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The Effect of Nitrogen Mustards on the Viscosity of Thymonucleate*

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The nitrogen mustards are compounds which have definite cytotoxic effects. The exact mechanism for this action is not known but some investigators have assumed that nuclear function is inhibited or destroyed. The present investigation is concerned with the effect of nitrogen mustards on the viscosity of thymus nucleate.

METHODS

The nitrogen mustards used in these experiments were the hydrochlorides of ethyl bis (β chloroethyl) amine, methyl bis (β chloroethyl) amine, and tris (β chloroethyl) amine.

Sodium nucleate was prepared from calf thymus by a modification of Hammarsten's procedure (5). After the preliminary extraction, filtration, and addition of $CaCl_2$, a precipitate was obtained by bringing the final ethanol concentration to 15 per cent. The precipitate was kept in the refrigerator overnight, and separated by centrifuging and washed with 15 per cent ethanol. The washed precipitate was dissolved in a saturated NaCl solution and allowed to stand in the refrigerator for 3 days. The solution was filtered through "Hyflo Super-Cel" under vacuum in a Buchner funnel. The sodium thymonucleate was precipitated by adding 95 per cent ethanol and was washed and redissolved in H_2O. It was reprecipitated and washed with ethanol and ether and dried in vacuo over P_2O_5. Despite the uniformity in procedure, the viscosity of the various batches of thymonucleate varied considerably.

The viscosity measurements were made according to the method described by Bingham and Jackson (1). A pressure of 20 cm. H_2O was used and the viscosimeter was kept in a constant temperature bath at 30° C. No attempt was made to express the values in terms of absolute viscosity.

Most of the experiments were performed by diluting 10 ml. of a 1 per cent thymonucleate solution with an equal quantity of buffer solution; the agent was either added to this mixture in solid form or in solution.

TABLE I: EFFECT OF VARIOUS CONCENTRATIONS OF NITROGEN MUSTARDS ON THE VISCOSITY OF A THYMONUCLEATE SOLUTION

	Minutes after mixing				
	10	30	60	90	
Agent *	Seconds				pH †
Control	367	375	383	390	6.0
0.002 M					
1	369	366	362	355	6.0
2	368	366	366	360	6.0
3	340	316	307	302	5.9
0.004 M					
1	369	364	355	340	5.9
2	369	366	360	350	5.9
3	310	251	233	224	5.7
0.01 M					
1	369	362	331	284	5.7
2	369	364	340	312	5.7
3	227	165	146	132	5.6

* The agents designated as follows:
(1) Ethyl bis (β chloroethyl) amine,
(2) Methyl bis (β chloroethyl) amine, and
(3) Tris (β chloroethyl) amine.
† Values for pH obtained after final viscosity reading.

RESULTS

Effect of concentration of nitrogen mustards.—To equal volumes (10 ml.) of a 1 per cent thymonucleate solution and an acetate buffer of pH 6.0 and ionic strength of 0.3, 1 ml. of an aqueous solution of the nitrogen mustards was added. The nitrogen mustards added were present so that the final molar concentrations were 0.01, 0.004, and 0.002.

The results are shown in Table I. At all concentrations the tris (β chloroethyl) amine causes appreciable

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decreases in viscosity of the nucleate solution. The effect of the remaining two agents is comparatively small.

Slight changes are seen in the hydrogen ion concentration after addition of the nitrogen mustard in these and in the following experiments. According to Vilbrandt and Tennent (7) small changes in pH below 5.0 and above 9.0 may influence the viscosity of nucleic acid solutions considerably. These findings were confirmed in this laboratory for the ionic strength (1 per cent NaCl) used by the investigators. It should be stated that increasing the ionic strength is responsible for more noticeable changes in viscosity within given ranges in pH. The differences in pH noted in Tables I to III are not sufficiently great to produce the changes noted after addition of nitrogen mustards.

