FUNGAL CELLULASES

XX.* SOME OBSERVATIONS ON THE INDUCTION AND INHIBITION OF THE CELLOBIASE OF *STACHYBOTRYS ATRA*

By M. A. JERMYN†

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Summary

The induction of cellobiase in washed suspensions of *Stachybotrys atra* mycelium harvested during the active phase of growth follows the classical pattern for induced protein synthesis, except that it does not appear to be dependent on continued growth of the organism. At 28°C under aerobic conditions there is no detectable lag period. Inducers are β-glucosides of polyhydroxylic aglycones, which may be another sugar residue or a chain of such residues. Such inducers are metabolizable by the mould; after their breakdown the enzyme rapidly disappears from the mycelium. Interference with RNA metabolism and information transfer depresses the induced synthesis of the enzyme; two amino acid analogues, β-chloroalanine and *p*-fluorophenylalanine, also inhibit this induction. Inhibition is also observed when respirable substances are present, and the inhibitor appears to arise from the metabolism of pyruvate.

The patterns of molecular structure which characterize effective acceptors for the transfer of a glycosyl residue by cellobiase differ greatly from those characterizing acceptors of aryl β-glucosidase.

I. INTRODUCTION

Glycosidases that will attack the glycosidic linkage in monomeric glycosides are a subclass of the "exoglycosidases", i.e. they will break bonds of the type Gly....OR but not of the type ....GlyOGly....OR, where R in each case may be the rest of a polyglycosyl chain. Some exoglycosidases from certain organisms do in fact have the restriction that R shall be such a chain, but none of this type have been found among the β-glucosidases of *S. atra*. Instead we have one enzyme for which R must be an aryl group, and another for which its nature is almost unrestricted. Since a crucial practical distinction between the two enzymes is the ability to hydrolyse cellobiose, the first has usually been referred to as "aryl β-glucosidase" or simply as "β-glucosidase" when the context permits and the second has been referred to as "cellobiase". For the second enzyme "non-specific β-glucosidase" or "exocellulase" would be equally appropriate.

However, the enzyme has been subjected to preliminary investigation in Part IX of this series by Youatt (1958) under the name "cellobiase", as also in a review

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† Division of Protein Chemistry, CSIRO Wool Research Laboratories, Parkville, Vic.

paper on the *S. atra* enzymes (Jermyn and Youatt 1959). The name will therefore be retained here, purely as a distinguishing label.

Youatt (1958) showed that the enzyme was produced during the growth of *S. atra* on cellulosic materials; Jermyn (1965) showed that it could be induced in washed *S. atra* mycelium to which cellobiose or certain glycitol β-glucosides had been added. Since Youatt's investigations also showed that the two β-glucosidases had little in common other than overlapping specificities it was of interest to determine how far the unusual pattern of induction for the aryl β-glucosidase was repeated for the cellobiase. Furthermore, since cellobiase and cellulase appear together during growth of the mould on cellulosic substrates it was necessary, for any understanding of the digestion of cellulose by the mould, to begin by discovering whether the two enzymes were in fact induced together, perhaps as part of the same operon. An alternative possibility would be that partial digests of cellulose contain such a variety of molecular species that the essentially independent induction of the two enzymes appears to take place simultaneously.

Youatt also showed that substances that inhibited aryl β-glucosidase frequently did not inhibit cellobiase and vice versa. Nor was it possible to demonstrate the ready transfer of a glycosyl group to simple hydroxylic acceptors by cellobiase that occurred with aryl β-glucosides. This paper will present further data that has been accumulated on mechanistic differences between the two enzymes.

II. Materials and Methods

The growth and harvesting of *S. atra* mycelium for induction experiments was as previously outlined (Jermyn 1965).

In experiments where a large number of samples had to be tested, or data were required on a comparative basis only, the colorimetric method of Jermyn (1961) was used. Where values for enzyme activity less subject to experimental error were required, e.g. in kinetic experiments, the manometric method of Youatt (1958) was used.

The same method, i.e. hydrolysis of *p*-nitrophenyl and other aryl glycosides, could theoretically be employed to measure both aryl β-glucosidase and cellobiase. However, the mould mycelium always contains a little, and quite often a large quantity, of the aryl β-glucosidase when it has not been specifically induced. Unless the cellobiase has been extensively purified, observed aryl β-glucoside hydrolysis will always be partly due to contaminating aryl β-glucosidase. Neither of the standard methods depending on the specific breakdown of cellobiose by the enzyme, involving as they do glucose oxidase which will have affinity for much the same molecular species as β-glucosidase and a third enzyme (catalase or peroxidase) also liable to disturbance, are altogether satisfactory for experiments involving inhibition. All methods involving cellobiose breakdown are useless for measuring transfer since the product of transfer to water and the aglucone are identical (glucose).

It might be thought that these difficulties could be overcome by using as substrate an alkyl β-glucoside, e.g. methyl β-D-glucopyranoside. This compound is not hydrolysed by the aryl β-glucosidase and actually behaves as a competitive inhibitor

of that enzyme (Jermyn 1955). Glucose release from the glucoside can be used as a measure of cellobiase activity in inhibition experiments. The glucoside is also a suitable substrate in incubation mixtures designed to be examined by paper chromatography for the presence of transfer products. However, methyl β-glucoside cannot be used in experiments, such as those devised for determining transfer ratios for aryl β-glucosidase, where the relative rates of liberation of glucose and aglucone must be measured, since minute amounts of methanol cannot be accurately estimated in complex incubation mixtures in the same way as phenols. A satisfactory substrate for use in transfer experiments involving unpurified cellobiase has not yet been found.

For experiments with alkanols as acceptors, cellobiose was used as substrate, and for experiments with polyols and sugars, methyl β-glucoside. This latter choice was determined by the positional overlapping on paper chromatography using dimethylformamide–butanol–water (Cramer and Steinle 1955) of cellobiose with disaccharides and polyolglucosides formed by transfer. Since the transfer products were all substrates of the enzyme, these transient intermediates were looked for by regular sampling of the incubation mixture and destruction of the enzyme in the sample by heating.

Most of the carbohydrate derivatives employed in this study were laboratory preparations previously described in other papers of this series. The synthetic procedure used in preparing the others will be indicated by naming the relevant author when a substance is first mentioned. In general, the physical properties of all organic compounds agreed with the literature.

Cellulose was dissolved in fuming hydrochloric acid at 25°C, and the product was fractionated into "soluble" and "insoluble" cellodextrins by pouring into ice–water after the desired interval. The water-insoluble material was washed well and finally purified from acid and low-molecular weight material by exhaustive dialysis. The degree of polymerization could be varied by varying the period of dissolution in fuming HCl between 5 and 60 min. "Soluble" cellodextrin was precipitated by making the filtrate from the insoluble dextrin 60% (v/v) in ethanol and allowing to stand a few hours at 0°C. The precipitate was filtered, washed several times with cold 60% ethanol, then suspended in ethanol containing 1% triethylamine. The last procedure was repeated twice more, and the product washed with several changes of ethanol and then ether. If the triethylamine and its hydrochloride had been completely washed out the product was non-hygroscopic and indefinitely stable. Judged by its chromatographic behaviour there were no detectable amounts of oligomers with $n < 7$.

III. General Observations on Induction

The slowness of either of the available methods for cellobiase compared to the speed and reproducibility of the *p*-nitrophenyl β-glucoside estimation of the aryl β-glucosidase means that it is inherently difficult to accumulate for the former enzyme the large quantities of results that were available for an analysis of aryl β-glucosidase induction. None the less, within these limits, enough information has been obtained to sketch in the outlines of the induction of cellobiase. The overall conclusions will be summarized first to allow comparison with the findings for the aryl β-glucosidase;

it will be seen that the two sets of results are different in almost every respect. The experimental details will follow.

(1) The synthesis of cellobiase begins (within the large experimental error) immediately a competent inducer is added to a suspension of washed mycelium. It rises to a maximum in some 4–6 hr at 28°C and then decreases steadily to zero.

(2) The enzyme is spontaneously secreted into the medium only under conditions where the cells are undergoing lysis.

(3) Cellobiose when used as inducer is taken into the cells quite rapidly; the cells can then be washed free of the old medium without immediate termination of induced enzyme synthesis, which continues for 15–60 min more.

(4) All effective inducers tested are metabolized by the mould.

