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Comparative Inductive Responses of Two β-Galactosidases of *Neurospora*

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The activities of the two β-galactosidases of *Neurospora* increased in response to the presence of lactose or galactose. D-Xylose preferentially induced higher levels of activity in one of these enzymes. The initial period of the inductive response was examined in cultures transferred from noninducing to inducing media. Variations in the ratio of activities of these enzymes during this induction period indicate that their induction was not coordinate. The induction of the β-galactosidases of *Neurospora* was less sensitive to low concentrations of inducers than was the induction of the β-galactosidase of bacteria; the time lag of induction was greater in the eucaryotic organism, and the maximal induction of active enzyme was less than that observed in bacteria.

The separate identities of two β-galactosidase enzymes of *Neurospora crassa* have previously been established by studies of differential induction of activity, by in vitro studies of differential activation and inactivation, by physical separation, and by differences in sedimentation rate (3, 6). The smaller enzyme, having maximal activity at *p*H 4.2 with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate, has been partially purified (5), and induced levels of activity of this enzyme were observed during growth on lactose, D-galactose, D-xylose, or L-arabinose. The larger of the two enzymes (3) shows maximal activity at *p*H 7.5 with the same substrate. They will be designated the "4.2 enzyme" and the "7.5 enzyme," with the designations corresponding to *p*H optima.

The occurrence of two inducible enzymes which act upon the same substrate provides an interesting system for the study of regulation. In addition to the comparison of two inducible enzymes in one organism, responses of the *Neurospora* enzymes may be compared to bacterial β-galactosidase (4) to delineate differences between procaryotic and eucaryotic inductive responses. The ability of both enzymes of *Neurospora* to hydrolyze lactose (Bates, Hedman, and Woodward, *unpublished data*) may suggest a functional relationship between the two and raises the question of coordinate control of their synthesis.

This study describes the inductive response of both *Neurospora* enzymes to the presence of various carbon sources and to mixtures of these. Descriptions of the time dependence of the initiation and termination of the inductive response are included. Portions of this work have been published as an abstract (Bates et al., Genetics **52**:427, 1965).

MATERIALS AND METHODS

Strains. The *Neurospora* strain used for these studies is designated L-5. This strain was isolated from ultraviolet-irradiated conidia of STA 4 wild type by selecting for large colonies on agar medium with lactose as sole carbon source.

Growth of cultures. Neurospora mycelium was grown in liquid standing cultures at 33 C. Low-form culture flasks (2,500-ml capacity) containing 250 ml of liquid medium were used. Vogel's minimal medium (8) was adjusted to *p*H 5.0 with concentrated HCl unless otherwise indicated. Concentrated aqueous solutions of the carbon sources were autoclaved separately from the remainder of the media and were added after cooling to give 1.5% carbon (w/v). The conidial inoculum was grown on minimal medium containing 1.5% sucrose-1.5% agar. Conidia were harvested in sterile water, and the suspension was filtered through four layers of sterile gauze to remove mycelial fragments. Total inoculum size for 250 ml of medium was approximately 2×10^7 viable conidia. Liquid standing cultures were harvested on a Büchner funnel, washed with cold deionized water, and blotted dry. Individual mycelial pads were then frozen, lyophilized, and weighed to determine total growth. Cells were disrupted for extraction of soluble proteins

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by grinding in a Wiley Mill (A. H. Thomas Co., Philadelphia, Pa.). With this procedure, both *Neurospora* β-galactosidases demonstrated reproducible specific activities for any particular carbon source.

Transfer of cultures to fresh medium, where indicated, was accomplished under aseptic conditions by collection of mycelia on a sterile sieve, followed by one wash with sterile deionized water. Much of the liquid trapped in the mycelium was gently expressed with a sterile pestle. The culture was then transferred to the fresh medium, shaken vigorously for even distribution, and returned to standing culture conditions.

