LINKED INDUCTION OF GLYCOSIDASES IN *STACHYBOTRYS ATRA*

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Summary

When phenyl β-D-thioglucopyranoside is added to a washed mycelial suspension of *S. atra*, strain CMI 32542, under conditions previously shown to result in the "inductive" synthesis of β-glucosidase, a series of other glycosidases and an esterase is concomitantly formed. The same enzymes are not formed in each experiment, but a series E_1, E_2, ... can be set up such that synthesis of E_n is not observed unless E_{n-1} is also synthesized. Induction by "homologous" inducers (e.g. phenyl β-D-thioxylopyranoside for β-xylosidase, phenyl α-D-thioglucopyranoside for α-glucosidase, etc.) is not observed. A certain slight increase in the activity of enzymes of this group is observed on prolonged (up to 24 hr) shaking of mycelial suspensions in the absence of any inducer. This increase is much less than that occurring in the presence of phenyl β-D-thioglucopyranoside, and the two phenomena can normally be easily distinguished.

Besides the obvious deduction from the fact that their simultaneous appearance is not obligatory, the non-identity of the glycosidases can also be inferred from their pH–activity curves. The double-peaked curve for β-galactosidase activity, of which only one section is given by the β-galactosidase produced during growth on lactose, suggests that two β-galactosidases are induced by the thioglucoside.

The prolonged presence of the thioglucoside appears to lift the barrier to the transcription of a segment of DNA rather greater than that covered by the normal concept of an operon. Repeated negative findings for some enzymes suggests that this segment is not, however, coextensive with the complete DNA complement. It is possibly a segment of it for which the normal repressor control is more than usually labile.

In contrast to former negative results for agents that interfere with transcription, cycloheximide and emetine have now been found to be potent inhibitors of the "induction", thus confirming that it does in fact involve protein synthesis *de novo*.

I. INTRODUCTION

It has previously been assumed (Jermyn 1965, 1967) that the induction of the aryl β-D-glucopyranosidase (β-glucosidase) of *Stachybotrys atra* is a unique phenomenon in this fungus. This enzyme was readily induced in washed mycelial suspensions in the absence of growth by the addition of a variety of β-D-glucopyranosides and β-D-thioglucopyranosides. However, when identical mycelial suspensions were incubated in the presence of glycosides derived from monosaccharides other than β-D-glucopyranose, no sign could be discovered of the induction of the appropriate "homologous" enzymes related to the potential inducers in the same way that β-glucosidase is to phenyl β-D-thioglucopyranoside. To see whether the fungus was in fact capable of producing such enzymes a strain of *S. atra*

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(CMI 32542) was selected that was capable of profuse growth on lactose as the carbon source in shake culture. Under these conditions a β-galactosidase was synthesized in quantity, but attempts using phenyl β-D-thiogalactopyranoside to induce β-galactosidase in the washed mycelium, whether grown on lactose or other carbon sources, were unsuccessful. In the course of certain control experiments it was noted, however, that β-galactosidase was produced in the presence of phenyl β-D-thioglucopyranoside when it was not produced in the presence of phenyl β-D-thiogalactopyranoside. The present paper records an attempt to delimit this phenomenon of "non-specific" induction.

It has been evident throughout this work that the induction of β-glucosidase in *S. atra* shows features which require some addition to the standard model put forward by Jacob and Monod (1961) for *Escherichia coli*. In particular, the resistance of the process to inhibitors of protein synthesis other than the ribosome-disrupting streptomycin has raised great difficulties in interpretation. There was no evidence to decide amongst inability of these inhibitors to gain access to the interior of the cell, absence of *de novo* protein synthesis, and an extreme of "species specificity". The finding that cycloheximide and emetine, which inhibit aminoacyl–*s*RNA transferase (Grollman 1966), are also potent inhibitors of the induction appears to clear away many of these difficulties.

II. Materials and Methods

(*a*) *Enzyme Determinations*

Since it was impractical to test more than a selection of the possible enzyme activities involved in the phenomenon of simultaneous induction, a decision was made to limit the testing to hydrolytic enzymes for which simple chromogenic reactions were available. The results to be discussed indicate that this was probably a reasonable limitation since a number of enzymes fulfilling these requirements but remote from the glycosidases in substrate requirement and mechanism did not appear to be involved in the induction, and there is no evidence that purely random testing, in the absence of any guiding hypothesis, of a wider selection of enzymes would yield any further information.

The example of the β-galactosidase, which was liable to be wrongly considered as absent if sought for under the optimum conditions for the β-glucosidase, showed that testing under a variety of pH conditions would be necessary to exclude the presence of a given enzyme. In general, enzyme activity was tested at intervals of 0·5 pH units over the pH range 3–8 in citric acid–sodium phosphate buffers, although, once the optimum conditions for an enzyme were known, subsequent testing for this enzyme was normally confined to this optimum. Phosphatase and sulphatase, which might be inhibited by this buffer, were tested over the pH range 3–9 using acetate and sulphopropylmorpholine (MPS) buffers. The pH range 5–9 was covered for proteinase using citric acid–sodium phosphate and MPS buffers. For sucrase the approximate pH optimum was 5, for amylase and phosphatase 6, and for sulphatase 7.

All glycosidases except sucrase were measured by the use of the appropriate *p*-nitrophenyl glycoside. Sucrase was measured by the determination of reducing sugar liberated from sucrose, and amylase by the fall in the blue colour with iodine using soluble starch (AnalaR, Hopkins and Williams) as substrate. The substrates for esterase were either *p*-nitrophenyl acetate or 3,5,3′,5′-tetrabromophenolsulphonphthalein diacetate. Phosphatase and sulphatase were measured using *p*-nitrophenyl phosphate and sulphate respectively. The routine substrate for proteinase estimation was *p*-dimethylaminobenzeneazocasein (Jermyn 1953); to check the negative results obtained with this substrate the other chromogenic substrates Azocoll (Koch-Light) and Azoalbumin (Sigma) were also tested.