TABLE II: EFFECT OF IONIC STRENGTH ON THE DEPOLYMERIZING ACTION OF TRIS (β CHLOROETHYL) AMINE ON THYMONUCLEATE

	Ionic strength	Minutes after mixing: 15 Seconds	30 Seconds	60 Seconds	pH †
Control	1.50	335	348	368	6.0
Agent		355	366	380	5.9
Control	1.00	310	322	330	6.0
Agent		294	297	305	5.9
Control	0.50	322	328	338	6.0
Agent		288	286	286	5.9
Control	0.30	306	308	314	6.0
Agent		267	258	250	5.9
Control	0.15	292	292	290	6.0
Agent		230	210	197	5.8
Control	0.06	300	298	295	6.0
Agent		208	183	164	5.8

† See this footnote on Table I.

Effect of ionic strength.—Since tris (β chloroethyl) amine caused an immediate decrease in the viscosity of thymonucleate, this agent was used to determine the effect of salt concentration.

Acetate buffers with an initial pH of 6.0 were prepared with ionic strengths of 1.5, 1.0, 0.5, 0.3, 0.15, and 0.06. The control was prepared by mixing 10 ml. of nucleate solution and 10 ml. of the respective buffers and 1 ml. of H_2O. Tris (β chloroethyl) amine hydrochloride (10 mgm.) was dissolved in 1 ml. of H_2O and added to the nucleic acid mixture immediately. The results are shown in Table II.

A lowering of the ionic strength results in a decreased viscosity in the control groups. The viscosity becomes progressively less for a given amount of agent as the ionic strength decreases.

Effects of compounds structurally related to the sulfur and nitrogen mustards.—To a mixture of 10 ml. thymonucleate (1 per cent) and 10 cc. acetate buffer having a final pH 6.0 and ionic strength of 0.15, the following compounds dissolved in 1 ml. H_2O were added to yield equimolar concentrations (1×10^{-4}).

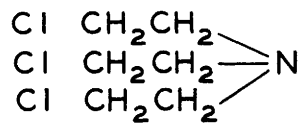
a. choline chloride	$(HOCH_2CH_2N(CH_3)_3)Cl$
b. guanidine hydrochloride	$NH=C(NH_2)_2HCl$
c. triethanol amine	$N(CH_2CH_2OH)_3$
d. thiodiglycol	$S(CH_2CH_2OH)_2$
e. dichloroethyl sulfide	$S(CH_2CH_2Cl)_2$
f. tris (β chloroethyl) amine	$N(CH_2CH_2Cl)_3$

The control solution was similarly prepared, but no agent was added.

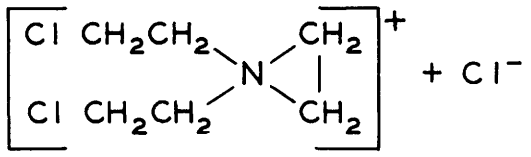
In Fig. 1, it is seen that the compounds a, b, c, and d increased viscosity above that of the control. Sulfur mustard e causes an appreciable initial decrease in viscosity which remains constant on standing. The nitrogen mustard f decreases the viscosity as previously shown. The importance of the chloroethyl group is apparent in these experiments.

Effect of transformation products.—Nitrogen mustards in aqueous solutions undergo characteristic intra molecular rearrangements to form a number of compounds. The rate of formation of these transformation products differs for each compound and is dependent on the hydrogen ion concentration and the inorganic and organic substances present in the solution. According to Gilman, Goodman, and Phillips (2), the various transformation products of a given nitrogen mustard differ in their pharmacodynamic action and toxicity.

The active physiological product formed appears to be an ethylene-imine ring derivative. For example, the compound tris (β chloroethyl) amine



undergoes a change to form the first imine ring.



Each chloroethyl radical is capable of forming an imine ring. The rate of formation of these rings can be slowed by using a $NaHCO_3$:NaOH solution of about pH 9.0 so that mixtures can be obtained in which a given ring is predominant (7).

