Roux's Arch Dev Biol (1984) 193:111–116

Roux's Archives of Developmental Biology

Regeneration of whole limbs from shank stumps in toad tadpoles treated with vitamin A

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Summary. Young tadpoles of the toad, *Bufo melanostictus* (Schneider), were immersed in 15 IU/ml vitamin-A palmitate solution for 3 days, only prior to amputation through the shank. In more than 65% of cases the resultant regenerates were whole limbs containing the skeletal elements from femur to phallanges; in several of them a new girdle had also differentiated. In others regeneration had progressed only up to the blastema stage and postblastemic development was inhibited. Opposite results were obtained when treatment was extended to another 3 days after amputation. A normal control-type regenerate consisting of parts distal to amputation level was not obtained in any case treated in either manner. The removed distal part of the shank was not restored in any treated case. It appears that, if suitably administered, vitamin A can make the limb regeneration blastema of amphibians completely equivalent to the original limb bud, probably by intensifying dedifferentiation of its cells. It is suggested that this chemical can be a useful tool to investigate the biochemical and genetic changes which occur during dedifferentiation and also whether through this process differentiated cells can really revert to a pluripotent state.

Key words: Amphibia – Anura – *Bufo* tadpoles – Limb regeneration – Vitamin A

Introduction

During limb regeneration in amphibians only the distal part actually removed by amputation is restored. This fact has been formalized into the rule of distal transformation of the blastema (Rose 1962). According to this rule the regeneration blastema never differentiates into more structures than are distal to its own level of origin along the proximodistal axis of the limb. The validity of this rule has been confirmed by a large number of studies. In the more recent studies on regeneration in urodeles, the position of blastemas along the limb axis was deliberately shifted by transplanting distal-level blastemas to more proximal levels of the same or contralateral limbs and vice versa. In all of these cases the grafted blastema always also gave rise to structures only distal to its own level of origin along the limb axis (Stocum 1975a, b, 1981; Wallace 1981). Earlier, on the basis of observed differentiation of some proximal limb elements from wrist-level blastemas of axolotls grafted into eye orbits, de Both (1970) and Dinsmore (1974) concluded that under certain conditions (increased mesenchymal mass) blastema can form more than distal structures, but their evidence has been seriously disputed (Stocum 1975a; Wallace 1981).

However, Faber (1976) postulated that under certain conditions it would not be surprising to find differentiation of even the shoulder girdle in regenerates developed from distal-level blastemas. He also had in mind the necessity of an increased amount of mesenchyme for such proximalization of the limits of structures differentiating from the blastema. That this indeed is possible has been demonstrated recently, although by use of a different procedure. By immersing frog and toad tadpoles (Niazi and Saxena 1978; Jangir and Niazi 1978; Jangir 1979; Sharma 1982; Alam 1983; Niazi 1983) and axolotls (Maden 1982) for some time after amputation, limb regenerates have been obtained that consisted of parts distal as well as proximal to the amputation level. Many of these regenerates developing in situ from limb stumps were complete limbs containing skeletal elements from girdle to phalanges in their normal sequence. In many anuran tadpoles more than one such whole limb regenerated from the same stump. It has been suggested that this radical change in the developmental capacity of the blastema might be due to greatly intensified dedifferentiation of the blastema cells caused by vitamin-A action. Treatment of tadpoles with this vitamin after limb amputation has been observed to increase the destruction of the stump tissues at and near the wound surface, but the integrity of tissues of more proximal limb parts did not seem to be affected. It is, therefore, suggested that the specific effect of vitamin A on the pattern of regenerates may possibly be due to a direct action of this chemical on the stump tissues exposed to it through the amputation wound and on the blastema cells originating from them (Saxena and Niazi 1977; Jangir and Niazi 1978; Sharma 1962). We report here that treatment with vitamin A *only after* amputation and direct contact of exposed mesodermal tissues of the stump in the vicinity of the cut end with

this chemical is not an essential prerequisite for the change in pattern of regenerates observed in the amphibians treated in this way.