In general, the total suspension was disrupted by ultrasonic disintegration and enzyme activity measured in the supernatant after centrifugation at 2000 *g*, thus making no distinction between intracellular and secreted enzyme. Where the cloudiness of the supernatant was troublesome, it was frozen at −20°C for 24 hr to coagulate the particulate matter, thawed, and recentrifuged; measured enzyme activities could not be shown to be significantly reduced by this treatment. However, as the data of Table 4 show, it does lead to significantly different partition of different enzyme activities between the particulate and non-particulate fractions. Clearing by freezing has therefore only been used routinely for those enzymse (β-glucosidase, β-xylosidase, etc.) known to be overwhelmingly concentrated in the non-particulate (supernatant) fraction.

(*b*) *Induction Experiments*

Induction experiments were carried out in exactly the same way as already reported for the induction of β-glucosidase by phenyl β-D-thioglucopyranoside (Jermyn 1965). "Inducers" were tested up to a maximum concentration of 10^{-1}M and a time of 24 hr before their action was reported as nil.

(*c*) *Materials*

All glycosides and thioglycosides used were laboratory preparations, with physical constants agreeing with those in the literature.

Phenyl 3-O-methyl-β-D-thioglucopyranoside

3-*O*-Methyl-D-glucose (1·0 g) was added portionwise with shaking to acetic anhydride (5 ml) containing 70% $HClO_4$ (0·05 ml). When dissolution was complete a solution of anhydrous HBr in acetic acid (10 ml; 45% w/v) was added. After working up in the usual way, the final chloroform solution was evaporated *in vacuo*, and the residue dissolved in acetone (5 ml). To this solution was added a mixture of acetone (5 ml), thiophenol (0·7 ml), and 2N NaOH (3 ml). Crystallization of the product began rapidly and was assisted by the cautious addition of more water (7 ml). The crystalline product (1·60 g; 75% yield) was filtered off after 2 hr, and recrystallized from methanol to give *phenyl 2,4,6-tri-O-acetyl-3-O-methyl-β-D-thioglucopyranoside* as glistening white needles, m.p. 133°C uncorr., $[\alpha]_D^0$ −68° (*c*, 2·8 in acetone). Calc. for $C_{19}H_{24}O_8S$: C, 55·3; H, 5·9; S, 7·8; $-OCH_3$, 7·5%. Found: C, 55·2; H, 5·7; S, 7·8; $-OCH_3$, 7·4%.

The triacetate was catalytically deacetylated with sodium in methanol to give a nearly quantitative yield of *phenyl 3-O-methyl-β-D-thioglycopyranoside*, recrystallized from water as white needles, m.p. 124°C (after vacuum drying at room temperature; freshly recrystallized and air dried the m.p. was 80°C in a preheated tube and the material was apparently a hydrate), $[\alpha]_D^{20}$ −52·2° (*c*, 10·5 in ethanol). Calc. for $C_{13}H_{18}O_5S$: C, 54·5; H, 6·3; S, 11·2%. Found: C, 54·4; H, 6·7; S, 11·1%.

3,5,3′,5′-Tetrabromophenolsulphonphthalein diacetate (diacetate of bromophenol blue) was prepared according to Orndoff and Sherwood (1923) and recrystallized from methanol until the filtrate of the suspension formed by pouring a dilute methanolic solution into water showed no optical absorption at 525 mμ above a blank. Although the solubility of this compound in water is limited ($\simeq 10^{-5}$M) it forms an excellent diagnostic substrate and may be used in suspension form. In agreement with Orndoff and Sherwood (1923), it is almost indefinitely stable in neutral media and the mixtures will sustain incubation for 24 hr at 28°C and pH 7–8 with negligible blank colour formation. It has the added advantage of giving the final alkaline colour of bromophenol blue at all pH values above 4, thus facilitating progress measurements.

N-(*3-sulphopropyl*)*morpholine*

Morpholine and propanesultone were reacted in water according to the method used earlier in similar reactions (Jermyn 1967). The aqueous solution was concentrated and the product precipitated by adding excess ethanol. Three recrystallizations from aqueous ethanol gave coarse white needles, m.p. 274–6°C, of N-(*3-sulphopropyl*)*morpholine*. Found: C, 40·3; H, 7·2; N, 6·8; S, 15·4%. Calc. for $C_7H_{15}NSO_4$: C, 40·2; H, 7·2; N, 6·7; S, 15·3%. For a 0·05M aqueous solution at 20°C, the value of pK_a determined from the titration curve was 7·10. It was observed

earlier that there is an increase of about one unit in pK_a value in passing from an *N*-sulphoethyl to the corresponding *N*-sulphopropyl compound (Jermyn 1967). This observation is confirmed when the pK_a value for MPS is compared with that (6·15) determined by Good *et al.* (1966) for the buffering agent *N*-(2-sulphoethyl)morpholine.

Stock MPS buffers used in the work on pH–activity curves of glycosidases were 0·05M, adjusted with NaOH. The final concentration in the standard method was thus 0·01M in MPS.

(*d*) *Selection of a* Stachybotrys *Strain*

As a variety of strains from the related genera *Memnoniella* and *Stachybotrys* were available, and since the strain of *S. atra* (DSL 1) used previously in these studies had never been found to produce more than traces of β-galactosidase, the screening of these new strains to find one capable of producing this enzyme in quantity was an essential preliminary to the proposed studies.

