STUDIES ON REGULATION OF NITRATE REDUCTASE INDUCTION IN GERMINATING RICE EMBRYOS

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PREFACE

The research work embodied in this dissertation has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi. The work is original and has not been submitted so far, in part or full, for any other degree or diploma of any University.

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* * *

INTRODUCTION AND PREVIOUS WORK

The phenomena of development and differentiation are shrouded in mystery. The various facets of this intensely interesting field are coming to view. The apparently conflicting observations from various developmental systems, and the resulting confusion, have added to a generalised appreciation of its fascinating complexity.

The problem of development is a problem of cell differentiation. The rationale for tracking down development is, therefore, to unravel the mechanisms of cell differentiation. One optimistically hopes that the latter process would be completely definable in molecular terms. Therefore the need for having a developmental system that may be easily amenable to analysis at the molecular level is so crucial. Inducible enzyme systems in developing plant embryos live up to our expectation in this respect.

This is due to the fact that the problem of cell differentiation is reducible to the problem of temporal control of gene expression. To study it, therefore, we should look at the behaviour of molecules, especially the enzyme systems, that show a temporal variation during differentiation under the influence of various experimental conditions. An ideal model system should be such that the variations of the enzyme system may be handled with the least influence on the developing organisms. Such a system may then be used as a handle to study the molecular processes that control its temporal variation.

The enzyme nitrate reductase, the first enzyme in the NO_3^--assimilatory pathway and inducible by its own substrate, provides a good system to work on in this field. The enzyme (molecular weight 500,000 to 600,000) present in the cytoplasm of higher plants requires NADH as the electron donor and contains FAD, cytochrome b557 and molybdenum as electron carriers as its integral components. It is not essential for germination and has been termed a "luxury enzyme".[1]

Attempts have been made earlier[2,3,4] to study the regulation of nitrate reductase in germinating embryos of Agrostemma githago, cotton (Gosypium hirsutum L.)[2] and barley[3]. However, events during early germination of these plants are not very well worked out. On the other hand, a considerable body of knowledge exists on the early germination events in wheat and rice. This is especially important because interpretation of behaviour of the enzyme system would be much aided by a knowledge of time course of various macromolecular events during the germination process.

Molecular Events During Germination Of Plant Embryos.

Germination of plant embryos is characterised by a rapid increase in polyribosome and rate of protein synthesis[5,6]. Protein synthesis is initiated within 15-20 minutes of imbibition when water content is as low as 30%[5,7,8]. Most of the proteins synthesised at this time are essential for germination[6]. In seeds imbibed for such short periods of time, all the conponents for in vitro protein synthesis can be isolated. But an interesting observation is that the protein synthesis occurring during imbition is insensitive to actinomycin-D[9,10]. As a matter of fact, germination of rice

embryos were shown to be independent of the synthesis of RNA up to 18-24 hours after the start of imbibition although RNA synthesis was defeeted as early as 9 hours. No new mRNA synthesis was observed during the early hours of germination in wheat. No increment in total RNA was observed during the first 12 hours of germination in Phaseolus Vulgaris, although radioactive precursors are incorporated at low level[5,9]. One recent report, however, demonstrates the occurrence of RNA synthesis as early as 30 minutes after imbition[bi] when the radioactive incorporation data were corrected for the intracellular pool size of the precursor molecules[12]. But the extent of new RNA synthesis detected during this period is very low compared to the amount of new protein synthesised.

A dramatic increase in RNA synthesis[13] at 12 hours of germination, as measured by incorporation of ^{3}H-adenosine into RNA when compensated for the change in ATP pool size, has been detected in Phaseolus spp. The rate of RNA synthesis is maximal at 14 hours, then declines by the 24th hour to about 60% of the maximal rate. The amount of RNA synthesised per axis also increases at 12 hours, reaching a maximum at 18 hour.

mRNA synthesis was supposed to commence from the begining of germination in wheat embryos after presoaking the seeds for 8 hours at 2° C[14]. In contrast, when they were germinated without presoaking, synthesis of rRNA was found to be the earliest transcriptional event[15].

In accordance with the second observation, and in contrast to the first, mRNA synthesis was detected after 12 hours of germination in rice [16]. On the basis of ^{32}P-labelled RNA isolated from germinating rice embryos and separated on SDS polyacrylamide gel, along with quantification of ribosomes and protein synthesis, sequence of events in rice embryo germination has been established, as follows [16].

protein	tRNA	rRNA	mRNA
0h ——— 0.5 h,	——— 6.0h	——— 12h	——— 24h
		DNA	maturation of ribosomes

The frustrating attempts to detect mRNA synthesis early in seed germination, and the resulting paradox, need not annoy us. Preformed mRNA in early germination has been reported from a number of laboratories [10,17-21].

Presence of conserved mRNA in wheat is suggested on the basis of observations that the RNA from ungerminated embryos can enhance amino acid incorporation in cell-free systems, that this RNA competes with labelled mRNA formed at later stages of germination on complimentary regions of wheat mRNA and that the inactive ribosomes of ungerminated embryos can be activated *in vitro*. An mRNA known to code for an enzyme appearing only during germination may be transcribed during embryogeny at a specific stage and be maintained at a translation-inhibited condition until germination [19,20,22]. The total RNA extracted from rye embryo and seeds of broad bean (*Vicia fava*), pea (*Pisum sativum*) and rape seed (*Brassica napus*)

exhibit low levels of template activity when incubated in wheat germ cell-free protein synthesizing system. RNA from pea, rape seed and rye embryos were fractionated by chromatography on oligo.dT cellulose columns[23]. Most of the template active RNA bound to the column at high ionic strength, indicating that it is polyadenylated. The remainder of the molecules would not bind to the column even when passed through it several times. The proteins synthesised *in vitro* from the template active RNA migrated as numerous bands in polyacrylamide gels and ranged in molecular weights from 10,000 to 70,000. The banding patterns obtained were quite different for the three species of seeds tested. It was concluded that the dry seeds contained a store of intact, long-lived, mRNA.

Since the stored mRNA in the dry seeds are not translated until defined periods in germination, either the mRNA may remain "masked", or the translational machinery may be inactive,or both the situations may be present simultaneously.

The ribonucleoprotein particles isolated from dry seeds can stimulate *in vitro* amino acid incorporation in experimental systems[24]. This observation supports the notion that either the translational machinary of ungerminated seeds are quiescent or that the stored mRNA in these systems are compartmentalised in the intact condition away from the translational system.

A membrane-associated messenger fraction from wheat germ separate from the ribosomal pellet has been isolated[24]. Only monoribosomes are present in dry wheat embryos and

these have no detectable amino acid incorporating acitivity[25]. The time-course of amino acid incorporation directed by messenger fraction occure with an initial lag. In contrast, kinetics of amino acid incorporation with polyribosome fraction isolated during germination, where mRNA and ribosomes are already attached, are linear from the onset of incubation. In addition, in contrast to the polysomal fraction, the messenger fraction needs ATP and a narrow concentration range of Mg ion. Finally, aurine tricarboxylic acid completely blocks messenger fraction activity. All these facts point to the inference that ribosome attachment is obligatory to messenger fraction function and that native messenger of ungerminated embryos probably occur unattached to ribosomes. Purified RNA isolated from the above messenger stimulates amino acid incorporation only to a limited extent, suggesting that other factors(proteins?) in addition to RNA are involved in the expression of template activity. Template activity, of course, is RNA-ase sensitive, but interestingly enough, it is also markedly sensitive to pronase and N-ethyl maleimide.

Initiation of protein synthesis in early phases of embryonic germination[7] is correlated with the rapid conversion of a considerable part of the monoribosome population into polyribosomes[26,8,27]. This event may be a consequence of

the mobilisation of preformed mRNA[6]. At any rate, the monosome to polysome conversion takes place in the absence of any transcription[7,25-28,18,9]. Use of cordycepin and αamanitin has shown that preformed mRNA has an important role to play in early germination and that polysome formation at this time is independent of both transcription and polyadenylation. Independence of polysome formation upon adenylation indicates that drawing on stored nuclear mRNA by polyadenylation is not involved. There is a little uncertainty in this experiment, which involves the measurement of relative incorporations of ^{3}H- uridine and ^{3}H-adenosine in the internal sequences of RNA in the presence or absence of cordycepin, because the difference in the relative pool sizes of the two precursors have not been taken into account. A direct evidence is at hand, however, which shows that about 60% of stored mRNA and about 36% of new mRNA are polyadenylated in cotton seeds during early germination.[55]

Summarising the above discussions, the following pertinent points emerge. The ungerminated embryos carry over from its earlier stages, a store of mRNA inactive in translation until a specified period in germination. A sharp rise in protein synthesis and a conversion of monosome to polysome are consequences of the presence of stored mRNA. New transcription, or polyadenylation of already formed

mRNA, are not involved in this. A rapid rise in protein synthesis leads to an onset of RNA synthesis in the germinating seeds and the first classes of RNA to be synthesised are rRNA and t RNA. mRNA for proteins that are detectable quite late in germination are already present in the embryo, and these are associated in dry seeds of wheat[30] with particles having sedimentation constants which are either higher (about 90S) or lower (about 45S) than that of the ribosomes(about 74S). Therefore, the stored mRNA may be associated with inactive ribosome precursors. Then comes the phase of the initiation of mRNA synthesis and a slow and steady increase in the appearance of new ribosomes.

α-Amanitin in 3 hour germinated embryos decreased the polysomal content and increased the monosomal fraction proportional to the concentration of the inhibitor[54]. Amount of active ribosomes completing a polypeptide chain also decreased proportionately. Such events showed a *pari passu* decrease in total cellular mRNA concentration. However, the amount of ribosome-associated mRNA decreased only marginally and, as such, cannot explain the extent of inhibition of protein synthesis and fall in polysomal content. Therefore it was concluded[54] that substrate level of mRNA is not a rate limiting determinant of protein synthesis. The same has been reported in *Xenopus*[59]. The results strongly suggest that newly synthesised mRNA may code for

a factor which activates the translation of preformed messengers and formation of polysomes.

Hardly anything is known about the mechanism of initiation of macromolecular synthesis at precisely timed sequences in germinating embryos. Some preliminary studies implicate the plant hormones, gibberellic acid and abscisic acid[31]. RNA synthesis was shown to be switched on only 12 hours after germination irrespective of the presence of these acids. But by the 24th hour, the total incorporation of ^{3}H-uridine was drastically reduced by abscisic acid, while markedly enhanced by gibberellic acid. On the other hand, a stimulation of ^{14}C-amino acids incorporation by gibberellic acid was noticed as early as 8 hours. There was no detectable influence of abscisic acid for the first 6 hours, but significant inhibition was noticed subsequently. Ribosomes isolated after six hours at various intervals from gibberellic acid treated embryos display 23-28% higher template activity whereas those from abscisic acid treated ones showed decreased activity from the untreated controls. It has also been shown from DNA-RNA hybridization experiments that newly synthesised ribosomes are not ~~newly~~ yet present at 12 hour and that the mRNA that are active at this period are not newly synthesised. Therefore the hormones may regulate the translation by a mechanism involving RNA synthesis[22]. This observation explains why abscisic

acid does not have any influence on germinating wheat embryo till about 6 hours. Such studies indicate the possible ways of attacking the problem of temporal control of macromolecular synthesis in germinating embryos, but clearly more studies are required.

Nitrate Reductase In Germinating Embryos

Nitrate reductase activity in germinating embryos may be induced with nitrate in a variety of plants such as cotton (Gossypium hirsutum), Agrostemma githago, sun flower (Helianthus annuyes), soybean (Glycine max) and barley. The enzyme is in no way specific to the germination period and may not be essential to the process.

