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STUDIES ON THE RELEASE OF  
 $\beta$ -GLUCOSIDASE BY THE PHYTOFLAGELLATE  
OCHROMONAS DANICA

by

DWIGHT H. MEYER

A dissertation submitted to the Graduate Faculty in  
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of New York

1974

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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## Abstract

# STUDIES ON THE RELEASE OF $\beta$ -GLUCOSIDASE FROM THE PHYTOFLAGELLATE, OCHROMONAS DANICA

by

Dwight Meyer

Advisor: Professor Sheldon Aaronson

A substantial amount of  $\beta$ -glucosidase was released by O. danica during photoheterotrophic growth in defined medium. Release was observed during log phase when lysis should be at a minimum and while the amount of  $\beta$ -glucosidase per cell was increasing. Further evidence that release of  $\beta$ -glucosidase was not dependent on cell damage was obtained by studying enzyme release during short term incubation in a resting medium. Cells from 5 day old cultures, washed and suspended in a dilute salt solution, released  $\beta$ -glucosidase at a nearly constant rate for at least 24 hours. Evidence that extracellular enzyme represented selective release in the absence of cell damage was: 1) The linear release suggests a normal cell process as loss of cell integrity caused by washing or by increasing suspension age would more likely result in non-linear release. 2) Release occurred while cell counts remained nearly constant. 3) Release of  $\beta$ -glucosidase was not accompanied by a proportional release of protein, thus resulting in a large increase of external specific activity with time. 4) The kinetics of release of material absorbing at 260 nm

was substantially different from  $\beta$ -glucosidase release. 5) Only a small amount of the cytoplasmic marker enzyme, phosphoglucose isomerase, was released compared to  $\beta$ -glucosidase, 6) A reduction in release during anaerobiosis suggested release was energy dependent. 7) Release of  $\beta$ -glucosidase was temperature dependent with a maximum between 15 and 25 C. 8) In contrast to release and secretion in mammalian systems, release was nearly stopped by the presence of cycloheximide, thus suggesting a dependence of release on protein synthesis.

I wish to acknowledge my indebtedness to Professor Sheldon Aaronson for his helpful advise and most importantly his unfailing support and encouragement.

A special kind of acknowledgement and appreciation is extended to my wife and children for their faith and many sacrifices which made this work possible.

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## Table of Contents

|              | <u>Page</u> |
|--------------|-------------|
| Introduction | 1           |
| Methods      | 6           |
| Results      | 13          |
| Discussion   | 69          |
| References   | 87          |

### List of Tables

|          |                                                                                                                          | <u>Page</u> |
|----------|--------------------------------------------------------------------------------------------------------------------------|-------------|
| Table 1  | Estimation of cell lysis needed to account for the extracellular $\beta$ -glucosidase in growing cultures.               | 16          |
| Table 2  | The effect of washing cells, with SIS, on release of $\beta$ -glucosidase.                                               | 22          |
| Table 3  | The effect of salts on release of $\beta$ -glucosidase.                                                                  | 24          |
| Table 4  | The effect of varying the SIS concentration on release of $\beta$ -glucosidase.                                          | 26          |
| Table 5  | The rate of $\beta$ -glucosidase release from cells in SIS and GM.                                                       | 27          |
| Table 6  | The effect of cell concentration on $\beta$ -glucosidase release in SIS.                                                 | 30          |
| Table 7  | Cellular enzyme activity.                                                                                                | 35          |
| Table 8  | The effect of SIS pH on the amount of enzyme activity appearing in the cell-free supernatant.                            | 37          |
| Table 9  | The influence of the incubation solution on the effect of BSA on enzyme activity appearing in the cell-free supernatant. | 40          |
| Table 10 | Change in total $\beta$ -glucosidase under dark and anaerobic incubation conditions.                                     | 43          |
| Table 11 | Temperature coefficients ( $Q_{10}$ ) of $\beta$ -glucosidase release.                                                   | 47          |
| Table 12 | The effect of metabolic inhibitors on $\beta$ -glucosidase release.                                                      | 49          |
| Table 13 | The effect of protein synthesis inhibitors on $\beta$ -glucosidase release.                                              | 54          |
| Table 14 | The effect of CHI on the release of protein and $A_{260}$ material.                                                      | 58          |
| Table 15 | The effect of CHI on $\beta$ -glucosidase release in the presence of IAA and DNP                                         | 59          |

|          |                                                                                       |    |
|----------|---------------------------------------------------------------------------------------|----|
| Table 16 | Ratio of radioactivity in protein released from pre and post-zero time labeled cells. | 64 |
| Table 17 | $\beta$ -Glucosidase release from cells with different levels of enzyme activity.     | 66 |
| Table 18 | Test for sedimentation of $\beta$ -glucosidase activity in cell-free supernatant.     | 68 |

### List of Figures

|           |                                                                                                                      | <u>Page</u> |
|-----------|----------------------------------------------------------------------------------------------------------------------|-------------|
| Figure 1  | Dependence of $\beta$ -glucosidase activity on pH.                                                                   | 10          |
| Figure 2  | $\beta$ -Glucosidase activity release and cell density during growth.                                                | 14          |
| Figure 3  | Cellular and extracellular $\beta$ -glucosidase activity per cell numbers.                                           | 15          |
| Figure 4  | Amount of $\beta$ -glucosidase activity and protein appearing in the cell-free supernatant during incubation in SIS. | 18          |
| Figure 5  | Amount of $\beta$ -glucosidase activity appearing in the cell-free supernatant, expressed as mU/ml.                  | 19          |
| Figure 6  | Change in cell density and $\beta$ -glucosidase activity during incubation in SIS.                                   | 20          |
| Figure 7  | Change in $\beta$ -glucosidase activity and protein in the cell-free supernatant.                                    | 29          |
| Figure 8  | Specific activity of $\beta$ -glucosidase in the medium and in the cells during incubation in SIS.                   | 31          |
| Figure 9  | Absorption spectra in the UV of the cell-free supernatant after 6 hours incubation in SIS.                           | 33          |
| Figure 10 | Change in $\beta$ -glucosidase activity and $A_{260}$ of the cell-free supernatant during incubation in SIS.         | 34          |
| Figure 11 | Enzyme activity and protein appearing in the cell-free supernatant during incubation in SIS at pH 7.                 | 39          |
| Figure 12 | The effect of anaerobic and dark incubation conditions on the release of $\beta$ -glucosidase.                       | 42          |
| Figure 13 | The effect of temperature on release.                                                                                | 46          |
| Figure 14 | Arrhenius plot of the effect of incubation temperature on $\beta$ -glucosidase release.                              | 48          |

|           |                                                                                                                   |    |
|-----------|-------------------------------------------------------------------------------------------------------------------|----|
| Figure 15 | The effect of DNP and IAA on the release of protein and $A_{260}$ material.                                       | 51 |
| Figure 16 | Release of enzyme activity in the presence of DNP and IAA.                                                        | 52 |
| Figure 17 | The effect of CHI on the release of $\beta$ -glucosidase.                                                         | 55 |
| Figure 18 | The effect of CHI on the total $\beta$ -glucosidase activity.                                                     | 56 |
| Figure 19 | Incorporation of radioactivity into cellular protein with and without CHI                                         | 60 |
| Figure 20 | Incorporation of radioactivity into extracellular protein with and without CHI.                                   | 62 |
| Figure 21 | Amount of radioactivity in the extracellular protein, represented as percent of total incorporated radioactivity. | 63 |

## INTRODUCTION

Some of the criteria for determining the existence of a truly extracellular enzyme come from the extensive studies of enzyme release from bacteria. These criteria are applicable to the investigation of released enzymes in other cell systems and were clearly defined by Pollock<sup>1</sup>. First, the enzyme must occur in the medium, separated from the cells. Second, the appearance of the enzyme in the medium must not depend upon irreversible damage to cell structure. To satisfy the second criterion, Pollock proposed three conditions that if met would indicate with near certainty that an enzyme is truly extracellular. These conditions are: 1) the enzyme is released when autolysis should be at a minimum (e. g. during log phase), 2) a substantial proportion of the total enzyme of the system (cells plus medium) is found in the medium free from cells, 3) release is observed in the absence of cell damage, as indicated by tests for the release of substances normally present only within the cell.

From characteristics of the release process, other evidence can also be used to support enzyme release by a specific process not involving cell damage, such as, energy dependence of release, dependence of release on new protein synthesis, and kinetics of release.

The occurrence of enzyme activity outside of cells is widespread and is not limited to the extracellular digestion by bacteria, fungi and metazoa. Dingle<sup>2</sup>, in a review of lysosomal enzyme secretion in

mammalian systems, stated that almost all tissue fluids contain free acid hydrolases. Enzymes have been reported free in cell and tissue cultures of higher plants<sup>3, 4, 5, 6</sup> and animals<sup>2, 7, 8</sup> and in the incubation medium of cell and tissue preparations of higher plants<sup>9, 10</sup> and animals.<sup>11, 12, 13, 14</sup> Extracellular enzyme activity has been found in the culture media of protozoa (see 15) and algae.<sup>12, 17, 18</sup> Enzyme activity has also been reported free in sea water<sup>19, 20, 21</sup> and in lake water.<sup>22, 23</sup>

Only a few papers report extracellular enzymes associated with phytoflagellates. Indirect evidence for extracellular hydrolytic enzymes was reported for Ochromonas variabilis based on the observed digestion of starch, protein and lipid.<sup>24</sup> Several organic phosphate esters which do not usually penetrate the phytoflagellate cell<sup>25</sup> were reported to be utilized by several phytoflagellates as a source of phosphate, thus, suggesting enzymes exterior to the cell surface.<sup>16, 25, 26</sup> Alkaline phosphatase activity has been described in the growth medium of a dinoflagellate.<sup>27</sup> Aaronson<sup>28</sup> and Shio<sup>29</sup> found acid phosphatase activity in the growth medium of light-grown Ochromonas danica and Shio<sup>29</sup> found cathepsin, ribonuclease, and deoxyribonuclease in the growth medium of light-grown O. danica and acid phosphatase and  $\beta$ -glucosidase in the culture medium of both light and dark-grown cells.

Although extracellular enzymes are associated with a large variety of cells and organisms, most of the reports give little or no critical evidence that the enzymes were found free of cells as the result of a selective release mechanism and not as the result of cell lysis or leakage.

The relatively limited number of reports of, and uncritical evidence for, extracellular enzymes in algae, protozoa and phytoflagellates may be 1) a reflection of infrequent occurrence, 2) because extracellular enzymes have not been frequently sought or 3) because some investigators attribute extracellular enzymes to cell death and autolysis.

If it was determined that  $\beta$ -glucosidase was released from O. danica by a selective process without cell damage, it would constitute the first example of a phytoflagellate to exhibit this phenomenon and would support the idea that many, if not all, cells are capable of releasing enzymes in the absence of cell damage. To understand the significance of the released  $\beta$ -glucosidase and other hydrolases in the biology of O. danica it must first be determined if the release represents a true secretion of enzyme from intact functional cells or if it is an artifact of non-specific release caused by cell lysis or leakage. This study was addressed primarily to this question of selective release. Some conditions and factors that influence enzyme release were also studied to see what clues they might offer to the mechanism and regulation of release and biological role of the released enzyme.  $\beta$ -glucosidase was selected for investigation of the nature of the release of enzyme activity because it was found to have the highest extracellular activity of the hydrolases found in the growth medium.<sup>29</sup>

The mechanism of enzyme secretion has only been well characterized for glandular tissue (see Wallach and Schramm,<sup>30</sup> and Jamieson and Palade<sup>31</sup>) and bacteria (see Lampen<sup>32</sup>). Although reports of exoenzymes

in other systems include non-hydrolytic enzymes,<sup>4, 6, 33, 34</sup> most reports of release are of acid hydrolases that are characteristic of lysosomes, thereby suggesting lysosomes as the source of the released enzyme. It is thought by some<sup>2, 35, 36</sup> that extracellular secretion of lysosomal enzymes is an integral part of the lysosomal activity of normal cells. Selective release of lysosomal enzymes has been suggested for phagocytizing leukocytes.<sup>11, 37</sup> Muller<sup>38</sup> has shown that a specific population of lysosomes is the source of released hydrolases in Tetrahymena pyriformis. Although a single source of released enzymes is indicated in these two examples, more than one mechanism of release might be operating. For example, Zurier et al.<sup>39</sup> outlined several possible mechanisms of release of lysosomal enzymes from leukocytes, including cell death, internal rupture of lysosomes followed by cell lysis, "regurgitation" of lysosomal enzymes through incompletely closed phagosomes, and "reverse endocytosis". In phytoflagellates evidence for the existence of lysosomes is limited, but their presence is likely.<sup>40</sup> Other possible mechanisms for the release of  $\beta$ -glucosidase from O. danica will be discussed.

The biological role of extracellular hydrolases, in most cases, is not understood. It is not likely that their role is limited to that of digestion to obtain soluble extracellular nutrients. Other roles for extracellular hydrolases are indicated in the literature, for example, regulation of cell activity and intercellular interaction, as exemplified by extracellular cyclic-AMP phosphodiesterase of the slime mold Dictyostelium.<sup>41, 42, 43</sup> Another possible role is that of protection,

as with penicillinase secretion by bacteria.<sup>1</sup> It has also been suggested that extracellular enzymes, including hydrolases, have a role in metabolism of extracellular macromolecules required for growth and morphogenesis, such as connective tissue of metazoa<sup>14, 44</sup> and cell walls of plants<sup>4</sup> and bacteria.<sup>33</sup>

O. danica has been reported to be capable of acting as a phagotroph, osmotroph, photoheterotroph or photoautotroph.<sup>45, 46</sup> This versatility in mode of nutrition induces speculation that the role of extracellular enzymes in O. danica would not be entirely nutritional, since it would seem these modes would satisfy its nutritional needs in the presence of a food source, and extracellular enzymes would not be of help in the absence of a food source.

## METHODS

### Cell cultures

Ochromonas danica Pringsheim was grown photoheterotrophically in the defined medium of Aaronson and Baker <sup>45</sup>at pH 5.0. Flasks (500 ml) containing 250 ml medium were inoculated with enough cells to give a final concentration of  $2 \times 10^4$  cells/ml from 2-3 day stock cultures maintained in culture tubes under the same conditions as the mass cultures. Mass culture flasks and maintenance culture tubes were incubated in constant temperature incubators at 25°C with no shaking and with continuous fluorescent illumination at 200-250 ft. cdl.

### Experimental procedures

Growth curves and cellular/extracellular enzyme determinations of growing cultures were carried out by aseptically removing samples from cultures at intervals up to 14 days. Cells were separated from the medium by centrifugation at  $7,700 \times g$  for 10 min. at 0-4°C in a Sorvall RC2-B with the SS34 rotor. Extracellular (supernatant medium) and cell samples were stored frozen until the end of the sample period (14 days) at which time assays were done.

Cells were counted with a hemacytometer.

For short term incubation experiments 5 day old cells were harvested at room temperature by centrifuging 30-35 ml aliquots of cell suspension in a Sorvall SS-1 table top centrifuge at  $2,400 \times g$  for 6 min.

The cell pellet was suspended in a salt incubation solution (SIS)\* containing 0.5 mM  $\text{CaCl}_2$ , 4.0 mM  $\text{MgSO}_4$  and 2.5 mM combined  $\text{KH}_2\text{PO}_4$  -  $\text{K}_2\text{HPO}_4$  to give pH 5.0 then centrifuged again at  $2,400 \times g$  for 6 min. The cell pellet was resuspended to the original volume in SIS or appropriate experimental solution. The suspension was transferred to 50 ml Ehrlenmeyer screw-cap flasks for incubation at  $25^\circ\text{C}$  with 200 ft. cdl. illumination. At time intervals up to 24 hrs., after gentle shaking to insure even cell distribution, 4-5 ml samples were removed. The samples were centrifuged at  $7,700 \times g$  for 10 min. at  $0-4^\circ\text{C}$ . Extracellular (supernatant) and cellular samples were assayed on the same day. For experiments in which percent recovery was to be determined, a sample of the suspension prior to centrifugation was also assayed.

### Enzyme assays

B-glucosidase activity was assayed with synthetic substrate p-nitrophenyl- $\beta$ -D-glucoside (PNPG). Activity against this substrate is associated with  $\beta$ -glucosidase ( $\beta$ -D-glucoside glucohydrolase, EC 3.2.1.21) which includes activity against aryl- $\beta$ -glucosides and  $\beta$ -disaccharides.<sup>47</sup> PNPG has also been found to serve as a substrate

---

\* The abbreviations used are: <sup>A</sup><sub>260</sub>, absorption at 260nm; BSA, bovine serum albumin; CAP, chloramphenicol; CHI, cycloheximide; DNP, dinitrophenol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylenebis (oxyethylenenitrilo) tetraacetic acid; GM, growth medium; IAA, iodoacetic acid; PM, puromycin; PNPG, p-nitrophenyl-B-D-glucoside; SA, sodium azide; SIS, salt incubation solution; TCA, trichloroacetic acid.

for yeast  $\beta$ -glucanases.<sup>48</sup>

The  $\beta$ -glucosidase assay used was a modification of the method of Beck and Tappel.<sup>49</sup> The final 1.0 ml incubation mixture contained 0.2 ml enzyme sample, 0.8 ml McIlvaine's citrate-phosphate buffer<sup>50</sup> pH 5.0, 5.3 mM PNPG (Sigma), 1.0 mM  $\text{CaCl}_2$ , and 0.1 % Triton X-100<sup>51</sup>. After incubation at 37°C for 30 min. the reaction was stopped by, and the color developed with the addition of 3.0 ml glycine-carbonate buffer, pH 10.7.<sup>52</sup> Absorbance was read at 410 nm in a Hitachi Perkin-Elmer spectrophotometer.  $\text{CaCl}_2$  was added to the reaction mixture because it was found that under some conditions, such as when enzyme was released into distilled water,  $\text{CaCl}_2$  was necessary for full  $\beta$ -glucosidase activity.

Phosphoglucose isomerase activity was assayed by a modification of the method of Reithel.<sup>53</sup> The final 1.0 ml assay mixture was obtained by combining 0.2 ml of diluted enzyme sample with 0.8 ml of 0.1 M Tris-acetate buffer pH 8.0 containing 2.0 mg/ml glucose-6-phosphate (Sigma) and 0.125% Triton X-100. Incubation was for 30 min. at 37°C. The reaction was stopped by addition of 4.0 ml of a freshly mixed solution containing 1.0 ml resorcinol (0.1% in 95% ethanol) and 3.0 ml concentrated HCl. Color was developed by heating the mixture to 80°C for 10 min. then quickly cooling to room temperature. The color developed was read at 530 nm.

Alkaline phosphatase was determined by a modification of the method of Bessey et al.<sup>54</sup> Conditions were the same as for the assay

of phosphoglucose isomerase except that p-nitrophenyl phosphate was the substrate, glycine-NaOH (pH 8.6) was the buffer and the mixture was incubated for 60 minutes at 37<sup>0</sup>.

For all the enzyme assays, reading blanks contained all components except the 0.2 ml enzyme sample which was added after the reactions were terminated. Controls run at various times in which substrate was added after incubation and those in which enzyme was subjected to 100<sup>0</sup>C for 20 min. always resulted in readings identical to the blank. All assays were performed in duplicate with essentially identical results. For  $\beta$ -glucosidase and phosphoglucose isomerase product formation was determined to be linear with incubation time and enzyme concentration. The effect of pH on  $\beta$ -glucosidase activity is shown in figure 1 for the pH range of 3.0 to 6.5 for extra-cellular enzyme and pH range 3.0 to 9.0 for cellular enzyme.

