KINETICS OF HYDROLASE PRODUCTION BY MICROORGANISMS

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ABSTRACT

The enzyme-forming systems (EFSs) concerned with the amylase of *Bacillus subtilis*, and the glucamylase and acid protease of *Aspergillus niger*, have been shown to be highly stable in the cultural conditions applied owing to the low decay rate of mRNAs specific for these respective enzymes. The quantity per cell of mRNAs for any of these enzymes is regarded as limiting the specific rate of enzyme production in the conditions used for enzyme production. Investigations on the repression, derepression and the preferential synthesis of these hydrolases have led to simple hypothetical relationships.

NOMENCLATURE

a; a' = constants ('differential' constants);
b; b' = constants ('repression' constants);
C = system constant related to the efficiency of turnover recovery from the decaying RNA to synthesize the specific mRNA in nongrowing phase;
E = enzyme concentration in a culture (units $\times$ ml^{-1});
E_0 = E value at time t_0;
f; g = system constants;
H = historical term or term of 'residual synthesis';
K, K', K'', K_1 = system constants;
k = rate constant for the decay of the above mRNA (h^{-1});
Q_m = specific activity to produce an intracellular metabolite atabolite or anabolite) (quantity $\times$ $mg^{-1}\,h^{-1}$);
Q_R = specific (per cell) activity to produce the mRNA in question (quantity $\times$ $mg^{-1}\,h^{-1}$);
R = quantity of RNA per mg of dry cell weight (quantity $\times$ mg^{-1});
R_0 = R-value at time t_0 (quantity $\times$ mg^{-1});
r = quantity per cell of the mRNA specific for a particular enzyme (quantity $\times$ mg^{-1});
t = time (h);
t_0 = time earlier than t by Δt, or at the start of experiment (h);
X = cell concentration ($mg\,ml^{-1}$);
α; β; γ; δ = system constants;
ε = specific rate of enzyme formation (units $\times$ $mg^{-1}\,h^{-1}$);

ε_0 = ε-value at time t_0 (units $\times$ $mg^{-1}\,h^{-1}$);
ε_m = ε-value at time t_m (units $\times$ $mg^{-1}\,h^{-1}$);
λ = rate constant for the decay of cellular RNA in nongrowing phase (h^{-1});
μ = specific growth rate (h^{-1});
$\tilde{\mu}$ = average μ-value in the time interval (h^{-1}).

INTRODUCTION

When dealing with fermentation kinetics and with the classification of processes, the establishment of the kinetics of enzyme formation is of crucial importance since all rate processes of product formation are determined by it. As the first approach to this problem, we made some kinetic studies on industrial fermentations for producing enzymes; these are convenient to deal with because the general characteristics of cultures have been intensively investigated. Recent developments in the knowledge of epigenetic systems have enabled us to form appropriate models, especially for the production of some extracellular enzymes.

ENZYME SECRETION IN MICROORGANISMS

Generally, most of the organisms known to produce extracellular enzymes are Gram-positive bacteria and fungi[1], and this is believed to be because of the high permeability of the cell wall of Gram-positive forms. It is known that the cell wall of Gram-negative bacteria has individual layers and a higher lipid content than that of the Gram-positive bacteria, and the wall may be less permeable to hydrophilic macromolecules such as enzymes[2]. It is generally postulated that microbial exo-enzymes are formed by polysomes attached to or within the cell membrane, and are secreted during or shortly after this process without existing in a free state within the cytoplasm.

In this report, the following extracellular hydrolases are considered: (i) *B. subtilis*—α-amylase; (ii) *Aspergillus niger*—glucamylase; (iii) *A. niger*—acid protease; (iv) *A. niger*—depolymeric polygalacturonase (DPG); (v) *Penicillium variable*—CMCase; (vi) *A. niger*—CMCase.

In all cases treated here, the hydrolases are inducible enzymes, and the following conditions are taken to be fulfilled:

(a) Under ordinary culture conditions, the inducer concentration is not rate-limiting for enzyme production.
(b) The increase of enzyme activity (ΔE) measured with culture filtrate (as made up to the original volume) in a time interval (Δt) represents the *de novo* formation of the enzyme in question. This has been confirmed using cultures for producing the above enzymes, and indicates that zymogen (or proenzyme protein) is absent, and that secretion lag is negligible. Therefore the enzyme-producing activity (ε) expressed in units per mg dry cell per h can be expressed by $(dE/dt)(1/X)$, where X is cell concentration.
(c) Enzyme concentration E (units ml^{-1}) of culture filtrate at any time and culture conditions applied represents the cumulative amount of enzyme

produced in the culture, i.e. the stabilities of hydrolases here dealt with are high enough to satisfy the relationship

$$\Delta E = \int_{t_0}^{t} \varepsilon X \, dt \tag{1}$$

$$\text{or } E = \int_{t_0}^{t} \varepsilon X \, dt + E_0 \tag{1a}$$

where E_0 is the E-value at $t = 0$.

Some cases described under (b) need further explanation.

(i) α-Amylase production by *B. subtilis*. The strain employed is a mutant derived from an industrial strain[3], whose α-amylase-producing activity is

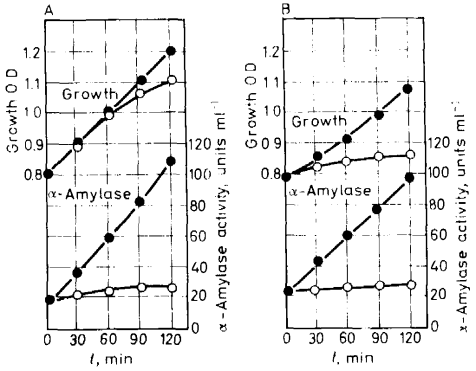

Figure 1. Effect of *p*-fluorophenylalanine and 5-methyltryptophan on α-amylase formation. *B. subtilis* strain KA64L was employed.

