A STUDY OF EXTRACELLULAR RIBONUCLEASE ACTIVITY IN *USTILAGO HORDEI*

by

Nils Torben Bech-Hansen

B.Sc., University of British Columbia, 1968

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in the Department

of

Botany

We accept this thesis as conforming to the

required standard

THE UNIVERSITY OF BRITISH COLUMBIA

January, 1970

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree tha permission for extensive copying of this thesis for scholarly purposes may be granted by the Head of my Department or by his representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Botany

The University of British Columbia
Vancouver 8, Canada

Date February 20, 1970

ABSTRACT

Extracellular ribonuclease activity was detected in culture media of sporidial and mycelial cultures of Ustilago hordei. The RNase activity was maximal at pH 5.0, 6.5 and 8.0. The secretion of the RNase activity was a function of the cell density. Release of pH 4.5 and pH 7.5 activity was coincident. Enrichment of the simple glucose and salts medium delayed the initial secretion of activity. The presence of RNA in the medium did not enhance the amount of activity released. Furthermore, since the presence of RNA in the medium was not required for the release of the RNase activity into the medium, it is suggested the synthesis and secretion is a constitutive function.

N-methyl-N'-nitro-N-nitrosoguanidine was used to produce auxotrophic strains. Selection methods for the detection of strains deficient in extracellular RNase activity are discussed.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

The author wishes to extend his sincere appreciation to Professor Clayton Person for his suggestions and criticisms during the investigation and especially in the preparation of this thesis.

Gratitude is also expressed to Dr. Michael Smith and Dr. Michael Shaw, co-supervisors, for their encouragement and helpful discussions. A special word of thanks to Dr. Smith who generously allowed me to use space and equipment in his lab, and who provided many useful criticisms during the preparation of this thesis.

To Dr. Peter Gilham, many thanks for initiating my interest in this study and offering helpful discussions.

The discussions and constructive criticisms of my fellow-colleagues in the Botany and Biochemistry Departments during the research and preparation of this thesis are gratefully acknowledged and appreciated.

My thanks to Margaret Shand for the technical assistance. To Barbara Szuts, Cathy Davison, Rita Rosbergen and Bruce Stewart for their assistance in the preparation of this thesis, thank you.

INTRODUCTION

Enzymes often occur in the culture fluids of bacteria and fungi. Sometimes their presence is a result of the death or lysis of a fraction of the cells in the culture but in other cases the enzymes are truly extracellular (Lampen, 1965). An extracellular enzyme or exoenzyme is, by definition, one which can be produced and released by the cell without any alteration to cell structure greater than that compatible with the cells' normal processes of growth and reproduction (Pollock, 1963).

Studies of extracellular enzymes produced by microbes have been reviewed extensively by Davies (1963), by Pollock (1963) and by Lampen (1965). Some general features are revealed from a comparison of those extracellular enzymes that have been studied (Pollock, 1963):

I) they are small in size - less than 80,000 M.W.;

II) they have little or no cysteine in their primary structure;

III) they often need calcium ions for activation and stabilization; and

Iv) they occur most frequently in gram-positive bacteria and fungi. Such generalizations are based mainly on studies of bacteria since relatively few studies have been carried out with fungi.

Extracellular enzymes are generally considered to be synthesized by the cell's regular protein synthesizing system. Studies of an extracellular penicillinase produced by Bacillus subtilis (Kushner and Polloch, 1961) showed that the liberation of the enzyme requires membrane synthesis. Beaton (1968), who studied Staphylococcus aureus, suggested that release of penicillinase involves alteration of membranous structures. Lampen (1965) in his hypothesis visualizes the formation and secretion of penicillinase as being associated specifically with the mesosomes. This proposal, based

on studies in prokaryotes, may not be totally compatible with the phenomena of secretion in eukaryotes. In Neurospora crassa it has been suggested that extracellular proteases located in membrane-bound vesicles are released extracellularly when they "cross the plasma membrane as intact particles by means of invaginations of the plasmalemma" (Matile et al., 1965). Electron micrographs have recently been obtained (Stein, 1970) which also support the idea that intracellular vesicles are involved in the secretion of cell products by U. hordei (Pers.) Lagerh. Probably the process of cell secretion in eukaryotes has been best studied in higher organisms, for example with pancreatic cells (production and release of zymogen). In this example zymogen is synthesized on the ribosomes of the endoplasmic reticulum (E.R.), moves into the intercisternal cavities of the E.R. and then to vesicles of the Golgi complex by transient connections. These vesicles then move to the cell surface where they fuse with the plasma membrane to release the zymogen extracellularly (De Robertis, 1965). In U. hordei where there is no Golgi complex (Stein, 1970), the production and release of extracellular products cannot follow this sequence of events. Although it is apparent that there is no single or standard mechanism of secretion used by all eukaryotic cells the process appears in all cases to involve movement, via vesicles, of proteins from their sites of synthesis to the plasma membrane.

Microbial ribonucleases, both intra- and extra-cellular, have been reviewed in detail by Egami and Nakamura (1969). These authors noted that, in general, intracellular RNases have no nucleic acid specificity, have a size of 30,000 - 40,000 M.W. units, are heat-labile and are exonuclease, in contrast to extracellular RNases which have base specificity, a size of 11,000 - 13,000 M.W. units, are heat-stable and are endonucleases.

Nishimura and Nomura (1959), who investigated the mode of formation of extracellular RNase in B. subtilis (strain H), found that the RNase activity

in the medium increased markedly when growth entered the stationary phase and then continued to increase at a constant rate during the stationary phase. In a later study they found that the extracellular RNase of B. subtilis differed from the intracellular RNase in such characteristics as optimum pH, heat stability and ion requirement (Nishimura and Maruo, 1960). This contrasts with the finding that one of the intracellular RNases of Neurospora crassa is very similar to the extracellular RNase (Takai et al., 1967).

The amount of extracellular RNase in the medium can be affected by the conditions of culture; it has been shown by Yanagida et al. (1964), who worked with U. zea, that where RNA or poly U (which was not a substrate for the RNase) was supplied as the sole source of carbon, the production of an extracellular guanyloribonuclease was enhanced. Glitz and Dekker (1964) also found that an extracellular guanyloribonuclease accumulated in the culture medium of U. sphaerogena when RNA was added as the sole carbon source. They therefore considered these to be inducible enzymes. In a subsequent study (Arima et al., 1968) four RNases were purified from the growth medium of U. sphaerogena: RNase U_1, a guanyloribonuclease; RNases U_2 and U_3, both of them puryloribonucleases; and RNase U_4, which was not base-specific. These workers reported a striking increase in the release of RNases U_1 and U_4, together with a small increase in RNases U_2 and U_3, following the addition of RNA as the sole source of phosphorus in the culture medium. In contrast with the results obtained in studies of U. zea and U. sphaerogena, RNA was reported not to enhance the formation or release of extracellular RNases by N. crassa (Takai et al., 1967).

The importance of RNA-degrading enzymes in growing cells is discussed in detail by Egami and Nakamura (1969), who summarize their probable physiological roles as follows:

I) metabolism of RNAs;

II) protection against penetration by phage RNA;

III) supply of nutrients by degrading extracellular RNA;

iv) activation of DNA-specific endonuclease I by removing inhibitory RNA.

Extracellular RNases often have base specificities and in considering the role of such RNases it is known that guanine-rich nucleotides tend to form aggregates and it is therefore suggested that RNases specific for guanylic acid phosphodiester bonds would yield digestion products which may diffuse through the cell membrane (Egami and Nakamura, 1969). On the whole, the microbial extracellular ribonucleases, because of their ease of purification and their stability, have found great favour with the biochemist while the general biological role of such extracellular products remains, for the most part, unresolved.

Species of the genus Ustilago are usually considered to be obligate parasites because they are entirely dependent on their host species during at least part of the life cycle. The association between Ustilago species and their hosts are usually highly specific and, although extracellular enzymes of several Ustilago species have been studied, there is as yet no evidence to indicate that the production of extracellular enzymes contributes in any particular way to the success of these species during their pathogenic phase.

One approach considered useful for evaluating the importance of extracellular enzymes of parasitic fungi involves the production of mutants deficient in the ability to release a specific activity and the study of the ability of such mutants to parasitize the host. Mutants in Ustilago hordei have to date been produced only by UV irradiation (Hood, 1966), but useful application of this method of mutation required the treatment of synchronously growing cultures. The chemical mutagen N-methyl-N'-nitro-N-nitrosoguanidine (NG or nitrosoguanidine) was used in the present study in attempts to produce RNase-deficient mutants. Holliday and Halliwell (1968) have previously

used NG with Ustilago maydis to produce extracellular DNase-deficient mutants. Mandell and Greenberg (1961) first reported the mutagenic action of NG in bacteria and this compound has subsequently been shown to be highly mutagenic. Adelberg et al. (1965) studied the action of NG in Escherichia coli and found that a high yield of mutations was obtained in conditions which gave over 50 percent survival rate. Mutagenic action of NG has similarly been studied in Salmonella typhimurium (Eisenstark et al., 1965), Schizosaccharomyces pombe (Loprieno and Clarke, 1965) and Arabidopsis thaliana (Müller and Gichner, 1964). Loprieno and Clarke (1965) in their study reported the following order for decreasing ratios of mutagenesis to lethality: NG > nitrosomethylurethane > ultraviolet light > nitrous acid. In vitro, NG acts on DNA and RNA to produce 7-methylguanine (Craddock, 1968; Lawley, 1968; McCalla, 1967) and a small amount of 3-methyladenine (Lawley, 1968).

The in vivo action of NG on DNA is specific for the replicating region of the bacterial genome (Cerdá-Olmedo et al., 1968), but this has yet to be shown in fungi. In another in vivo study, Baker and Tessman (1968) found different mutagenic specificities in phages S13 and T4 following treatment with NG. With S13 (as in Salmonella typhimurium) both transitions of GC to AT and of AT to GC were induced in about equal frequences, whereas GC to AT transitions predominated in phage T4. The molecular environment of the replication region of the DNA (which should depend on the specific mechanism of replication, the nature of the DNA polymerase and the base composition of the DNA) is suggested to be responsible for the differences. The authors point out that care should be taken in making generalizations about the specificity of a particular mutagen (e.g. beyond saying that NG is an alkylating agent).