As a screening test, the ability to grow in serial transfer on McQuade (Jermyn 1965) mineral medium using lactose as the sole carbon source was used. Such growth is unlikely in the absence of an effective β-galactosidase. Of the strains tested, only three were able eventually to sustain a growth rate on lactose comparable to that observed on glucose, sucrose, or starch. When these three strains [CMI 32542 and 42311 and *S. atra*, var. *genuina* (Centraalbureau voor Schimmelcultures, Baarn)], had attained such a growth rate that the logarithmic phase was reached in 2–3 days in shake culture at 28°C, the cultures were sampled at various stages from inoculation to senility. The samples of mycelium were disrupted ultrasonically without previous washing, the resulting suspensions centrifuged, and β-galactosidase estimated in the resulting supernatants. The levels of β-galactosidase were found to be 5–10 times higher in CMI 32542 at all stages than in the other two strains. This strain was therefore used in all subsequent work.

III. Results

(*a*) *Properties of the β-Galactosidase Induced by Growth on Lactose*

The properties of this enzyme are of no particular interest in themselves and are not in any way different from the general run of glycosidases. They are summarized in Table 1 where they are compared with those of the β-xylosidase of CMI 32542 and also of aryl β-glucosidase and of the *exo*-β-glucosidase (cellobiase) as they have been determined in previous work. The equivalence of the pH–activity curves for the β-glucosidase from CMI 32542 [Fig. 1(*a*)] and DSL 1 suggests that any differences in this enzyme between the two strains can only be very minor.

The properties of the β-galactosidase of *S. atra* agree in a general way with those of the β-galactosidase of *E. coli* (Kuby and Lardy 1953) and those of the cellobiase of *S. atra* (Youatt 1958) rather than with the aryl β-glucosidase. The enzyme is a general *exo*galactosidase, removing a terminal β-galactosyl group from any appropriate substrate.

Two points of general interest may be noted. Firstly, the pH–activity curve [Fig. 1(*b*)] shows a sharp optimum at pH 6·8 and practically zero activity at pH 5·0. Since this last pH is the standard one for the determination of β-glucosidase activity, it is likely that some past reports we have made, based as they were on the substitution of *p*-nitrophenyl β-D-galactopyranoside as a potential substrate for *p*-nitrophenyl β-D-glucopyranoside with all other conditions remaining unchanged, of the absence of β-galactosidase activity in certain circumstances were in error. Secondly, *ortho*-substitution as against *para*-substitution in otherwise identical aryl glycosides leads to an increase in V and K_m for the β-galactosidase and β-xylosidase and a decrease for

the β-glucosidase. Thus the conjecture of Veibel and Yang (1952) that depression by *ortho*-substitution is a constant feature of fungal β-glucosidases cannot be carried over to the other β-glycosidases even when the comparison is made for a single organism.

TABLE 1

COMPARISON OF THE ENZYMIC AND INDUCTIVE PROPERTIES OF THE β-GALACTOSIDASE OF *S. ATRA* CMI 32542 AND CERTAIN OTHER GLYCOSIDASES OF *S. ATRA*

All kinetic data were determined at the pH optimum of the enzyme concerned

Inductive or Enzymic Property	β-Galactosidase from Strain CMI 32542	β-Xylosidase from Strain CMI 32542	β-Glucosidase from Strain DSL 1	Cellobiase from Strain DSL 1
Produced by growth on homologous carbon source	Yes	No data	Yes	Yes
Induced by homologous thioglucoside	No	No	Yes	No
Induced by phenyl thioglucoside	Yes	Yes	Yes	No
Induced by homologous disaccharide	No	No data	No	Yes
Oligosaccharide substrates	Lactose	No data	None	Cellobiose
Alkyl glycoside substrates	Yes	No data	No	Yes
Aryl glycoside substrates	Yes	Yes	Yes	Yes
pH optimum	6·8	7·2	5·0	5·5
Activated by alkali metal cations	Yes	No	No	Yes
Inhibited by 1,5-gluconolactone	Slightly	Slightly	$K_i = 5 \times 10^{-6}$M	No
Inhibited by 1,4-galactonolactone	$K_i = 10^{-4}$M	No	Slightly	No
K_m against *o*-nitrophenyl glycoside	$1{\cdot}1 \times 10^{-3}$M	$9{\cdot}3 \times 10^{-5}$M	$2{\cdot}1 \times 10^{-4}$M	No data
K_m against *p*-nitrophenyl glycoside	$2{\cdot}7 \times 10^{-3}$M	$2{\cdot}2 \times 10^{-4}$M	$3{\cdot}2 \times 10^{-5}$M*	5×10^{-3}M
V_o/V_p †	2·35	1·45	0·18	No data

* K_m for the α-glucosidase and *p*-nitrophenyl α-D-glucopyranoside = $4{\cdot}8 \times 10^{-3}$M.

† Ratio of the values of V for the *o*- and *p*-nitrophenyl glycosides at the same enzyme concentration.

(*b*) *Synthesis of β-Galactosidase during Growth on Lactose*

The induced synthesis of the β-galactosidase during growth on lactose, like its properties, showed no points of novelty. Once the lag period had passed, enzyme production was proportional to mycelial weight. On changing from growth on starch medium to serial transfer on lactose medium, the proportionality constant (enzyme/mycelial weight) rose steadily to a maximum after four or five transfers. This behaviour suggests the diluting out of some type of repressing factor.

