Extracellular Ribonuclease Formation in *Bacillus subtilis* and its Stimulation by Actinomycin D

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1. Extracellular ribonuclease is produced linearly for at least 3 hr. by washed post-logarithmic-phase cells of *Bacillus subtilis* suspended in a medium containing maltose (1%) and casein hydrolysate (0.5%). 2. Low concentrations of actinomycin D (less than 2 µg./ml.) stimulate ribonuclease formation, the maximum effect being observed with a concentration of 1 µg./ml. Concentrations greater than 2 µg./ml. are inhibitory. There is no parallel stimulation of α-amylase formed under the same conditions, and [¹⁴C]uracil incorporation into a perchloric acid-insoluble form is inhibited. 3. The actinomycin D-induced stimulation is not due to the presence of an activator, nor is the inhibition due to the release of an inhibitor by the cells. The effect is on the amount of ribonuclease produced in the medium. 4. Extracellular ribonuclease formation is partially inhibited by anaerobiosis, 2,4-dinitrophenol, sodium azide and by chloramphenicol and puromycin. 5. High concentrations of antibiotic do not completely inhibit ribonuclease formation, but a basal amount of enzyme representing 20 min. synthesis in an uninhibited system is always produced. This 'antibiotic-insensitive' enzyme could possibly represent preformed enzyme 'in the pipe-line' en route to secretion. 6. The stimulated appearance of ribonuclease in the presence of 1 µg. of actinomycin D/ml. is shown to be dependent on enzyme synthesis. The mechanism of this effect is discussed.

It has been shown by Nishimura & Nomura (1959) that ribonuclease [polyribonucleotide 2-oligonucleotidyltransferase (cyclizing) (*B. subtilis*), an enzyme not listed in the Report of the Commission on Enzymes (see Whitfeld & Witzel, 1963)] accumulates in the culture medium of *Bacillus subtilis*. Enzyme appearance begins as the cultures approach the stationary phase of growth, much as is the case with α-amylase (α-1,4-glucan 4-glucanhydrolase, EC 3.2.1.1) synthesis. However, no detailed study of the formation of ribonuclease by *B. subtilis* has been reported.

The present paper reports a study of ribonuclease synthesis by washed-cell suspensions of *B. subtilis* and the effect of actinomycin D on this process; it has been found that production of the enzyme is greatly stimulated by critical concentrations of the drug. A preliminary report has been published (Coleman & Elliott, 1964).

EXPERIMENTAL

Materials

Inhibitors. Actinomycin D was a gift from Professor A. W. Johnson. Chloramphenicol was a product of Parke,

Davis and Co. Ltd., Sydney, New South Wales, Australia. Puromycin was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., as the dihydrochloride. Aqueous solutions of these antibiotics were prepared at a concentration of 1.0 mg./ml.

2,4-Dinitrophenol and sodium azide were obtained from British Drug Houses Ltd., Poole, Dorset.

Radioactive compounds. [2-¹⁴C]Uracil (6.5 mc/m-mole) was obtained from the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.). An aqueous solution was prepared containing 20 µC/ml.

L-[U-¹⁴C]Valine (25.9 mc/m-mole) was obtained from The Radiochemical Centre, Amersham, Bucks. The material was dissolved in water to give a solution containing 20 µC/ml.

Yeast RNA. This was prepared by the method of Crestfield, Smith & Allen (1955).

Other chemicals. All other chemicals were of the highest purity commercially available.

Culture medium. This contained: (NH₄)₂HPO₄ (34 mm); KCl (5 mm); MgSO₄ (1 mm); CaCl₂ (0.125 mm); ZnSO₄ (0.0125 mm); sodium citrate (4.25 mm); FeCl₃ (0.5 mm); trace-metal solution (0.25 ml./l.); casein hydrolysate (British Drug Houses Ltd.) (0.5%); maltose (1%); the solution was adjusted to pH 7.2 with 10% (v/v) H₃PO₄. The trace-metal solution was essentially that of Pollock & Kramer (1958) and consisted of the following mixture of salts, dissolved in 1 l. of water; CoCl₂·6H₂O, 0.1 mg.; MnCl₂·4H₂O, 1.0 mg.; CuSO₄·5H₂O, 0.01 mg.; ammonium molybdate, 0.1 mg.

Suspending medium. This was the same as the culture medium except that FeCl_3 was omitted (enzyme synthesis by washed cells suspended in fresh culture medium was the same in the presence and absence of FeCl_3).

