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THREONINE DEHYDRATASE OF *BACILLUS LICHENIFORMIS*

II. REGULATION DURING DEVELOPMENT

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SUMMARY

The specific activity of the enzyme threonine dehydratase (threonine hydrolyase (deaminating), EC 4.2.1.16) of *Bacillus licheniformis* decreases rapidly at the end of growth to a 4-fold lower level where it remains during development. The high specific activity of the enzyme is believed to be dependent on the maintenance of the physiological state that exists in growing cells. Experimental conditions that maintain this physiological state (addition of glucose or chloramphenicol) will prevent the decrease in specific activity of the enzyme, whereas conditions that allow the depletion of intracellular nutrients (mitomycin C) or mimic this effect (anaerobic shock) will cause a decrease in the specific activity of the enzyme.

L-Isoleucine added to the medium can prevent the initial increase in specific activity. Other than the change in specific activity, no differences in physical or kinetic properties can be observed for the enzyme isolated from cells at different stages of the life cycle.

Quantitation of the enzyme protein by an immunological procedure indicates that the amount of enzyme protein is constant per unit weight of cells during all phases of the life cycle of the organism. It is postulated that the variation in the specific activity of the enzyme is related to the metabolic activity of the cell.

INTRODUCTION

The function of amino acid biosynthetic enzymes during sporulation is not clear, because some amino acids need not be synthesized since they are provided from the breakdown of cellular material. *Bacillus licheniformis*, unlike some other Bacillus species does not contain any special storage compounds and spores are formed from the oxidation, following turnover, of endogeneous protein[1]. The control, by end product inhibition, of allosteric enzymes is well known[2,3] but changes occurring during

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development are not considered in the control models proposed. The biosynthetic threonine dehydratase (or threonine deaminase; threonine hydro-lyase (deaminating), EC 4.2.1.16) has been investigated extensively in non-sporeformers (for references see ref. 4), but only one report is published for a Bacillus species[5]. The special problem of intracellular development may necessitate new types of regulation[6] and we felt that an investigation of this enzyme during the whole life cycle of the organism would be important.

This report describes the fate of threonine dehydratase during growth and sporulation. The effects of different growth conditions and metabolic inhibitors on the enzyme activity were investigated. The enzyme protein was quantitated by an immunological procedure. The purification and some properties of this enzyme in *B. licheniformis* have been reported in the previous paper[4].

EXPERIMENTAL PROCEDURE

Materials. Freund's complete adjuvant and Noble special agar were Difco products. All other materials and chemicals have been described[4].

Methods. The assay of threonine dehydratase, the protein determination, the growth of the organism and the methods used to extract and purify the enzyme have all been described[4]. Crude extracts of small cell samples were prepared by suspension of the cells in 1 ml of 1.0 M potassium phosphate at pH 8.0 followed by sonic oscillation for 20 sec (ref. 4).

Purification of the enzyme. Purification of the threonine dehydratase from both 4-h and 7-h cultures was achieved using the method described in the previous paper[4]. Approximately the same overall increase in specific activity was obtained and the ratio of each increase during individual steps was similar. Thus, except for the 4-fold difference in specific activity, no differences could be detected in the enzyme from 4- or 7-h extracts.

Production of antibodies. Young adult female rabbits (New Zealand) were bled by heart puncture and the normal serum was separated after 3 h at 3° by centrifugation and stored at −20°. The purified threonine dehydratase was filtered through a Sephadex G-100 column equilibrated and eluted with 0.15 M NaCl and was emulsified with an equal volume of Freund's complete adjuvant. The emulsified enzyme was injected intradermally in the area of the inguinal lymph nodes in amounts of 1 ml (0.75 mg protein) or 4 ml (2.5 mg protein). The animals were bled from the ear vein after 10 days and antibody determined by the capillary precipitin test. At the same time, a booster injection of the emulsified enzyme was administered, also in the inguinal area. 2 weeks after the booster injection 35 ml of blood were collected by heart puncture and the antiserum stored at −20°.

