Kinetic Studies of the Induction of Nitrate Reductase and Cytochrome *c* Reductase in the Fungus *Aspergillus nidulans*

By D. J. COVE
Department of Genetics, University of Cambridge

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In an earlier paper (Cove, 1966) it was reported that the kinetics of appearance of nitrate reductase (NADPH–nitrate oxidoreductase, EC 1.6.6.3) on the addition of nitrate to a growing culture of *Aspergillus nidulans* were different in certain respects from those found for many *Escherichia coli* enzymes. When urea is used as an initial nitrogen source, a further difference is found: enzyme synthesis is no longer continuous. This interruption of synthesis does not appear to be due to synchronous cell division in the culture, nor to be due to accumulation of ammonia. Fluctuations in the intracellular concentration of nitrate, though appearing to be partly responsible for the discontinuity of enzyme syntheses, cannot account for all the observations. Two related hypotheses are put forward to explain this discontinuity of synthesis; each suggests that nitrate reductase is intimately concerned with its own synthesis. One possibility is that the enzyme when it is not in the form of a complex with nitrate is a co-repressor of its own synthesis, and the other that the enzyme is its own repressor.

Pateman, Cove, Rever & Roberts (1964) proposed that nitrate reductase (NADPH–nitrate oxidoreductase, EC 1.6.6.3) in the ascomycete fungus *Aspergillus nidulans* possessed cytochrome *c* reductase (NADPH–cytochrome *c* reductase, EC 1.6.2.3) activity. Cove & Coddington (1965) have since provided direct evidence of this by showing that a 300-fold purification of nitrate reductase fails to remove cytochrome *c* reductase activity. Preliminary investigations (Cove, 1966) of the kinetics of induction of nitrate reductase in *A. nidulans* showed certain differences from the kinetics found in most bacterial systems. The present paper describes further detailed kinetic studies of the induction of both nitrate reductase and cytochrome *c* reductase in wild-type and mutant strains of *A. nidulans*.

MATERIALS AND METHODS

Except where listed below the materials and methods were as described by Cove (1966).

Experimental organism. Details of the strains of *A. nidulans* used are given in Table 1.

Culture of mycelium. The method used was basically the aerated-culture technique described by Cove (1966). Some experiments were, however, carried out with 50l. of medium in a 60l. polypropylene reagent bottle. In these cases the temperature was regulated by circulating water from a water bath through a stainless-steel coil immersed in the culture vessel, which was stirred magnetically and had air passed into it at a rate of 90l./min.

Assay of cytochrome c reductase. The method described by Cove & Coddington (1965) was used.

Procedure for induction experiments. Mycelium was grown in aerated culture in either 17·5l. or 50l. of nitrogen-less minimal medium to which was added biotin to give 0·01 mg./l. and a nitrogen source to give a concentration of 10mg.atoms of N/l. The culture was sampled 1hr. after inoculation and the number of viable sporelings/l. was determined by diluting the sample appropriately, plating on to complete medium, and counting the number of colonies that developed after incubation. The required quantity of inducer was added as a concentrated solution at the appropriate time. Samples (200ml.) of the growing culture were drawn from the bottle as required.

RESULTS

Figs. 1–6 give the time-course of appearance of nitrate reductase or cytochrome *c* reductase or both under various conditions of induction. Blotted weight of mycelium is also plotted. Details of the experiments are given in the legends to the Figures.

DISCUSSION AND CONCLUSIONS

Cove (1966) has described the kinetics of nitrate reductase appearance on the addition of nitrate to wild-type mycelium, when this is grown with 10mM-sodium L-glutamate as an initial nitrogen source. These are shown for reference in Fig. 1(*a*). It was pointed out that these kinetics differed from those found for many bacterial enzyme systems in