Methods

Growth of the organism. The organism and growth conditions were as described by Coleman & Elliott (1962), except that the culture medium was inoculated from a suspension of washed spores by means of a platinum loop.

Washed-cell experiments. Cells were harvested after 25 hr. growth, when the culture was entering the stationary phase, by centrifugation at 1800g for 45 sec. The cells were washed in suspending medium, centrifuged and finally suspended to the original cell density of 1.6 mg. dry wt./ml. The entire process was carried out in 5 min. or less at 30°, at which temperature all solutions and apparatus had been equilibrated.

Samples (5 ml.) of the cell suspension, containing the additions indicated in the text, were placed in 100 ml. conical flasks and shaken at 30°. At the appropriate times cells were removed by centrifugation and the supernatant fractions taken for ribonuclease and α -amylase assay.

α -Amylase estimations. These were carried out by the procedure of Coleman & Elliott (1962). All values given are corrected for a zero-time blank of about 5 units/ml.

Ribonuclease estimation. Ribonuclease was assayed by a method essentially that described by Josefsson & Lagerstedt (1962).

A sample (0.5 ml.) of suitably diluted enzyme solution was placed in a 10 ml. conical centrifuge tube together with 1.0 ml. of 0.15 M-tris buffer, pH 8.2, containing EDTA (0.25 mM). The mixture was equilibrated at 25°, after which 1.0 ml. of aq. 0.8% yeast-RNA solution, also equilibrated at 25°, was added. After 30 min. incubation the reaction was stopped by the addition of 0.5 ml. of 0.75% uranyl acetate in 25% (w/v) perchloric acid. The mixture was rapidly cooled in an ice-water bath, left for 10 min. and the precipitate removed by centrifuging at 3°. A portion (0.5 ml.) of the supernatant was diluted in 3.5 ml. of water and the extinction measured at 260 m μ in a 1 cm. cuvette. All values are corrected for a zero-time blank of approx. 0.4 unit/ml.

A unit of ribonuclease activity is defined arbitrarily as the amount of enzyme that, under the standard conditions of the assay procedure, produces an increase in extinction at 260 m μ of 1.00. The assay gave a linear relationship between enzyme concentration and ΔE_{260} up to 0.5.

Measurement of [^{14}C]uracil incorporation. To 2.5 ml. samples of cell suspension, containing the appropriate amounts of actinomycin D, 0.1 ml. of [^{14}C]uracil solution (2 μC) was added. After 5 min. incubation with shaking in air at 30° 2.5 ml. of cold 0.1 N- HClO_4 was added. Duplicate 0.4 ml. samples were pipetted from this into 3 ml. of cold 0.1 N- HClO_4 containing an excess of unlabelled uracil (0.5 mg./ml.). The resulting preparations were kept at 0° for about 5 min. and then filtered through a 2.5 cm.-diam. Oxoid membrane filter. The filter was washed with 20 ml. of cold 0.1 N- HClO_4 containing unlabelled uracil (0.5 mg./ml.) and then with 7.5 ml. of 1% (v/v) acetic acid; it was prepared for counting as described by Elliott (1963).

Measurement of L-[^{14}C]valine incorporation. To 5.0 ml. samples of cell suspension, containing appropriate concen-

trations of actinomycin D, 0.1 ml. of L-[^{14}C]valine solution (2 μC) was added. The suspensions were then incubated with shaking at 30° and at various times during the incubation 0.1 ml. samples were pipetted into tubes containing 4 ml. of 0.5% Difco casein hydrolysate in 5% (w/v) trichloroacetic acid. The tubes were heated in a water bath at 90° for 30 min., cooled and the contents filtered through 2.5 cm.-diam. Oxoid-membrane filters. The filters were then treated with 20 ml. of 5% trichloroacetic acid containing 0.5% of Difco casein hydrolysate and then with 7.5 ml. of 1% acetic acid; they were prepared for counting as described by Elliott (1963).

RESULTS

Enzyme formation by washed-cell suspensions. Washed *B. subtilis* cells, harvested after logarithmic growth had ceased, produce ribonuclease in the extracellular medium in a linear fashion, when shaken aerobically with maltose and amino acids (Fig. 1). This is in striking contrast with the progress of α -amylase production in the same experiments, which invariably shows a marked, apparently 'biphasic' course (also shown in Fig. 1). The reason for this difference between the courses of formation of the two enzymes is unknown. During the 3 hr. incubation period the cell mass of the organism approximately doubled: over a large number of similar experiments the cell mass was shown to increase by $90 \pm 20\%$.