Purification of antibodies. The globulin fraction of the antiserum was precipitated by adding ammonium sulfate with constant stirring to 35% saturation. After 2 h, the suspension was centrifuged at room temperature and the precipitate suspended in 0.15 M NaCl, filtered through a Sephadex G-200 column that was equilibrated and eluted with 0.15 M NaCl, and stored at −20°.

Ouchterlony plates. Micro-Ouchterlony plates were prepared with 1.5% Noble special agar made up in a buffer containing 4.38 g sodium tetraborate and 2.14 g $NaH_2PO_4 \cdot H_2O$ per l of distilled water, and adjusted to pH 8.2 with KOH. 2 ml of agar

were placed on a clean microscope slide and a pattern was cut with a 15 μl center well and six 5-μl outer wells. After filling the wells, the plates were kept at room temperature for 12 h in petri dishes containing filter paper soaked in water.

Serological precipitation reactions. Enzyme in crude extract or in partially purified form was added to antiserum or purified antibodies. All dilutions were made with 0.15 M NaCl. After 20 min at room temperature the precipitated material was removed by centrifugation and the supernatant solution was tested immediately for enzyme activity and protein content.

Sucrose gradient density centrifugation. The method of BRITTEN AND ROBERTS[7] was used to prepare 5–20% sucrose density gradients of 4.5 ml containing 1.0 M potassium phosphate at pH 8.0. After layering fractions of 0.1–0.3 ml of the enzyme preparation on top of the gradient the samples were subjected to centrifugation at 39 000 rev./min for 16 h at 3° in a SW-39 rotor of a Spinco model L centrifuge. The tubes were punctured at the bottom and 10-drop samples were collected for immediate assay of enzyme activity and protein content.

Anaerobic shock and ultraviolet light treatment. Cell cultures (25 ml) were grown at 37° in 125 erlenmeyer flasks on an Eberbach waterbath shaker. Control cultures were kept under these conditions throughout. For the ultraviolet light irradiation experiments cell cultures were poured into sterile petri dishes at the desired time, agitated by swirling and irradiated with a 30-W General Electric germicidal lamp (G 30 T 8) at a distance of 40 cm. 75-sec irradiation resulted in about 1% survival as determined by plate counts on nutrient agar. After irradiation the cultures were returned immediately to the erlenmeyer flasks in the 37° water-bath shaker. For the anaerobic shock experiments the culture flasks were placed at 37° without agitation and exposed to a steady stream of nitrogen. After 30 min the cultures were returned to the 37° waterbath shaker.

RESULTS

1. *Specific activity of enzyme.* The specific activity of threonine dehydratase was followed during the stages of growth and development by taking samples from a 15-l culture (Fig. 1). During the first half of the log phase of growth, the specific activity was fairly constant, but then increased rapidly to a much higher level, where it remained until growth ceased. At the end of growth the activity dropped rapidly to about its original level where it remained during development. At least 90% of the cells subsequently (at 14–18 h) formed spores. If cultures that contained high specific activity enzyme were used for the inoculum, the specific activity of the enzyme was high throughout growth.

The *in vitro* addition of amino acids, nucleotides, cofactors and metabolic intermediates to the enzyme that exhibited low specific activity did not cause an activation. Changes in the pH or ionic strength of the solution or addition of high specific activity enzyme extract did not activate the enzyme. The specific activity of the enzyme could not be elevated by heating, as reported for the ornithine transcarbamylase of yeast[8].