A large number of attempts were made to obtain growth-independent induction of the β-galactosidase parallel with the "induction" of β-glucosidase, but without success. These experiments were made with mycelium grown on starch, sucrose,

glucose, and lactose; at physiological ages ranging from 2 to 7 days of growth in shake culture at 28°C; and with lactose, methyl β-D-galactopyranoside, and isopropyl and phenyl β-D-thiogalactopyranosides. Since the growth-dependent synthesis of the β-glucosidase when the mould is grown on salicin as carbon source is quite parallel to the growth-dependent synthesis of β-galactosidase (Jermyn, unpublished observations), there is obviously a wide gap in mechanism between these two inductions in the Jacob–Monod pattern and the growth-independent synthesis triggered by the presence of phenyl β-D-thioglucopyranoside. The thioglucoside-induced β-xylosidase is formed in negligible quantities during growth on salicin.

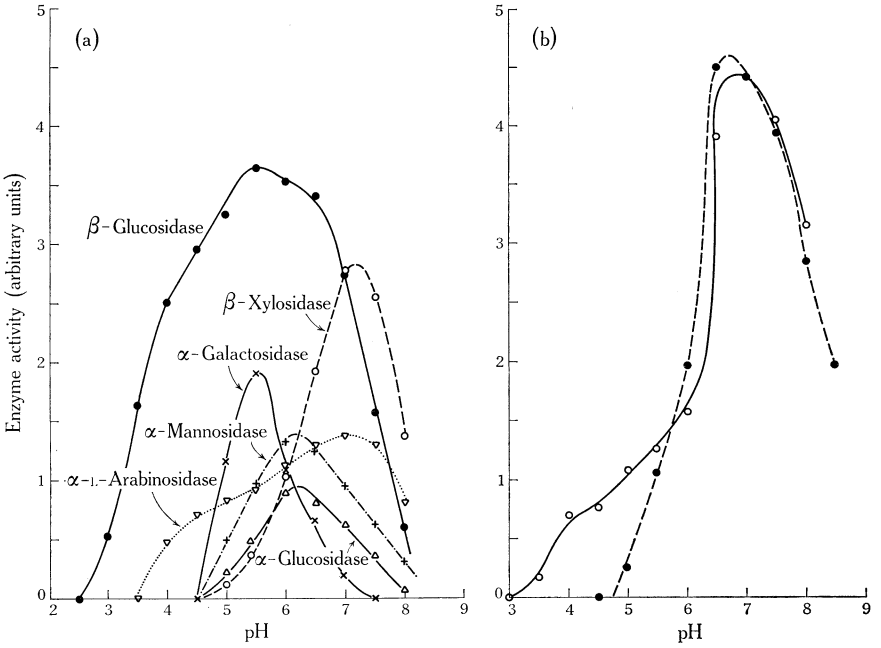

Fig. 1.—(*a*) pH-activity curves for a number of glycosidases present in the supernatant obtained from ultrasonic disintegration of a phenyl β-D-thioglucopyranoside-induced *S. atra* mycelial suspension. Substrate throughout, 10^{-3}M *p*-nitrophenyl glycoside; buffer, citric acid–sodium phosphate. The levels of enzyme activity (expressed in arbitrary but identical units) have not been adjusted relative to one another and are thus true comparative values in a randomly chosen sample. (*b*) pH–activity curves for β-galactosidase present in the supernatant of ultrasonically disrupted mycelium from *S. atra* CMI 32542. ● Sample obtained from mycelium after growth for 3 days on lactose medium. ○ Sample obtained from starch-grown mycelium induced for 24 hr with phenyl β-D-thioglucopyranoside (1 mg/ml).

(*c*) *pH–Activity Curves for the Enzymes Induced by Phenyl Thioglucoside*

It might be supposed, in spite of the known specificities of the β-glucosidase and β-galactosidase and the fact that the co-appearance of none of the glycosidases that can be simultaneously induced by phenyl β-D-thioglucopyranoside is invariable, that these activities are the common properties of a single enzyme. As an additional piece of evidence that this is not so, the pH–activity curves of those enzymes known to be simultaneously induced were determined. All of these except that of β-galactosidase are shown in Figure 1(*a*). They show little in common and, since

none of the substrates are ionizable, it is difficult to suppose that a single enzyme acting on these substrates could produce this set of curves.

The pH–activity curves for the β-galactosidase induced by growth on lactose and that induced by phenyl thioglucoside are set out in Figure 1(*b*). The straightforward interpretation of these data is to suppose that phenyl thioglucoside induces the formation of two β-galactosidases, one corresponding to that induced by growth on lactose and a second, an "acid β-galactosidase", with a pH optimum around 4·5.

Since β-xylosidase appears to be the most consistent product of the induction by phenyl thioglucoside, and its level has been used as a measure of induction in later sections of this paper, the proof of its non-identity with β-glucosidase is of some importance. Besides the evidence from pH–activity curves and the occasional non-appearance of β-glucosidase in extracts containing β-xylosidase, a third datum is the great difference in stability in acid solutions. Figure 2 shows that at pH 4·1 β-glucosidase is stable at 22°C, and β-xylosidase is rapidly inactivated. The kinetics of this inactivation are approximately second order.

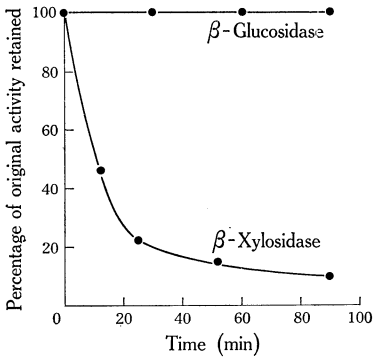

Fig. 2.—Comparative loss of activity of the β-glucosidase and β-xylosidase in the same sample at pH 4·1 and 22°C in 0·01M sodium acetate buffer.