The 3 nitrogen mustards were dissolved in the alkaline buffer and were allowed to stand at room temperature for varying lengths of time until the desired ring was present. A 1 per cent nucleate solution was mixed with an equal amount (10 ml.) of the alkaline solution of nitrogen mustard which contained 20 mgm. of the

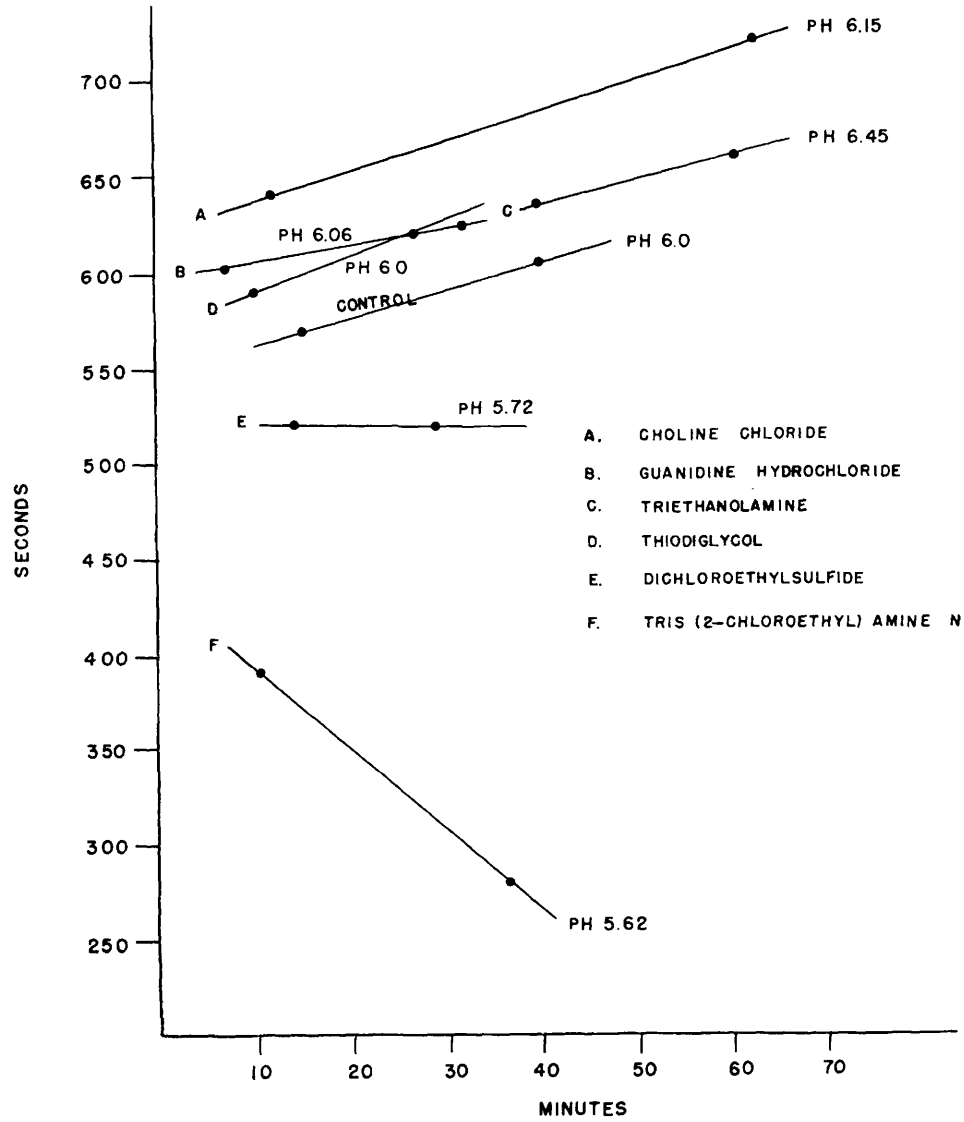


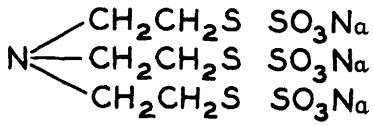
FIG. 1.—Effect of sulfur mustard and a nitrogen mustard and related compounds on the viscosity of a nucleic acid solution.

respective agents. After mixing, the pH of the solution decreased to about 8.0.