Replacement medium contained 200 μg ml^{-1} of leucine. A: experiment with added *p*-fluorophenylalanine (1 mg ml^{-1}); B: experiment with added 5-methyltryptophan (1 mg ml^{-1}). ●, Control; ○, with added inhibitor.

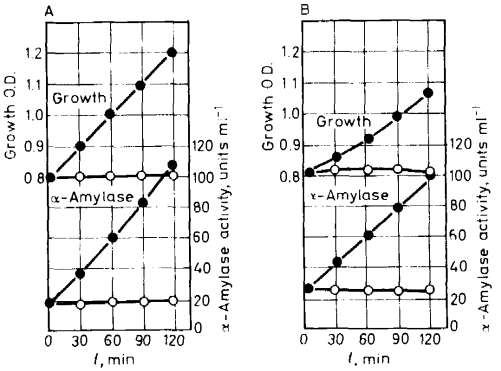

Figure 2. Effect of chloramphenicol and puromycin on α-amylase formation by *B. subtilis*.

General conditions were those of *Figure 1*. A: experiment with added chloramphenicol (10 μg ml^{-1}): B: experiment with added puromycin (100 μg ml^{-1}). ●, Control; ○, with added inhibitor.

considered to be more than ten-fold higher than that of the strains used in basic investigations in this country. Replacement cultures were carried out with the medium which was prepared by adding to the minimal medium (containing per litre, 50 g soluble starch, 5.6 g NH_4NO_3, 2.8 g sodium citrate, 1.3 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, pH 6.8), 5 gl^{-1} and 2 gl^{-1}, respectively, of peptone and yeast extract powder. As shown in *Figure 1* and *Figure 2*, amino acid analogues (5-fluorophenylalanine; 5-methyltryptophan) at a concentration of 1 mg ml^{-1}, chloramphenicol (10 $\mu g\, ml^{-1}$) and puromycin (100 $\mu g\, ml^{-1}$), which strongly inhibited protein synthesis, stopped α-amylase production (ΔE) almost immediately.

(ii) Acid protease production by *A. niger*. An adenine-requiring strain (U20–2–5) was employed. The basic medium used to harvest fully developed mycelia consisted of (per litre) 150 g glucoses, 30 g peptone, 7 g yeast extract and the mineral salt mixture (4 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g $CaCl_2 \cdot 2H_2O$, 0.1 g $ZnSO_4 \cdot 7H_2O$ per litre in final concentration). Replacement cultures were carried out with a modified basal medium at 30°C. The addition of 5-fluorotryptophan (4 mg ml^{-1}) stopped acid protease synthesis completely after a short period of lag.[4]

(iii) Glucamylase production by *A. niger* U20–2–5. Glucamylase production by washed induced mycelia in replacement cultures was active (5 units in 16 h mg^{-1}), but was immediately stopped by adding NaN_3 (10^{-3}M) or 2,4-dinitrophenol (5×10^{-3}M).[5]

STABILITY OF SPECIFIC mRNA

To provide a framework for consideration of the kinetics of enzyme production, the problem was examined in terms of the generally accepted hypothesis that an mRNA specific for an enzyme is the rate-limiting component of the enzyme-forming system under ordinary culture conditions. The quantity of a specific mRNA per unit quantity of cells is denoted as r, which is concerned with the active form capable of initiating the synthesis of a specific enzyme as well as of coding the complete chain. The quantity r can therefore be expressed in terms of an enzyme-producing activity, ε. It is also assumed that the translation rate per unit amount of the mRNA maintains a fixed value throughout the course of the culture in our conditions unless otherwise noted. Accordingly,

$$\varepsilon \simeq r. \tag{2}$$

The mRNAs for most of the endogenous enzymes are regarded as having a very short life. In the case of *E. coli* growing at a doubling time of 50 minutes the half-life of ordinary mRNAs is estimated to be about 2 minutes. Also in the case of penicillinase (an exo-enzyme) production by *Bacillus* sp., the specific mRNA is known to be short-lived: the inhibition of transcription by actinomycin D results in a prompt inhibition of penicillinase formation[6].

In marked contrast to the above, the mRNAs for a variety of extracellular hydrolases here dealt with have been shown to be very long-lived. Actinomycin D was applied as the inhibitor of RNA synthesis in order to see how long hydrolase production (the action of specific mRNA) lasts after cessation of RNA synthesis. Examples are given as follows.

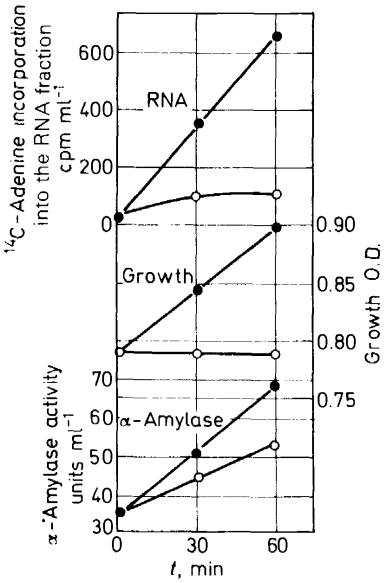

Figure 3. Effect of actinomycin D on the ^{14}C-adenine incorporation into the RNA fraction of *B. subtilis* strain KA64L cells.

For replacement culture the minimal medium was used with 100 μg ml^{-1} of leucine and ^{14}C-adenine (5.3×10^4 cpm ml^{-1}; 10 μg ml^{-1}) added. Actinomycin D was added to a concentration of 0.6 μg ml^{-1} (–○–). Control: —●—

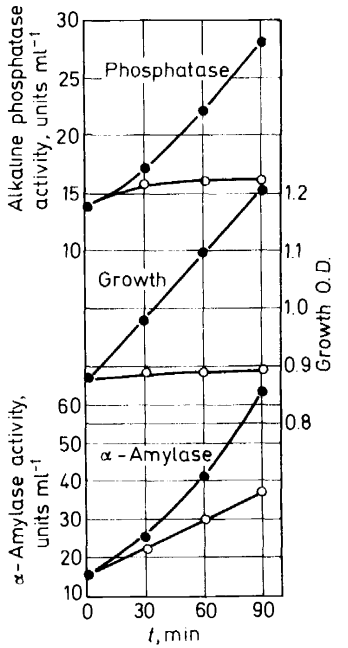

Figure 4. Effect of actinomycin D on alkaline phosphatase formation making a comparison with α-amylase formation. (*B. subtilis* KA64L).