One unit of enzyme activity is defined as the amount of enzyme necessary to release one micromole of product per min. Specific activity is defined as units of activity per microgram protein. Activity in units per ml refers to activity per ml supernatant for extra-cellular activity and units per ml of cells resuspended to original concentration for cellular activity. Units of activity per cells was calculated by dividing activity per ml by the number of cells per ml.  $\beta$ -glucosidase and alkaline phosphatase activity was based on a molar extinction coefficient of 18,000 for p-nitrophenol<sup>55</sup> and for phosphoglucose isomerase the molar extinction coefficient of the fructose-1-phosphate

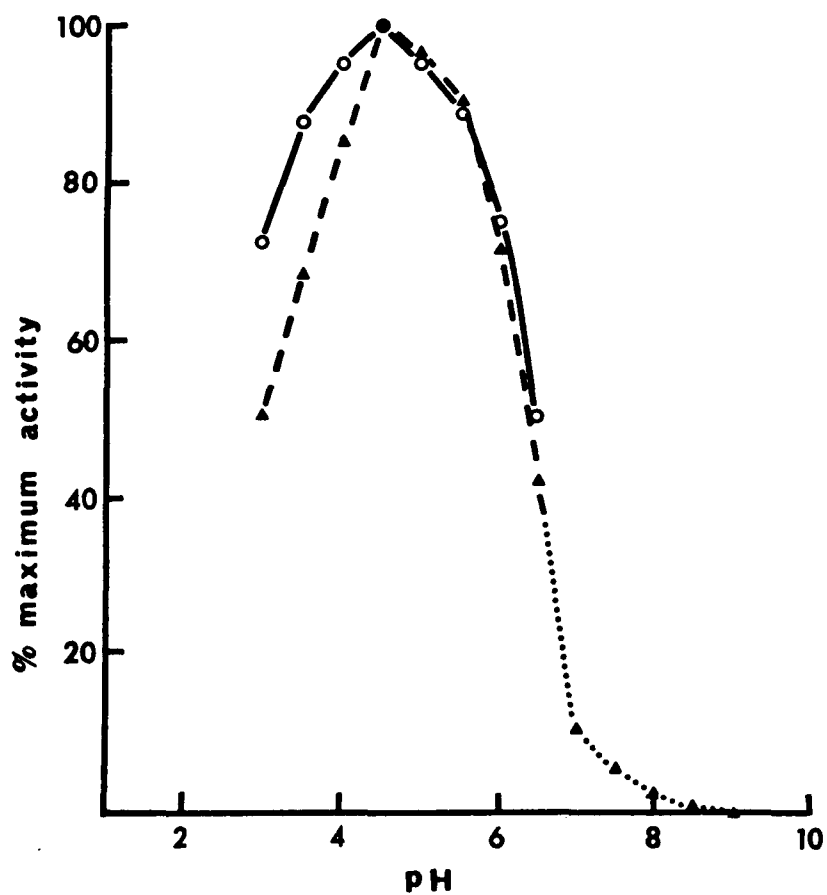


Figure 1. Dependence of  $\beta$ -glucosidase activity on pH.  
Activity of cells (solid triangles) and cell-free medium (open circles) of 5 day old cultures. Citrate-phosphate buffer, pH 3.0 - 6.5; Tris-HCl buffer, pH 7.0 - 9.0.

derivative was determined to be 16,000.

Cells were assayed for internal enzyme with Triton X-100. Centrifuged cells were suspended in distilled water to proper dilution and the suspension was added directly to the enzyme assay mixture containing 0.1% Triton X-100. Cellular  $\beta$ -glucosidase activity by other methods (as percent of the Triton X-100 method) were: homogenizing with glass beads in a Sorvall Omnimixer, 51%; freeze-thawing, 105%; treatment with n-butanol, 95%. Without Triton X-100 cellular activity for B-glucosidase was 28% and phosphoglucose isomerase less than 2% of the activity with 0.1% Triton X-100. Increasing Triton X-100 concentration to 1.0% did not change the cellular activity of either enzyme. It was also determined that Triton X-100 did not affect extracellular enzyme activity in the cell free medium or cellular enzyme activity obtained by breaking cells in the Omnimixer.

#### Protein determination

Protein was determined by the method of Lowry et al.<sup>56</sup> as modified by Hartree.<sup>57</sup> Bovine serum albumin(BSA) was used as the standard.

#### <sup>3</sup>H-Leucine incorporation into cell and medium TCA precipitate

Washed cells were suspended in autoclaved SIS containing 1.0-1.5 uci/ml L-leucine 4,5-<sup>3</sup>H (Schwarz/Mann, sp. act. 15 ci/m mole). Cell suspensions were incubated at 25°C with constant illumination as previously mentioned. At intervals 3.0 ml samples were removed and

treated as follows: Samples were centrifuged at  $16,300 \times g$  for 5 min. at  $0^{\circ}\text{C}$  in the B-4 swinging bucket rotor in the Sorvall RC2-B. Cells and supernatant were separated. The cells were washed once with 3.0 ml SIS then extracted twice with 3.0 ml acetone; the acetone pellet was suspended in 3.0 ml water and then 3.0 ml of 20% TCA; the mixture was heated in a  $90^{\circ}\text{C}$  waterbath for 10 min., centrifuged and washed once with 10% TCA and once with 90% ethanol. Protein was precipitated from the incubation medium (supernatant from first centrifugation) by adding carrier protein (2.0 mg bovine serum albumin/3.0 ml medium) followed by 3.0 ml of 20% TCA. The TCA precipitate was washed twice with 3.0 ml of 10% TCA. Cellular and medium TCA precipitates were solubilized in NCS solubilizer (Amersham) and added to 10 ml of Permafluor (Amersham) liquid scintillation mixture in glass counting vials for counting in a Nuclear Chicago Mark II scintillation counter.

Bacterial contamination of the incubating cell suspension was monitored by plating aliquots on nutrient agar and found to be virtually bacteria-free through 24 hours.

## RESULTS

### Release of $\beta$ -glucosidase in growing cultures

Cells and growth medium (GM) separated from cells were assayed for  $\beta$ -glucosidase at intervals as described in Methods. Figure 2a shows that extracellular  $\beta$ -glucosidase activity, as percent of total (cellular plus extracellular), increased at a rate similar to the increase in cells (figure 2b) during log phase. External enzyme continued to increase at a reduced rate while growth passed through stationary to the death phase. When calculated on a per cell basis (figure 3) cellular enzyme activity increased dramatically from day 1 to day 3, then declined rapidly from day 4 until about day 9 from which time it appeared to level off. Extracellular activity on a per-cell basis increased approximately linearly during that time (figure 3) even when the cellular activity was increasing.

Calculations were made to arrive at an estimate of the percent of cells that would have to lyse to account for the release of extracellular  $\beta$ -glucosidase if lysis was the only source of the external enzyme. Table 1 indicated that during the period of 3 to 7 days approximately 37% to 108% of the cells at a given time would have to lyse each subsequent generation time to account for the observed external enzyme. The increase from 37 to 108 was the result of an observed increase in generation time (table 1). Calculated on a basis of 24 hours absolute time rather than generation time, the value is an average of 44%.

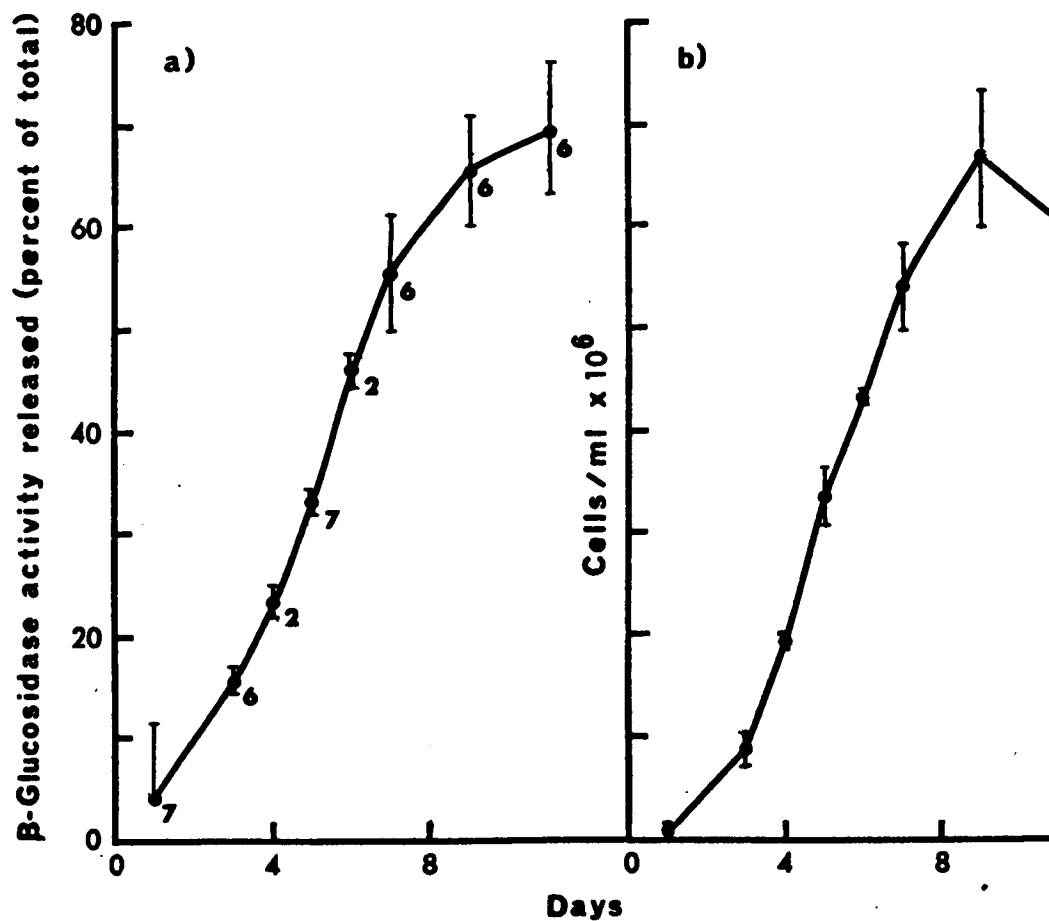


Figure 2. β-Glucosidase activity release and cell density during growth. a) β-Glucosidase activity released with culture age. b) Cell density with culture age. Data for a) and b) from the same experiments. Vertical bars represent standard deviation. Number of experiments next to data points.

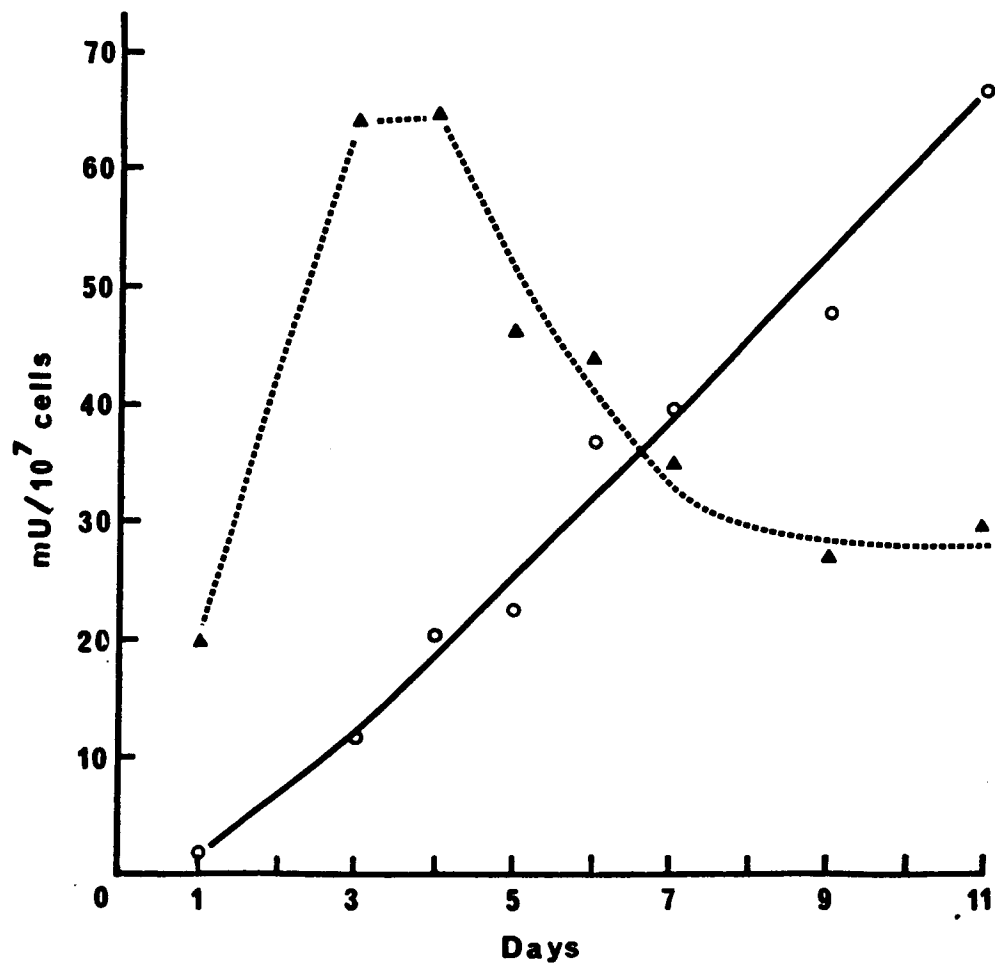


Figure 3. Cellular and extracellular  $\beta$ -glucosidase activity per cell numbers. Data from same experiments as for figure 3. Solid triangles, cellular activity; open circles, extracellular activity.

Table 1: Estimation of cell lysis needed to account for the extracellular  $\beta$ -glucosidase in growing cultures. Calculated as percent of initial cellular  $\beta$ -glucosidase activity at the beginning of the measured interval released into the culture medium. GT= generation time.

| Measured interval (days) | Mean GT during interval (hours) | a<br>% of initial cell enzyme released/GT | b<br>% of initial cell enzyme released/day |
|--------------------------|---------------------------------|-------------------------------------------|--------------------------------------------|
| 3 - 4                    | 16.9                            | 38                                        | 53                                         |
| 4 - 5                    | 27.8                            | 37                                        | 32                                         |
| 5 - 6                    | 43.4                            | 88                                        | 49                                         |
| 6 - 7                    | 61.5                            | 108                                       | 42                                         |

a Calculated according to the formula:  $\frac{(E_n - E_0) / g}{C_0} \times 100$

$E_n$  = extracellular enzyme at end of interval

$E_0$  = extracellular enzyme at beginning of interval

$g$  = number generations per interval (time interval divided by GT)

$C_0$  = cellular enzyme at beginning of interval

b Calculated according to the formula:  $\frac{(E_n - E_0)}{C_0} \times 100$

### Release of $\beta$ -glucosidase into SIS

Investigation of  $\beta$ -glucosidase release by cells incubating in a resting medium was prompted by several factors. 1) Preliminary experiments revealed that the Lowry method could not be used for protein determination in the GM because of interference from components in the medium. Controls with fresh GM produced higher absorbance readings than GM separated from cells. Glucose and amino acids at concentrations equivalent to those in GM produced color by the Lowry method. Various substances including sugars have been reported to interfere with the Lowry method.<sup>56, 58, 59, 60, 61</sup> 2) A non-growth condition was thought likely to circumvent some of the problems of interpreting the kinetics of release caused by an increase in cell number. 3) It would reduce the possibility that substances accumulating in the medium after several days of growth might influence release. 4) It would facilitate adjustment of the medium and assessment of the effect on release of changes in the medium.

Five day old cultures of O. danica washed once and incubated in SIS released  $\beta$ -glucosidase activity into the surrounding SIS nearly linearly for at least 24 hours. In figure 4 this is expressed as percent of the total system activity (cells plus medium). The amount released increased 10 fold, from 3.5% to 35% during the 24 hours. Figure 5 shows the same data expressed in units of activity released.

Figure 6 (lower panel) shows the percent change in cellular and

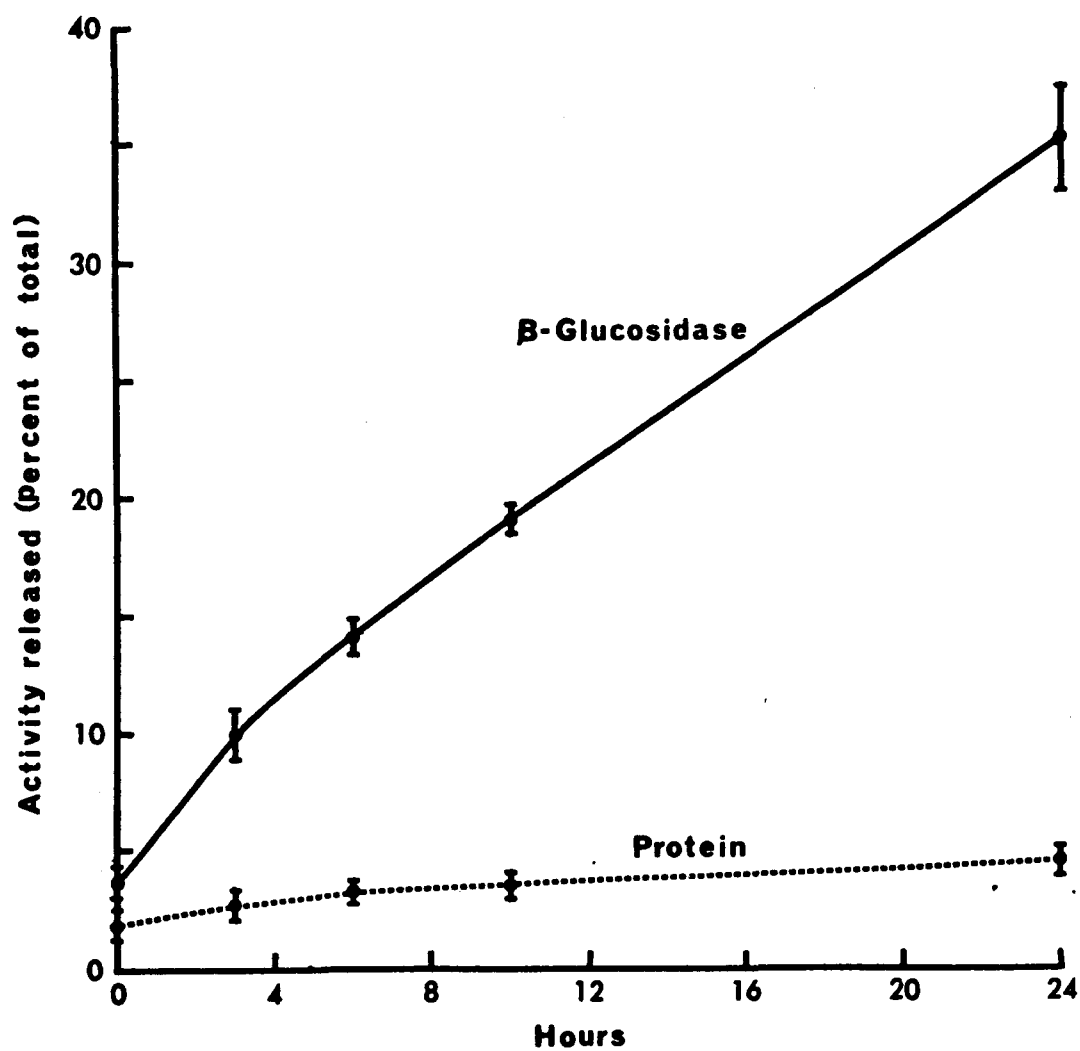


Figure 4. Amount of  $\beta$ -glucosidase activity and protein appearing in the cell-free supernatant during incubation in SIS. Vertical bars represent the standard deviation of 6 experiments.