Development of nitrate reductase in germinating cotton embryos has an early phase occurring in the dark[32]. This reaches a peak at about 24th hour of germination. This early rise of induced nitrate reductase activity decays and then shows a slow, light dependent, rise reaching a maximum at the third day of germination. An interesting observation is that the early phase of the enzyme activity is insensitive to actinomycin-D whereas both the phases are sensitive to cycloheximide. The early phase, where the activity is inducible at about the 12th hour of germination is reminiscent of the early burst in enzyme synthesis characterised by Ihle & Dure(1972).

Sunflower and soybean germinating embryos did not show the early phase of nitrate reductase activity[32], whereas

barley[4] and Agrostemma spp.[34] showed continuous levels of the enzyme after its induction in early germination. The induced nitrate reductase activity in both the systems is not inhibited by actinomycin-D.

Insensitivity towards actinomycin-D does not give us any new insight into the matter. First of all, one is never certain about the degree of effective-ness of the inhibitor towards RNA synthesis. In the cotton system, actinomycin-D has been shown to suppress mRNA synthesis by about 65%[35]. It is therefore quite possible that nitrate reductase mRNA escapes inhibition by actinomycin-D. Secondly, presence of preformed mRNA for germinating enzymes are well known and nitrate reductase may be another case at hand. There is, of course, the point that nitrate reductase is not essential to germination and as such, presence of its preformed mRNA is unexpected, if not unusual.

Interpretation of the action of actinomycin-D is complicated by the observation that means of applying the inhibitor affects its influence. Presoaked seeds behave differently from seeds germinated without presoaking[3]. An interesting finding is that actinomycin-D under these conditions frequently stimulated nitrate reductase activity by about 50% of the control[3].

In the absence of a direct demonstration of the existence of preformed mRNA of nitrate reductase in germinating embryos or a direct elimination of the possibility of already

synthesized nitrate reductase in seeds, nothing conclusive may be said. But the effect of cycloheximide (which completely blocks nitrate reductase activity) and the insensitivity towards actinomycin-D have been taken to be indications of the presence of preformed mRNA in translation inhibited condition[3]. This conclusion is strengthened by the fact that protein synthesis in the first three days of germination in cotton is not inhibited by actinomycin-D, in fact it is slightly stimulated[28,10]. Likewise, synthesis of carboxy peptidase-C (unique to germination) is not inhibited by acinomycin-D in early germination[19]. It was shown that actinomycin-D inhibited rRNA and tRNA synthesis in these systems by about 95% and mRNA by about 70%.

Nitrate reductase inducible by cytokinin in germinating embryos of Agrostemma spp. has been shown to be a direct influence on the de-novo synthesis of the enzyme by the cytokinin, rather than through an indirect induction via an increase in intracellular availability of NO_3^- ion[34,36]. It has also been shown that induction of this enzyme in excised embryos of Agrostemma either by NO_3^- or by benzyladenine(a cytokinin) is insensitive to actinomycin-D[3,37]. This suggests that the cytokinin may regulate the translation of nitrate reductase mRNA.

However, conclusive evidence of the effect of the cytokinin could not be demonstrated in cotton embryos[32].

Apart from the actinomycin-D paradox, a body of confusion exists in our understanding of the mechanism of induction of nitrate reductase by NO_3^- itself. The enzyme is also repressible by ammonium ion, an intermediate product of nitrate metabolism. Both induction as well as repression appear to require synthesis of RNA and protein in some systems[1,38,39,40]. The model for the mechanism of induction is almost always along the "operon" type of theory, in which NO_3^- is assumed to function as a coinducer and NH_4^+ as corepressor. But the levels of action of these two ions are far from established. One report is consistent with the promotion of transcription of nitrate reductase mRNA by NO_3^-[1], whereas another[41] in Neurospora shows that NO_3^- promotes translation of nitrate reductase mRNA. The latter workers also argue that NH_4^+ inhibits mRNA transcription.

Use of 6-methyl purine[42] gave a circumstantial evidence of NO_3^- promoting on the transcriptional level. On the other hand, rapid reversible inactivation by NH_4^+ ion does not seem to be dependent on RNA or protein synthesis[43]. However, the evidences are mainly based on inhibitor studies. Also, nothing is known about the levels of action of NO_3^- and NH_4^+ in germinating embryo.

The regulation of the enzyme level in vivo may be accomplished by regulation of the synthesis of the enzyme or

of its degradation. Several mechanisms may be involved in the rapid loss of enzyme activity in response to various conditions. One may postulate a specific or nonspecific degradation of an active enzyme. This will necessarily lead to an irreversible loss of the enzyme. In addition, an inhibitory protein binding to an active protein may lead to a reversible inactivation. Reports of an enzyme which irreversibly inactivates nitrate reductase are at hand[44-49]. Whatever the mechanism of regulation, the lower limit of the half life of nitrate reductase in higher plants has been estimated to be about 4.3 hours[50]. This estimate is on the basis of triple labelling with ^{15}N-, ^{14}C- and ^{3}H- amino acids and measurement of ~~density~~ density shift and incorporated radioactivity in the nitrate reductase band in isopycnic density gradients. However, individual experiments show half lives for corn leaves[51] as 4h, corn roots as 2-3h[1] and for barley leaves [52]as 9-12h. The turn-over rate of nitrate thus approaches that of the precursor amino acid pools. Therefore, the lower estimate of 4.3h is deemed preferable to the other values mentioned[50,53].

MATERIAL AND METHODS.

Plant Material: The seeds of rice (Oryza sativa var. Pusa 33) obtained from IARI, New Delhi, were first dehusked in a commercial dehusker and stored dry in a desiccator at room temperature. The seeds were washed thoroughly in tap water,

& followed by three rapid washes in distilled water. About 10 gm. of dehusked seeds were kept in dark for germination in about 20 ml of appropriate medium in a petri dish at 25° C in a BOD incubator for the required period of time at 50% relative humidity.

For radioactive work, the embryos were excised manually from the seeds soaked in chilled water and blotted dry. These were stored at -20° C until use in stoppered vials and each batch was used within 5 days of excision. Fifteen embryos were placed per 5ml petridish and processed as before.// Isolation of Embryos. For enzymatic ~~see~~ studies, the embryos were isolated in bulk by floatation in 70% (w/v) sucrose according to a method modified from Johnston and Stern (1957)[60]. About 10gm of seeds were blended with crushed ice in a waring blendor for exactly 10 seconds. The crushed pieces were washed with cold distilled water through a mesh and the resulting meterial on the mesh were dumped on to the cold 70% (w/v) sucrose solution. Whatever material floated on, were collected again on the mesh. About 70% of these were broken piece of embryos along with some endosperm contaminants. This method produced about 30% yield on weight basis.

For measurement of Km, the germinted seeds were chilled over ice and the embryos were excised manually in order to reduce endosperm contamination to a minimum. All the operations were carried out at 4°C .

Enzyme Extraction: The embryos were blotted dry and ground in 2 ml of extraction buffer (0.1 M Tris-HCl, pH8, 5 at 4°C)

with a mortar and pestle at 4°C in the cold room. The extract was centrifuged at 15,000 rpm for 15 minutes in K-24 refrigerated high speed centrifuge at 0-4°C temperature. The supernatant was used for enzyme assay.

Enzyme Assay: For induction studies, the enzyme was assayed according to the procedure of Hagenan and Hucklesby (1971)[61] with some modifications. The reaction mixture contained 1.0 ml of 0.1 M Potassium Phosphate buffer ($_pH$7.5), 0.2 ml of 0.1 M KNO3 and 0.2 ml of 2.0 mM NADH (freshly prepared). NADH was replaced by distilled water in the control. The reaction was started by the addition of 0.2 ml of the crude enzyme extract. The reaction ~~we~~ mixture was incubated at 30°C in a water bath for 30 minutes, at the end of which the reaction was stopped by the addition of 0.1 ml of 1.0 M Zinc acetate. The precipitate obtained was removed by centrifugation either in a Remi table top centrifuge(model T-8) at maximum speed or in K-24 at 7,000 rpm for 5 minutes. To the supernatant, 1 ml of sulphanilamide (1% in 1.5N HCl) and 1.0 ml of 0.02% NED were added. This resulted in appearance of pink colour. Absorbance reading was taken after allowing 15 minutes for full colour development. Optical density of each tube was taken against the respective control at 540 nm using Bausch and Lomb Spectronic 20 spectrophotometer. The amount of nitrite was estimated from the standard curve (Fig. 1).

A Standard Curve for NO_2^- Estimation

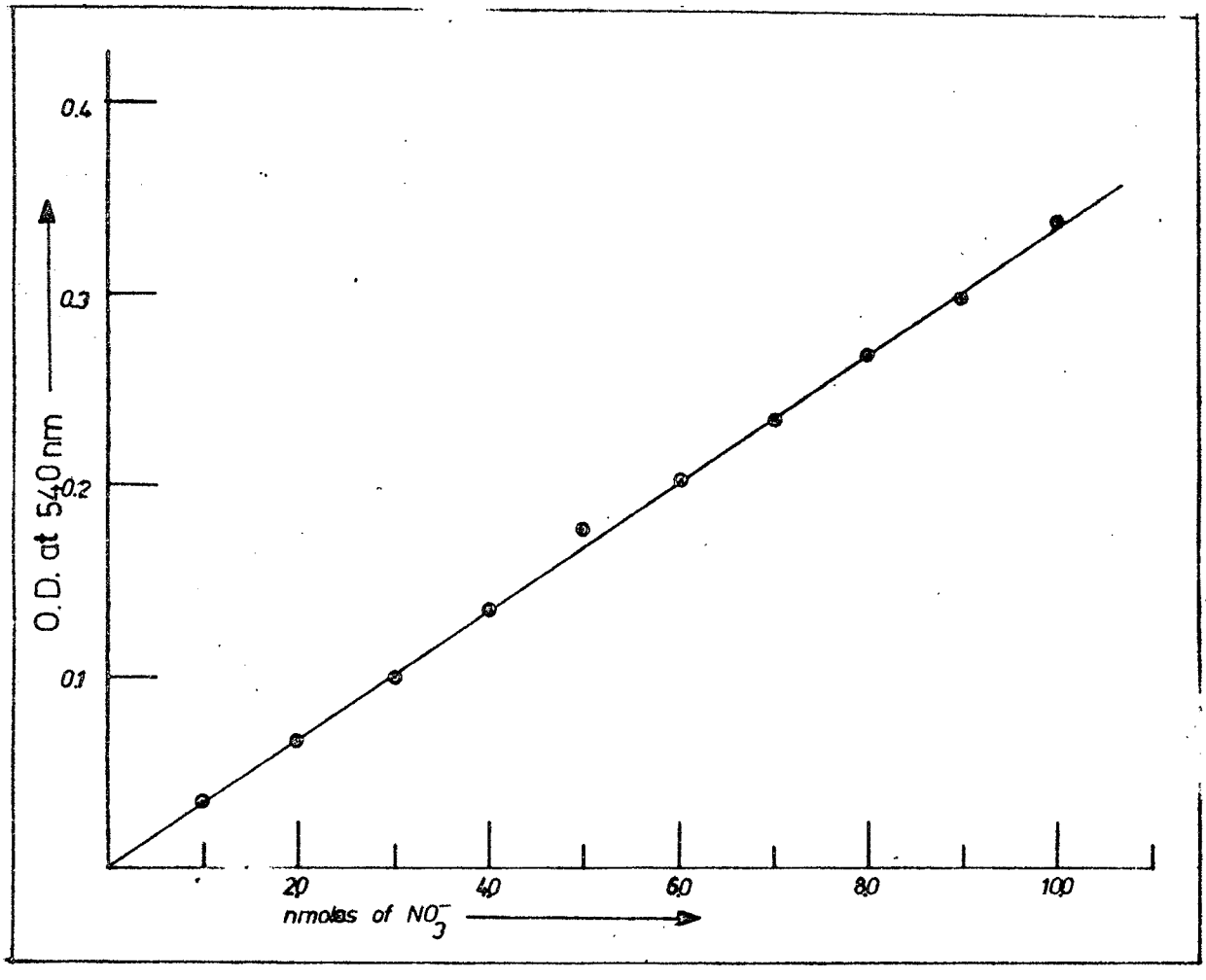

Fig.1

For measurement of Km, direct monitoring of the disappearance of NADH was carried out at 340 nm in a Gilford model 250 spectrophotometer. The control was without KNO_3 in order to account for nitrate independent NADH-oxidoreductases that may be present in the crude extract. The temperature of the 1.0 cm light path cuvette was maintained at 30°C and the reaction was started therein by the addition of NADH. Decrease in absorbance with time at 340 nm was followed and recorded in the chart recorder and three samples were handled simultaneously by means of analogue multiplexer unit. Under such conditions, the method is not very sensitive. Therefore high sensitivity region of the absorbance scale was employed which gave a considerable instrumental noise. Individual points for the first 5 minutes in the record were therefore analysed by the least square method of curve fitting and the slope of the best fit straight line was calculated theoretically. This gave the velocity of the reaction in arbitrary units of absorbance per unit of time.