(*d*) *Linked Induction*

Of the enzymes tested for in the supernatants from ultrasonically disrupted *S. atra* mycelium only α-D-arabinosidase (using *p*-nitrophenyl α-D-arabinopyranoside as substrate) and proteinase could not be demonstrated. The absence of the first of these is not surprising in view of the extreme rarity of this enzyme in nature, but that of proteinase is puzzling. The wrong substrates may have been used, or the enzymes may have needed activating, or they may not have been released from the particulate fraction by ultrasonic disintegration; it is most unlikely that they were not present initially. The absence of any effective proteinase activity may also be gauged from the fact that other enzyme activities in the supernatants were stable for some days at refrigerator temperatures in the absence of microbial growth.

Allowance had to be made for the fact that in CMI 32542 the observed changes in the levels of enzyme activities of washed mycelium shaken at 28°C are not the same as in DSL 1. In both strains there was a slight decline in levels in the first few hours of shaking, but this was followed for CMI 32542 by a slow rise over the period

8–24 hr to a level for most enzymes about 1·5–2·5 times the minimal level. A number of examples can be seen in Figure 3. It would appear that the repressor mechanisms become "leaky" under the influence of prolonged starvation. However, if an enzyme was induced at all, its level of activity after 24 hr was usually several times the final level in the control. A few doubtful cases have been marked as such in the tables.

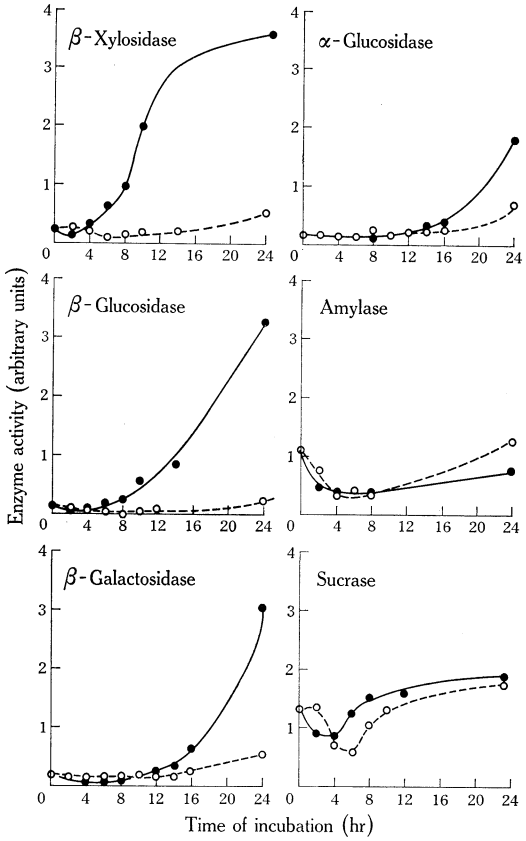

Fig. 3.—A sample of a washed mycelial suspension from *S. atra* CMI 32542 was divided into halves, both of which were shaken at 28°C, one of them with the addition of phenyl β-D-thioglucopyranoside (1 mg/ml). Samples were taken from both at intervals, disrupted ultrasonically, and the supernatants tested for the enzyme activities shown in the figure. O Non-induced mycelium.

The most obvious feature of the simultaneous induction process was a sequence which could be deduced by applying two mutually concordant criteria. These were the frequency with which the enzyme was induced and the relative amount of the enzyme induced. If enzyme A were induced more frequently than enzyme B, then in any experiment in which both enzyme A and enzyme B were induced, relatively more of enzyme A was induced than of enzyme B. The correlation can be seen directly by inspection of Table 2, which records the data for frequency of induction, and Table 3, which records actual numerical data for an experiment in which all the

TABLE 2

FREQUENCY WITH WHICH CERTAIN ENZYMES ARE INDUCED BY PHENYL β-D-THIOGLUCOPYRANOSIDE (1 MG/ML) IN SHAKEN SUSPENSIONS OF WASHED MYCELIUM OF *S. ATRA* CMI 32542 AT 28°C

The fractions are the number of positive inductions divided by the number of experiments in which this particular activity was tested for. All experiments were carried out under conditions that were as nearly as possible identical and with mycelium that had been harvested 72 hr after inoculation, washed exhaustively with laboratory-deionized water, and resuspended in deionized water. The thioglycoside was added after the shaken suspensions had come to temperature equilibrium with the constant-temperature room. The success or failure of an induction was judged by the appearance or non-appearance of a given enzyme after shaking for 24 hr. The number of trials quoted for each activity is not identical, since certain enzyme tests were added during the course of the series as the required substrates became available or as their importance for the interpretation of the preceding results was realized

Enzyme	Frequency*	Enzyme	Frequency*
β-D-Xylosidase	17/17	α-D-Galactosidase	3+2d/17
β-D-Glucosidase	14+2d/17	Sucrase	0/12
Esterase	12/16	Amylase	0/12
β-D-Galactosidase	10+2d/17	Phosphatase	0/17
α-L-Arabinosidase	6/11	Sulphatase	0/17
α-D-Glucosidase	6/15	α-D-Arabinosidase	Not found
α-D-Mannosidase	4+3d/15	Proteinase	Not found

* d = doubtful, i.e. 1–3 times as much enzyme activity was present in induced as in control mycelium.

