SYNTHESIS AND SECRETION OF INVERTASE IN RELATION TO THE GROWTH OF MYROTHECIUM VERRUCARIA

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Release of enzymes by bacteria and fungi is not only of common occurrence but is probably frequently essential for growth on substrates unavailable to the cell because of insolubility or impermeability (lipoids, polysaccharides, proteins). Furthermore, host penetration by plant pathogens is commonly ascribed to solution of primary cell walls by enzymes secreted from the fungus hyphae. Although surface located enzymes could be involved in these situations, no enzymes attacking such substrates have, as yet, been demonstrated to occur at the cell surface (Rosenberg and Wilbrandt, 1952; Goddard and Stafford, 1954). While many extracellular enzymes appear only after autolysis has set in, it is usually tacitly assumed that earlier liberation is from living cells. Undoubtedly enzymes are released from living cells, although this has not been unequivocally demonstrated, nor has the mechanism been investigated. The only recent discussion of the problem encountered is that of Lamanna and Mallette (1953) who suggest surface synthesis or film penetration as possible mechanisms. These authors note, however, that "the exclusive origin of extracellular enzymes directly from living bacteria has not been shown clearly." Other recent textbooks on mycology or bacteriology, as well as surveys of cellular permeability and secretion, essentially avoid any discussion of mechanisms of enzyme secretion by bacteria and fungi.

Release of protein exotoxins from bacterial cells has been studied by several workers. Stone (1954) concluded that tetanus toxin could diffuse from within the cell, while Elberg and Meyer (1939) have shown botulinus toxin to be released only after inception of autolysis. Non-dialyzable peptides are released by microorganisms during growth (Morton and Broadbent, 1955, and references cited therein; Drews *et al.*, 1953) and by germinating bacterial spores (Strange and Powell, 1954). The synthesis of other extracellular polymers, such as cellulose, by cells may be effected by mechanisms similar to those involved in enzyme secretion.

During experiments with spores of the fungus *Myrothecium verrucaria*, considerable invertase was found to be released during germination in a sucrose yeast extract medium. Release of the enzyme appeared to be closely related to its synthesis and to growth. The present paper reports certain physiological aspects of these phenomena as they occur during spore germination.

METHODS

Spores of *M. verrucaria* strain QM 460 were obtained from cultures containing a sheet of filter paper (Whatman no. 2) as carbon source, 1.5 per cent agar (Difco), and the following inorganic nutrient solution: NH_4NO_3, 3.0 g; KH_2PO_4, 2.59 g; K_2HPO_4, 2.21 g; $MgSO_4 \cdot 7H_2O$, 2.22 g; and distilled H_2O, 1000 ml (Mandels, 1951). Washed spores were suspended in the above inorganic salts solution or in buffer as indicated. Growth in either medium is essentially the same for the incubation periods used. Sucrose (1 or 2 per cent) and yeast extract (1 per cent, Difco) were added and the cultures incubated in Erlenmeyer flasks at 30 C on a reciprocal shaker. Spore concentrations were between 0.5 and 1.0 mg dry weight/ml. Aseptic precautions were not observed, since significant growth of contaminants does not occur during the experimental period. Significant pH changes did not occur in the cultures.

Invertase activity was followed in the original culture, in supernatants, in dialyzed supernatants, or in washed spores removed from the cultures as indicated. Secreted invertase is that in culture supernatants. Synthesized invertase is either the sum of activity in the supernatants and in the washed sporelings, corrected for original activity of the spores (figures 2 and 3), or the increase in activity in the intact cultures (figures 1 and 4). Washed sporelings were resuspended in original volume. Activity is expressed

as mg reducing sugar (as glucose) formed per ml per hour, as determined by the dinitrosalicylic acid method (Sumner and Somers, 1944). Where dialyzed supernatants or washed spores were used, 1 per cent sucrose was added, the medium being buffer as indicated. Where sugar determinations were made in solutions of varying pH, alkali was added to neutralize the buffer.

Growth was measured by determination of increase in cell volume of aliquots centrifuged in hematocrit tubes in triplicate (Mandels and Darby, 1953). Dry weight changes of aliquots were measured after filtration, washing and drying at 80 C on tared sintered glass crucibles (Corning, fine porosity).

Properties of the secreted enzyme were determined by using portions of dialyzed (against distilled water), lyophilized supernatants from sucrose, yeast extract, inorganic salts solution cultures incubated 3 to 4 hr as described above.

RESULTS

Course of synthesis, secretion and growth. M. verrucaria spores swell and increase in dry weight when suspended in a medium containing sucrose and yeast extract (figure 1). Invertase activity increases slowly shortly before germination and **then** increases rapidly. Extracellular invertase

Figure 2. Effect of pH on synthesis, secretion and growth. Spores incubated in 0.05 M citrate-phosphate buffers containing 1% sucrose, 1% yeast extract. Cell volumes measured at 4.5 hr; at 5 hr suspensions centrifuged, supernatants dialyzed 16 hr against buffer pH 5.1; sporelings washed and suspended in buffer pH 5.1. Sucrose—to give 1%—added to dialyzed supernatants and to washed sporelings, and reducing sugars measured after incubation at 30 C under toluene; invertase in mg reducing sugar/ml × hr. Initial cell volume, 9 μL/5 ml.