General conditions were those of *Figure 2.* ●, Control; ○, with added actinomycin D (0.6 μg ml^{-1}).

(a) α-Amylase production by *B. subtilis*. Actinomycin D at a concentration of 0.6 μg ml^{-1} inhibited almost completely the incorporation of ^{14}C-adenine into the polysome fraction of cells. The inhibition of growth and incorporation became complete after 30 minutes. Meanwhile in this condition, α-amylase formation proceeded at a fairly high rate (*Figure 3*). In a parallel experiment, the synthesis of cell proteins monitored by ^{14}C-leucine incorporation was shown to be markedly suppressed by actinomycin D. *Figure 4* indicates that the mRNA in alkaline phosphatase is much less stable than that for α-amylase: the former enzyme is produced in close association with growth.

(b) Production of acid protease and glucamylase by *A. niger*[4, 9]. DNA and

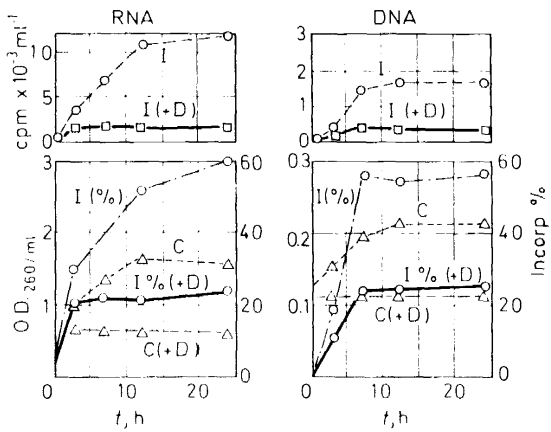

Figure 5. Incorporation of ^{14}C-adenine into RNA and DNA in the presence of actinomycin D (150 μg ml^{-1}). (*A. niger*).

Replacement medium: 500 μM 8-^{14}C-adenine (specific activity 0.36 μCi μmol $^{-1}$), 0.5% amino acids mixture, 7% maltose, 0.3% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% $CaCl_2 2H_2O$, initial pH 3.6.

Symbols: Actinomycin D (150 μg/ml) added, (+D): Total quantity per ml, C: Incorporation (cpm), I: Percentage of incorporation, I(%).

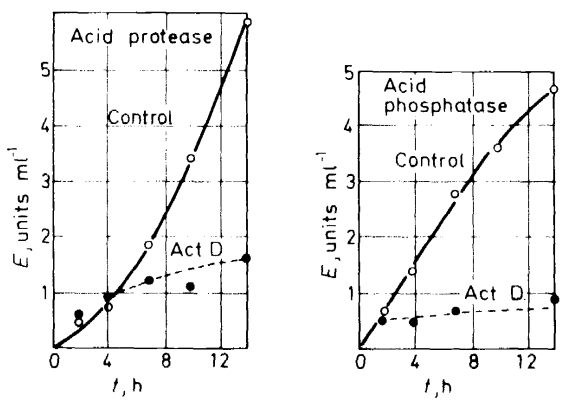

Figure 6. Effect of actinomycin D on enzyme production at 30 °C. (*A. niger*).

RNA syntheses in mycelia by *A. niger* U20-2-5 (adenine-requiring strain) were stopped completely by actinomycin D (150 μg ml^{-1}) after inhibition lags of 4 hours and 2 hours, respectively. The incorporation of ^{14}C-

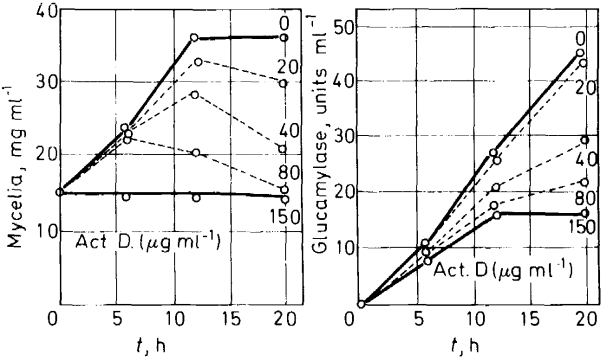

Figure 7. Effect of actinomycin D upon growth and glucamylase formation.

Mycelia (15 mg dry wt./ml) harvested from a 56 h-culture with the standard medium were shifted to the following medium containing actinomycin D. Medium: 750 μM adenine, 7% maltose, 1% monosodium glutamate, 0.5% NH_4NO_3, 0.3% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% $CaCl_2 \cdot 2H_2O$, initial pH 3.6. Concentrations of actinomycin D (Act.D μg ml^{-1}) are listed in the *Figure*.

adenine was stopped thereafter (*Figure 5*). The rate of acid protease production was not decreased in this condition for 4 hours; thereafter the rate fell off, but a low rate of enzyme production seemed to continue for a long time (*Figure 6*). In a replacement culture, growth was inhibited completely by adding 150 μg ml^{-1} of this drug whereas glucamylase-producing activity was maintained without appreciable decrease for about 10 hours (*Figure 7*). The same results on actinomycin D were obtained in the case of CMCase production by *A. niger* and *P. variabile*, and DPG production by *A. niger*.

PROCESS PATTERNS

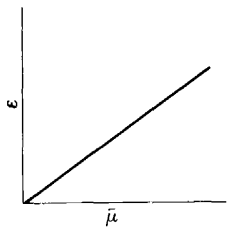

Figure 8. Plot of ε expressed by the short-life mRNA against μ.