Protein Estimation Protein was estimated by the procedure of Lowry et al (1951)[62] as follows: 0.2 ml of cold 10% TCA was added to the crude extract (0.2 ml) to precipitate the protein. This was kept at 4° C overnight, at the end of which the precipitate was centrifuged, the supernatant discarded and the former dissolved in 1.0 ml of 1.0N NaOH. From this solution, 0.2 ml was added to 2.5 ml of solution A (containing 10 parts of 2% Na_2CO_3 in 0.1 N NaOH, 0.1 part of 1% $CuSO_4$, 0.1 part of 2% Na-K-tarterate) and mixed throughly. After 15 minutes, 0.2 ml of 1:1 diluted Folin's reagent was added shaken well and kept at room temperature for 20 minutes. Absorbance reading was taken at 500 nm by using Bausch & Lomb

Standard Curve for Protein (BSA) Estimation

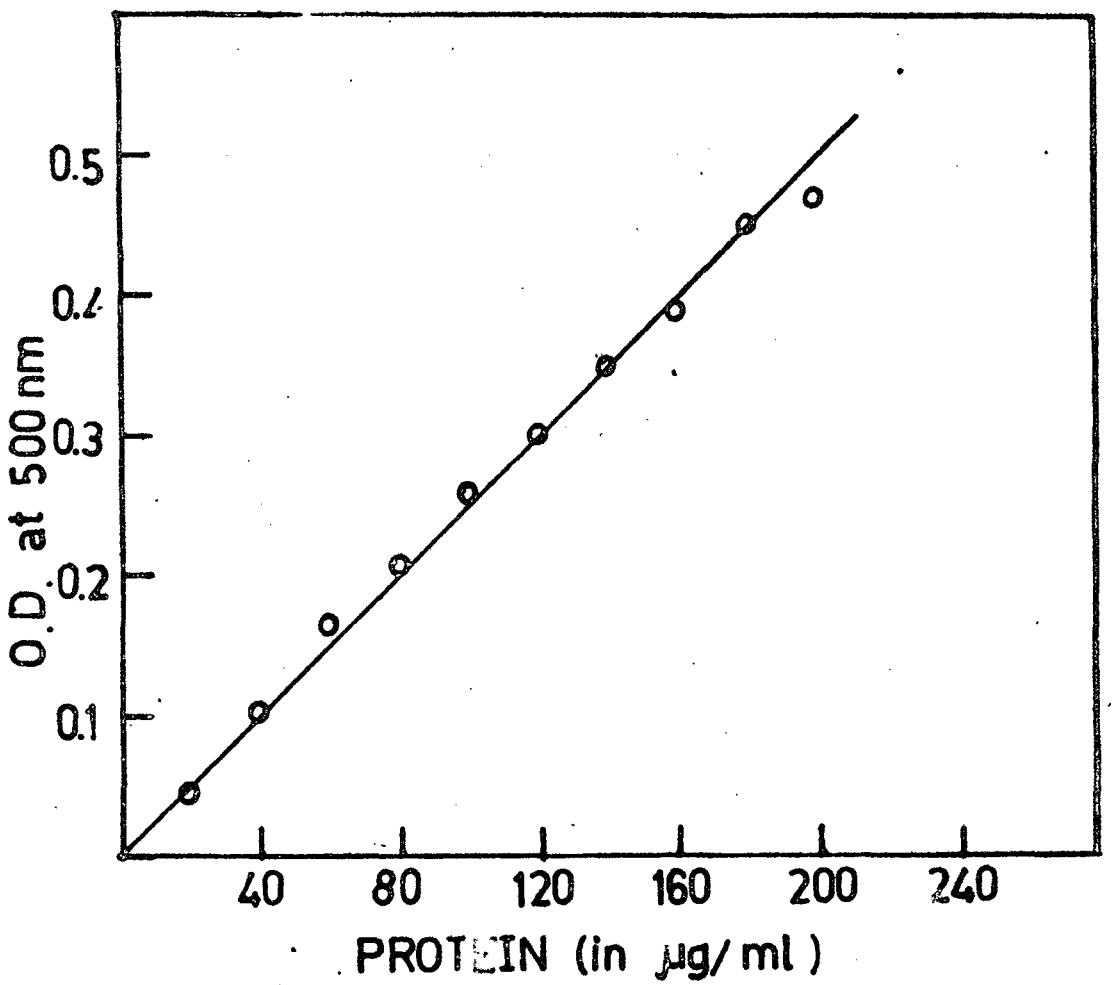

Fig.2

spectronic 20. The amount of protein was calculated from the standard curve obtained with BSA (Fig.2)

Enzyme Unit : The activity of nitrate reductase has been expressed as the amount of nitrite formed/hour/mg protein.

Calculation of Nitrate Reductase Activity: After having calculated the dilution factors and multiplying the observed concentration of nitrate and protein by their respective dilution factors, the specific activity was calculated as follows:

$$\text{Specific activity} = \frac{\text{Nanomoles of nitrite/hour}}{\text{microgram of protein}} \times 1000.$$

expressed in units of nanomoles of nitrite formed/hour/mg protein.

Radiolabelling Experiments: About 25-30 embryos, excised manually and stored at - 20°C in stoppered bottles, were placed in a 5 ml petri dish in 2.0 ml of Hepes buffer (0.1M, pH 7.0) containing 50 µg/ml of chloramphenicol in order to prevent bacterial growth. For single labelling, 10 µCi of ^{14}C - leucine (specific activity= 102 mCi/m mole) was added per petri dish and kept for the required period in the BOD incubator. For dual label studies, the embryos were set for germination for a specific period, 5.0 µCi of ^{14}C- leucine was added and kept for eight hours. At the end of this period, the embryos were first rinsed with distilled water, followed by a wash with Hepes buffer (0.1 M, pH 7.0) containing 10 mM cold leucine and again with distilled water, blotted

dry, and placed in 0.5 ml of HEPES buffer of the same strength and pH . ^{3}H- leucine (specific activity=7,700- mCi/m mole) was then added to a final activity of 100 μCi/ml and it incubated for 30 minutes.

The embryos were then washed in cold distilled water, blotted dry, homogenised manually in a glass homogeniser with 2.5 ml of cold 5% TCA. The homogenised tissue was then kept in a boiling waterbath for 20 minutes to set free any radioactive leucyl-tRNA that may precipitated by TCA, then settled in ice. The precipitate was then washed through GF/C- filter in a millipore assembly under pressure, first with 5% TCA (cold) containing 10 m moles of nonradioactive leucine (10 ml), then with cold 10%TCA (25-30 ml) followed by 20 ml cold, distilled alcohol. The glass fibre discs were dried in an oven at 60° C overnight and counted in 8 ml of Toluene based scintillation fluid (0.4g PPO, 0.025g POPOP, 100 ml Toluene) by Packard liquid scintillation counter model 1650.

For single labelling, counting efficiency calculated by the channel ratio method was found to be 80% and,

$$d.p.m. = \frac{c.p.m.}{\text{Channel ratio}}$$

For dual labelling, following method was adopted:

a) ^{14}C- standard was counted in ^{3}H -channel (A) and in ^{14}C- channel (B) respectively.

b) The ratio, (cpm in channel A/cpm in channel B)=c, was calculated.

c) All multiply labelled samples were counted in both the channels.

d) ^{3}H- count was calculated as,

^{3}H-counts in cpm= cpm in ^{3}H- channel- x. (cpm in- ^{14}C- channel)

No correction for ^{14}C- counts in ^{14}C- channel was required as ^{3}H-spill-over in ^{14}C- channel was negligible.

Chemicals: KH_2PO_4, EDTA, EDTA, sulphanilamide, BSA, cycloheximide, chloramphenicol and HEPES were obtained from Sigma Chemicals, USA. Folin's reagent was obtained from V.P. Chest Institute, Delhi. Actinomycin-D was obtained from Calbiochem, USA. All other chemicals used were of analytical grade. 3H & 14C- leucine were obtained from BARC, Trombay.

RESULTS

Induction Kinetics Of Nitrate Reductase:

Embryos were germinated in petri plates either in water or in 0.02M KNO_3. After given periods of germination, the seeds were washed in chilled water and the embryos were harvested as described in Materials and Methods. Nitrate reductase was assayed and specific activity plotted against time (in hours) of germination counting from the time of first soaking of seeds. The results are presented in Fig3. Appreciable nitrate reductase activity was found only after about 16 hours of germination. The activity then rapidly rose to a maximum value within 12 hours. The maximum value obtained was quite uniform in almost all batches excepting one where it was quite

Kinetics of Nitrate Reductase Induction by NO_3 ions.