2. *Quantitation of enzyme.* It was possible that the disappearance of enzyme activity was due to degradation of the enzyme by the protease shown to be induced during differentiation[9]. Thus, an immunological procedure was devised to measure the

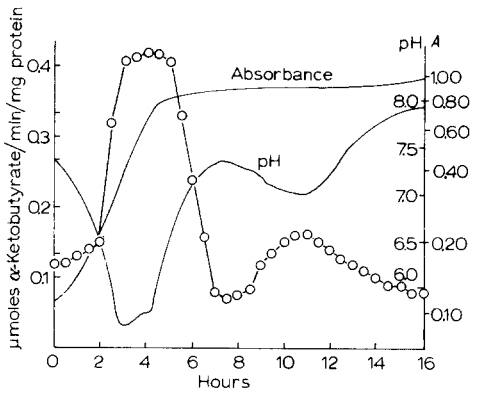
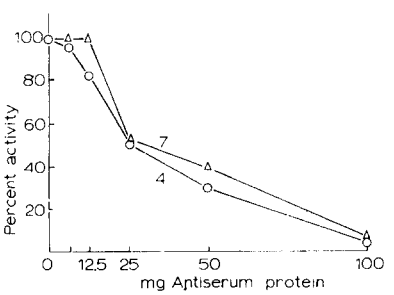


Fig. 1. The specific activity profile during development. Crude extracts were obtained from small samples (see *Methods*) of a 15-l culture and assayed as described under *Methods*. ○—○, specific activity.

amount of enzyme protein present at the different stages of the life-cycle of the organism. Preparations of the 50-fold purified enzyme with high specific activity (4 h in Fig. 1) and with low specific activity (7 h in Fig. 1) were used as antigens. The antisera and the purified antibodies but not the normal sera precipitated enzyme isolated from all stages of the life-cycle of the organisms.

Micro-Ouchterlony plates containing antiserum in the inner well and enzyme from cultures of different ages in the outer wells showed precipitation lines of identity. These lines were absent using normal serum. Extract from any age of the culture and antiserum against high and low specific activity also showed lines of identity. The line was absent when the antibodies were precipitated from the antiserum with enzyme



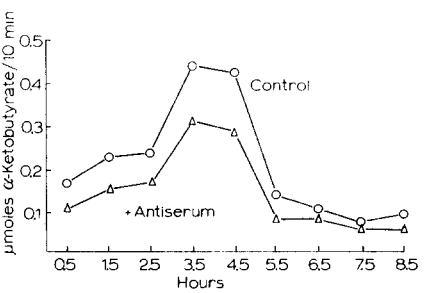


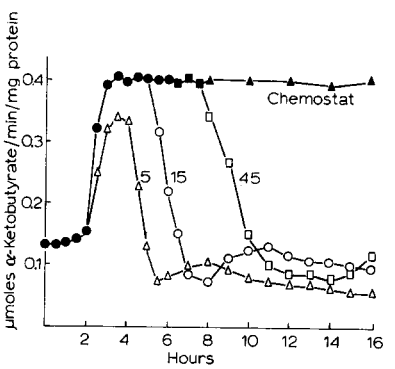
Fig. 2. Precipitation of purified enzyme with antibodies. Purified enzyme was diluted 1:5 with 0.15 M NaCl and mixed with an equal volume of antiserum containing the protein indicated. The supernatant fluid (see *Serological precipitation reactions*) was assayed as described under *Methods*. The line marked 4 indicates purified enzyme from 4-h cultures (Fig. 1) with a specific activity of 8.9 and a 100% activity of 0.46 μmole α-ketobutyrate per 10 min. The line marked 7 indicates purified enzyme from 7-h cultures (Fig. 1) with a specific activity of 2.7 and a 100% activity of 0.14 μmole α-ketobutyrate per 10 min. Both enzyme preparations contained the same amount of protein (5 mg/ml).

Fig. 3. Precipitation of enzyme in crude extract with antibodies. Crude extracts were prepared from samples of a 15-l culture at the times indicated and were adjusted to 3 mg protein per ml. For the control curve, samples were diluted 1:5 and assayed as described under *Methods*. For the antiserum curve, samples were mixed with an equal volume of purified antibody (625 μg protein per ml) and the supernatant fluid (see *Serological precipitation reactions*) was assayed as described under *Methods*.

and the remaining supernatant solution was employed in the Ouchterlony tests. The amount of protein precipitated during the incubation of antisera with either the 4-h or 7-h purified enzyme preparation was negligible, showing that the antigen–antibody reaction was quite specific.