Protein extraction and assays. Soluble protein was extracted from the lyophilized, ground mycelium into 0.0125 M tris(hydroxymethyl)aminomethane chloride buffer (*p*H 8.0) at a ratio of 15 ml of buffer per g of mycelial powder. The powder was suspended by agitation for 10 sec on a Vortex mixer and was then packed in ice and agitated for 1 hr on a reciprocal shaker at approximately 25 cycle/min. Extracted samples were centrifuged for 20 min at 30,000 rev/min (71,000 × *g*) at 2 C. The clear sample above the sedimented cell debris and below the thin lipid layer was withdrawn with a capillary pipette and chilled. This sample was the crude extract used for enzyme and protein assays.

Both β-galactosidase enzymes were assayed by their ability to release *o*-nitrophenol from the substrate ONPG. The 7.5 enzyme was assayed by continuous recordings of increase of absorbance at 420 mμ at 37 C in a double-beam spectrophotometer. For this assay, crude extracts were diluted 1:5 with 0.01 M phosphate (Na) buffer, *p*H 7.5. The reaction mixture for assay was: 0.5 ml of phosphate buffer, 0.1 ml of 0.01 M aqueous ONPG, and 0.1 ml of diluted enzyme preparation which initiated the reaction. The reference cell contained 0.6 ml of buffer and 0.1 ml of ONPG. Activity was determined from the slope of the linear portion of the recording.

The 4.2 enzyme was assayed by incubation for 10 min at 37 C in a mixture of 1.5 ml of 0.075 M acetate buffer (Na), *p*H 4.2, and 0.3 ml of 0.01 M aqueous ONPG. The reaction was initiated by addition of 0.3 ml of crude extract. After 10 min of incubation, the reaction was terminated by placing the tubes in boiling water for 2 min. Samples were then chilled, and 0.5 ml of 1.0 M Na_2CO_3 was added. Precipitated protein was removed by centrifugation for 20 min at 15,000 rev/min (29,000 × *g*) at 2 C. Absorbance was determined at 420 mμ, with corrections for separate substrate and sample blanks.

Enzyme activities are presented in arbitrary units. One unit is defined as an increase of 0.001 absorbance unit per min in the indicated assay mixtures.

Total protein content of crude extracts was determined by the method of Lowry (7) as modified by Eggstein and Kreutz, described by Bailey (2). Bovine plasma albumin was used as standard.

Purity of chemicals. All inorganic chemicals were reagent grade. Lactose was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. D-Galactose and D-xylose were obtained from Sigma Chemical Co., St. Louis, Mo., and ONPG was obtained from Calbiochem, Los Angeles, Calif. Sucrose, D-arabinose, L-arabinose, D-ribose, raffinose, D-melibiose, and maltose were obtained from Mann Research Laboratories, Inc., New York, N.Y.

RESULTS

In preliminary studies to establish suitable conditions for growth, cultures were grown on Vogel's medium with the *p*H adjusted with HCl or KOH to various values in the *p*H range 3.5 to 7.0. With sucrose as carbon source, little variation of specific activity of either enzyme was observed over the *p*H range 4.0 to 6.0, although growth decreased markedly near the extreme values of *p*H. With lactose as carbon source, specific activities of the 4.2 and 7.5 enzymes varied with *p*H of the medium. These studies led to selection of *p*H 5.0 medium as a compromise between growth, which was slight at *p*H 3.5 or *p*H 7.0, and induced activity, which was high for the 7.5 enzyme at either *p*H extreme and maximal for the 4.2 enzyme at the lower *p*H.