The macromolecular action of NG in vivo is not restricted to DNA (Cerdá-Olmedo and Hanawalt, 1967). Besides causing alteration of DNA which can be recognized and repaired by the dark repair mechanism, NG can inactivate

proteins and inhibit protein synthesis. To a lesser extent NG can also inhibit RNA synthesis, resulting in a reduction in the rate of synthesis of proteins and the production of small amounts of non-functional protein. NG may also cause misreading of the genetic code, which can be suppressed by streptomycin.

Whether NG or diazomethane is the reactive species *in vivo* is still an unanswered question. The former view is supported by Mandell and Greenberg (1960) and McCalla (1967) and the latter by Cerdá-Olmedo and Hanawalt (1968),

As well as having an efficient procedure for inducing mutations, one must also have a selection method for detecting those mutants which are of specific interest. For a detailed consideration of the problems involved see the Discussion.

In the present study, one approach that was considered for the selection of RNase-deficient mutants involved the use of a derivative of RNA which altered the attack of base-specific RNases. The modification of ribonuclease degradation products by the addition, in this case, of a water-soluble carbodiimide to a dinucleotide substrate was first reported by Gilham (1962). N-cyclohexyl-N'-B-(4-methylmorpholinium)ethylcarbodiimide p-toluene-sulfonate (CMC-p-toluenesulfonate) was shown to react specifically with the bases guanine, uracil and thymidine (Ho and Gilham, 1967).

Pyrimidine-specific RNases are restricted to degradation at cytosine when the RNA substrate has previously been reacted with CMC-p-toluene-sulfonate. Similarly it should be feasible to limit the action of RNases of other base specificities (P.T. Gilham, personal communication).

The practical application of this method of limiting RNase action was demonstrated by Lee *et al.* (1965) for pancreatic RNase in the preparation of trinucleotides containing a terminal cytidine, by Naylor *et al.* (1965) for RNase on CMC-modified polynucleotides, and by Sanger *et al.* (1968) in the

restriction of RNase A on modified 5s RNA for partial hydrolyses to facilitate base sequence analysis.

After establishing the presence of ribonuclease activity in the culture medium of the barley-smut fungus (Ustilago hordei (Pers.) Lagerh.), this thesis was undertaken to gain information about the possible role of the enzymes responsible for RNase activity. (Protease and amylase activities were also detected but these were not studied.) The study is divided into two parts: one, the study of the RNase activity in the non-pathogenic phase of the life cycle (nature of activity, secretion pattern, control of secretion); and two, chemical mutagenesis of U. hordei in attempts to produce mutants deficient in RNase activity which would be useful in considering the importance of extracellular RNases in the host parasite relationship of U. hordei and its host, cultivated barley (Hordeum vulgare L.).

MATERIALS AND METHODS

1. BIOLOGICAL MATERIAL

The two standard monosporidial (i.e. haploid) lines of Ustilago hordei (Pers.) Lagerh. used in this study were developed by Hood (1966) and designated by him as E_3^- and I_4^+. Both these lines were used in enzyme studies while E_3^- only was used in mutagenesis experiments.

A mycelial culture derived from a complementation test (Dinoor and Person, 1969) of two sporidial lines derived from a single teliospore, Met$^-$ Pan$^-$ Arg$^-$ A and Met$^+$ Pan$^+$ Arg$^-$a (Drs. Jean Mayo and C.O. Person, personal communication), was studied for production of extracellular RNase.

2. CULTURING

Liquid cultures were grown in a New Brunswick psycrotherm incubator at 22° C on a shaking table (100 RPM). Agar plates were also incubated at 22° C.

In order to measure cell density of cultures at different stages of growth, cultures were grown in liquid culture in 125 ml Erlenmeyer flasks fitted with Klett-Summerson colorimetric tubes (side-arm flasks). By turning the flasks sideways, the side-arm filled with culture medium whose turbidity was then measured on a Klett-Summerson photoelectric colorimeter fitted with a red filter. This allowed one to follow a culture from a cell density of 10^6 cells/ml (0-5 K.U.) through to stationary phase which occurred at a cell density near 2×10^8 cells/ml (400-450 K.U.).

3. CULTURE MEDIA AND SELECTION PLATES

Culture media, minimal and complete, were prepared according to the procedures outlined by Hood (1966). Minimal medium contained 20 ml Vogel's salt solution (see Appendix A) and 10 g glucose per one liter of distilled water. Complete medium was a minimal medium enriched with 5 g Difco yeast extract, 5 g salt-free casein hydrolysate (N.B. Co.), 50 mg tryptophan and 10 ml vitamin solution (see Appendix A) per one liter of minimal medium. For solid medium 2.0% Difco bacto agar was added.

Supplemented minimal medium was prepared according to Holliday (1961). The individual growth factors were added to minimal medium as required: amino acids, 100 mg; purines and pyrimidines, 10 mg; and vitamins, 1 mg per liter.

Initial screening of auxotrophs was carried out on agar plates of minimal medium supplemented with yeast extract, vitamins, or casein hydrolysate, in amounts normally used for complete medium.

Auxotrophs were stored on agar slants (complete medium plus 2.5% agar) at 4° C and transferred every four weeks.

RNA plates containing 0.5% yeast RNA were prepared by adding Millipored 10% RNA solution to autoclaved regular minimal medium containing one percent agar.

4. ASSAYS

(a) Ribonuclease activity was determined quantitatively by measuring the absorbance of acid-soluble degradation products according to the assay for RNase T_1, described by Takahashi (1961) and modified by Arima et al. (1968).

The reaction mixture contained 0.1 ml of enzyme solution, 0.25 ml of 0.2 M Tris buffer, pH 7.5, or 0.2 M sodium acetate buffer, pH 4.5, 0.1 ml of 2×10^{-2} M EDTA, 0.3 ml of distilled water and 0.25 ml of yeast sodium ribonucleate (Schwarz), 10 mg/ml freshly prepared before use. The hydrolysis was allowed to proceed for 30 min at 37° C after the addition of RNA solution and was stopped with 0.25 ml of 0.75% uranyl acetate in 25% perchloric acid. The reaction mixture was centrifuged and 0.2 ml of supernatant solution was diluted in 4.8 ml of distilled water and the absorbance at 260 nm was read on a Unicam SP 800 spectrophotometer fitted with silica cells of one centimeter light path. The amount of enzyme that under the standard assay conditions and thirty minutes of hydrolysis would produce an increase in absorbance of one at 260 nm was defined as one enzyme unit (Takahashi, 1961).

An alternative, qualitative assay for RNase activity similar to that used by Holliday and Halliwell (1968) for DNase and earlier by Jeffries et al. (1957) was employed in assaying for activity released from colonies of U. hordei. Since factors such as agar concentration, per cent RNA and depth of agar influenced this plate assay, the assay was carried out under standard optimal conditions, using plates containing 1% agar, 0.5% RNA and 8 ml of minimal medium per 8.5 cm diameter Petri plate. These RNA plates were spotted or spread with sporidia and grown for about five days at 22° C or they were spotted with culture medium and the plates were floated in a 37° C water bath for thirty minutes. To stop the hydrolysis, the plates were flooded with 10% trichloroacetic acid (TCA). To test the validity of this assay, ten microliters of purified pancreatic RNase (100-0.01 micrograms/ml, approximately 20,000 units/ml; Worthington) were spotted on such RNA plates. Where there was no RNase, a white precipitate formed while in areas of RNase spotting cleared areas were visible. This assay proved to be a rapid and sensitive method for detecting activity that may be due to RNases.

(b) Phosphodiesterase (PDE) I and II (both described by Razzell, 1967) were assayed for by the method of Razzell (M. Smith, personal communication). For PDE I the stock solution contained 0.1 ml M Tris, pH 9.3, 0.05 ml 0.2 M $MgCl_2$, 0.10 ml para-nitrophenyl thymidine-5' phosphate, 5 micromoles per ml (i.e. 8.3 O.D./ml measured at 272 nm in 0.01 M HCl), pH 9.3, and 0.75 ml water. To 0.1 ml of stock warmed 2 minutes at 37° C about 20 microliter of enzyme solution was added and incubated for one hour. The reaction was stopped with 0.25 ml 0.3 M NaOH and the contents of the tube mixed by inverting several times, made up to 1 ml with water and absorbance at 400 nm was determined. For phosphodiesterase II the stock solution contained 0.25 ml M ammonium acetate, pH 5.9, 0.05 ml 0.02 M sodium EDTA, 0.1 ml 2,4-dinitrophenyl thymidine-3' phosphate, 10 micromoles per ml, and 0.55 ml water. The assay procedure was as for PDE I. The use of 2,4-dinitrophenyl thymidine-3' phosphate, a modification of Von Tigerstrom and Smith (1969), allows direct quantitative measurement at pH 5.9 and 360 nm, since the 2,4-dinitrophenoxide anion has maximum absorbancy near the assay pH. This is an improvement over the use of p-nitrophenyl thymidine-3' phosphate as a substrate, since p-nitrophenoxide has little absorbance at pH 5.9.

(c) Phosphomonoesterase, acid and alkaline, were measured by the method of Arima et al. (1968).

5. CHEMICAL MUTAGENESIS, DETECTION AND SCREENING OF MUTANTS

To induce mutations, wild type, log phase sporidia (E_3^-) grown in liquid minimal medium were pelleted and resuspended at a concentration of about 10^8 cells per ml in citrate buffer at pH 5.0 or 5.7 in 40 ml Nalgene tubes. Sufficient NG stock solution was added to give a final concentration of 0.1 mg NG per ml of medium. Standard NG treatment lasted for fifteen minutes at 22 - 24° C. Stock solution of 2 mg per ml of NG was made fresh with each

treatment.

Treated cells were immediately washed with citrate buffer and then with complete medium before being suspended in the treatment volume of complete medium and transferred to 50 ml Erlenmeyer flasks to be incubated for about ten hours before plating.

Samples of cultures treated in this way were diluted to give 50 to 100 colonies per plate when spread on complete medium. Colonies of these plates were replicated, using the method of Lederberg and Lederberg (1952), as follows: to minimal plates to determine auxotrophs, to RNA plates to detect RNase-deficient colonies and, as a final step, to complete plates to confirm that transfer of each colony had been made onto all previous plates. After five to ten days, each plate was examined and the auxotrophs were tentatively identified; these were further defined by their ability to respond to casein hydrolysate, to vitamin solution or to yeast extract. Final classification of an auxotroph was based on a positive response to a single compound added to minimal plates.

Arima et al. (1968) reported four extracellular RNases in Ustilago sphaerogena, two with sharp optima at pH 4.5 and two others with a broader optima at pH 7.5. These observations were taken as the basis for beginning this study. RNA plates were made with a pH of 6.0 or greater in order to assay for deficiency of the RNases with the broad optima (i.e. U_1 and U_4).