Figure 3. Effect of temperature on synthesis, secretion and growth. Procedure same as for figure 2, except 0.05 M citrate-phosphate buffer at pH 5.2. Cultures shaken in water baths at indicated temperatures ± 0.01 C. Invertase of sporelings and supernatants determined at 30 C. Initial cell volume, 5 μL/3 ml.
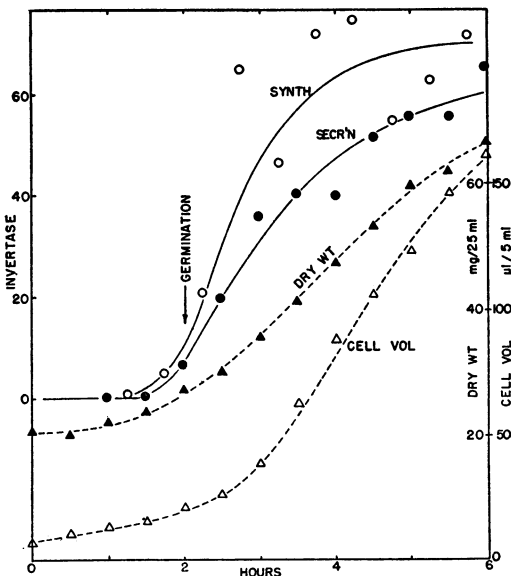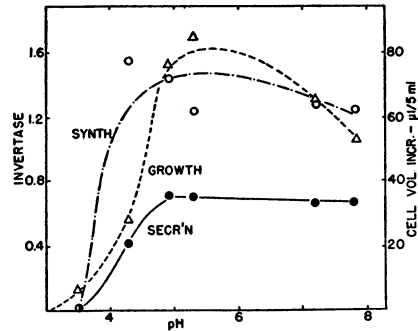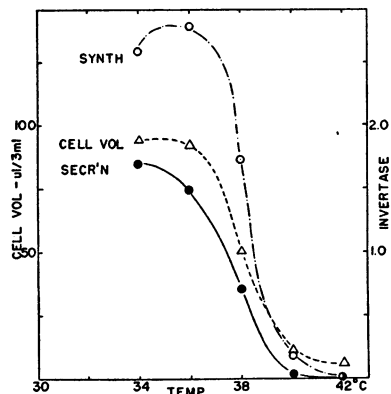

Figure 1. Course of invertase synthesis and secretion, and of growth. Nutrient salts + 2% sucrose + 1% yeast extract; 0.8 mg spores/ml; invertase in mg reducing sugar/ml × hr.

does not appear until after synthesis has been initiated. After 3 hr, the invertase activity has increased about 3.5 times and about 60 per cent of the synthesized enzyme (45 per cent of the total) is found in the filtrates. Since 90 to 100 per cent germination occurs in this period, and few if any of the cells die, the enzyme must be released from intact cells during germination. Ex-
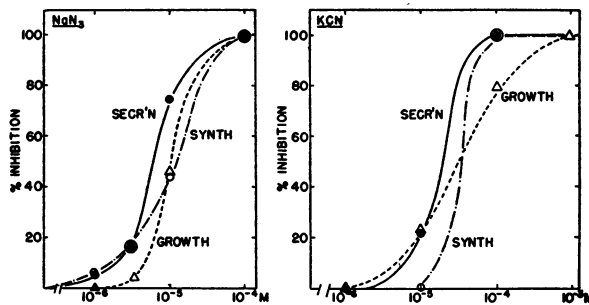
Figure 4. Effect of azide and cyanide on synthesis, secretion and growth. Supernatants not dialyzed; 0.05 M KH₂PO₄, 2% sucrose, 1% yeast extract.

periments have shown that invertase is not released from intact spores incubated in buffer or nutrient salts solution. Neither does release occur if spores germinated in sucrose yeast extract medium are washed and suspended in nutrient salts solution. The decreases in rate of growth and synthesis after about 4 hr are due to depletion of critical constituents of the yeast extract.

Effect of environmental factors on synthesis, secretion and growth. Neither synthesis, secretion, nor growth occurs in the absence of oxygen (N₂ or H₂ atmosphere). The effects of pH and also of temperature are quite similar upon all three processes (figures 2 and 3). The quantitative differences, indicated at about pH 4.3, are difficult to evaluate precisely without more extensive data on the time course of the processes. This also applies to the small differences noted in the effects of temperature. It is interesting to note the relative pH independence of synthesis, secretion and growth from about pH 5 to 8.
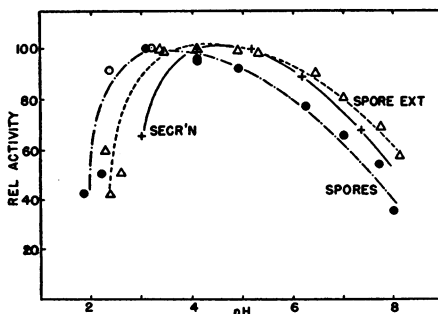

Figure 5. Effect of enzyme source on pH characteristics of invertase. Secreted enzyme —— +; 0.05 M citrate-phosphate. Spores ——·——; ○, 0.05 M citrate-HCl; ●, 0.05 M phosphate. Spore extract - - - -; △, 0.05 M citrate-HCl; ▲, 0.05 M phosphate.