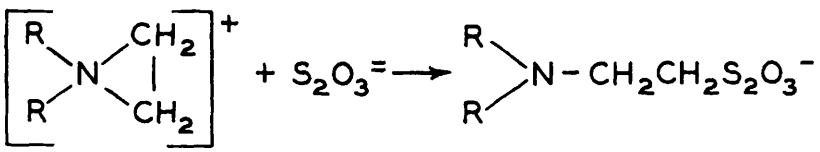
The results for a typical set of experiments are shown in Fig. 2. In each case the solution containing a preponderance of the 1st imine ring has the greatest effect in reducing the viscosity of the nucleic acid. In the case of the tris (β chloroethyl) amine, the 2nd imine ring is more effective than the 3rd imine ring.

In the presence of thiosulfate a relatively stable end product is formed.

A molecule of thiosulfate is required for each imine ring formed. Thus, in the case of the tris (β chloroethyl) amine the final product is



which is a stable, non-toxic compound.



Equal volumes (10 ml.) of 1 per cent nucleic acid and phosphate buffer (pH 7.5, ionic strength 0.3) solutions which contained various amounts of sodium thiosulfate were mixed. Tris (β chloroethyl) amine hydrochloride (50 mgm.) was dissolved in 1 ml. H_2O and added. In a preliminary experiment in which 3 moles of thiosulfate were added to react with the 3 imine rings of the agent, the usual decrease in viscosity was completely inhibited.

In Fig. 3, the effect of varying the amounts of thiosulfate on viscosity is shown. The reduction in available thiosulfate is accompanied by a decreased viscosity of thymonucleate. This appears to be proof that the imine ring is directly concerned with depolymerization of nucleic acid.

Table III: The Effect of Thiocyanate on the Depolymerizing Effect of Tris (β chloroethyl) Amine on Thymonucleate

NaSCN mgm.	Agent mgm.	Minutes after mixing: 10	30	60	pH †
		Seconds			
50.3	0	271	271	271	7.1
75.0	50	233	205	190	6.7
50.3	50	232	204	180	6.7
30.0	50	162	135	110	6.7
20.0	50	159	128	120	6.7
0.0	50	139	119	105	6.7

† See this footnote on Table I.