6. THE PREPARATION OF CMC-RNA

The water-soluble carbodiimide derivative of RNA was prepared by the method of Ho et al. (1967). The reactants, CMC-p-toluenesulfonate (Aldrich) and yeast sodium ribonucleate (Schwarz), were reacted for 26-30 hours.

RESULTS

1. EVIDENCE FOR EXTRACELLULAR RIBONUCLEASES IN U. HORDEI

(a) Initial qualitative and quantitative assays for RNase activity in the medium of a stationary phase E_3^- culture gave positive results, both at pH 4.5 and 7.5. Assaying across pH range 3.0 to 9.2 demonstrated two pH maxima, one at pH 5.0 and another at pH 8.0 (Fig. 1). In addition, a third maximum was obtained at pH 6.5; this maximum has not been reported previously.

Some preliminary attempts at identifying the number of RNases released by U. hordei were made (see Discussion).

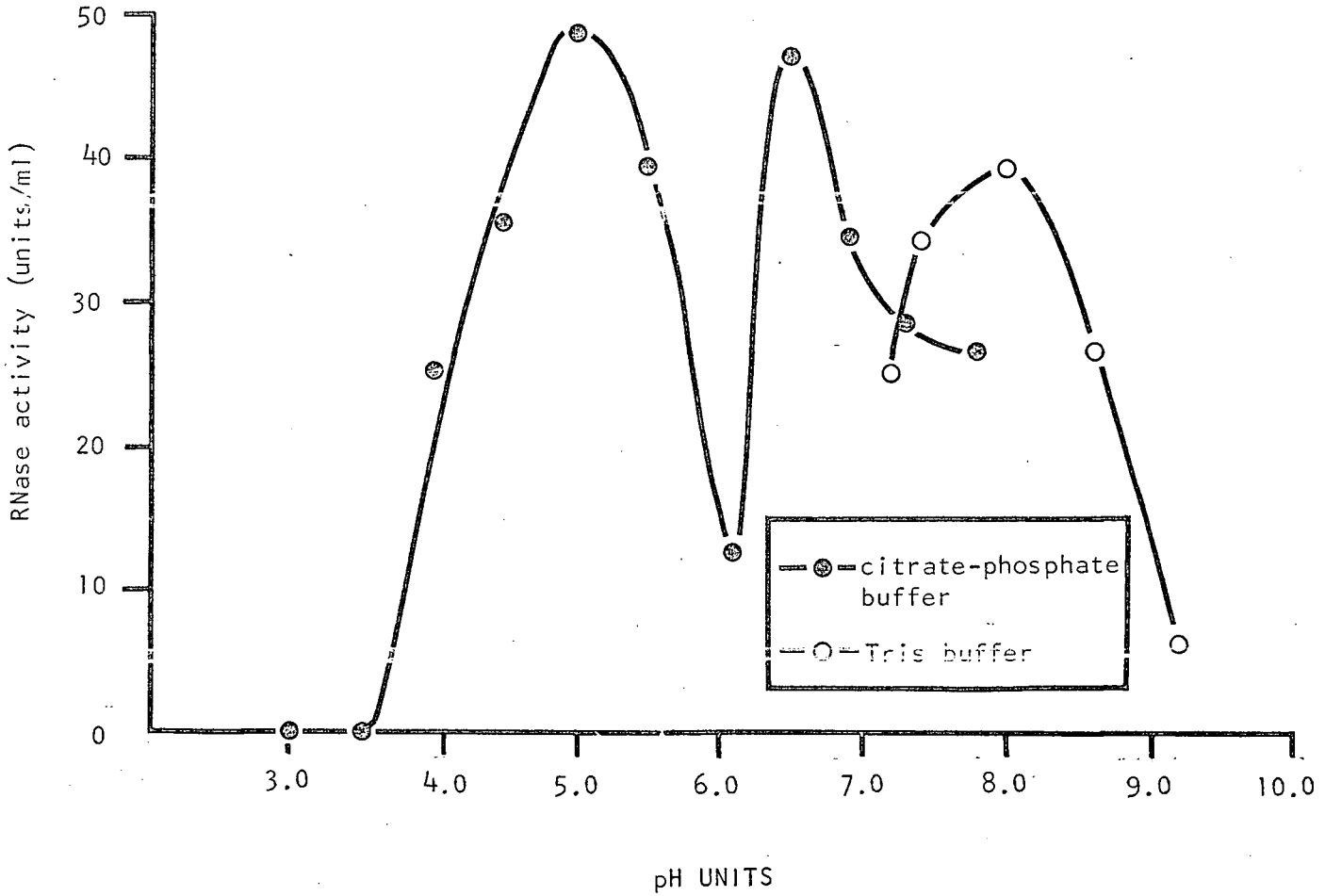
(b) Evidence that the RNase activity was due to the release of extracellular RNases came from observation of culture samples under the light microscope. In samples taken during the period of maximal increase in RNase activity no rupture cells or cell fragments were seen. U. hordei characteristically does not lyse but rather, becomes mycelial towards the end of its log phase of growth.

(c) A slight amount of activity (0.1 mM/ml/hr) due to phosphodiesterase I was found to be associated with the cell surface, which, on centrifuging out the cells and assaying the cell-free culture medium was not detectable. Phosphodiesterase II activity was not detected on the cell surface or in the culture medium.

(d) A ten day old liquid culture of strain E_3^- in minimal medium, that had been in the stationary phase of growth for five days, was assayed for phosphomonoesterase (PME) activity. Activity was detected only under acid conditions (pH 5.5) and primarily in cells containing culture medium (0.54 mM/hr/ml); a small amount was found in cell free medium (0.04 mM/hr/ml), possibly due to cell death or lyses.

Figure 1: The RNase activity of cell-free minimal culture medium, in which strain E_3^- had been grown to stationary phase, measured across the pH range 3.0 to 9.2.

FIGURE I


RNase activity (units/ml)
50
40
30
20
10
0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
pH UNITS
citrate-phosphate buffer
Tris buffer

2. RELEASE OF RIBONUCLEASE ACTIVITY

(a) Figure 2 presents representative graphs to show the release of RNase activity for E_3^- and I_4^+ grown in complete and in minimal medium. A sharp increase in RNase activity occurred in late log phase and by early stationary phase maximal release was achieved. The release of extracellular enzymes during this phase of growth has been observed not only with RNases but also with many other extracellular enzymes, and may be regarded as a rather general characteristic of extracellular enzymes of microorganisms (Egami and Nakamura, 1969).

The release of activity into minimal medium was more gradual and began earlier after entry of the culture into the log phase of growth, in contrast to cultures of complete medium. The increases in ribonuclease activity measured at the two pH's (4.5 and 7.5) parallel each other during the period of release (Fig. 3). This suggests that the period of synthesis and release are co-incidental for the enzymes involved. The total amount of RNase activity was greater in complete medium suggesting that conditions in this medium were more favourable for synthesis or release of RNase.

(b) A stable mycelial stain grown in complete medium released ribonuclease activity in a pattern similar to a sporidial culture in complete medium (Fig. 4). Total RNase activity reached a level similar to that of sporidial cultures. The constitutive release of RNase activity, in both sporidial and mycelial (possibly dikaryotic) cultures would suggest that such is the case for at least all of the non-parasitic part of the life cycle of U. hordei.

(c) To determine whether the addition of RNA to the culture medium had an inducible effect (i.e. increase the amount of RNase release), the release of RNase activity from strain E_3^-, grown in minimal medium plus 0.5% yeast RNA, was measured (Fig. 5). This figure shows, firstly, that the maximal level

Figure 2: Growth (-o-) of strain E_3^- and I_4^+ cultured in either minimal or complete medium; and the release of RNase activity at pH 7.5 (---o---). a) E_3^-/M; b) E_3^-/C; c) I_4^+/M and d) I_4^+/C.

FIGURE 2

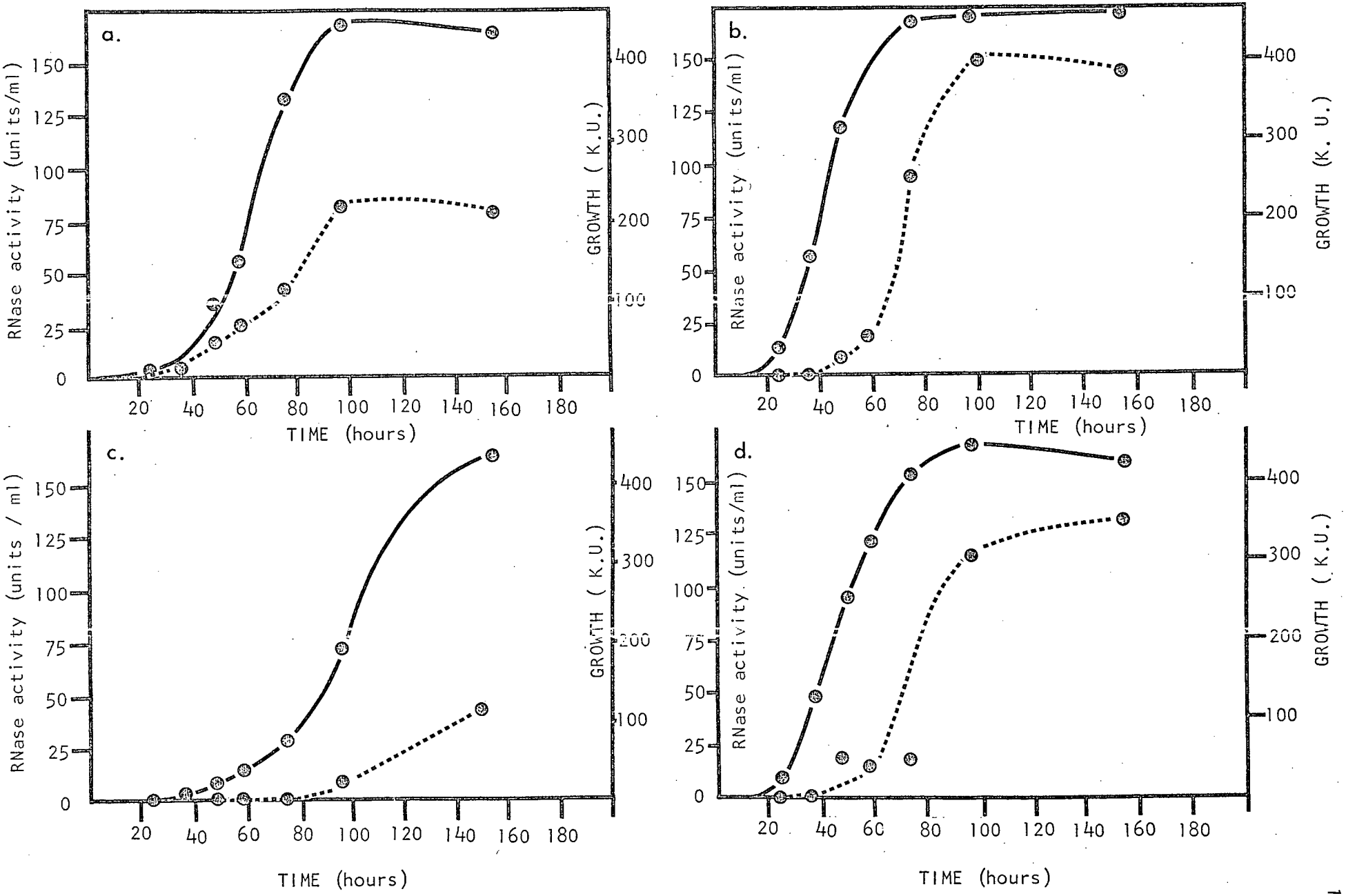

a.
b.
c.
d.
RNase activity (units/ml)
GROWTH (K.U.)
TIME (hours)
0
25
50
75
100
125
150
100
200
300
400
20
40
60
80
100
120
140
160
180