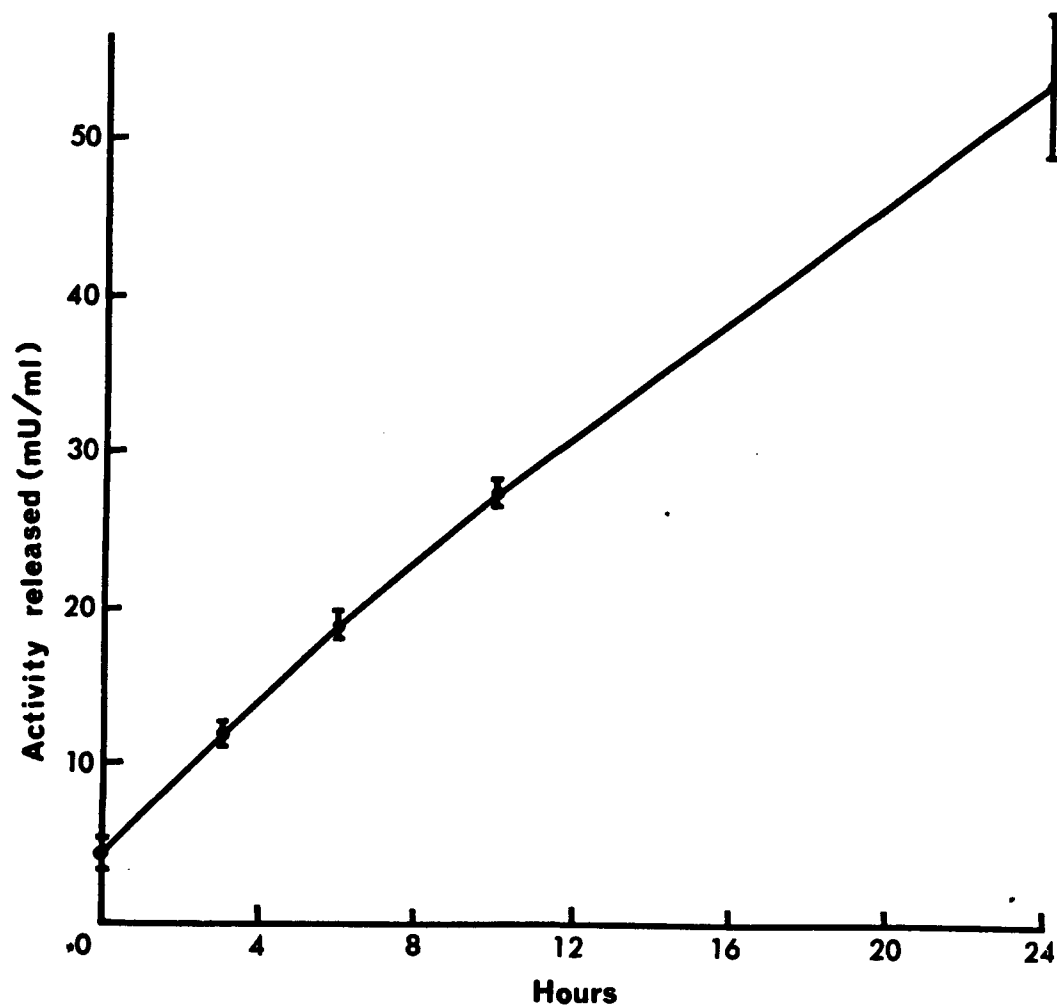


Figure 5. Amount of B-glucosidase activity appearing in the cell-free supernatant, expressed as mU/ml. Same experiments as shown in figure 4.

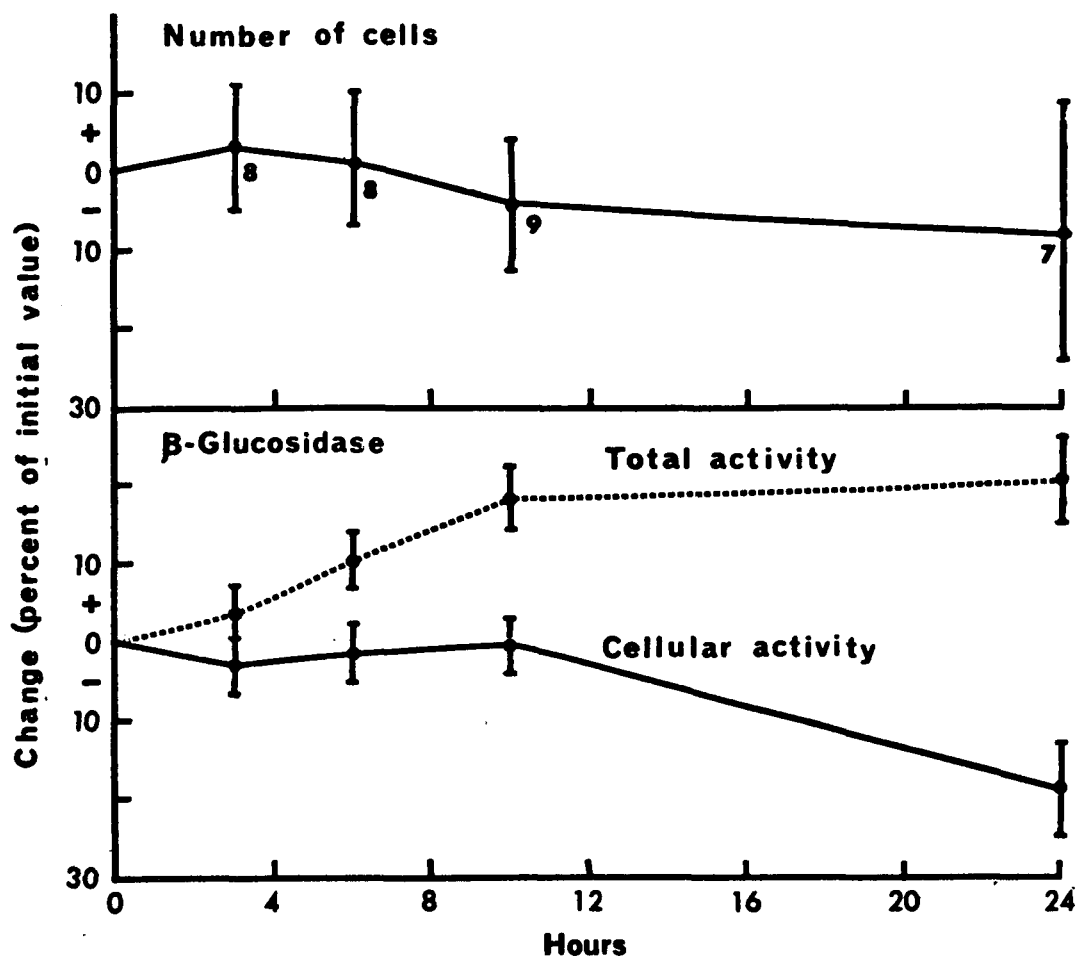


Figure 6. Change in cell density and  $\beta$ -glucosidase activity during incubation in SIS. Vertical bars represent standard deviation. a) change in cell density. Number of determinations next to data points. b) Change in total and cellular  $\beta$ -glucosidase activity. Same 6 experiments as shown in figure 4.

total  $\beta$ -glucosidase activity. Changes in the level of enzyme activity in the cells had three phases. There was a consistent slight drop in activity followed by recovery to the original level and then a moderate decline between 10 and 24 hours. The total activity (combined cellular and extracellular) increased significantly during the first 10 hours and then nearly leveled off.

Percent recovery, that is, the activity calculated by adding cellular and medium activity as a percent of activity in a complete suspension sample (cells not separated from medium), was determined in three experiments to be  $102 \pm 2$ ,  $99 \pm 5$  and  $106 \pm 3$  (mean  $\pm$  SD).

Cell counts taken during some of the experiments are shown in figure 6 (upper panel) and indicate cell number remains nearly constant or declines slightly (mean decline 8% at 24 hrs) during the course of the experiments.

Under the light microscope it was noted that shortly after washing and resuspension in SIS the cells were mostly rounded and exhibited little motility, by 3 hours the cells had recovered their normal tear drop shape and increased motility. The tear drop shape and high motility (estimated 50-80%) continued through 24 hours of incubation.

As can be seen in figures 4 and 5  $\beta$ -glucosidase activity was found in the SIS, separated from cells, at time zero. To see if this was because the cells were not washed sufficiently or the effect of the washing treatment (e. g. lysis or leakage) samples of cells were subjected to several washes. Table 2 shows that each subsequent

Table 2: The effect of washing cells, with SIS, on release of B-glucosidase.

| Cell<br>Suspension<br>sample | Wash<br>number | Activity in the cell-free supernatant<br>(mU/ml) |                                       |     |
|------------------------------|----------------|--------------------------------------------------|---------------------------------------|-----|
|                              |                | Activity<br>in wash                              | Incubation time after wash<br>(hours) |     |
|                              |                |                                                  | 2                                     | 4   |
| A                            | 1              | 3.3                                              |                                       |     |
|                              | 2              | 2.2                                              |                                       |     |
|                              | 3              | 4.6                                              |                                       |     |
|                              | 4              | 3.8                                              | 5.8                                   | 8.3 |
| B                            | 1              | 4.6                                              | 6.8                                   | 9.9 |

wash contained enzyme activity, indicating the cells lost enzyme from the washing treatment. Subsequent incubation revealed that release with time from the sample washed 4 times was not appreciably different from the sample washed once (table 2). Thus washing did not appear to affect release beyond the time of washing.

That the centrifugal force during separation of cells and medium did not cause cell damage and enzyme release was indicated by comparison of enzyme activity remaining in the supernatant medium after various speeds of centrifugation. At speeds lower than the 8,000 rpm used routinely, the amount of activity remaining was greater (because some cells were not sedimented). At 12,000 rpm there was no appreciable difference in the amount of activity remaining compared to 8,000 rpm.

Changes in the ionic make up of the incubation solution were made to determine the suitability of SIS in this study and to serve as a preliminary assessment of the effect of the ionic make up on release of  $\beta$ -glucosidase. Results of the effect of different incubation solution on the ratio of extracellular to total enzyme activity are compared with SIS, as percent of the ratio in SIS, in table 3. Release in the presence of individual components of SIS and in the presence of EDTA is shown in the first experimental series. Release into  $MgSO_4$  is somewhat reduced compared with that in SIS, while in  $CaCl_2$  release is slightly increased. Release in the presence of potassium phosphate is greater than in SIS, by up to 65% at 10 hours. Release in the presence of EDTA

Table 3: The effect of salts on release of  $\beta$ -glucosidase. Values given are the mean percent of release in SIS (ratio of extracellular to total activity)  $\pm$  SD of 3 exps.

| Incubation solution | Addition Omission                                        | 1st series              |              |              |             |              |
|---------------------|----------------------------------------------------------|-------------------------|--------------|--------------|-------------|--------------|
|                     |                                                          | Incubation time (hours) |              |              |             |              |
|                     |                                                          | 0                       | 2            | 4            | 6           | 10           |
| SIS (control)       |                                                          | 100                     | 100          | 100          | 100         | 100          |
| H <sub>2</sub> O    | + MgSO <sub>4</sub> 4.0mM                                | 97 $\pm$ 3              | 88 $\pm$ 11  | 78 $\pm$ 7   | 83 $\pm$ 6  | 92 $\pm$ 3   |
|                     | + CaCl <sub>2</sub> 0.5mM                                | 102 $\pm$ 4             | 115 $\pm$ 4  | 111 $\pm$ 5  | 104 $\pm$ 2 | 114 $\pm$ 11 |
|                     | + KH <sub>2</sub> -K <sub>2</sub> HPO <sub>4</sub> 2.5mM | 122 $\pm$ 22            | 138 $\pm$ 14 | 132 $\pm$ 10 | 137 $\pm$ 7 | 165 $\pm$ 3  |
|                     | + EDTA 4.0 mM                                            | 132 $\pm$ 16            | 142 $\pm$ 9  | 150 $\pm$ 3  | 159 $\pm$ 2 | 170 $\pm$ 5  |
|                     |                                                          | 2nd series              |              |              |             |              |
|                     |                                                          | Incubation time (hours) |              |              |             |              |
|                     |                                                          | 0                       | 3            | 6            | 10          | 24           |
| SIS (control)       |                                                          | 100                     | 100          | 100          | 100         | 100          |
| H <sub>2</sub> O    | none                                                     | 147 $\pm$ 35            | 113 $\pm$ 6  | 106 $\pm$ 5  | 127 $\pm$ 5 | 146 $\pm$ 11 |
| SIS                 | - MgSO <sub>4</sub>                                      | 102                     | 109          | 104          |             |              |
|                     | + MgSO <sub>4</sub> 8.0mM                                | 102                     | 104          | 98           |             |              |
|                     | + MgSO <sub>4</sub> 16.0mM                               | 102                     | 107          | 99           |             |              |
|                     | - CaCl <sub>2</sub>                                      | 106                     | 87           | 92           |             |              |
|                     | + CaCl <sub>2</sub> 4.0mM                                | 102                     | 112          | 107          |             |              |

is also greater than in SIS, by up to 70% at 10 hours. The second experimental series shows that release into distilled water is quite variable; it is greater than in SIS at time zero by 47%, but with a large standard deviation and at time 6 hours there is virtually no difference from the control, while again at 24 hours 46% more enzyme is released. Variation of the magnesium concentration in the presence of the other components of SIS had virtually no effect on release as shown by the second experimental series (table 3). In a solution with the components of SIS plus additional calcium (4.0 mM  $\text{CaCl}_2$  in table 3) release was slightly greater than in SIS whereas with no  $\text{CaCl}_2$  present release was slightly less than in SIS.

Variation in the total concentration of SIS components affected release somewhat as shown in table 4. Concentrations of 10 times and 0.1 times the standard SIS increased release by an average 12 and 18% of standard respectively.

To compare release in SIS with release in GM and to check the effect of washing on release in GM an experiment was conducted in which one sample of 5 day old cells was washed and suspended in SIS, one sample washed with SIS and suspended in fresh GM, one sample washed with SIS and suspended in the GM from which cells had been removed and one sample was left in the original GM with no washing. The results, calculated as release per hour per  $10^6$  cells, are given in table 5 and indicate that release in SIS is not appreciably different from that in GM and the washing had no effect.

The amount of cells that would have to lyse to account for the

**Table 4: The effect of varying the SIS concentration on release of  $\beta$ -glucosidase.**

Values given are the mean (2 exps. ) percent of release in SIS control (calculated from the ratio of extracellular to total activity).

| Concentration<br>of SIS  | Incubation time (hours) |     |     |     |     |
|--------------------------|-------------------------|-----|-----|-----|-----|
|                          | 0                       | 2   | 4   | 6   | 10  |
| 0.1 x standard           | 118                     | 129 | 120 | 108 | 114 |
| 1.0 x standard (control) | 100                     | 100 | 100 | 100 | 100 |
| 10.0 x standard          | 109                     | 106 | 111 | 112 | 121 |

Table 5: The rate of  $\beta$ -glucosidase release from cells in SIS and GM. Values represent mU activity released per hour per  $10^6$  cells for the time intervals.

| Incubation medium         |       |       |        |         | Mean for the intervals |
|---------------------------|-------|-------|--------|---------|------------------------|
|                           | 0 - 3 | 3 - 6 | 6 - 10 | 10 - 24 |                        |
| SIS <sup>a</sup>          | 12.6  | 10.0  | 10.0   | 9.1     | 10.4                   |
| Fresh GM <sup>b</sup>     | 11.1  | 7.4   | 10.2   | 11.2    | 10.0                   |
| Original GM <sup>c</sup>  | 14.8  | 5.4   | 13.7   | 14.3    | 12.0                   |
| Untreated GM <sup>d</sup> | 11.9  | 11.4  | 7.4    | 14.1    | 11.2                   |

<sup>a</sup>Washed in SIS and incubated in SIS

<sup>b</sup>Washed in SIS and incubated in fresh GM

<sup>c</sup>Washed in SIS and incubated in the original GM from which the cells were harvested.

<sup>d</sup>Cells were left in GM suspension without harvesting.

$\beta$ -glucosidase activity released in SIS was calculated the same way as for release in growing cultures (table 1). An equivalent of approximately 41% of the cells in SIS at time zero would have to lyse over the 24 hour incubation period. This value is remarkably similar to the average 44% calculated for growing cultures.

To see if the enzyme released into the SIS was activated or degraded with time, cells were removed from samples after 3 hours incubation and the cell-free SIS was assayed for B-glucosidase at intervals (figure 7 upper panel). There was only a slight reduction in activity during incubation; mean activity after 21 hours was 8% below the initial activity.

A release experiment was conducted, in which samples at 0.5 and 2.0 times the control cell concentration were incubated in SIS, to see if cell concentration had an effect on release. Table 6 shows that differences in cell concentration resulted in nearly proportional differences in release, indicating cell concentration did not influence release.

#### Release of protein into SIS

Protein was released at a much lower rate than  $\beta$ -glucosidase (figure 4) which led to a considerable increase in the specific activity of  $\beta$ -glucosidase in the medium (figure 8). Specific activity in the cells was lower than in the medium even at time zero and remained nearly constant throughout the incubation period (figure 8).

To see if released protein was degraded in the SIS, cells were

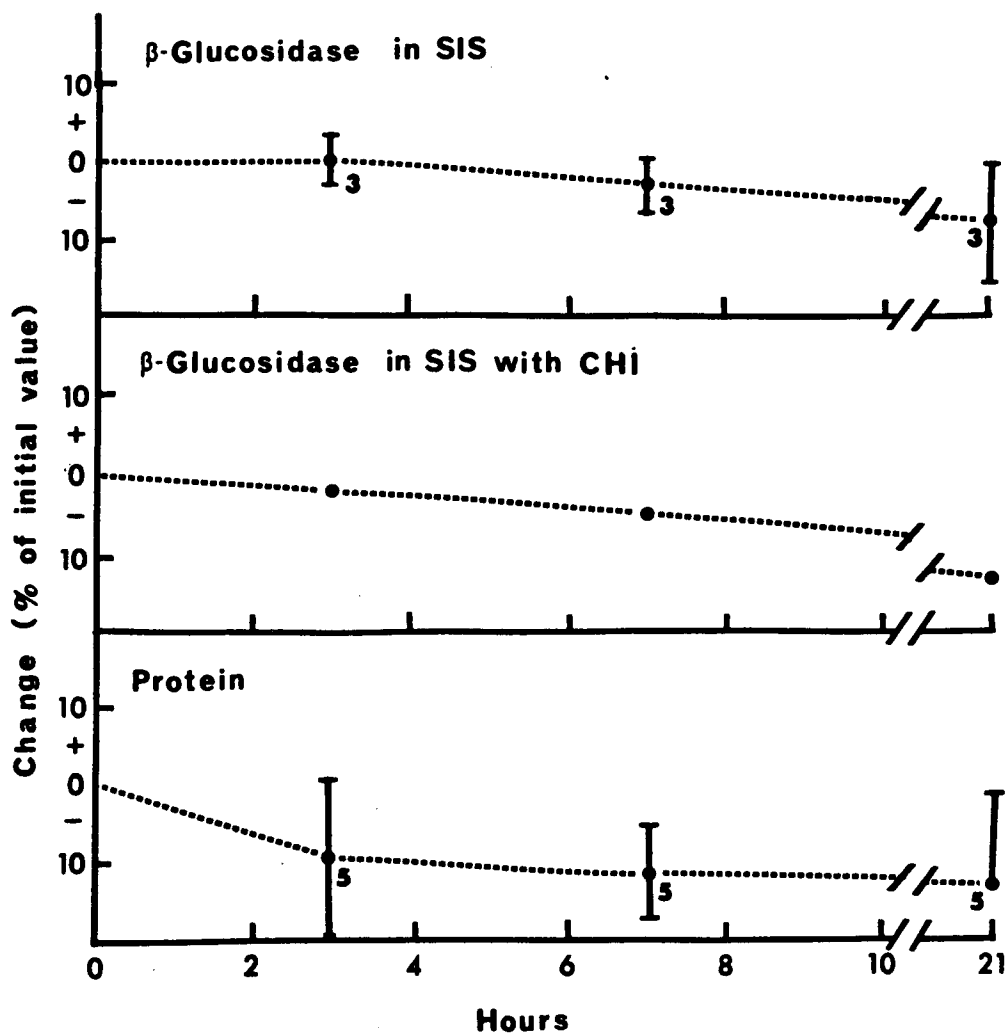


Figure 7. Change in  $\beta$ -glucosidase activity and protein in the cell-free supernatant. Conditions as described in the text. Vertical bars represent standard deviation. Number of determinations shown next to data points.