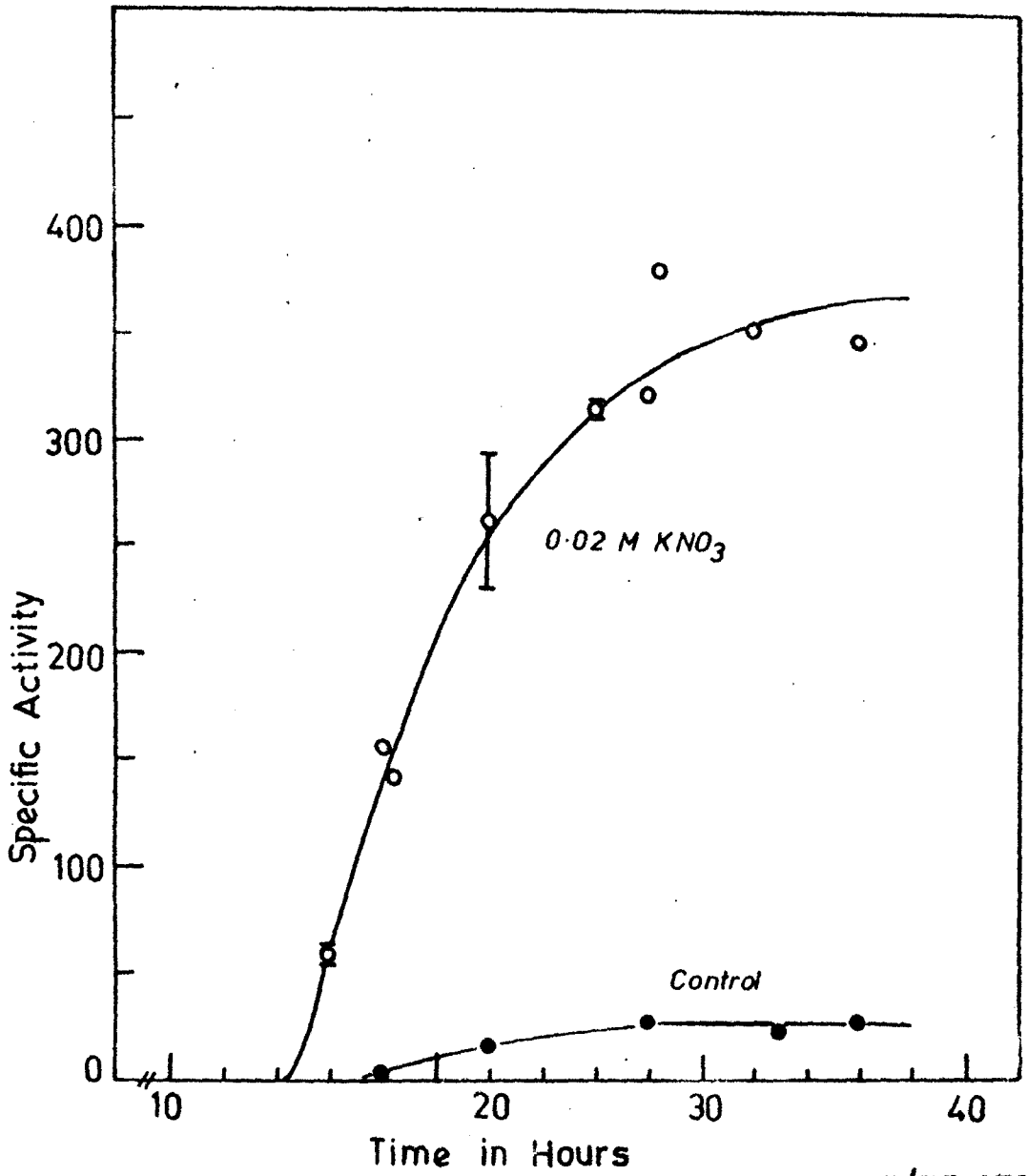

Fig. 3

Specific Activity expressed in nMoles of NO_2^-/mg protein/hr

Time denotes hours of germination after imbibition

low. This batch of seeds were rejected for further experiments. In one experiment, the level of the enzyme was noticed to drop by about 34% of the maximum after 33 hours of germination. But this drop was not repeatable. The enzyme activity after 36 hours of germination was ignored because considerable microbial growth was noticed after this period.

If the seeds were washed after 22 hours and reincubated in fresh medium, the increase in enzyme activity was observed to be only marginal.

EFFECT OF ACTINOMYCIN-D & CYCLOHEXIMIDE:

In order to test the possibility whether nitrate reductase activity required de novo protein and RNA synthesis, the seeds were germinated in 0.02 M KNO_3 containing either 15 µg/ml cycloheximide or 20 µg/ml actinomycin-D for 24 hours. The results are given in Table.I. As is evident, cycloheximide completely blocks the induction of the enzyme but actinomycin-D does not seem to have any effect at the concentration used.

KINETICS OF INDUCTION IN PRESENCE OF ACTINOMYCIN-D:

In order to rigorously verify whether actinomycin-D has no effect upon nitrate reductase activity in germinating rice embryos, the time course of induction was followed in presence of 0.02M KNO_3 and two concentrations of actinomycin-D (12 µg/ml and 24 µg/ml) The results are presented in Fig. 4. Most surprisingly, a uniform increase in nitrate reductase level was found in both the experiments above the control with 0.02 M NO_3^- only. The activity with actinomycin-D

Table I. EFFECT OF ACTINOMYCIN-D AND CYCLOHEXIMIDE ON NITRATE REDUCTASE ACTIVITY IN GERMINATING RICE EMBRYOS

Treatment	Specific Activity of NR at 24 h of germination (h^{-1} (in nmoles of NO_2 produced x h^{-1} x mg protein^{-1})
With 0.02 M KNO_3	307.13
With 0.02 M KNO_3 + 20 ug/ml Act-D	315.50
With 0.02 M KNO_3 15ug/ml Cycloheximide	35.07

The seeds were grown for 24 hours in either 20 µg/ml Actinomycin-D or in 15 µg/ml Cycloheximide, along with 0.02 M KNO_3 or without the inhibitors. Following this period, the embryos were isolated and nitrate reductase assayed. Specific activity expressed as nmoles NO_2^-/mg protein/Hr.

Effect of Actinomycin-D on the Induction of Nitrate Reductase

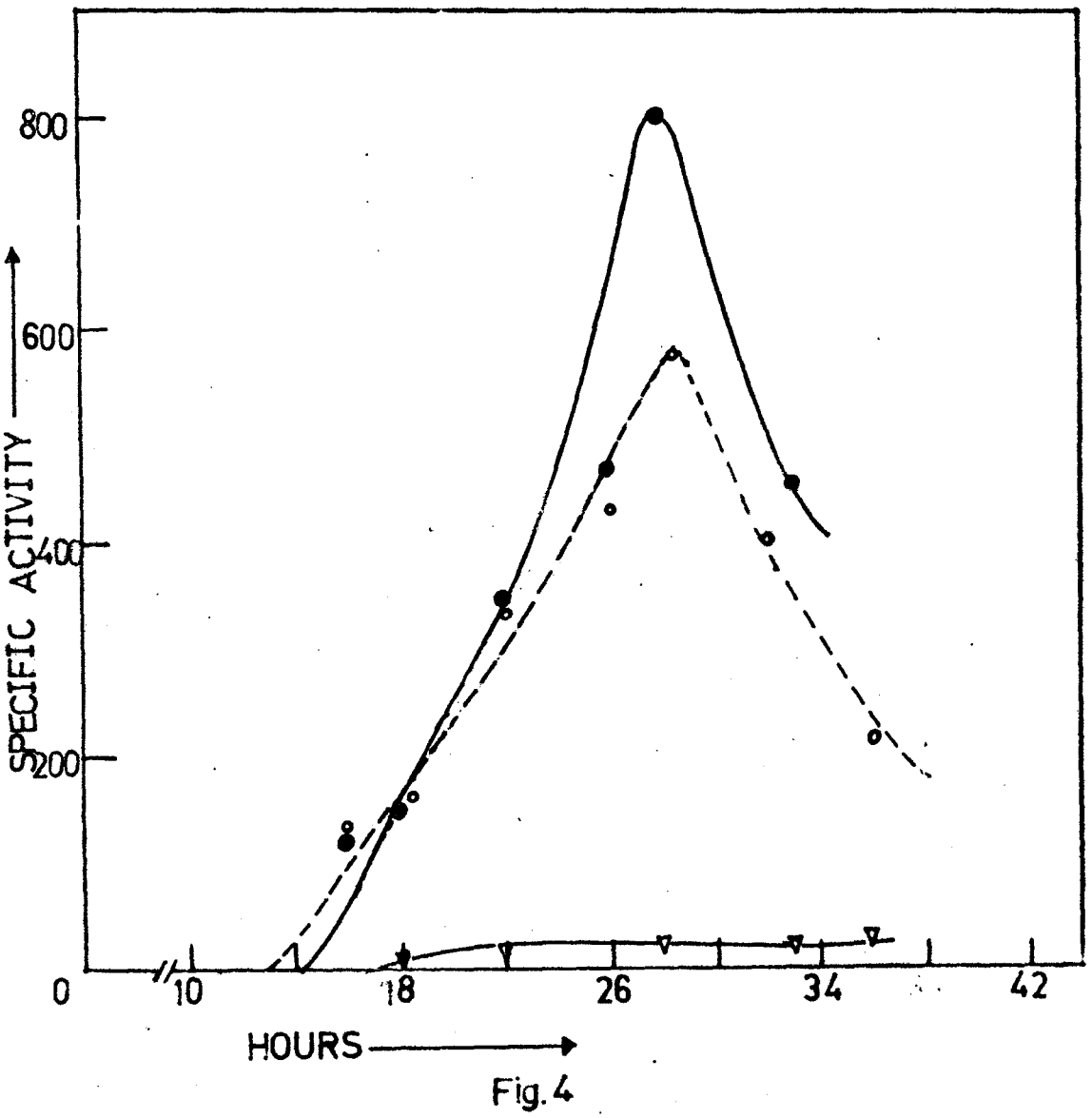

Fig. 4

—°—°— 12 µg/ml Actinomycin-D with 0.02 M KNO_3

—•—•— 24 µg/ml Actinomycin-D with 0.02 M KNO_3

—▽—▽— Control

Specific Activity expressed in nMoles NO_2^-/mg protein/hr

Time denotes hours of germination after imbibition

taken at about 20 hour of germination regardless of the concentration of actinomycin-D used, but rapidly fell to much lower values within 8 hours.

In order to test whether the peak activity could be delayed or shifted with respect to the hours of germination, the enzyme was first induced with only NO_3^- and then transferred to fresh induction medium containing actinomycin-D in addition to NO_3^- at the 20th hour. The results are presented in Table II. This experiment seems to be inconclusive. It seems that there is a slight rise in nitrate reductase activity 4 hours following transfer to fresh NO_3^- with 24 µg/ml actinomycin-D. However, it rapidly falls down afterwards. In the set having less actinomycin-D, no such peak was noticed, although a gradual rising tendency was obvious. In order to bring out the essential features of the effects of actinomycin-D on the induction of nitrate reductase, the percentage activity with respect to controls in NO_3^- only are plotted against time in Fig. 5.

MEASUREMENT OF RATE OF PROTEIN SYNTHESIS.

The paradoxical effects of actinomycin-D on nitrate reductase activity may be through an indirect effect on the overall rate of protein synthesis by direct modulation on amino pools. Therefore, the rate of protein synthesis at various times during germination, in presence and absence of actinomycin-D were worked out. The rationale behind the experiment is that if the embryos are labelled for long periods with ^{14}C-amino acids and then washed free, followed by a brief pulse with ^{3}H- amino acid, the ratio of tritium to ^{14}C

Table II. EFFECT OF TRANSFER TO ACTINOMYCIN-D ON THE INDUCTION OF NITRATE REDUCTASE

Hours after transfer to fresh medium with Act-D	Hours of Germination	With Act-D conc. 12.0 µg/ml		With Act-D conc. 14.0 µg/ml	
		Sp.Act	% Act.	Sp.Act.	% Act.
0.5	28.5	327.16	100	346.72	n100
4.0	32.0	364.15	111	459.60	132.55
7.0	35.0	443.88	135.67	204.91	59.09

The seeds were first germinated for different periods in 0.02 M KNO_3, then transferred to either a solution having 0.02 M KNO_3 and either 24 µg/ml or 12 µg/ml Actinomycin D. After growth in this period for specified hours, the embryos were isolated and the enzyme assayed.

