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***Trypanosoma cruzi*: effect of pH on in vitro formation of metacyclic trypomastigotes**

H. UCROS, B. GRANGER, S. M. KRASSNER

Summary

The formation of metacyclic trypomastigotes of *Trypanosoma cruzi* in cultures initiated by freshly isolated bloodstream trypomastigotes was studied using three strains of parasites (Peru, CL, and Y) and two different media (L-15 and Grace's medium). Serum (10% newborn calf serum) and various sugars were required for in vitro development of metacyclic forms. This development was best at an alkaline pH (9.0); lower pH (e.g. 5.5–6.0) favored prolonged persistence of bloodstream trypomastigotes. Medium pH remained relatively stable during the 15–17 days of culture. The culture vessel employed proved very important; large numbers of metacyclic parasites were found in microtiter well cultures but not in test tube cultures. This latter result suggests that the diffusion rates of gases may be important for metacyclic trypomastigote formation in vitro.

Key words: metacyclic trypomastigote formation; *Trypanosoma cruzi*; pH; trypanosome culture.

Introduction

Trypanosoma cruzi is an arthropod-transmitted protozoal parasite of man and other animals which undergoes specific differentiation in its life cycle. Although readily studied in culture, the physiologic conditions required to initiate differentiation of *T. cruzi* culture and/or insect stages to the infective metacyclic trypomastigote stage are incompletely understood. Working with culture forms of *T. cruzi*, Sullivan found high levels of metacyclogenesis in Grace's medium, at an acid pH (Communication at 29th Annual Meeting,

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Amer. Soc. Trop. Med. Hyg., 1980). Lanar (1979), however, using a variant of L-15 medium reported that pH was not a factor in initiating metacyclic trypomastigote formation. This also contradicted an earlier study by Fernandes et al. (1969) which suggested that a drop in pH in cultures of *T. cruzi* due to accumulation of organic acids was one of the main stimuli for transformation to the metacyclic trypomastigote stage. In Lanar's study, *T. cruzi* was cocultivated with a *Triatoma infestans* embryo cell line. He found that the insect cells produce a labile factor(s) important for differentiation to the metacyclic trypomastigote stage.

In order to carry out in vitro immunological tests on *T. cruzi* we decided to improve the culture system devised by Lanar (1979) by dispensing with the insect cells. Medium (L-15) alone did not permit consistent metacyclic trypomastigote formation and when it did occur it was very slow. We therefore tested a number of media and confirmed Sullivan's finding (see above) that the best culture and transformations of *T. cruzi* (Peru, CL and Y strains) took place in Grace's insect tissue culture medium. Since both Grace's and L-15 are defined media we initiated experiments to determine nutritional and other requirements for in vitro transformation of bloodstream trypomastigotes to the metacyclic trypomastigote stage. L-15 was used as a basal medium and nutrients present in Grace's medium but absent from L-15 were added to basal medium to determine their effect upon transformation. Our initial step was to determine an optimal pH for the investigation. To our surprise we found that, under certain circumstances, an alkaline pH favored metacyclic trypomastigote formation. The results of these experiments are reported in this paper.