(5) Observations (1) to (4) may be explained by supposing a balance within the cell between the synthesis of enzyme under the influence of inducer and its destruction by other cell processes; when the inducer has been metabolized away, induced synthesis stops and the enzyme disappears. For the aryl β-glucosidase, in contrast, we have a long lag period, the necessary continued presence of an inducer that need not be either taken into the cell or metabolized, and the immunity of the synthesized enzyme to the processes of cellular destruction.

(6) The existence of a cross-over point between aryl β-glucosidase and cellobiase induction at the level of complexity of pentityl and hexityl β-glucosides has already been established (Jermyn 1965). β-Glucosides with sugar aglycones, although there are some exceptions, appear generally to act as inducers of cellobiase. Soluble cello-oligosaccharides are also inducers of this enzyme, but insoluble cellodextrins, native cellulose, and solubilized cellulose derivatives are not.

(7) Aryl β-glucosidase induction was dependent on no more than a minimum metabolism at the level of endogenous oxidative phosphorylation (Jermyn 1965); cellobiase induction is quite sensitive to the blocking of various metabolic chains.

(8) Cellobiase induction is not very sensitive to the presence of glucose and most other metabolizable sugars; this is in contrast to the very apparent "glucose effect" that aryl β-glucosidase induction shares with most other inducible enzyme systems.

(9) Cellobiase induction does not appear to be promoted by an external nitrogen source or supply of amino acids. Of the amino acid analogues tested for their ability to inhibit the induction, only β-chloroalanine and *p*-fluorophenylalanine showed effects reversible by the corresponding natural amino acid in contrast to the aryl β-glucosidase induction which was specifically inhibited by *S*-aminoethylcysteine.

(10) Cellobiase induction is inhibited by agents that interfere with the intracellular transmission of information; aryl β-glucosidase induction is sensitive only to streptomycin.

IV. Results

(*a*) *General Course of Cellobiase Induction*

Since cellobiase is not in general released into the medium, the enzyme had to be released by disruption of the mycelium. This was accomplished by ultrasonic

Table 1

ULTRASONIC DISRUPTION OF A SUSPENSION OF *S. ATRA* MYCELIUM THAT HAD BEEN INDUCED WITH CELLOBIOSE

The temperature of the suspension remained at 1–2°C during disruption; particulate matter was removed by immediate centrifugation at the end of the disruption period

Period of Disruption (min)	Enzyme Activity in Supernatant (arbitrary units)	Period of Disruption (min)	Enzyme Activity in Supernatant (arbitrary units)
0	0·026	10	0·638
2	0·254	20	0·596
5	0·335	30	0·449

disintegration at ice-water temperatures in a Mullard ultrasonic generator fitted with a titanium probe. Table 1 shows that there was a maximum in the release of

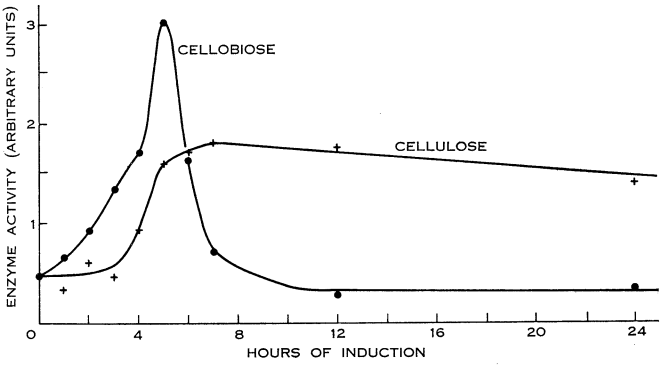

Fig. 1.—Course of induced cellobiase production in a washed mycelial suspension of 3-day-old, sucrose-grown *S. atra*, to 500 ml of which cellulose powder (Whatman standard grade; 2 g) or cellobiose (100 mg) were added. The suspensions were then shaken aerobically at 28°C. The blanks have not been subtracted in the cellobiase estimations, to allow a realistic assessment of experimental errors.

soluble enzyme into the medium (after about 10 min under our conditions) after which the measured activity slowly fell. Ten minutes was therefore adopted as the standard disruption period.

The time course of the induction of cellobiase at 28°C in washed mycelium is compared for identical mycelial samples in the presence of added cellobiose and cellulose in Figure 1. In the presence of cellobiose, the induced enzyme rises to a steep maximum in 5 hr, experimental error being here too great to say whether or not there is any definite lag period, and then falls steeply to zero again. In the presence of cellulose there is no induction for 2–3 hr; the amount of induced enzyme then rises to a certain level and remains there. It will be shown in Part XXI (Jermyn 1967) that there is a lag of 2–3 hr in the production of induced cellulase under these circumstances; the observed course of cellobiase induction in the presence of cellulose

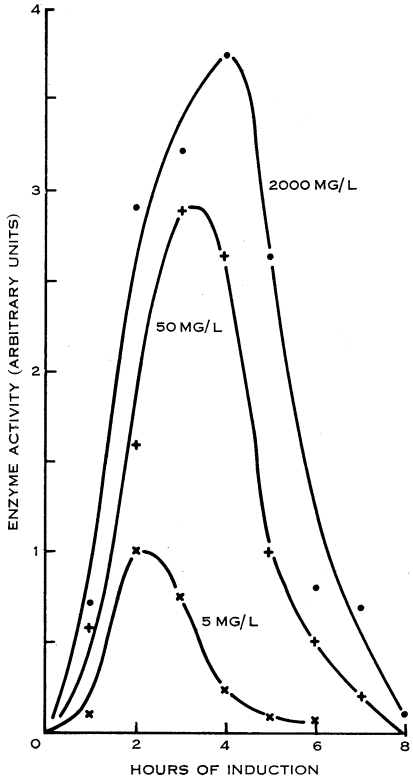

Fig. 2.—Course of induced cellobiase production in a washed mycelial suspension of 4-day-old, sucrose-grown *S. atra*, divided into portions made up to different concentrations of cellobiose and shaken aerobically at 28°C.

can then be explained by supposing that cellulose is not itself an inducer, but that the oligocellosides produced by the action of induced cellulase on cellulose are the true inducing agents. The processes involved—induction of cellulase, action of cellulases on cellulose, catabolism of oligocellosides, induction of cellobiase, catabolism of cellulase and cellobiase—reach a steady state. The course of cellobiase induction in the presence of cellobiose represents only three of these processes—induction of cellobiase, catabolism of cellobiose and of cellobiase—that cannot reach a steady state. This is because cellobiose has only a transitory existence in the system in comparison with the slowly degraded cellulose. Manometric experiments suggest that respiratory stimulation would have ceased after 4–5 hr at the concentrations of cellobiose and

mycelium used in the experiment of Figure 1; by implication the cellobiose would have been used up.

Figure 2 shows that the lower the initial concentration of cellobiose, the earlier comes the point of maximum cellobiase production; there is no simple relationship, probably because there is no simple relationship between rate of metabolism and concentration of metabolite.

An arbitrary time of induction (4 hr) was therefore taken as optimum for experiments in which the time course of induction was not important. Similarly a concentration of inducer cellobiose (2 g/l) was chosen in inhibition etc. experiments, as giving a near-maximum induction which would not be much affected by small variations in the activity of the mycelium. Figure 3 shows that for two separate inducers and a 4-hr induction period the total amount of induction is only slightly

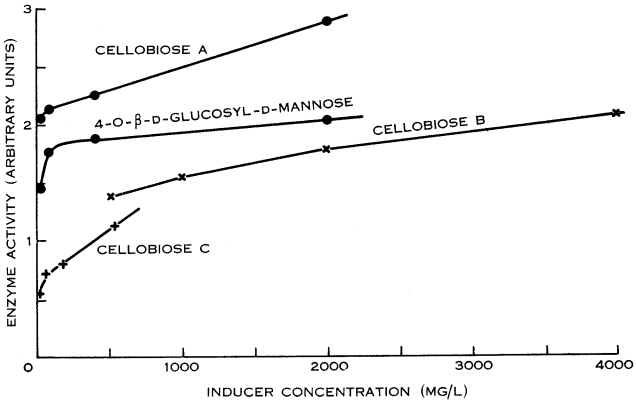

Fig. 3.—Effect of cellobiose concentration on amount of induced cellobiase produced after 4 hr aerobic shaking at 28°C in washed mycelial suspensions of *S. atra*. The three experiments were done at widely separated intervals. The experiment involving 4-*O*-β-D-glucosyl-D-mannose was carried out on a second sample of the same mycelium as in "cellobiose A".

affected by inducer concentration over a wide range of concentrations. The amount of induction drops sharply with inducer concentration over just that range (<20 mg/l) where metabolic effects are important. It is also this range that is important if the rate of variation of the induced enzyme formation with inducer concentration is to be studied and it must be concluded that no such experiments can usefully be carried out with cellobiose or with 4-*O*-β-D-glucosyl-D-mannose (Brauns 1926) which is also capable of being metabolized by the mould. The search for a metabolically inert inducer will be discussed later, but the results may be anticipated by saying that no such inducer has so far been found, and accordingly it has not seemed worth while to carry out critical experiments on the effects of inducer concentration.