**Table 6: The effect of cell concentration on  $\beta$ -glucosidase release in SIS. Values are percent of release in the control calculated from mU/ml in the cell-free supernatant.**

| Cell concentration | Incubation time (hours) |     |       |     |     | Mean |
|--------------------|-------------------------|-----|-------|-----|-----|------|
|                    | 1/2                     | 3   | 5 1/2 | 10  | 24  |      |
| 0.5 x control      | 62                      | 54  | 57    | 60  | 55  | 58   |
| Control            | 100                     | 100 | 100   | 100 | 100 | 100  |
| 2.0 x control      | 206                     | 183 | 190   | 198 | 187 | 193  |

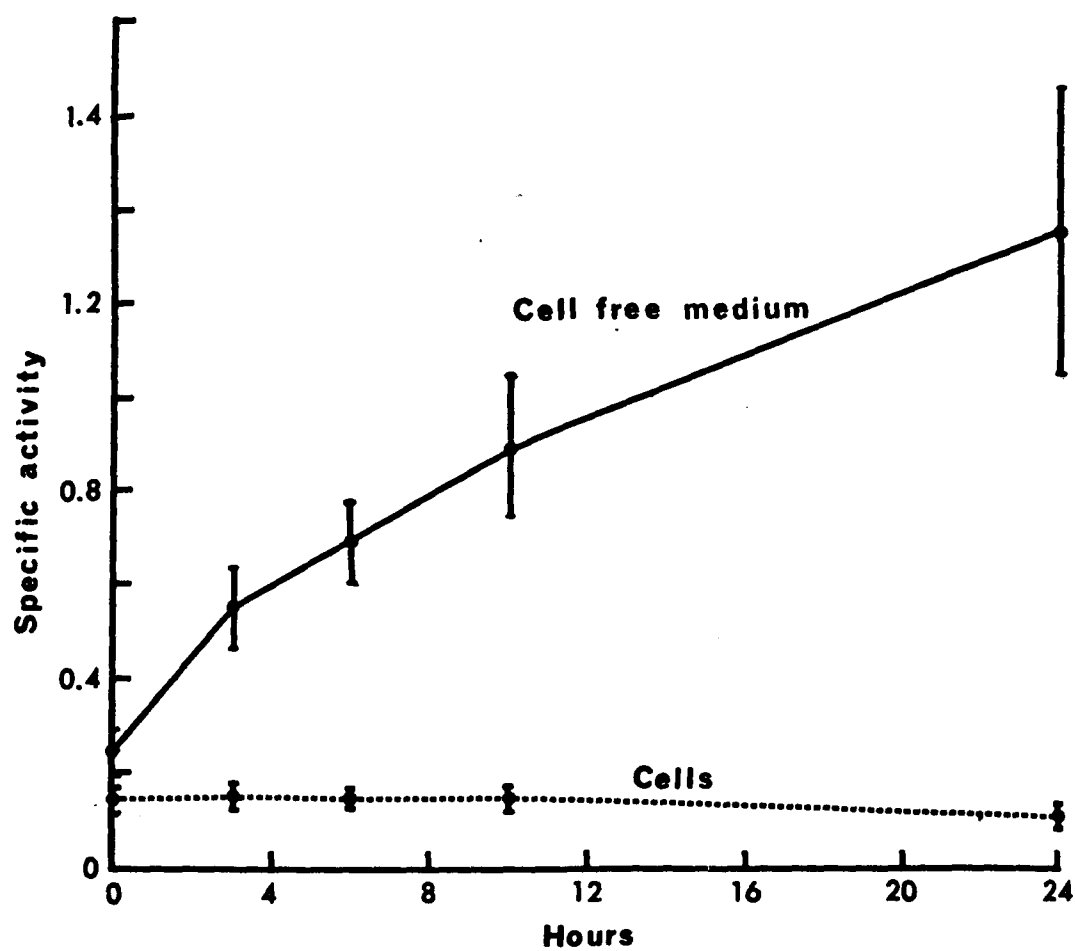


Figure 8. Specific activity of  $\beta$ -glucosidase in the medium and in the cells during incubation in SIS. Same experiments as in figure 4.

separated from the medium after 3 hours incubation and protein in the cell-free SIS was determined at the intervals shown in figure 7 lower panel. The results show a mean reduction in protein content at 21 hours of 12% of the initial value.

#### Release of UV-absorbing material

As a possible indicator of cell lysis or leakiness, measurements of absorbance at 260 nm were routinely made of the media separated from cells. The UV absorption spectrum of the cell-free SIS medium separated from cells after 6 hours incubation is shown in figure 9.

Figure 10 shows that in SIS  $A_{260}$  material was released nearly proportionately with  $\beta$ -glucosidase for the first 3 hours but then the rate of release declined rapidly relative to enzyme release.

#### Release of alkaline phosphatase and phosphoglucose isomerase

Further evidence that  $\beta$ -glucosidase is released by a specific selective process not involving lysis or efflux of enzyme as a result of an artifactual leakiness to large molecules was sought by assaying the medium for enzymes that would not be expected to be released or might be released at a different rate than glucosidase.

Alkaline phosphatase was not found in SIS separated from cells after 5 1/2 hours incubation, while glucosidase was being released. However, alkaline phosphatase activity was found in the medium when the cells were apparently damaged by metabolic inhibitors (figure 15).

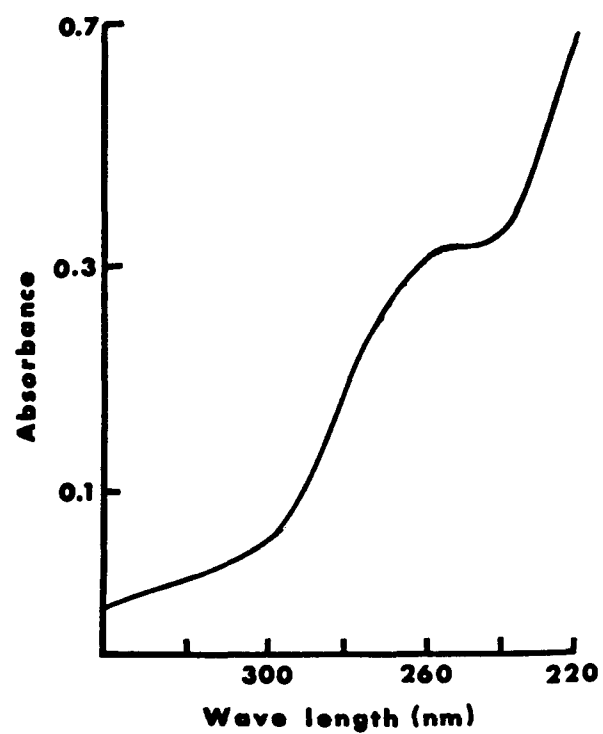


Figure 9. Absorption spectrum in the UV of the cell-free supernatant after 6 hours incubation in SIS.

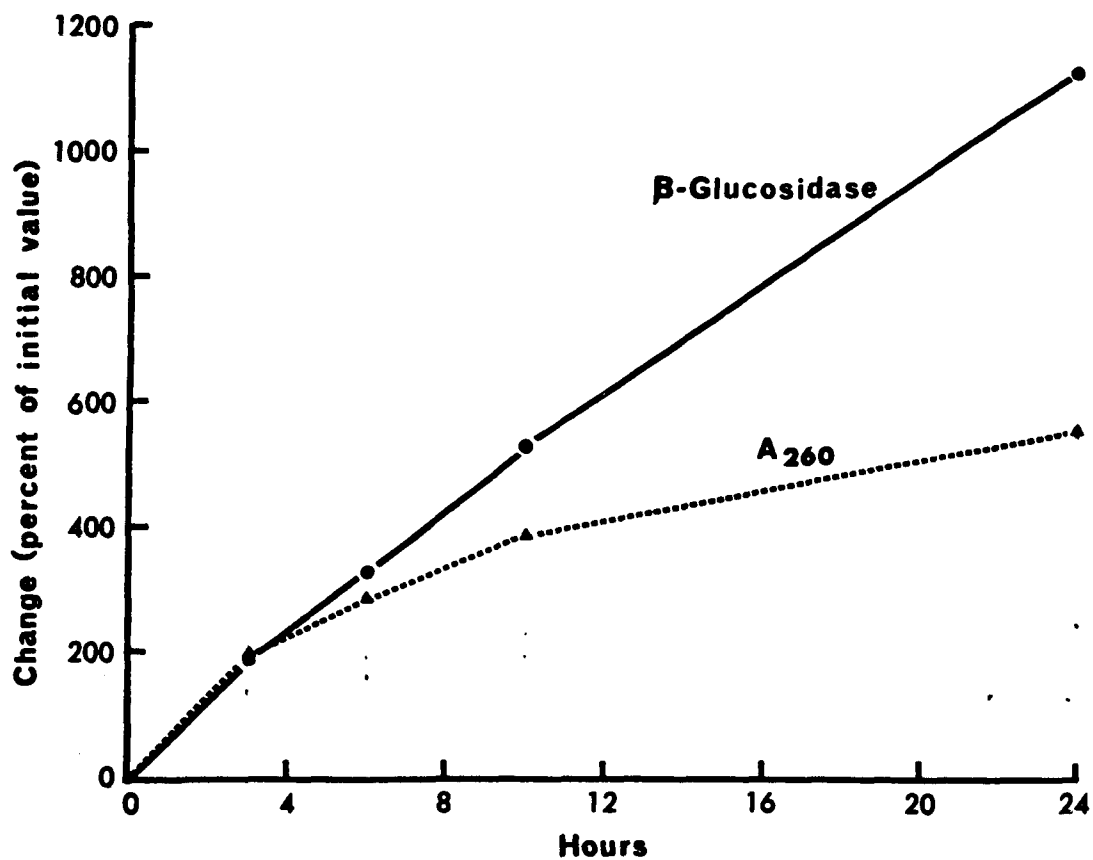


Figure 10. Change in  $\beta$ -glucosidase activity and  $A_{260}$  of the cell-free supernatant during incubation in SIS.

Table 7: Cellular enzyme activities. Values give mean activities

$\pm$  SD in cells of 5 day old cultures washed once with SIS. The number of determinations is indicated in parenthesis.

| Enzyme                   | mU/10 <sup>7</sup> cells |     |
|--------------------------|--------------------------|-----|
| $\beta$ -Glucosidase     | 49 $\pm$ 8               | (5) |
| Phosphoglucose isomerase | 351 $\pm$ 58             | (5) |
| Alkaline phosphatase     | 13 $\pm$ 2               | (4) |

A better lysis/leakage marker enzyme was sought for the following reasons: 1) Alkaline phosphatase was one of the enzymes reported in the growth medium of O. danica.<sup>28</sup> 2) Dr. Patni in our laboratory has found uncertainties in the assay of alkaline phosphatase at low concentrations in GM. 3) The activity of cellular alkaline phosphatase was determined to be very low relative to cellular  $\beta$ -glucosidase, on a per cell basis (table 7). 4) The cellular location of alkaline phosphatase is unknown.

Phosphoglucose isomerase was found to have high cellular activity relative to  $\beta$ -glucosidase (table 7). Since glycolytic pathway enzymes are located in the cell cytosol<sup>62</sup> release of this enzyme might be expected to take place under conditions which render the cell leaky to cytoplasmic enzymes, whereas markers located in cell organelles might not be released unless there was complete cell breakdown.

Preliminary results indicated no phosphoglucose isomerase was released into SIS for incubation periods up to 24 hours. To see if this was the result of instability and loss of activity of any enzyme that might have been released, experiments were conducted in which crude phosphoglucose isomerase, in the form of cell homogenate (prepared by forcing cells through a Millipore filter), was added to SIS with and without cells at the beginning of incubation. It was found that phosphoglucose isomerase activity was rapidly lost with time in SIS at pH 5 but not pH 7. After 6 hours of incubation 92% of the isomerase

**Table 8: The effect of SIS pH on the amount of enzyme activity appearing in the cell-free supernatant. Values are percent of total activity (cells plus supernatant).**

| SIS pH | <u>Phosphoglucose isomerase</u> |     |     |     |     | <u>B-Glucosidase</u> |      |      |      |      |
|--------|---------------------------------|-----|-----|-----|-----|----------------------|------|------|------|------|
|        | Incubation time (hours)         |     |     |     |     |                      |      |      |      |      |
|        | 0                               | 3   | 6   | 10  | 24  | 0                    | 3    | 6    | 10   | 24   |
| 5      | 0.4                             | 0.1 | 0.1 | 0.4 | 0.4 | 3.2                  | 9.2  | 13.1 | 18.6 | 35.9 |
| 6      | 1.8                             | 2.7 | 3.0 | 3.5 | 5.7 | 3.6                  | 10.4 | 14.7 | 20.3 | 39.3 |
| 7      | 2.2                             | 4.7 | 5.9 | 7.2 | 9.5 | 5.0                  | 11.6 | 17.0 | 21.5 | 39.4 |
| 8      | 2.0                             | 4.5 | 5.7 | 7.0 | 9.6 | 5.2                  | 11.7 | 17.2 | 20.8 | 38.9 |

originally added to SIS pH 7 with cells remained in the medium; without cells 96% remained. Thus phosphoglucose isomerase was stable in the presence of cells in SIS at pH 7.

The effect of pH of SIS on the extracellular phosphoglucose isomerase activity during incubation without added enzyme was then determined and the results are shown in table 8. Increasing the SIS pH to 7 resulted in increasing activity in the cell-free medium. An increase from pH 7 to 8 did not result in a further increase in phosphoglucose isomerase activity. This result along with the aforementioned stability of added enzyme at pH7 indicated that all phosphoglucose isomerase released at pH 7 is detected. An increase in pH increased initial release of  $\beta$ -glucosidase but had no effect on the rate of release (table 8). The pH 7 data from table 8 is plotted in figure 11 and shows that, although phosphoglucose isomerase was released during incubation in SIS, it was significantly less than  $\beta$ -glucosidase release under the same conditions.

Further evidence that phosphoglucose isomerase and  $\beta$ -glucosidase were released at different rates reflecting different release mechanisms and evidence that phosphoglucose isomerase is released when cells are leaky or damaged was shown by the effect of bovine serum albumin (BSA). The presence of BSA was found to stabilize phosphoglucose isomerase activity in SIS without significantly affecting  $\beta$ -glucosidase release. Table 9 shows that increase in extracellular enzyme, expressed as percent of initial value, is

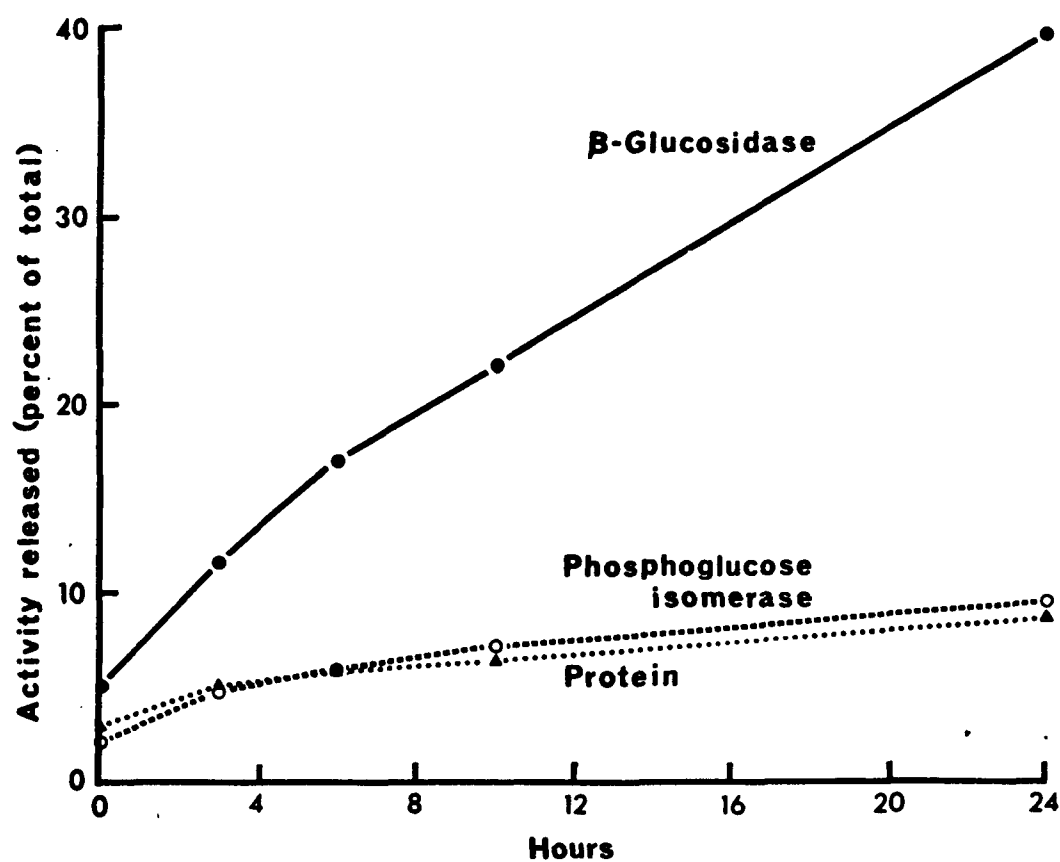


Figure 11. Enzyme activity and protein appearing in the cell-free supernatant during incubation in SIS at pH 7.

**Table 9: The influence of the incubation solution on the effect of BSA on enzyme activity appearing in the cell-free supernatant.** The incubation solutions contained 8.0 mg/ml BSA. Values are enzyme activity appearing in the cell-free supernatant (mU/ml).

| Enzyme                          | Incubation solution | Incubation time (hours) |      |      |      |
|---------------------------------|---------------------|-------------------------|------|------|------|
|                                 |                     | 0                       | 2    | 4    | 6    |
| <b>β-glucosidase</b>            | SIS                 | 2.2                     | 5.6  | 7.9  | 10.1 |
|                                 | H <sub>2</sub> O    | 17.3                    | 26.5 | 31.7 | 35.2 |
| <b>Phosphoglucose isomerase</b> | SIS                 | 11                      | 13   | 14   | 16   |
|                                 | H <sub>2</sub> O    | 181                     | 466  | 532  | 615  |

much greater for  $\beta$ -glucosidase than for phosphoglucose isomerase in SIS in the presence of 8mg/ml BSA. Table 9 also shows that cells incubated in distilled water with BSA become damaged and release large amounts of enzymes, particularly phosphoglucose isomerase. Further indication that phosphoglucose isomerase is released and detectable during cell damage was shown by release in the presence of metabolic inhibitors (figure 15). The results given in table 9 also indicate the importance of SIS in protecting the cells from leakiness and cell damage.

#### Evidence for energy dependent release of $\beta$ -glucosidase

Cells were deprived of aerobic oxidative energy by constant bubbling of nitrogen through the incubating cell suspension. Since O. danica also has a photosynthetic capability, the possibility of obtaining energy from this source was circumvented by incubation in the dark. Under these dark anaerobic conditions the release of  $\beta$ -glucosidase in SIS was reduced by up to 42% of that of the control under light aerobic conditions (figure 12). This value of 42% is the result of calculations after subtracting the initial release which is common to all samples and not the result of selective release, as previously shown (table 2). Under dark aerobic conditions release is greater than under dark anaerobic conditions but less than the control (figure 12). With light anaerobic conditions release was slightly enhanced the first 3 hours, but then the rate of release paralleled that of the dark aerobic conditions. From the results it

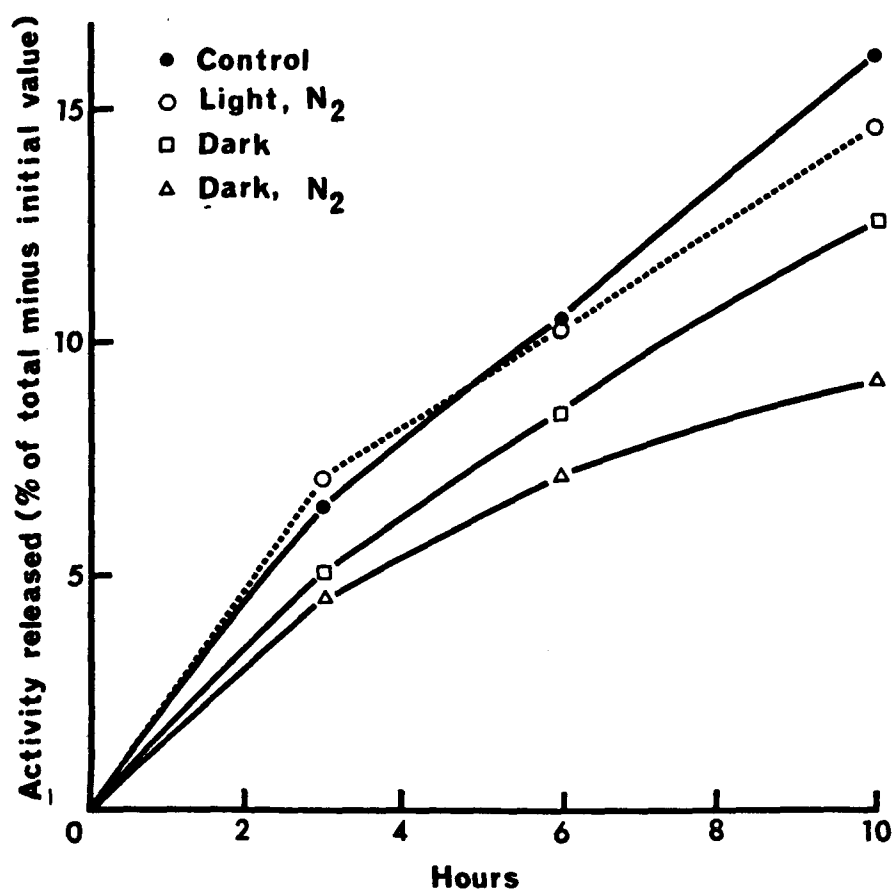


Figure 12. The effect of anaerobic and dark incubation conditions on the release of  $\beta$ -glucosidase. Conditions as described in Methods and the text.