Figure 3: Growth of strain E_3^- in minimal medium (a) and in complete medium (b). RNase activity was measured at both pH 4.5 and pH 7.5.

FIGURE 3

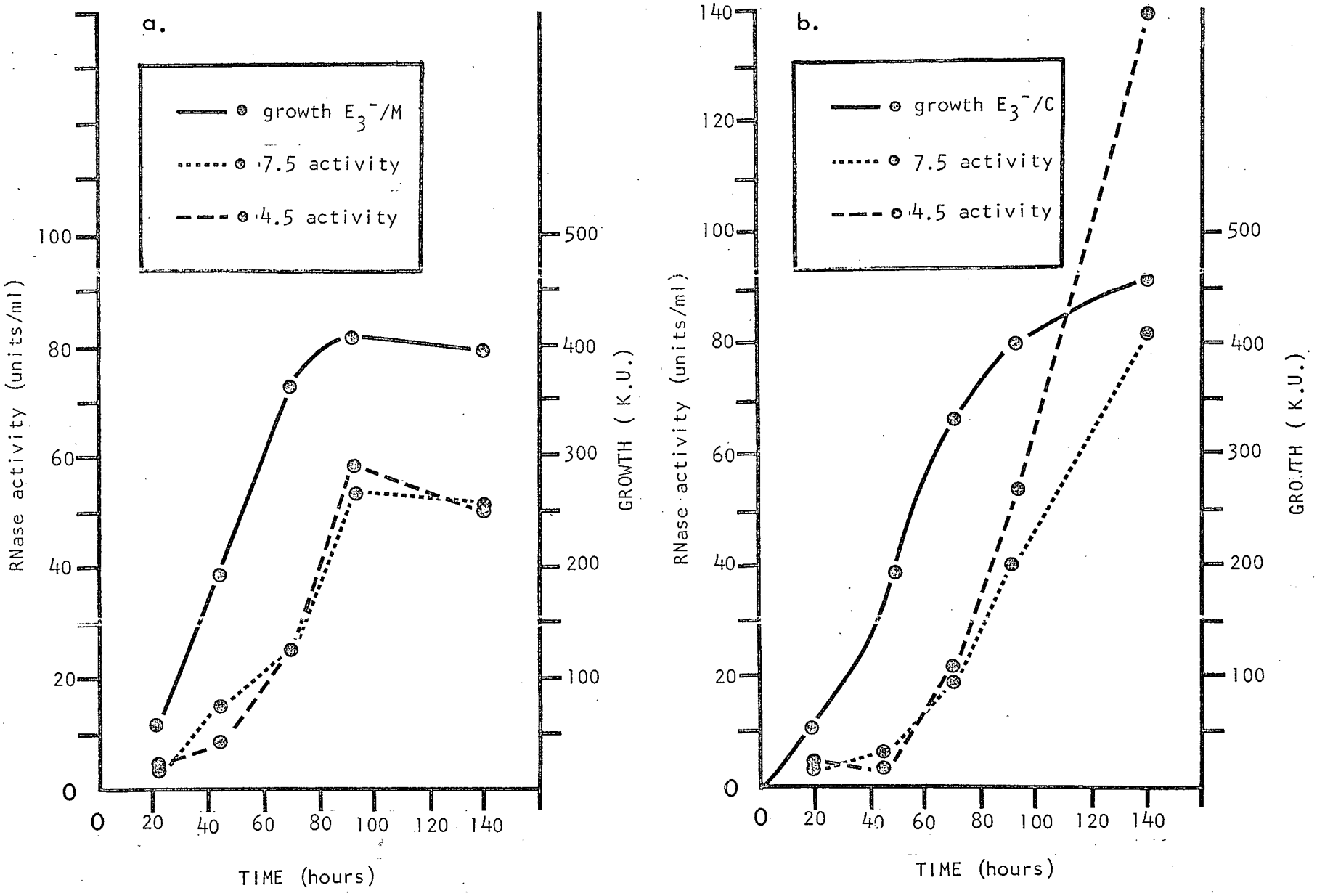

a.
growth E_3^-/M
7.5 activity
4.5 activity
RNase activity (units/ml)
GROWTH (K.U.)
TIME (hours)
0
20
40
60
80
100
120
140
500
400
300
200
100
b.
growth E_3^-/C
7.5 activity
4.5 activity
RNase activity (units/ml)
GROWTH (K.U.)
TIME (hours)

Figure 4: The growth curve of the stable mycelial strain (Myc/C) and the release of pH 4.5 and pH 7.5 RNase activity.

FIGURE 4

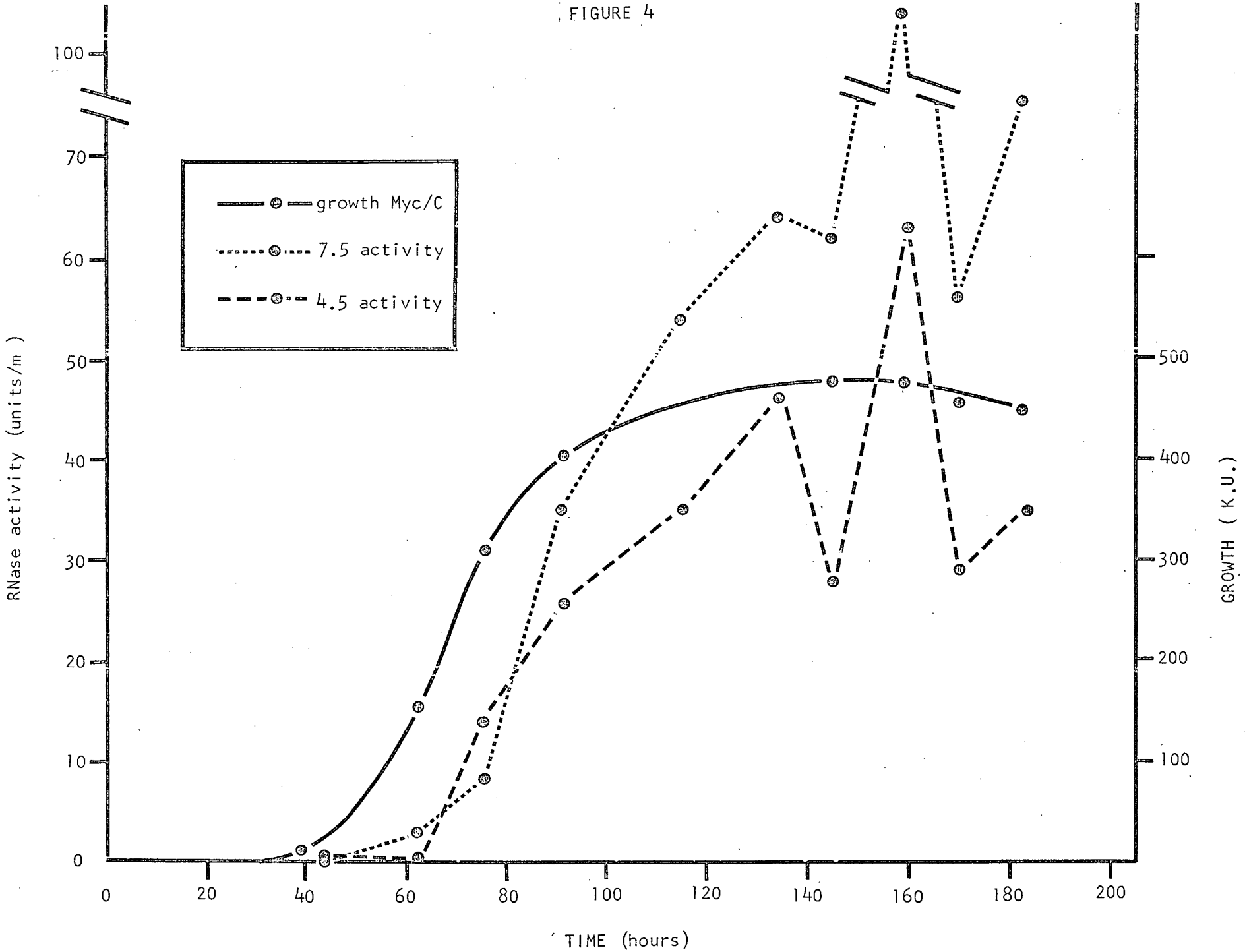
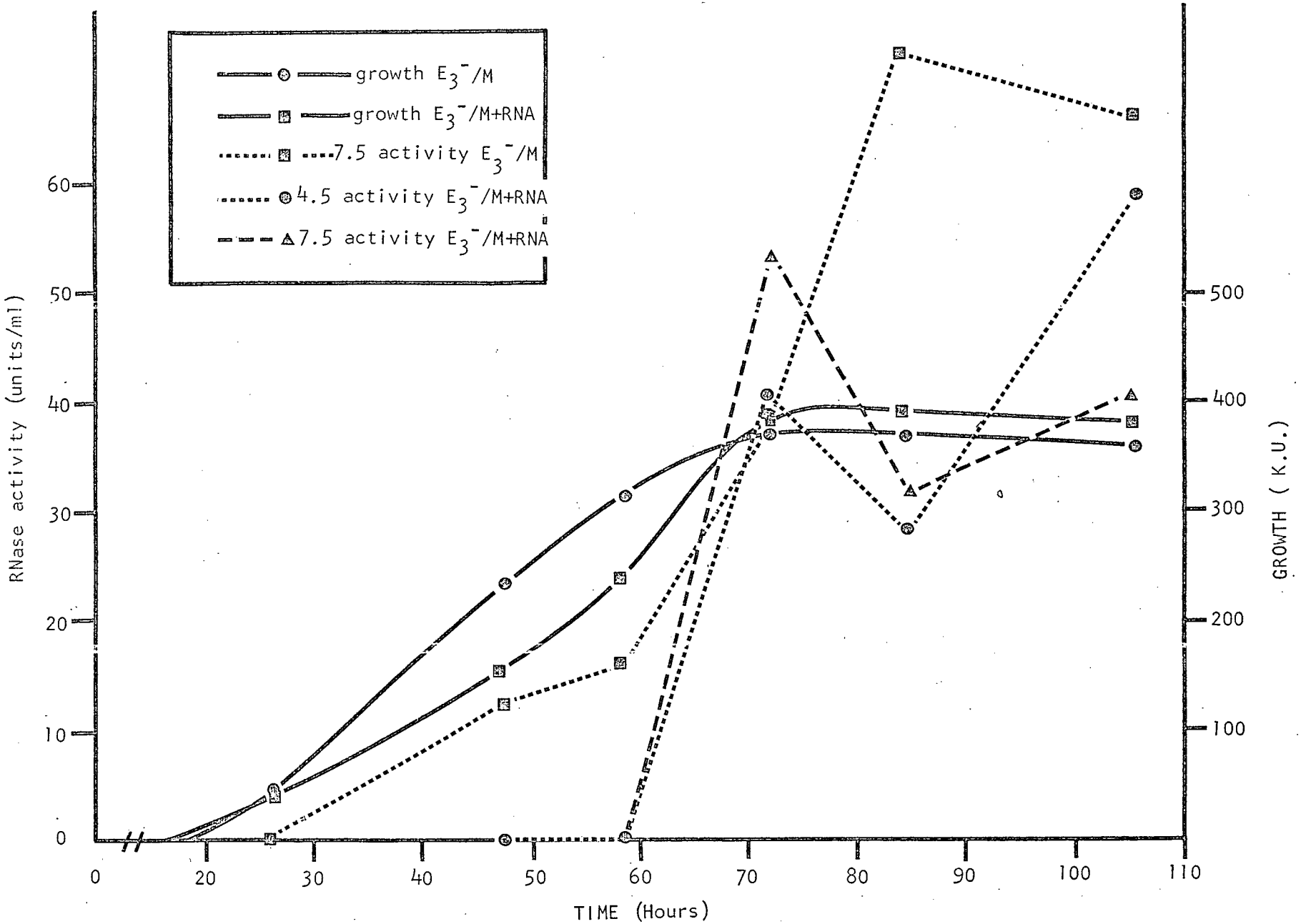