Specific Activity expressed as nmoles NO_2/ mg protein/hour

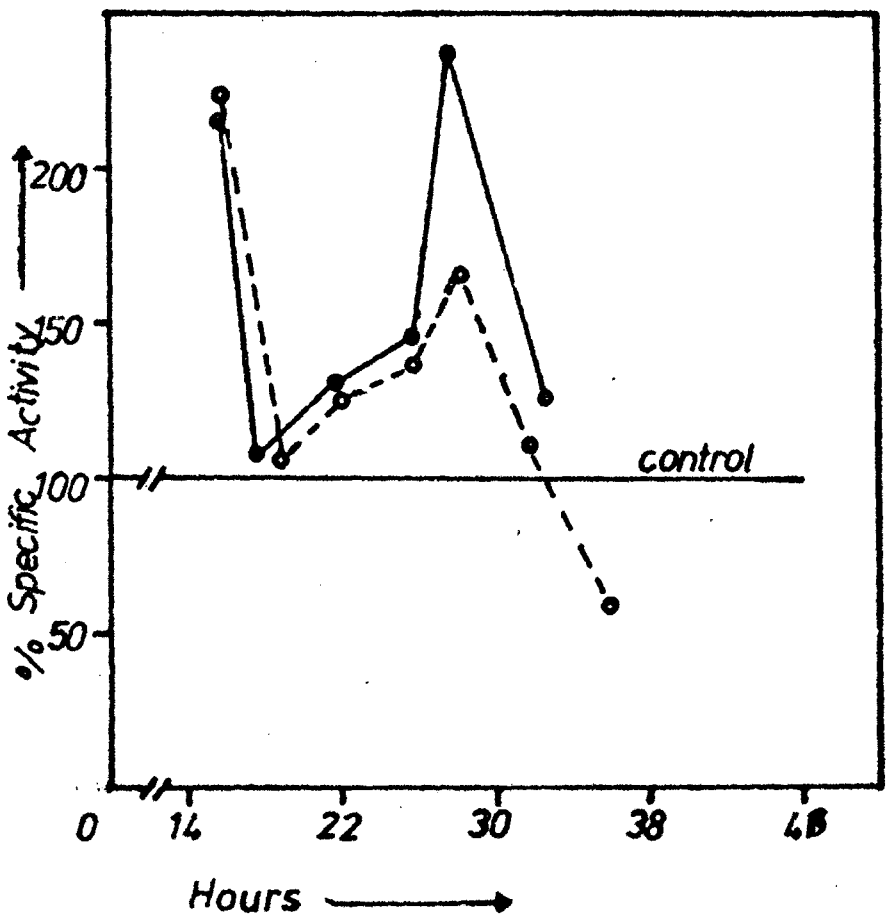

Fig. 5

Percentage Specific Activity of Nitrate Reductase from embryos treated with 12 µg/ml (—°—°—°—) and 24 µg/ml (• • •) -Actinomycin-D and 0.02 M KNO_3 with respect to those treated with 0.02 M KNO_3 only (control) . (Calculated from Figs. 3 and 4)

incorporated in TCA precipitable, heat resistant (to eliminate the possibility of counting any labelled amino acyl tRNA that may be formed) material will be a direct function of protein synthesis rate and an indirect function of the total mRNA pool size.

Uniform labelling time was established as ≥ 8 hours after labelling the embryos for different periods of time with ^{14}C-leucine after 18 hours of germination as described in the section on Materials & Methods. The results are presented in fig. 6.

The double labelling experiments were carried out as detailed in Materials & Methods. The results are given in Table III. In Table IV, the results are expressed as percentages of the controls.

DETERMINATION OF DECAY CONSTANT OF NITRATE REDUCTASE

Nitrate reductase was induced by 0.02M KNO_3 during germination. At the end of 24 hours, cycloheximide at a concentration of either 25.0 µg/ml or 50.0 µg/ml was added to the medium after washing the embryos free of KNO_3. Embryos were isolated at intervals after 15 minutes and specific activity of the enzyme determined. The results are plotted in Fig.7(a) as specific activity against time after addition of cycloheximide. For calculation of the decay constant, the results are plotted as log of specific activity versus time and the constant was calculated from the slope of the line devided by 2.303, assuming the decay to be a first order process (Fig.7)(b). Its value was found to be 0.79 hr^{-1}.

DETERMINATION OF Km:

Km of the enzyme was determined as described in the Materials & Methods. A plot of the velocity of the reaction (in arbitrary units of $\Delta OD.min^{-1}.10^{-1}$) versus substrate concentration in molarity of KNO_3 is presented in Fig.8 (a) for enzymes isolated from embryos germinating for 18

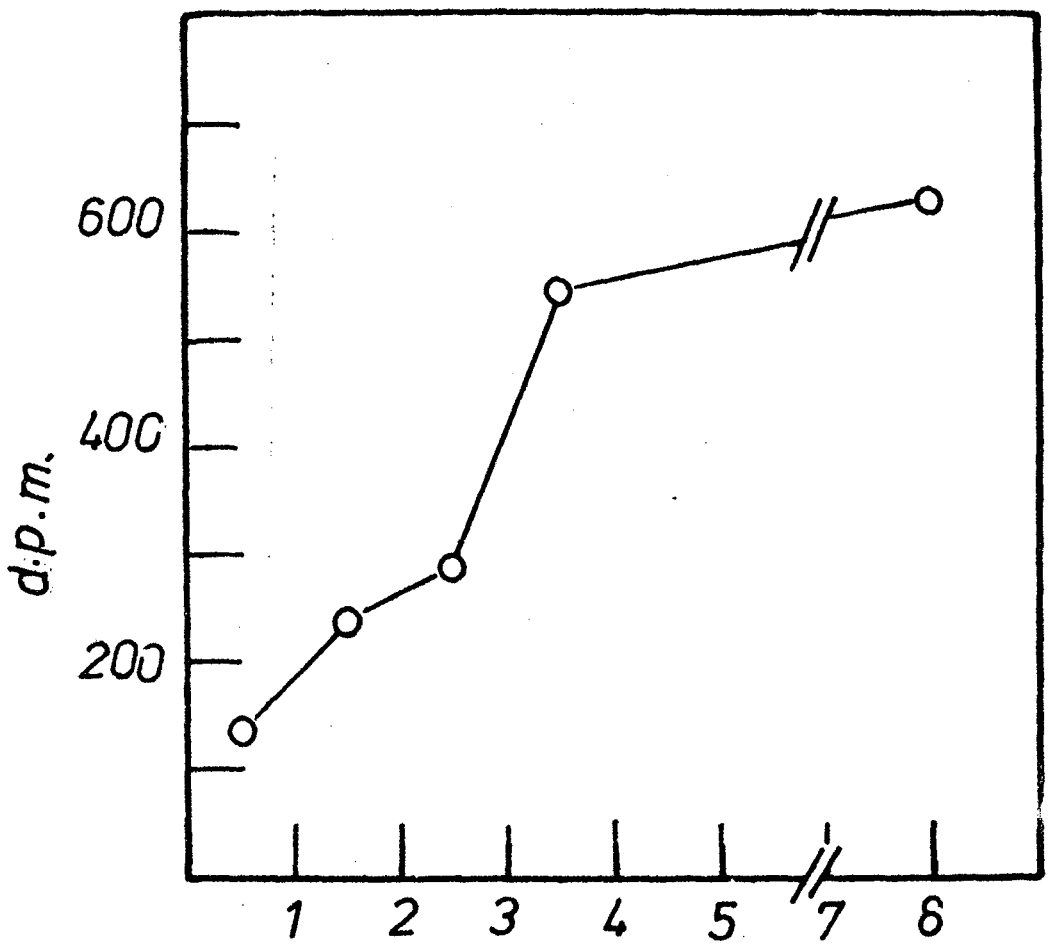

Fig. 6

Activity of ^{14}C-leucine (in dpm) in embryos plotted against time of incubation in ^{14}C-leucine.
0 hour denotes hour of germination after transfer to radioactive medium.

Table III. DETERMINATION OF THE RATE OF PROTEIN SYNTHESIS

Hours of germination	$^{3}H/^{14}C$ ration per 30 embryos incubated with		
	HEPES buffer (Control)	HEPES buffer with 0.02 M KNO_3	HEPES buffer 0.02 M KNO_3 & 12 µg/ml Act-D
20	0.49	0.135	0.225
25	0.66	0.942	1.126
30	1.10	1.30	1.80

Table IV

Hours of germination	$^{3}H/^{14}C$ ratio as percentage of control		
	Control	with KNO_3 only	with KNO_3 & Act-D
20	100	27.551	45.918
25	100	142.727	170.606
30	100	118.181	163.636

Isolated embryos grown in appropriate media for specified periods were transferred to fresh media with ^{14}C-leucine (5 µCi/µl) and grown for 8 hours. Following this period, the embryos were washed free of ^{14}C-leucine by washing with non-radioactive leucine and distilled water, then transferred to appropriate media co ntaining ^{3}H-leucine (100 mCi/µl) and grown for 30 minutes. At the end of this period, TCA precipitable radioactivity was counted as detailed in Materials and Methods.

Decay Curve of Nitrate Reductase

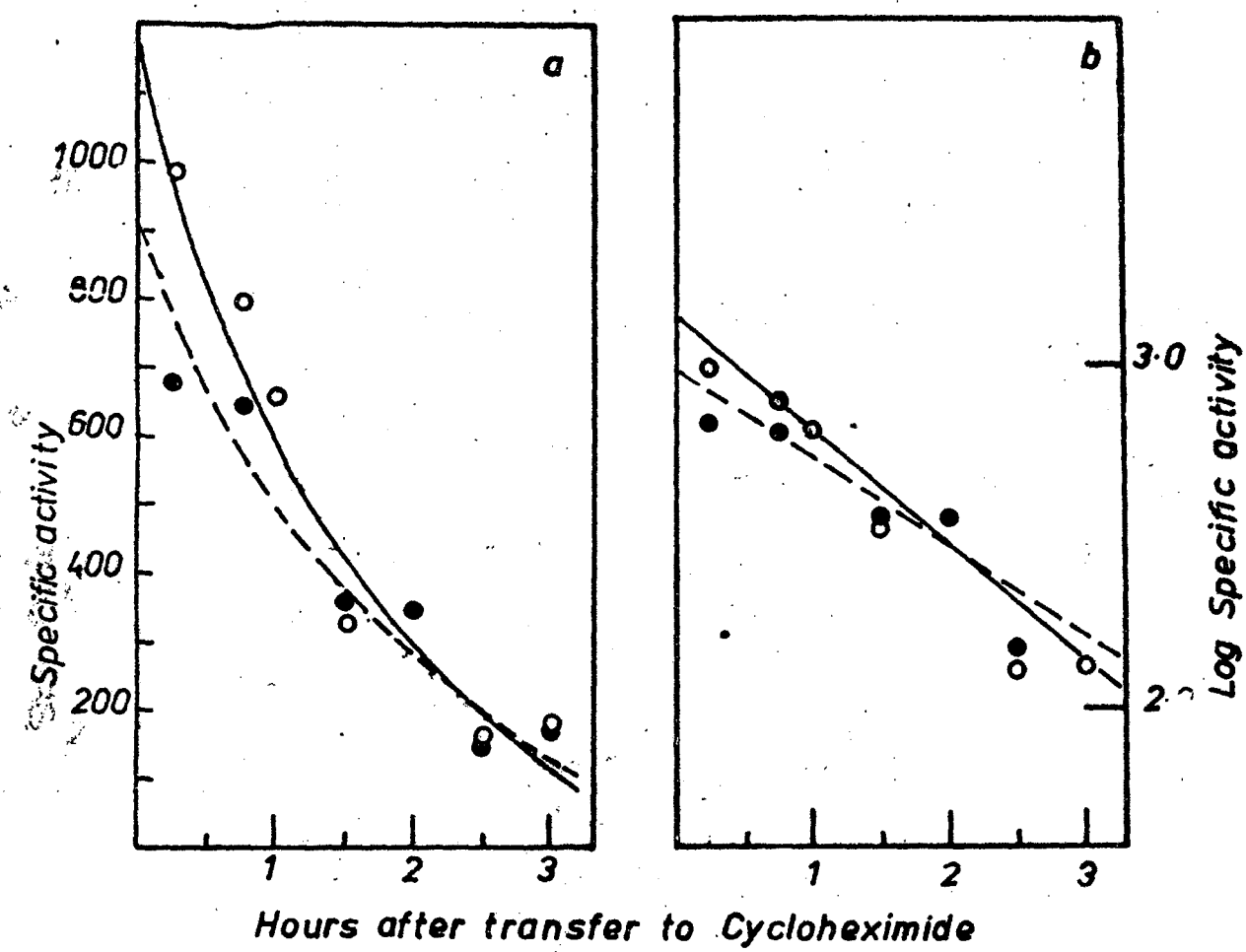

Fig. 7

Specific activity expressed as nMoles of NO_2^-/mg protein/hr
Time denotes hours after transfer to cycloheximide