For a comparison of the amount of enzyme protein present when the specific activity was high (4 h, Fig. 1) with the amount when the specific activity was low (7 h, Fig. 1), the enzyme was purified and adjusted to the same protein level. After mixing increasing amounts of antiserum with enzyme, the supernatant solution was checked for remaining enzyme activity (Fig. 2). The same percentage of activity had been precipitated from both enzyme solutions, indicating that the same amount of enzyme protein was present. The enzyme in crude extract was used to establish the quantity of enzyme protein during growth and development. After adjusting the protein content of all samples to the same level, the activity of the supernatant solution was compared before and after the addition of antiserum (Fig. 3). The same percentage of activity was lost from each sample again indicating that the same amount of enzyme protein was present. Greater amounts of antiserum lowered the inactivation curve, but the percentage of activity remaining was constant. Samples prepared from cultures containing isoleucine (see Fig. 5) also contained the same amount of enzyme protein during the life-cycle investigated. Thus, it is concluded that the changes in the specific activity of threonine dehydratase during growth and sporulation (Fig. 1) are due to changes in the activity of the enzyme and not to its relative amount.

3. *Effects of nutrients.* Since the amount of enzyme protein remained constant during the life cycle we set out to determine the physiological factors that control the enzyme activity. As pointed out before it was not possible to activate the enzyme with low specific activity *in vitro* by either addition to or changes in the environment. The effect of different concentrations of glucose in the growth medium showed that the specific activity of the enzyme, once at a higher level, remained elevated as long as growth was supported by glucose, but dropped rapidly at the end of growth (Fig. 4).



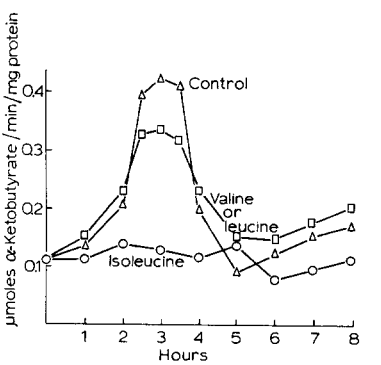


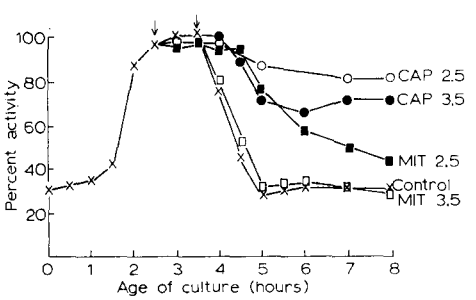
Fig. 4. Effect of glucose concentration on the specific activity. Cells were grown in salts medium (200 ml) *plus* the indicated glucose concentrations (mM). The cells in the chemostat were kept at 20 mM glucose. Crude extracts were prepared from small samples (see *Methods*) and assayed as described under *Methods*.

Fig. 5. Effects of single amino acids on enzyme activity. Cells were grown in salts–glucose medium (200 ml) *plus* $2 \cdot 10^{-2}$ M of the indicated amino acids. Crude extracts were prepared from small samples (see *Methods*), filtered and assayed as described under *Methods*.

Cells grown in a chemostat and supplied with a constant flow of 20 mM glucose could be kept growing continuously and showed an elevated specific activity throughout (Fig. 4). Cells grown on 50 mM glutamate as the sole carbon source had also a high level of specific activity until the end of growth and then dropped to the lower level (data not shown).

B. licheniformis does not grow on single amino acids of the aspartic acid family as the sole carbon source. The effects of the L isomers of isoleucine, leucine, valine, and threonine were tested by adding these amino acids ($2 \cdot 10^{-2}$ M) to the glucose–salts medium. Only isoleucine showed a significant effect on the specific activity of the enzyme by preventing the increase (Fig. 5). The addition of the amino acids in combination also showed that only when isoleucine was present was the increase in the specific activity of the enzyme prevented. None of these combinations, including isoleucine *plus* valine *plus* leucine, caused a repression of the enzyme.