Table 1. *Growth characteristics and origins of strains used*

Genotype	Ability to use as N source: NO_3^-	NO_2^-	Hypoxanthine	Other characteristics	Origin or reference
bi-1	+	+	+	Requires biotin	See Cove (1966)
yw-3	+	+	+		From a cross between *bi*-1 *Acr*-1 *w*-3 and *ribo*-1 *an*-1 *paba*-1 *y meth*-1, both obtained from the Department of Genetics, University of Glasgow
bi-1 *Mer*-1	+	+	+	Resistant to methylammonium chloride	From *bi*-1 by treatment with diethyl sulphate
yw-3 *cnxG*-2	−	+	−		From *yw*-3 by u.v. irradiation (Pateman *et al.* 1964)
bi-1 *niiA*-2	−	−	+		From *bi*-1 by treatment with diethyl sulphate (Rever, 1965; Pateman *et al.* 1967)

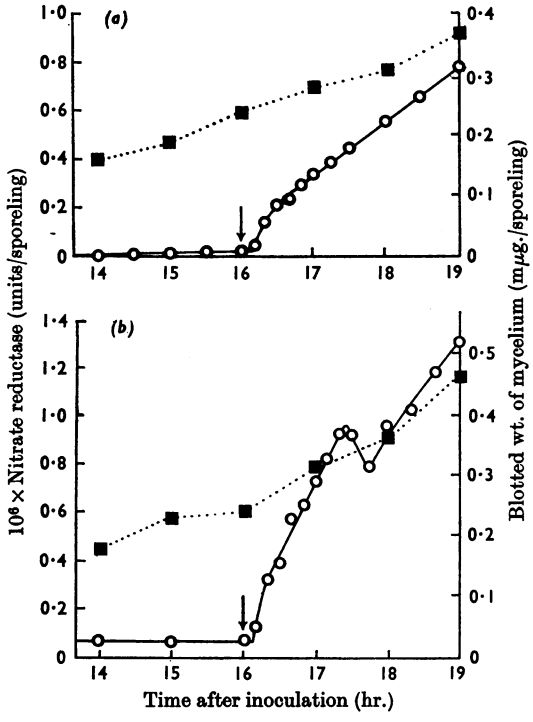

Fig. 1. Kinetics of induction of nitrate reductase in strain *bi*-1. Mycelium was grown in 17·5l. of medium in aerated culture with 10mM-sodium L-glutamate (*a*) or 5mM-urea (*b*) as a nitrogen source. Then 14·87g. of $NaNO_3$ dissolved in 100ml. of sterile distilled water was added 16hr. after inoculation. ○, Nitrate reductase; ■, growth weight.

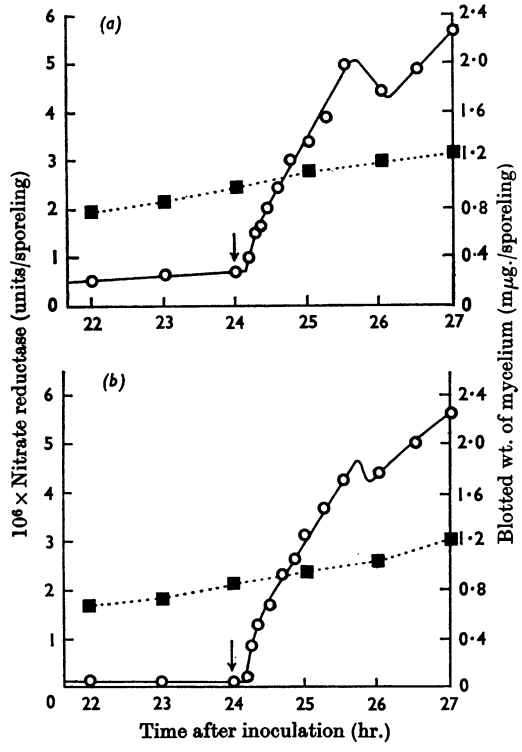

Fig. 2. Kinetics of induction of nitrate reductase in strain *bi*-1 *niiA*-2 (*a*) and strain *bi*-1 *Mer*-1 (*b*). Experimental details were similar to those described for Fig. 1(*b*), except that the $NaNO_3$ was added 24hr. after inoculation. ○, Nitrate reductase; ■, growth weight.

two respects. First, the initial rate of nitrate reductase synthesis by each sporeling was maximal, decreasing for about 0·5hr. Thereafter a constant rate of enzyme synthesis by each sporeling was established, even though the mass of each sporeling increased during the experiment. The present paper describes experiments carried out to investigate these kinetics further. It was found that urea at

