FUNGAL CELLULASES

XXI.* INDUCTION OF THE CELLULASE OF *STACHYBOTRYS ATRA*

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[*Manuscript received May* 17, 1966]

Summary

The use of Modocoll M (ethyl hydroxyethyl cellulose) as a soluble inducing agent for the cellulase of *Stachybotrys atra* is described. Some characteristics both of the kinetics of the induction and its inhibition have been determined. There is a lag period of 2·5 hr in the induction at 28°C for peptone-grown mycelium, and added sugars appear to be competitive with the inducer. These two characteristics have previously been noted as peculiarities in the induction of aryl β-glucosidase. The induction is also dependent on the continued presence of long-chain polymer. Inhibition experiments suggest that the normal processes of induced protein synthesis are operating in all other particulars, although the details of this inhibition are different from those observed in cellobiase induction. The way in which cellobiase and cellulase induction may be integrated during natural growth on cellulose is discussed.

I. INTRODUCTION

"Cellulase" will be taken in this paper to mean the endo-β-glycosidase that attacks the internal bonds in cellulose. This is without prejudice to the question of whether there is an anterior enzyme (the C_1 of Saunders, Siu, and Genest 1948) which brings about the initial disruption of native cellulose. Thomas (1956) has shown that cellulase is an induced enzyme in *S. atra*.

Youatt (1960) has shown that celluloses from different sources have widely differing efficiencies in bringing about adaptive formation of cellulase, and that cellulose from the same initial source varies in its effect according to its history. Thomas (1956) found that low concentrations of cellulase formed in *S. atra* cultures growing on cellulose powder are lost to assay procedures through adsorption on the cellulose. It may be deduced from these observations that cellulose itself is a very unsuitable inducer for observing the kinetics of the induction process.

A search was therefore instituted for a suitable soluble inducer for the cellulase of *S. atra*. When one was discovered (Modocoll M — ethyl hydroxyethyl cellulose), it was used to make a preliminary determination of some parameters of the induction process.

II. MATERIALS AND METHODS

S. atra was grown on McQuade mineral medium containing 1% peptone in shake culture at 28°C and harvested after 3 days growth according to procedures outlined earlier (Jermyn 1965).

The mycelium was disrupted in a Mullard ultrasonic disintegrator and the product centrifuged for 10 min at 2000 *g*. Cellulase in the supernatant was determined

* Part XX, *Aust. J. Biol. Sci.*, 1967, **20**, 193–220.

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viscometrically by the method of Thomas (1956). The fungal culture was grown by serial transfer in flasks sealed with metal caps. After a few such transfers there was a minimum of adventitious cellulose in the medium and the amount of endogenous, apparently "uninduced" cellulase came to a limiting low level.

This mycelium was harvested by filtering and washing on a coarse sintered-glass funnel which was kept dust free. Filtering and washing the mycelium on paper, even hardened (e.g. Whatman No. 541) grades, led to considerable formation of induced cellulase. Filtering was very slow even on the coarsest glass (grade 0) filters since the extracellular slime clogged them very readily, and generally proceeded best under gravity alone.

All other substances and procedures used were as already set out (Jermyn 1965).

TABLE 1

DEPENDENCE OF CELLULASE INDUCTION IN WASHED MYCELIAL SUSPENSIONS OF *S. ATRA* ON THE CARBON SOURCE IN THE GROWTH MEDIUM

S. atra was grown on mineral medium containing one of the carbon sources indicated (1%). After the desired time it was filtered, washed, resuspended in the original volume of distilled water, and cellulose powder (Whatman Standard; 4 g/l) added. After 5 hr of aerobic shaking at 28°C, the suspension was subjected to ultrasonic disintegration, and cellulase determined in the supernatant

Carbon Source	Days of Growth	Cellulase Activity (units/ml)	Carbon Source	Days of Growth	Cellulase Activity (units/ml)
Glucose	1	0·013	Peptone	1	0·005
	2	0·049		2	0·013
	3	0·032		3	0·074
	4	0·022		4	0·033
Starch	1	0·018	Glycerol	1	0·009
	2	0·043		2	0·017
	3	0·028		3	0·045
	4	0·019		4	0·029
Sucrose	1	0·011			
	2	0·040			
	3	0·021			
	4	0·011			

III. RESULTS

Two general points may be made about cellulase induction in *S. atra*. First, in contrast to aryl β-glucosidase and cellobiase induction, the physiological age of the mycelium is not critically important. Except for extremely old or partly autolysed medium, some induction always takes place when a suitable inducer is added to a washed suspension of mycelium, although the amount of induced enzyme formed does of course depend on the age of the mycelium and the carbon source on which it was grown. Second, although cellulase is secreted into the medium in growing

cultures, this does not occur with washed mycelium and disruption of the cells was always necessary for cellulase to be released, at any rate over the few hours of the induction experiments.