—•—·—•— 25 µg/ml
———— 50 µg/ml

Determination of K_m of Nitrate Reductase

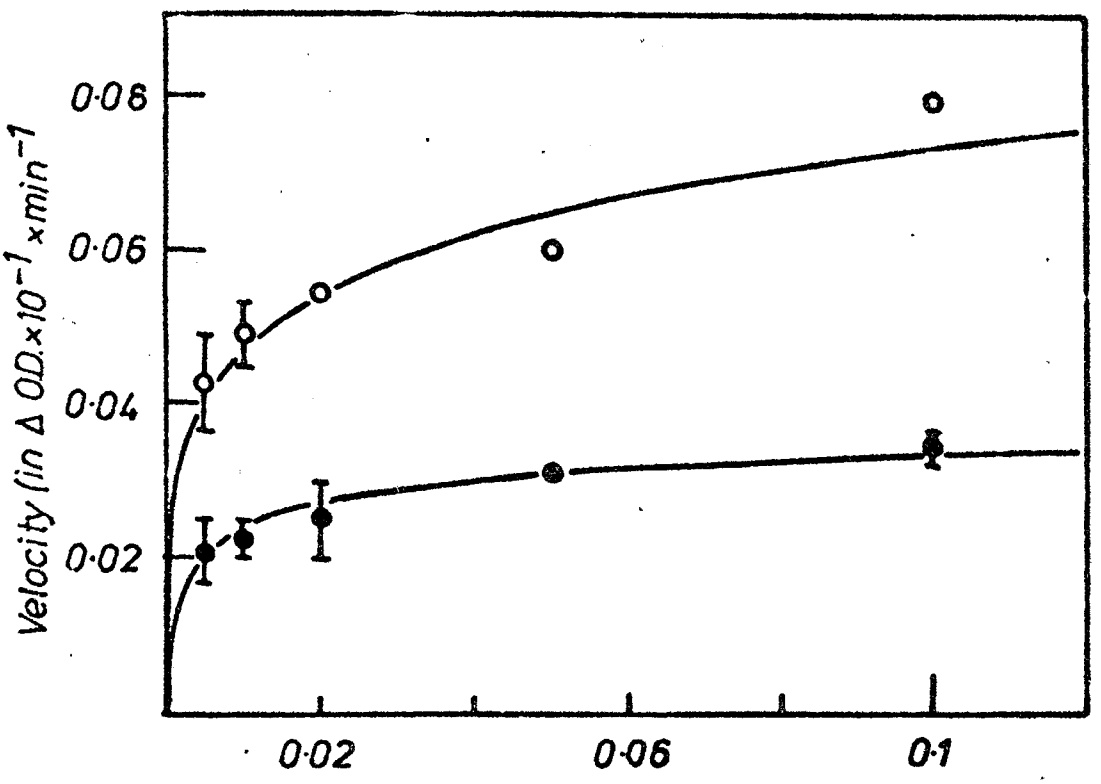

Fig.8a

25 hours

18 hours

hours and 25 hours respectively. Other details are presented in Tables V & VI. Km was calculated theoretically from the formula.

$$Km = (+)\frac{\text{Slope of the least square line}}{\text{Intercept of the least square line on Y-axis}}, \text{(in arbitrary units)}$$

which has been derived from MICHAELIS-MENTEN equation (See Appendix I for the derivation).

The Km of enzyme at 18 hour of germination (3.2924×10^{-3} arbitrary units) was found to be within 95% of that of the enzyme at 25 hours of germination (3.1343×10^{-3} arbitrary units). A Lineweaver-Burke plot of the data is given in Fig. 8(b).

DETERMINATION OF MICHAELIS-MENTEN CONSTANT (K_m)

Table V

Enzyme extracted after 18 hrs of germination

Substrate concn. in M KNO_3 (S)	Velocity (v) in ΔO.D x 10^{-7} x min Mean v	Standard deviation	1/v	1/S	Intercept of the least square line on 1/v axis	Slope of the least square line	K_m (in arbitrary unit)
0.005	0.0420	± 0.0007	23.6	200			
0.01	0.0496	± 0.0045	20.1	100			
0.02	0.0541	± -	18.5	50			
0.05	0.0599	± 0.0011	16.7	20			
0.10	0.0795	-	12.6	10			
					14.634	0.0482	3.2924×10^{-3}

Table VI Enzyme extracted after 25 hrs.

0.005	0.0205	± 0.0045	48.7	200			
0.01	0.0224	± 0.0024	44.5	100			
0.02	0.0251	± 0.0012	39.8	50			
0.05	0.0314	± 0.0009	31.8	20			
0.10	0.0344	± 0.0026	29.0	10			
					31.391	0.0984	3.1343×10^{-3}

Determination of K_m of Nitrate Reductase

(Lineweaver-Burke Plot)

● 25 hours

○ 18 hours

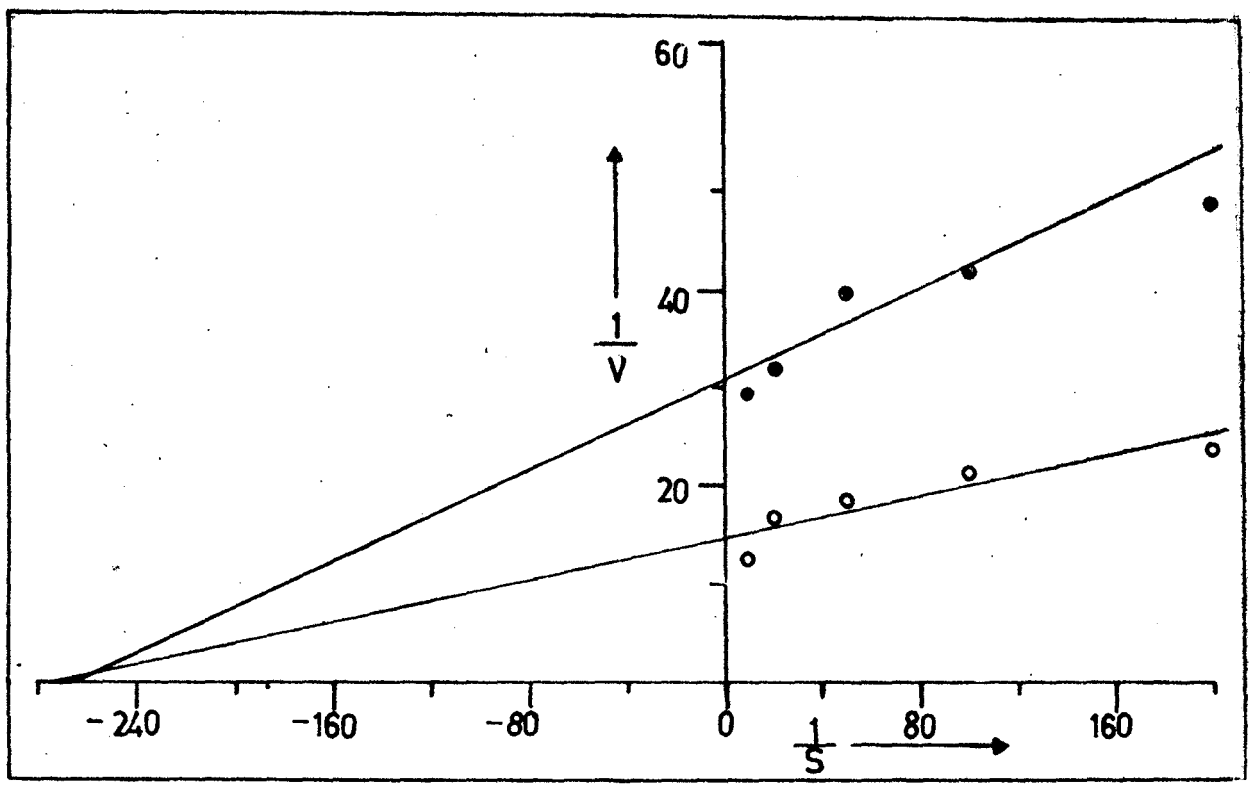

Fig.8b

DISCUSSIONS

Molecular events in germinating rice embryos have been well worked out[16]. New mRNA synthesis seems to commence only after 12 hours of germination. At about this time, new ribosomes also start maturing. Nevertheless, translation of proteins start within a few minutes of imbition and continue unabated throughout the period, rising in its rate continuously. The early translatory activity thus seems to be taking place on preformed mRNA. Some residual mRNA synthesis may also be occuring in the early period of germination which may escape inhibition by actinomycin-D. This possibility has never been rigorously excluded. In fact, this has been noticed as a rule in some recent works[54,55].

In the light of the above observations, it is natural to expect that an enzyme that is not required for germination should make its appearance only after about 12 hours of germination in rice. If it is inducible, it should be so only after this period. We also expect such an enzyme to be sensitive to inhibition by actinomycin-D.

The present study shows that nitrate reductase in germinating rice embryo is inducible by nitrate only after about 15 hours of germination (Fig.3). The enzyme activity reaches a plateau after about 28 hours.

But surprisingly enough, actinomycin-D did not have any inhibitory effect on the enzyme activity (Table I), whereas cycloheximide did reduce it to about 10% of the control level.

This result by itself is not conclusive. Uptake of actinomycin-D has not been measured. Also, actinomycin-D might have been inactivated by this period within the cell. The mRNA for nitrate reductase may also escape inhibition by actinomycin-D at the concentration used (20 µg/ml). Such possibilities can never be convincingly settled unless direct measurement on the rate of nitrate reductase mRNA synthesis is done. But due to difficulties inherent in such studies, and due to unavailibility of the purified enzyme from this source, direct measurement could not be done. It was hoped however, that some information may be obtained from the effect of actinomycin-D on the kinetics of induction of this enzyme.

As indicated in Fig.4, a paradoxical effect of actinomycin-D was observed when the induction kinetics was followed in its presence. The time of onset of the induction remained approximately the same. But immediately after induction, the activity rose to a higher value than the control and reached a peak at about 28 hours of germination only to fall rapidly even below that of the control by the 36th hour. The

The following points are clear: the possibility that the inhibitor concentration was suboptimal is ruled out because actinomycin-D at the higher concentration (24 µg/ml) showed at least 75% more stimulatory activity than at the lower concentration (12 µg/ml). Actinomycin-D also did not influence the time of onset of induction; therefore, some developmental clock work mechanism may be involved which paves the way for induction of nitrate reductase at a precise hour of germination. The question may be asked whether the time of peak activity of the enzyme in presence of actinomycin-D is due to developmental competence or not. Therefore, the embryos were germinated in nitrate and transferred to actinomycin-D after 20 hours. The results presented in table II are inconclusive due to experimental limitation. However, a actinomycin-D at the higher concentration did indeed seem to produce a rise and a fall within 7 hours of the transfer.