(*b*) *Effects of Variations in the History of the Mycelium*

It was shown (Jermyn 1965) that changes in the metabolic age and growth substrate of the sample of mycelium used had profound effects on the inducibility of

the aryl β-glucosidase. Even when the best allowance possible had been made for these effects, erratic variations of unknown origin in the inducibility of the mycelium occurred. The cellobiase induction is not particularly sensitive to the carbon source in the medium and induction could be shown in mycelium grown on glycerol, glucose, sucrose, starch, cellobiose, salicin, and peptone. However, freshly harvested mycelium grown on any of these media except sucrose was occasionally and unpredictably non-inducible. This phenomenon could usually be eliminated by "starving" the mycelium for a few hours, i.e. by shaking a suspension aerobically in the absence of added carbon source. It was presumably due to a build-up of inhibitory metabolites. Since

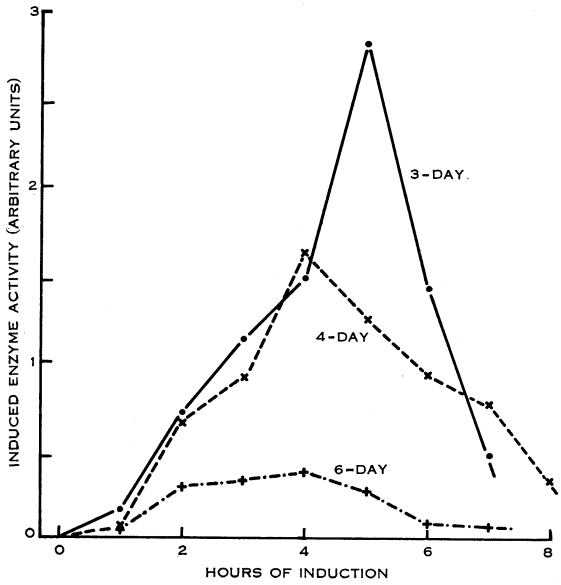

Fig. 4.—Effect of physiological age on cellobiase induction. *S. atra* growing in shake culture in 4% sucrose medium at 28°C was partially harvested at intervals and the washed mycelium resuspended and tested for cellobiase induction under the same conditions. The suspension was adjusted to contain the same wet weight of mycelium per standard volume in each case.

this procedure added an unknown amount to the "physiological age" of the organism, the use of sucrose-grown mycelium, which was almost invariably immediately inducible, was preferred.

Some carbon sources gave rise to mycelium that gave excessive blanks in cellobiase determinations, i.e. the supernatants of the ultrasonic disruption process alone reacted with the glucose oxidase–catalase (or peroxidase) systems. This was particularly true of glucose-grown mycelium; it was not due to the presence of glucose since the effect disappeared very slowly on starving the mycelium. A possible explanation would be that enzymes in such supernatants slowly release glucose from some endogenous substrate.

The physiological age of the mould has a considerable effect on cellobiase inducibility. Figure 4 shows the effect of harvesting mycelium at different stages of the growth of *S. atra* on 4% sucrose medium and testing its inducibility with cellobiose. The falling inducibility of the older mycelium is not due to the accumulation of inhibitory intermediate metabolites; starving the mycelium does not restore the lost ability. The earlier occurrence of the peak of induced enzyme activity and the slower fall from the peak as the mycelium grows older indicate that various biochemical processes may occur at different relative rates within the mycelium at different physiological ages.

TABLE 2

TIME COURSE OF CELLOBIASE INDUCTION IN *S. ATRA*

A washed suspension of 4-day-old sucrose-grown mycelium was divided into three portions which were shaken aerobically at 28°C. Cellobiose (2000 mg/l) was added to one portion, and phenyl β-thioglucoside (1000 mg/l) was added to another. There was no addition to the third portion. At hourly intervals (staggered by 15 min) samples were withdrawn, subjected to ultrasonic disruption, and centrifuged. The cellobiase in the supernatant was measured by the colorimetric method

Sampling Period (hr)	Cellobiase Activity (units/0·5 ml)		
	No Addition	Cellobiose Present	Phenyl β-Thioglucoside Present
0	0·264	0·162	0·279
1	0·210	0·255	0·294
2	0·255	0·315	0·240
3	0·225	0·630	0·252
4	0·366	1·269	0·234
5	0·375	0·957	0·258
6	0·315	0·972	0·225
7	0·315	0·642	0·162
23	0·192	0·360	0·216
Mean±S.D.	0·280±0·068		0·240±0·038

(c) *Non-induced Cellobiase Activity*

Both the manometric (Youatt 1958) and colorimetric (Jermyn 1961) methods for estimating cellobiase are differential methods, measuring the change in a three-enzyme system of the rate of either oxygen uptake or of colour production from *p*-phenylenediamine when substrate cellobiose is added. Since the "cellobiase" preparations are supernatants from the ultrasonic disintegration which contain all sorts of endogenous substrates and enzymes, apparent cellobiase activity may be due to other things than the splitting of the added cellobiose to give glucose. In fact, such supernatants of non-induced mycelium always give a positive reading for "cellobiase". Although this can theoretically be allowed for by controls in induction experiments, it is of interest to consider whether the cellobiase measured is actually the effect of endogenous enzyme.

Table 2 illustrates rather poor induction in sucrose-grown mycelium in the presence of cellobiose and a non-induction in the presence of phenyl β-thioglucoside, all against the background of a fairly high endogenous "cellobiase". It illustrates the scatter of results that make it difficult to give a quantitative estimate of small amounts of induced enzyme formation. The scatter of results is in the actual event less in the presence of phenyl β-thioglucoside than in the absence of any additive. The table, however, illustrates the point that induction occurs in mycelium containing a considerable amount of apparent endogenous "cellobiase". Induction has in fact been demonstrated with levels of "cellobiase" 10 times greater than that illustrated. The meaning of this observation depends on the interpretation to be given to the apparent endogenous enzyme.

TABLE 3

COMPARISON OF DIFFERENT METHODS OF ESTIMATING INDUCED CELLOBIASE IN *S. ATRA*

Six flasks of mineral medium were divided into pairs, to which were added sucrose (2%), cellobiose (1%), or cellulose (1%), and inoculated. One of each pair was harvested after 3 days and one after 5 days. The washed mycelium was resuspended in 0·4 vol. of water, disrupted ultrasonically, and centrifuged. The supernatant was either tested directly for cellobiase or dialysed (16 hr against running tap water at 13°C) and tested. The units have been adjusted so that the two methods should give numerically similar results on the same enzyme sample

Growth Substrate	3-day Mycelium				5-day Mycelium			
	Manometric Cellobiase (units/20 ml)		Colorimetric Cellobiase (units/0·5 ml)		Manometric Cellobiase (units/20 ml)		Colorimetric Cellobiase (units/0·5 ml)	
	Crude	Dialysed	Crude	Dialysed	Crude	Dialysed	Crude	Dialysed
Sucrose	0·24	0·18	3·1	0·20	0·14	0·05	0·04	0·04
Cellobiose	0·96	0·45	7·1	0·32	0·17	0·09	0·15	0·07
Cellulose	0·80	0·44	22·8	0·50	0·97	0·51	15·2	0·48

Table 3 sets out an experiment from which it can be deduced that actively growing cultures contain materials destroyed or removed by dialysis that simulate cellobiase in both methods, but more significantly in the colorimetric one. These materials largely disappear in mature cultures (the cellulose culture is of course in active growth for many days). There is "cellobiase" stable to dialysis in all growing cultures. Since it is highest in ones grown on inducing carbon sources, it is almost certainly true cellobiase. This cellobiase decreases when growth stops. The nature of the material that simulates (or perhaps stimulates) cellobiase is uncertain. It is certainly not glucose nor an oxidant for the peroxidase substrate, since these would be allowed for in the blanks. Nor do dialysates show any "cellobiase" activity.