(*a*) *Effect of Carbon Source for Growth*

Table 1 sets out an experiment of the type that led to the choice of peptone as a carbon source for the growth of *S. atra* for cellulase induction experiments. It can be seen that the advantage of peptone as carbon source is relative and not absolute and it would have been possible to use *S. atra* harvested after growth on any

TABLE 2

TESTS FOR THE ABILITY OF VARIOUS SUBSTANCES TO INDUCE CELLULASE IN *S. ATRA*

To portions of a washed mycelial suspension of 3-day-old peptone-grown *S. atra* were added various putative inducing agents at a final concentration of 4 g/l. After aerobic shaking at 28°C for 4–5 hr the samples were disrupted ultrasonically and centrifuged, and cellulase assayed in the supernatant

Inducer	Source and Description	Cellulase Activity (units/ml)
Blank	—	0·002
Laminarin	Gift of Prof. T. Dillon	0·002
Phytomonas tumefaciens polysaccharide	Laboratory preparation	0·002
Mannan A	Ivory nut; laboratory preparation	0·001
Xylan	Pear cell wall; laboratory preparation	0·002
Cellulose powder	Whatman; standard grade	0·038
Cellodextrin 1	Laboratory preparation from Whatman powder; degree of polymerization $\simeq 200$	0·031
Cellodextrin 2	As above; degree of polymerization $\simeq 30$	0·025
Sodium carboxymethyl cellulose	I.C.I.; high viscosity; degree of substitution $\simeq 0{\cdot}6$	0·002
Sodium cellulose sulphate	Eastman; medium viscosity; degree of substitution $\simeq 0{\cdot}5$	0·021
Modocoll M	Mo och Domso AB; degree of substitution $\simeq 0{\cdot}8$	0·057
Diethylaminoethyl cellulose	Eastman; degree of substitution $\simeq 0{\cdot}15$ (insoluble)	$< 0{\cdot}001$
ECTEOLA cellulose	Whatman; degree of substitution $\simeq 0{\cdot}05$ (insoluble)	$< 0{\cdot}001$
Cellulose phosphate	Whatman; degree of substitution $\simeq 0{\cdot}3$ (insoluble)	0·002

of the other carbon sources in the later experiments. Peptone also functions as source of presynthesized amino acids for the mould, and this may have some influence on the physiological state of the mycelium that favours the induction.

(*b*) *Effect of Various Inducers*

Table 2 shows the comparative effect of a number of putative inducers on the induction of cellulase. Polymers containing β-1,4-linked monose residues other than glucose (xylan, mannan A), a polymer with β-1,3-linked glucose residues

(laminarin), and one with β-1,2-linked glucose residues (*P. tumefaciens* polysaccharide) were all ineffective. Of the substituted celluloses, only the water-soluble cellulose sulphate and Modocoll M were effective as inducers and a number of experiments agreed in showing that Modocoll M was 2–3 times as effective as cellulose sulphate. This last conclusion is valid only for the samples of substituted cellulose actually used; it is quite possible that samples of other chain lengths or degrees of substitution would give different answers. Modocoll M was therefore chosen as a standard inducer for further experiments.

(c) *General Nature of the Inductive Process*

Figure 1 illustrates a number of points about the inductive process:

(1) There is a lag period in the induction of about 2·5 hr in aerobic shaken suspension at 28°C. A similar lag period was observed when powdered cellulose was used as inducer.

(2) Once the induced synthesis of enzyme begins it is linear with time.

(3) The induced synthesis is dependent on the presence of long-chain material. The obvious interpretation of the halt in the synthesis of cellulase at the lower Modocoll M concentration is that the action of the enzyme has degraded the long-chain inducer. This interpretation is easily checked by taking a Modocoll M solution, allowing a cellulase preparation to act on it for various lengths of time, destroying the enzyme by heating, and checking the resulting preparation for inducing activity. It was found that when the intrinsic viscosity of the Modocoll M solution had fallen to 50% of its initial value, inducing ability was completely lost. End-group determination by the dinitrosalicylic acid method (Bernfeld 1955) showed that less than 2% of the linkages had been ruptured. If the mean degree of polymerization of Modocoll M is taken as 1000–2000, then a degree of polymerization of at least 50–100 seems to be required for an effective inducer.