Materials and methods

The experiments were performed on tadpoles of the common Indian toad, *Bufo melanostictus* (Schneider). At the start of the experiments they were at stages 30/31 and 34 according to the normal table for this species (Khan 1965). At stage 30/31 the tiny hind limbs are at the paddle stage, with a knee bend marking the boundary between the stylopodial and zeugopodial segments. In tadpoles of stage 34 all three limb segments (stylo-, zeugo- and autopodia) are clearly demarcated and rudiments of all five toes are separated from each other by indentations. One group of each stage was reared in water throughout the 18 days of the experiment. Two groups of each stage were immersed in a solution of 15 IU/ml vitamin-A palmitate, made by dissolving the oily preparation Arovit (Roche, India) in a small quantity of ethanol and then diluting it with an appropriate quantity of water. Three days after their sojourn in this solution these tadpoles were narcotized in 1:4000 aqueous solution of MS 222 (Sandoz) and their left hind limbs were amputated through the shank. Following this operation one group of each stage was reared in water for the next 15 days and the other two groups were placed in the vitamin solution for another 3 days before also being transferred to water for the remaining period. Left hind limbs of control tadpoles were similarly amputated on the same day as the treated ones. The tadpoles were reared in large bowls at room temperature (30°–32° C), their medium was changed every day and they were fed boiled spinach leaves.

On the 15th day after amputation the tadpoles were fixed in Bouin's solution, their removed limbs severed, and many of them were stained in toto with victoria blue according to the method of Bryant and Iten (1974). A few were sectioned and those which could not be photographed properly were drawn with the help of a camera lucida.

Results

Controls

Regeneration occurred in all tadpoles, both young and older ones. All regenerates were well formed with about half of them possessing all five toes and the others lacking one or two toes. As expected all regenerates were of the normal type, i.e. only the part of the limb that had actually been removed, distal to the amputation level, had been restored (Table 1; Fig. 1).

Tadpoles treated

Regeneration also occurred in all cases of the four groups of tadpoles treated. However, in 35% of tadpoles given this treatment only before amputation and in more than 80% of those exposed to vitamin A after amputation, regeneration did not progress beyond the blastema stage. Some of these persistent blastema-like regenerates were sectioned and were found to contain only undifferentiated mesenchyme. In the remaining cases regenerates attained various degrees of morphogenesis, differentiation and growth.

Treatment only prior to amputation. In 13 of 40 both young (stage 30/31) and older (stage 34) tadpoles regenerates had persisted as blastema-like outgrowths. In 7 of these one and in the other 6 two such regenerates had developed from each stump (Table 1; Figs. 2, 3). In the remaining 27 cases regenerates had developed better, although in a few differentiation and growth were poor. However, structurally they were fundamentally different from the control regenerates. In at least external morphology each of them appeared to be a whole limb consisting of stylopodium, zeugopodium and autopodium, even if skeletogenesis in these segments was poor or had not occurred at all. Therefore, in Table 1 they are all included in the column of whole-limb regenerates. According to their proximodistal organization and the degree of differentiation and growth attained, they appeared to be of four broad types:

1. In 15 cases (10 young and 5 older tadpoles) regenerates were well formed and had grown almost as well as the controls. Each was a complete limb with stylopodial, zeugopodial and autopodial segments. In 3 of these cases multiple regeneration had occurred, with the development of two whole limbs from each stump (Table 1). The regenerates contained all the skeletal elements from girdle to phalanges (Figs. 4, 5). In many cases, however, the girdle element was too poorly differentiated to stain well with victoria blue; in 6 cases this element was represented only by a mass of almost undifferentiated mesenchyme, surrounding the head of the femur of the regenerate (Fig. 6).