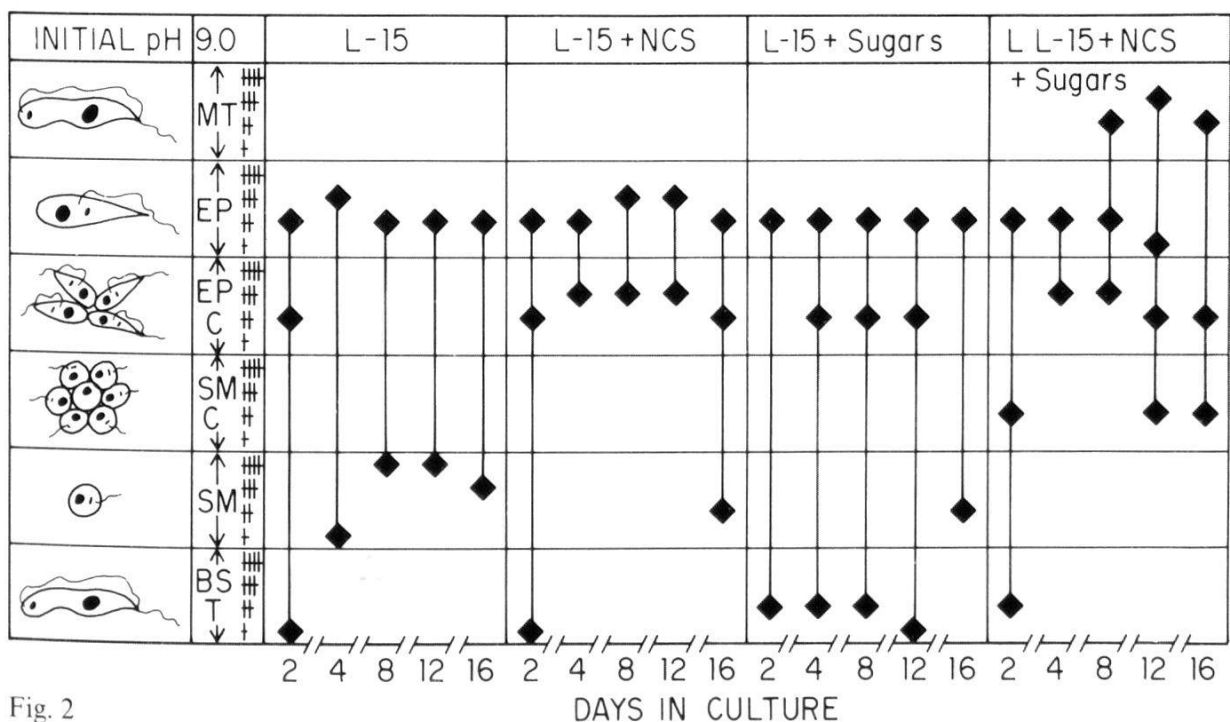
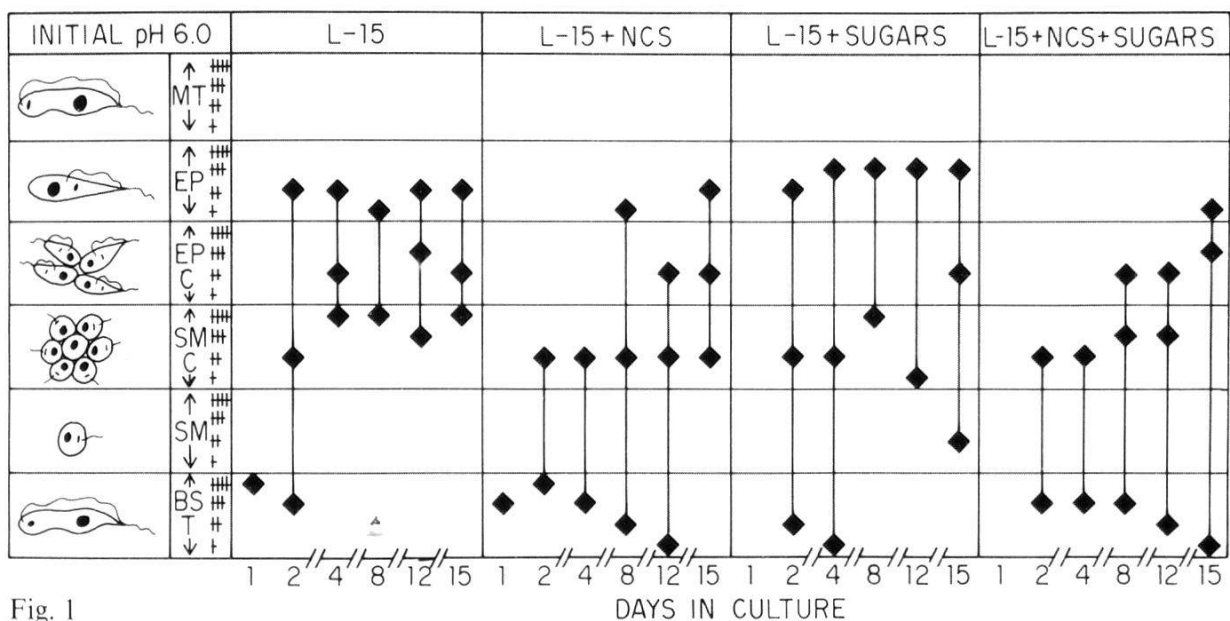
Materials and Methods