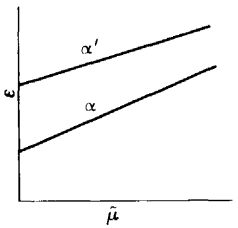

Figure 9. Plot of equations 8 and 9, in which the values can be obtained through shift-up and shift-down experiments.

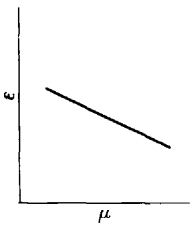

Figure 10. Diagrammatic representation of the negative correlation.

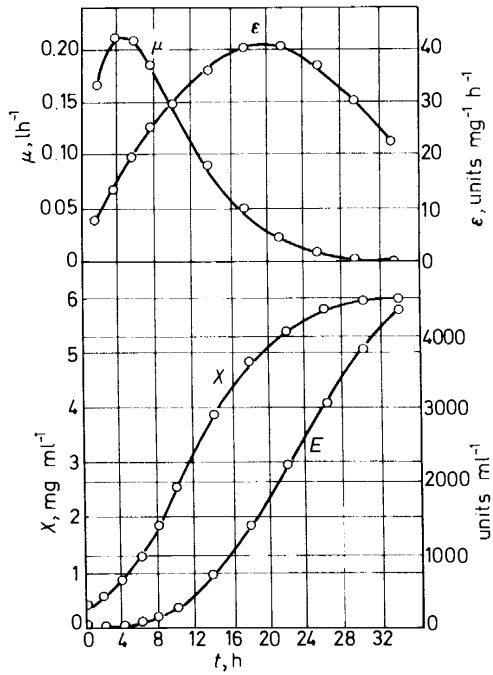

Figure 11. Time course of α-amylase production by *B. amylosolvens* Terui, presented by Terui and Konno (1961). The organism resembles *B. subtilis* in physiological characteristics.

The process patterns of product formation may be divided into several classes: (i) those exhibiting approximately constant differential rates, which are depicted diagrammatically in *Figures 8* and *9*, and (ii) those showing negative correlation with growth as depicted in *Figure 10*.

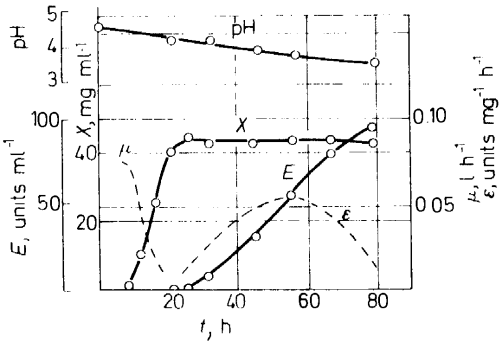

Figure 12. Time course of acid protease production in a culture with the basal medium inoculated with spores of *A. niger* U20-2-5.

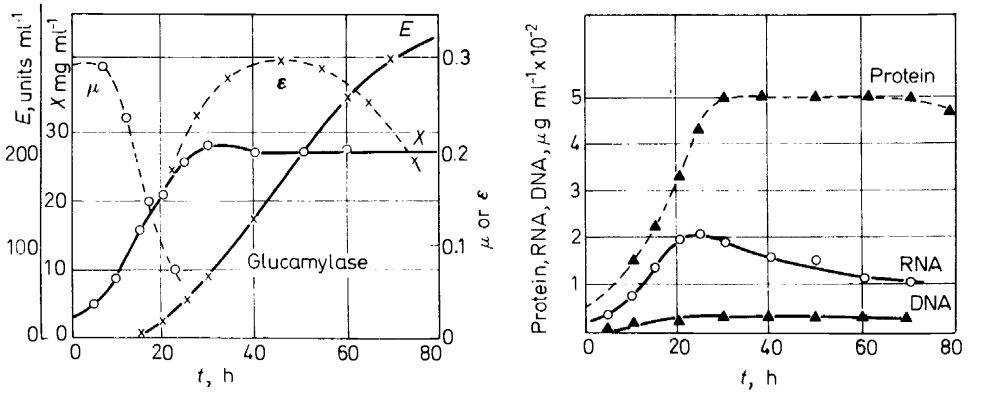

Figure 13. Representative time course of the culture of *A. niger U20-2-5*. Submerged culture was conducted at 30°C with the synthetic adenine-limited medium.

According to *Figure 9*, the following relationship is approximately true (cf. the next paragraph):

$$\varepsilon = \alpha\mu + \beta \qquad (3)$$

Where β is considered to be dependent on the stability of the mRNA and μ is the specific growth rate. Some examples are shown in *Figures 11–14*.

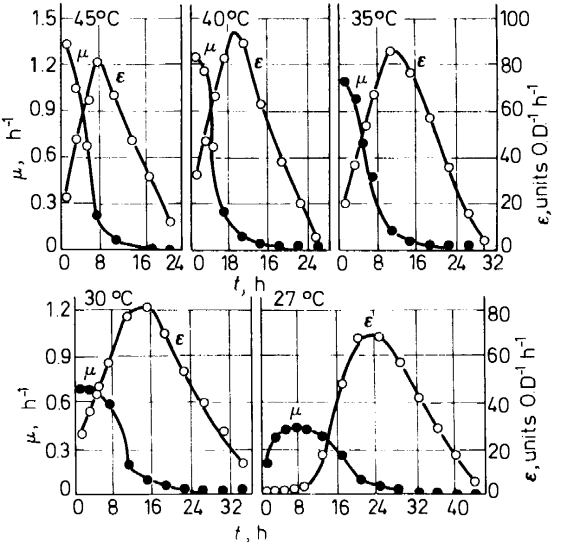

Figure 14. Time courses of α-amylase production by *B. subtilis* KA63 at various temperatures. Flasks dipped in water baths were shaken reciprocally.