Effect of actinomycin D on ribonuclease formation. Actinomycin D has an unusual effect on the rate of

Fig. 1. Progress of ribonuclease (○) and α -amylase (●) synthesis by washed *B. subtilis* cells suspended in new medium. Experimental details are given in the Methods section. The 100% values were 12.4 units/ml. for ribonuclease and 158 units/ml. for α -amylase.

Fig. 2. Effect of increasing concentrations of actinomycin D on the formation of ribonuclease and α -amylase and on [¹⁴C]uracil incorporation by washed cells of *B. subtilis*. Increase in both ribonuclease (○) and α -amylase (●) was measured over 1 hr., and incorporation of [¹⁴C]uracil into a perchloric acid-insoluble form (△) was measured over a 5 min. period. Experimental details are given in the Methods section. The 100% values were 4.0 units/ml. for ribonuclease, 32 units/ml. for α -amylase and 2786 counts/min. for [¹⁴C]uracil incorporation.

appearance of ribonuclease in the extracellular medium of washed-cell suspensions. At low drug concentrations, enzyme appearance is markedly stimulated, reaching a peak with 1 μg. of actinomycin D/ml. As the concentration of antibiotic is increased to 5 μg./ml. ribonuclease appearance progressively falls (Fig. 2). The actinomycin D-insensitive portion shown in Fig. 2 does not result from incomplete inhibition of synthesis but is due to 'blank' formation of the enzyme that occurs initially, and is discussed below. For comparison the effect of actinomycin D on α -amylase formation in the same experiment is also shown in Fig. 2; in this case concentrations of the drug of less than 0.5 μg./ml. had no effect, but thereafter the inhibition of enzyme synthesis progressively increases in parallel to the inhibition of [¹⁴C]uracil incorporation into a perchloric acid-insoluble form (Fig. 2). Inhibition of L-[¹⁴C]valine incorporation into protein was found to parallel closely the inhibition of [¹⁴C]uracil incorporation.

It was possible that the apparent effects of actinomycin D on ribonuclease formation were spurious and due to effects on the enzyme assay. Actinomycin itself was found to have no effect on this but it was also conceivable that at a concentration of 1 μg./ml. it caused the release of an activator from the cell. That this is not the case was shown

Fig. 3. Effect of 1 μg. of actinomycin D/ml. on the progress of (a) ribonuclease and (b) α -amylase formation. Experimental details are given in the Methods section. In each case enzyme synthesis in the presence of actinomycin D (●) is compared with that occurring in its absence (○).

by the fact that when supernatants from 'no actinomycin D' experiments were mixed with those from '1 μg. of actinomycin D/ml.' experiments, the activity observed was the mean of the two assayed separately. Similarly it was shown that the supernatants from incubations made with 5 μg. of the drug/ml. did not contain a ribonuclease inhibitor. The effect of actinomycin D is therefore on the amount of enzyme produced in the extracellular medium.

The stimulatory effect of 1 μg. of actinomycin D/ml. is not exerted immediately. An exposure of the cells to the drug for 30 min. is required before the maximal rate of ribonuclease appearance is observed (Fig. 3a); after this time the rate of

ribonuclease appearance in the extracellular medium is stimulated threefold. Fig. 3(b) shows the strikingly different effect of the drug in the same experiments on α -amylase production.

A curious and unexplained phenomenon occurs with actinomycin D at a concentration of $10\text{ }\mu\text{g./ml.}$ in that after addition of the antibiotic a small rise in extracellular ribonuclease takes place, but this is unstable and is lost over a 2 hr. incubation period (Fig. 4). This is in total contrast with the situation with the ribonuclease produced normally or that produced in the presence of $1\text{ }\mu\text{g.}$ of actinomycin D/ml., which is completely stable for at least 4 hr. either in the presence or absence of cells and also in the presence of high actinomycin D concentrations alone. Whether the high concentrations of drug cause some non-specific damage to the cells resulting in a slight release of a different and less stable ribonuclease activity is not known.

Is ribonuclease formation new protein synthesis? The stimulatory action of actinomycin D raised the question whether extracellular ribonuclease formation represents a synthesis of a truly extracellular enzyme or whether it represents leakage of pre-formed enzyme from the cell. This question applies with particular force to that fraction of the ribonuclease whose production is dependent on the presence of drug, since it could very easily be argued that

slight cell damage caused by actinomycin D might result in the release of intracellular enzyme. The fact that further increase in actinomycin D concentration is inhibitory does not answer this question conclusively, as is discussed below.