2. In 4 cases (2 young and 2 older tadpoles) the girdle element was absent, but each regenerate consisted of all three limb segments and contained a complete femur or

Table 1. Hind limb regeneration in *Bufo melanostictus* tadpoles treated with 15 IU/ml vitamin-A palmitate prior to or both prior to and after amputation through the shank

Stage of tadpoles at start	Treatment	Limbs amputated (no.)	Number of different types of regenerates				
			Normal	Whole limb		Blastema	
				Single	Double	Single	Double
30/31	Nil (controls)	20	20				
	3 days prior	25		17	2	3	3
	3 days prior + 3 days post	15		3		8	4
34	Nil (controls)	20	20				
	3 days prior	15		7	1	4	3
	3 days prior + 3 days post	15		2		7	6

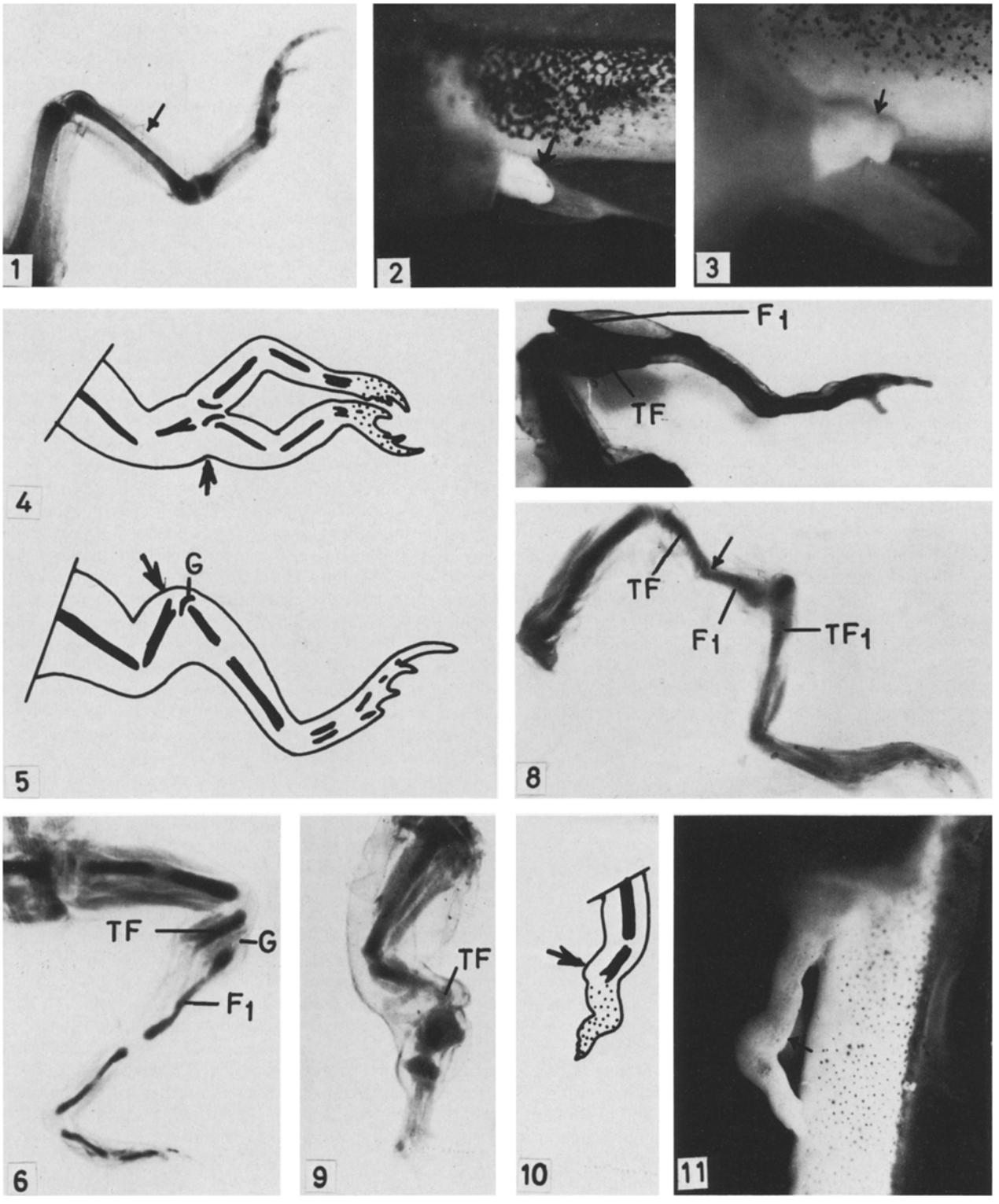

Fig. 1. A control regenerate

Figs. 2–10. Limb regenerates of tadpoles treated with vitamin A for 3 days, only prior to amputation through the shank. **Figs. 2, 3.** Inhibited persistent blastemas. **Figs. 4–10.** Whole limb regenerates. Distal, removed part of shank stump and of tibia-fibula (TF) was not restored. **Figs. 4, 5.** Camera lucida drawings of two whole limbs regenerated from one stump (**Fig. 4**) and of a single such regenerate (**Fig. 5**). Regenerates contained entire limb skeleton including a new girdle (G). **Fig. 6.** Regenerating girdle (G) represented by undifferentiated mesenchyme around head of new femur (F_1). **Fig. 7.** Regenerate containing a complete new femur (F_1) and the more distal elements, but no girdle. **Fig. 8.** Regenerate containing distal half of femur (F1), complete new tibia-fibula (TF_1) and autopodial elements. **Fig. 9.** A malformed whole-limb regenerate with unidentifiable cartilages. **Fig. 10.** Camera lucida drawing of a rudimentary whole-limb regenerate containing undifferentiated mesenchyme

Fig. 11. A whole limb regenerate of a tadpole treated with vitamin A for 3 days before and 3 days after amputation through the shank. (*Arrows* indicate approximate level of amputation)