Table 10: Change in total  $\beta$ -glucosidase under dark and anaerobic incubation conditions. Values given are percent of initial value.

| Incubation conditions          | Incubation time (hours) |     |     |     |
|--------------------------------|-------------------------|-----|-----|-----|
|                                | 0                       | 3   | 6   | 10  |
| Light                          | 100                     | 100 | 100 | 110 |
| Light + bubbled N <sub>2</sub> | 100                     | 92  | 95  | 118 |
| Dark                           | 100                     | 100 | 100 | 110 |
| Dark + bubbled N <sub>2</sub>  | 100                     | 98  | 90  | 101 |

may be suggested that the deprivation of both oxygen and light reduced the rate of release. Monitoring of the pH of the medium during incubation showed that the pH did not change appreciably and addition of bicarbonate to the medium of the dark anaerobic sample did not influence the results. Calculation of the percent change in total  $\beta$ -glucosidase (table 10) showed that although dark anaerobic conditions resulted in a 10% reduction in total activity at 6 hours that amount was regained by 10 hours; thus indicating  $\beta$ -glucosidase synthesis was not appreciably affected and the reduction in release cannot be attributed to reduced synthesis.

The effect of temperature on release is shown in figure 13. Release of  $\beta$ -glucosidase shows a similar temperature dependent pattern at pH 5 and pH 7 in SIS (upper 2 panels, figure 13). Experiments at pH 7 were conducted so that the release of phosphoglucose isomerase could be monitored. There is an apparent temperature optimum between  $15^{\circ}$  and  $25^{\circ}\text{C}$ , since the change in release rate with temperature is less above and below that temperature range. The temperature coefficients ( $Q_{10}$ ) were calculated and are given in table 11.  $Q_{10}$  in the temperature range  $5-25^{\circ}$  varies between 2.4 and 2.6 for pH 5 and 1.8 to 2.8 for pH 7, whereas, in the range  $25-37^{\circ}$  the  $Q_{10}$  is 1.3 (pH 5) and 1.1 (pH 7). An Arrhenius plot of the rate of  $\beta$ -glucosidase release is shown in figure 14. Two slopes are clearly indicated, one for the temperature range  $5-25^{\circ}$  and a smaller slope for the temperature range  $25-37^{\circ}$ . The activation energy for each

slope was calculated, and for the range 25-37<sup>0</sup> was found to be 4.5 kcal and for the range 5-25<sup>0</sup> it was 15.2 kcal. Release of protein and phosphoglucose isomerase, for the pH 7 experiments (lower left 2 panels, figure 13), increased slightly with increasing temperature but not appreciably compared to  $\beta$ -glucosidase release; only at 37<sup>0</sup> and 10 hours was there an increase in released phosphoglucose isomerase to an extent suggestive of cell damage. Increase in release of A<sub>260</sub> material, as percent change (lower right panel, figure 13), was released in a pattern somewhat similar to the release of  $\beta$ -glucosidase.

In the search for evidence that  $\beta$ -glucosidase release was energy dependent the effect of three inhibitors of ATP synthesis was looked at. Representative results with three concentrations of the inhibitor of oxidative phosphorylation, dinitrophenol (DNP), the respiratory chain poison, sodium azide (SA), and the glycolytic pathway inhibitor, iodoacetic acid (IAA) are shown in table 12. The lowest concentrations tested for DNP ( $5 \times 10^{-6}$  M) and IAA ( $10^{-5}$  M) resulted in release essentially the same as in the controls. Higher concentrations of DNP and IAA and the three concentrations of SA resulted in release of  $\beta$ -glucosidase at a greater rate than the control. The drop in percent of control seen in some cases (table 12), for example with DNP at  $5 \times 10^{-4}$  M, is a reflection of the increased level of released activity in the control without a corresponding increase in the experimental release.

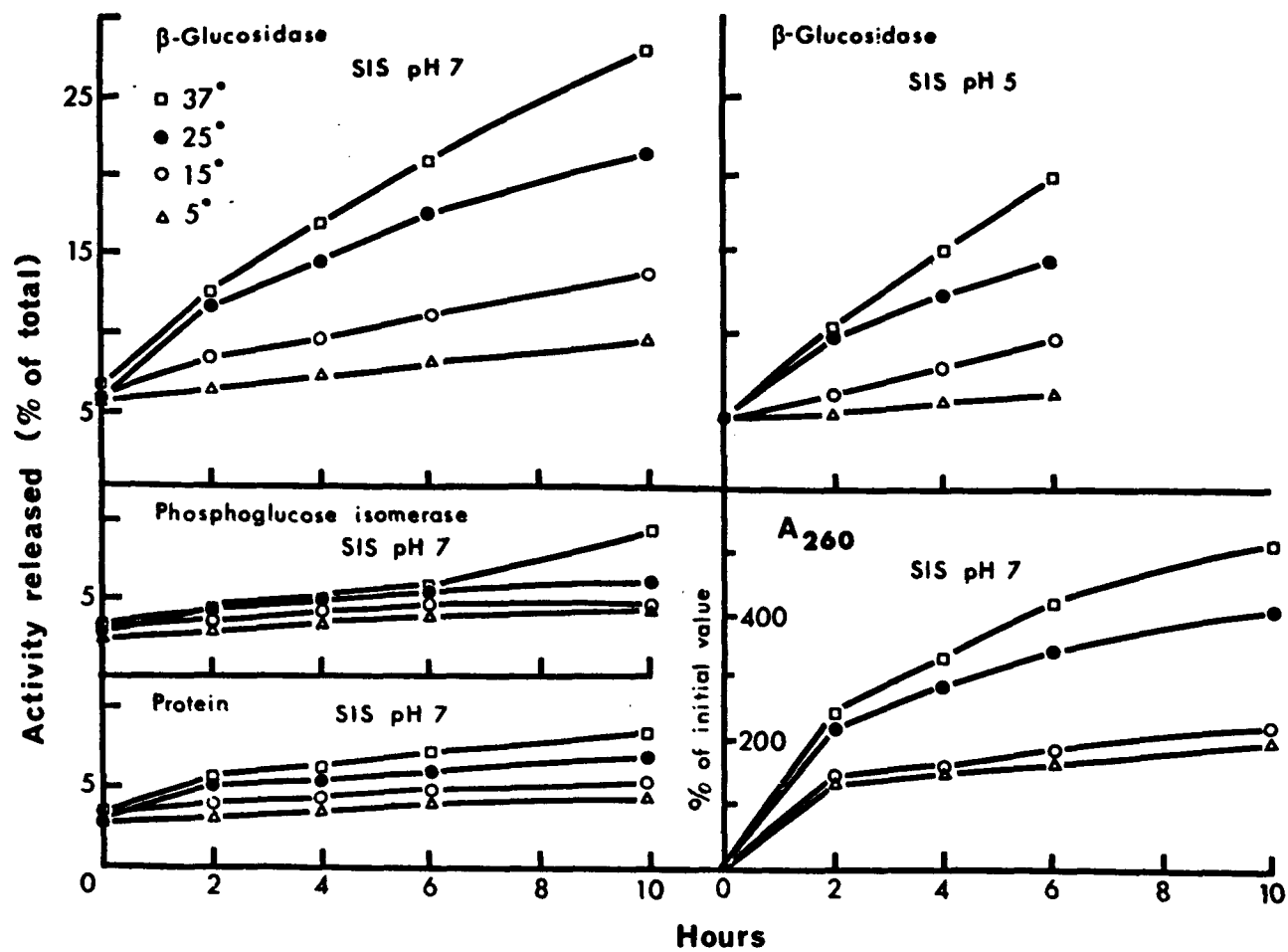


Figure 13. The effect of temperature on release.

Table 11: Temperature coefficients ( $Q_{10}$ ) of  $\beta$ -glucosidase release.

| pH of SIS | Temperature range ( $^{\circ}\text{C}$ ) | $Q_{10}$ |
|-----------|------------------------------------------|----------|
| 5         | 5 - 15                                   | 2.6      |
|           | 15 - 25                                  | 2.4      |
|           | 25 - 37                                  | 1.3      |
| 7         | 15 - 25                                  | 2.8      |
|           | 25 - 37                                  | 1.1      |

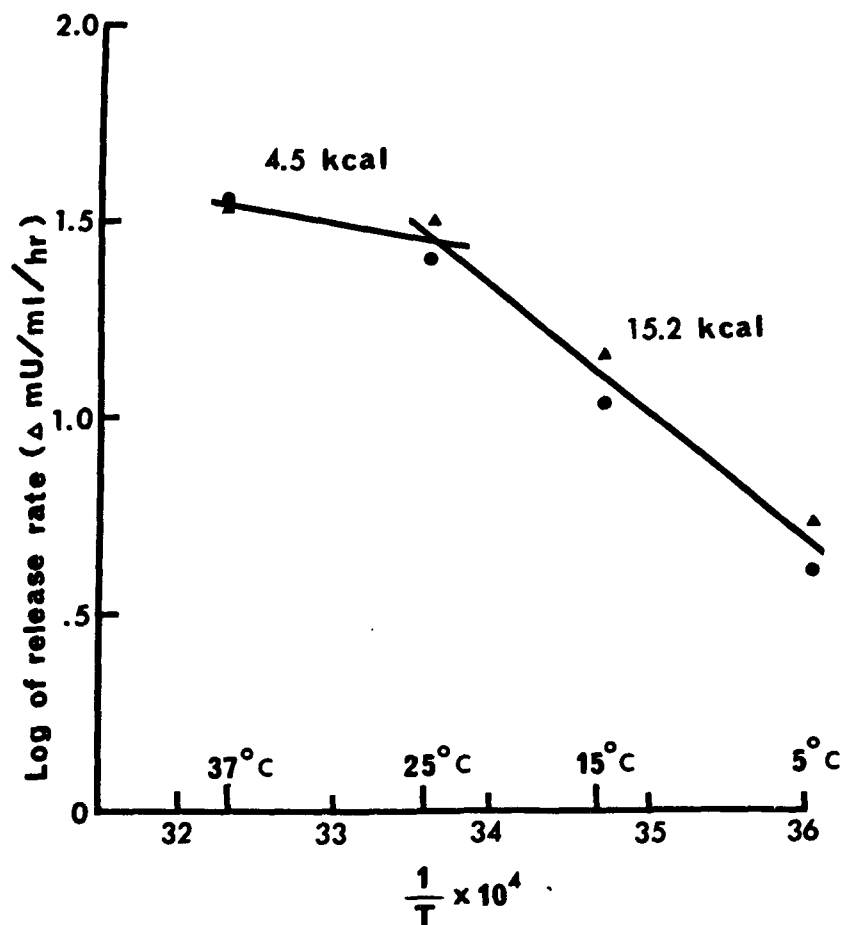


Figure 14. Arrhenius plot of the effect of incubation temperature on  $\beta$ -glucosidase release. Incubation was in SIS at pH 5 (circles) and pH 7 (triangles). Activation energy ( $E_a$ ) was calculated from the expression:

$$\ln k = - \frac{E_a}{R} \left( \frac{1}{T} \right)$$

where  $k$  is the rate of release,  $R$  the gas constant and  $T$  the absolute temperature.

Table 12: The effect of metabolic inhibitors on  $\beta$ -glucosidase release.

Values represent percent of control (SIS without drug), calculated from activity (mU/ml) appearing in the cell-free supernatant.

| Inhibitor | Concentration (M)  | Incubation time (hours) |       |       |
|-----------|--------------------|-------------------------|-------|-------|
|           |                    | 1/2                     | 2 1/2 | 4 1/2 |
| DNP       | $5 \times 10^{-6}$ | 112                     | 104   | 107   |
|           | $5 \times 10^{-5}$ | 192                     | 192   | 186   |
|           | $5 \times 10^{-4}$ | 660                     | 358   | 293   |
| SA        | $5 \times 10^{-5}$ | 150                     | 137   | 120   |
|           | $5 \times 10^{-4}$ | 253                     | 236   | 220   |
|           | $5 \times 10^{-3}$ | 316                     | 299   | 320   |
| IAA       | $10^{-5}$          | 93                      | 98    | 99    |
|           | $10^{-4}$          | 108                     | 274   | 273   |
|           | $10^{-3}$          | 388                     | 273   | 223   |

DNP at  $10^{-4}$ M and IAA at  $2 \times 10^{-4}$  M were determined to have no effect on enzyme activity.

For DNP at  $5 \times 10^{-5}$ M and IAA at  $10^{-4}$ M cellular enzyme activity was determined and found to be reduced in proportion to activity released resulting in virtually no change in total activity over the time period studied.

The increase in released  $\beta$ -glucosidase activity in most of the inhibitor experiments was thought likely to be the result of loss of cell integrity. This was supported by the following: 1) Microscopic observations showed the cells to be abnormally shaped (round vs normal tear drop shape) and in the severest cases of increased release, cells took on a crenated, dead appearance and apparent cell debris became noticeable. 2) Protein and  $A_{260}$  material were released in greater amounts than in the controls as seen in figure 15, which gives the results of one experiment showing the percent of release of protein and  $A_{260}$  material above control for IAA and DNP. It also shows that the release of  $A_{260}$  material and protein is very similar under these conditions. 3) The marker enzymes phosphoglucose isomerase and alkaline phosphatase were released into the medium (SIS, pH 5) in the presence of DNP and IAA (figure 16).

#### Effect of protein synthesis inhibitors on $\beta$ -glucosidase release

If protein synthesis is required for  $\beta$ -glucosidase release then agents which inhibit protein synthesis should also inhibit release.

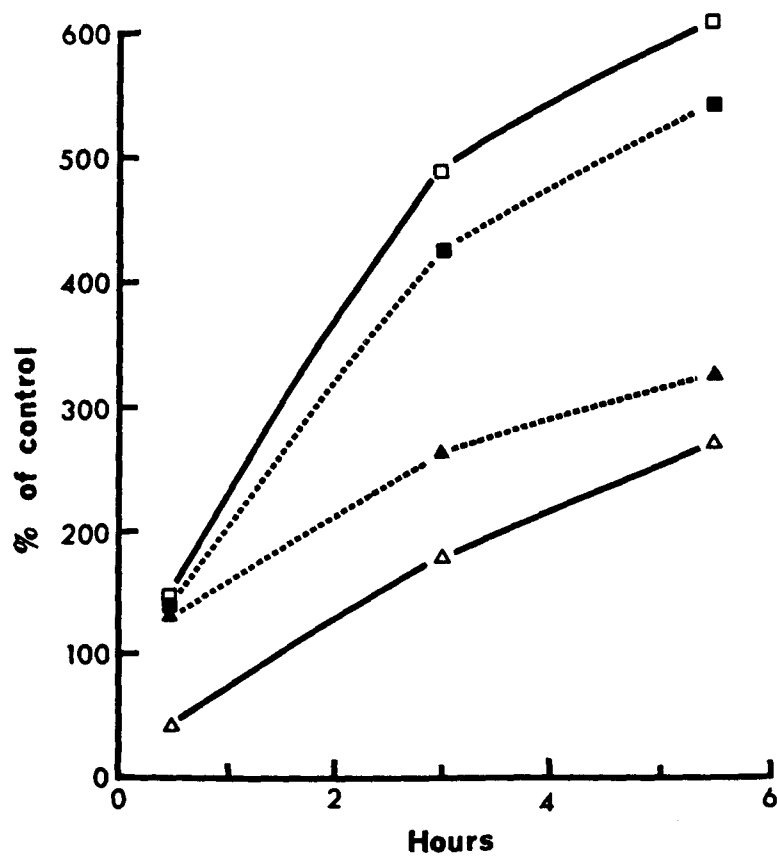


Figure 15. The effect of DNP and IAA on the release of protein and A<sub>260</sub> material. Triangles, DNP; squares, IAA; solid lines, A<sub>260</sub>; dashed lines, protein.

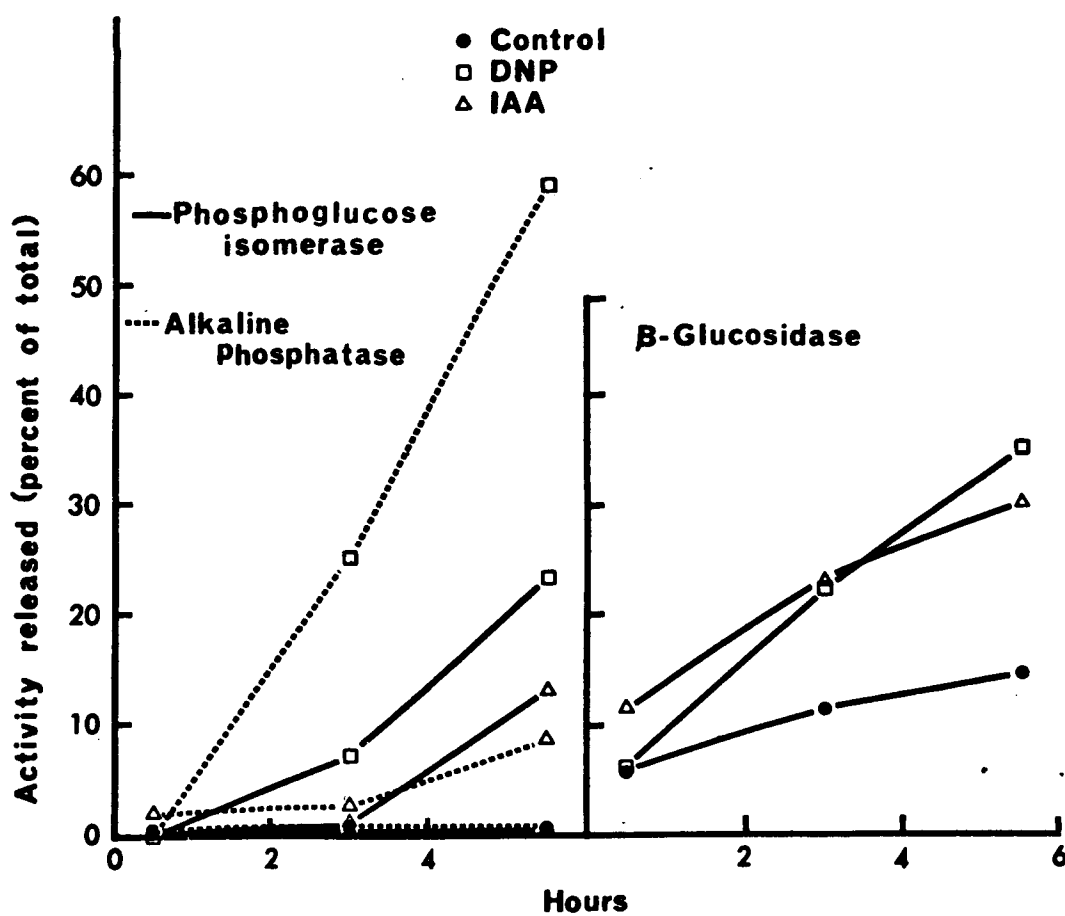


Figure 16. Release of enzyme activity in the presence of DNP and IAA. Cells were incubated in SIS pH 5. Drug concentrations: DNP  $5 \times 10^{-5}M$ , IAA  $10^{-4}M$ .