The only previously reported work bearing on this question was that of Nishimura & Nomura (1959), who found that the intracellular ribonuclease in stationary-phase cultures amounts to less than 0.2% of the extracellular production. They therefore concluded that ribonuclease is a true exoenzyme. This apparently forceful argument is, however, totally invalidated by the discovery of a powerful inhibitor of extracellular ribonuclease in extracts of *B. subtilis* cells (Smeaton, Elliott & Coleman, 1965). If, for example, the cells in a normal 3 hr. incubation experiment, containing ribonuclease in the extracellular medium, are lysed *in situ* instead of removing them by centrifugation, the resulting solution is virtually devoid of ribonuclease activity. It is clearly not possible to deduce what the true intracellular content of ribonuclease is by assaying cell-free extracts.

The inhibition by actinomycin D at a concentration of $10\text{ }\mu\text{g./ml.}$ cannot be regarded as proof that extracellular ribonuclease formation is in fact newly synthesized. It could equally well be that the effect of high concentrations of actinomycin D is due to disorganization of the cell that allows the inhibitor to come into contact with intracellular ribonuclease, from which it might be presumed to be normally separated by some form of 'compartmentation'.

From these considerations it was clearly necessary to establish whether ribonuclease formation in the extracellular medium involves protein synthesis or not. To do this a study of its general characteristics was made.

(a) Dependence of ribonuclease on energy supply. *B. subtilis* is an aerobic organism and, as expected if ribonuclease production involves protein synthesis, appearance of the enzyme in the extracellular medium was inhibited by anaerobiosis, azide and dinitrophenol (Table 1). The incompleteness of the inhibition is discussed below. Checks were made that these agents did not affect the assay; for anaerobiosis additional checks were made to establish that the inhibition was not due to release of the ribonuclease inhibitor by cell lysis. To do this a normal 2 hr. incubation supernatant containing ribonuclease was used to suspend fresh cells and the suspension was shaken anaerobically for a further 3 hr. There was no loss of activity, and indeed a slight rise occurred, in keeping with the small formation of the enzyme that occurs under anaerobic conditions (see Table 1).

(b) Effect of chloramphenicol and puromycin on the formation of ribonuclease. Both these anti-

Fig. 4. Effect of $10\text{ }\mu\text{g.}$ of actinomycin D/ml. on the progress of ribonuclease formation. The progress of enzyme synthesis in an uninhibited system (○) is shown, together with the effect of adding $10\text{ }\mu\text{g.}$ of actinomycin D/ml. (●) at zero time and after 1 hr. (arrows) on the subsequent course of enzyme formation. Experimental details are given in the Methods section; inhibition was achieved by the addition of 0.05 ml. of actinomycin D solution containing 1 mg./ml. to each 5 ml. of incubation mixture.

Table 1. *Effect of anaerobiosis and respiratory inhibitors on the formation of ribonuclease*

Experimental details are given in the Methods section. Enzyme formation was measured over a 1 hr. period and related to that formed in an uninhibited system, which amounted to 3.4 units/ml. (100%).

Inhibitor	Inhibition of ribonuclease formation (%)
Anaerobiosis	70
2,4-Dinitrophenol (1 mM)	51
Sodium azide (1 mM)	19
Sodium azide (10 mM)	75

Fig. 5. Effect of chloramphenicol on the formation of ribonuclease (○) and α -amylase (●) and on the incorporation of L-[14 C]valine (Δ). Experimental details are given in the Methods section; enzyme synthesis in each case was measured over a 1 hr. period. The 100% values were 3.3 units/ml. for ribonuclease, 37 units/ml. for α -amylase and 932 counts/min. for L-[14 C]valine incorporation.

biotics inhibited the appearance of extracellular ribonuclease. Chloramphenicol inhibited α -amylase and ribonuclease synthesis in a parallel fashion (Fig. 5); puromycin inhibits both α -amylase and ribonuclease synthesis at a concentration of 20 μg./ml. (Fig. 6). Inhibition of ribonuclease formation approximates to that of general protein synthesis, as measured by L-[14 C]valine incorporation (Fig. 6).