The duration of enzyme-producing activity after the end of growth indicates the extraordinary stability of the EFS, and in some instances we can see the distinct negative slope of the plot of ε versus μ, which implies that the enzyme formation is repressed by the metabolite whose flux is greater at larger μ.

KINETIC MODELS FOR GROWING PHASE

Based on the conditions (a), (b), (c) on p. and equation 2, and the experimental results with the production of hydrolytic exo-enzymes, kinetic models were constructed[10]. We have seen in the preceding paragraph that there are at least three distinct process patterns. If the lag phase of growth is disregarded, the process models will be built by a simple procedure.

Consideration of the processes connected with growth-associated enzyme production has led to the following relationship

$$dr/dt = a'\mu - kr \quad \text{or} \quad d\varepsilon/dt = a\mu - k\varepsilon \tag{4}$$

where k is the monomolecular decay constant (h^{-1}) of mRNA of the corresponding enzyme-forming system, and a' and a are constants.

Equation 4 shows that ε increases with the rate of mRNA formation, and that this is proportional to μ, and the rate of decrease is proportional to the monomolecular decay of existing mRNA; the rate of the latter has already been mentioned.

Equation 4 can be solved to give

$$\varepsilon = \exp(-k\Delta t)\,(a\int_{t_0}^{t} \mu \exp(k\Delta t)\,dt + \varepsilon_0) \tag{5}$$

where ε_0 is the ε-value at $t = t_0$.

Shortening sufficiently the time interval ($\Delta t = t - t_0$), e.g. to 1 or $\frac{1}{2}$ hour, the following approximation can be used

$$\varepsilon - \varepsilon_0 \exp(-k\Delta t) = (a/k)\{1 - \exp(-k\Delta t)\}\tilde{\mu} \tag{6}$$

where $\tilde{\mu}$ is the average μ-value in that time interval.

Pertaining to the ordinary bacterial enzymes operating within the cells, the corresponding mRNAs are known to be very unstable in ordinary culture conditions: e.g. a half-life of 2 minutes means a k-value higher than 21. Then equation 6 can be simplified to

$$\varepsilon \simeq (a/k)\mu$$

On the other hand, data have recently appeared concerning enzyme formation by the action of long-life mRNA in microbes. In such cases, the fixed value of $\exp(-k\Delta t)$ in a series of replacement culture experiments may become significant so that equation 6 takes the form

$$\varepsilon = \alpha\mu + H. \tag{8}$$

Here, H is the historical term or the term of 'residual synthesis' which is varied in the course of culture time, but it is regarded as a constant in a set of replacement culture experiments started with the same culture.

With a system having an extremely small value of k, we obtained by replacement culture experiments (*Figure 9*)

$$\varepsilon = \alpha'\mu + H. \tag{9}$$

Here, $\alpha' = a\Delta t$, in which Δt has a fixed value, since

$$\lim_{k\to 0} (1/k)(1 - \exp(-k\Delta t)) = \Delta t$$

Therefore H becomes equal to ε_0, the initial activity (cf. *Figure 11*).

A characteristic feature common to such process types expressed by equation 3 may be observed in experiments in a chemostat. The steady-state equation can be derived directly from equation 4

$$\bar{\varepsilon} = (a/k)\mu, \tag{10}$$

where $\bar{\varepsilon}$ and $\bar{\mu}$ are the steady-state values.

With regard to the negative correlation slope between ε and μ, this is assumed to be due mainly to metabolite repression, though the mechanism of the actual process is still obscure.

By introducing the simplest correlation kinetics for a decreasing exponential function, which is expressed by

$$d\varepsilon = -b d\mu \quad \text{or} \quad d\varepsilon/dt = -b d\mu/dt, \tag{11}$$

into equation 4, we obtain a versatile relationship

$$d\varepsilon/dt = a\mu - b(d\mu/dt) - k\varepsilon, \tag{12}$$

in which b is called the repression constant. When a is regarded as zero,

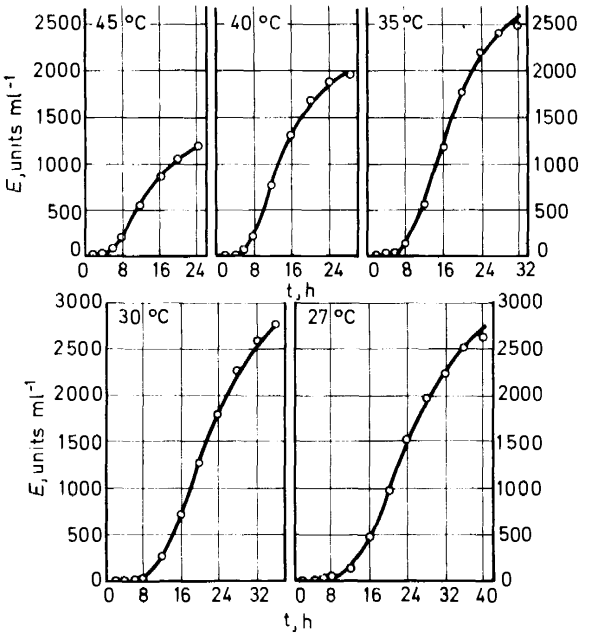

Figure 15. Tests for the fitness of the kinetics model to cultures shown in *Figure 14*.

Table of system constants (*B. subtilis*).

Constants	Temperature °C				
	45	40	35	30	27
k	0.175	0.14	0.11	0.09	0.075
a (units O.D.$^{-1}$ h^{-1})	12	11	11	12	14
b (units O.D.$^{-1}$)	91	91	91	107	145

$$d\varepsilon/dt = -b(d\mu/dt) - k\varepsilon. \tag{12a}$$

Another possible expression for 'the growth-associated repression' was considered by Goodwin in 1966, but we have found that an expression much more complicated than equation 12 is not necessary as far as the present problems are concerned.

We considered the respective kinetics from which equation 12 can be deduced, in the following manner.