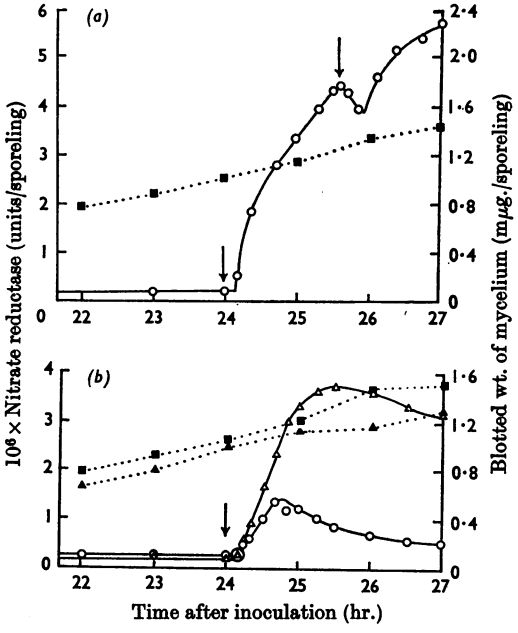

Fig. 3. Kinetics of induction of nitrate reductase in strain *bi*–1. In (*a*) experimental details were similar to those described for Fig. 2(*a*), except that a further 14·87g. of $NaNO_3$ was added 25½hr. after inoculation. ○, Nitrate reductase; ■, growth weight. In (*b*), experimental details were similar to those described for Fig. 2(*a*), except that instead of $NaNO_3$ either 5·7ml. of methyl nitrate or 17·5ml. of *n*-propyl nitrate was added 24hr. after inoculation. ○ and △, Nitrate reductase in the methyl nitrate and *n*-propyl nitrate experiments respectively; ■ and ▲, growth weight in the methyl nitrate and *n*-propyl nitrate experiments respectively.

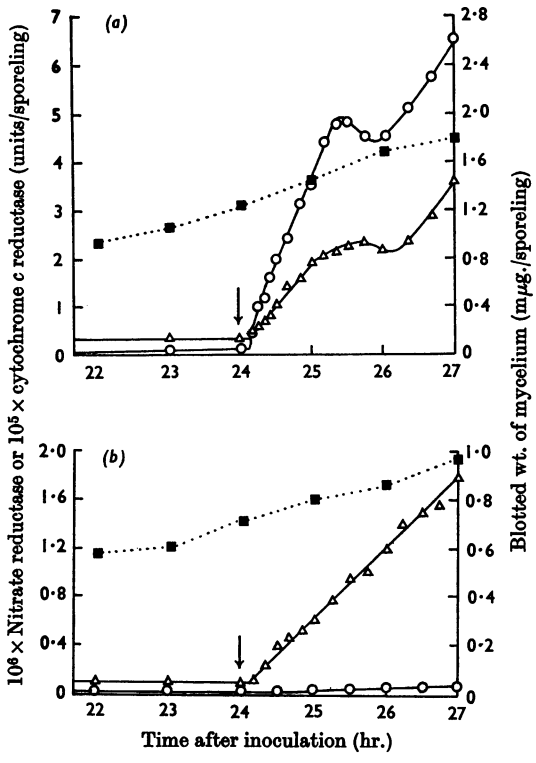

Fig. 4. Kinetics of induction of nitrate reductase and cytochrome *c* reductase in strain *bi*–1 (*a*) and strain *yw*–3*cnxG*–2 (*b*). Mycelium was cultured in 50l. of medium in aerated culture with 5mM-urea as a nitrogen source. Then 42·5g. of $NaNO_3$ dissolved in 200ml. of sterile distilled water was added 24hr. after inoculation. ○, Nitrate reductase; △, cytochrome *c* reductase; ■, growth weight. The lower values obtained for enzyme activities in (*b*) are accounted for by the lower growth rate of strain *yw*–3, and strains derived from it, compared with that of strain *bi*–1.