Neurospora grown with sucrose, D-glucose, D-fructose, maltose, raffinose, or glycerol as sole carbon source produced a low, basal level of both β-galactosidase activities (Table 1). Of the other carbon sources studied, the maximal induction of both activities was produced by D-galactose. This response was significantly greater than that produced by lactose. A different type response was produced by D-xylose, which had previously been shown to induce the 4.2 enzyme

TABLE 1. *Specific activities of 4.2 and 7.5 β-galactosidase enzymes from standing cultures grown on single carbon sources*[a]

Carbon source	Specific activity (units/mg of protein)		Total growth (dry wt in g)
	4.2 enzyme	7.5 enzyme	
Sucrose	11	17	0.67
D-Glucose	11	15	0.65
D-Fructose	14	16	0.70
Maltose	12	19	0.72
Raffinose	14	28	0.48
Glycerol	11	18	0.72
D-Xylose	102	17	0.32
L-Arabinose	144	49	0.44
Lactose	68	82	0.84
D-Galactose	172	170	0.33
D-Arabinose	Insufficient material for assay		0.02
D-Ribose	Insufficient material for assay		0.04
D-Melibiose	Insufficient material for assay		0.02

[a] Cultures were grown for 96 hr.

(Bates et al., Genetics **52**:427, 1965). D-Xylose did not alter the basal level of the 7.5 enzyme. L-Arabinose, which induced primarily the 4.2 enzyme, produced only slightly elevated levels of the 7.5 enzyme.

Responses to mixtures of carbon sources are summarized in Table 2. When carbon sources which when used alone yield the basal activity level of both enzymes were mixed with galactose, the inductive responses of the enzymes were decreased. Of these, glycerol interfered least. In the mixtures with lactose, only sucrose decreased the level of induced activity.

The effects of sucrose, lactose, and galactose were selected for further study. Cultures grown on each of these sugars were harvested in a series of various times after inoculation. The results are summarized in Fig. 1 for sucrose, in Fig. 2 for lactose, and in Fig. 3 for galactose. The two upper curves of each figure represent specific activity of the 4.2 and 7.5 enzymes, and the lower curve of each figure represents total growth expressed in grams (dry weight).

In cultures grown on sucrose (Fig. 1), the level of the 7.5 enzyme activity was markedly dependent upon the age of the culture. The maximal level of this response remained far below levels obtained with "inducing" sugars. Less dependence upon age of the culture was shown by the 4.2 enzyme.

With lactose and galactose (Fig. 2 and 3), induction reached a maximum in very young cultures. The subsequent decrease of specific activity was perhaps due to dilution of synthesized enzyme by other soluble proteins produced

TABLE 2. *Specific activities of 4.2 and 7.5 β-galactosidase enzymes from standing cultures grown on carbon source mixtures*[a]

Carbon source (1.0% + 0.5%)	Specific activity (units/mg of protein)		Total growth (dry wt in g)
	4.2 enzyme	7.5 enzyme	
Lactose + glycerol	96	119	0.92
Lactose + D-glucose	73	103	0.70
Lactose + D-fructose	74	95	0.86
Lactose + sucrose	46	68	0.54
Lactose + D-xylose	101	58	0.67
Lactose + D-galactose	115	135	0.79
D-Galactose + lactose	160	224	0.55
Glycerol + D-galactose	131	82	0.89
D-Xylose + D-galactose	160	38	0.55
D-Fructose + D-galactose	72	54	0.69
Sucrose + D-galactose	71	68	0.65
D-Glucose + D-galactose	76	62	0.65

[a] Cultures were grown for 96 hr.

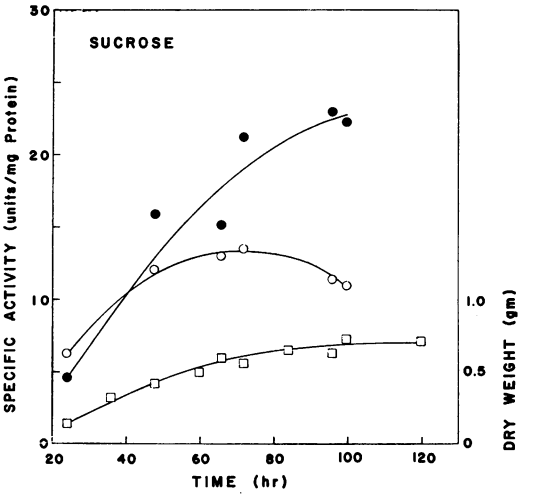

FIG. 1. *Continuous growth of standing cultures with sucrose as sole carbon source.* ●, *7.5 enzyme;* ○, *4.2 enzyme;* □, *total dry weight of culture.*