growth Myc/C
7.5 activity
4.5 activity
RNase activity (units/m)
GROWTH (K.U.)
TIME (hours)
0
10
20
30
40
50
60
70
100
100
200
300
400
500
0
20
40
60
80
100
120
140
160
180
200

Figure 5: The release of RNase activity by strain E_3^- grown on minimal medium supplemented with 0.5% yeast RNA. This graph also includes, for comparison, the growth of strain E_3^- and its release of RNase activity in minimal medium.

FIGURE 5


growth E_3^-/M
growth E_3^-/M+RNA
7.5 activity E_3^-/M
4.5 activity E_3^-/M+RNA
7.5 activity E_3^-/M+RNA
RNase activity (units/ml)
GROWTH (K.U.)
TIME (Hours)
0
10
20
30
40
50
60
100
200
300
400
500
0
20
30
40
50
60
70
80
90
100
110

of RNase activity release by early stationary phase of the E_3^- culture in RNA medium was noticeably less than that of a culture of E_3^- in minimal medium. Secondly, a significant delay in the release of the RNase activity, relative to the start of log phase growth, was noted, as had similarly been noted with E_3^- culture in complete medium (e.g. Figs. 2 and 3).

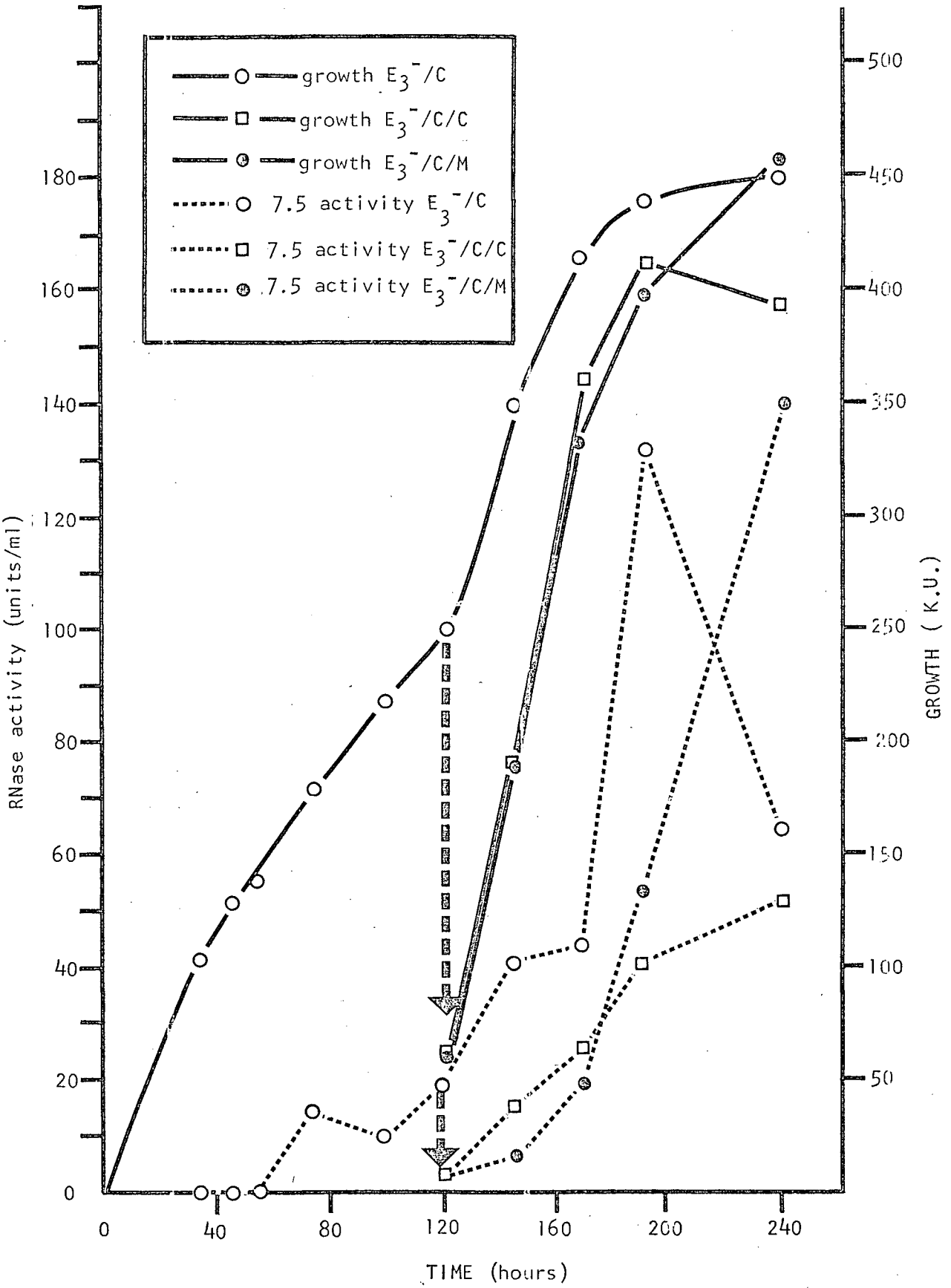
(d) Considering the release of RNase activity relative to the growth of the culture, it appeared (taking into account the lag period between the inoculation of the culture and first detecting RNase activity) that the quantity of RNase activity was a function of cell density rather than of the age of the culture. A dilution experiment in which culture E_3^- in completr medium was grown to mid-log phase and then diluted to a one-fifth cell concentration was carried out. The dilution was performed by taking one volume of culture sample and adding it to four volumes of either complete or minimal medium. Growth and release of RNase activity was followed by the standard methods, and these results are presented in figure 6. The rates of release of activity (i.e. the slope of the activity lines) of the original and the diluted cultures are similar. The greatest rate of release in both cases occurred at the point of maximal cell density.

3. RNA AS A CARBON SOURCE FOR U. HORDEI

Sporidia of Ustilago hordei were not able to grow on agar plates or in liquid culture which contained only yeast RNA (5%) and Vogel's salt solution, suggesting that the RNA cannot serve the cell as a carbon source in the same way as glucose.

Figure 6: The dilution experiment. From a culture of strain E_3^- in complete medium (E_3^-/C), a sample was diluted into four times its volume of minimal (E_3^-/C/M) or complete medium (E_3^-/C/C) at 121 hr. Growth was followed for both the original and the diluted cultures. Release of RNase activity was measured at pH 7.5.

FIGURE 6


growth E3⁻/C
growth E3⁻/C/C
growth E3⁻/C/M
7.5 activity E3⁻/C
7.5 activity E3⁻/C/C
7.5 activity E3⁻/C/M
RNase activity (units/ml)
GROWTH (K.U.)
TIME (hours)
0
20
40
60
80
100
120
140
160
180
50
100
150
200
250
300
350
400
450
500
0
40
80
120
160
200
240

4. CHEMICAL MUTAGENESIS

(a) Table I presents the assembled information of four different NG treatment trials. The younger the culture sampled for treatment, the greater the killing effect and probably the higher the mutation rate (though the data are incomplete on this point). This is in agreement with the report that NG acts at the replication points (Cerdá-Olmedo et al., 1968), and therefore an active log phase culture as in trial NG IV is predicted to be affected by the treatment, more than an end-of-log or early-log phase culture such as that used in trial NG I or III.

(b) Among approximately 14,000 colonies from NG-treated culture samples, no RNase-deficient mutants were detected.