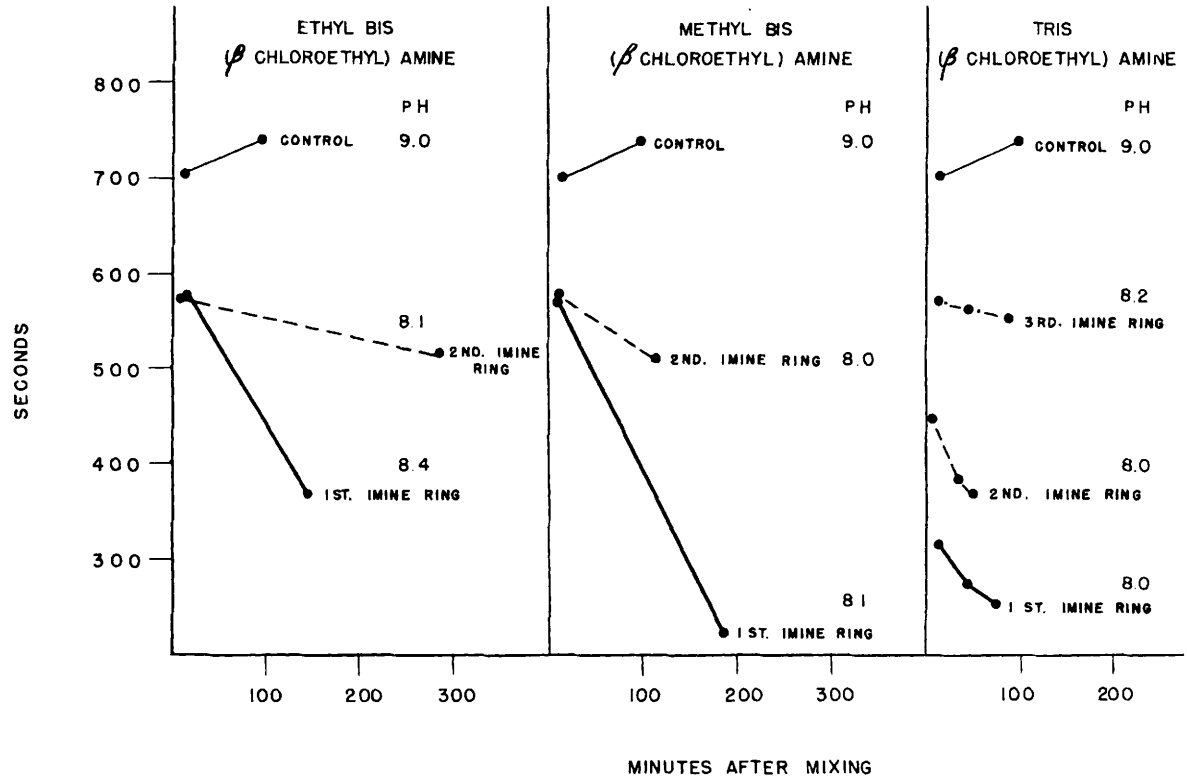


Fig. 2.—Effect of transformation products of three nitrogen mustards on the viscosity of a nucleic acid solution.

Sodium thiocyanate is known to react with the imine ring. However, the competition of thiocyanate for the imine ring is not as great as that of thiosulfate. According to theory, 3 moles of NaSCN should react with and form stable salts with the 3 potential imine rings of tris (β chloroethyl) amine. It should require 50.3 mgm. NaSCN to neutralize 50 mgm. of the hydrochloride. The results of adding varying amounts of SCN to a thymonucleate mixture similar to that described for thiosulfate are shown in Table III. This ion is partially effective in reducing the depolymerizing effect of the nitrogen mustards. It will be noted that excessive amounts of thiocyanate (75 mgm.) do not prevent the decrease in the viscosity of thymonucleate solutions.

DISCUSSION

There is no doubt that the sodium thymonucleate is a highly polymerized structure made up of many molecules of tetranucleotides (4). An enzyme such as desoxyribonuclease (6) causes a rapid depolymerization with an accompanying decrease in viscosity. No compound is as effective as this enzyme. However, as

far as can be ascertained, transformation products of the nitrogen mustards are more effective than any other chemical compound in depolymerizing nucleic acid.

dilution of small amounts of the injected agent in the blood stream. Gilman and Philips (3), in a recent review, stressed the high degree of specificity of these agents on lymphoid tissue, bone marrow, and intestinal