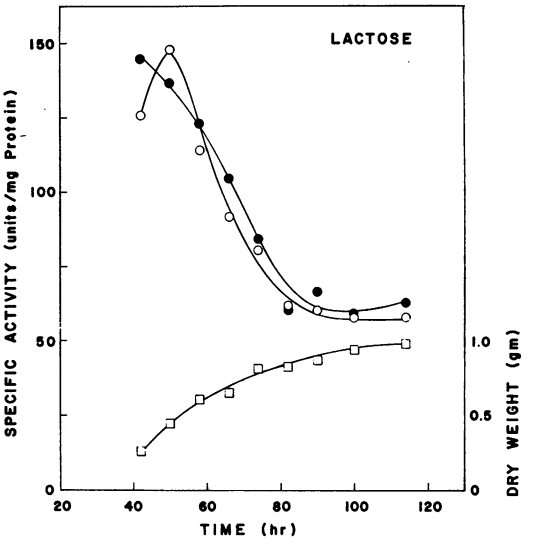

FIG. 2. *Continuous growth of standing cultures with lactose as sole carbon source.* ●, *7.5 enzyme;* ○, *4.2 enzyme;* □, *total dry weight of culture.*

in proportion to total dry weight of the culture. This suggests that production of both activities ceased at 45 hr with lactose and at 60 hr with galactose. Through the entire period studied, galactose induced higher activities of both enzymes than did lactose. The time required for growth to reach the maximal rate was dependent upon the carbon source, as seen in the lower curves of Fig. 1 to 3. Sucrose produced growth

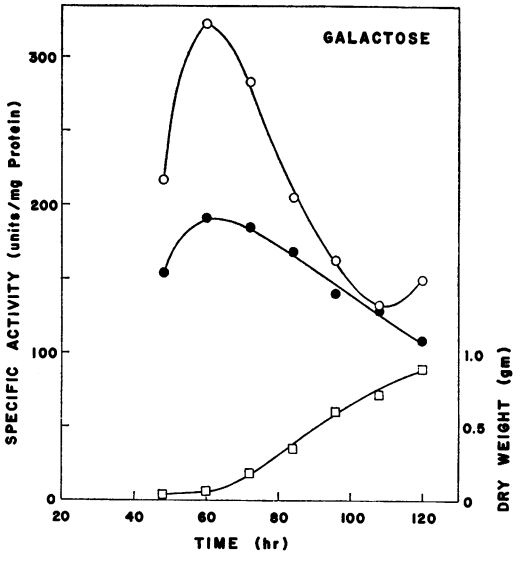

FIG. 3. *Continuous growth of standing cultures with* D*-galactose as sole carbon source.* ●, *7.5 enzyme;* ○, *4.2 enzyme;* □, *total dry weight of culture.*

more rapidly than did lactose, and lactose produced growth more rapidly than did galactose.

The interval between inoculation and expression of the maximal inductive response cannot be studied in detail by this continuous growth method because of limitations in preparation of small amounts of mycelium. A transfer procedure was therefore developed to study the early stages of the inductive response. Cultures were grown on sucrose for 48 hr and transferred to fresh lactose or galactose media (Fig. 4 and 5). After transfer to lactose (Fig. 4), induction occurred rapidly, with specific activities of both enzymes reaching a maximum approximately 48 hr after transfer. After transfer to galactose (Fig. 5), a longer delay occurred before onset of induction, and specific activities of the two enzymes reached a maximum approximately 96 hr after transfer. Before detectable growth occurred, induced activities reached approximately 30% of the maximal observed response. Control transfers from sucrose to sucrose showed an initial loss of 20% of total dry weight within the 1st hr after transfer and a return to the normal rate of growth within 8 hr after transfer.