The parasites

The cloned Peru strain of *T. cruzi* was derived from the stabilate LUMP-722 obtained from Lumsden (Lanar, 1979). Characteristics of this strain have previously been described (Marsden, 1967). The Peru strain has been maintained in our laboratory by bimonthly subcutaneous injection of 10^5 bloodstream trypomastigotes in 8–12 week old BALB/c mice since 1974. The blood was collected 11 days after injection by heart puncture with a heparinized syringe and diluted in Hanks Balanced Salts Solution (DIFCO) supplemented with 0.5% albumin. The diluted blood was centrifuged at low speed ($120 \times g/15$ min) to remove most of the host cells. The number of parasites was determined by collecting $1.0 \mu\text{l}$ of blood in heparinized Microcaps (Drummond Scientific Co.), diluting the blood with $9.0 \mu\text{l}$ 0.89% NH_4Cl and counting the number of trypomastigotes with a hemocytometer. Two other cloned strains of *T. cruzi* were used for one series of experiments. These were the Y strain, obtained from Dr. W. Leon (University of Rio de Janeiro, Brazil) and the CL strain, obtained from Dr. R. Hoff (Harvard University, Massachusetts, USA). The CL strain was transferred and maintained as above while the Y strain was transferred every 7 days.

Culture media

Twenty μl containing $1.5\text{--}2.0 \times 10^4$ bloodstream trypomastigotes were added to microtiter wells containing 200 μl of medium and maintained at 27°C . The two media tested were: i. L-15 (GIBCO) supplemented with 0.025 mg/ml hemin (Sigma Chemical Co.) and ii. Grace's Anteraea



Figs. 1 and 2. Effect of pH, newborn calf serum (NCS) and sugars (glucose 2.0 $\mu\text{g}/\text{ml}$ and sucrose 2.66 $\mu\text{g}/\text{ml}$) on the in vitro formation of Peru strain *T. cruzi* metacyclic trypomastigotes in L-15 medium. The number of organisms found in each stage is shown plotted against days in culture. Cultures were initiated with freshly isolated bloodstream trypomastigotes added to medium in microtiter wells. Parasite isolation procedures, culture methods and determination of stages observed are described in Materials and Methods. All tests were done in triplicate a minimum of three times. MT = metacyclic trypomastigote stage; EP = epimastigote stage; EPC = epimastigote clumps; SMC = staphylomastigote clumps; SM = staphylomastigote stage; BST = bloodstream trypomastigote stage. + = one organism in 4-8 fields ($100\times$ magnification); ++ = one organism in each field ($100\times$ magnification); +++ = 10 or more organisms in each field ($100\times$ magnification); ++++ = 50 or more organisms in each field ($200\times$ magnification). Fig. 1: initial pH 6.0; Fig. 2: initial pH 9.0.

Medium (KC Biological Inc.). The final pH was adjusted with sterile 2.0% (v/v) HCl or NaOH. The pH range tested was 5.5–9.0; outside this range it was difficult to maintain a constant pH during the course of an experiment. In some experiments the media were also supplemented with 10.0% newborn calf serum (NCS) (GIBCO). For some experiments L-15 was supplemented with 20 μ l of a sugar solution containing glucose (2.0 g/100 ml) and sucrose (26.6 g/100 ml); Grace's medium contains fructose (400 mg/l), glucose (700 mg/l) and sucrose (26.68 mg/l) while L-15 contains 900 mg/l galactose. Neutral red was added to all media to allow rough monitoring of pH. For accurate measurement of pH, small amounts of test media were measured daily with a Beckman 3560 Digital pH meter equipped with a microelectrode. All experiments were done a minimum of three times with each variable tested in triplicate. In some tests 0.1 ml bloodstream trypomastigotes (1.0×10^5) were added to 1.0 ml medium in 16 \times 125 mm screw-capped plastic test tubes.

Examination of cultures

Cultures were inspected every day for at least 15 days with an inverted phase-contrast microscope. Occasionally parasites from cultures were also stained by mixing a small amount of culture with an equal volume of 1.0% (v/v) sucrose albumin and smears made. These were air dried, fixed in methanol, and stained with Giemsa.

