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The Assimilation of Amino Acids by Bacteria

22. THE EFFECT OF 8-AZAGUANINE UPON ENZYME FORMATION IN *STAPHYLOCOCCUS AUREUS**

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In a previous paper in this series Gale & Folkes (1955) showed that enzyme formation in disrupted *Staphylococcus* was dependent upon nucleic acids. They found that the formation of catalase, glucosylase and β -galactosidase was stimulated by staphylococcal deoxyribonucleic acid (DNA) and that staphylococcal ribonucleic acid (RNA) enhanced the formation of catalase and glucosylase. The formation of β -galactosidase was not affected by RNA, synthesis of this enzyme being dependent upon a supply of purines and pyrimidines and being abolished by ribonuclease. Pardee (1954), studying the formation of β -galactosidase by mutants of *Escherichia coli* that require uracil, found that enzyme formation took place only in the presence of added uracil and, if the supply of uracil was limiting, enzyme formation ceased when the uracil was exhausted. He concluded that RNA synthesis was essential for induced enzyme formation to occur. Similar conclusions have been drawn by Spiegelman, Halvorson & Ben-Ishai (1955) as a result of studies upon inducible enzyme formation in *E. coli* and in yeast.

Many workers (see reviews by Wright, 1951; Bendich, 1955) have shown that analogues of purines and pyrimidines are capable of preventing the growth of a large number of micro-organisms. It has also been found that certain analogues of this

type are capable of inhibiting the formation of inducible enzymes. Chantrenne & Courtois (1953) showed that catalase formation in baker's yeast was inhibited by 2-thiouracil; Spiegelman *et al.* (1955) demonstrated that β -galactosidase synthesis in *E. coli* could be prevented by 5-hydroxyuridine, and Creaser (1955b) found that 8-azaguanine and 2:6-diaminopurine reduced the rate of β -galactosidase formation in *Staphylococcus aureus*. Matthews (1954) showed that 8-azaguanine was incorporated into the nucleic acid of tobacco mosaic virus, and Lasnitzki, Matthews & Smith (1955) found that this analogue could become incorporated into the RNA of several tissues and organisms, but not into the DNA. Several workers have shown that the inhibitory effect of 8-azaguanine can be reversed by guanine, guanosine or xanthine (see Wright, 1951; Bendich, 1955).

The present work describes investigations into the necessity for RNA synthesis during enzyme formation in *Staph. aureus*, 8-azaguanine being used as an inhibitor of induced enzyme formation. A preliminary account of some of the work has already appeared (Creaser, 1955c).

METHODS

Organism. Two strains of *Staph. aureus* (*Micrococcus pyogenes* var. *aureus*) have been used in this work. Except where stated the strain Dunn was used; in some experiments the Duncan strain, as used by Gale & Folkes (1955), was employed. Growth and harvesting of the organism was normally carried out as described previously (Creaser, 1955a).

* Part 21: Gale & Folkes (1955).

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In some cases a growth medium of casein hydrolysate (33 g./l.), Marmite (1 g./l.) and glucose (1 g./l.) was used. Aerobic growth was obtained in the apparatus described by Mitchell (1949); anaerobic growth was carried out in a McIntosh-Fildes jar under an atmosphere of $H_2 + CO_2$ (95:5).

Incubation mixtures. Two systems were used for the development of enzymic activities in washed suspension. System *A* comprised salts, amino acids, galactose (20 mg./ml.), glucose (1 mg./ml.) and washed *Staph. aureus* (2 mg./ml.). System *B* differed from the above in that the galactose was omitted and the glucose concentration was 10 mg./ml. Both systems were maintained at 37° and were aerated. Details of the amino acid and salts solutions used have been given previously (Creaser, 1955a).

Estimation of enzymic activity. Cells were centrifuged from their growth or incubation media at 3000 g, washed once in water and made into an aqueous suspension which was then used for the assay of enzymic activity. Glucozymase was estimated by the method of Gale & Folkes (1955) and β -galactosidase was measured as described by Creaser (1955a). Catalase activity was determined manometrically as follows: 1.0 ml. of the cell suspension under test was placed in the main compartment of a Warburg manometer cup with 1.0 ml. of phosphate buffer (0.2M, pH 7.0). In the side arm was 1.0 ml. of a '10 vol.' solution of H_2O_2 . The manometers were assembled and equilibrated at 25°. At zero time the contents were mixed and the gas output was recorded at intervals of 30 sec. It was found that the rate of gas output was constant over the period from 1 to 5 min. after the start of the reaction, and this constant rate was taken as a measure of the catalase activity of the cells under test.