part of a femur, followed by all of the more distal elements (Figs. 7, 8).

3. Four regenerates (3 of young tadpoles and 1 of an older tadpole) were poorly developed. They did appear as whole limbs in external morphology, but contained a series of badly formed cartilages, which could not be identified with any certainty (Fig. 9).

4. The remaining 4 regenerates (3 of young tadpoles and 1 of an older tadpole) were very rudimentary, appearing to be divisible into stylo-, zeugo- and autopodial segments, but containing only undifferentiated mesenchyme (Fig. 10).

Treatment prior to and post-amputation. Of a total of 30 cases, 25 regenerates had persisted as blastema-like outgrowths, and in 10 of them 2 such regenerates had developed from each stump (Table 1). The remaining 5 regenerates were definitely recognizable whole limbs, but had not grown well. Two of these appeared to contain skeletal elements from girdle to phalanges, still insufficiently differentiated to stain well with victoria blue. One of these is shown in Fig. 11. Histogenesis in the other 3 was even poorer, so that in toto staining did not reveal the presence of any cartilage in the regenerated portion at all.

The regenerating limbs of treated tadpoles also differed from those of controls in certain other significant features:

1. The distal part of the original shank segment removed by amputation was not restored during regeneration in any case. This can be seen in Figs. 4–6 and 8–10, which show that the stump tibia and fibula had remained incomplete and were not continuous with the skeleton of the regenerates.

2. The regenerates did not always develop from the exact distal end of the stump. Some of them were seen to have grown out from some distance proximal to the amputation level and lateral to the stump skeleton (see Figs. 6, 7).

3. In most cases the anterior-posterior and dorsal-ventral axes of the regenerates did not appear to coincide with those of the stump.