Experiments with a limited number of inhibitors produced no evidence indicating separation of synthesis, secretion and growth at various concentrations of azide or of cyanide (figure 4). Fluoride is without effect on growth or synthesis up to 10⁻² M, while iodoacetate inhibits both growth and synthesis to about the same extent —25 per cent at 10⁻³ M and 100 per cent at 10⁻² M. Effects on secretion were not studied in these cases. None of the inhibitors tested affects invertase activity at the concentration used.

Properties of secreted invertase. Several experiments were carried out to determine the similarities or differences between the enzyme associated with the spores and that released during germination. Identity of the two enzymes is indicated by the pH characteristics of the enzyme for intact resting spores, spore extracts, or secreted enzyme (figure 5).

The substrate specificity of the secreted enzyme is qualitatively the same as that of intact spores or of spore extracts, i.e., both sucrose and raffinose are hydrolyzed but not melezitose, melibiose, or turanose (Mandels, 1954). Quantitatively, however, the ratio of rate of hydrolysis of sucrose to that of raffinose is about 4 for the dialyzed, secreted enzyme, whereas it is about 2 for intact spores or spore extracts (undialyzed).

DISCUSSION

Data presented show that invertase is released from viable, actively growing (germinating) spores of *M. verrucaria*. Within 3 hr after suspension in a sucrose-yeast extract medium, a 3.5-fold increase in invertase is observed, about 60 per cent of which is found in the medium. Since essentially 100 per cent germination occurs under these conditions, and since the sporelings consist of only one cell and are still growing (figure

3, Mandels and Darby, 1953), it can be concluded that the enzyme comes from viable cells.

The release of invertase has not been shown to meet the characteristics of secretion as defined by Danielli (1952) and Robertson (1950) with respect to rate of release, independence from concentration gradients, or inhibition by enzyme poisons. We shall, however, consider the process as being one of secretion, since the process appears to involve the elaboration and discharge of enzyme from the cell and hence is intimately associated with cellular metabolism. Adequate data are not available for definitive postulation of the mechanism of secretion. Several mechanisms can be evaluated, however, with respect to the data available on secretion of invertase and current concepts of membrane structure.

In considering the permeability of animal membranes to proteins, Davson and Danielli (1943) point out that significant penetration by simple diffusion is impossible if the membrane is assumed to be a thin liquid layer. Consequently, they assume protein penetration to occur through differentiated areas. In certain specialized animal membranes, proteins appear to pass through pores.

The morphological structure of the plasma membrane in relation to permeability problems has been discussed by Frey-Wyssling (1948). Elastic properties of the plasma membrane have been demonstrated by several workers who have further shown that, when the membrane is stretched by allowing naked protoplasts to swell by endosmosis, the semipermeability of certain cells disappears suddenly and the cells begin to leak.

Neither of these mechanisms accounts for the absence of enzyme release from sporelings removed from the germination medium or from sporelings germinated in the absence of sucrose (glycerol + yeast extract).

Directly pertinent to postulation of the secretory mechanism involved are: (1) the surface location of the enzyme, (2) the independence of synthesis and secretion on pH during growth, and (3) the dependence of secretion upon synthesis. Substantial evidence attests to the surface location of invertase in *M. verrucaria* spores (Mandels, 1953a, 1953b). We assume a similar location in germinated spores. From these considerations, we postulate that the processes responsible for synthesis are located just within the external surface of the plasma membrane in such a man-

ner as to be unaffected by the pH of the external medium. During synthesis, some of the enzyme molecules are released from the cell, the remainder being affixed to the cell membrane. The mechanism of enzyme transport through the membrane may involve film penetration (Rideal, 1945) as proposed by Lamanna and Mallette (1953).

While generalization cannot be made from a single example, it is possible that secretion of enzymes, or other proteins or peptides, may be limited to those which are surface located. Secretion of all surface located enzymes during growth does not occur, however, for the surface located ascorbic acid oxidase of *M. verrucaria* spores (Mandels, 1953b) is not released during germination. The synthesis of other extracellular polymers, such as cellulose, may be effected by mechanisms similar to those involved in enzyme secretion. Thus, Hestrin and Schramm (1954) have postulated that the enzyme complement concerned in cellulose production by *Acetobacter xylinum* may be on the external surface of the cells.

SUMMARY

Invertase is secreted by spores of the fungus *Myrothecium verrucaria* during germination in a sucrose-yeast extract medium. The secretion occurs from growing cells and appears to be closely related to synthesis and growth as indicated by effects of pH, temperature, and inhibitors. The properties of the secreted enzyme indicate it to be similar to that of the intact spores. The surface location of the enzyme in the spores is believed to be of significance to the mechanism of secretion.

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