Results

Differentiation in medium L-15

The effect of serum and sugars on in vitro transformation of bloodstream trypomastigotes was determined by adding the parasites to microtiter wells containing one of the following media: L-15, L-15 + 10.0% NCS, L-15 + sugars (glucose and sucrose), and L-15 + 10.0% NCS + sugars. Media were adjusted to a pH range 6.0–9.0 to find the optimal pH for parasite differentiation. The results of experiments at pH 6.0 and pH 9.0 are depicted in Figs. 1 and 2.

In vitro metacyclic trypomastigote formation in L-15 was found only at alkaline pH; optimal formation was at pH 9.0 (Fig. 2). The presence of both serum and sugars was required for differentiation into metacyclic trypomastigotes. Long-term persistence of the bloodstream trypomastigote stage was enhanced by NCS at pH 6.0 (Fig. 1) but not at pH 9.0 (Fig. 2).

All of the other in vitro stages of *T. cruzi* (staphylomastigotes [amastigote-like forms described by Lanar, 1979], staphylomastigote clumps, epimastigote clumps and epimastigotes) were found in L-15 at the various pH tested; however, free staphylomastigotes were readily seen only at pH 9.0 in media lacking NCS and sugars (Fig. 2). Staphylomastigote clumps were present in the different configurations of L-15 at pH 6.0–8.0 (data not shown for pH 7.0 and pH 8.0); at pH 9.0 serum and sugars were necessary for their presence and even then the clumps were smaller than those found at more acid pH. In those wells not showing metacyclic trypomastigote formation the most advanced in vitro stages usually found were free swimming epimastigotes. Epimastigote clumps at pH 6.0 were much larger than those at more alkaline pH.

Surprisingly, the pH of the various media did not change appreciably during the 15–16 days of culture. The pH of cultures started at 6.0 rose to 6.8 by

the second day and stayed at 7.03–7.15 for the remainder of the experiment. Cultures started at pH 7.0 remained at 7.57–7.73 for the duration of their study. Cells in L-15 started at pH 8.0 ranged between pH 8.10–8.15 for the 16 days in culture while organisms started in L-15, pH 9.0, were exposed to pH 8.75–8.80 for the 16 days of study. At no time did we find a marked drop in the pH of the different configurations of L-15 media.

When bloodstream trypomastigotes were added to 1.0 ml media in screw-capped plastic test tubes they did not transform into metacyclic trypomastigotes during the 16 days of culture. This was true at all pH levels tested (6.0–9.0). NCS and sugars had no effect on the lack of metacyclic stage formation. Tests of the pH in the tube cultures showed no difference from the pH seen in microtiter cultures; pH remained relatively unchanged.

Bloodstream parasites were added to microtiter wells and tubes containing L-15 (adjusted to pH 9.0) and serum plus one of the following sugars: glucose (2.0 $\mu\text{g}/\text{ml}$), fructose (0.4 $\mu\text{g}/\text{ml}$), sucrose (2.66 $\mu\text{g}/\text{ml}$) and trehalose (0.4 $\mu\text{g}/\text{ml}$). Metacyclic trypomastigotes were readily found within a week in all the microtiter wells containing the test sugars. The number of metacyclics formed in the presence of trehalose was lower than that formed in the presence of the other sugars tested but we do not know if this difference is significant. As was the case for previous tests, pH was stable for the 15 days of culture (pH 8.65–8.90), and no metacyclic form was noted in tube cultures.

Differentiation in Grace's medium

Bloodstream trypomastigotes were added to microtiter wells and tubes containing Grace's medium or Grace's medium + NCS at pH 5.5 or 9.0. Large numbers of metacyclic forms were seen only in microtiter wells containing Grace's medium + NCS (initial pH 9.0) (Fig. 3). In one experiment a very few metacyclic-like organisms were formed at pH 5.5 on the 16th day. The pH in all wells remained relatively unchanged during the course of study; when the initial pH was 9.0, the pH remained between 8.75–8.86 and when the initial pH was 5.5, it ranged from 5.67–5.83. As we found in experiments with L-15, bloodstream trypomastigotes persisted longer at the more acid level (initial pH 5.5). Metacyclic forms were not noted in any of the tube cultures.