Discussion

The nature of the effect of vitamin A, which causes the observed specific change in the pattern of limb regeneration in amphibians, is not yet understood. However, it can now be stated with certainty that this effect is not restricted locally to the stump tissues and blastema cells originating from them at and near the amputation surface and exposed to this chemical directly through the open wound. Immersion of tadpoles in vitamin-A solution only prior to amputation is also found to result in proximalization of the structural limit of limb regenerates developing from shank stumps in 76% of young and 54% of the older tadpoles. Obviously, the changed pattern of regenerates resulting from amputation of these pretreated limbs must have been due to the effect of the vitamin, absorbed through the skin or the digestive tract or both, accumulating and retained in the tissues of the intact limbs. The vitamin retained in the tissues may have exerted its specific influence on the stump tissues only after these pretreated limbs were amputated, so initiating regenerative processes; or, it may be speculated that the vitamin might have already altered the state of morphological and cytochemical differentiation of tissues of the intact limbs in some manner, so that the cells released from them after amputation could give rise to blastema with increased morphogenetic potencies, capable of differentiating into limb parts not only distal but also proximal to its level of origin.

It has been suggested that proximalization of the limits of developmental capacity of blastema manifested in the production of whole-limb regenerates in anuran tadpoles may be due to enhanced dedifferentiation of its cells and neutralization of any regulatory influence emanating from the limb stump (Niazi and Saxena 1978; Jangir and Niazi 1978; Jangir 1979; Sharma 1982; Alam 1983). Alteration of positional information assumed to be encoded in the surface of blastema cells by vitamin-A action is also indicated as a possibility, although only in a speculative manner (Maden 1982). Vitamin A is important in cell differentiation and organogenesis and is known to cause a variety of effects on tissues and cells in vivo and in vitro. These include dissolution of cartilage and bone, reversion of chondrocytes to mesenchymal morphology, changes in the synthesis of proteoglycans and cell-surface glycoproteins, labilization of cellular, mitochondrial and lysosomal membranes and changes in their permeability, increased production and release of hydrolases, appearance of phagosomes, enhancement of mitotic activity, alteration in the synthetic pathways of differentiated epithalial cells, promotion of growth factor receptors on the surface of epidermal cells etc. (Deluca and Zila 1975; Dhoually and Hardy 1978; Vasan 1981; Wiley et al. 1983; Jetten 1980; reviews: Fell and Rinaldini 1965; Roels 1969). It is not possible at present to say which of these or other effects of vitamin A on limb tissues are involved in changing the pattern of limb regeneration in amphibians in the characteristic manner.

It has been observed that vitamin-A treatment of tadpoles after amputation promotes blastema formation in all cases, but continued exposure beyond this stage inhibits postblastemic processes of redifferentiation and growth (Saxena and Niazi 1977; Jangir and Niazi 1978). In agreement with the known facts on the stage-dependent susceptibility of embryonic cells to teratogens, it was suggested by Jangir and Niazi (1978) that cells of the fully-formed blastema are at a developmental stage which is sensitive to the toxic action of vitamin A, and if exposed to it at that stage the blastema fails to differentiate and its cells may even become necrotic. In the present study 35% of the cases were of inhibited blastema-like regenerates even when no treatment at all was given after amputation. It seems that the inability of some blastemas of treated tadpoles to redifferentiate cannot be attributed to the inhibitory action of the vitamin, which becomes effective only after the blastema is formed. In this connection the recent findings of Vasan (1981) are noteworthy, who observed that not only the morphological appearance of the sternal chondrocytes of chick embryos cultured in retinoic acid containing medium was changed to mesenchymal form, but probably their synthetic processes were also irreversibly altered so that they failed to redifferentiate into cartilage when transferred to normal medium. It may be that in cases of persistent blastemas of treated tadpoles vitamin A may have altered the synthetic processes of blastema cells to such a degree that they became incapable of differentiating again.