(c) Sixty-nine colonies were picked from plates of NG-treated cells for their inability to grow on minimal medium, and one for having a peculiar colony morphology. Table II summarizes the preliminary screening of these isolates. Fifty-five of the isolates had sharp nutritional deficiencies and were considered to be auxotrophs. All 55 responded strongly to yeast extract and of these: seven were possibly adenine-deficient (NG 48 and NG 64 definitely); seven were vitamin-deficient (probably niacin or riboflavin); one responded weakly to cytosine (NG 66), while another (NG 40) responded to all of the bases except guanine, suggesting that it is a nucleic acid mutant of some kind. NG 69, a morphological mutant, had the appearance of a length of randomly coiled rope.

5. CMC-P-TOLUENESULFONATE MODIFIED RNA

To measure the extent to which guanines in the RNA had been blocked by the CMC addition reaction, the RNase T_1 (10 mg/ml) hydrolysis of CMC-RNA

Table I. NG treatments of strain E_3^- of *U. hordei*.

Treatment												
				Cell Density								
				Sampled			Post-treatment growth	Cell Survival				
Trial	Duration (minutes)	pH	KU	Viability count	During treatment (calc.)	period (hrs.)	Colony count	Percent (calc.)[b]	Auxotrophs	Percent auxotrophs	Isolate numbers
NG I	12	5.0	330	1.1×10^8	$.55 \times 10^8$	8.5	2.3×10^7	18	5	-	1-6
NG II	15	5.0	280	$.8 \times 10^{8a}$	$.8 \times 10^8$	12.5	1.5×10^7	5	I) 2	-	7-14
									II)[c] 15	1.5	15-31
NG III	15	5.7	350	2×10^8	1×10^8	9	3.3×10^7	12	33	.43	32-70
NG IV	15	5.7	106	1.5×10^7	$.75 \times 10^8$	9.5	1.4×10^6	0.7	-	-	-

a) Estimated;

b) Calculation based on the assumption that 10 hrs of post-treatment growth represents 1.5 generations;

c) A sample of 1024 colonies from 'complete' plates with treated cells.

Table II. Preliminary screening of possible mutant isolates.

NG isolate	Minimal medium	Minimal medium plus supplement								
		Yeast extract	Vitamin solution	Amino acids[b]	Bases					
					Cytosine	Uracil	Thymine	Guanine	Adenine	Description
1	-	+	-	-	-	-	-	-	1	Adenine[c]
2 + 3	-	+	-	-	-	-	-	-	-	
4	1	+	+	-	1	1	1	1	1	leaky, yellow
5 + 6	-	+	-	-	-	-	-	-	1	Adenine[c]
7	-	+	-	-	-	-	-	-	-	Adenine[c]
8	-	+	-	1	-	-	-	-	-	
9 - 11	1	+	-	1	1	1	1	1	1	leaky
12	+	+	1	1	+	+	+	+	+	wild
13	1	+	-	1	1	1	1	1	1	leaky
14	+	+	1	-	+	+	+	+	+	wild
15 - 20	-	+	-	1	-	-	-	-	-	
21	-	+	-	-	-	-	-	-	1	

Table II Continued

NG isolate	Minimal medium	Minimal medium plus supplement								Description
		Yeast extract	Vitamin solution	Amino acids	Bases					
					Cytosine	Uracil	Thymine	Guanine	Adenine	
22 + 23	-	+	+	-	-	-	-	-	-	Vitamin[d]
24 + 25	-	+	-	l	-	-	-	-	-	
26	l	+	l	l	l	l	l	l	l	leaky
27	-	+	-	-	-	-	-	-	-	
28	-	+	+	-	-	-	-	-	-	Vitamin[d]
29	-	+	-	l	-	-	-	-	-	
30	l	+	l	-	l	l	l	l	l	leaky
31	-	+	-	-	-	-	-	-	-	
32	-	+	-	l	-	-	-	-	-	
33	+	+	-	l	l	l	l	l	l	wild
34	l	+	-	l	l	l	l	l	l	leaky
35	+	+	l	l	+	+	+	+	-	wild
36	-	+	+	-	l	-	-	-	-	Vitamin[d]
37	-	+	+	-	-	-	-	-	-	Vitamin[d]

Table II Continued

NG isolate	Minimal medium	Minimal medium plus supplement								Description
		Yeast extract	Vitamin solution	Amino acids	Bases					
					Cytosine	Uracil	Thymine	Guanine	Adenine	
38	-	+	-	1	-	-	-	-	-	
39	-	+	-	-	-	-	-	-	1	Adenine[c]
40	-	+	-	1	+	1	1	-	+	n.e.[c]
41 - 47	-	+	-	1	-	-	-	-	-	
48	-	+	-	-	-	-	-	-	+	Adenine
49 - 51	-	+	-	1	-	-	-	-	-	
52	-	+	-	+	-	-	-	-	-	
53	-	+	-	1	-	-	-	-	-	
54	-	+	-	+	-	-	-	-	-	
55	-	+	-	1	-	-	-	-	-	
56	-	+	-	+	-	-	-	-	-	
57	-	+	-	1	-	-	-	-	-	
58	-	+	+	-	-	-	-	-	-	Vitamin[d]
59	-	+	-	1	-	-	-	-	1	Adenine[c]
60 + 61	-	+	-	1	-	-	-	-	-	

Table II Continued

NG isolate	Minimal medium	Minimal medium plus supplement: Yeast extract	Vitamin solution	Amino acids	Bases: Cytosine	Uracil	Thymine	Guanine	Adenine	Description
62	l	+	-	l	l	l	l	l	l	leaky
63	-	+	-	l	-	-	-	-	-	
64	-	+	-	-	-	-	-	-	-	Adenine
65	-	+	+	-	-	-	-	-	-	Vitamin[d]
66	-	+	-	-	l	-	-	-	-	Cytosine[c]
67	-	+	-	l	-	-	-	l	-	
68	l	+	-	l	l	l	l	-	-	leaky
69	+	+	+	l	+	+	+	+	+	wild, morphological[e]
70	-	+	-	l	-	-	-	-	-	

a) + wild type growth; l weak growth response; - no response.

b) casein hydrolysate plys tryptophan.

c) probable but not definite.

d) probably niacin or riboflavin, since these are common to yeast extract and vitamin mixture.

e) appearance like a length of randomly coiled rope.

was compared with RNase hydrolysis of unmodified RNA. Two preparations of CMC-RNA had, respectively, 94 and 99 percent decrease in the formation of acid soluble products. (The inhibition of RNase T_1 activity, due to unreacted CMC-p-toluenesulfonate in the CMC-RNA product, was not evaluated). CMC-RNA is probably less acid-soluble than unmodified RNA for it was found that pH 4.5 plates which contain RNA are clear while containing CMC-RNA they formed a white precipitate even before the addition of trichloroacetic acid. RNase degradation of this white precipitate was still possible for cleared areas formed against the precipitate wherever RNase-containing solution was spotted.

DISCUSSION

It was mentioned earlier that one consideration of this study was to establish whether extracellular RNases were produced by Ustilago hordei and, if so, to determine whether they are in any way similar to those produced by U. sphaerogena (Arima et al., 1968).

The results indicate: i) that extracellular ribonucleases are in fact present after growth of U. hordei sporidia and mycelia in culture media; and ii) that the presence of ribonucleases in the growth medium is not entirely due to release from ruptured cells.

The existence of three pH maxima (Fig. 1) suggests that at least three enzymes are present; two of these maxima (i.e. those at pH 5.0 and pH 8.0) compare with the pH optima shown by ribonucleases of U. sphaerogena. Whether each of these optima corresponds to a single RNase (in U. sphaerogena, two were reported for each pH optimum; Arima et al., 1968) can only be established by biochemical methods. Preliminary studies (author), using gel filtration on Sephadex G-75 (method of Arima et al., 1968) suggested that there may be two RNases with pH maxima at 4.5 (see Appendix B).

Changes in the cellular environment resulted in noticeable effects on the pattern of RNase secretion. Release of RNase activity from strain E_3^- could be delayed by the addition to minimal medium of RNA or complete medium components.

Several lines of evidence would suggest that phosphate content in the medium is a possible controlling factor for the synthesis and secretion of extracellular enzymes. The fungus Aspergillus oryzae grown on phosphate-free medium, both synthesized and released its extracellular amylase more readily (Yurkevich et al., 1967).

Similarly, the production of extracellular phytase by a number of

Aspergillus isolates was strongly repressed by low levels (2 mg/100 ml) of inorganic phosphorus; it was, however, possible to overcome the phosphate-induced repression by increasing the ratio of carbon to phosphorus in the growth medium. The authors (Shieh and Ware, 1968) concluded that since phytase in produced when concentrations of inorganic phosphate are limiting, the organism has the capacity to obtain inorganic phosphate from organic phosphates when this becomes necessary.

Arima et al. (1968), who worked with U. sphaerogena, replaced all phosphates in the culture medium with RNA and, following this, observed a significant increase in RNase U_1, together with a smaller increase in U_4 and still smaller increases in U_2 and U_3. If these increases were due to RNA being a poor phosphate source (i.e. the enzymes being produced as a response to limiting concentrations of inorganic phosphate), it is interesting to speculate on the reason for the differing levels of enzyme production. In future investigations of synthesis and release of extracellular RNases of U. hordei it would be of interest to investigate the effects of limiting phosphate concentrations.

The secretion of enzymes by microorganisms leads to speculation as to the function of such an activity. In the case of U. sphaerogena, the secretion of four extracellular RNases, possibly different from all different types of base specificities, can lead to the speculation that selection, at least in part, has been acting in the evolution of such a particular biological feature. However, to support such a speculation one must be able to attribute some role to the enzymes (i.e. some selective advantage associated with their presence).

Extracellular enzymes are most often degradative enzymes and the view can be taken that they are "scavenger" enzymes, i.e. enzymes that make certain material in the cell's environment usable which would otherwise be wasted.

The inability of U. hordei sporidia and mycelia to grow on RNA plus salts indicates that the RNA and its degradation products are apparently not being used as carbon sources. Furthermore, if the RNases of U. hordei did function as scavenger enzymes to make use of any RNA in the culture medium, they would probably do so in conjunction with phosphomonoesterases (PMEs), for it is generally considered that nucleotides (which represents the final products of RNase degradation) do not pass readily through cell membranes; the phosphomonoesterases, acting at the cell surface to convert nucleotides to nucleosides, could thus mediate the movement of RNA degradation products into the cell. In this connection it will be recalled that acid PME activity was detected primarily on the cell surface rather than in the culture medium from which growing cells had been removed.

