possibility that part of the enzymic staining found in the grey matter would be localized in interneuronal processes or terminals, where a concentration of the enzyme could bring it above the method's sensitivity threshold. Indeed, in contrast to histochemical observations on mammalian tissue (see, however, Hard & Peterson, 1950), the amphibian spinal cord revealed sites of definite AChE activity in the neuropil areas of the spinal grey, particularly in a region of the dorsal horn bordering the dorsal column ('dorsal neuropil'), and in the marginal zone ('lateral neuropil'). Elements of high AChE content in the neuropil zones could be pre- or post-synaptic, or both, and the distinction is fairly difficult at the present stage. One category, at least, of post-synaptic structures belonging to cholinergic cells, viz. motoneuron dendrites, can be expected to be present in both medial and lateral neuropil areas and contribute to the AChE reaction. However, the enzymic staining at these sites does not seem to be localized exclusively in motoneuron expansions; indeed, the retrograde reaction following ventral root section does not result regularly in a decreased AChE activity in the neuropil areas, although it was shown that the axon reaction, as assessed by other methods, usually involves the whole dendritic arborizations and terminations (Cerf & Chacko, 1958). Among the various presynaptic contributions to the medial (and maybe also to the lateral) neuropil, afferent terminals from dorsal roots, and collaterals from dorsal column fibres are presumably not the site of the histochemical reaction either; it is interesting to note that the dorsal root fibres showed practically no enzyme activity, in contrast to the ventral root fibres, and that the dorsal columns appeared quite blank on the sections. From their very low ACh and choline-acetylase contents, it is generally admitted that mammalian sensory fibres are non-cholinergic (see Feldberg, 1957), although a definite but low AChE activity has been reported in dorsal roots (Koelle, 1951, 1954, 1955). In our experiments on amphibian tissue, the results of the histochemical investigation of

root AChE also appear in agreement with the accepted view of the enzyme's specific role in cholinergic fibres. Other presynaptic elements contributing to the neuropil areas, i.e. axon collaterals from the white matter could be the site of part of the enzymic activity. Indeed, the sections show a mild histochemical reaction in the antero-lateral column, although part of this reaction might be localized in motoneuron dendritic processes infiltrating the tracts. Finally, even if interneuron (and dorsal horn) cell-bodies do not appear to show noticeable AChE activity, the enzyme might be concentrated in interneuronal dendritic or axonal processes participating in synaptic contacts in the neuropil zones. Thus, it is as yet very difficult to assign to definite structures the enzymic activity found in the spinal cord besides the motoneurons, and even more so to relate the histochemical findings to the nature of chemical mediation at synaptic sites; in the neuropil areas, positive identification of stained elements as axons or dendrites arising locally, or as entering fibres, must certainly await denervation studies.

The participation of neuroglial elements in the histochemical reaction is not easily assessed. The morphological characters of glial cells are not defined with the enzymic staining process as the motoneurons are. Whether neuroglial processes, which form the background framework in the amphibian spinal cord and are morphologically closely related to the neuronal elements (Silver, 1942*b*), are also involved in the histochemical reaction cannot be stated with certainty. However, the Schwann cells and their processes around the ventral root fibres do show distinct AChE activity, whereas in mammals, on the contrary, a non-specific enzyme has been detected histochemically in spinal gliocytes and in Schwann cells (Koelle, 1950, 1954). In the human brain, non-specific ChE only has been found in glial tissue by Cavanagh *et al.* (1954). As mentioned above, a phylogenetic trend in the appearance among vertebrates of the non-specific enzyme in nervous and non-nervous structures seems to apply to neuroglia. The same remark appears to hold good in the case of blood vessels which show intense AChE activity in the amphibian spinal cord, when on the contrary the predominant presence of a non-specific enzyme has been reported, for instance, in the smooth muscle fibres of blood vessels and in the capillary walls of rat brain (Koelle, 1954). Shen *et al.* (1955) also found almost exclusively AChE in frog brain capillary walls; they suggested for the enzyme a function of auxiliary 'scavenger' in the disposal of any local excess of neurally liberated ACh.

SUMMARY

Histochemical localization of cholinesterase activity in frog and in toad spinal cord, using Koelle's thiocholine technique, revealed the presence of the specific enzyme, acetylcholinesterase, exclusively. Acetylcholinesterase was found principally in perikarya of the motoneurons, and to a lesser degree in the neuropil areas of the grey matter and the perimedullary plexus. The sensory fibres in the posterior column were devoid of enzymic activity, and only a few dorsal root fibres were histochemically stained; the ventral root fibres showed a marked histochemical reaction. Schwann cells in the ventral roots revealed an acetylcholinesterase reaction. Acetylcholinesterase was also present in the walls of blood vessels in the spinal cord.

During axon reaction, the motoneuron cell-bodies showed a definite decrease in acetylcholinesterase activity.