The normal existence of some cellobiase in most metabolically active mycelium can be assumed from the usual immediate respiratory stimulation caused by the addition of this sugar to mycelial samples in manometric experiments.

(*d*) *Existence of the Lag Period*

The existing data suggest that there is an acceleration of enzyme synthesis during the first stages of cellobiase induction. However, the data from any single experiment are not sufficiently accurate to demonstrate whether there is a true lag period in the induction. The experiment detailed in Table 4 was therefore set up

TABLE 4

AN EXPERIMENT TO INVESTIGATE THE EXISTENCE OF A LAG PERIOD IN A CELLOBIASE INDUCTION

Each sample was estimated in triplicate by the manometric method [see Section IV(*d*) for details]

Incubation Period (hr)	Cellobiase Activity (units/20 ml)			
	3-day-old Mycelium		4-day-old Mycelium	
	Sample 1	Sample 2	Sample 1	Sample 2
0	0·04	0·04	0·02	0·01
	0·04	0·05	0·01	0·01
	0·05	0·05	0·01	0·01
0·25	0·08	0·09	0·04	0·03
	0·08	0·07	0·05	0·04
	0·07	0·07	0·05	0·05
0·5	0·17	0·15	0·10	0·08
	0·15	0·15	0·11	0·08
	0·14	0·13	—	0·08
1·0	0·41	0·27	0·20	0·19
	0·36	0·28	0·22	0·22
	0·34	0·34	0·27	0·26
2·0	0·96	0·90	0·66	0·58
	1·06	0·84	0·62	0·55
	1·10	0·80	0·58	0·60

having regard to the facts that the manometric method was more accurate than the colorimetric one and that dialysis was obviously necessary before small amounts of cellobiase could be distinguished in the presence of disturbing factors.

A sample of *S. atra* grown on 4% sucrose was harvested, half after 3 days growth and half after 4 days. The washed mycelial suspension for each day was each divided into two, to each of which was added cellobiose (2000 mg/l). There was an interval of 3 hr between the beginning of the two experiments during which the second sample was shaken aerobically at 28°C. At intervals after the addition a sample was

withdrawn, disrupted ultrasonically, centrifuged briefly, and the supernatant dialysed for 16 hr against running tap water at 12°C. The dialysates were kept frozen at −20°C until enzyme estimations on them were made.

The conclusion follows that, for 3-day and 4-day-old sucrose-grown mycelium (the basic material used in almost all experiments), if there is a lag phase it is complete in a few minutes at 28°C.

(e) *Inducers for Cellobiase*

The known inducers may be divided into various groups.

(i) *Oligosaccharides*

Cellobiose, 4-*O*-β-D-glucopyranosyl-D-mannose, gentiobiose (6-*O*-β-D-glucopyranosyl-D-glucose), laminaribiose (3-*O*-β-D-glucopyranosyl-D-glucose, the gift of Dr. B. W. Stone), sophorose (2-*O*-β-D-glucopyranosyl-D-glucose, the gift of Dr. N. K. Richtmeyer), celtrobiose (4-*O*-β-D-glucopyranosyl-D-altrose, Richtmeyer and Hudson 1936), and 4-*O*-β-D-glucopyranosyl-L-fucose (the gift of Dr. P. A. J. Gorin) were all about equally effective at equimolar concentrations. All of these sugars were metabolizable, judging from respiratory stimulation in manometric experiments.

Lactose, maltose, trehalose, turanose, melibiose, sucrose, 4-β-D-mannopyranosyl-D-mannose (and the corresponding mannodextrins), 4-β-D-xylopyranosyl-D-xylose (and the corresponding xylodextrins), raffinose, melezitose, and stachyose were inactive as inducers. The manno- and xylobioses (and the corresponding "dextrins", $n = 3$–6) were prepared by carbon column chromatography of hydrochloric acid hydrolysates of ivory-nut mannan and pear xylan.

(ii) *Substances Related to Cellulose*

Cellotriose, tetraose, pentaose, and hexaose were about equally effective (on a molecular basis) as cellobiose. The soluble cellodextrins behaved similarly to cellobiose in induction experiments, if care were taken to see that they were actually in solution in the incubation mixture. The insoluble cellodextrins showed the same behaviour as cellulose. This was true of products of average degree of polymerization of from 30 to 200 (by the 3,5-dinitrosalicylic acid method of Bernfeld 1955); insolubility rather than retention of the native structure of cellulose seemed to be the important characteristic.

Long-chain soluble cellulose derivatives were ineffective. This applied to carboxymethylcellulose of various degrees of substitution, cellulose sulphate, and ethyl hydroxyethyl cellulose. After a long lag period cellobiase did appear in experiments involving ethyl hydroxyethyl cellulose but, as this substance is a fairly effective inducer of cellulase, this result was to be expected.

It is uncertain whether the inactivity of soluble cellulose derivatives was due to their chain length, substitution, or a combination of both factors. All that can be said with certainty is that increasing chain length does not appear to affect inducer action up to $n = 10$ (the approximate limit of aqueous solubility in cellosides).

(iii) *Glycosides, etc.*

Phenyl α-cellobioside was an effective inducer of cellobiase. Methyl β-cellobioside was also an inducer, but after the normal lag period (about 4 hr) there was also a burst of aryl β-glucosidase production. Since the cellobiase also acts on the test substrate, *p*-nitrophenyl β-glucoside, this event had to be deduced from a sharp rise in β-glucosidase unaccompanied by increased cellobiase activity (cf. the cases discussed in Jermyn 1965). The implication is that cellobiase (induced or endogenous) acts on methyl β-cellobioside to give the aryl glucosidase inducer, methyl β-glucoside. For *p*-nitrophenyl β-cellobioside, no cellobiase could be induced but aryl β-glucosidase appeared after the lag. This is possibly related to the dependence of cellobiase induction on an intact respiration; it will be shown elsewhere that certain *p*-nitrophenyl glycosides inhibit the respiration of *S. atra*.

No simple glycoside of D-glucose, D-galactose, D-mannose, D-fructose, D-xylose, D- and L-arabinose, lactose, or maltose was found to be an inducer. Nor were the β-primeveroside macrozamin (primeverose is 6-*O*-β-D-xylopyranosyl-D-glucose) and the β-rutinoside rutin (rutinose is 6-*O*-β-L-rhamnopyranosyl-D-glucose).

Since 3-*O*-methylglucose is a "gratuitous" inducer of the aryl β-glucosidase, some hundred compounds comprising substituted glucoses and other monoses and their substituted derivatives were tried as inducers of cellobiase, but without success. This of course does not preclude the existence of gratuitous induction, since there are almost endless possibilities. In view of the probability of unexpected physiological variations, a negative conclusion was never drawn in any induction experiment unless a parallel control with cellobiose and the same mycelium gave clear-cut positive induction.

(iv) *Sulphur Compounds, etc.*

Since many aspects of cellobiase induction could not be investigated without an unmetabolized inducer, attention was turned to thio-analogues of inducers. Thioglycosides have been the agents of choice in many investigations of glycosidase induction.

Phenyl, *p*-cresyl, and ethyl β-thioglucosides had no inductive effect. ββ′-Diglucopyranosyl sulphide (Schneider and Wrede 1917) was without effect, in spite of its doubled β-thioglucoside linkage. Nor was it an inhibitor of the inductive effect of cellobiose even when present in a hundredfold excess. The octa-acetate of ββ′-diglucopyranosyl disulphide (Wrede 1919) was deacetylated *in situ*, since the deacetylated compound is amorphous and hygroscopic, but the product showed no inductive effect, nor did *bis*(6-deoxyglucopyranose-6-)disulphide (Wrede 1921). An even closer approach to the structure of a known inducer was provided by Hutson's (1964) synthesis of the methyl glycoside of the thio-analogue of gentiobiose. The octa-acetate of this compound was deacetylated without attempting to isolate the non-crystalline glycoside; the product showed no inducing ability when gentiobiose used as a parallel control was highly effective. Nor did the presence of the thio-analogue inhibit the inductive effect of gentiobiose.

It may be concluded that the replacement of oxygen in the glycosidic linkage by sulphur leads to non-inducers.

Replacement of oxygen by an imino group, at least in diglucosylamine (Irvine, Thompson, and Garrett 1913), led to a molecule that was not an inducer. Nor was *N*,*N*′-diglucosylurea (Benn and Jones 1960), which is considered to have the β,β′-structure, any more effective.