Demonstration of this effect would provide a strong case for characterizing the release process as selective release from intact cells.

Table 13 gives the results of the effect of chloramphenicol (CAP), puromycin (PM) and cycloheximide (CHI) on release of  $\beta$ -glucosidase. At 0.4mg/ml CAP had no apparent effect on release of  $\beta$ -glucosidase. A 10 fold increase in CAP concentration resulted in an average 33% greater release compared to the control. Puromycin, used at only one concentration (100ug/ml), also enhanced release after about 4 hours. In contrast CHI inhibited release. At 50ug/ml CHI inhibition of release was greater than at 10ug/ml, however, inhibition at 100 and 200ug/ml was not appreciably greater than at 50ug/ml (table 13). The data from the 100ug/ml experiment of table 13, plotted in mU/ml released  $\beta$ -glucosidase, is compared with the control in figure 16. The response to CHI appears to be immediate and approaches complete inhibition of release by 10 hours.

The change in total  $\beta$ -glucosidase activity for the same experiment (figure 18) shows a decrease in total activity by about 10% at 10 hours with CHI, whereas, in the control total activity increased by 25% at 10 hours.

These results indicate that both synthesis and release are inhibited by CHI.

To see if the decrease in extracellular activity was an effect of CHI on enzyme activity, enzyme activity in the SIS medium with CHI after separation from cells was determined over a period of

Table 13: The effect of protein synthesis inhibitors on  $\beta$ -glucosidase release. Values given are percent of control (SIS without drug), calculated from activity released (mU/ml).

| Inhibitor | Concentration | Incubation time (hours) |       |       |       |
|-----------|---------------|-------------------------|-------|-------|-------|
|           |               | 1/2                     | 2 1/2 | 4 1/2 | 6 1/2 |
| CAP       | 0.4 mg/ml     | 112                     | 104   | 108   | 103   |
|           | 4.0 mg/ml     | 138                     | 123   | 133   | 138   |
| PM        | 100 ug/ml     | Incubation time (hours) |       |       |       |
|           |               | 1/2                     | 3     | 5 1/2 | 10    |
| CHI       | 10 ug/ml      | Incubation time (hours) |       |       |       |
|           |               | 0                       | 3     | 6     | 10    |
|           | 10 ug/ml      | 100                     | 73    | 73    | 74    |
|           | 50 ug/ml      | 105                     | 73    | 60    | 45    |
|           | 100 ug/ml     | 110                     | 71    | 59    | 45    |
|           | 200 ug/ml     | 103                     | 67    | 56    | 43    |

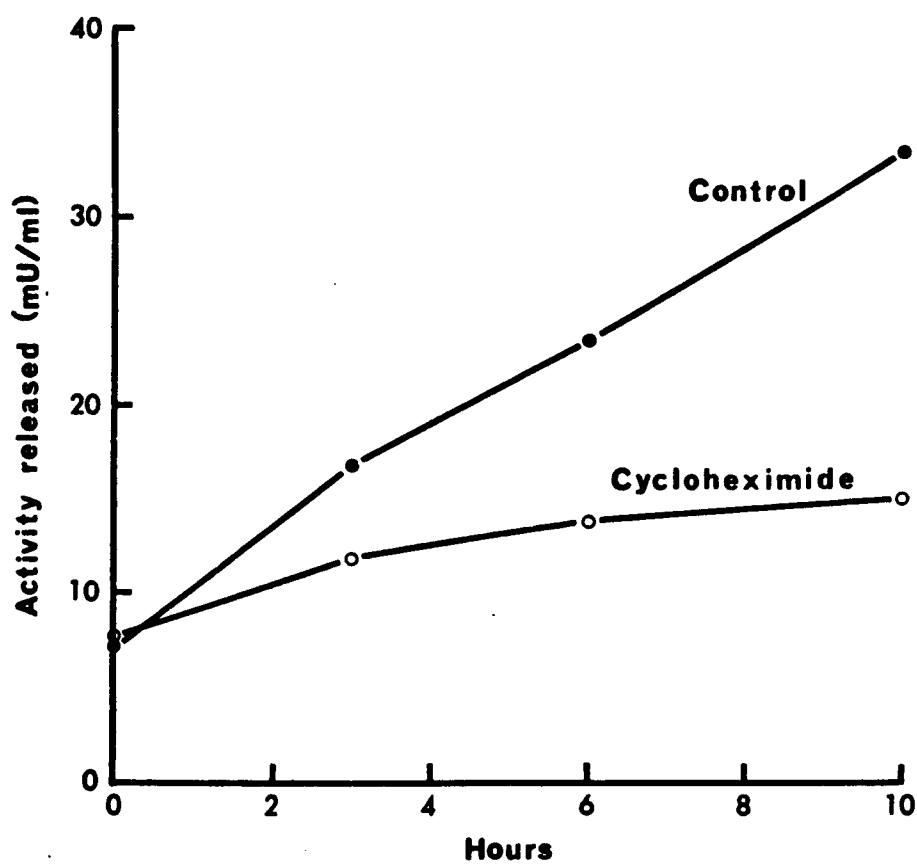


Figure 17. The effect of CHI on the release of B-glucosidase. CHI at 100 ug/ml.

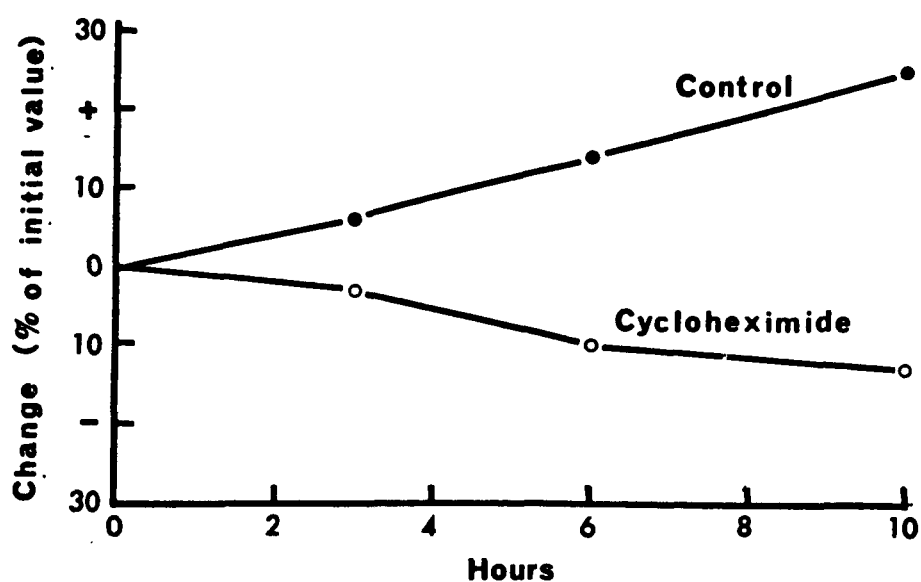


Figure 18. The effect of CHI on the total  $\beta$ -glucosidase activity. The same experiment as shown in figure 17.

time. The results given in figure 7 (middle panel) show that  $\beta$ -glucosidase activity remaining in the medium after separation from cells was not appreciably different from medium without CHI (figure 7 upper panel).

Table 14 gives the data on release of protein and  $A_{260}$  material in the presence of CHI. It shows that release of protein and  $A_{260}$  material, although quite variable, on the average is only slightly reduced from release in the control.

If CHI inhibits a selective release process, then release which is not due to this mechanism but is a result of cell damage should be unaffected by CHI. The results of an experiment in which CHI was added along with DNP and IAA to SIS are given in table 15 and show that CHI had essentially no effect on  $\beta$ -glucosidase release with DNP and little effect with IAA (5% less release than in IAA without CHI at 5 1/2 hours). However, release was inhibited 48% by CHI without IAA or DNP.

#### Incorporation of $^3\text{H}$ -leucine into cellular and extracellular TCA precipitate

This series of experiments were done to 1) measure protein synthesis during incubation in SIS, 2) check the release of protein, 3) check if CHI inhibited protein synthesis and 4) attempt to determine if the released protein comes from newly synthesized protein or older synthesized protein.

Radioactive leucine was incorporated into cellular protein during

Table 14: The effect of CHI on the release of protein and A<sub>260</sub> material. Values give percent of control (without CHI). CHI present in the SIS incubation medium at 100ug/ml.

| Material<br>released      | Incubation time (hours) |    |    |    |
|---------------------------|-------------------------|----|----|----|
|                           | 0                       | 3  | 6  | 10 |
| Protein                   | 108                     | 93 | 97 | 89 |
| A <sub>260</sub> material | 91                      | 93 | 86 | 92 |

Table 15: The effect of CHI on  $\beta$ -glucosidase release in the presence of IAA and DNP. Activity released calculated as mU/ml in the cell-free supernatant after subtracting the initial value.

| Incubation                  |                 | Activity released<br>(mU/ml) |       | %<br>Difference |
|-----------------------------|-----------------|------------------------------|-------|-----------------|
| SIS + addition              | Time<br>(hours) | - CHI                        | + CHI |                 |
| None                        | 3               | 5.6                          | 3.3   | - 41            |
|                             | 5 1/2           | 10.3                         | 5.4   | - 48            |
| DNP ( $5 \times 10^{-5}$ M) | 3               | 10.9                         | 10.8  | - 1             |
|                             | 5 1/2           | 18.2                         | 18.7  | + 3             |
| IAA ( $10^{-4}$ M)          | 3               | 20.5                         | 17.8  | - 13            |
|                             | 5 1/2           | 32.3                         | 30.7  | - 5             |

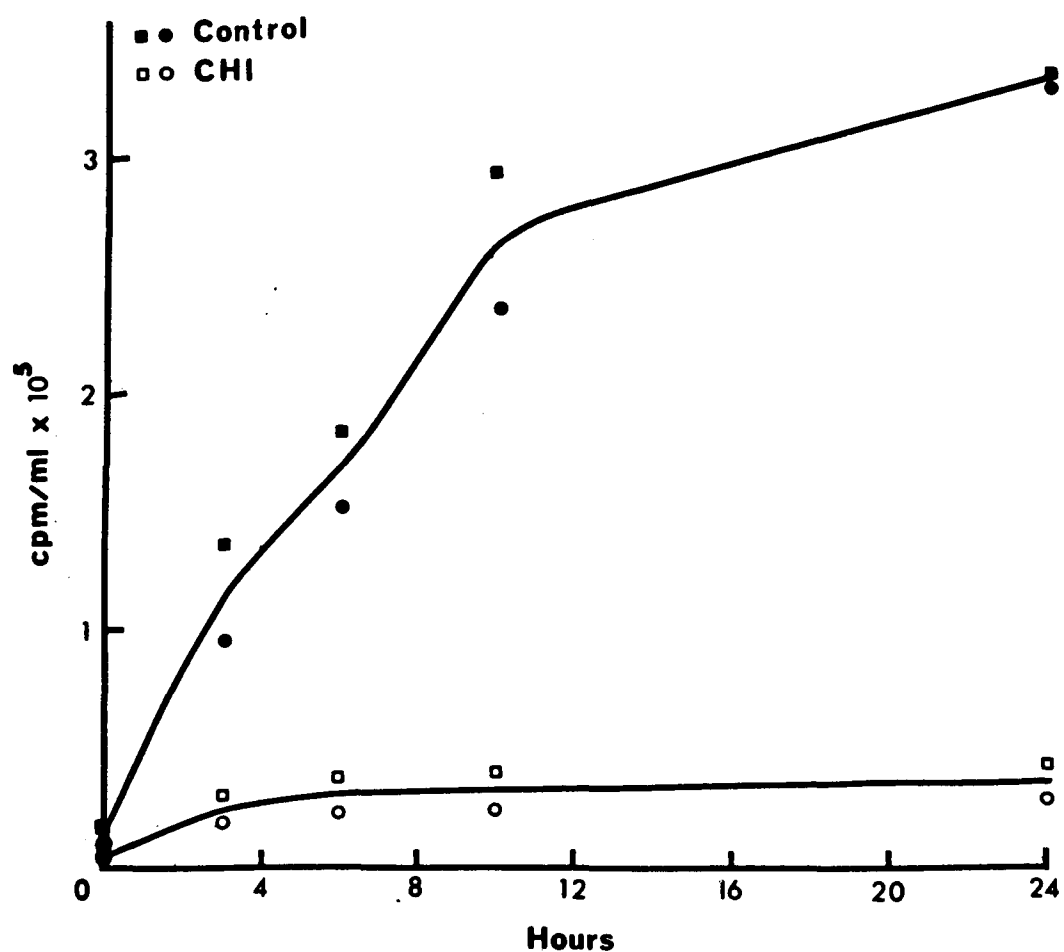


Figure 19. Incorporation of radioactivity into cellular protein with and without CHI. Results of two experiments (indicated by round and square symbols). CHI at 100 ug/ml (open symbols).

incubation in SIS, rapidly for about 10 hours and then continued at a slower rate between 10 and 24 hours (figure 19). Incubation of cells in the presence of CHI caused an apparent immediate reduction in incorporation and stopped further incorporation after 6 hours (figure 19).

There was very little incorporation of tritiated leucine into the extracellular TCA precipitate compared to cellular incorporation (figure 20). However, the small amount of incorporation was approximately linear for 24 hours. CHI nearly stopped incorporation after time zero.

The small incorporation of extracellular label and the low rate of appearance of precipitable label in unlabeled medium with pre-labeled cells (figure 21) shows that protein is released at a low rate during incubation in SIS.

If newly synthesized protein is the only source of released protein or enzyme then prelabeled cells should release relatively few counts when transferred to SIS containing cold leucine. Table 16 gives the extracellular precipitable cpm/ml of the media from post-zero time labeled cells, pre-zero time labeled cells and the ratio. A rapid increase in the ratio would indicate mostly newly synthesized protein was released. The results show a small ratio and only a small increase in the ratio after 3 hours. This indicates that newly synthesized and earlier synthesized protein were released. This suggestion is supported by counterindicating aspects of the

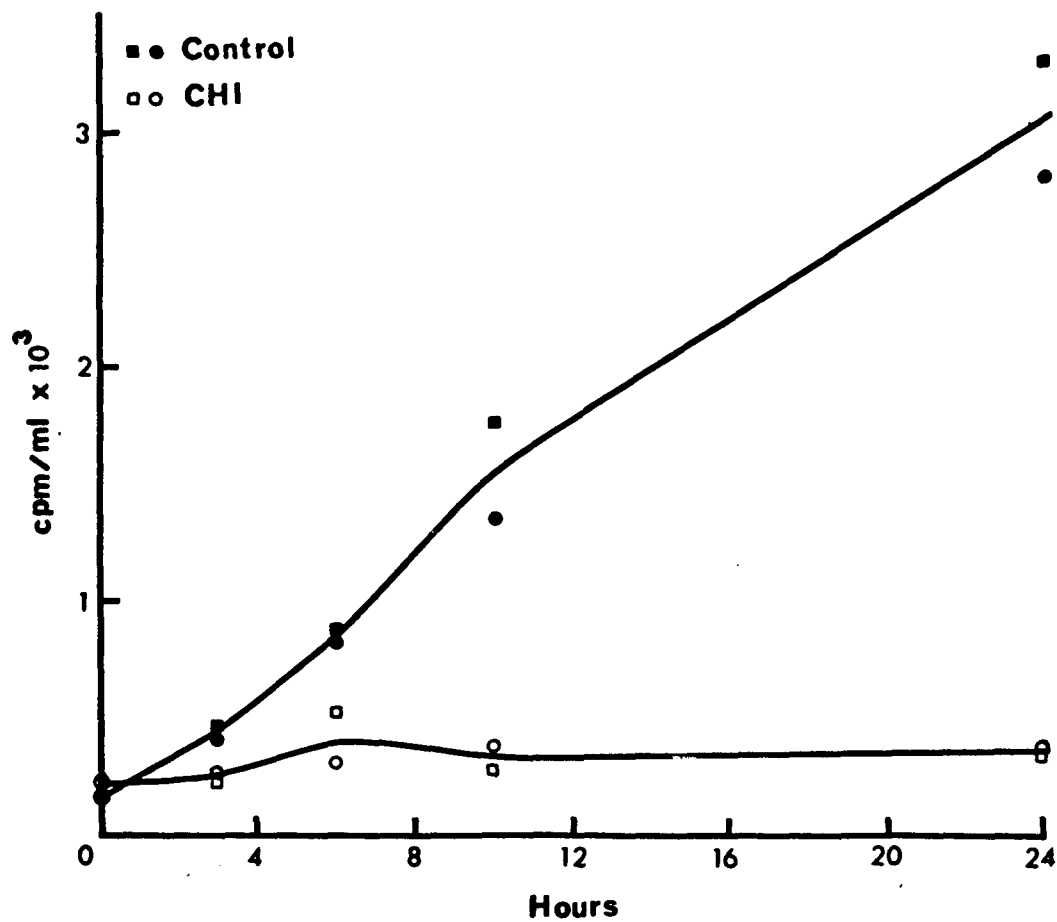


Figure 20. Incorporation of radioactivity into extracellular protein with and without CHI. Same experiments as shown in figure 19.

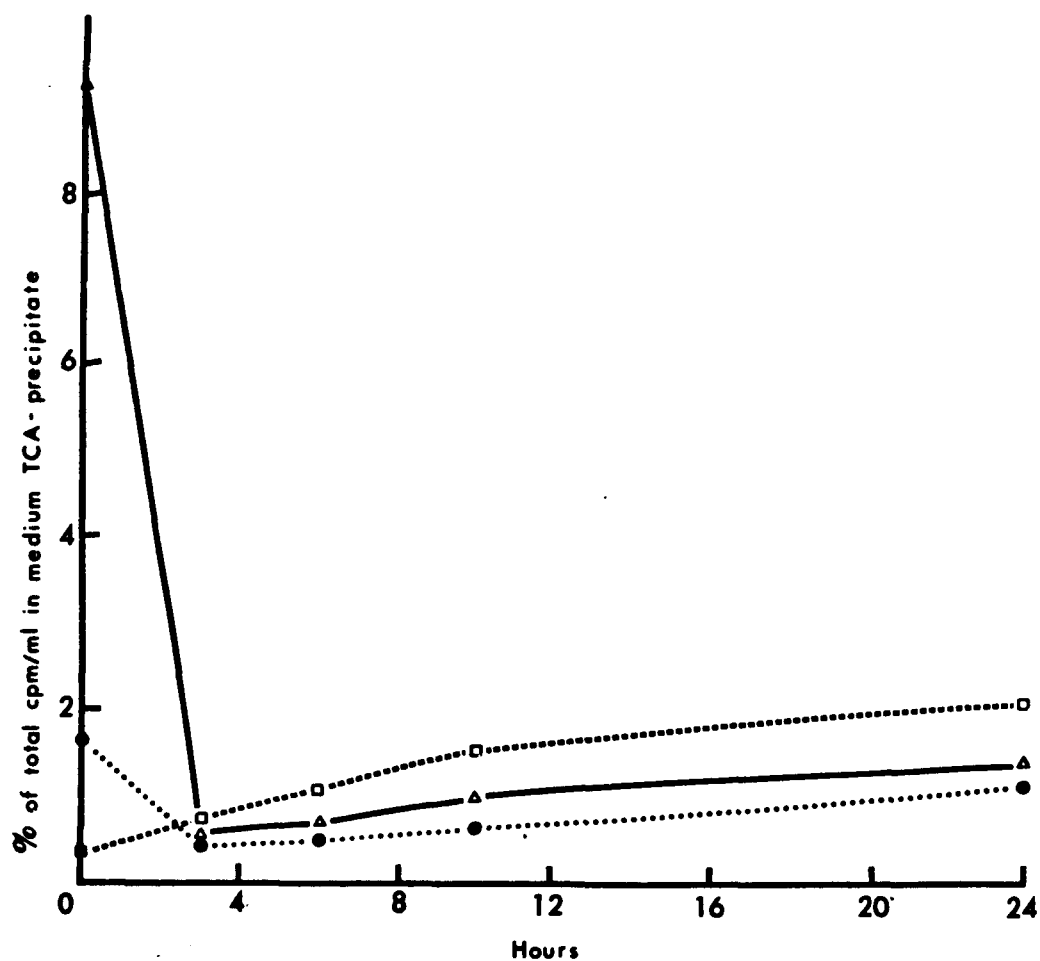


Figure 21. Amount of radioactivity in the extracellular protein, represented as percent of total incorporated radioactivity.