In order to demonstrate that the effects of pH and of culture enclosure on metacyclic trypomastigote formation were not strain dependent, we repeated the above tests using CL and Y strains of *T. cruzi*. The results were essentially the same as those found with Peru strain parasites. Metacyclic formation was seen only in the presence of serum and at pH 9.0 when cells were cultured in microtiter wells. Metacyclic formation was not found in tube cultures. In vitro growth of CL strain parasites was much slower than that of Peru strain organisms (parasitemia in mice was also characteristically less). These characteristics for Y strain *T. cruzi* were intermediate between those of the CL and Peru strains. For this reason the numbers of metacyclic trypomastigotes arising in CL and Y

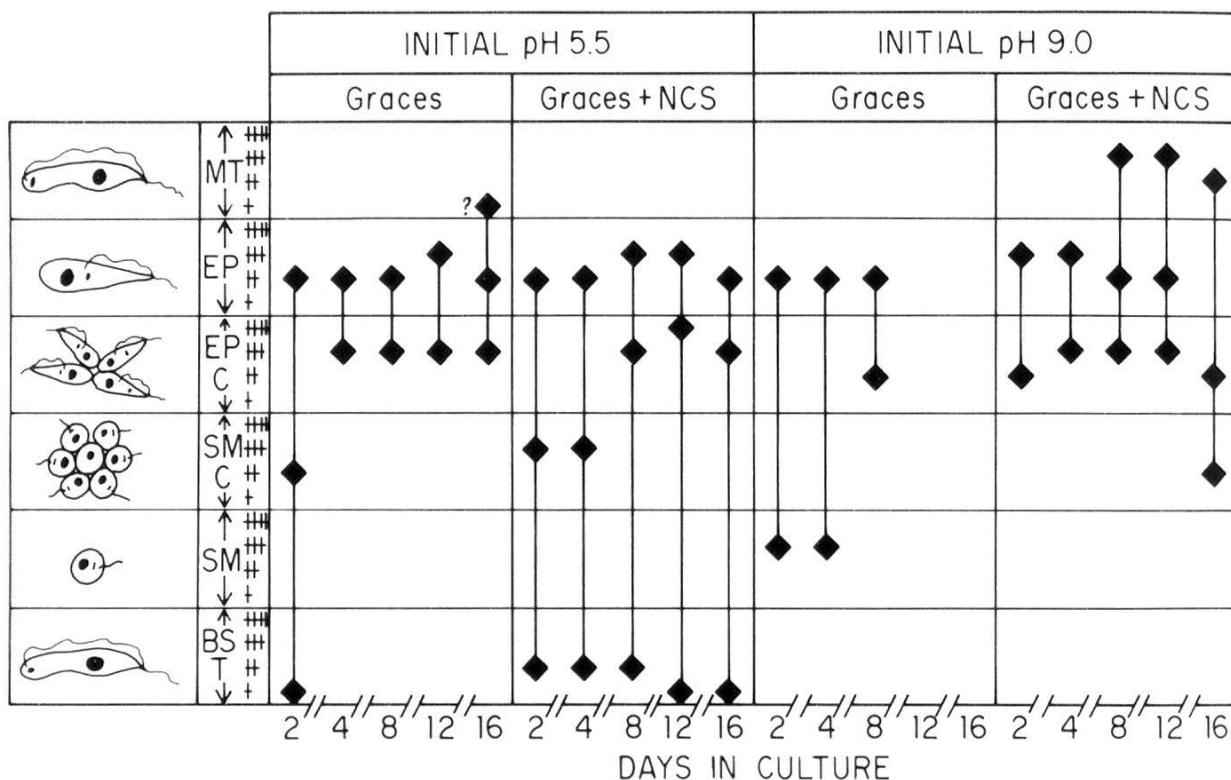


Fig. 3. Effect of pH and NCS on transformation of Peru strain *T. cruzi* bloodstream trypomastigotes in microtiter wells containing Grace's medium. The number of organisms found at each stage is plotted against the days in culture. Isolation procedures, culture conditions, and determinations of stages are described in Materials and Methods. All tests were done in triplicate a minimum of three times. Abbreviations as in Fig. 1.

strain cultures were considerably lower than those found with the Peru strain. In the case of CL strain cultures, metacyclic forms disappeared after 10 days of culture.