The similarity of the response of the two enzymes to lactose and galactose is most apparent in the initial portion of the curves and in the interval near the maximal response, which occurred at approximately the same time for both. The two activities did not, however, show a constant ratio throughout the course of induction as do bacterial proteins whose synthesis is directed by a single polycistronic messenger (1, 9). Deviations from coordinate response were more apparent in cultures induced by galactose than in those induced by lactose.

The transfer procedure was also useful for

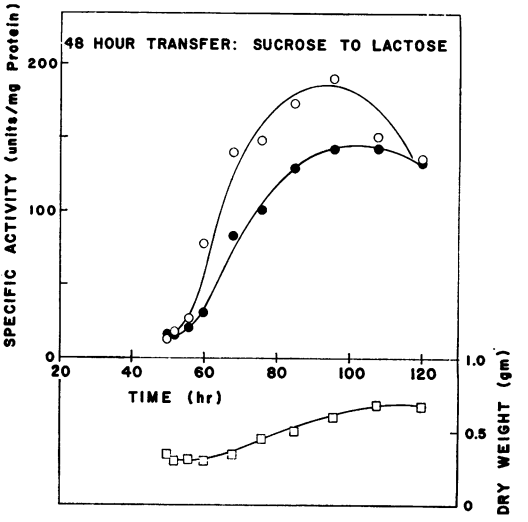

FIG. 4. *Standing cultures grown 48 hr on sucrose, then washed and transferred to lactose media.* ●, *7.5 enzyme;* ○, *4.2 enzyme;* □, *total dry weight of culture.*

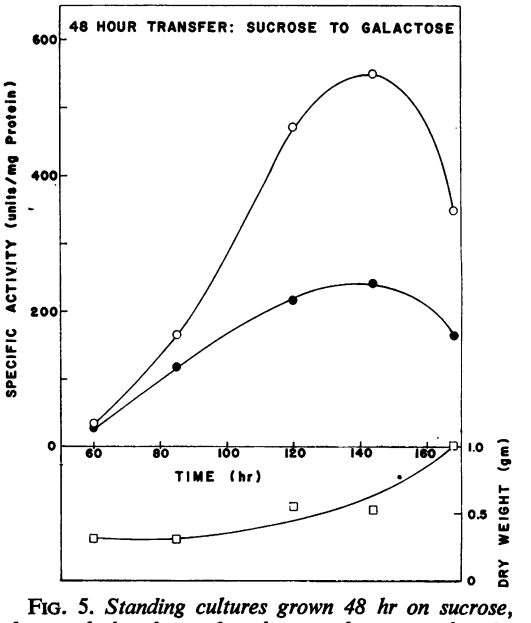

FIG. 5. *Standing cultures grown 48 hr on sucrose, then washed and transferred to* D*-galactose media.* ●, *7.5 enzyme;* ○, *4.2 enzyme;* □, *total dry weight of culture.*

study of the termination of the inductive response. Cultures grown on lactose or on galactose were transferred to sucrose by the procedure previously described. The resulting specific activities and growth as a function of time are presented in Fig. 6 and 7. Lactose cultures were transferred after 52 hr of growth and galactose cultures were transferred after 94 hr of growth. These times provided total growth corresponding to that of 48-hr sucrose cultures, as indicated in the growth curves of Fig. 1 to 3.

After transfer from lactose to sucrose, the specific activity of the 4.2 enzyme decreased approximately in proportion to subsequent growth, indicating dilution of this activity by newly synthesized protein. The specific activity of the 7.5 enzyme decreased more rapidly, as did activities of both enzymes after transfer from galactose to sucrose. This effect indicates possible inactivation in addition to dilution, although the in vivo stabilities of both enzymes have yet to be determined.