Q_R is the activity (expressed by specific rate) to synthesize the specific mRNA. The inhibiting action of a repressor is assumed to be proportional in quantity to the μ-limiting metabolite within the cells and to block the activity of the gene with very high affinity, i.e.

$$Q_R = g\mu - fQ_m \tag{13}$$

— where Q_m is the activity (specific rate) of production of the above-mentioned metabolite within the cells; g and f are constants. Equation 13 means that the active entity is produced at a rate proportional to μ, from which one must subtract the rate of blocking expressed by Q_m in order to obtain Q_R.

It seems reasonable to assume the relation between Q_m and μ is as follows

$$d\mu/dt = \gamma Q_m - \delta\mu \tag{14}$$

where γ and δ are constants. This means that a metabolite produced at a rate of Q_m is consumed at a rate proportional to μ and the remaining amount

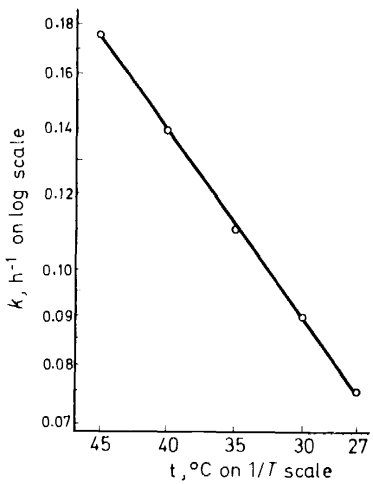

Figure 16. Arrhenius' plot for the assumed decay constant of mRNA. The values of k at various temperatures are given in the table attached to *Figure 15*.

controls the rate of increase of μ. In a steady state or in the logarithmic phase, $Q_m \propto \mu$. Combining equations 13 and 14, we have

$$Q_R = a'\mu - b'(d\mu/dt), \tag{15}$$

where a' and b' are constants.

The overall rate of change in r is therefore

$$dr/dt = Q_R - kr = a'\mu - b'(d\mu/dt) - kr. \tag{16}$$

Combining equation 16 with equation 2, we obtain equation 12 which is solved in the form

$$\varepsilon = \exp(-k(t-t_0))\left[a\int_{t_0}^{t}\mu\exp(k(t-t_0))\,dt + b\int_{t_0}^{t}(-d\mu/dt)\exp k(t-t_0)\,dt\right] + \varepsilon_0 \qquad (17)$$

where ε_0 is the ε-value at $t = t_0$.

The applicability of equation 12 or equation 17 was tested with a variety of cultures. *Figure 14* shows the processes of *B. subtilis* α-amylase production at various temperatures. A test for the fitness of equation 17 is shown in *Figure 15* (solid lines are theoretical), from which the Arrhenius activation energy for the *k-step* (11 kcal mol^{-1}) was obtained (*Figure 16*). *Figure 17* is an example of acid protease production by fully derepressed mycelia. Meta-

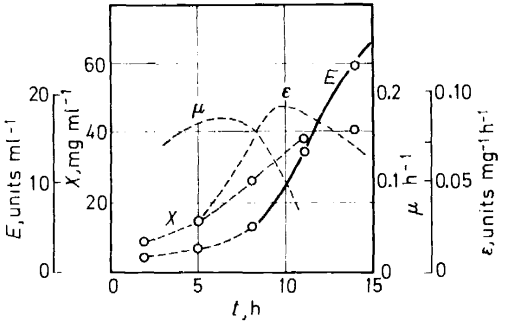

Figure 17. Time course of acid protease production in a culture with the basal medium inoculated with fully derepressed mycelia of *A. niger* U20-2-5 (adenine-limited culture).
Theoretical time course of *E* according to equations *1* and *12* is shown by a solid line. Constants: $a = 0.10$; $b = 0$; $k = 0.09$.

bolite flux within the cells seems to be absent ($b = 0$). Other examples indicated that the kinetic model, equation 17, conforms with experimental results (*Figure 19*, below).

KINETIC MODEL FOR ENZYME PRODUCTION IN THE NONGROWING PHASE

The so-called preferential synthesis of an enzyme is considered to be due mainly to the duration of the specific mRNA.

In the case of mould cultures for hydrolase production, mycelial activity in the nongrowing phase plays an important role in increasing yield. The kinetics of enzyme production in this phase was studied employing *A. niger* U20-2-5, whose growth could be limited by adenine. It was revealed that the mRNA for glucamylase or acid protease in this phase is partly formed by that carried over from the growing phase and partly that formed *de novo* through turnover utilization of the decaying RNA.

A kinetic model for this process was built tentatively on the assumption

that the rate of turnover synthesis might be limited by the rate of RNA decomposition; the rate constant is denoted as λ

$$\lambda R = \lambda R_0 \exp\{-\lambda(t - t_0)\} = K' \exp\{-\lambda(t - t_0)\} \quad (18)$$

where R_0 and R are the RNA contents (quantity mg^{-1}) at t_0 (starting time for the experiment) and t, respectively (*Figure 19*). If the rate of turnover synthesis of RNA is proportional to that of the mRNA specific for glucamylase or acid protease, it will follow that

$$(dr/dt)_{\text{by turnover}} = CK' \exp\{-\lambda(t - t_0)\} = K'' \exp\{-\lambda(t - t_0)\} \quad (19)$$

where C, K' and K'' are system constants, the first being related to the coefficient of turnover utilization to produce mRNA. But the actual rate of increase in cellular mRNA content (r) should be

$$dr/dt = (dr/dt)_{\text{by turnover}} - kr = K'' \exp\{-\lambda(t - t_0)\} - kr \quad (20)$$

where k is the rate constant for the mRNA decay. Equation 20 is solved in the form

$$r = K[\exp\{-\lambda(t - t_0)\} - \exp\{-k(t - t_0)\}] \quad (21)$$

in which,

$$K = K''/(k - \lambda) = \lambda CR_0/(k - \lambda) \quad (22)$$

The incorporation of ^{14}C-guanine into the RNA of nongrowing *A. niger* mycelia (*Figure 18*) was analysed kinetically as depicted in *Figure 19*. This