(4) Once formed the cellulase is stable in the presence of intact mycelium. This is in direct contrast to cellobiase which rapidly disappears after the inducer has been metabolized by the mould (Jermyn 1967). In the experiment illustrated in Figure 1, the enzyme activity was still at the same level in the experiment involving the lower concentration of Modocoll M after a further 20 hr.

(5) Experiments to determine the effect of inducer concentration on rate of induced enzyme formation are not possible with this experimental arrangement. Figure 1 shows that the concentration range where a decrease in the initial rate of synthesis of the enzyme follows a decrease in inducer concentration must lie at a level well below the minimum concentration (200 mg/l) illustrated there. However, if Modocoll M concentration is dropped by a further factor of 10 (to 20 mg/ml) a very small increase in cellulase is demonstrable between 2·5 and 3 hr followed by no further increase greater than that seen in the control. The inducer is apparently too rapidly degraded by the first traces of the enzyme that it induces for any conclusions to be drawn.

(6) An additional point not illustrated in Figure 1 is that the induction cannot proceed in the absence of oxygen; a nitrogen atmosphere completely inhibits it. Like the cellobiase induction, but unlike that of aryl β-glucoside, it is insensitive to daylight.

(*d*) *Effect of Metabolizable Substances and Metabolic Inhibitors*

No external source of nitrogen tested, whether as ammonia, nitrate, asparagine, casein hydrolysate, or a synthetic amino acid mixture had any effect in promoting the induction.

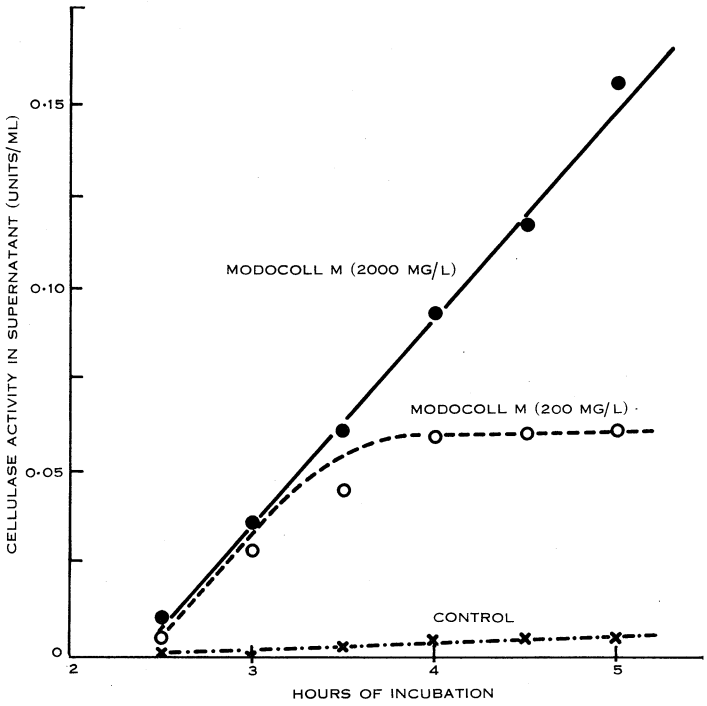

Fig. 1.—Effect of various concentrations of Modocoll M on cellulase induction in *S. atra*. A sample of washed 3-day-old, peptone-grown *S. atra* mycelial suspension was divided into portions, of which one was retained as a control; to the others was added Modocoll M at the concentrations indicated. All were shaken aerobically at 28°C. At intervals, samples were withdrawn, subjected to ultrasonic disintegration, centrifuged, and the cellulase in the supernatant assayed.

Table 3 shows a sighting experiment in which a number of readily metabolizable substances are tested for their effect on cellulase induction. This is seen to be in every case inhibitory; cellobiase induction thus shows a "glucose effect". Before attempting an explanation for this observation, Table 4 showing the effect of a variety of metabolic inhibitors may be considered.