TABLE 5

EFFECT OF VARIOUS CONCENTRATIONS OF D-GLUCOSE ON THE INDUCTION OF CELLOBIASE BY CELLOBIOSE

Portions of a washed suspension of 3-day-old sucrose-grown *S. atra* mycelium shaken aerobically at 28°C for varying periods without any additions, in the presence of cellobiose, or in the presence of cellobiose plus varying concentrations of glucose

Period of Shaking (hr)	Cellobiase Activity (units/0·5 ml)					
	No Additions	Cellobiose (2000 mg/l)	Cellobiose (2000 mg/l) plus Glucose at Concentrations of:			Cellobiose (2000 mg/l) plus Methyl α-Glucoside (2000 mg/l)
			100 mg/l	500 mg/l	2500 mg/l	
0	0·321	0·326	0·447	0·287	0·450	0·480
2	0·423	1·005	0·927	0·684	0·585	0·900
4	0·378	1·284	1·254	1·113	0·798	1·284
6	0·360	1·587	1·587	1·593	1·197	1·728

TABLE 6

EFFECT OF VARIOUS CONCENTRATIONS OF D-GLUCOSE, D-XYLOSE, OR D-GALACTOSE ON CELLOBIASE INDUCTION

Portions of washed suspensions of 3-day-old sucrose-grown *S. atra* mycelium were shaken aerobically for 4 hr at 28°C in the presence of cellobiose (2000 mg/l)

Concentration of Additive (mg/l)	Cellobiase (units/0·5 ml) Formed in the Presence of:		
	D-Glucose*	D-Galactose	D-Xylose
0	0·906	2·091	2·091
20	—	1·605	2·097
100	0·876	1·359	1·607
500	0·735	1·275	1·554
2500	0·420	0·984	0·783

* See Table 5.

(v) *Summary*

In summary only compounds with the structure β-glucosyl-*O*-(polyol aglycone) have so far been shown to act as inducers, and these are all metabolizable.

(*f*) *Effect of Added Metabolites on the Induction*

A priori, the possible existence of a "glucose effect" in the induction of cellobiase raises some interesting problems. Since the enzyme is unstable in the cell and glucose is the product of its action, a "glucose effect" would mean feedback control of the level of enzyme in the cell. However, since such a control would operate to produce a constant supply of glucose for metabolism, it is unlikely that a "glucose effect" would be so sharply marked, in terms of effective concentrations, as it is where its function is to close off certain metabolic pathways so long as glucose is present to maintain the glycolytic or related pathways.

TABLE 7

EFFECT OF VARIOUS SUGARS ON THE FORMATION OF INDUCED CELLOBIASE

Portions of washed suspensions of 3-day-old sucrose-grown mycelium were shaken for 4 hr aerobically at 28°C either in the presence of cellobiose (2000 mg/l) only or in the presence of cellobiose plus one of the sugars listed (concn. 2000 mg/l). The values are expressed as percentages of the induced enzyme formed in the control containing cellobiose only, and are means of three experiments

Sugar	Cellobiase Activity (as % of control)	Sugar	Cellobiase Activity (as % of control)
D-Glucose	29	Trehalose	46
D-Fructose	5	L-Sorbose	−6
D-Mannose	32	D-Arabinose	31
L-Rhamnose	90	Lactose	121
D-Galactose	47	D-Lyxose	63
D-Xylose	79	Turanose	60
L-Fucose	106		

Experimentally the results set out in Table 5 answer the questions as well as they can be answered in the absence of systems designed to maintain steady concentrations of the metabolites. The higher the initial concentration of glucose, the lower the initial rate of cellobiase synthesis; as glucose is eliminated by metabolism the rate of cellobiase synthesis comes to the same level in every flask. Methyl α-glucoside, which is a rather poor respiratory substrate, will yield glucose to the system at about the same rate as cellobiose; it has virtually no effect on the induction process.

Table 6 compares glucose with two other metabolizable monoses. The results are obviously comparable. Table 7 extends the comparison in an even more skeletonized form to a variety of sugars. It may be remarked that the two methyl pentoses, L-rhamnose and L-fucose, do not seem to be metabolized by *S. atra*; endogenous

β-galactosidase is quite lacking in sucrose-grown cells and in this case lactose is metabolically unavailable also.

The only case of nearly total repression encountered is that of the two ketoses; further studies showed that the quoted values for fructose (+5%) and sorbose (−6%) are in fact zero within experimental error. The effect is not due to inhibition of glucose oxidase in the assay system by carried-over sugars. If the crude supernatants from the centrifugation of disrupted mycelium, in which induced cellobiase should have appeared, were dialysed to remove the ketoses before assay, there was no apparent increase in cellobiase. In another experiment, L-sorbose (100 mg/l) was

TABLE 8

INFLUENCE OF CERTAIN METABOLITES ON THE FORMATION OF INDUCED CELLOBIASE IN *S. ATRA*

In two separate experiments portions of a washed mycelial suspension of 3-day-old sucrose-grown *S. atra* were shaken aerobically at 28°C for 4 hr in the presence of cellobiose (2000 mg/l). Additives were at 2000 mg/l, and acids had been adjusted in concentrated solution to pH 5 before addition. The values give the increase in cellobiase over a control experiment

Experiment 1		Experiment 2	
Additive	Induced Cellobiase Formed (units/0·5 ml)	Additive	Induced Cellobiase Formed (units/0·5 ml)
Glycerol	0·432	Oxalacetate	3·222
Acetate	1·602	Maleate	2·907
Citrate	1·851	Acetate	3·303
Asparagine	1·488	Glutamine	2·940
Malate	0·927	Aspartate	3·240
Succinate	1·662	Glutamate	3·072
Aconitate	1·776	Malate	1·002
α-Oxoglutarate	1·764	Fumarate	2·166
Pyruvate	0·330	Pyruvate	0·454
None (control)	1·788	None (control)	3·084

found to totally inhibit induction by cellobiose (2000 mg/l) over a 6-hr period; in two parallel experiments these concentrations allowed 7 and 20% of the control induction. The nature of the effect is not clear and it may be quite different from that investigated for the other sugars.

It is usually believed that some member of a metabolic chain proceeding from glucose exerts the "glucose" effect, rather than the sugar as such. Since other sugars than glucose show this effect, and with them may be included glycerol (Table 8), it would be better classified as a "metabolite effect". Phosphoglycerate seems to be the first catabolite common to all the substances showing the effect, and their metabolism would be identical beyond this point. A search for nearer precursors of the active metabolite has uncovered a group of three—pyruvate, fumarate, and malate (Table 8).

No other substances belonging to the Krebs cycle were effective; and fumarate was erratically effective, showing inhibition in two of five experiments and little or none in three others. Pyruvate and malate were consistently inhibitory. Malate is connected with pyruvate independently of the Krebs cycle by the enzyme NADP–malate dehydrogenase (E.C. 1.1.1.40; Ochoa, Mehler, and Kornberg 1948). The NAD–malate dehydrogenases seem to be excluded by the ineffectiveness of oxalacetate.

We may therefore postulate that the enzyme sequence

$$\text{fumarate} \xrightarrow{\text{fumarase}} \text{L-malate} \xrightarrow[\text{dehydrogenase}]{\text{NADP–malate}} \text{pyruvate}$$

explains the observations and points to pyruvate as the second stage on the pathway to the active metabolite. It is of course an essential stage in the catabolism of sugars and glycerol.

(*g*) *Effects of Metabolic Inhibitors*

In general the induction of aryl β-glucosidase showed no dependence on an intact cellular metabolism; the results could be interpreted as meaning that no more than a minimal level of endogenous oxidative phosphorylation was needed (Jermyn 1965). This is in strong contrast with the induction of cellobiase where a respiratory metabolite and intact respiration are required.

Table 9 illustrates the effects of a number of metabolic inhibitors on the cellobiase inhibition. It has been constructed so that we have first, on passing from left to right, substances having no effect on aryl β-glucosidase induction, i.e. nystatin, a general fungistatic agent; tetramethyl thiuram disulphide, which disturbs thiol–disulphide equilibra; malonate, which blocks the Krebs cycle; and fluoride and arsenate, which inhibit phosphorylation. Then follow substances that block both inductions, i.e. azide and cyanide, which interact with haem groups, and tributyl tin acetate, a potent inhibitor, and dinitrophenol, a less potent inhibitor, of oxidative phosphorylation.