a concentration of 5mM supported a more rapid rate of growth than 10mM-sodium L-glutamate. The kinetics of appearance of nitrate reductase on the addition of nitrate to mycelium grown for 16hr. on urea as an initial nitrogen source are shown in Fig. 1(*b*). Similar results have been obtained when nitrate was added after 12, 13½, 15, 16½, 18, 19½, 20, 21, 24, 28, 32, 36 and 40hr. The implication of these findings is discussed below. As in the experiments with glutamate the initial rate of synthesis was maximal, and again decreased until a constant rate was established. This rate was greater, however, and was maintained for only about 1hr. Thereafter there was a characteristic fall in the enzyme content of a sporeling, although synthesis was resumed after a short lag. It has already been shown (Cove, 1966) that *A. nidulans* nitrate reductase is unstable *in vivo*, and so it is likely that this fall in enzyme content was due to a temporary interruption of enzyme synthesis.

Thus culture on urea introduces another characteristic difference from other described systems, the inability to maintain continuous enzyme synthesis in the presence of the co-inducer.

This latter phenomenon will be considered first. A possible explanation might be that the cells in the cultures studied undergo some form of division cycle, and that protein synthesis is only possible at certain times during this cycle. Knutsen (1965) has reported that the synthesis of nitrite reductase in synchronous cultures of *Chlorella pyrenoidosa* is only possible at certain times in the cells' mitotic cycle. As indicated below, under certain conditions some mutant strains of *A. nidulans* do not show this interrupted synthesis of nitrate reductase, and this alone provides strong evidence against an

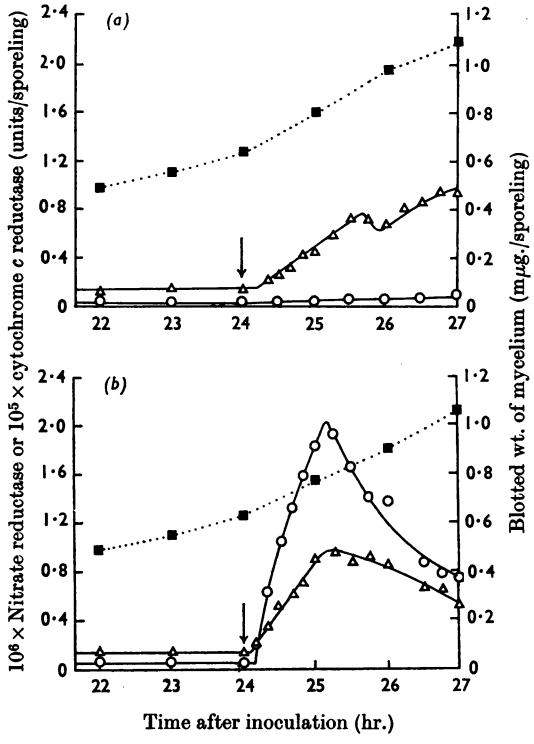

Fig. 5. Kinetics of induction of nitrate reductase and cytochrome *c* reductase in strain *yw–3cnxG–2* (*a*) and strain *yw–3* (*b*). Experimental conditions were similar to those described for Fig. 4(*a*), except that only 425mg. of $NaNO_3$ was added. ○, Nitrate reductase; △, cytochrome *c* reductase; ■, growth weight.