Conditions as described in Methods and in the legend of table 17.

Triangles, post-zero time labeled cells; squares, pre-zero time labeled cells; solid circles, no preincubation, cells suspended in label at zero time.

Table 16: Ratio of radioactivity in protein released from pre and post-zero time labeled cells. Cells were preincubated 3 hours in SIS with  $^3\text{H}$ -leucine (pre-zero time labeled) or SIS without leucine (post-zero time labeled), washed once and suspended at zero time in SIS with unlabeled leucine (pre-zero labeled) or SIS with  $^3\text{H}$ -leucine (post-zero labeled) for incubation and sampling as described in Methods and at times indicated.

| Incubation<br>time<br>(hours) | Radioactivity in<br>released protein<br>(cpm/ml) |                           | Ratio |
|-------------------------------|--------------------------------------------------|---------------------------|-------|
|                               | post-zero<br>labeled cells                       | pre-zero<br>labeled cells |       |
| 0                             | 298                                              | 544                       | 0.55  |
| 3                             | 397                                              | 1,087                     | 0.37  |
| 6                             | 940                                              | 1,550                     | 0.61  |
| 10                            | 1,482                                            | 1,971                     | 0.75  |
| 24                            | 3,359                                            | 2,836                     | 1.19  |

labeling experiments. First, the early increase in incorporated label in the medium when unlabeled cells ~~were~~ introduced into labeled medium indicates new, but not exclusively new, protein is released. Second, calculations of extracellular label as percent of total incorporation, plotted in figure 21, shows that after 3 hours the rate of increase in percent of extracellular incorporated label ~~was~~ similar for the pre-zero and post-zero time labeled cells. This indicates old, but not exclusively old, protein is released. (The high percentage of extracellular label at time zero for the cases in which cells are suspended in labeled medium at time zero (figure 21) is unexplained. Perhaps it is an artifact of  $^3\text{H}$ -leucine adsorbing to protein released from the cells by the washing/suspension shock.

#### $\beta$ -glucosidase release from cells with a low level of enzyme activity

Stock cultures of O. danica were inadvertently maintained for a period of time in subdued light. Inoculation of flasks from these cultures and grown at the standard 200 ft. cdl. resulted in 5 day cultures with markedly less  $\beta$ -glucosidase activity than previous or subsequent 5 day cultures inoculated from stock maintained at 200 ft. cdl. The release of enzyme from two of these low level cultures incubated in SIS was looked at and in table 17 compared with two standard level cultures. Although the cellular enzyme activity in the low level cultures was 66% below that of the standard level cultures, the rates of release calculated on a percent basis were remarkably similar. Thus, the level of enzyme in the cells did not seem to affect release in terms

Table 17:  $\beta$ -Glucosidase release from cells with different levels of enzyme activity.

| Cellular<br>activity<br>(mU/10 <sup>7</sup> cells) | Activity released (% of total) |      |      |      |      |
|----------------------------------------------------|--------------------------------|------|------|------|------|
|                                                    | Incubation time (hours)        |      |      |      |      |
|                                                    | 0                              | 3    | 6    | 10   | 24   |
| 53                                                 | 3.4                            | 9.4  | 13.7 | 18.4 | 36.5 |
| 18                                                 | 3.6                            | 10.5 | 14.5 | 19.4 | 32.5 |

of percent of total. This is consistent with the idea that extracellular  $\beta$ -glucosidase comes from an enzyme pool in common with intracellular enzyme.

#### Test for particle bound $\beta$ -glucosidase activity in the medium

To see if  $\beta$ -glucosidase was released from the cells in membrane bound vesicles or other sedimentable form, a set of experiments were conducted in which cells were incubated for 24 hours, the cells were then separated from the SIS medium at relatively low speed. The cell-free medium was then centrifuged at speeds at which lysosome like material would be expected to sediment. The results, given in table 18, show that centrifugation at high speed removed little or no  $\beta$ -glucosidase activity from the cell-free medium and that mannitol, for osmotic protection of vesicles, did not increase sedimentation of activity. These results indicate  $\beta$ -glucosidase was released in soluble form.

Table 18: Test for sedimentation of  $\beta$ -glucosidase activity in cell-free supernatant. Cells were incubated for 24 hours before separation from the media. Values represent activity remaining in solution after high speed centrifugation, as percent of that remaining after removing cells by centrifugation at 500 x g for 5 min. at 0-4<sup>0</sup>C.

| Wash              | Incubation        | % activity remaining<br>after centrifuging |                     |
|-------------------|-------------------|--------------------------------------------|---------------------|
|                   |                   | 12,100xg<br>10 min.                        | 23,300xg<br>20 min. |
| SIS               | SIS               | 98                                         |                     |
| SIS               | Mannitol          | 99                                         |                     |
| SIS               | Mannitol          | 100                                        |                     |
| SIS +<br>mannitol | SIS +<br>mannitol | 99                                         |                     |
| SIS +<br>mannitol | SIS +<br>mannitol | 96                                         | 102                 |

## DISCUSSION

### Released $\beta$ -glucosidase as an extracellular enzyme

This study shows in a variety of ways that  $\beta$ -glucosidase is released from intact undamaged O. danica cells. Pollock's<sup>1</sup> criteria for determining if an enzyme is truly extracellular have been satisfied for the released  $\beta$ -glucosidase.

The first criterion, that the enzyme must occur in the medium, separated from the cells, was demonstrated for  $\beta$ -glucosidase when cells were incubated in both growing and resting condition. This confirms the report of Shio,<sup>29</sup> who first found  $\beta$ -glucosidase in the growth medium.

Three conditions recommended by Pollock, to satisfy the second criterion, that appearance of the enzyme in the medium must not depend upon irreversible cell damage, are 1) enzyme release should be demonstrated when autolysis should be at a minimum (e. g. during log phase), 2) a substantial proportion of the total system should be found in the cell free medium, 3) tests of the cell free incubation medium for the presence of substances normally present within the cells should indicate the absence of gross cell damage during release.

The first condition was demonstrated by accumulation of  $\beta$ -glucosidase activity in the GM during the logarithmic phase of growth (figure 2). Contrary to what would be expected if release was the result of cell death due to culture ageing, the rate of increase

in percent of total activity found in the medium decreased during stationary and the beginning of the death phase (figure 2) and increased while the cellular level increased (figure 3). It might be possible, however, to account for the enzyme released by assuming that a portion of each generation spontaneously lysed. In actively growing cultures, lysis of some cells may be masked by growth of others<sup>1</sup>. Conceivably, the increase in generation time (table 1) could be the result of no change in the rate of cell division but a reflection of a continually increasing rate of lysis. This possibility seems remote, however, when considering the calculations (table 1) which indicate no increase in the proportion of cell lysis per absolute time and the high proportion of cell lysis needed to account for the released enzyme. Rogers<sup>63</sup> has suggested an organism could evolve in which a small proportion of the cell population would produce and, through controlled autolysis, liberate an enzyme into the medium. This possibility is highly unlikely<sup>1</sup> and an early, rapid increase in enzyme activity in the medium, similar to that shown here, was used as an argument against such a possibility in the case of extracellular  $\beta$ -lactamase from *E. coli*.<sup>64</sup>

Consideration of the data on the percent of enzyme released during growth (figure 2) and the amount of lysis needed to account for the released enzyme (table 1) shows that a substantial proportion of  $\beta$ -glucosidase is extracellular, which satisfies the second condition.

Although tests of the cell-free incubation medium (SIS) were not

negative for protein,  $A_{260}$  material and phosphoglucose isomerase, their relatively low rate of release compared to  $\beta$ -glucosidase, provides substantial evidence that the majority of the released  $\beta$ -glucosidase is the result of a selective release process in the absence of cell damage, in agreement with the third condition.

On the basis of a low rate of protein release with a resultant high and increasing specific activity of enzymes in the medium, Muller<sup>38</sup> reported that enzyme release in Tetrahymena is a specific process, not due to disintegration of cells. Similarly for O. danica a low rate of protein release was shown by protein determination (figure 4) and a low release rate of radioactive protein from pre-labeled cells (figure 21). Also the data on specific activity of  $\beta$ -glucosidase (figure 8) showed a higher and increasing specific activity in the medium.

Although release of UV absorbing material has frequently been used as an index of cell permeability and lysis (e. g. 65, 66, 67, 68, 69, 70), release of UV absorbing material is not necessarily associated with cell lysis.  $A_{260}$  material was found to accompany the release of extracellular enzymes from yeast without release of intracellular enzymes during osmotic shock.<sup>68</sup> Induced leakage of UV material from fungi by low concentrations of triterpeneglycosides did not destroy growth capacity.<sup>69</sup> Although  $A_{260}$  material was released from O. danica in this study, it appeared to be a normal process, since it was released in all media, including GM (data not

given), and since release increased with temperature (figure 13). The value of the  $A_{260}$  data is that the rate of release changed relative to  $\beta$ -glucosidase release (figure 10) and in contrast to  $\beta$ -glucosidase release was virtually unaffected by CHI (table 14). Thus release of  $\beta$ -glucosidase and  $A_{260}$  did not run parallel, indicating  $\beta$ -glucosidase release is not dependent on non-specific cell leakiness.

To my knowledge the use of cytoplasmic enzymes as markers of cell lysis has not been previously reported in observations of enzyme release from phytoflagellates or other protozoa or algae. LDH has been used as an intracellular marker in studies of lysosomal enzyme release from leukocytes.<sup>11, 37, 39, 71, 72</sup> Some of these reports show from 5% to 10% of the total LDH was released within the first hour of incubation.<sup>37, 39, 71</sup> This release of marker enzyme is comparable to the release of phosphoglucose isomerase in this report (figure 11). The low rate of release of phosphoglucose isomerase compared to  $\beta$ -glucosidase clearly indicates selective  $\beta$ -glucosidase release. It should be pointed out that the release of phosphoglucose isomerase was very similar to the release of protein at the same SIS pH (figure 11); this would be expected if both substances can serve as markers of lysis or leakage.

Determination of lysis or leakage by the release of marker substances is highly dependent on the stability of the marker. If the marker is degraded upon release, lysis would be underestimated. I am unaware of any reports where the stability of marker substances

was determined. Proteinase release has been reported for Tetrahymena (see 55). Rothstein and Blum<sup>73</sup> in further studies on enzyme release from Tetrahymena reported proteinase degradation of extracellular hydrolase activity and applied correction factors to the release rates of the enzymes, but degradation of extracellular protein was not studied. Proteinase activity has been found in the culture medium of O. danica.<sup>29</sup> It is thus conceivable that the apparent low rate of protein release and increase in external  $\beta$ -glucosidase specific activity were artifacts of protein breakdown. Since extracellular  $\beta$ -glucosidase activity was stable (figure 7) this possible artifact could only be the result of selective breakdown of protein other than  $\beta$ -glucosidase. The selective breakdown of protein is made plausible when one considers that most extracellular protein and lysosomal hydrolases described to date are glycoproteins<sup>74, 75, 76</sup> which appear to be more resistant to degradation than other proteins.<sup>75, 76, 77</sup> However, the stability of the extracellular protein (figure 7) rules out the possibility that the low rate of protein release is an artifact. The stability of phosphoglucose isomerase activity in the presence of intact metabolizing cells, a feature Pollock<sup>1</sup> considered important for a marker substance, coupled with an observed stability of  $A_{260}$  material in the incubation medium (data not given) and the aforementioned stability of protein, all serve to indicate that there was little or no undetected marker substances released into the medium in this study.

In addition to these studies which satisfy Pollock's criteria, other

features of the release process investigated substantiate the thesis that  $\beta$ -glucosidase in the medium is a truly extracellular enzyme.

One of these additional factors is the near linearity of  $\beta$ -glucosidase release. Although there is an initial period of 2-3 hours in which  $\beta$ -glucosidase release is slightly greater than later, which can be attributed to a shock effect as indicated by a somewhat greater release of marker substances during this period, after this period  $\beta$ -glucosidase release is quite linear. If release was only the result of cell damage caused by a washing and suspension shock then release would be expected to diminish substantially with time. On the other-hand, if release was the result of an increasing loss of cell integrity caused by starvation or some property of the ageing incubation medium, then release should increase with time. It seems highly unlikely that both initial shock and later loss of viability would be coordinated to maintain a relatively linear release.

The marked reduction of  $\beta$ -glucosidase release under dark anaerobic conditions indicated release was energy dependent, a feature that would clearly indicate release by a specific process.

The results showing temperature dependence of  $\beta$ -glucosidase release support the thesis that release is from intact cells. The  $Q_{10}$  for many chemical reactions is about 2, whereas, for most physical processes the  $Q_{10}$  is less than 1.5.<sup>78</sup> This is consistent with interpreting the temperature effects on  $\beta$ -glucosidase release as indicating that in the temperature range 5-25<sup>0</sup> ( $Q_{10}$  of 2.4), release

was a biological process, while release in the range 25-37<sup>0</sup> ( $Q_{10}$  of 1.2) was the result of a physical process, such as diffusion. A  $Q_{10}$  of 1.4 was reported for movement of  $\alpha$ -amylase through the cell wall of barley aleurone.<sup>79</sup> Similarly it can be argued that the activation energy of 15.2 kcal in the 5-25<sup>0</sup> range, which is slightly above the 12 kcal typical for enzymatic processes<sup>106</sup>, indicates a biological process, while the 25-37<sup>0</sup> activation energy of 4.5 kcal is less likely to represent a biological process. Secretion of alkaline phosphatase in Micrococcus sodonensis was reported to have an activation energy of 55 kcal.<sup>84</sup> Enzyme activities and other biological processes show a wide range of values for  $Q_{10}$  and activation energy and may show decreasing values with increasing temperature.<sup>78</sup> Therefore, the real significance of the effect of temperature on enzyme release may not be the absolute values for  $Q_{10}$  and activation energy as much as the change in these values at the higher temperature range. It seems unlikely that  $\beta$ -glucosidase release by cell lysis or leakage would show a reduction in  $Q_{10}$  or activation energy with increasing temperature. That release at 37<sup>0</sup> is in part due to cell damage and not a biological process is supported by the increase in phosphoglucose isomerase release at 10 hours (figure 13) and by the likelihood that incubation at 37<sup>0</sup> is somewhat damaging since the related organism, O. malhamensis does not normally grow at temperatures above 35<sup>0</sup>.<sup>80</sup>

Perhaps the most convincing argument for secretion of  $\beta$ -glucosidase is the observation of the effect of CHI. This clearly

shows that CHI stops the synthesis of  $\beta$ -glucosidase (figure 18) and protein (figure 19) and release of  $\beta$ -glucosidase (figure 17) and protein (figure 20). If release was the result of cell damage then it would seem reasonable to expect release to continue in the presence of CHI at approximately the same rate as without CHI until the cellular  $\beta$ -glucosidase pool was significantly reduced. The rapid and nearly complete cessation of  $\beta$ -glucosidase release in the presence of CHI, before significant reduction in the cellular level of enzyme, clearly indicates cell damage is not the source of released enzyme. The only possible alternative which would allow cell damage as the explanation, is to assume that CHI in addition to stopping protein synthesis also stopped the cell lysis or leakage that was taking place without CHI. This unlikely explanation is ruled out by the data showing that CHI did not appreciably effect the enhanced release caused by cell damage during treatment with metabolic inhibitors, while CHI significantly reduced release in the control (table 15).

#### Characteristics of $\beta$ -glucosidase release

This study also provides information of possible interest with regard to factors and conditions that might be expected to influence the release of an extracellular enzyme.

The experiments on the effect of the ionic composition of the incubation solution on  $\beta$ -glucosidase release were preliminary. The results do indicate that omission of calcium and magnesium caused

enhanced release of  $\beta$ -glucosidase. It is likely that this enhancement is the result of increased leakage. This interpretation is supported by a corresponding enhancement in the release of  $A_{260}$  material and protein. That calcium or magnesium may be important for maintenance of stable non-leaky cells is not unexpected in view of the importance of these ions in other systems. The effect of lack of external calcium includes alteration of cell permeability and disruption of intracellular systems or leakage of intracellular constituents.<sup>80</sup> Magnesium was found important in maintaining impermeability of the surface membrane of 3T3 mouse cells.<sup>82</sup> A decrease in calcium concentration in the medium of bone cultures stimulated the release of  $\beta$ -glucuronidase.<sup>7</sup> On the other hand, omission of calcium or magnesium in some systems reduces release or secretion of enzymes.<sup>13, 81, 83, 84</sup> My results which show a slight reduction in release without calcium but with the presence of magnesium (table 3), may indicate a calcium requirement for selective release of  $\beta$ -glucosidase, however, the results are equivocal and require further investigation.

There is evidence that some energy for  $\beta$ -glucosidase release was provided by photosynthesis. This is suggested by the reduction in release under dark aerobic conditions compared to light aerobic conditions (figure 12) and the fact that release under light anaerobic conditions was not reduced to the same extent as dark anaerobic conditions. There is no evidence to indicate if photophosphorylation provides energy or if photosynthesis provides oxygen for oxidative

phosphorylation. Since under dark anaerobic conditions release continued up to 24 hours (data not shown), it seems that glycolysis can also provide energy for selective release. In line with this, Bauduin et al.<sup>85</sup> found the anaerobiosis inhibited, but not completely, induced secretion in rat pancreas and increased the role of glycolysis in providing the tissue energy supply by a factor of 3.

Although energy dependence of secretion in other systems has been shown by the inhibitory effects of metabolic inhibitors, there are examples in the literature of effects similar to the loss of cell integrity caused by metabolic inhibitors in this study. Schramm's laboratory<sup>86</sup> reported that although DNP and anaerobic conditions inhibited secretion of amylase from parotid slices, oxidative phosphorylation was required to maintain the amylase within the zymogen granules when the cells were not actively secreting. Vaes<sup>7</sup> observed that IAA stimulated the release of  $\beta$ -glucuronidase from bone cells and attributed this to a toxic effect. Treatment of pancreas with sodium oxamate (a glycolytic inhibitor) resulted in an increased spontaneous release of the chymotrypsinogen to 200% of the control.<sup>85</sup>

A dependence on new enzyme synthesis for  $\beta$ -glucosidase release is indicated by the inhibitory effect on release by CHI. The same effect of CHI on release of acid phosphatase,<sup>87</sup> invertase<sup>88</sup> and  $\beta$ -glucanase<sup>48</sup> in yeast has been interpreted as proof that the release is a result of de novo synthesis.<sup>48, 87</sup>  $\beta$ -glucosidase

synthesis during release, in this report, was evident during the first 10 hours when total  $\beta$ -glucosidase activity of the system increased, however, between 10 and 24 hours, loss of activity from the cells was approximately paralleled by its appearance in the medium, resulting in little or no increase in total activity. Results somewhat similar to these two different phases of total enzyme level have been observed in Tetrahymena. Muller<sup>38</sup> found that total  $\beta$ -glucosidase increased slightly and total  $\alpha$ -glucosidase activity declined during the 5 hours in which Tetrahymena were incubated in a dilute salt solution; at the same time both enzymes were accumulating in the medium. Rothstein and Blum<sup>73</sup> found, in contrast, that the total  $\alpha$ -glucosidase activity increased under the same conditions. Thus in Tetrahymena and in O. danica enzyme release occurs with and without increasing total enzyme activity (assumed net synthesis). Therefore, to assume that enzyme synthesis is taking place to support release, while total enzyme activity remains the same or declines, it must also be assumed there is enzyme degradation while synthesis is taking place. That this may be so in O. danica is indicated by a decline in total activity in the presence of CHI (figure 18) in contrast to the control.

If the correct interpretation of the inhibitory effect of CHI is that it stops release by stopping synthesis of new  $\beta$ -glucosidase required for release, then two possibilities present themselves.