Response to low concentrations of inducers was examined by growing cultures on glycerol for 48 hr and adding, at that time, lactose, galactose, ONPG, or *o*-nitrophenol. Cultures were harvested after 48 hr of exposure to inducer. Activities of the two enzymes (Table 3) showed

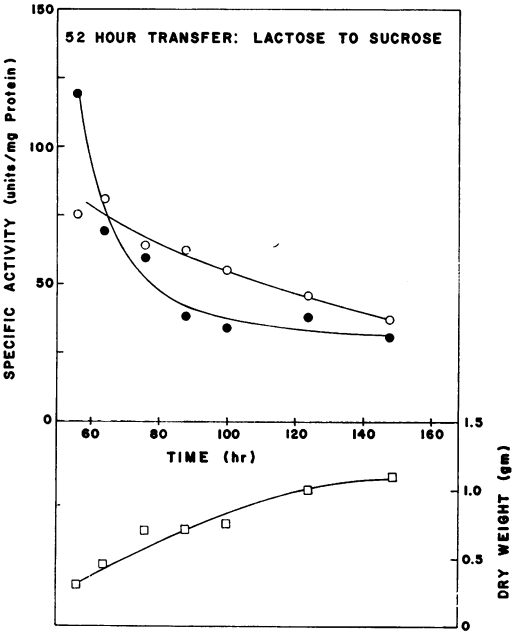

FIG. 6. *Standing cultures grown 52 hr on lactose, then washed and transferred to sucrose media.* ●, *7.5 enzyme;* ○, *4.2 enzyme;* □, *total dry weight of culture.*

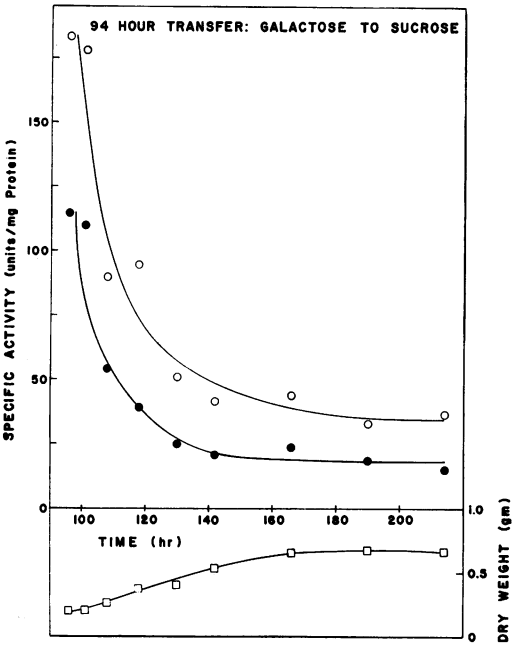

FIG. 7. *Standing cultures grown 94 hr on* D-*galactose, then washed and transferred to sucrose media.* ●, *7.5 enzyme;* ○, *4.2 enzyme;* □, *total dry weight of culture.*

TABLE 3. *Specific activities of 4.2 and 7.5 β-galactosidase enzymes from cultures grown on 1.0% glycerol with inducers added*[a]

Inducer added[b]	Specific activity (units/mg of protein)		Total growth (dry wt in g)
	4.2 enzyme	7.5 enzyme	
D-Galactose, 2.1×10^{-4} M	17	16	0.41
D-Galactose, 2.1×10^{-3} M	57	26	0.50
D-Galactose, 2.1×10^{-2} M	125	48	0.45
D-Galactose, 8.3×10^{-2} M	159	104	0.60
Lactose, 2.1×10^{-4} M	16	17	0.40
Lactose, 2.1×10^{-3} M	26	30	0.44
Lactose, 2.1×10^{-2} M	48	51	0.49
Lactose, 4.2×10^{-2} M	93	66	0.73
ONPG, 2.1×10^{-3} M	16	12	0.21
ONP, 2.1×10^{-3} M	14	15	0.21

[a] Inducers added at 48 hr and cultures were left an additional 48 hr before harvest. Concentrations of 8.3×10^{-2} M D-galactose and 4.2×10^{-2} M lactose correspond to 1.5% of these sugars.