TABLE 3

COMPARISON OF THE RELATIVE AMOUNTS OF CERTAIN ENZYMES INDUCED IN A SUSPENSION OF WASHED MYCELIUM OF *S. ATRA* CMI 32542 SHAKEN AT 28°C IN THE PRESENCE OR ABSENCE OF PHENYL β-D-THIOGLUCOPYRANOSIDE FOR 24 HR

Enzyme activity expressed as colorimetric enzyme units per millilitre

Enzyme	Initial Activity	Activity after 24 hr		Ratio of Specific to Unspecific Induction
		Thioglucoside Absent	Thioglucoside (1 mg/ml) Present	
β-Xylosidase	0·130	0·376	12·9	52
β-Glucosidase	0·94	1·66	22·0	30
Esterase	0·42	0·79	9·70	25
β-Galactosidase	0·038	0·243	2·50	12
α-L-Arabinosidase	0·041	0·099	0·522	8·3
α-Glucosidase	0·084	0·219	0·790	5·2
α-Mannosidase	0·217	0·362	0·655	3·0
α-Galactosidase	0·097	0·150	0·293	3·7
Sucrase	0·52	0·72	0·76	
Amylase	0·223	0·253	0·157	
Sulphatase	0·302	0·345	0·271	
Phosphatase	0·44	1·45	1·40	

relevant enzymes were induced. The order is perfectly clear for the first members of the sequence (β-xylosidase, β-glucosidase, esterase) but for the final members (α-mannosidase, α-galactosidase) it had to be deduced from the averaged results of the few occasions on which they appeared and consequently might be subject to some revision. An observation to which no exception was found was that an enzyme was never induced unless all enzymes prior to it in the sequence were also induced.

TABLE 4

COMPARISON OF ACTIVITIES OF CERTAIN ENZYMES IN PARTICULATE AND NON-PARTICULATE FRACTIONS IN SUPERNATANTS FOLLOWING ULTRASONIC DISINTEGRATION OF INDUCED *S. ATRA* MYCELIUM

A sample of the supernatant was frozen for 24 hr at −20°C, thawed, and centrifuged for 10 min at 2000 *g*. The new supernatant was decanted from the pellet, which was twice resuspended in water, centrifuged down, and finally made up to the original volume. Enzyme activities are expressed as colorimetric units per millilitre

Enzyme	Activity		Ratio of Activities (A/B)
	Non-particulate Fraction (A)	Particulate Fraction (B)	
β-Xylosidase	1·10	0·063	20
β-Glucosidase	0·624	0·014	45
α-Mannosidase	0·358	0·152	2·4
β-Galactosidase	0·609	0·007	90
α-Galactosidase	0·145	0·127	1·2
α-Glucosidase	0·365	0·120	3·0
Phosphatase	0·27	0·44	0·6
Sulphatase	0·089	0·238	0·4

An observation that clearly distinguishes between the enzymes that are inducible by phenyl thioglucoside and those that are not was provided by the results of experiments in which an attempt was made to clear particularly cloudy supernatants from the ultrasonic disintegration process by the freezing and thawing technique. Those enzymes that are high in the inductive sequence are "soluble" and associated with the non-particulate fraction; the enzymes that are low on the inductive sequence show intermediate behaviour (Table 4). This observation has been repeated a number of times, including an experiment in which the supernatant was freed of all heavy cellular debris by prolonged centrifugation at 2000 *g*. The nature of the subcellular particles with which the non-inducible hydrolytic enzymes are associated is not clear.

It might be suspected that enzymes have been determined as inducible or non-inducible solely on the basis of whether they are "soluble" or not. A series of induction experiments was therefore carried out in which no attempt was made to fractionate by centrifugation the samples prepared by ultrasonic treatment of the aliquots of mycelium plus medium withdrawn after various time intervals. The samples were added directly to the enzyme assay mixture and particulate matter was removed by rapid centrifugation before optical densities were measured at the completion of incubation. A comparison was made of β-glucosidase, β-xylosidase, phosphatase, and sulphatase; no sign of induction of the second pair could be found under conditions where the first pair were abundantly induced. The suspicion can

thus be rebutted for sulphatase and phosphatase at least, and there is no reason to suppose that they are not typical.

Phenyl β-D-thioglucopyranoside has been used as the inducer in these experiments since it does not appear to be metabolized by *S. atra* (Jermyn and Drew 1967). However, phenyl β-D-glucopyranoside is an active inducer of β-glucosidase, though less effective than the *S*-glucoside, and acts by the same mechanism (Jermyn 1965). For complete confidence that the process being studied here is the same as that described in earlier papers, the *O*-glucoside should therefore be shown to bring about the same type of simultaneous induction as the *S*-glucoside. Table 5 shows that the

TABLE 5

COMPARATIVE EFFECT OF VARIOUS PHENYL THIOGLYCOSIDES AND OF PHENYL β-D-GLUCOPYRANOSIDE ON THE SIMULTANEOUS INDUCTION OF A NUMBER OF ENZYMES IN SUSPENSIONS OF WASHED *S. ATRA* CMI 32542 MYCELIUM SHAKEN AT 28°C IN THE PRESENCE OF 1 MG/ML OF THE POTENTIAL INDUCER

The course of the induction was followed for 24 hr, but only the final (24 hr) values are shown here for clarity. All enzyme activities are expressed as colorimetric units per millilitre

Inducer	Activity of Induced Enzymes				
	α-Gluco-sidase	β-Galacto-sidase	β-Xylo-sidase	β-Gluco-sidase	Esterase
Nil (zero time)	0·092	0·250	0·172	2·96	0·680
Nil (24 hr)	0·108	0·151	0·233	2·20	0·450
Phenyl thioglycoside					
α-D-Glucopyranoside	0·113	0·118	0·229	2·02	0·400
3-*O*-Methyl-β-D-glucopyranoside	0·380	0·890	0·779	9·83	3·40
β-D-Xylopyranoside	0·122	0·190	0·262	2·33	0·620
β-D-Galactopyranoside	0·132	0·233	0·300	2·38	0·840
β-D-Glucopyranoside	0·522	1·53	2·50	25·6	7·96
6-*O*-Acetyl-β-D-glucopyranoside	0·102	0·190	0·244	2·47	0·710
6-*O*-Benzoyl-β-D-glucopyranoside	0·134	0·222	0·211	1·93	0·520
Phenyl β-D-glucopyranoside	0·277	0·520	0·608	6·74	2·07

inductive process appears to be identical for the initial members of the induction series at least using the two types of glucoside. That induction could not be observed with the *O*-glucoside further down the series than α-glucosidase is probably a consequence of the feebler inductive powers of the *O*-glucoside rather than any difference of mechanism.