The simplest inhibiting procedure of all is to exclude oxygen by passing nitrogen through the culture. As in the presence of high concentrations of certain metabolic blocking agents (Table 9), there is in this case also a decrease in "endogenous" cellobiase. There is no trace of the light-sensitivity encountered in the aryl β-glucosidase induction.

(*h*) *Amino Acid Analogues*

A full list of amino acid analogues tested may be found in Part XII of this series (Jermyn 1965). Of these only DL-*p*-fluorophenylalanine (to a slight degree), L-*p*-nitrophenylalanine, and DL-2-chloroalanine inhibited the induction.

The action of DL-2-chloroalanine can be reversed by alanine (Table 10), and appears to be a simple case of blocking protein synthesis at some stage by incorporation of the analogue. The blockage may well be at the level of transfer RNA since β-chloroalanine is not known to be incorporated into proteins. The effects of the substituted phenylalanines present a more complex case, since tyrosine, alone of the

TABLE 9

EFFECT OF VARIOUS METABOLIC BLOCKING AGENTS ON THE INDUCTION OF CELLOBIASE

Washed suspensions of 3-day-old sucrose-grown *S. atra* mycelium were shaken aerobically for 4 hr at 28°C in the presence of cellobiose (2000 mg/l). Cellobiase was assayed colorimetrically

Nystatin Concn. (units/ml)	Cellobiase Activity (units/ 0·5 ml.)	Inhibitor Concn. (p*I*)	Cellobiase Activity (units/0·5 ml) in Presence of:							
			Tetramethyl Thiuram Disulphide	Malonate	Arsenate	Fluoride	Azide	Cyanide	Dinitro-phenol	Tributyl Tin Acetate
		1		1·67	0·09	0·04				
66	0·72	1·5		3·21	0·45	0·06				
6·6	1·46	2		3·87	1·52	0·09	0·16	0·10	0·14	
0·66	1·86	2·5	1·94	4·42	1·56	0·31	0·18	0·12		
0·07	2·16	3		4·21	1·68	0·86	0·36	0·11	0·13	
0·007	2·40	3·5	2·08			0·96	0·88	0·13		
		4					1·05	0·18	0·68	
		4·5	2·14					0·28		
		5						0·84	0·94	
		5·5	2·42					0·87		
		6						0·96		0·90
		6·5	2·80							
		7								1·36
		7·5	2·88							
No inhibitors	3·33		3·33	4·31	1·97	1·00	0·91	0·94	0·91	2·42
Controls	0·73		0·73	0·68	0·51	0·24	0·15	0·09	0·15	0·42

natural amino acids tested, substantially inhibits the induction. The effect of phenylalanine by itself was consistently in the direction of the result of the single experiment reported in Table 10, i.e. there was evidence of slight inhibition. Nevertheless, phenylalanine reverses the effects of *p*-fluorophenylalanine (Table 10), in the expected way. On the other hand, tyrosine not only does not reverse the effect of *p*-fluorophenylalanine but its effect actually appears to be cumulative with that of *p*-nitrophenylalanine (Table 10). No explanation can be advanced for these

TABLE 10

SELECTED DATA ON THE EFFECT OF CERTAIN AMINO ACID ANALOGUES ON THE INDUCTION OF CELLOBIASE

Washed suspensions of 3-day-old sucrose-grown *S. atra* mycelium were shaken aerobically for 4 hr at 28°C in the presence of cellobiose (1000 mg/l). Cellobiase was assayed colorimetrically

Experiment 1		Experiment 2	
Addition	Cellobiase Activity (units/ 0·5 ml)	Addition	Cellobiase Activity (units/ 0·5 ml)
DL-β-Chloroalanine (6×10^{-4}M)	0·10	DL-*p*-Fluorophenylalanine (6×10^{-4}M)	1·23
+ L-Alanine (10^{-4}M)	0·35	L-*p*-Nitrophenylalanine (5×10^{-4}M)	0·57
+ L-Alanine (2×10^{-4}M)	0·70	L-Phenylalanine (2×10^{-3}M)	2·63
+ L-Alanine (4×10^{-4}M)	0·90	L-Tyrosine (2×10^{-3}M)	1·47
+ L-Alanine (8×10^{-4}M)	0·94	DL-*p*-Fluorophenylalanine (6×10^{-4}M)	
L-Alanine (8×10^{-4}M)	0·92	+ L-Phenylalanine (6×10^{-4}M)	1·59
No additions	0·95	+ L-Phenylalanine (6×10^{-3}M)	3·10
Control	0·21	L-*p*-Nitrophenylalanine (5×10^{-4}M)	
		+ L-Phenylalanine (6×10^{-3}M)	0·52
		+ L-Tyrosine (6×10^{-3}M)	0·37
		No additions	2·96
		Control	0·73

observations at this time; inhibition by *p*-fluoro- and *p*-nitrophenylalanine are probably not closely related phenomena. The framing of a testable hypothesis is not made any easier by the failure of two other phenylalanine analogues, β-2-furyl- and β-2-thienylalanine, to show any inhibitory effect.

(*i*) *Inhibitors of Information Transfer*

This seems the simplest heading under which to group a number of agents the effects of which are set out in Table 11. The conclusion to be drawn is that the whole process of transfer of information from DNA to assembled protein is necessary for the induction; actual *de novo* synthesis of DNA (absence of mitomycin inhibition) is not required.

Table 12 contrasts the effects of certain agents on the induction of cellobiase and aryl β-glucosidase. The latter induction has been shown to be grossly affected by streptomycin only (Jermyn 1965) and the conclusion was drawn that so long as ribosomal organization was maintained (Spotts and Stanier 1961) the cell carried all

TABLE 11

EFFECT OF CERTAIN INHIBITORS ON CELLOBIASE INDUCTION IN *S. ATRA*

Washed mycelial suspensions of 3-day-old sucrose-grown *S. atra* containing cellobiose (2000 mg/l) were divided into portions containing different concentrations of inhibitors, shaken aerobically for 4 hr at 28°C, and tested colorimetrically for cellobiase

Chloramphenicol Concn. (M)	Cellobiase Activity (units/0·5 ml)	Puromycin Concn. (M)	Cellobiase Activity (units/0·5 ml)	Tetracycline Concn. (M)	Cellobiase Activity (units/0·5 ml)	Streptomycin Concn. (M)	Cellobiase Activity (units/0·5 ml)	Mitomycin Concn. (units/ml)	Cellobiase Activity (units/0·5 ml)
10^{-2}	1·49	2×10^{-3}	1·15	3×10^{-3}	0·04	10^{-2}	0·64	2·0	3·42
10^{-3}	2·12	2×10^{-4}	1·32	3×10^{-4}	0·19	10^{-3}	0·59	0·2	3·21
10^{-4}	2·28	2×10^{-5}	1·39	3×10^{-5}	0·48	10^{-4}	1·08	0·02	3·14
10^{-5}	2·43	2×10^{-6}	1·46	3×10^{-6}	0·56	10^{-5}	2·05	0·002	3·63
10^{-6}	2·48	2×10^{-7}	1·60			10^{-6}	2·60	0·0002	3·11
10^{-7}	2·68	2×10^{-8}	1·69						
No addition	3·11		2·42		1·13		2·88		3·64
Control	0·48		0·57		0·25		0·66		0·99

the necessary information for producing the enzyme and it was difficult to fit the observations into the "repressor" scheme. In contradistinction, the induction of cellobiase appears to follow a "conventional" pattern.

If the conclusion that these agents are acting by interference with information transfer is valid, it follows that removal of the inhibited mycelium to a new incubation medium containing inducer but no inhibitor should allow induction to proceed

TABLE 12

DIFFERENTIAL EFFECTS OF CERTAIN INHIBITORS ON β-GLUCOSIDASE AND CELLOBIASE INDUCTION IN *S. ATRA*

Washed mycelial suspensions of 3-day-old sucrose-grown *S. atra* were divided into seven portions. Three of these contained phenyl β-glucoside (1 mg/ml), and three cellobiose (1 mg/ml), and the seventh was a control. For each inducer, the first flask contained an inhibitor at 0·2 mg/ml, the second the inhibitor at 0·002 mg/ml, and the third no addition. After shaking aerobically for 5 hr at 28°C the flasks containing phenyl β-glucoside were tested for aryl β-glucosidase activity, those containing cellobiose for cellobiase activity (colorimetrically), and the control for both

Inhibitor	Inhibitor Concn. (mg/ml)	Cellobiase Activity (units/0·5 ml)	β-Glucosidase Activity (units/ml)
Streptomycin	0·2	0·20	6·6
	0·002	1·21	43·0
	0	1·05	46·0
	Control	0·13	3·2
Tetracycline	0·2	0·05	19·1
	0·002	0·48	21·2
	0	1·16	20·9
	Control	0·18	2·6
Puromycin	0·2	0·91	32·0
	0·002	1·45	29·8
	0	3·33	30·7
	Control	0·46	0·9
Chloramphenicol	0·2	1·28	22·2
	0·002	1·88	24·3
	0	1·95	25·5
	Control	0·21	1·4

normally. Figure 5 illustrates that this is indeed so; it contrasts this behaviour with the permanent effects of exposure to tetramethyl thiuram disulphide, which disrupts the metabolism of the fungus.