So far as the parasitic phase of the life cycle is concerned, it is difficult to visualize any important function for the extracellular RNases. While it is possible that extracellular ribonucleases may contribute in some special way to the success of U. hordei during the parasitic phase, the data do not relate to the parasitic phase. The success or failure of RNase-deficient mutants would have provided useful information concerning the role of extracellular RNases during the pathogenic phase: loss of pathogenicity would have suggested a vital role, whereas no loss of pathogenicity would have suggested that their role during the parasitic phase is dispensible.

In considering the synthesis and translocation of extracellular RNases, it is interesting to speculate whether the many membrane-bound vesicles seen in electron micrographs of U. hordei sporidia and mycelia (personal communication, Jane Robb and Carla Stein, Dr. C. Person's laboratory) have a role in moving extracellular RNases within the cell to the cell surface. This could be investigated by histochemical methods using fluorescence-labelled antibodies against the purified extracellular ribonucleases. If the vesicles were involved

in the movement of extracellular RNases the fluorescence-labelled antibodies (see Shugar and Sierakowska, 1967 for details) should be concentrated in the vesicles. Likewise, vesicles which have been isolated from cells (e.g. by the method of Matile, 1967) should contain RNase activity.

It is not possible to decide whether the failure to obtain ribonuclease-deficient mutants was due to the fact that no mutants were produced, or to the fact that the mutants having been produced went undetected. In favour of the first of these possibilities is the fact that the total number of mutations obtained was small. Under these conditions, the recovery of specific mutants would be influenced by the relative stabilities of specific genetic loci. If the change to ribonuclease-deficiency occurs only rarely it is possible that the total size of the mutant sample was too small to include this type of mutant. This point could be clarified in future work by choosing conditions in which mutagenicity is enhanced, for example by treating cells in mid-log phase when they are more susceptible to killing by NG (cf. trial NG IV). Studies with bacteria have shown that NG-induced mutagenesis is effected at the region, or "point", of DNA replication (Cerdá-Olmedo et al., 1968). If this observation holds also for U. hordei, it should be possible to find a stage, either in the growth of a culture or in the cell cycle of synchronized cultures, in which even higher yields of NG-induced mutations can be obtained.

In considering the second possibility (i.e. that ribonuclease-deficient mutants were in fact produced but not detected), it should be noted that if the excreted ribonucleases perform an indispensible role within the cell before they are released, it is not likely that RNase mutants (incapable of forming the needed enzyme) could be recovered. The screening procedures were, however, based on the assumption that cells deficient in extracellular ribonucleases could nevertheless survive and reproduce. The selection of

RNase-deficient mutants is complicated by the fact that there are at least two and possibly more (as many as five if the enzyme activity shown at pH 6.5 is significant) enzymes. The problem of detecting loss of activity of single enzymes would be less complicated if the production of two or more enzymes were controlled by a single genetic locus, and it would be a much simpler problem if all extracellular RNase activity were controlled by a single genetic locus. However, since extracellular RNase activity seems not to represent a strictly inducible system, it is probable that the extracellular RNases are not under coordinated control by a single locus. It was therefore necessary to employ a selection procedure in which identification of mutants was based on the loss of activity of single enzymes. The method used in this study was selective only on the basis of pH optima. RNA-containing plates at pH 6.0 (or higher) were used to screen for loss of enzymes with wide alkali optima (see Materials and Methods). But where there is overlapping of pH optima of different RNases, or where there is activity of nonspecific diesterases, the method becomes ineffective, and it is probable that these two factors did interfere with the effectiveness of the screening. An alternative to this method would be to shift the pH of the RNA-containing plates to below 4.0, thus selecting for deficiency of enzymes with optima at pH 5.0 and, at the same time, minimizing the effects of enzymes with optima at pH 6.5 and higher.

A second method of selection, based on all optima and base specificity, had been considered but because of the inavailability of the compound CMC-p-toluenesulfonate was not used. The rationale of this method, based on the presence of the RNases reported by Arima et al. (1968) is outlined in Table III.

A disadvantage of this method, as with the first, is that it does not identify ribonuclease activity on the basis of individual cistrons and that definite and positive results are thus contingent on a multiple mutational event.

Table III. Selection method for detecting extracellular RNase deficient mutants using a CMC derivative of RNA.

Deficiency in RNase activity	Response to substrate[a]			
	RNA		CMC-RNA	
	pH 4.5	pH 7.5	pH 4.5	pH 7.5
1. Total loss	-	-	-	-
2. pH 4.5 loss (U_2 and U_3)	-	+[b]	-	<<<<+[c]
3. pH 7.5 loss	+[d]	-	<+[e]	-

a) + enzyme activity present;

- no enzyme activity;

<+ less activity than when using unmodified RNA substrate;

<<<+ very much less than when using unmodified RNA substrate.

b) U_1 and U_4 present.

c) U_4 acting at cytidine and adenine.

d) U_2 and U_3 present.

e) U_2 and U_3 acting at guanine.

The disadvantage of both these methods could possibly be overcome by arranging to screen for mutants whose survival is dependent on the capacity for ribonuclease activity. It is known that the wild-type (i.e. non-mutant) strain of U. hordei cannot grow when provided with RNA as the only source of carbon. But if it were possible to synthesize a strain which has a specific requirement for a preformed nucleoside that could be obtained through degradations of RNA (either by a combination of RNase and PME, or of PDE and PME activity), such a strain should be capable of showing a growth response on RNA-supplemented medium. If a positive growth response were obtained, it should then be possible to select for RNase mutants on the basis of failure to show the response. This approach, if it should prove practicable, would thus select for RNase mutants on the basis of auxotrophy; the auxotrophs would, of course, require further screening.

The method just outlined could perhaps be modified so as to eliminate the possible involvement of both PME and PDE activity. The modification would require development of a hypothetical strain having a requirement for substance "X", which could be liberated, through action of its extracellular RNase, from a nucleoside 3'-(X) phosphate. If one were to assume the existence of a system in which RNases U_1 through to U_4 (Arima et al., 1968) were all present, the base specificity of endonucleases could then be used to some advantage since it would allow for selection, in sequence, for the U_4, the U_2 and U_3 and finally for the U_1 deficiency (see outline of method, Table IV). A mutant that is deficient for U_1 (and therefore for all four RNases), if one were obtained, could be used as a parental strain in attempting to develop, through selection of back mutations, those cultures that remained deficient for specific RNases. As a final modification, it may be possible to find a compound which, acting as compound "X" in this system, would be toxic

Table IV. Selection scheme for the detection of three classes of extracellular RNase deficient strains using model substrates.

Model substrate	Genotype	Enzyme response[a]			
		U_1	U_2	U_3	U_4
1. Uridine 3'-(X)P	wild	n/a[b]	n/a	n/a	+
"	U_4^-	n/a	n/a	n/a	-
2. Adenosine 3'-(X)P	wild	n/a	+	+	+
"	$U_2^- + U_3^-$	n/a	-	-	+
3. Guanosine 3'-(X)P	wild	+	+	+	+
"	U_1^-	-	+	+	+

a) + ability to release compound X;
- inability to release compound X.

b) not applicable since assuming absolute base specificity as suggested for each enzyme (U_1 - U_4) by Arima *et al.* (1968).

to the cells following its release by RNase. This would automatically eliminate all cells excepting those that were entirely deficient in production of extracellular RNases and those which had become resistant to the toxic compound.

The application of this general method using a model compound nucleoside 3'-(X)P is contingent on the following conditions:

I) that the endonucleases are able to use the model compound as an efficient substrate,

II) that the model compound is stable and can be synthesized with reasonable ease and therefore in quantity, and

III) that the enzymes have absolute base specificity.

The type of auxotrophs which were obtained in the NG study had been in many cases previously obtained by Hood (1966) using U.V. irradiation. The only definite nucleic acid mutants (i.e. the adenine deficients) obtained here, were, in fact, the only nucleic acid mutant obtained by Hood. The mutants with vivitamin requirement were suggested to be either for niacin or for riboflavin. Of these two, Hood obtained only niacin mutants in his study. If the NG vitamin mutants are found to be niacin requiring, they should be considered in relationship to the hypothesis of Hood's that two metabolic pathways lead to synthesis of niacin.

The majority of the mutants are still unidentified, though characteristically all responded to yeast extract; this observation was also made by Hood for his unclassified mutants. Attempts to produce ribonuclease-deficient strains of U. hordei should be continued for such mutants would be useful:

1. to the biochemist interested in RNases for sequence analysis, enzymology and evolutionary comparisons,
2. to the biologist interested in the role, the synthesis and the secretion of extracellular RNases,

3. to the geneticist interested in the organization of genetic information and possible controls for the release of such information, and
4. to the plant pathologist for the investigation of a possible role of RNases in host-parasite relationships.

SUMMARY AND CONCLUSIONS

Ribonuclease activity was detected in culture medium in which Ustilago hordei had been grown. Some evidence has been presented to suggest that the detected ribonuclease activity was not due to release from ruptured cells. Also, the activity was not due to phosphodiesterases since these were not detected in the cell-free medium and only minor amounts were found to be associated with cell surfaces.

The release of RNase (as measured by RNase activity) was influenced by the cellular environment. With complete medium or with minimal medium enriched with yeast RNA the release of RNase was delayed. It is postulated that phosphate starvation will encourage the early release of RNase activity. The pH 4.5 and pH 7.5 RNase activity appears to be released concurrently.

The level of RNase activity detected in the culture medium was a function of the cell density, while the maximal level of RNase activity was in turn determined by the richness of the culture medium.

The detection of RNase activity cannot by itself be taken as an indication that RNases play the role of "scavenger" enzymes, since they could do this only in conjunction with phosphomonoesterases. With the detection of acid phosphomonoesterase activity on the cell surface, it is reasonable to attribute a "scavenger" role to the extracellular ribonucleases.

Among haploid cells of U. hordei treated with the chemical mutagen, nitrosoguanidine, fifty-five biochemically deficient mutants were isolated. Preliminary screening of these mutants showed that some were adenine and others vitamin mutants. The remaining auxotrophs, everyone of which responded to a yeast extract supplement, remain undetermined as to their specific requirement.

The level of mutagenesis was not as high as that reported with U.V. irradiation in U. hordei (Hood, 1966) so a more extensive study should be made of optimal conditions for the use of NG in this organism. Applying the results of such a study and using the selection methods outlined in the Discussion, it should be possible to produce and identify strains deficient in extracellular ribonucleases with a satisfactory level of efficiency.