Although the word "linked" has so far been used to characterize the induction process, this does not, however, carry the implication that the enzymes concerned actually appear together after the same time interval. Figure 3 demonstrates that, in fact, even making the most generous possible allowances for error, they do not do so. The qualification is particularly important in this case since the *S. atra* system is inherently incapable of yielding the fine temporal analysis that *E. coli* does in such hands as those of Kaempfer and Magasanik (1967) where periods of a minute are capable of subdivision.

The time of onset of induced enzyme synthesis is not under nearly as close control in CMI 32542 as it is in DSL 1, nor, because of the much higher endogenous enzyme

levels, is it capable of such precise definition. But the appearance of induced β-xylosidase after 4–5 hr at 28°C in the presence of thioglucoside is the first and most precisely timed of the events. Induced β-glucosidase appears next, after an irregular lag of a further 1–3 hr, and unambiguous induced synthesis of the other enzymes in the sequence is even more retarded but is usually evident by about 15 hr.

Further synthesis of induced enzyme usually does not occur after about 24 hr and the level of both induced and endogenous enzymes falls slowly after this time. Nevertheless the mycelium is far from being in a state of autolysis, and samples that have been starved by simply shaking in distilled water up to 48 hr are still capable of induced enzyme synthesis.

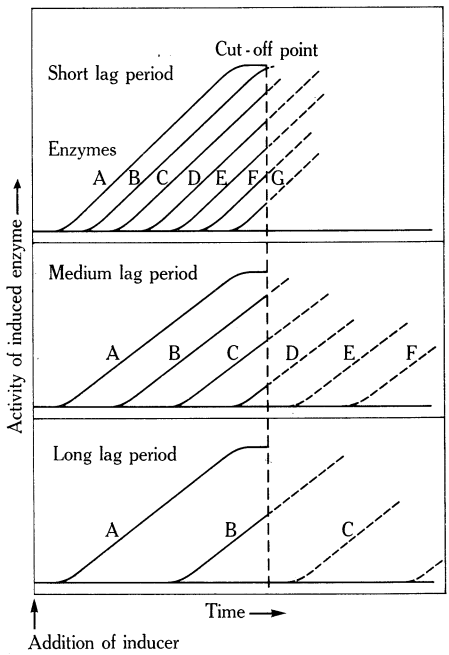

Fig. 4.—A scheme to unify the observations on the linked enzyme syntheses in *S. atra*. The nature of the processes controlling neither the length of the lag period nor the timing of the cut-off point is specified.

The difficulty of defining the exact time of induced enzyme synthesis and the general experimental variability make it uncertain that the temporal order in which the enzymes appear is the same sequence as that defined by frequency and effectiveness of induction, but it seems probable that the three criteria do agree in a general way, and that all are aspects of a single phenomenon. A scheme that will fit the observed results without "explaining" them is set out in Figure 4. The cut-off time beyond which further inductive synthesis is impossible may well be the outcome of the onset of a metabolic process that leads to the destruction of the inducing thioglucoside. It has been shown earlier that removal of the inducer leads to immediate cessation of inductive enzyme synthesis (Jermyn 1965).

(e) *Further Experiments with Alternative Inducers*

The experiments described in the last section suggest that so far as there is a "primary" product of the induction by phenyl β-D-thioglucopyranoside, it is

β-xylosidase rather than β-glucosidase. This raised the possibility that phenyl β-D-thioxylopyranoside, or, with rather less likelihood, some other of the "homologous" inducers, might serve as an efficient replacement for the thioglucoside. A number of trials gave negative results for mycelium known to be inducible with the thioglucoside for the following: phenyl β-D-thioxylopyranoside, phenyl α-D-thioglucopyranoside, phenyl α-D-thioglucofuranoside, phenyl α-D-mannopyranoside, phenyl α-D-galactopyranoside, and phenyl α-L-arabinopyranoside. Neither the early enzymes in the sequence nor the corresponding "homologous" enzyme was produced in any case.

The trial of phenyl 3-*O*-methyl-β-D-thioglucopyranoside as an inducer was based on the observation that 3-*O*-methyl-D-glucose can not only reverse "metabolite inhibition" of induction by phenyl β-D-thioglucopyranoside but can act as an inducer itself under some circumstances (Jermyn 1965). The 6-*O*-acylated thioglucosides, the synthesis of which will be described elsewhere, were included for comparison. The 3-*O*-methylthioglucoside was found to be an agent for the simultaneous induction only a little less potent than the thioglucoside itself; the 6-*O*-acylated thioglucosides were ineffective (Table 5). This is a striking reversal of the specificity observed for the enzymic activity of the β-glucosidase, for which *p*-nitrophenyl 3-*O*-methyl-β-D-glucopyranoside is a non-substrate (Jermyn 1958) and 6-*O*-acylated β-D-glucopyranosides are excellent substrates (Jermyn 1958, and unpublished observations).

(*f*) *Emetine and Cycloheximide as Inhibitors of Induction*

Grollman (1966) has shown that emetine is similar to cycloheximide in its action (Siegel and Sisler 1964) in most phyla of organisms. Though completely different in overall size and shape, the molecules of the two compounds share certain structural features in common, and alike inhibit aminoacyl–*s*RNA transferases. Both substances are effective inhibitors of the inductive syntheses initiated by the thioglucoside, which must accordingly be considered to involve *de novo* protein synthesis. As Grollman's (1966) results would predict for the comparative effect of the two on protein synthesis in general, cycloheximide is a considerably better inhibitor than emetine (Table 6).