Fig. 7, which shows the time-course of ribonuclease formation at a high concentration of puromycin (40 μg./ml.), clearly demonstrates that the 'inhibitor-insensitive' appearance of enzyme (corresponding in amount to 20 min. of normal synthesis)

Fig. 6. Effect of increasing concentrations of puromycin on the formation of ribonuclease (○) and α -amylase (●) and on the incorporation of L-[14 C]valine (Δ). Experimental details are given in the Methods section; enzyme synthesis in each case was measured over a 1 hr. period. The 100% values were 3.3 units/ml. for ribonuclease, 37 units/ml. for α -amylase and 932 counts/min. for L-[14 C]valine incorporation.

Fig. 7. Effect of 40 μg. of puromycin/ml. on the progress of ribonuclease formation. Experimental details are given in the Methods section. Enzyme synthesis in the presence of puromycin (●) is compared with that occurring in its absence (○).

is confined to the first 60 min. of incubation, after which ribonuclease formation is totally inhibited; this puromycin-insensitive fraction is equal in

Table 2. *Effect of amino acids on ribonuclease formation*

Two batches of cells were washed and suspended separately in medium with and without casein hydrolysate; other experimental details are given in the Methods section.

Incubation time (hr.)	Increase in ribonuclease (units/ml.)	
	No casein hydrolysate	With 0.5% casein hydrolysate
1	1.0	3.6
2	2.1	6.9
3	3.6	10.9

Table 3. *Effect of anaerobiosis on actinomycin D-stimulated ribonuclease formation*

Experimental details are given in the Methods section. Enzyme formation was measured over a 1 hr. period. In the control experiment the 100% value represents an increase in ribonuclease of 3.8 units/ml.

System	Ribonuclease formed (% of control)
Aerobic, no addition (control)	100
Aerobic + actinomycin D (1 μ g./ml.)	153
Anaerobic, no addition	22
Anaerobic + actinomycin D (1 μ g./ml.)	26

amount to both the actinomycin D-insensitive chloramphenicol-insensitive and the energy-independent fractions. It presumably represents either elution of adsorbed enzyme or possibly preformed enzyme 'in the pipe-line' en route to extracellular secretion. There is, however, no evidence for the latter suggestion or indeed that synthesis and secretion are separate events.

(c) Requirement of amino acids for ribonuclease formation. Ribonuclease formation was stimulated by the presence of amino acids (Table 2).

Evidence that the actinomycin D-stimulated appearance of ribonuclease is dependent on enzyme synthesis. The above results clearly show that normal ribonuclease production involves protein synthesis. The question remains whether the same is true for the actinomycin D-stimulated enzyme production, or whether the latter is due to cell damage causing release of preformed ribonuclease. That it is in fact dependent on an energy supply is indicated by the fact that ribonuclease appearance in the presence of 1 μ g. of actinomycin D/ml. shows the same sensitivity to anaerobiosis as the normal production (Table 3). Moreover, as shown in Fig. 8, the stimulated production is sensitive to chloramphenicol during the entire course of incubation with actinomycin D at 1 μ g./ml.

Fig. 8. Effect of the addition of 100 μ g. of chloramphenicol/ml. at different times on the subsequent progress of ribonuclease formation in the presence of 1 μ g. of actinomycin D/ml. Experimental details are given in the Methods section. Chloramphenicol was added at zero time, 1 hr. and 2 hr. (arrows); 0.1 ml. of chloramphenicol solution containing 5 mg./ml. was added to each 5 ml. of incubation mixture. Enzyme synthesis in the presence of chloramphenicol (●) is compared with that occurring in its absence (○).

Fig. 9. Time-course of L-[14 C]valine incorporation into the 'hot-trichloroacetic acid-precipitable' fraction of washed *B. subtilis* cells in the presence of actinomycin D. Experimental details are given in the Methods section. L-[14 C]-Valine incorporation in the presence of 1 μ g. of actinomycin D/ml. (●) and 10 μ g. of actinomycin D/ml. (Δ) is compared with that occurring in its absence (○).

It seems therefore that the actinomycin D-stimulated appearance of ribonuclease involves protein synthesis. As shown in Fig. 9, in the

presence of actinomycin D at this concentration there is still considerable protein synthesis, as measured by L-[¹⁴C]valine incorporation into the 'hot-trichloroacetic acid-precipitable' fraction of the bacterial cells.