One puzzling feature of Table 11 is the ability of very low concentrations of puromycin and chloramphenicol to inhibit the induction strongly, and the very limited further inhibition caused by increasing the concentrations of these agents beyond this point. Since puromycin is believed to interfere with the attachment of

activated aminoacyl-*S*-RNA to the ribosome–messenger RNA complex and chloramphenicol with the formation of this complex, it is difficult to conceive of a system of protein synthesis that will partially bypass the influence of both these reagents.

(*j*) *Metabolites and Antimetabolites of RNA Metabolism*

If the synthesis of messenger RNA is involved in cellobiase induction then these substances should affect the process. Before the effect of antimetabolites can be understood, the extremely erratic behaviour of added metabolites must be

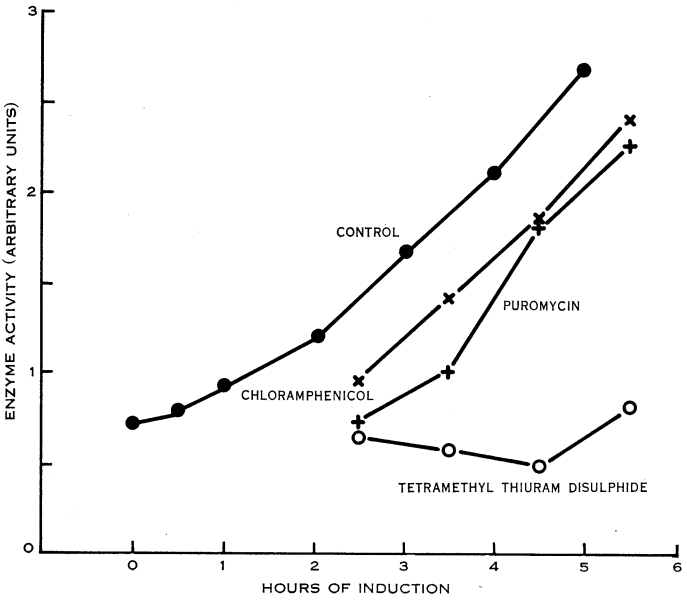

Fig. 5.—Effect of inhibitors on cellobiase induction. A washed suspension of 3-day-old, sucrose-grown *S. atra* mycelium containing cellobiose (2000 mg/l) was divided into four portions containing (1) no addition, (2) chloramphenicol (2×10^{-3}M), (3) puromycin (2×10^{-3}M), and (4) tetramethyl thiuram disulphide (2×10^{-3}M) and shaken aerobically at 28°C. After 2 hr, portions (2), (3), and (4) were filtered rapidly, washed in a number of changes of water, resuspended in cellobiose solution (2000 mg/l) containing no additions, and the shaking continued.

considered. Thus adenine was consistently inhibitory (six out of seven experiments; indifferent in one) but the degree of inhibition at the same concentration varied from mild to almost total. In six experiments, thymine was three times inhibitory, once indifferent, and twice activating. For adenosine the corresponding results were two, four, two.

It is not surprising therefore that the results for such antimetabolites as 2,6-diaminopurine, 5-nitrouracil, and 6-azaxanthine also varied erratically from experiment to experiment. The physiology of the mould is too little understood to devise conditions where the concentration of any given metabolite consistently controls the rate of induced enzyme synthesis. Nevertheless, in any given experiment, striking effects could be achieved by the addition of at least one metabolite or

antimetabolite. It was apparent that cellobiase synthesis was dependent on nucleic metabolism, although there were no predictable effects.

Table 13 shows the results of two experiments that are merely set out as examples, i.e. it is not to be expected that diaminopurine or adenosine would always give effects of this amount or even this sign. But Table 13 does show that the expected antagonisms (diaminopurine and guanine; aminouracil and uracil) occur and that inhibitory effects show the usual sort of relationship between degree of inhibition and concentration of inhibitor.

TABLE 13

SELECTED DATA ON THE EFFECT OF CERTAIN RNA METABOLITES AND ANTIMETABOLITES ON THE INDUCTION OF CELLOBIASE

Washed suspensions of 3-day-old sucrose-grown *S. atra* mycelium were shaken aerobically for 4 hr at 28°C in the presence of cellobiose (2000 mg/l). Cellobiase was estimated colorimetrically. All additives were at the rate of 100 mg/l in experiment 1

Experiment 1		Experiment 2	
Addition	Cellobiase Activity (units/0·5 ml)	Addition	Cellobiase Activity (units/0·5 ml)
2,6-Diaminopurine	0·652	Adenosine (200 mg/l)	0·433
2,6-Diaminopurine + adenine	0·601	Adenosine (40 mg/l)	0·520
2,6-Diaminopurine + guanine	1·075	Adenosine (4 mg/l)	0·754
Adenine	0·292	Adenine (1000 mg/l)	0·676
Guanine	0·943	Adenine (100 mg/l)	0·916
5-Aminouracil	0·496		
5-Aminouracil + uracil	0·988		
5-Aminouracil + thymine	0·670		
Uracil	0·832		
Thymine	0·589		
None	1·144	None	1·126
Control	0·112	Control	0·202

(*k*) *Transfer by Cellobiase and Acceptors for the Enzyme*

The conditions of both the manometric and colorimetric cellobiase estimations were specified in such a way that there would be large excesses of the second and third enzymes in the system, so that production of glucose from cellobiose was rate-limiting in the system. None the less, if the rate of glucose production is so small that the resulting concentration of this secondary substrate is too low to saturate any appreciable fraction of the glucose oxidase molecules, the rate of hydrogen peroxide production must decrease disproportionately even in the presence of excess of the enzyme. Similar remarks apply to the relationship between hydrogen peroxide and peroxidase. At a low enough level of cellobiase activity, therefore, the rate of change of the final measured quantity (oxygen uptake or phenazine formation) in the two systems will no longer be proportional to that of cellobiose breakdown. Hence the

connection between cellobiose concentration and enzyme activity measured in this way cannot be expected to follow Michaelis–Menten kinetics over any wide concentration range.

Figures 6 and 7 show, however, that a Lineweaver–Burk plot for a dialysed cellobiase sample gives a distribution of points that conforms to a straight line within experimental error. A mean "Michaelis constant" of 9×10^{-4}M has been calculated for the cellobiose concentration range (10^{-2}–10^{-3}M) and assay method used in these experiments. The value for this constant must not be taken as meaningful outside these limits. Whether it is possible to use experiments of this sort to examine the effects of various additives on the kinetics of cellobiase depends on the effects of the additives on glucose oxidase and catalase (the manometric assay was used throughout except for volatile alcohols).

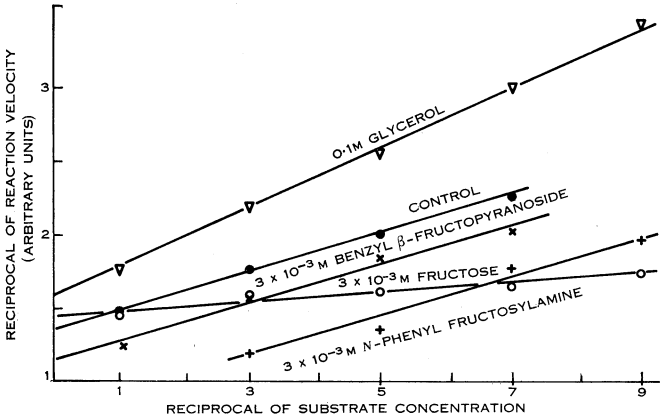

Fig. 6.—Lineweaver–Burk plots showing the effect of selected additives on the decomposition of cellobiose by the cellobiase of *S. atra* (pH 5·4, 28°C). Unit cellobiose concentration, 10^{-2}M).