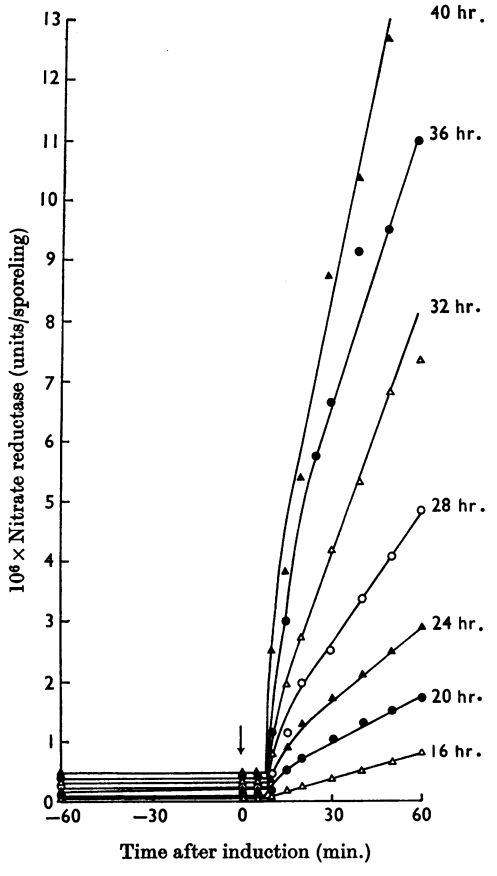

Fig. 6. Comparison of the kinetics of induction of nitrate reductase obtained in strain *bi–1* in a series of experiments differing in the time of addition of $NaNO_3$. Experimental conditions were basically similar to those described for Fig. 1(*b*), except that the $NaNO_3$ was added in each experiment at the time after inoculation indicated to the right of the graphs.

explanation based on a cell cycle. In addition to this, no decrease in growth rate, as measured by mycelial mass, or mycelial protein content could be detected during the period when nitrate reductase was not synthesized. Further, as stated above, similar kinetics of induction of nitrate reductase in *Aspergillus* have been found for many different times of induction. A lag period of about 8min. has been found in every experiment. If the intermittent synthesis were due to a synchronous mitotic cycle, then there should be times at which induction would be delayed, and so the homogeneity of induction times that is found is difficult to reconcile with an explanation relying on a synchronous mitotic cycle. However, combined cytological and enzymological studies have been started to test this possibility.

Alternative explanations for this interrupted synthesis have therefore been sought. As nitrate reductase synthesis has been shown to be subject to repression by ammonium (Cove, 1966), an increase in the intracellular concentration of this ion, brought about by the reduction of nitrate, might cause the observed interruption of enzyme synthesis. Two experiments were carried out to see whether this was likely. In the first a mutant strain, *bi–1niiA–2*, was used. This strain, whose characteristics are more fully described elsewhere (Rever, 1965; Pateman, Rever & Cove, 1967), is unable to grow on either nitrate or nitrite as a sole source of nitrogen, but is able to utilize ammonium. It possessed normal nitrate reductase activity, and so was presumably deficient in some function required to convert nitrite into ammonium. The addition of nitrate to this strain should not there-

fore affect the ammonium concentration. Fig. 2(*a*) shows the induction kinetics for nitrate reductase obtained with this strain, and the graph is essentially similar to that obtained with a wild-type strain. The second experiment used a different mutant strain, *bi*–1 *Mer*–1. This had been selected as being resistant to the methylammonium ion, when nitrate was supplied as a nitrogen source. Such a strain was found to be insensitive to repression by ammonium, i.e. to have a de-repressed phenotype. Fig. 2(*b*) shows the induction kinetics for this strain, and again these are similar to those for the wild-type. Thus enzyme synthesis was found to be discontinuous both in a strain unable to synthesize ammonium from nitrate and in a strain whose nitrate reductase synthesis was insensitive to ammonium repression, and it therefore seems unlikely that this interruption in synthesis is due to a rise in the intracellular concentration of ammonium.

Another possible explanation is that, as a result of enzyme activity, the concentration of nitrate fell below that required to maintain induction. Fig. 3(*a*) shows the kinetics of nitrate reductase appearance in a wild-type strain when additional nitrate was added 90min. after the initial inducing treatment. The addition of this extra nitrate failed to prevent the interruption of synthesis. However, if uptake were the rate-limiting process, this treatment would not necessarily prevent the intracellular concentration of nitrate falling below the level required for induction.

The intracellular concentration of inducer may be kept independent of the enzyme concentration in two ways. Either some appropriate analogue of nitrate that does not serve as a substrate for the enzyme may be used as a gratuitous inducer, or a mutant strain having an altered inactive nitrate reductase protein may be used. Various analogues of nitrate were tried as both nitrogen sources and inducers. It was shown that neither methyl nitrate nor *n*-propyl nitrate can act as nitrogen source, or as substrate for nitrate reductase, but both were capable of nitrate reductase induction. Fig. 3(*b*) shows the induction kinetics obtained with these substrates. In neither case is synthesis maintained, but the kinetics differ from those obtained with nitrate ions in that the interruption of synthesis appears to be permanent. Another difference in the kinetics obtained with these two co-inducers is that no fall-off in the rate of synthesis occurs before the establishment of a constant rate. For these reasons it is perhaps dangerous to interpret these experiments as showing that interruption of enzyme synthesis is not due to decrease in co-inducer concentration.