Discussion

In this paper we have described the effect of pH, NCS and various sugars on the formation of metacyclic trypomastigotes in cultures inoculated with freshly isolated bloodstream trypomastigotes. It is clear that both serum and sugars were necessary for the development of metacyclic forms and that, with our culture conditions, optimal metacyclic development occurred at an alkaline pH (9.0). The culture vessel employed was important; metacyclic parasites were found in microtiter well cultures but were not seen in tube cultures.

Our observations with NCS are in agreement with those of many other workers showing a stimulating effect of serum on *T. cruzi* development and growth. O'Daly (1976) found that transformation of the parasite to the metacyclic form was induced by several proteins obtained from fetal calf serum. Zeledon et al. (1977) have suggested that these types of proteins or polypeptides may be present in the urine or excreta of triatomid bugs.

The role played by sugars in *T. cruzi* transformation is probably as a source of energy required for growth and development. It is also possible that some end product of carbohydrate metabolism acts as a trigger for *T. cruzi* transformation. In our hands, trehalose was not as vigorous in stimulating formation of metacyclic stage parasites as the other sugars tested. However, these forms were seen when trehalose was added to test medium. This is of interest because trehalose is a common sugar constituent in insects.

Several studies have shown that insect cells secrete substances favoring the formation of metacyclic trypomastigotes of *T. cruzi*. Lanar (1979) found that a material secreted by *Triatoma infestans* embryonic cells promoted the formation of metacyclic forms. Formation of this stage has also been promoted by insect tissue culture and moth hemolymph (Wood and Pipkin, 1969). In another report Wood and Souša (1976) showed that moth hemolymph could be replaced by a *Rhodnius* extract. Identification of the insect substances responsible for promoting metacyclic stage development is still forthcoming, although Zeledon et al. (1977) have long espoused the view that high levels of uric acid or its salts were the key substance in the intestines of insects.

Our results, showing a strong positive effect by alkaline pH on the formation of metacyclic trypomastigotes, differ markedly from the data in previous studies. Fernandes et al. (1969) concluded that a drop in pH in the culture medium, probably resulting from the accumulation of organic acids, was required for initiation of metacyclic stage formation. Sullivan (see above) also found that with Grace's medium, metacyclic stage formation occurred at acid pH. Lanar (1979), however, concluded that pH was not a factor in in vitro differentiation of *T. cruzi*. At this time it is not possible to reconcile these disparate findings. One possible explanation is that Sullivan initiated his experiments with culture stage parasites whereas our experiments were initiated with bloodstream trypomastigotes. In our system, the data clearly show that an acid pH does not favor in vitro metacyclic stage development; rather, it favors the prolonged retention of bloodstream trypomastigotes (see Figs. 1 and 2).

There is little information in the literature concerning the range of hindgut pH of triatomid bugs. Wigglesworth (1931), using pH indicator dyes, studied the pH of *Rhodnius prolixus* malpighian tubules. These organs are responsible for excretion of uric acid into the posterior portion of the insect gut. Wigglesworth found that they were slightly acid (pH 6.6) in their lower segment. Uric acid is, of course, slightly acid as well (pH 6.5). Zeledon et al. (1977) reported that the pH of the urine from another triatomid bug, *Dipetalogaster maximus*, was quite alkaline (pH 8.5) except immediately after a blood meal when it dropped to pH 7.5. More work needs to be done, directly measuring pH of triatomid hindgut contents before and after a bloodmeal.

The results from our experiments comparing metacyclic stage development in microtiter wells and in culture tubes suggest that differential diffusion of gases to and from the cells in culture medium may be important in in vitro

transformation. Diffusion rates would be more rapid in the microtiter wells because of lesser medium depth and larger surface to volume ratios. Another possible explanation for our results is suggested by the work of Camargo (1964), who concluded that transformation to the metacyclic stage in vitro was probably due to the depletion of some nutrient in the culture medium or in the metabolic pool of the parasite. We maintained the same initial parasite density in microtiter wells and in the tubes. If cells grew more rapidly in the wells they would more quickly exhaust the supply of any limiting nutrients, an event which, according to Camargo's view, would trigger transformation. Certainly there have been numerous studies showing that metacyclic trypomastigote formation usually occurs at the end of exponential growth (see, e.g., Camargo, 1964).

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