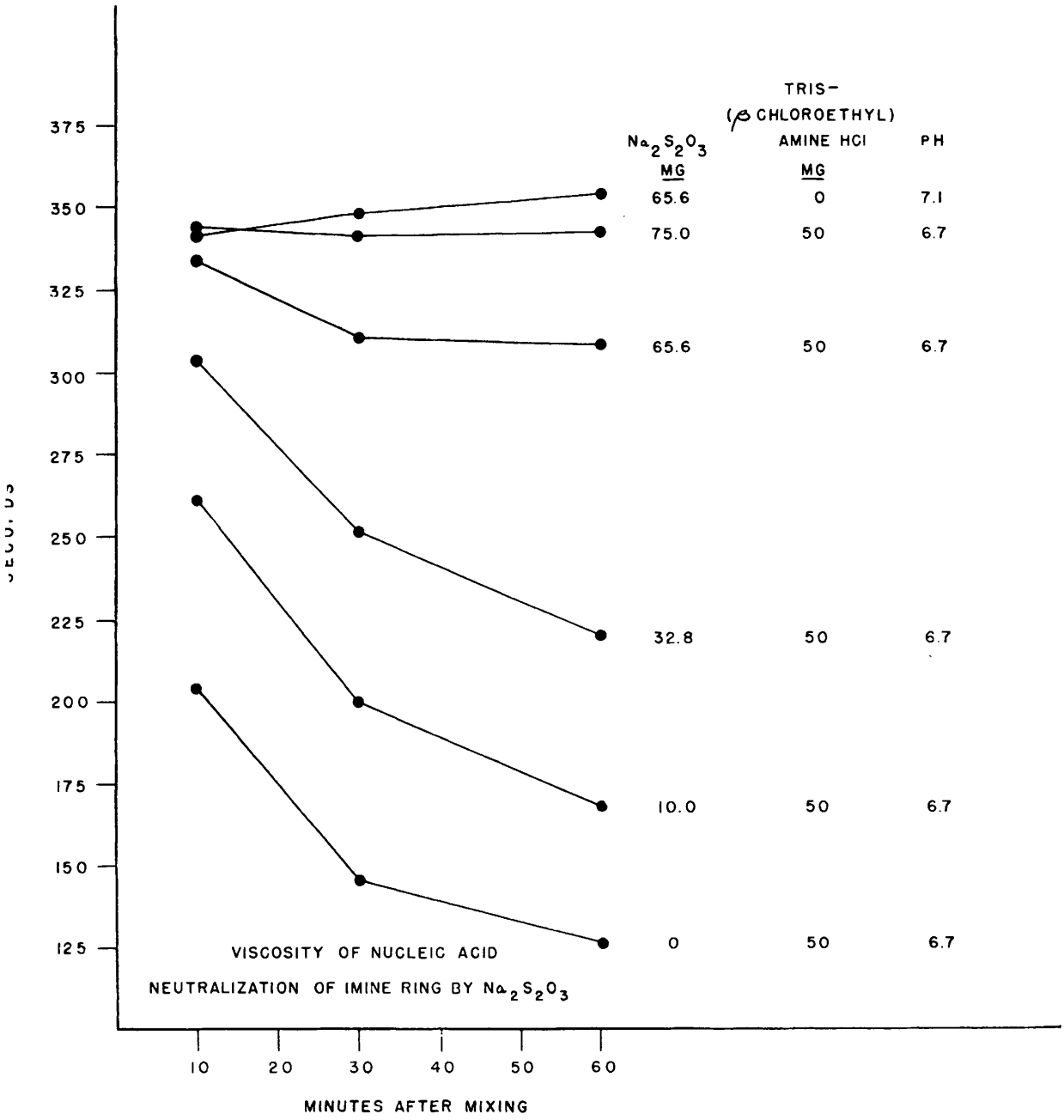


FIG. 3.—Neutralization of transformation products of a nitrogen mustard by sodium thiosulfate.

The toxicity of the nitrogen mustards may be explained in part by the action of the transformation products on the nucleoproteins of the cells. The objection to this hypothesis could be based on the high

mucosa. They also pointed out that the mustards can exhibit a primary nucleotoxic action. These agents have been studied for their therapeutic effects in the treatment of neoplasms of lymphoid tissue.

SUMMARY

1. The influence of 3 nitrogen mustards, ethyl bis (β chloroethyl) amine, methyl bis (β chloroethyl) amine, tris (β chloroethyl) amine, on the viscosity of thymus nucleate has been investigated.

2. The viscosity of thymus nucleate solutions is decreased by these mustards. The greatest decreases in viscosity are noted in solutions of low ionic strength. The degree and rate of change in viscosity depends on the formation of active transformation products.

3. Evidence is presented to show the importance of the chloroethyl radical in the depolymerizing action of the mustards.

4. The ethylene-imine ring transformation product of the nitrogen mustards appears to be responsible for the depolymerizing effect. This effect is most pronounced when the first imine ring predominates.

5. In the presence of thiosulfate, the effect of the ethylene-imine rings is completely inhibited. Thiocyanate partially inhibits the action of these rings.

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