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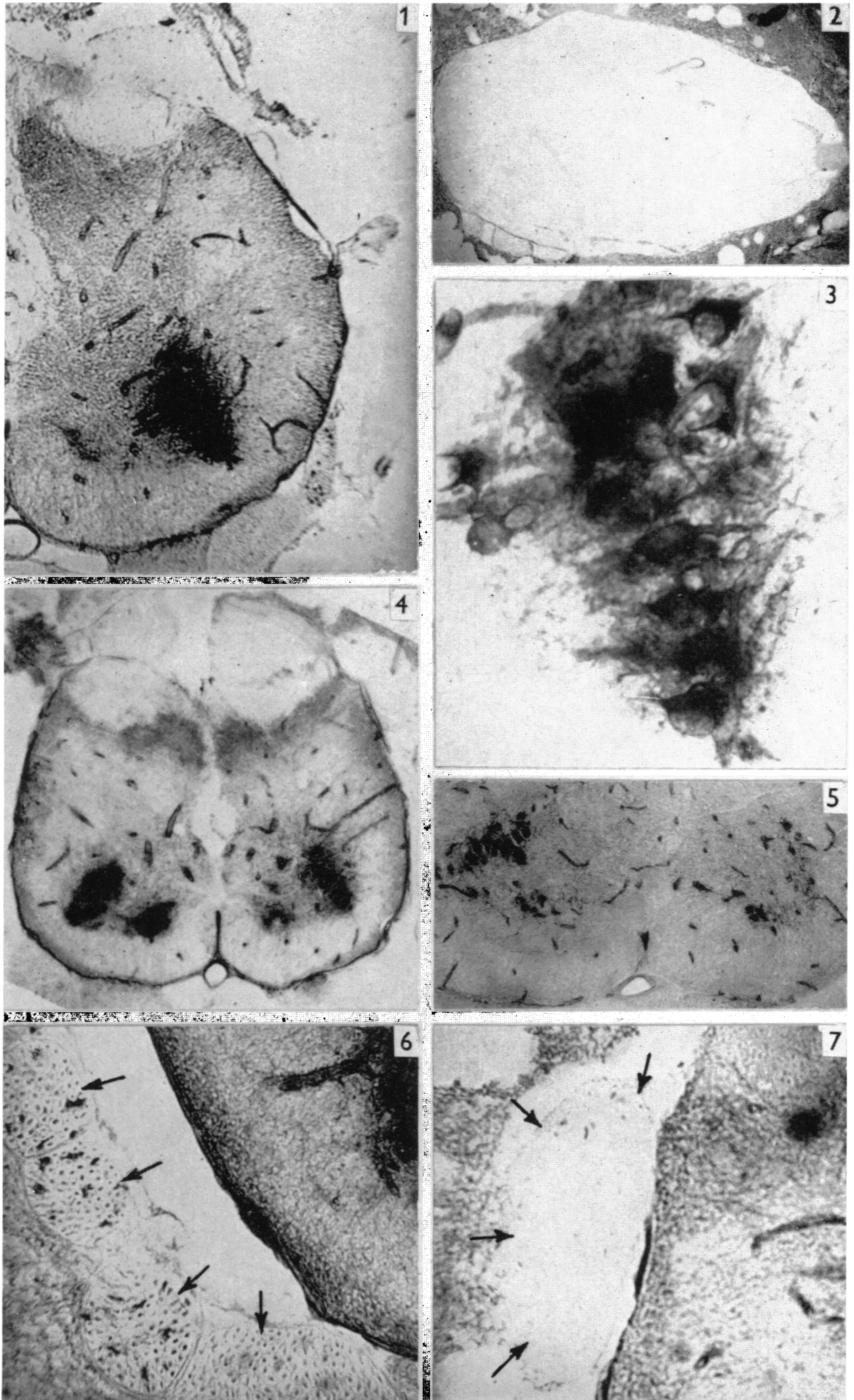

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EXPLANATION OF PLATE

All figures are photomicrographs of transverse sections through the lumbar enlargement of the toad spinal cord, some sections including spinal roots.

Fig. 1. Half section, showing a positive enzymic reaction in the motor nuclei and, to a lesser degree, in the neuropil areas. Blood vessels and leptomeninges also show a fair degree of activity. Koelle's histochemical method for the detection of acetylcholinesterase, using acetylthiocholine as substrate and no inhibitor (group I). Magnification ×47.

Fig. 2. Negative reaction throughout the spinal cord. Section incubated in acetylthiocholine substrate solution with incorporation of B.W. 284 (group II). Magnification ×19.

Fig. 3. Detail of motor nucleus, showing AChE activity extending from the cell-bodies into the proximal part of the dendritic processes. Section incubated in acetylthiocholine substrate solution with incorporation of DFP (group III). Magnification ×173.

Fig. 4. Toad in which the 8th, 9th and 10th ventral roots were sectioned on one side. Note the decreased AChE activity on the operated side (right), evident in the motor nucleus and much less marked in the marginal zone ('lateral neuropil'). Same technique as in fig. 1. Magnification ×34.

Fig. 5. Other example of unilateral retrograde reaction in the toad spinal cord, showing the decreased histochemical reaction in the motor nucleus on the operated side (right). Same technique as in fig. 1. Magnification ×45.

Fig. 6. Ventral spinal roots and part of ventro-lateral column. In the roots (pointed out by arrows), AChE activity is seen in both the axonal fibres and Schwann cells; the myelin sheaths are devoid of enzymic activity. The histochemical reaction in the marginal zone ('lateral neuropil') is particularly evident in this section. Same technique as in fig. 1. Magnification ×95.

Fig. 7. Dorsal spinal roots (pointed out by arrows) and part of lateral column. Only a very small proportion of axons show a faint histochemical reaction. Same technique as in fig. 1. Magnification ×92.

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