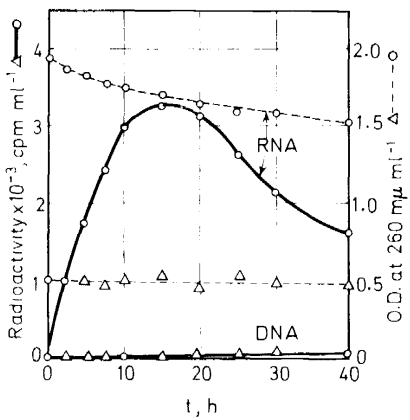

Figure 18. Incorporation of ^{14}C-guanine into RNA and DNA in nongrowing mycelia (*A. niger*). Mycelia used were harvested from a 48 h culture with the complex medium. Replacement cultures were conducted with the basal medium added with 50 μM 8-^{14}C-guanine (specific activity: 0.9 μCi μmol^{-1}). Cell concentration in the adenine-deficient replacement cultures was *ca.* 20 mg ml^{-1} throughout.

indicates that the overall RNA synthesis by turnover conforms with equation 21. In a parallel run of experiments, it was shown that the time course of ^{14}C-guanine incorporation is almost parallel to the glucamylase activity

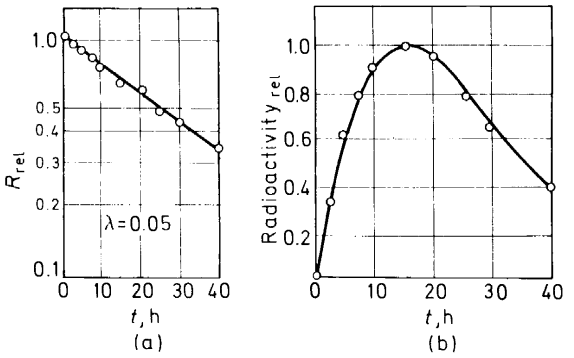

Figure 19. Kinetics of the incorporation of ^{14}C-guanine in nongrowing mycelia (*A. niger*). (a) Monomolecular degradation of cellular RNA. (b) Fitness of equation 21 to the results of incorporation tests.

Theoretical plots (with $k = 0.09$ and $\lambda = 0.05$) are depicted by solid lines: experimental values are shown by open circles. Conditions: cf. *Figure 18.*

curve obtained from the results of induction experiments in which uninduced stationary-phase mycelia grown with sorbose as carbon source were fed with maltose. Therefore, the kinetic model of glucamylase (or acid protease) formation in the nongrowing phase is built up as follows:

$$\varepsilon = \varepsilon_m \exp\{-k(t - t_m)\} + K_1[\exp\{-\lambda(t - t_m)\} - \exp\{-k(t - t_m)\}] \quad (23)$$

where ε_m is the enzyme-producing activity in the beginning of the maximum stationary phase, and K_1 an empirical constant.

Figure 20 shows the conformity of equation 23 and 17 with the entire course of glucamylase production by *A. niger*.

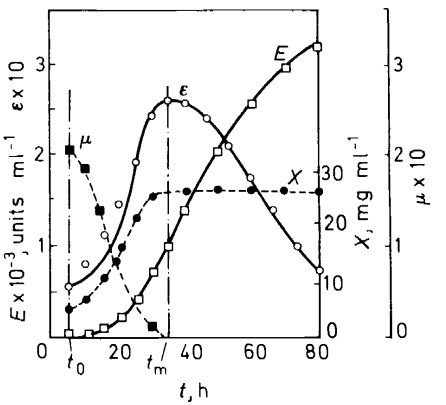

Figure 20. Conformity of the kinetic models, equations 17 and 23, with the time course of glucamylase production by *A. niger* (adenine-limited culture).

The basal synthetic medium was used. The theoretical plots (solid line) are drawn with the following values: $k = 0.05$; $\lambda = 0.1$; $K_1 = -0.4$; $b = 4.7$; $\varepsilon_0 = 0.088$; $E_0 = 3.0$; $t_0 = 5$; $t_m = 35$. Open circles and squares are experimental values of ε and E, resp.

EFFECT OF GLUCOSE

General considerations

The inhibitory effect of glucose on the formation of various enzymes was observed with a variety of microorganisms. Magasanik[12] reported that glucose interferes generally with the formation of catabolic enzymes in bacteria, yeast and other microorganisms. Extensive studies of this phenomenon at the molecular level have been made on the formation of β-galactosidase of *E. coli*. According to Magasanik[13], glucose interferes with β-galactosidase synthesis in the following three ways.

(i) Inducer exclusion: glucose excludes the inducer from cells that do not contain a high level of permease controlled by the *y* gene. (ii) Transient repression:glucose represses β-galactosidase strongly but transiently when added to cells growing on another source of carbon. (iii) Catabolite repression: glucose represses the enzyme weakly but permanently during balanced growth.

In case (i), the effect may be overcome by increasing the level of inducer or by increasing the specific permease through induction in the absence of glucose[14].

As to case (ii), investigations have been conducted with the cells previously exposed to a gratuitous inducer (IPTG). The addition of glucose (10mM) to such cells brings about a strong, but temporary inhibition of β-galactosidase formation. Prevost and Moses[15] suggested that a phosphorylated metabolite of glucose may be the effector of transient repression. On the other hand, it is reported that glucose analogues (e.g. 2-deoxyglucose) repress transiently β-galactosidase in the wild strain of *E. coli*. Though the mechanism of this phenomenon is still obscure, it is strongly suggested by the work of Kundig, Tyler and Magasanik that such a repression is triggered by an interaction of exogenously added glucose with the phosphotransferase system (EI and EII) during its passage through the cell membrane. After a period of strong repression, cells escape from repression. We have little knowledge of the mechanism of escape.