4. *Effects of inhibitors, irradiation and anaerobic shock.* The treatment of cells with chloramphenicol (100 μg/ml; twice the minimum inhibitory concentration), mitomycin C (3 μg/ml; twice the minimum inhibitory concentration) and ultraviolet light irradiation caused the cessation of growth. Both of these antibiotics inhibit amino acid incorporation into protein by more than 90% in *B. licheniformis*[1]. The addition to the growth medium of chloramphenicol during growth or shortly before the end of growth prevented the rapid decrease in enzyme activity (Fig. 6), while the addition of mitomycin C at the end of growth did not interfere with the normal decrease in the enzyme activity (Fig. 6). Mitomycin C added during growth (2.5 h) caused a postponement of the decrease of about 1 h (Fig. 6). Cells treated by ultraviolet light irradiation at 2.5 h maintained a high level of enzyme activity until the exhaustion of nutrients and then showed the same decrease as the mitomycin C-treated cultures (Fig. 7). Irradiation at the end of growth (3.5 h) had no effect on the enzyme activity. Cultures anaerobically shocked at 2.5 h lost their enzyme activity rapidly but regained



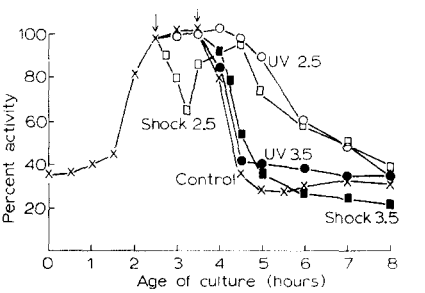


Fig. 6. Effects of metabolic inhibitors on enzyme activity. Cells were grown in the glucose–salts medium (25 ml) and chloramphenicol (CAP, 100 μg/ml) or mitomycin C (MIT, 3 μg/ml) was added at the indicated times. Both inhibitors caused the cessation of growth. Crude extracts were prepared from small samples and assayed as described under METHODS. The 100% activity equalled 0.54 μmole α-ketobutyrate per 10 min.

Fig. 7. Effect of anaerobic shock and ultraviolet light treatment. Cells were grown and treated at the indicated times as described in EXPERIMENTAL PROCEDURE. Anaerobic shock (Shock) and ultraviolet light irradiation (UV) caused cessation of growth which was resumed in the culture shocked anaerobically when returned to aeration. Crude extracts were prepared and assayed as described under METHODS. The 100% activity equalled 0.48 μmole α-ketobutyrate per 10 min.

it after shaking in air was resumed and showed the same decrease in enzyme activity as the other cultures treated at 2.5 h (Fig. 7). Thus, glucose addition, continuous culture, and chloramphenicol addition to batch cultures were the only conditions found that would prohibit a decrease in activity. All other treatments permitted the loss of activity at a time that could be correlated only with the depletion of nutrients in the medium.

Although data are not shown in this paper, the time of exhaustion of nutrients from the culture medium has been determined in our laboratory repeatedly[10,11]. Growth was always limited by the supply of glucose or glutamic acid and exhaustion of nutrients was coincident with the cessation of growth. In control cultures and in experiments in which growth was inhibited, the exhaustion of nutrients was coincident with the rise of the pH in the culture medium (Fig. 1)[10,11].

5. *Enzyme properties during development.* The properties of threonine dehydratase from growing cells were reported in the preceding paper[4]. These properties were compared with those of enzyme preparations from cells harvested at different stages of the life-cycle. The different enzyme preparations showed essentially the same properties with respect to stability, sensitivity to isoleucine inhibition, activation and desensitization. The filtration of the enzyme from different cultures through a 2.4 cm × 30 cm Sephadex G-200 column indicated that the enzyme in all preparations had a molecular weight of over 200 000. Sedimentation of the enzyme in a sucrose density gradient showed that the enzymes from cultures of different ages appeared at the same position (Fig. 8) indicating the same molecular weight of the enzyme preparations. The enzyme activity coincided with the protein fraction, indicating a fair degree of purity of the enzyme preparation. Because of the instability of the enzyme it has not been possible to determine the relative homogeneity of the purified enzyme in more precise terms.