The possibility, however, exists that this stimulatory effect of actinomycin-D on the induction kinetics is indirect through an inhibition of overall rate of protein synthesis, without much specific effect on nitrate reductase itself. This may be significant because actinomycin-D is known to exert different degree of effect on the synthesis of different RNA species[35]. An attempt was, therefore, made to measure the overall rate of protein synthesis, in the presence and absence of actinomycin-D and in uninduced embryos. The results from the pulse labelling experiments show that there is in fact, a slight increase in overall rate of protein synthesis

in presence of inhibitor (Tables III&IV) at 25 and 30 hours of germination. While this result is in general agreement with the conclusion of others[28,10], it fails to support the contention that actinomycin-D effect may be indirect.

The above considerations, along with the observation that stimulatory activity of actinomycin-D declines sharply after 28 hours to fall even below the control level by about 40% within 36 hours of germination, strongly suggest that preformed mRNA may be involved in the induction upto at least 28 hours but that, after this period, a switching of the available template occurs and new mRNA (actinomycin-D sensitive) are preferred. Therefore the phase of nitrate reductase activity during germination may be divided into an early quiescent (or preparatory) period up to about 15 hours, an intermediate actinomycin-D insensitive period (upto 28 hours) and a late actinomycin-D sensitive period.

Development of a Model:

Following points compiled from the previous works and the present study are used in an attempt to develop a coherent picture of the state of affairs.

1) Preformed mRNA provides the majority of the templates for translatory activity up to 12 hours of rice embryos germination[16].

2) A small proportion of mRNA may be synthesised during this period and may be required for initiation of translation of at least some of the preformed mRNAs[54].

3) New mRNA synthesis commences at about 12 hours of germination[16].

4) Assembly of new ribosomes commences also at 12 hours and there is a slow and steady increase in the number of available ribosomes following this period.

5) Nitrate reductase cannot be induced before 15 hours of germination, following which it reaches a plateau rapidly.

6) The initial 16 hours of nitrate reductase induction period is actinomycin-D insensitive, but sensitive to cycloheximide.

7) The latter period of the enzyme activity is sensitive to both actinomycin-D and cycloheximide.

Any model for describing the behaviour of nitrate reductase in developing rice embryo must incorporate all the above points.

Thus it may be assumed that at zero time of germination, the only template available for nitrate reductase is stable and preformed mRNA. This population of mRNA will decay with a certain rate, which will vary with the period of germination, depending on the increasing concentration of various RNAses in the cell.

New mRNA concentration at zero hour of germination is also zero, following which, it will show an increase with a first order rate constant.

The effective concentrtion of preformed, or newly synthesised, mRNA for nitrate reductase will also determine the rate of synthesis of the enzyme with a first order rate constant at any instant.

The effective concentration of assayable enzyme will in turn depend on its own overall rate of synthesis and its rate of inactivation. The rate of inactivation will also depend upon its own concentration among other things.

The newly synthesised mRNA will also decay with a first order rate constant depending on its own concentration.

In addition, the rate of synthesis of the enzyme may also be expected to depend on the number of available ribosomes etc. - which may increase with time.

The Model:

The above conclusions are used to put together the state of affairs in the form of a model (Fig. 11).

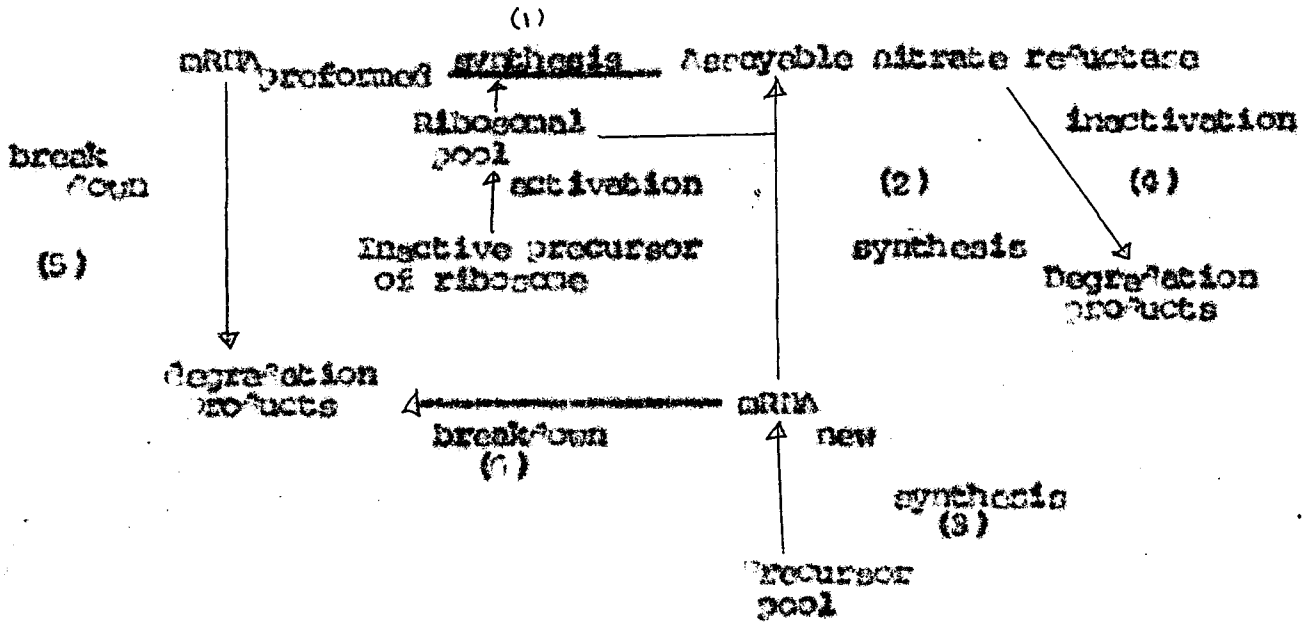

Fig. 11

Mathematical formalism of the Model:

Symbols: R_o = Initial concentration of preformed m RNA for nitrate reductase at zero hour of germination.

R_s = Concentration of preformed mRNA at any instant of time ($R_s < R_o$).

R_n = Concentration of newly synthesised mRNA for nitrate reductase at any instant of time.

C = Pool size of precursors for the synthesis of new messenger RNA and is a constant.

N = Concentration of assayable an enzyme at any instant of time.

K_1 = Overall rate constant for the synthesis of the enzyme upon its preformed mRNA template.

K_2 = Overall rate constant for the synthesis of the enzyme upon its new template.

K3 = Overall rate constant for the synthesis of new mRNA from its precursor pool.

$K_5 = K_6$ = Degradation rate constants for the preformed and new mRNA respectively.

K_4 = Rate constant for the inactivation of nitrate reductase enzyme. This includes both nonspecific and specific degration of the enzyme.

A and α are constants which describe some independent developmental processes that must influence the transcription and translation of mRNA.

β = time constant for the activation of ribosomes.

A series of differential equations may now be constructed to describe the behaviour of the model system.

Channel (5) of the model in fig. (11) is adequately described by the equation:

$$\frac{d R_s}{d t} = 0 - k_5 R_s \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (i)$$

Channels (3) & (6) are taken care of by the equation:

$$\frac{d R_n}{dt} = k_3 \cdot C - k_6 R_n \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (ii)$$

Channels (1), (2), and (4) may be described by the equations:

$$\frac{dN}{dt} = -k_4 N + A e^{-\alpha t} (R_s + R_n) + e^{\beta t} \quad \ldots\ldots\ldots\ldots\ldots (iii)$$

While the first two equations are self explicatory, the third merits some explanation.

The first negative term in equation (iii) describes the rate of inactivation of the enzyme, which is obviously proportional to its own concentration.

The second term describes the rate of synthesis of the enzyme, which is proportional to the total amount of template available at any instant of time. For the sake of generality, provision has

~~has~~ been made of any developmental process, taking place independently, which may have a steady time dependent negative influence on the synthetic rate by including the term, $A \cdot e^{-\alpha t}$.

The third term describes the rate of availability of active ribosomes.

A solution to eqn. (i) leads to the equation,

$$R_s = R_o \cdot e^{-k_5 t} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (iv)$$

and that of equation (ii) leads to the equation,

$$R_n = \frac{k_3 C}{k_6} \cdot (1 - e^{-k_6 t}) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots (v)$$

(see Appendix II)

Using the equations (iv) and (v), the solution of equation (iii) may be obtained as:

$$N = \frac{A \cdot R_o}{k_4 - k_5 - \alpha} \cdot (e^{-(\alpha + k_5)t} - e^{-k_4 t}) + \frac{A \cdot k_3 C}{k_6 (k_4 - \alpha)} (e^{-\alpha t} - e^{-k_4 t})$$

$$- \frac{A\, k_3\, C}{k_6 (k_4 - \alpha - k_5)} \cdot (e^{-(\alpha + k_6)t} - e^{-k_4 t}) + \frac{1}{k_4 + \beta} (e^{\beta t} - e^{-k_4 t}) \ldots (vi)$$

(see Appendix III)

Equation (vi), therefore, should describe completely the behaviour of nitrate reductase activity with time during germination.

Let us now try to describe what happens when actinomycin-D is added to the system. Actinomycin-D may be assumed in this case to shut off all transcriptions. Thus, the channels (3), (6) and (2) of fig. (9), are blocked.

Under such conditions the general equation (iii) reduces to,

$$\frac{dN}{dt} = -k_4 N + k_1 R_s + e^{\beta t} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots (vii)$$

where R_s is governed by equation (iv)

A solution to equation (vii) may be written as:

$$N = \frac{k_1 R_o}{k_4 - k_5} \cdot (e^{-k_5 t} - e^{-k_4 t}) + \frac{1}{k_4 + \beta} (e^{\beta t} - e^{-k_4 t}) \ldots\ldots\ldots\ldots (viii)$$

(see Appendix IV)

Equation (viii) now describes the nitrate reductase activity in germinating embryo in presence of actinomycin-D according to the model of Fig. 11.

Numerical Solutions:

Numerical solution of equation (vi) were done by computer using the following values of the constants,

$k_3 = 3.6\ h^{-1}$ (ref. 58)
$k_4 = 0.79\ h^{-1}$ (Fig. 7b)
$k_5 = k_6 = 0.06\ h^{-1}$ (refs. 1 & 57)

Values of C, R_o, and A have all been taken arbitrarily to be unity.

Figure ~~8(b)~~ shows the computed values of N in ~~arbiterfly~~ arbitrary units of concentrations plotted against hours of germination for different values of α/β as described by eqn (vi)

The extraordinary resemblance between these curves and the experimentally obtained one in fig.3 is immediately apparent.

The theoretical curves show a lag of 7 to 8 hours, which of course is inaccurate because of rough estimation of the various constants. But that it shows a lag, and that it reaches a plateau, are themselves strong reminders of the adequacy of the model.

Further support in favour of the model comes when numerical solution of eqn(viii) is plotted for the same values of β as in fig.9(a&b) and with $k_1 = 100\ h^{-1}$ (ref. 62). The theoretical plot (Fig. 10), though without the lag, shows a peak, followed by a decline.

It seems that, provided the model is fairly adequate, one does not need to postulate any repressor or activator for the nitrate reductase enzyme induction. The kinetic parameters are enough to provide the actinomycin-D induced peak in the enzyme activity.