Adams, Mast, and Free (1960) have shown that high concentrations of D-arabinose have a depressing effect on glucose oxidase, possibly by competition with substrate glucose, although the mechanism proposed by Gibson, Swoboda, and Massey (1964) for the enzyme seems to preclude competitive inhibition. High concentrations of organic solutes may be expected to affect the activity of both glucose oxidase and catalase. However, both these effects are only of importance if the glucose oxidase–catalase portion of the system is so depressed that the cellobiase portion is no longer rate-limiting.

Experimentally, using the glucose oxidase plus catalase portion of the system with added glucose as a check on the effect of additives, it was found that none of the sugar derivatives had any observable effects at the concentration used. Organic solutes had a certain depressing effect, but as this did not exceed 25% for 1M glycerol, it was judged not to affect the overall efficiency of the system.

Figures 6 and 7 set out some kinetic data for the effect of various additives on the manometric cellobiase assay, selected to show various types of effect observed.

Except in one particular point the plots cannot be interpreted directly. This follows from the fact that successful transfer by cellobiase to an acceptor other than water halves the amount of glucose liberated by enzyme action; cellobiose breakdown by the enzymic pathway using added acceptor must go at least twice as fast as that by the pathway using water before there can be a net activation by added acceptor. The *prima facie* interpretation of the Lineweaver–Burk plots for the added sugar derivatives is that these are particularly active acceptors; of the rest it is impossible to hazard an explanation on the basis of kinetic data.

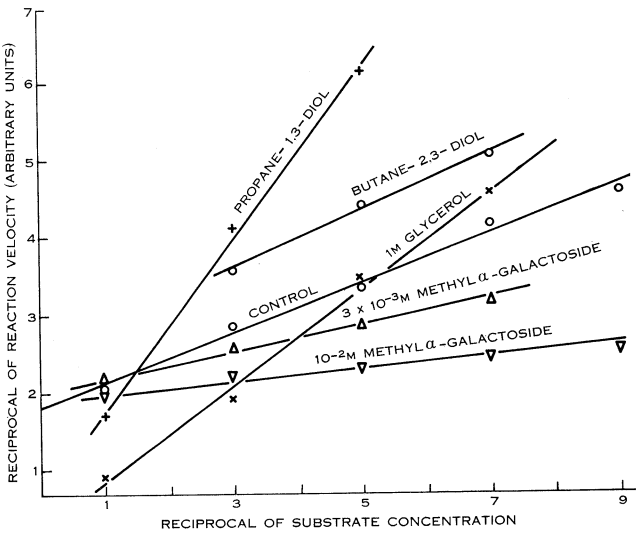

Fig. 7.—Lineweaver–Burk plots showing the effect of selected additives on the decomposition of cellobiose by the cellobiase of *S. atra* (pH 5·4, 28°C). Unit cellobiose concentration, 10^{-2}M).

A series of transfer experiments were run with cellobiase preparations made by dialysing and lyophilizing the supernatants from ultrasonically disrupted mycelia of induced *S. atra*. These preparations held their activity indefinitely when stored at −20°C and allowed incubation mixtures containing high enzyme activities to be made up. The following results were obtained:

(1) No trace of transfer products was ever seen in experiments involving cellobiose plus alkanols.

(2) No trace of transfer products could be seen from experiments involving either cellobiose or β-methylglucoside and straight-chain polyols.

(3) Transfer to galactose and galactosides or fructose and fructosides from β-methyl glucoside could be readily demonstrated. The combinations β-methyl glucoside plus fructose were tested under a variety of conditions. One and the same extra ketose-positive spot was found on paper chromatography in all experiments; it corresponded in position to a disaccharide. No other transfer products were seen.

(4) No trace of autotransfer products were seen in experiments involving β-methyl glucoside. Several such products were visible in small amounts in incubation mixtures in which cellobiase was 35–75% hydrolysed.

With these results established a return can be made to the interpretation of Figures 6 and 7. As predicted, the fructose and galactose derivatives are excellent acceptors of a glucose residue transferred by cellobiase. The observations made with the polyols must be interpreted without invoking transfer except that the results with butane-2,3-diol and cellobiase look very like the results obtained in Part XIX (Jermyn 1966) for aryl β-glucosidase and benzyl β-fructopyranoside, i.e. the acceptor centre is being blocked to give "anticompetitive" inhibition.

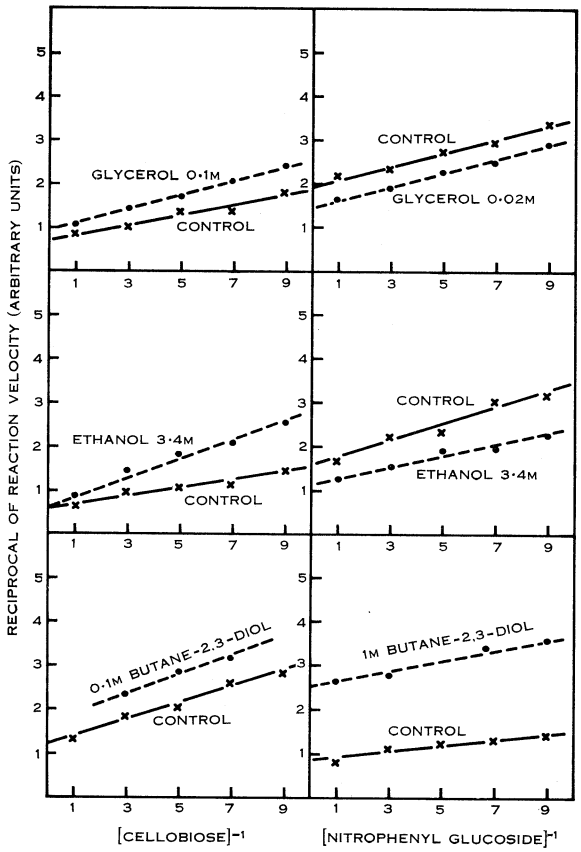

Fig. 8.—Lineweaver–Burk plots selected to show the contrasting effects of certain additives on the decomposition of cellobiose by cellobiase and *p*-nitrophenyl β-glucoside by aryl β-glucosidase. Temperature 28°C and pH 5·0 for β-glucosidase assay; temperature 28°C and pH 5·4 for cellobiase assay. Unit concentration of cellobiose, 10^{-2}M, and of the β-glucoside, 10^{-3}M.

Figure 8 compares observations with cellobiase and aryl β-glucosidase; behaviour can be quite opposite (glycerol), much the same (butane-2,3-diol; the diol is such as poor acceptor for aryl β-glucosidase that it behaves effectively as an anticompetitive inhibitor), and unrelated (ethanol).

V. Discussion

The Jacob–Monod (1961) account of induced enzyme formation has now been generally accepted as the "classical" picture; in these terms, the induction of the cellobiase of *S. atra*, so far as its characteristics have been studied here, is so "classical" that it scarcely warrants further discussion in terms of mechanisms. The induction fits neatly into the pattern of cellulose metabolism in *S. atra*. So long as there is a flow of breakdown products from cellulose, the enzyme continues to be induced; when the production of these substances ceases, or the mould is transferred to other carbon sources, high concentrations of the enzyme are no longer maintained. Since, functionally, the enzyme reduces cellobiose and cellotriose, produced by the random splitting of cellulose chains by cellulase but not themselves substrates of that enzyme, to metabolizable glucose, the advantages of the control mechanism are clear.

No functional significance for the aryl β-glucosidase has been found, although the maintenance of the almost universal synthesis of this enzyme in the lower fungi (Jermyn 1959) against selective pressures argues that there must be some. The unusual character of the induction in *S. atra* suggests that this significance may not be connected with any of the obvious metabolic sequences. The studies on the acceptor pattern of this enzyme in Parts XIV–XIX of this series were partly motivated by the hypothesis that it might be primarily a synthetic rather than a degradative enzyme. However, the kind of acceptor specificity for sugars that was envisaged as necessary for such a function could not be demonstrated. On the basis of the few experiments reported here it now appears as if such a specificity may be associated with the cellobiase instead. In the metabolism of the intact cell one function of the cellobiase may be to synthesize oligosaccharides from cellobiose as a plentiful source of preformed glycosidic linkages, even if an energetically very inefficient one compared with the nucleoside diphosphate sugars. This would leave the natural function of the aryl β-glucosidase even more puzzling.

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VII. References

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