6. *Absence of the biodegradative threonine dehydratase.* UMBARGER AND BROWN[12] discovered two threonine dehydratases in *Escherichia coli*. During aerobic growth on a minimal medium the biosynthetic enzyme was produced, but during anaerobic growth on a rich medium the AMP-stimulated biodegradative enzyme was synthesized. In other systems investigated only one of these enzymes has been detected. All of our data in this and the previous paper[4] indicate that *B. licheniformis* contains only one

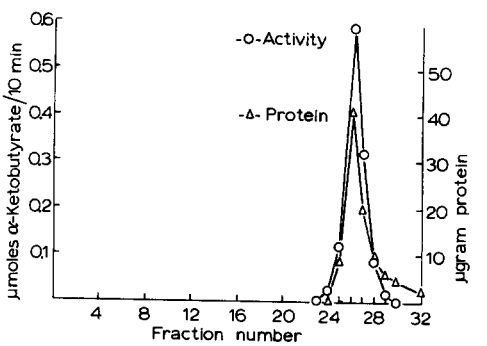


Fig. 8. Sedimentation of enzyme in a sucrose gradient. Purified enzyme (0.1 ml) with a specific activity of 12.8 was layered on a gradient that was prepared, fractionated, and assayed as described in EXPERIMENTAL PROCEDURES.

threonine dehydratase in cells grown under different conditions and in all stages of development. Inactivation studies in crude extracts and the absence of separation during purification[4] support this conclusion.

DISCUSSION

We have shown that *B. licheniformis* contains a single threonine dehydratase enzyme during growth and sporulation. Although all kinetic and physical characteristics of the enzyme investigated are the same throughout, the specific activity decreases about 4-fold during the onset of sporulation. A quantitative immunological method showed that the amount of enzyme protein does not change during the *in vivo* change in specific activity (Fig. 3). Our data indicate that the control of the change in enzyme activity is related to the physiological state of the cell, since the decrease in specific activity is coincident with the exhaustion of nutrients from the culture medium that occurs at the end of growth. This exhaustion of nutrients is mimicked in cultures that are exposed to an anaerobic shock (Fig. 7) and permitted in cultures treated with ultraviolet light (Fig. 7) or mitomycin C (Fig. 6). The decrease in specific activity can be prevented by nutrient addition (Fig. 4) or by treating the cells with chloramphenicol (Fig. 6).

In other *Bacillus* species a similar loss of enzyme activity has been observed with different enzymes[13,14]. The loss of aspartokinase activity in *B. licheniformis* has recently been reported from our laboratory[15]. In addition, a decrease in enzyme activity caused by glucose was observed for one of the malate dehydrogenases[16] and the fructose-1,6-diphosphate phosphatase[17] in yeast cells incubated in an acetate medium. The decrease in ornithine transcarbamylase activity in yeast has been observed in a medium containing arginine[8]. In a cellular slime mold, UDP-galactose polysaccharide transferase changes in activity during development[18]. We are not aware of the disappearance of enzyme activities with similar kinetics in any non-sporulating organism.