Predictions From the Model:

Apart from predicting the general behaviour of mRNA of

Fig. 9 (a) & (b) Computer simulation graphs of equation V

Fig. 10 Computer Simulation graphs of equation VIII

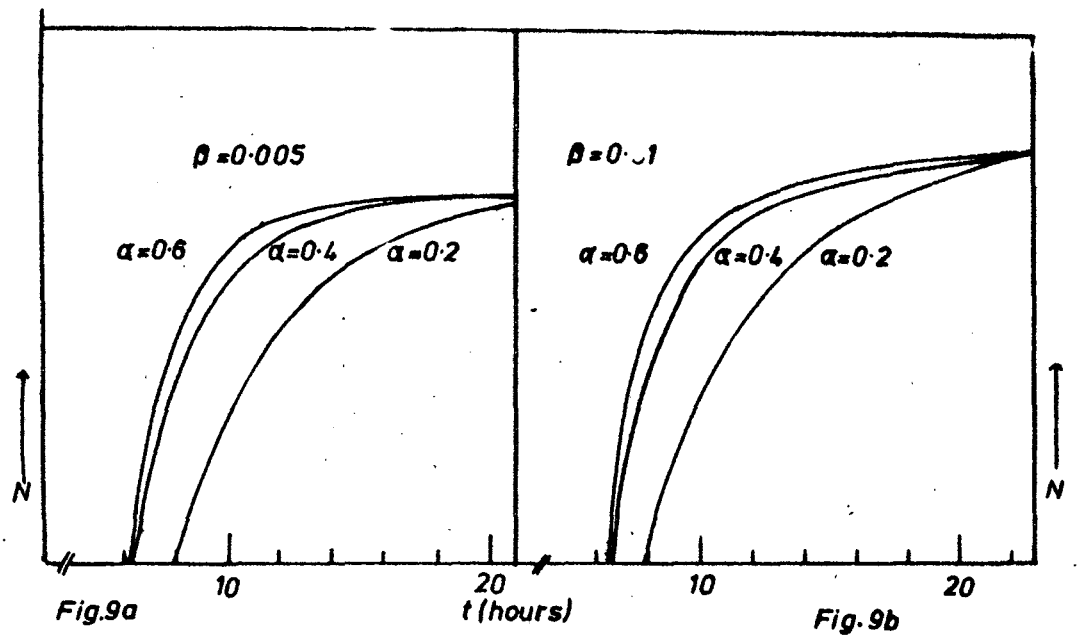

Fig.9a

Fig. 9b

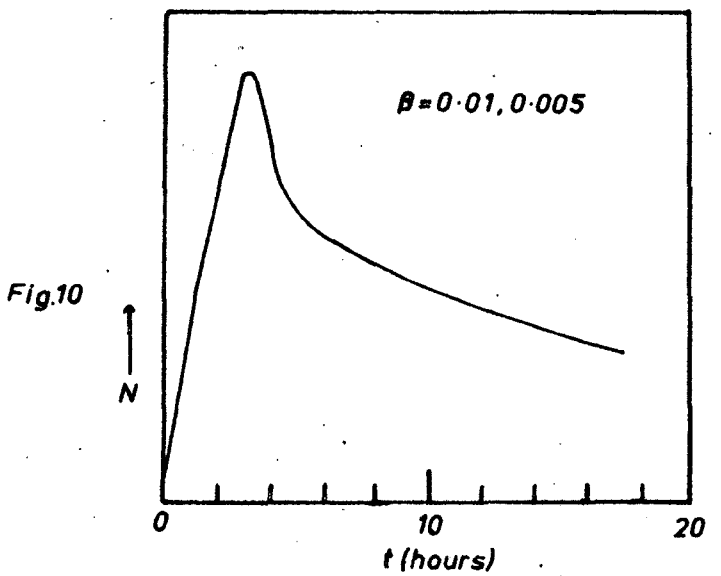

Fig.10

of nitrate reductase from equations (iv) & (v), the model does not presuppose any repressor or activator of Jacob-Monod type. It has been shown that simple kinetic parameters are enough to explain the apparently paradoxical results obtained with transcription inhibitor.

The model strongly favours the site of action of NO_3^- as an inducing agent at the translational level, rather than at the transcriptional or post translational level. It also implies a switching over of the preferred template for translation of the enzyme from preformed mRNA to newly synthesised mRNA. The model also predicts the presence of a developmental clockwork mechanism which gives rise to the delay. As a matter of fact, eqn(viii) does not give the delay because assumption has been made that actinomycin-D suppresses the developmental clock-work mechenism also (i.e. the $A.e^{-\alpha t}$ factor). This assumption may not be justified because of two reasons: either actinomycin-D is unable to inhibit transcription required to maintain the process, or that the clockwork mechanism does not involve new transcription. But what may be the nature of this clock work mechanism remains in the realm of speculation. Fundamentally, it may be argued that translation of preformed mRNA of specific kinds may set the pace in early germination.

The possibility of increased availability of intracellular NO_3^- was for the enzyme induction during appropriate phases of germination has not been excluded in this study. If, however, the uptake of NO_3^- becomes an increasing function of time of germination, then this factor also may have been taken care of by the term $e^{\beta t}$ in the model.

The model, however makes a number of gross assumptions apart from using arbitrary values of some of its parameters. But these values, it is recognised, will only change the scale of concentration without affecting the general shapes of the curves. The main assumption is that the degradation rate constant for

the two kinds of RNA are the same. Another assumption is that the rate of translation of the two mRNAS may not be different. This may be particularly true if the preformed mRNA and the new mRNA are transcribed form two different genes. In that case, the possibility of difference in efficiency of the two enzymes may also play a role in the assayable enzyme activity profile with time. But a measurement of Km of the enzyme at two different periods of germination (18 hours and 25 hours) did not show any significant difference within the experimental sensitivity limits. Thus the argument that two different enzymes may be involved during germinating, does not hold ground in the present state of limited understanding. A rigorous verfication of this point by isolating the enzyme on polyacrylamide gel or by some such means, should not be difficult. Possibility of isozymes having almost similar Km values is strong.

SUMMARY

The present study focusses on the behaviour of a substrate inducible enzyme, nitrate reductase, during the germination period (upto 36 hours) of rice embryo. The enzyme is inducible only after about 14 hours of germination. Cycloheximide suppressed the induction, whereas treatment with actinomycin-D showed a transitory superinduced phase at the two concentrations used. Previous works on rice embryogenesis coupled with the present study, have led to the development of a kinetic model which does not presuppose any inducer or inhibitor of the classical "Jacob-Monod type". Computer simulation of the model closely predicted the experimental findings and any deviation from the results obtained, may be ascribed to experimental limitations. The analysis suggests that the super-inducibility by actinomycin-D may be simply a non-specific kinetic effect. However, due reservations are required because an impure enzyme was used throughout the study even while being alive to the famous warning, "Do not waste clean thoughts on dirty enzymes".

Appendix - I

For the points,

$$(x_1, y_1), (x_2, y_2), (x_3, y_3), \ldots\ldots\ldots (x_n, y_n)$$

the gradient of the least square is given by,

$$m = \frac{nD - BC}{nA - B^2}$$

and the intercept on Y-axis,

$$c = \frac{AC - BD}{nA - B^2}$$

where,

$$A = \sum x_i^2$$
$$B = \sum x_i$$
$$C = \sum y_i$$
$$D = \sum x_i y_i$$

Let the equation of the least square line thus obtained, be,

$$Y = mX + c \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (a)$$

In the special case, when

$$X_i = \frac{1}{S_i} \quad \text{and} \quad Y_i = \frac{1}{V_i},$$

where, V_i is the velocity of the enzymatic reaction at the corresponding substrate concentration S_i, then the equation (a) becomes the Lineweaver-Burke transformation of the Michaelis-Menten's equation. Therefore $\left(-\frac{1}{K_m}\right)$ is given by the condition when,

$$Y_i = 0$$

i.e., when, $X_i = -\frac{c}{m}$.

Therefore, $K_m = \frac{m}{c}$

Appendix-II

$$\frac{dR_n}{dt} = k_3 C - k_6 R_n$$

i.e., $$\frac{dR_n}{dt} + k_6 R_n = k_3 C$$

Thus, $$R_n e^{\int k_6 dt} = \int \left[k_3 C e^{\int k_6 dt} \right] dt + I$$

Therefore, $$R_n = e^{-\int k_6 dt} \left[\int k_3 C e^{\int k_6 dt} dt + I \right]$$

$$= \frac{k_3 C}{k_6} + I . e^{-k_6 t}$$

Defining, $R_n = 0$, at $t = 0$,

we have, $$I = -\frac{k_3 C}{k_6}$$

Therefore, $$R_n = \frac{k_3 C}{k_6} \left(1 - e^{-k_6 t}\right)$$

Appendix-III

$$\frac{dN}{dt} = -k_4 N + A e^{-\alpha t}(R_s + R_n) + e^{\beta t}$$

where, $\frac{dR_s}{dt} = -k_5 R_s$

i.e., $R_s = R_0 e^{-k_5 t}$, $(R_0 = R_s \text{ at } t = 0)$

and R_n is given by the solution in Appendix I.

Putting the solution of R_s and R_n, we get,

$$\frac{dN}{dt} = -k_4 N + A e^{-\alpha t}\left[R_0 e^{-k_5 t} + \frac{k_3 C}{k_6}(1 - e^{-k_6 t})\right] + e^{\beta t}$$

i.e., $$N = e^{-\int k_4 dt}\left[\int\left\{AR_0 e^{-(\alpha+k_5)t} + \frac{Ak_3C}{k_6}(e^{-\alpha t} - e^{-(\alpha+k_6)t}) + e^{\beta t}\right\} e^{\int k_4 dt}\, dt + I\right]$$

Integrating and simplifying, we get,

$$N = \frac{AR_0}{k_4 - k_5 - \alpha} e^{-(\alpha+k_5)t} + \frac{Ak_3C}{k_6(k_4-\alpha)} e^{-\alpha t} - \frac{Ak_3C}{k_6(k_4-\alpha-k_6)} e^{-(\alpha+k_6)t} + \frac{1}{k_4+\beta} e^{\beta t} + I.e^{-k_4 t}$$

Defining, $N = 0$, at $t = 0$, and calculating the value of I, we get,

$$N = \frac{AR_0}{k_4 - k_5 - \alpha}\left(e^{-(\alpha+k_5)t} - e^{-k_4 t}\right) + \frac{Ak_3C}{k_6(k_4-\alpha)}(e^{-\alpha t} - e^{-k_4 t}) - \frac{Ak_3C}{k_6(k_4-\alpha-k_6)}\left(e^{-(\alpha+k_6)t} - e^{-k_4 t}\right) + \frac{1}{k_4+\beta}(e^{\beta t} - e^{-k_4 t}).$$

Appendix-IV

$$\frac{dN}{dt} = k_4 N + k_3 R_3 + e^{\beta t}$$

where, $\frac{dR_3}{dt} = -k_3 R_3$

i.e., $R_3 = R_0 e^{-k_3 t}$ $\quad [R_0 = R_3 \text{ at } t=0]$

Proceeding as above,

$$N = e^{-\int k_4 dt}\left[\int (k_3 R_0 e^{-k_3 t} + e^{\beta t}) e^{\int k_4 dt} \cdot dt + I\right]$$

Integrating and simplifying, we get,

$$N = \frac{k_3 R_0}{k_4 - k_3} e^{-k_3 t} + \frac{1}{k_4 + \beta} e^{\beta t} + I e^{-k_4 t}$$

Defining, $N=0$, at $t=0$, and calculating the value of I,

we have,

$$N = \frac{k_3 R_0}{k_4 - k_3}\left(e^{-k_3 t} - e^{-k_4 t}\right) + \frac{1}{k_4 + \beta}\left(e^{\beta t} - e^{-k_4 t}\right)$$

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