Whatever the changes caused by vitamin A in tissues and cells which affect the pattern of regeneration may be, they are perhaps of a graded nature. Changes more radical

than those that usually occur during dedifferentiation in regenerating limbs may in some manner reactivate the entire set of pattern determinants (or genes) without irreversibly altering on damaging the synthetic machinery of cells. In such cases the blastema may acquire the capacity to form a complete limb with skeletal elements from girdle to phalanges. If the changes are slightly less radical, the regenerate may come to possess not all but at least some proximal parts. During the present study both these types of regenerates, structurally fully or partially proximalized, were obtained. Maden (1982) also reported that the extent of structural proximalization of limb regeneration in axolotls was correlated with intensity of treatment. If, however, the effect exerted by vitamin A is too drastic or the treatment too prolonged, the cells may become irreversibly altered or damaged and be unable to redifferentiate, or they may die. It may be recalled that in the groups treated for 6 days (3 days before and 3 days after amputation) the percentage of inhibited blastema-like regenerates was more than 80% as compared to 35% in tadpoles treated only before amputation. In the former, the extent of differentiation and growth attained by the few regenerates that attained some recognizable morphology was also of a significantly lower level.

During regeneration the distal portion of the amputated segment of the limb is restored. This part is developed from the basal region of the blastema. It was surprising that the distally removed portion of the shank, including that of its skeleton, was restored in none of the vitamin-treated cases. This was the case whether regenerates persisted as blastemas or grew into well-formed limbs. Also, the anterior-posterior and dorsal-ventral axes of treated regenerates did not always appear to coincide with those of the stump. These observations seem to support the rule of distal transformation of the blastema. It seems as if the blastema of vitamin-treated tadpoles was a completely autonomous structure, equivalent to the original limb bud "grafted" on the cut end of the shank stump, destined to develop according to its own potentialities without regard to what was present or lacking in the stump of the original limb proximal to it. Being destined to form a complete limb from girdle to toes, such a blastema would not be expected to form any structure proximal to it, and hence no restoration of the distal part of the shank which had been removed by amputation.

During the last decade, the cellular basis of the limitation of the developmental capacity of the blastema to differentiate into only distal structures in amphibian limb regeneration has been sought within the framework of the positional information theory (Wolpert 1971). It is assumed that there is a continuous graded series of positional values along the proximodistal axis of the whole limb, whose information is encoded in some manner in cells located along the axis. In regeneration the blastema cells arising from stump tissues at a given amputation level are supposed to remember the positional value of that particular level. It is further assumed that this memory of positional value of their level of origin serves as the basis for generating the positional values of all the more distal but for some unknown reason not of the proximal levels in the blastema cells, and therefore only distal parts are regenerated (Stocum 1975a, 1981; French et al. 1976; Faber 1976; Carlson 1983). The results of the present study suggest that vitamin-A treatment of at least young tadpoles of the toad, *Bufo melanostictus*, perhaps erases the memory of positional values in cells of even intact limbs prior to amputation, permitting development of a blastema at any level along the proximodistal axis capable of forming a complete limb in situ.

Dedifferentiation is a prerequisite for regeneration, but little is known about the nature of biochemical and genetic changes which take place in the blastema cells during this process. Vitamin A is known to alter the synthetic processes in the embryonic as well as differentiated cells and tissues. It is now also found that in amphibians vitamin-A treatment can increase the developmental capacity of the regeneration blastema formed on the amputated limb to the level of the original limb bud so that the blastema becomes capable of differentiating into a complete limb and also the girdle. The consistency with which this result can be obtained suggests that this chemical could be a reliable tool for investigating the nature of biochemical and genetic changes that occur in the blastema cells during dedifferentiation. It may also be useful in studies to resolve the controversial question as to whether differentiated cells can really dedifferentiate enough to revert to an embryonic or pluripotent state without suffering irreversible damage to their ability to redifferentiate.

Acknowledgement. The study was supported by grant no. F.23-1231/80 (SR-II) from the University Grants Commission of India.

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Received September 14 / Accepted November 15, 1983