The examples cited[8,13–18] and other reports also dealing with sporulating systems[19,20] indicate that the loss of catalytic activity of enzymes may involve a general type of control necessary and operative in cells capable of development[6]. The life cycle of Bacillus and other sporulating organisms consists of at least two different physiological and metabolic segments. During growth, all of the biosynthetic enzymes needed for the synthesis of amino acids are produced, but at the onset of differentiation some of these enzymes are no longer necessary since the end products are either available or not required. Many examples are known where enzyme activities either disappear, appear, or change quantitatively during sporulation[21]. Similarly, cells growing on glucose must contain all of the glycolytic enzymes but during sporulation the metabolic flow of carbon changes to a pattern of gluconeogenesis[1]. Since the control of many "key" enzymes is important under these circumstances[22], the cell must, under non-growing (sporulating) conditions, use either a mechanism of feedback inhibition for stopping the activity of biosynthetic enzymes and allowing the reverse flow of carbon, or inactivate the enzymes by some other mechanism. In the case of threonine dehydratase, the end product inhibitor, isoleucine, may serve this function since it prevents the increase in enzyme activity when added to the growth medium (Fig. 5). The intracellular concentration of isoleucine, however, increases only slightly during sporulation[23], and does not correspond to the variation in enzyme activity. In the case of

phosphofructokinase, the inhibitor, ATP[2], should increase many-fold to prevent glycolysis, but pool studies have shown that the level of ATP is very low during differentiation[24]. Thus, a mechanism of selective enzyme inactivation to control net carbon flow seems to be necessary and quite possible.

This selective inactivation could be accomplished[25] *via* proteolysis by an induced hydrolytic or inactivating enzyme. A protease is produced in sporulating *B. licheniformis*[9] but it has not been shown to be selective. In addition it is difficult to envision a degradation that ceases after 75% of the activity has been lost. The pH of the medium does not influence the enzyme activity in *B. licheniformis* and cannot be invoked to explain the pH-dependent glucose effect observed for the threonine dehydratase in *E. coli*[26]. The decrease in the enzyme activity cannot be brought about by dilution, since the cells do not divide during sporulation. The effect on the enzyme activity of the amino acids added to the medium cannot be explained readily. Isoleucine prevents the increase in the specific activity of the enzyme (Fig. 5) even in the presence of valine and leucine and does not cause a repression. The addition of isoleucine, leucine and valine to the medium of *E. coli* and *S. typhimurium* led to the discovery of multivalent repression[27]. Since no multivalent repression in the presence of glucose was observed for the *B. licheniformis* enzyme the *in vivo* control of threonine dehydratase must be different in some aspect. The decrease of the activity of the yeast ornithine transcarbamylase[8] is believed to be due to a specific regulatory protein which binds to and inactivates the enzyme, since the protein synthesis inhibitor cycloheximide added to the medium prevented the decrease. However, it was pointed out[16], that the effect of cycloheximide might be an artifact, since interference with glucose metabolism might lower the effective concentration of glucose catabolites which initiate the repression process. The enzyme activity of the UDP-galactose polysaccharide transferase of a cellular slime mold is released into the medium at a specific stage of development. It is postulated[18] that this elimination of the enzyme is a reflection of a mechanism of control. We have not been able to detect the threonine dehydratase in the culture medium during development.

Our data *plus* information from the literature lead to two hypotheses concerning the control of the decrease in the specific activity of enzymes in developing systems. The first hypothesis would invoke the function of a stable mRNA that would code for the synthesis of an inactivating or binding protein. This is suggested because the decrease in activity is chloramphenicol sensitive. This hypothesis is not favored for the following reasons:

1. The quantity of enzyme protein as measured by an immunological procedure does not decrease (Fig. 3). (An inactivation without loss of antigenicity of the enzyme cannot be ruled out.)

2. The time of decrease of enzyme activity is always correlated with the exhaustion of nutrients (glucose or glutamic acid) from the culture medium.

3. Cells exposed to an anaerobic period during growth lose enzyme activity rapidly but regain it when aeration is resumed (Fig. 7).

4. A hydrolase activity would not be expected to function in a manner consistent with the data presented in the previous three points.