Concerning case (iii), experiments by Nakada and Magasanik[18], Loomis and Magasanik[19], Tyler and Magasanik[17], and Jacquet and Kepes[20] indicated that catabolite repression affects gene expression at the level of transcription. Involvement of 3′,5′-cyclic AMP in this phenomenon first suggested by Makman and Sutherland[21] was further investigated by Ullman and Monod[22] and Perlman and Pastan[23] who indicated that the presence of this compound almost completely overpowered catabolite repression of both transient and permanent type. It is known that catabolite repression depends not only on the nature of the carbon source, but also on the availability of nitrogen source, and that generally a remarkable repression is observed by using a rapidly metabolized carbon source together with a slowly metabolized nitrogen source. Contesse *et al.*[24] postulated, on the basis of their kinetic studies on m RNA synthesis, that under the condition of catabolite repression, the formation of a 'competent' complex between DNA (specifically promoter DNA) and RNA polymerase is rendered less frequent or less efficient. Their results also confirmed that catabolite

repression operates at the level of initiation of transcription, and indicated that it deals also with the process of RNA chain elongation. It is suggested from their results that cyclic AMP, whose intracellular level is lowered on addition of glucose, exerts directly a positive control over the initiation of transcription at the level of promoter of a glucose sensitive operon.

Effect of glucose on the production of extracellular hydrolases

The effect of added glucose on the production of the above-mentioned extracellular hydrolases depends upon the EFS of the organism. Among these, α-amylase production by *B. subtilis* and the production of glucamylase and acid protease by *A. niger* were shown to be only slightly sensitive or almost insensitive to glucose. In some other instances the glucose effect is remarkable. In the cases where the glucose effect is remarkable in the growing phase, it is not always clear whether added glucose exerted the so-called catabolite repression or whether it excluded inducer from the cells. I should like to show some conspicuous effects of glucose upon hydrolase production in the nongrowing phase. The production of DPG by *A. niger*[25] and CMCase production by *P. variabile*[26] were strongly inhibited by glucose. 30mM glucose completely stopped the preferential synthesis of these enzymes by the action of the existing EFS which is insensitive to actinomycin D (*Figures*

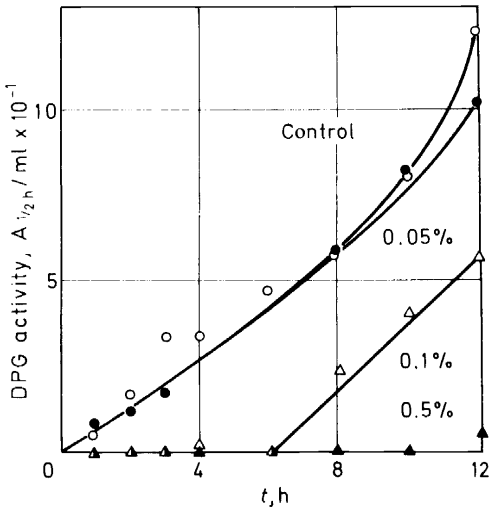

Figure 21. Inhibition of DPG production by glucose. Fully-induced 48 h-mycelia were used for replacement cultures. Figures indicate glucose concentration.

21 and *22*). This seems to indicate that the repression by glucose in these cases might be exerted at the translational level.

It is of interest that the EFS for CMCase of *A. niger* is quite insensitive to glucose (*Figure 23*). It is important to survey the effect of glucose on the production of many other industrial enzymes to confirm whether this takes place at the transcriptional or at the translational level.

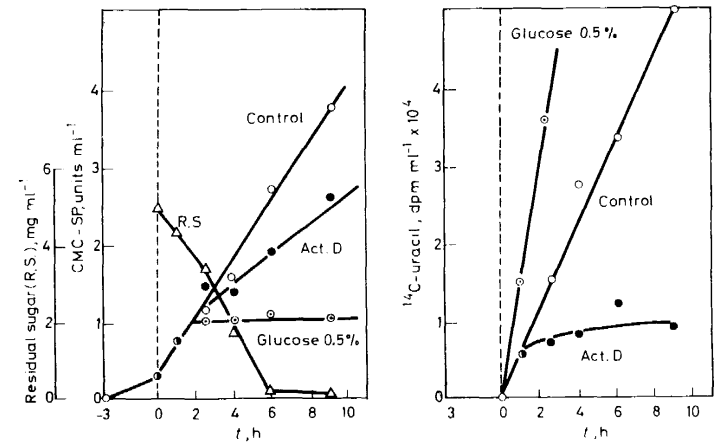

Figure 22. Effect of actinomycin D on the production of CMC-SP by *Pen. variabile*. (SP: saccharifying power).

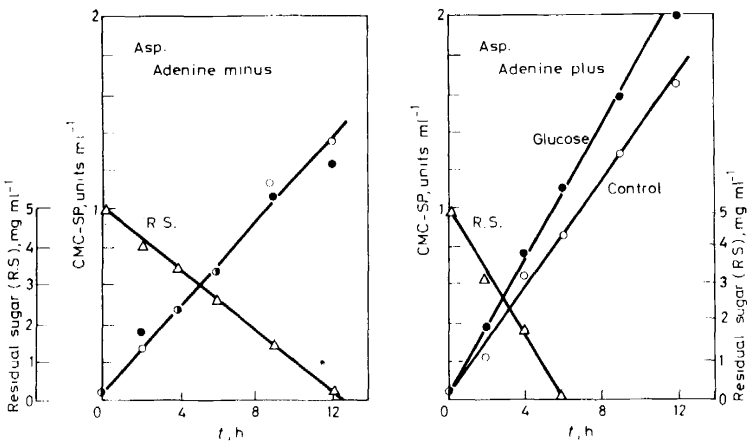

Figure 23. Effect of glucose (0.5%) added to replacement cultures on CMCase production. (*Asp. niger* U20-2-5).

Replacement cultures were started with 36-h mycelia.

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