Fluoride which inhibits glycolysis and arsenate which inhibits both glycolytic and, to a lesser extent, oxidative phosphorylation are ineffective in inhibiting the

induction of cellulase. Dinitrophenol, an uncoupler of oxidative phosphorylation, is only partly effective at 10^{-3}M and azide at 10^{-4}M. Tributyl tin acetate, an inhibitor of oxidative phosphorylation, and cyanide, which inhibits not only this

TABLE 3

EFFECT OF CERTAIN METABOLITES ON CELLULASE INDUCTION IN *S. ATRA* BY MODOCOLL M

To portions of a washed mycelial suspension of 3-day-old peptone-grown *S. atra* were added Modocoll M (4 g/l) plus certain metabolizable substances (4 g/l). After 4 hr of aerobic shaking at 28°C, the samples were disrupted ultrasonically and centrifuged and cellulase assayed in the supernatant. These conditions were taken as standard for the experiments reported in Tables 4 to 10. In this and succeeding tables "control" refers to an experiment with added Modocoll M only, "blank" to an experiment with no additions whatever

Addition	Cellulase Activity (units/ml)	Addition	Cellulase Activity (units/ml)
Sucrose	0·004	Cellobiose	0·003
D-Glucose	< 0·001	D-Galactose	0·002
Glycerol	0·006	D-Gluconate*	0·007
D-Xylose	< 0·001	Soluble starch	< 0·001
Control	0·072	Blank	< 0·001

* Potassium salt.

cellular function but many others, are both exceedingly effective inhibitors. It would seem that endogenous energy supplies at a mitochondrial level are suffcient for induced cellulase synthesis. The exact internal sources of this energy cannot be

TABLE 4

EFFECT OF VARIOUS METABOLIC INHIBITORS ON CELLULASE INDUCTION IN *S. ATRA* BY MODOCOLL M

Values are given as a percentage of induced enzyme synthesis in the presence of Modocoll M alone

Metabolic Inhibitor Added	Inhibitor Concn. (M)	Induced Enzyme Synthesis (%)	Metabolic Inhibitor Added	Inhibitor Concn. (M)	Induced Enzyme Synthesis (%)
Azide	10^{-3}	14	Cyanide	10^{-3}	0
Azide	10^{-4}	51	Cyanide	10^{-4}	16
Fluoride	10^{-2}	97	2,4-Dinitrophenate	10^{-3}	56
Arsenate	10^{-2}	105	2,4-Dinitrophenate	10^{-4}	90
Tributyl tin acetate	10^{-5}	0			

specified on the basis of a few simple experiments. Table 5 shows that inhibitors of glycolysis do not reverse the glucose effect. As in the induced synthesis of aryl β-glucosidase in *S. atra*, it seems unlikely that the normal metabolism of glucose is playing a direct effect in the glucose effect in this case.

In Table 6, the interrelationship between cellobiose and Modocoll M in the induction is set out. Cellobiose was chosen for these experiments because endogenous or induced cellobiase would have converted it during the lag period to a mixture

TABLE 5

EFFECT OF ADDITION OF GLUCOSE AND TWO GLYCOLYTIC INHIBITORS, EITHER ALONE OR IN COMBINATION, ON CELLULASE INDUCTION IN *S. ATRA* BY MODOCOLL M

Addition	Cellulase Activity (units/ml)
Glucose (10^{-3}M)	0·014
Fluoride (10^{-2}M)	0·114
Arsenate (10^{-2}M)	0·098
Glucose (10^{-3}M)	
+Fluoride (10^{-2}M)	0·012
+Arsenate (10^{-2}M)	0·016
Control	0·104
Blank	0·008

of the original cellobiose and hydrolytically produced glucose approximating to the "natural" metabolic inhibitor arising from growth on cellulose. Data from the cellobiase induction experiments suggest that some cellobiose would be present until

TABLE 6

EFFECT OF VARIOUS CONCENTRATIONS OF MODOCOLL M ON CELLULASE INDUCTION IN *S. ATRA* IN THE PRESENCE OR ABSENCE OF CELLOBIOSE

Modocoll M (mg/l)	Cellulase Activity (units/ml)	
	No Cellobiose	Cellobiose (100 mg/ml)
5000	0·099	0·109
3000	0·109	0·094
800	0·103	0·066
320	0·083	0·038
Blank	0·021	

up to 5–6 hr after addition to the induction medium, while the standard period used in cellulase induction experiments such as those of Table 6 is 4 hr. The experimental scatter is not sufficient to hide the fact that the relationship between the inducer and the inhibitor is essentially a competitive one.