The alternative method of investigation was therefore pursued. Certain strains of *Aspergillus* are known that, it is thought, are unable to synthesize a cofactor necessary for both nitrate reductase and xanthine dehydrogenase activity. The wild-type nitrate reductase protein also possesses another activity, cytochrome *c* reductase (Pateman *et al.* 1964; Cove & Coddington, 1965). In strains unable to synthesize the cofactor, the nitrate reductase protein, though having no or very low nitrate reductase activity, retains this cytochrome *c* reductase activity. Most of such strains synthesize cytochrome *c* reductase, and hence nitrate reductase protein, constitutively (Pateman *et al.* 1964), a point discussed more fully by Pateman *et al.* (1967). Some strains retain inducible synthesis of the protein, and one of these, *yw*–3 *cnxG*–2, was used for these studies. Figs. 4(*a*) and 4(*b*) show the kinetics of appearance of both nitrate reductase and cytochrome *c* reductase activities on the addition of 10mM-nitrate in a wild-type strain, and in the cofactor mutant *yw*–3 *cnxG*–2. In the wild-type the appearance of cytochrome *c* reductase activity parallels that of nitrate reductase activity. In the mutant virtually no nitrate reductase activity is detectable and the cytochrome *c* reductase continues to be synthesized without interruption for at least 3hr. In both the wild-type and mutant strain no initial fall-off in rate of synthesis of cytochrome *c* reductase is observable. Thus it seems likely that variations in nitrate concentration must be at least partly responsible for the inability of the wild-type to maintain continued enzyme synthesis. In view of the indications, from the experiments described above with gratuitous inducers, that variations in concentration of co-inducer were not wholly responsible for this interruption, further experiments were carried out with strain *yw*–3 *cnxG*–2. If the cessation of synthesis is brought about only because of a fall in intracellular nitrate concentration, then lowering the concentration of nitrate used to induce in strain *yw*–3 *cnxG*–2 should not affect the shape of the curve, only perhaps the rate of synthesis. Fig. 5(*a*) shows that when 0·1mM-nitrate is used as an inducer in the mutant strain continued synthesis is not maintained. It might be argued that, with this low concentration of nitrate, the amount of nitrate reductase activity present in the mycelium, although very small, would be sufficient to deplete the intracellular nitrate pool. As a control to check this, 0·1mM-nitrate was used to induce the wild-type. The kinetics obtained are shown in Fig. 5(*b*). Synthesis continues for about 70min. and then stops, presumably because all the nitrate has been reduced, as no resumption of synthesis occurs. The nitrate reductase concentration in the wild-type mycelium at the time when synthesis stops is 2500 enzyme units/g. blotted weight. At the time when linear enzyme synthesis is interrupted in the

mutant, the concentration is only 70 enzyme units/g. blotted weight. It therefore seems very improbable that the amount of nitrate reductase activity present in the mutant could be sufficient to decrease the intracellular nitrate pool enough to interrupt induction.

If, as therefore seems likely, variations in the intracellular concentration of inducer cannot wholly account for the intermittent synthesis of enzyme, another factor must be invoked. Two alternative but related hypotheses can be put forward that could account for this situation. The addition of nitrate to a growing culture will not only give rise to variations in the intracellular nitrate concentration but will also alter the concentration of nitrate-induced enzymes, including nitrate reductase. If the nitrate reductase molecule not in the form of a complex with nitrate were directly involved in the regulation process then the kinetic observations described above might be accounted for in either of two ways. The enzyme when not in the form of a complex with nitrate might act as a co-repressor of nitrate reductase synthesis, or it might act directly as the repressor. On either hypothesis, enzyme synthesis would lead to repression when the enzyme concentration rose to such a level that the intracellular nitrate concentration was no longer sufficient to saturate it. It would be resumed when the nitrate concentration again increased as a result of the fall-off in nitrate reductase content. The continuous synthesis, found when glutamate instead of urea is used as a nitrogen source, would occur because the lower rate of synthesis of enzyme would not allow the nitrate pool to be depleted sufficiently rapidly for free enzyme to accumulate. The observations obtained with the mutant strain *yw–3cnxG–2* are also consistent with these hypotheses. When 10mM-nitrate is added, as no significant depletion occurs as a result of enzyme activity, there would be sufficient nitrate present to keep all the repressor inactivated. With only 0·1mM-nitrate, however, excess of free enzyme would again accumulate, and its synthesis would be interrupted.