It would be difficult to suppose that two such widely different substances as cycloheximide and emetine would be transported across cell membranes with anything like equal ease, so that if transport difficulties do play any part in the observed non-effectiveness of certain inhibitors of protein synthesis in hindering the induction, one of the two aminoacyl–*s*RNA transferase inhibitors might well be ineffective. That both are effective at the same relative levels observed by Grollman (1966) for a variety of systems is some slight indication that the lack of interference with the inductive process in *S. atra* by substances such as puromycin and chloromycetin (Jermyn 1965) that inhibit the later stages of transcription in other organisms may be due to a "species specificity" that precludes them from interaction with the relevant enzymes in *Stachybotrys*.

IV. Discussion

When the information presented here is combined with what is already known about the induction (Jermyn 1965), formerly considered entirely as an induction of

β-glucosidase, a somewhat altered picture emerges. A group of substances including the aryl glucosides and thioglucosides, an aryl 3-*O*-methylthioglucoside, and 3-*O*-methylglucose promote the appearance, after a considerable lag period and in the absence of sensible growth, of a group of enzymes by a process that involves new protein synthesis. Any coincidence between the structure of the inducer and the function of the enzyme, as in the relationship between β-glucosidase and phenyl β-D-thioglucopyranoside that first attracted attention, appears to be merely fortuitous. The enzymes appear to be too many and too varied to form a single operon, and in this connection it must be remembered that only relatively few enzymes that can be determined by simple methods have been looked for, and of these most have been found to be part of the "cluster". In any case, members of a single operon would be expected to be far more closely linked in the inductive process than the group studied here.

TABLE 6

INHIBITION BY EMETINE OR CYCLOHEXIMIDE OF β-XYLOSIDASE INDUCTION BY PHENYL β-D-THIOGLUCOPYRANOSIDE

Washed mycelium of *S. atra* CMI 32542 were shaken for 8 hr at 28°C in the presence of thioglucoside (1 mg/ml) and inhibitor

Conditions	Enzyme Activity (units/ml)	Conditions	Enzyme Activity (units/ml)
Experiment 1		Experiment 2	
Initial (zero time)	0·23	Initial (zero time)	0·09
Control (no inducer)	0·36	Control (no inducer)	0·10
Control (no inhibitor)	4·92	Control (no inhibitor)	3·99
Streptomycin (10^{-3}M)	0·36	Streptomycin (10^{-3}M)	0·14
Tetracycline (10^{-2}M)	4·13	Tetracycline (10^{-2}M)	4·20
Emetine		Cycloheximide	
3×10^{-3}M	0·64	3×10^{-5}M	0·38
1×10^{-3}M	0·85	1×10^{-5}M	1·54
3×10^{-4}M	1·60	3×10^{-6}M	4·08
1×10^{-4}M	3·18	1×10^{-6}M	4·04

A working hypothesis that will cover some of the observed facts is to suppose that the repressors for the various transcriptions of DNA that eventually lead to the various enzyme syntheses are the product of a single operon and furthermore that these repressors are unstable. The eventual slow synthesis of enzymes that begins in the starved mycelium suggests not only that the repressors are unstable, but also that there is not a sufficient stock of them in the cell to counter even a period of diminished repressor synthesis. The function of the inducer would then be to bring about the repression of the "master" operon, leading to the cessation of the synthesis of the secondary repressors, the slow inactivation of those already synthesized, and the eventual commencement and acceleration of the various enzyme syntheses.

The most important objection to this hypothesis is that the course of events is altogether too well timed. In the hundreds of separate experiments that were the basis of earlier conclusions (Jermyn 1965), the onset of induced enzyme synthesis in washed log-phase mycelium of *S. atra* DSL 1 at 28°C took place at 3·75 hr ±10 min

after the addition, and the variations are to be explained by looseness in the initial temperature equilibration. Even in the more loosely controlled CMI 32542, the first inductive event, the appearance of β-xylosidase, occurs regularly at 4·5 hr ± 45 min after addition of inducer. These observations are incompatible with a statistical fluctuation in the number of repressor molecules per cell in different cultures.

The data of Jermyn (1965) on the temperature dependence of the lag period for β-glucosidase induction in DSL 1 suggested that this period was controlled by a single finite process with a defined energy of activation, hence a fixed rate, and a fixed time span between initiation and completion. The two intracellular processes that fit this description are the transcription of DNA and the translation of *m*RNA, both of which could release a product on completion that could theoretically initiate a number of enzyme syntheses. Unfortunately the time scale seems to be quite wrong; transcription of an operon appears to take 1–2 min in *E. coli* and translation an even shorter time (Kaempfer and Magasanik 1967). This may be compared with 3·75 hr at 28°C for β-glucosidase induction with DSL 1 and about 4·5 hr for β-xylosidase induction with CMI 32542. It is difficult to suggest any finite process other than cell duplication that takes as long as this, and cell duplication is precluded by the conditions of the experiment.

The nature of the process intervening between the first contact of cell and inducer (which must be continuous, but does not necessarily involve uptake into the cellular interior; see Jermyn 1965) and the eventual release of the *m*RNA molecules corresponding to some or all of the observed sequence of enzymes thus remains mysterious. However, it now appears that the problems to be solved lie in this part of the "inductive" process. The relationship of the "induction" studied here with the conventional induction of β-galactosidase by growth on lactose or β-glucosidase by growth on salicin is also obscure but it is probable that the two sets of problems are interdependent.

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