(e) *Effect of Amino Acid Analogues*

A set of amino acid analogues (Jermyn 1965) was tested for their ability to inhibit the induction of cellobiose. Only *p*-fluorophenylalanine showed sufficient

TABLE 7

EFFECT OF CERTAIN SELECTED AMINO ACID ANALOGUES ON CELLULASE INDUCTION IN *S. ATRA* BY MODOCOLL M

Amino Acid Analogue (50 mg/l)	Cellulase Activity (units/ml)
S-Aminoethyl-L-cysteine	0·116
β-Chloro-DL-alanine	0·118
DL-*p*-Fluorophenylalanine	0·010
DL-β-Furylalanine	0·085
DL-β-Thienylalanine	0·092
DL-*p*-Nitrophenylalanine	0·104
Sarcosine	0·117
DL-Citrulline	0·120
Control	0·111
Blank	0·011

inhibitory power to merit further investigation. Table 7 shows how sharply its effect contrasts with that of certain other phenylalanine analogues and also of

TABLE 8

EFFECT OF ADDITION OF PHENYLALANINE AND *p*-FLUOROPHENYLALANINE IN VARIOUS RATIOS ON CELLULASE INDUCTION IN *S. ATRA* BY MODOCOLL M

Addition	Cellulase Activity (units/ml)
L-Phenylalanine (500 mg/l)	0·079
DL-*p*-Fluorophenylalanine (50 mg/l)	0·008
DL-*p*-Fluorophenylalanine (5 mg/l)	0·013
DL-*p*-Fluorophenylalanine (50 mg/l) +L-Phenylalanine (100 mg/l)	0·004
DL-*p*-Fluorophenylalanine (5 mg/l) +L-Phenylalanine (100 mg/l)	0·033
DL-*p*-Fluorophenylalanine (5 mg/l) +L-Phenylalanine (500 mg/l)	0·074
Control	0·070
Blank	0·006

β-chloroalanine and *S*-aminoethylcysteine, which are effective in repressing cellobiase and aryl β-glucosidase respectively. The origin of this striking trichotomy cannot

even be guessed at; it seems unlikely that a specific amino acid analogue could block the synthetic mechanism for a specific protein. Lysine, alanine, and phenylalanine are all present at reasonable concentrations in the amino acid pool of actively growing *S. atra* (Jermyn 1965). It seems unlikely that competition between natural amino acids and their analogues for the protein-synthesizing machinery of the cell can account for the observed results, and there is no reason to suppose that such competition would lead to differential effects on the synthesis of unrelated proteins by various analogues. An alternative explanation would be to suppose that the analogues are actually incorporated and that one or more lysine, alanine, or phenylalanine residues are crucially involved in the activity or structural integrity of the respective enzyme proteins. Two clues, possibly leading to contradictory conclusions, are the facts that *S*-aminoethylcysteine has been shown to be incorporated into the proteins of *S. atra* and that *p*-fluorophenylalanine does show some activity in inhibiting the inductive synthesis of cellobiase.

Table 8 shows that the inhibitory effect of *p*-fluorophenylalanine on cellulase induction is in fact completely reversed by phenylalanine in the expected manner.

TABLE 9

EFFECT OF ADDITION OF GUANINE AND 8-AZAGUANINE EITHER SEPARATELY OR TOGETHER IN TWO DIFFERENT EXPERIMENTS ON CELLULASE INDUCTION IN *S. ATRA* BY MODOCOLL M

Addition	Cellulase Activity (units/ml)	
	Experiment 1	Experiment 2
Guanine (50 mg/l)	0·058	0·097
Guanine (5 mg/l)	0·097	0·102
8-Azaguanine (50 mg/l)	0·124	0·029
8-Azaguanine (5 mg/l)	0·117	0·043
Guanine (50 mg/l) +Azaguanine (5 mg/l)	0·104	0·055
Guanine (5 mg/l) +Azaguanine (50 mg/l)	0·087	0·026
Control	0·131	0·091
Blank	0·004	0·012

(*f*) *Effect of Purine and Pyrimidine Analogues*

Purine and pyrimidine analogues show the same sort of effect on cellulase induction as they have on cellobiase induction (Jermyn 1967). There was in general an effect to be found in any given experiment, but the nature and sign of this effect varied from experiment to experiment. In general, it may be said that conditions have not been established where the availability of any given purine or pyrimidine is consistently limiting in determining the rate of protein synthesis.

None the less, the results imply that the synthesis of RNA is involved in the inductive synthesis of cellulase.