5. The 7-h enzyme can be purified in a manner parallel to that of the 4-h activity.

An alternative and preferred hypothesis to explain the loss of activity of threonine dehydratase and also of the other enzymes cited above, involves a situation

similar to catabolite repression[28] that we choose to term "metabolite control". During growth on glucose, metabolites accumulate to a relatively high level[29]. The metabolite(s) would be tightly bound[30] and function allosterically to activate selected "key" enzymes. "Key" enzymes would be those that function at the first step in amino acid biosynthetic pathways and those important to the control of metabolism in glycolysis and the citric acid cycle[2]. If the cells were grown on amino acids or acetate, *etc.*, these metabolites would not accumulate and the "key" enzymes would assume a much lower activity or would not be active at all.

To follow this reasoning further, cells growing on acetate would synthesize the enzymes necessary for gluconeogenesis and these systems would be active in the absence of the metabolite(s) that is needed for the activity of the above "key" enzymes. Upon the addition of glucose, the accumulated metabolite(s) would be tightly bound and allosterically inactivate the gluconeogenic enzymes. This phenomenon, coupled with the rationale for the control of the activity of threonine dehydratase, would also explain the results obtained for the malate dehydrogenase[16] and fructose-1,6-diphosphate phosphatase[17] in yeast without the need of enzyme degradation by a specific enzyme induced by a catabolite of glucose[16]. Thus, the accumulated metabolite(s) would activate the glycolytic enzymes and inactivate the gluconeogenic enzymes, in a reversible manner. Such reversibility, *in vivo*, is seen in the anaerobic experiments (Fig. 7).

Since sporulation ensues following the exhaustion of nutrients from the growth medium, the "metabolite control" would be quite advantageous to the cell. The organism would have a selective mechanism for reversing the flow of metabolites. Also, it would allow the cell to separate all of the activities necessary for growth from those important in an efficient biosynthetic metabolism leading to the synthesis of a spore. Our data are consistent with this hypothesis, using the following reasoning: The amount of enzyme protein remains at a proportional level throughout growth and sporulation. The base level of enzyme activity is controlled by the metabolite(s). In the case of threonine dehydratase, both isoleucine and the metabolite(s) of glucose are important. If the energetics of the cell are changed by adding glucose in unlimited supply (Fig. 4) or by allowing metabolites to accumulate after chloramphenicol addition[31] the activity of the enzyme does not decrease (Fig. 6). Although chloramphenicol would be expected to inhibit the synthesis of an inactivating protein we prefer to eliminate this conclusion for the reasons listed earlier. The effect of this antibiotic on metabolic pools[31] is consistent with the other data obtained. The induction of a new inactivating protein[8] is not indicated as cells killed with ultraviolet light irradiation but still metabolizing[32] exhibit a decrease in enzyme activity only after the exhaustion of nutrients from the medium (Fig. 7). The postponement of the decrease of the enzyme activity in the ultraviolet light treatment (2.5 h) is correlated with the decrease of nutrients in the culture containing fewer cells. The exhaustion of nutrients can be mimicked by subjecting the cells to a period of anaerobic shock that is assumed to lower the supply of nucleotide triphosphates and change the energetics from a situation favorable for growth to one unfavorable for growth. This condition, again, leads to the loss of enzyme activity (Fig. 7), that is regained upon resumption of aeration. The activity is finally lost after the exhaustion of nutrients. The use of mitomycin C, an inhibitor of DNA replication, permits the loss of enzyme activity (Fig. 6), only after the exhaustion of nutrients. It has been shown, that mitomycin C does not increase

the nucleotide pool in *E. coli*[33], and inhibits mRNA and protein synthesis only after prolonged exposure[34,35]. Since there is growing concern about the lack of specificity of actinomycin D[36], it was not used in these studies.

Thus, it is clear that the total metabolic and physiological condition of the cell controls the activity of threonine dehydratase. Because of this, we feel that the term "metabolite control" best describes this situation in sporulating cells. The relationship to catabolite repression[28,29] is obvious but yet distinctively different. "Metabolite control" may be a regulation mechanism unique to systems of development and is simpler than the proposed "inactivation–repression" which involves the induction of a special enzyme[16]. "Metabolite control" is also more efficient since there is no need for the synthesis of special regulatory proteins[8].

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