[b] ONPG = *o*-nitrophenyl-β-D-galactopyranoside; ONP = *o*-nitrophenol.

a similar dependence upon lactose concentration. Specific activity of the 7.5 enzyme showed similar responses to varying concentrations of galactose and of lactose. The 4.2 enzyme showed a greater response to low levels of galactose than did the 7.5 enzyme. At low concentrations of either galactose or lactose, induction of 4.2 activity was higher on galactose.

Other possible inducers were tested by transferring sucrose cultures grown 48 hr to 10^{-3} M solutions of the inducers. Phenyl-β-D-galactopyranoside, methyl-β-D-galactopyranoside, methyl-β-D-thiogalactopyranoside, isopropyl-β-D-thiogalactopyranoside, and phenyl-β-D-thiogalactopyranoside were tried. None induced as well as lactose solutions of the same concentration, and induction was less than twofold in all cases.

DISCUSSION

In contrast to the basal activity levels of both *Neurospora* β-galactosidase enzymes during growth on sucrose and various other noninducing carbon sources, lactose or D-galactose will induce relatively high levels of activity of both enzymes. The induction of maximal activity in standing cultures during growth on D-galactose was about 10-fold for the 7.5 enzyme and about 15-fold for the 4.2 enzyme.

During growth of cultures on lactose or on D-galactose, the ratios of the two activities were not constant, but showed variation with time as demonstrated by comparison of the shapes of the plots of specific activities as a function of time. Variation in the ratios of the activities is also obvious in the tabulated values for specific activities as a function of galactose concentration. These variations demonstrate that production of the two enzyme activities was not coordinate under these conditions. However, the specific activities may not necessarily be directly related to synthesis of the corresponding proteins.

Induction of activity of the *Neurospora* β-galactosidases contrasts in three ways with β-galactosidase induction in bacteria. Bacteria typically respond to the presence of inducer within minutes, whereas increased activities of the *Neurospora* enzymes could not be readily demonstrated until a minimum of 2 hr after exposure to inducer, and the maximal response in this eucaryote required 40 hr or longer. The maximal response in bacteria is 1,000-fold or more (4), whereas 15-fold was the maximum observed in these *Neurospora* standing cultures. Relative sensitivities of the induction process was indicated by the barely detectable response shown by the *Neurospora* enzymes to inducer concentrations of 10^{-3} M, whereas this concentration can induce bacterial β-galactosidase.

In the studies in which *Neurospora* cultures were transferred from noninducing to inducing media (Fig. 4 and 5), there was a pronounced lag in growth concomitant with induction. The relationship between this growth lag and the induction process has yet to be elucidated. Did induction occur at the expense of growth, or did the transfer process cause a cessation in growth which in turn elicited a lengthened induction process? It is noteworthy, however, that in transfer from inducing to noninducing media (Fig. 6 and 7) there was little or no lag in growth.

The relative insensitivity of *Neurospora* β-galactosidases to small amounts of inducers places severe limitations upon the study of various synthetic β-galactosides as inducers. The galactosides and thiogalactosides studied at a concentration of 10^{-3} M elicited barely detectable increases in activity of the *Neurospora* enzymes, yielding no information regarding the relative efficiency of these inducers except that none produced a greater response than lactose at the same concentration. Thus, low levels of these gratuitous inducers cannot be used for kinetic studies of induction of these enzymes, and interpretation of the kinetic responses obtained with lactose and galactose is complicated by metabolism of these sugars.

ACKNOWLEDGMENT

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