Table 9 illustrates the confusing nature of the evidence to be considered by setting out some observations made with 8-azaguanine. The conclusion would seem to be that maintenance of a certain level of guanine in the cells of *S. atra* is necessary for the inductive synthesis of the cellobiase. Either too high or too low a concentration, presumably in relation to the other building blocks for RNA, leads to a fall in enzyme synthesis.

(*g*) *Effect of Inhibitors of Information Transfer*

If the normal mechanisms of protein synthesis are functioning, then substances that interfere with the transcription of genetic information should inhibit the inductive synthesis of cellulase. Table 10 shows that this is indeed the case.

TABLE 10

EFFECT OF ADDITION OF CERTAIN INHIBITORS OF INFORMATION TRANSFER ON CELLULASE INDUCTION IN *S. ATRA* BY MODOCOLL M

Inhibitor	Inhibitor Concn. (M)	Cellulase Activity (units/ml)
Chloramphenicol	5×10^{-3}	0·064
	5×10^{-4}	0·107
Puromycin	2×10^{-3}	0·033
	2×10^{-4}	0·071
Tetracycline	2×10^{-3}	0·012
	2×10^{-4}	0·028
Streptomycin	2×10^{-3}	0·015
	2×10^{-4}	0·047
Control		0·141
Blank		0·008

IV. DISCUSSION

The induced synthesis of cellulase seems to follow the same pattern as the induced synthesis of cellobiase (Jermyn 1966), except in two particulars. These are the long lag period before the appearance of induced cellulase and the apparent action of added sugars as competitors of the inducer rather than as the source of inhibitory metabolites. Both these features also appear in the induced synthesis of aryl β-glucosidase (Jermyn 1965). The peculiar features of the latter induction thus appear to be not altogether so exclusive as they seemed when they were first discovered. There may be any number of steps between the first contact of an inducer with the cell and the final triggering of the protein synthetic mechanism, and different inductions may combine a selection of these steps in different ways.

If it is accepted that the presence of macromolecules is necessary for cellulase induction, then this induction must be due to the presence of inducers external to the cell. Tracer experiments (Jermyn 1965) showed that inducing phenyl β-thioglucoside did not appear to be taken up by starch-grown *S. atra* in the aryl β-glucosidase

induction. Both features common to the two inductions (lag period, competitive action of sugars) could well be associated with reactions at a cell surface.

A striking feature of the observations on cellulase and cellobiase induction is the degree to which the products of the reactions of these enzymes control their induction in simple feedback loops. It must be remembered that, in nature, the mould growing on moist cellulose finds itself in an environment which is at best semi-aqueous, so that there will be little diffusion of the products of enzyme action and high effective concentrations are maintained. The induction of cellulase leads to the production of cello-oligosaccharides which in turn induce cellobiase, the products of which inhibit further cellulase formation. Since cellulase is fairly stable after induction a steady state will be set up giving a fairly uniform rate of cellulase action.

The induction of cellobiase is limited by the presence of metabolites, and here again a steady state will be established. Furthermore since it is unstable in the cell, it will tend to decrease in amount if its induction ceases. This will lead to a lifting of the repression of cellulase synthesis by the products of cellobiase action, and hence the supply of more inducers for cellobiase, so that an overall equilibrium between the two sets of inducers will be established. The depression of respiration of uninduced cells in the presence of cello-oligosaccharides (Jermyn and Drew 1967) also takes its place here; it will also tend to equilibrium by preventing the exhaustion of metabolites in the absence of the machinery for producing more.

V. Acknowledgment

I am indebted to Mr. C. Tsiganos for much of the experimental work in this paper.

VI. References

Bernfeld, P. (1955).—In "Methods in Enzymology". (Eds. S. P. Colowick and N. O. Kaplan.) p. 149. (Academic Press, Inc.: New York.)

Jermyn, M. A. (1965).—*Aust. J. Biol. Sci.* **18**, 387.

Jermyn, M. A. (1966).—*Aust. J. Biol. Sci.* **19**, 1153.

Jermyn, M. A. (1967).—*Aust. J. Biol. Sci.* **20**, 193.

Jermyn, M. A., and Drew, P. G. (1967)—*Enzymologia* **31**, in press.

Saunders, P. R., Siu, R. G. H., and Genest, R. P. (1948).—*J. Biol. Chem.* **174**, 697.

Thomas, R. (1956).—*Aust. J. Biol. Sci.* **9**, 159.

Youatt, G. (1960).—*Aust. J. Biol. Sci.* **13**, 188.