BIBLIOGRAPHY

Adelberg, E.A., M. Mandel, and G.C.C. Chen. 1965. Optimal conditions for mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine in Escherichia coli K12. Biochem. Biophys. Res. Comm. 18: 788-795.

Arima, T., I. Uchida, and F. Egami. 1968. I. Studies of extracellular ribonucleases of Ustilago sphaerogena; purification and properties. Biochem. J. 106: 601-608.

Arima, T., I. Uchida, and F. Egami. 1968. II. Characterization of substrate specificity with special reference to purine-specific ribonucleases. Biochem. J. 106: 609-613.

Baker, R., and I. Tessman. 1968. Different mutagenic specificities in phages S13 and T4: In vivo treatment with N-methyl-N'-nitro-N-nitrosoguanidine. J. Mol. Biol. 35: 439-448.

Beaton, J.D. 1968. An electron microscope study of the mesosomes of a penicillinase-producing Staphylococcus. J. gen. Microbiol. 50: 37-42.

Cerdá-Olmedo, E., and P.C. Hanawalt. 1967. Macromolecules action of nitrosoguanidine in Escherichia coli. Biochim. Biophys. Acta 142: 450-464.

Cerdá-Olmedo, E., P.C. Hanawalt, and N. Guerola. 1968. Mutagenesis of the replication point by nitrosoguanidine: map and pattern of replication of the Escherichia coli chromosome. J. Mol. Biol. 33: 705-719.

Craddock, V.M. 1968. The reaction of N-methyl-N'-nitro-nitrosoguanidine with deoxyribonucleic acid. Biochem. J. 106: 921-922.

Davies, R. 1963. Microbial extracellular enzymes, their uses and some factors affecting their formation. In Biochemistry of Industrial Microorganisms, Chap. 4. Ed. by C. Rainbow & A.H. Rose. London, Academic Press.

DeRobertis, E.D.P., W.W. Nowinki, and F.A. Saez. 1965. Cell Biology. 4th ed. W.B. Saunders & Co. Philadelphia.

Dinoor, A., and C. Person. 1969. Genetic complementation in Ustilago hordei. Can. J. Botany 47: 9-14.

Egami, F., and K. Nakamura. 1969. Microbial Ribonucleases. Springer-Verlag New York, Inc.

Eisenstark, A., R. Eisenstark, and R. van Sickle. 1965. Mutation of Salmonella typhimurium by nitrosoguanidine. Mut. Res. 2: 1-10.

Gilham, P.T. 1962. An addition reaction specific for uridine and guanosine nucleotides and its application to the modification of ribonuclease action. J. Am. Chem. Soc. 84: 687-688.

Glitz, D.G., and C.A. Dekker. 1964. Studies on a ribonuclease from Ustilago sphaerogena. I. Purification and properties of the enzyme. Biochemistry 3: 1391-1399.

Glitz, D.G., and C.A. Dekker. 1964. Studies on a ribonuclease from Ustilago sphaerogena. II. Specificity of the enzyme. Biochemistry 3: 1399-1406.

Ho, N.W.Y., and P.T. Gilham. 1967. The reversible chemical modification of uracil, thymine and guanine nucleotides and the modification of the action of ribonuclease on ribonucleic acid. Biochemistry 6: 3632-3639.

Holliday, R. 1961. The genetics of U. maydis. Genet. Res. Comb. 2: 204-230.

Holliday, R., and R.E. Halliwell. 1968. An endonuclease-deficient strain of Ustilago maydis. Genet. Res. Comb. 12: 95-98.

Hood, C.H. 1966. UV-irradiation sensitivity and mutation production in the haploid sporidia of Ustilago hordei. Ph.D. Thesis, University of Alberta, Edmonton.

Jeffries, C.C., D.F. Holtman, and D.G. Guse. 1957. Rapid method for determining the activity of microorganisms on nucleic acids. J. Bact. 73: 590-591.

Kushner, D.J., and M.R. Pollock. 1961. The location of cell-bound penicillinase in Bacillus subtilis. J. gen. Microbiol. 26: 255-265.

Lampen, J.O. 1965. Secretion of enzymes by microorganisms. In Symp. of Gen. Microbiologists 15: 115-133.

Lawley, P.D. 1968. Methylation of DNA by N-methyl-N-nitrosourethane and N-methyl-N-nitroso-N'-nitroguanidine. Nature 218: 580-581.

Lederberg, J., and E.M. Lederberg. 1952. Replica plating and indirect selection of bacterial mutants. J. Bacteriol. 63: 399-406.

Lee, J.C., N.W.Y. Ho, and P.T. Gilham. 1965. Preparation of ribotrinucleotides containing terminal cytidine. Biochim. Biophys. Acta 95: 503-504.

Loprieno, N., and C.H. Clarke. 1965. Investigations on reversions to methionine independence induced by mutagens in Schizosaccharomyces pombe. Mutation Res. 2: 312-319.

McCalla, D.R. 1968. Reaction of N-methyl-N'-nitro-N-nitrosoguanidine and N-methyl-N-nitroso-p-toluenesulfonamide with DNA in vitro. Biochim. Biophys. Acta 155: 114-120.

Mandell, J.D., and J. Greenberg. 1960. A new chemical mutagen for bacteria, 1-methyl-3-nitro-1-nitrosoguanidine. Biochem. Biophys. Res. Comm. 3: 575-577.

Matile, P. 1965. Intracelluläre lokalisation proteolytischer enzyme von Neurospora crassa. I. Funktion und subcelluläre Verteilung proteolytischer Enzyme. Z. Zeleforsch. 65: 884-896.

Matile, P., M. Jost, and H. Moor. 1965. Intracelluläre Lokalisation proteolytischer Enzyme von Neurospora crassa. II. Identifikation von proteasehaltigen Zellstrukture. Z. Zellforsch. 68: 205-216.

Matile, P., and A. Wiemken. 1967. The vacuole as the lysosome of the yeast cell. Archiv für Mikrobiol. 56: 148-155.

Müller, J., and T. Gichner. 1964. Mutagenic activity of 1-methyl-3-nitro-1-nitrosoguanidine on Arabidopsis. Nature 201: 1149-1150.

Naylor, R., N.W.Y. Ho, and P.T. Gilham. 1965. Selective chemical modifications of uridine and pseudouridine in polynucleotides and their effect on the specificities of ribonuclease and phosphodiesterases. J. Am. Chem. Soc. 87: 4209-4210.

Nishimura, S., and M. Nomura. 1959. Ribonuclease of Bacillus subtilis. J. Biochem. (Tokyo) 46: 161-167.

Nishimura, S., and B. Maruo. 1960. Intracellular ribonuclease from Bacillus subtilis. Biochim. Biophys. Acta 40: 355-357.

Pollock, M.R. 1963. Exoenzymes. In The Bacteria 4: 121. Ed. by I.C. Gunsalus and R.Y. Stanier. N.Y. Academic Press.

Razzell, W.E. 1967. Polynucleotidases in animal tissues. Experentia 23: 321-325.

Shieh, T.R., and J.H. Ware. 1968. Survey of microorganisms for the production of extracellular phytase. Appl. Microbiol. 16: 1348-1351.

Shugar, D., and H. Sierakowska. 1967. Mammalian nucleolytic enzymes and their localization. Prog. N.A. and Mol. Biol. Ed. J.N. Davidson & W.E. Cohn, 7: 369-429.

Stein, C.W. 1970. An electron microscope study of a mycelial mutant of Ustilago hordei. M.Sc. Thesis, University of British Columbia.

Subba Rao, P.V., K. Moore, and G.H.N. Towers. 1967. Purification and properties of phenylalanine ammonia-lyase from Ustilago hordei. Can. J. Biochem. 45: 1863-1872.

Takahashi, K. 1961. Chromatographic purification and properties of ribonuclease T_1. J. Biochem. (Tokyo), 49: 1-8.

Takai, N., T. Uchida, and F. Egami. 1967. Ribonuclease, phosphodiesterases and phosphomonoesterases of Neurospora crassa in various culture conditions. J. Japan. Biochem. Soc. (Seikagoku) 39: 285-290.

Veleminský, J., T. Gichner, and V. Pojorny. 1967. The action of 1-alkyl-3-nitro-1-nitrosoguanidine on the M. generation of barley and Arabidopsis thaliana (L.) Heynk. Biologia Plantarum (Praha), 9: 249-261.

Vogel, H.J. 1956. A comment on growth medium for Neurospora crassa. Microbiol. Gen. Bull. No. 13.

Von Tigerstrom, R.G., and M. Smith. 1969. Preparation of the 2,4-dinitrophenyl esters of thymidine 3'- and thymidine 5'-phosphate and their use as substrates for phosphodiesterases. Biochemistry 8: 3067-3070.

Yanagida, M., T. Uchida, and F. Egami. 1964. Culture of Ustilago zeae with RNA or poly U as phosphorus source. J. Agr. Chem. Soc. Japan 38: 531-535.

Yurkevich, V.V., G.T. Kozureva, and M.I. Dergacheva. 1967. Secretion of amylase by the fungus Aspergillus oryzae. Prikladnaya Biokhimiya i Mikrobiologiya 3: 157-161.

APPENDIX A.

1. Vogel's (1956) salt solution contained: 123 g sodium citrate, 250 g monobasic potassium phosphate, 100 g ammonium nitrate (anhyd.), 10 g magnesium sulphate, 5 g calcium chloride, 5 ml trace element solution in 750 ml distilled water with 2 ml chloroform.

2. The trace element solution contained: 5 g citric acid, 5 g zinc sulphate, 1 g ferrous ammonium sulphate, 0.25 g copper sulphate, 0.05 g manganese sulphate, 0.05 g boric acid, 0.05 g sodium molybdate, 1 ml chloroform all in 95 ml distilled water. Both these solutions were stored at room temperature.

3. The vitamin solution contained: 100 mg thiamin, 50 mg riboflavin, 50 mg pyridoxine, 200 mg calcium pantothenate, 50 mg para-amino-benzoic acid, 200 mg nicotinic acid, 200 mg choline chloride, 400 mg inositol and 50 mg folic acid per one liter distilled water.

APPENDIX B.

Flash-evaporation of standard minimal medium, from a stationary culture, to one-thirtieth the original volume produced a syrupy liquid which affected the column loading and eluting. In future attempts to establish the number of RNases that are released by U. hordei, it is suggested that cultures be grown on a glucose-limiting minimal medium. This would allow for greater concentration of the medium and this provides a more definite RNase activity elution profile.