- 1) Most of the internal  $\beta$ -glucosidase is not available for release

and thus is in a separate pool from the releasable enzyme. 2) There is one common pool but synthesis is required to maintain a critical pool size for release to occur. A single  $\beta$ -glucosidase pool is indicated by the results showing that the same proportional amount of enzyme (% of total) is released at two different levels of cellular enzyme activity (table 17); it seems highly unlikely that a cellular pool and a pool for release would be reduced exactly proportionately. In further support of a one pool hypothesis are the results of the radioactive experiments which indicate that old and newly synthesized protein are released. Thus, a single enzyme pool is likely. However, it also seems likely from the previous discussion on enzyme synthesis and turnover that release can occur with a reduced pool size, which would rule out the critical enzyme pool size hypothesis. Thus a more attractive interpretation of the effect of CHI is that it stops the synthesis of some factor, such as a protein, that is required for the selective release mechanism, and not that it inhibits release by stopping de novo enzyme synthesis.

Although this study has shown that  $\beta$ -glucosidase is released by a selective process, non-selective release (lysis/leakage) does account for a portion of the released enzyme, as indicated by release of a small amount of marker substances. The non-selective component of the released  $\beta$ -glucosidase can be estimated, for a given time, based on the release of phosphoglucose isomerase. For example at 24 hours ca. 10% of the total isomerase is in the medium,

which would be 11% of the cellular isomerase. Assuming a comparable portion of  $\beta$ -glucosidase is released by the same non-selective process, then 11% of the cellular  $\beta$ -glucosidase would represent the non-selective component of the extracellular  $\beta$ -glucosidase. At 24 hours this comes to ca. 20% of the extracellular  $\beta$ -glucosidase. However, there is reason to believe this is an overestimate of non-selective release. The 10% estimate of lysis/leakage based on phosphoglucose isomerase is artificially high because 1) there was a drop in total isomerase activity over the 24 hour period 2) the 10% includes the accumulation of isomerase during the first 3 hours when release of markers was higher than normal because of the shock period as previously discussed.

#### Mechanism of $\beta$ -glucosidase release

Since the bulk of the extracellular  $\beta$ -glucosidase is not the result of non-selective release, the question arises as to the mechanism that accounts for the selective release.

Three possible types of release mechanism could be operating in O. danica. 1) Transport in vesicles traversing the plasma membrane, 2) Direct but selective transport across the plasma membrane, 3) A form of reverse endocytosis (exocytosis).

Extracellular  $\beta$ -glucosidase could be derived from whole vesicles or lysosomes that become external by exocytosis or blebbing. Heyn<sup>89</sup> has described EM observations of Avena coleoptiles that indicate sacs, containing vesicles and/or lysosomes, fuse with the plasma membrane and expel the vesicles by a type of exocytosis. Blebbing of the surface

of O. danica has been reported; EM micrographs reveal surface projections of various sizes containing cytoplasm, portions of organelles, myelin like figures, and vesicles.<sup>90, 28</sup> Examination of a high-speed centrifugate derived from cell-free culture medium revealed vesicles of various sizes, which suggested the blebs pinch off and separate from the cell.<sup>90</sup> The results presented here (tabel 18) show that extracellular  $\beta$ -glucosidase was not in a sedimentable form clearly indicating that external vesicles were not the source of enzyme. This is consistent with the report by Shio<sup>29</sup> that only about 10% of the extracellular hydrolase activity was associated with the sediment after high speed centrifugation of O. danica culture media at various culture ages.

Direct transport of enzyme across the plasma membrane could be like that in bacteria or by selective diffusion. In bacterial enzyme secretion it is generally held that secreted enzymes are formed by membrane-bound polysomes and transferred by a vectorial process into and through the plasmalemma.<sup>32</sup> Indirect evidence for secretion of amylase through the intact cell membrane of pigeon pancreas was reported,<sup>91</sup> however, the evidence was meager and no reports in support of this view have appeared. The present study provides no evidence that  $\beta$ -glucosidase is transported directly across the membrane. It seems unlikely that O. danica would have a procaryotic type mechanism, and it also seems unlikely that diffusion would be selective.

The mechanism of exocytosis involves the discharge of the contents of a membrane bound organelle to the outside of the cell

after fusion of the organelle membrane with the plasma membrane; subsequently the organelle membrane becomes part of the plasma membrane. A feature of this mechanism is the simultaneous release of all soluble unbound contents of the organelle. Thus, parallel release of more than one acid hydrolase would imply exocytosis of lysosomes. This mechanism seems the most likely for  $\beta$ -glucosidase release and is supported by the following observations in this laboratory and reports in the literature.

That acid phosphatase is released in parallel with  $\beta$ -glucosidase in O. danica during incubation in SIS is suggested by unpublished work in this laboratory by Dr. Patni (personal communications) and during this study (data not shown). In addition, Shio<sup>29</sup> found that  $\beta$ -glucosidase and acid phosphatase release was similar in growing cultures.

Evidence for lysosomes in O. danica is indicated by ultra-structure observations in this laboratory (Dr. Aaronson, personal communications). Cytochemical<sup>92</sup> and biochemical<sup>93</sup> evidence for lysosomes has been reported for the related O. malhamensis.

Exocytosis is the mechanism of enzyme secretion in eucaryotes (see 94, 95). Triggering of this mechanism varies with cell type and conditions. Mammalian secretory tissue respond to chemical agents, for example pancreas cells respond to catecholamines and release large quantities of zymogen granule contents. Leukocytes, on the other hand, respond to a phagocytic challenge, with slight differences in mechanism depending on the nature of the challenge. If the challenge

is capable of being incorporated, "regurgitation during feeding" takes place.<sup>39</sup> In this process lysosomes join with the invaginating phagosomes and leak their contents to the outside before the phagosomes have sealed at the cell surface. If the challenge is a non-phagocytosable surface, phagosomes do not develop but the lysosomes fuse with the surface membrane (exocytosis).<sup>39</sup> Release of  $\beta$ -glucosidase is not a phagocytic response, since O. danica were incubated in a dilute salt solution without a phagocytic challenge. Although autophagy and cannibalism are possible, the extent and consistency of release seems to rule this out. It has been reported that a phagocytic challenge failed to effect release in Tetrahymena.<sup>38, 73</sup>

Unstimulated secretion of protein has been reported for fasting rat pancreas and it was suggested to result from "fortuitous contacts of young secretory granules with the apical cell membrane."<sup>96</sup> It is also pertinent to note that in pancreas stimulated secretion is not inhibited by CHI,<sup>96, 97</sup> while one phase of unstimulated protein secretion is.<sup>96</sup> From these considerations and the previously discussed effects of CHI on O. danica one might suggest that  $\beta$ -glucosidase release involved lysosomal enzyme release by a mechanism like unstimulated secretion in pancreas.

#### Biological role of released $\beta$ -glucosidase

Since this study has shown that release of  $\beta$ -glucosidase is a specific process probably requiring energy and since energy would certainly be required for the synthesis of the released enzyme, it

seems logical to assume that selection would have eliminated selective release of enzyme if the released enzyme had no beneficial biological function.

From the discussion it seems reasonable to assume that several lysosomal acid hydrolases are released as a group, therefore, it is likely that the role of these enzymes is related in some biological way.

The role that comes immediately to mind is that of extracellular digestion to obtain soluble nutrients. The attractiveness of this hypothesis, however, is diminished by a consideration that these enzymes released in the natural habitat would be so diluted as to make them ineffective.<sup>38</sup> Circumstances can, however, be imagined where this could be the role, such as when a population is very dense the accumulation of hydrolases could facilitate the breakdown of dead cells and perhaps be of considerable survival value by providing for cryptic growth.

Aaronson<sup>98</sup> has described particle aggregation by O. danica and suggested that the cell surface is adhesive and serves as a trap for picking up substances for possible endocytosis. It may be that hydrolases on the cell surface have a role in modifying this material for uptake, initiating digestion or detoxification of substances or organisms. Considering the exocytosis mechanism of secretion and the same mechanism for deposit of cell surface material such as cell walls,<sup>99, 4</sup> it seems reasonable to suggest that the role of these hydrolases is on the cell surface, while viewing the loss of the enzymes

from the surface into the media as a price to pay for some essential function at the surface. It has been reported that surface-located glycosidase, for example, are of common occurrence in micro-organisms and higher animals and plants.<sup>100</sup>

An intriguing possibility is that the hydrolases may modify the cell surface and assist in the regulation of endocytosis, taxis, intercellular communication and growth by hydrolyzing active or blocking substances bound to receptor sites on the surface. Evidence has been reported for galactosyl transferases and galactosyl acceptors on the surface of cultured animal cells<sup>101, 102</sup> and it was suggested they might have a role in intercellular communication. Poste<sup>103, 104</sup> has demonstrated that release of enzymes by lysosomal activation in animal cells in culture by various agents led to digestion of the cell surface coat but not cell death. Berger<sup>105</sup> has shown that protease treatment of normal cells can bring about a change in the cell surface and cause the initiation of cell growth similar to that of transformed cells.

Evidence is accumulating that release of lysosomal hydrolases is part of the lysosomal function in normal cells.<sup>2, 35, 104</sup> Demonstration, here, of release of an acid hydrolase from O. danica by a selective non-lethal mechanism, perhaps can be looked at as the addition of a representative of the phytoflagellates to an expanding range of organisms exhibiting this phenomenon, which may eventually be considered universal.

## REFERENCES

1. Pollock, M. R., in "The Bacteria," (Gunsalus and Stanier eds.), Vol. IV p. 121. Academic Press, New York (1962).
2. Dingle, J. T., in "Lysosomes in Biology and Pathology," (Dingle and Fell eds.), Vol. II p. 421. North-Holland, New York (1969).
3. Gentile, A. C., in "Tissue Culture," (C. V. Ramakrishnan ed.), p. 358. Junk (1965).
4. Northcote, D. H., Proc. Roy. Soc. 173, 21 (1969).
5. Van Huystee, R. B., and Turcon, G., Can. J. Bot. 51, 1169 (1973).
6. Olson, A. C., Evans, J. J., and Jansen, E. F., Plant Physiol. 44, 1594 (1969).
7. Vaes, G., J. Cell Biol. 39, 676 (1968).
8. Glick, J. L., Goldberg, S. R., and Pardee, A. B., Cancer Res. 26, 1774 (1966).
9. Chrispeels, M. J., Planta 108, 283 (1972).
10. Clutterbuck, V. J., and Briggs, D. E., Phytochemistry. 12, 537 (1973).
11. Weissmann, G., Dukor, P., and Zurier, R. B., Nature New Biology 231, 131 (1971).
12. Karlsson, B. W., and Carlsson, E. I., Life Sciences Pt. II 9, 291 (1970).
13. Stewart, J. E., and Schotz, M. C., J. Biol. Chem. 246, 5749 (1971).
14. Greenwald, R. A., and Sajdera, S. W., Proc. Soc. Exp. Biol. Med. 142 924 (1973).
15. Muller, M., in "Protozoa," (G. W. Kidder ed.), Vol. I p. 351. Academic Press, New York (1967).
16. Kuenzler, E. J., and Perras, J. P., Biol. Bull. 128, 271 (1965).
17. Belmont, L., and Miller, D. A., J. Exp. Bot. 16, 318 (1965).

18. Lewin, J. C., and Lewin, R. A., Can. J. Microbiol. 6, 127 (1960).
19. Goldschmidt, H., Chemiker - Ztg 83, 442 (1959).
20. Overbeck, J., and Babenzien, H. D., Natur wissenschaften 50, 571 (1964).
21. Perry, M. J., Mar. Biol. 15, 113 (1972).
22. Berman, T., Nature 224, 1231 (1969).
23. Reichardt, W., Overbeck, J., and Steubing L., Nature 216, 1345 (1967).
24. Pringsheim, E. G., J. Micros. Sci. 93, 71 (1952).
25. Provasoli, L., Ann. Rev. Microbiol. 12, 279 (1958).
26. Kuenzler, E. J., J. Phycol. 1, 156 (1965).
27. Strickland, J. D. H., and Solorzano, L., in "Some Contemporary Studies in Marine Science," p. 665. (1966).
28. Aaronson, S., Limnol. Oceanog. 16, 1 (1971).
29. Shio, H., M. A. Thesis, Queens College of CUNY (1971).
30. Wallach, D., and Schramm, M., Eur. J. Biochem. 21, 433 (1971).
31. Jamieson, J. D., and Palade, G. E., J. Cell Biol. 50, 135 (1971).
32. Lampen, J. O., Biotechnol. & Bioeng. Symp. No. 3, 37 (1972).
33. Brown, A. M. and Gascoigna, J. A., Nature 187, 1010 (1960).
34. Weinshilboum, R. M., Nguyen, B. Thoa, Johnson, D. G., Kopin, I. J., and Axelrod, J., Science 174, 1349 (1971).
35. Weiss, L., "The Cell Periphery Metastasis and other Contact Phenomena," North - Holland, Amsterdam (1967).
36. Poste, G., Exp. Cell Res. 67, 11 (1971).
37. Wright, Daniel G., and Malawista, S. E., J. Cell Biol. 53, 788 (1972).

38. Muller, M., J. Cell Biol. 52, 478 (1972).
39. Zurier, R. B., Hoffstein, S., and Weismann, G., J. Cell Biol. 58, 27 (1973).
40. Aaronson, S., in "Lysosomes in Biology and Pathology," (J. T. Dingle ed.), Vol. 3 p. 18. North - Holland, Amsterdam (1973).
41. Chang, Y., Sci. 161, 57 (1968).
42. Pannbacker, R. G., and Bravard, L. J., Science 175, 1014 (1972).
43. Chassy, B. M., Science 175, 1016 (1972).
44. Layman, D. L., and Ross, R., Arch. Biochem. Biophys. 157, 451 (1973).
45. Aaronson, S., and Baker, H., J. Protozool. 6, 282 (1959).
46. Allen, M. B. Ann. Rev. Microbiol. 23, 29 (1969).
47. Barman, T. E., in "Enzyme Handbook," Vol. II p. 578. Springer - Verlag, New York (1969).
48. Biely, P., Farkas, V., and Bauer, S., FEBS Letters 23, 153 (1972).
49. Beck, C., and Tappel, A. L., Biochim. Biophys. Acta 151, 159 (1968).
50. McIlvaine, T. C., J. Biol. Chem. 49, 183 (1921).
51. Wattieux, R., and de Duve C., J. Biochem. 63, (1956).
52. Aronson, N. N., and de Duve C., J. Biol. Chem. 243, 4564 (1968).
53. Reithel, F. J., in "Methods in Enzymology," (Colowick and Kaplan eds.), Vol. IX p. 565. Academic Press, New York (1966).
54. Bessey, O. A., Lowry, O. H., and Brock, M. J., J. Biol. Chem. 164, 321 (1946).
55. Muller, M., J. Protozool. 16, 428 (1969).
56. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. 193, 265 (1951).

57. Hartree, E. F., *Anal. Biochem.* 48, 422 (1972).
58. Rosenthal, H. L., and Sobieszczanska, W. A., *Anal. Biochem.* 34, 591 (1970).
59. Vallejo, C. G., and Lagunas, R., *Anal. Biochem.* 36, 207 (1970).
60. Potty, V. H., *Anal. Biochem.* 29, 535 (1969).
61. Gregory, J. D., and Sajdera, S. W., *Science* 169, 97 (1970).
62. Edwards, N. A., and Hassell, K. A., "Cellular Biochemistry and Physiology," McGraw-Hill, London (1971).
63. Rogers, H. J., in "The Bacteria," (I. C. Gunsalus and R. Y. Stanier eds.), Vol. II p. 257 Academic Press, New York (1961).
64. Tanco, J. M., del Pilar Miravalles, M., and Arcos, J. M., *Arch. Microbiol.* 72, 16 (1970).
65. Neu, H. C., and Heppel, L. A., *J. Biol. Chem.* 239, 3893 (1964).
66. Thomas, T. D., Lyttleton, P., Williamson, K. I., and Batt, R. D. *J. Gen. Microbiol.* 58, 381 (1969).
67. Drew, M. C., and McLaren, A. D., *Physiol. Plant.* 23, 544 (1970).
68. Schwencke, Farias, G., and Rojas, M., *Eur. J. Biochem.* 21, 137 (1971).
69. Olsen, A., *Physiol. Plant.* 24, 534 (1971).
70. Bigger, C. H., White, M. R., and Braymer, H. D., *J. Gen. Microbiol.* 71, 159 (1972).
71. Zurier, R. B., Hoffstein, and Weissmann, G., *Proc. Nat. Acad. Sci. USA* 70, 844 (1973).
72. Davies, P., Allison, A. C., and Haswell A. D., *J. Biochem.* 134, 33 (1973).
73. Rothstein, T. L., and Blum, J. J., *J. Cell Biol.* 57, 630 (1973).
74. Lampen, J. O., *Antonie van leeuwenhoek* 34, 1 (1968).
75. Goldstone, A., and Koenig, H., *Life Sci.* 9, 1341 (1970).

76. Schanel, L., Blaich, R., and Esser, K., Arch. Mikrobiol. 77, 140 (1971).
77. Strumeyer, D. H., and Malin M. J., Biochem. J. 118, 899 (1970).
78. Trumbore, R. H., "The Cell Chemistry and Function," C. V. Mosby, Saint Louis (1966).
79. Varner, J. E., and Mense, R. M., Plant Physiol. 49, 187 (1972).
80. Hutner, S. H., Baker, H., Aaronson, S., Nathan, H. A., Rodriguez, E., Lockwood, S., Sonders, M., and Peterson R. A., J. Protozool. 4, 259 (1957).
81. Rasmussen, H., Science 170, 404 (1970).
82. Culp, L. A., and Black, P. H., Biochemistry 11, 2161 (1972).
83. Lockwood, D. H., and Williams-Ashman H. G., J. Cell Physiol. 77, 7 (1971).
84. Glew, R. H., and Heath, E. C., J. Biol. Chem. 246, 1566 (1971).
85. Bauduin, H., Colin, M., and Dumont, J. E., Biochim. Biophys. Acta 174, 722 (1969).
86. Babad, H., Ben-zvi, R., Bdolah, A., and Schramm, M., Eur. J. Biochem. 1, 96 (1967).
87. Van Rijn, H. J. M., Boer, P., and Steyn-Parve, E. P., Biochim. Biophys. Acta. 268, 431 (1972).
88. Liras, P., and Gascon, S., Eur. J. Biochem. 23, 160 (1971).
89. Heyn, A. N. J. Ultrastruc. Res. 37, 69 (1971).
90. Aaronson, S., Behrens, U., Orner, R., and Haines, T. H., J. Ultrastruc. Res. 35, 418 (1971).
91. Dandrifosse, G. Comp. Biochem. Physio. 34, 229 (1970).
92. Stoltze, H. J., Lui, N. S. T., Anderson, O. R., and Roels, O. A., J. Cell Biol. 43, 396 (1969).
93. Lui, N. S. T., Roels, O. S., Trout, M. E., and Anderson, O. R., J. Protozool. 15, 536 (1968).
94. Abrahams, S. J., and Holtzman, E., J. Cell. Biol. 56, 540 (1973).

95. Helminen, H. J., and Ericsson, J. L. E., J. Ultrastruc. Res. 33, (1970).
96. Kramer, M. F., and Poort, C., J. Cell. Biol. 52, 147 (1972).
97. Jamieson, J. D. and Palade, G. E., J. Cell. Biol. 48, 503 (1971).
98. Aaronson, S., Arch. Mikrobiol. 92, 39 (1973).
99. Whaley, W. G., Dauwalder, M., and Kephart, J. E., Science 175, 596 (1972).
100. Kliss, F. M., Physiol. Plant. 25, 253 (1971).
101. Roth, S., McGuire, E. J., and Roseman, S., J. Cell Biol. 51, 536 (1971).
102. Roth, S., and White, D., Proc. Nat. Acad. Sci. 69, 485 (1972).
103. Poste, G., Microbios. 3, 105 (1971).
104. Poste, G., Exp. Cell Res. 67, 11 (1971).
105. Burger, M. M., Nature 227, 170 (1970).
106. Pollard, E. C., Advan. Chem. Phys., 7, 201 (1964).