The concept of an enzyme functioning as its own repressor may at first sight seem curious. It is not difficult, however, to envisage that selection might favour the evolution of a further site on an enzyme that bound to its own structural gene, or its messenger, in such a way as to block gene transcription or translation in the absence of its substrate and so become its own repressor. Such a control system could be regarded perhaps as primitive, but might give rise to the better-known two-element systems by duplication and subsequent divergent evolution of the two genes.

The second aspect of the kinetics of induction of nitrate reductase to be considered is the constancy of the rate of enzyme synthesis achieved about 30min. after induction and which in certain circumstances can be maintained for at least 5hr. This rate is expressed in enzyme units/sporeling/unit time. But, as has been pointed out in an earlier paper (Cove, 1966), growth is occurring and so therefore the mass of a sporeling and the number of its nuclei increase during the experiment even though the rate at which it produces enzyme remains constant. A possible explanation of this would be that only a certain number of nuclei within a sporeling are capable of producing nitrate reductase, e.g. those near the hyphal tips. Increase in nuclear number need not add to this class of nuclei, and hence a constant rate of synthesis would be maintained. This explanation would predict, however, that this rate would be the same irrespective of the time of induction and the sporeling mass at the time of induction. Fig. 6 shows the induction kinetics obtained in a number of experiments in which the time of induction ranged from 16 to 40hr. after inoculation. The constant rates attained increase with time, and are in fact approximately proportional to fungal mass at the time of induction. It is therefore likely that all the nuclei produced during growth are capable of enzyme synthesis. and so some alternative explanation must be sought. At the present time it is not possible to suggest a hypothesis that is consistent with all the data.

The final points to be discussed concern the rate of enzyme synthesis in the immediate period after induction. A lag period of 8min. in cultures kept at 25°, during which no synthesis can be detected, has been shown to exist irrespective of the time of induction, or of whether glutamate or urea is used as the initial nitrogen source. In every experiment where enzyme synthesis has been followed as nitrate reductase activity, and nitrate has been used as an inducer, the initial rate of appearance of enzyme is maximal, and this decreases during the next 30min. until a constant rate of synthesis is attained (see Figs. 1*a*, 1*b*, 2*a*, 2*b*, 3*a*, 4*a*, 5*b* and 6). However, when methyl nitrate or *n*-propyl nitrate is used as inducer, or when the enzyme is followed as cytochrome *c* reductase activity, a constant rate of synthesis is established at once (see Figs. 3*b*, 4*a*, 4*b*, 5*a* and 5*b*). The absence of any detectable nitrate reductase activity from many strains that differ from the wild-type by a single gene mutation (Cove & Pateman, 1963) makes it unlikely that the kinetics of induction result from the combined appearance of two different enzymes. Nevertheless the only simple explanation for these facts that can at the present time be put forward is that the initial fast rate of enzyme synthesis, found in wild-type strains induced with nitrate, is due to a period of synthesis of a second nitrate reductase that has

no cytochrome *c* reductase activity and that is not induced by nitrate analogues.

The kinetics of induction of nitrate reductase in *Aspergillus*, differing markedly as they do from those found for many bacterial systems, pose problems many of which at the present time remain unsolved. Work that has been carried out on a number of mutant strains is described elsewhere (Pateman *et al.* 1967), and the data obtained are discussed in the light of the hypotheses, put forward above, to explain the intermittent synthesis of the enzyme.

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