DISCUSSION

The present work shows that extracellular ribonuclease production is clearly due to synthesis of the enzyme during the period of its secretion rather than to non-specific release of preformed enzyme. It follows from this that it is a 'truly extracellular' enzyme in the sense defined by Pollock (1962). In this respect it resembles α -amylase formation. The fact that ribonuclease is produced in a precisely linear fashion while the same cells synthesise α -amylase with a markedly 'biphasic' time-course is a problem that has no answer at present.

The most striking feature of ribonuclease formation is its stimulation by actinomycin D. This effect had no previous parallel, but since this work was done Pollock (1963) has independently reported a twofold stimulation of inducible penicillinase by actinomycin D in *B. subtilis*.

At this stage it is only possible to speculate on the reason for the effect, but there are four obvious possibilities that might account for the stimulation of the rate of appearance of extracellular ribonuclease. First, it could be that the drug somehow increases the rate of liberation of the enzyme; this seems unlikely, and might imply that under normal conditions a massive accumulation of ribonuclease occurs inside the cell because of limited release rate. The presence of ribonuclease inhibitor has so far prevented the experimental testing of this idea; with α -amylase it has been well established that negligible or zero amounts of the enzyme are found inside the cell. The second possibility is that the messenger RNA for ribonuclease is stable, and that actinomycin D has the effect of suppressing the synthesis of other proteins whose messenger-RNA molecules are short-lived and thus available protein-synthesis intermediates are channelled into ribonuclease formation. Apparently against this idea is the inhibition of the formation of extracellular ribonuclease by high concentrations of actinomycin D, but this is not a conclusive argument because at higher concentrations the drug may have effects other than that of preventing RNA synthesis. It is known, for example, that actinomycin D can inhibit DNA polymerase at concentrations higher than those needed for RNA-polymerase inhibition (Kirk, 1960; Elliott, 1963), and, more importantly, Acs, Reich & Valanju (1963) have shown that the drug can accelerate the breakdown of preformed messenger

RNA. It is possible therefore that a general cytotoxic effect may be responsible for the inhibition at high drug concentrations. A third possibility is that actinomycin D has a lower affinity for the ribonuclease gene than for the α -amylase and other genes. Clearly this question cannot be decided conclusively, but what evidence is available does not support this idea. Since actinomycin D combines with the guanine residues of DNA (Goldberg, Rabinowitz & Reich, 1962; Reich, 1964) the ribonuclease gene would have to be lower in guanine content than the α -amylase gene. When the amino acid compositions of *B. subtilis* ribonuclease and α -amylase are translated into the base compositions of the corresponding messenger RNA molecules by using the code data of Speyer, Lengyel, Basilio & Ochoa (1962), it is found that the guanine contents of the ribonuclease and α -amylase genes account for 14.5 and 14.2% respectively of the total number of base residues. It must be admitted that such a calculation is fraught with possible errors, in that no account is taken of the degeneracy of the code and the assumption is made that the ribonuclease of our strain of *B. subtilis* is the same as that of Nishimura & Ozawa (1962) [for α -amylase it has been shown that the enzyme of the strain used here has essentially the same amino acid composition as that produced by the strain used by Akabori, Okada, Fujiwara & Sugae (1956)]. An alternative basis for a selective action of actinomycin D could be the small size of the ribonuclease gene (mol. wt. of the protein approx. 10000), which might be taken to mean a small 'gene target' for the drug, but this is hardly a satisfying explanation. The second and third possibilities discussed above carry the inherent implication that the rate of ribonuclease synthesis is limited not by the rate of messenger-RNA synthesis but by a step subsequent to this, e.g. at the level of supply of activated amino acids. The fourth obvious possibility is that actinomycin D selectively inhibits the production of a ribonuclease-repressor protein (which must be supposed to decay within 30 min.; see Fig. 3a).

Moog (1964) has reported the stimulation of the synthesis of alkaline phosphatase in the cells of the mouse duodenum by the administration of actinomycin D *in vivo*. The drug appears to cause the acceleration of a normal process of biochemical differentiation that occurs as the animal 'switches' from infant to adult type. The author speculates that possibly the actinomycin D selectively inhibits the production of a repressor protein. Pollock (1963) has also dealt at length with the possible reasons for penicillinase stimulation by actinomycin D.

It seems clear that no satisfactorily established explanation for the action of actinomycin D in stimulating the synthesis of a particular enzyme is

yet available. If it is assumed, as seems eminently reasonable, that actinomycin D does not stimulate ribonuclease messenger-RNA formation, then it would appear that the rate of synthesis of this enzyme is not controlled primarily at the level of the gene.

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