Identification of nucleic acid components. For this purpose 500 mg. dry wt. of cells was treated with trichloroacetic acid (TCA) at a final concentration of 50 mg. of TCA/ml. and held at 5° for 2 hr. The precipitate which formed was sedimented at 3000 g, washed once with TCA solution (50 mg./ml.) and re-sedimented, the supernatant fluids being discarded. The precipitate was dissolved in 5.0 ml. of *N*-NaOH by incubation at 37° for 15 hr. The solution was then made *N* with respect to HCl and left at 5° for 2 hr. The precipitate which formed (DNA fraction) was sedimented at 3000 g, washed once with *N*-HCl and re-sedimented. The supernatant fluids (RNA fraction) were pooled and both fractions were lyophilized. The two dried materials were then hydrolysed with 72% (w/w) $HClO_4$ at 100° for 2 hr., and chromatographic analysis for purines and pyrimidines was performed as described by Wyatt (1951).

Incorporation of radioactive uracil. Randomly labelled [^{14}C]uracil (specific activity 2.5 mc/m-mole) was prepared from residues obtained from the Radiochemical Centre, Amersham, and in some experiments was added at a concentration of 10 μ g./ml. to the systems described above. After incubation, the cells (3–7 mg. dry wt.) were treated as above as far as the stage of drying the two fractions. These were then dissolved in *N*- NH_3 soln. and measured samples were applied to polythene planchettes (2 cm.²). A drop of 2% (w/v) cetyltrimethylammonium bromide was added, the planchette contents were dried at 37° and their radioactivity was assayed with a Geiger-Müller tube in association with a Panax-type 100c counter. Observed counts were corrected for background radiation and expressed as μ mg. of pyrimidine incorporated/mg. dry wt. of cells.

RESULTS

Enzyme formation in washed suspension

It was found previously that β -galactosidase formation would occur in system *A* described above (Creaser, 1955a). Also, formation of catalase and glucozymase could be observed in both *A* and *B*. In both systems catalase and glucozymase were formed linearly for at least 3 hr.; β -galactosidase was formed in system *A* after a lag of 30–60 min. Formation of all three enzymes was dependent upon a supply of amino acids in the incubation mixture and was prevented by the addition of 10 μ g. of chloramphenicol/ml. No significant amount of bacterial growth (measured turbidimetrically) could be observed during the course of the experiments described below.

Inhibition of enzyme formation by 8-azaguanine

Upon addition of 8-azaguanine to the incubation mixtures detailed above there was an inhibition of the formation of catalase and β -galactosidase (Fig. 1). This inhibition was constant from one

Fig. 1. Effect of 8-azaguanine concentration on the formation of catalase and β -galactosidase. Cells formed catalase for 3 hr. in system *B* and β -galactosidase in system *A* for 3 hr. Percentage inhibition of enzyme formation is calculated as

$$100 - \left[100 \times \frac{\text{increased enzymic activity in the presence of 8-azaguanine}}{\text{increased enzymic activity in the absence of 8-azaguanine}} \right]$$

●, Catalase formation; ○, β -galactosidase formation.

experiment to another, no significant enzyme formation occurring in the presence of 100 $\mu\text{g.}$ of 8-azaguanine/ml. On the other hand, the formation of glucosylase exhibited marked variations in sensitivity to inhibition by 8-azaguanine, but in all cases was much less sensitive to inhibition than was the formation of either catalase or β -galactosidase.

Spiegelman *et al.* (1955) showed that 5-hydroxyuridine would inhibit the formation of β -galactosidase in *E. coli* when added after induction. Fig. 2 shows that a similar effect was observed when β -galactosidase formation in *Staph. aureus* was inhibited by 8-azaguanine. If 100 $\mu\text{g.}$ of 8-azaguanine/ml. was added at the start of the experiment there was little enzyme formation. If the analogue was added during the course of the experiment enzyme formation was inhibited and soon ceased. Similar results were obtained when catalase formation was studied.

Mechanism of action of 8-azaguanine

Cells of *Staph. aureus*, in which the formation of β -galactosidase had been suppressed by the addition of 100 $\mu\text{g.}$ of 8-azaguanine/ml. to the system, were fractionated by the procedure described above. The purine-pyrimidine composition of the two fractions was then determined from the chromatographic properties and the ultraviolet-absorption spectra of the components observed. It was found that the RNA fraction contained adenine, guanine, 8-azaguanine, cytosine and uracil, and the DNA fraction contained adenine, guanine, cytosine and thymine. The incorporation of 8-azaguanine into the RNA but not the DNA fraction agrees with the findings of Lasnitzki *et al.* (1955). When the RNA fraction was not hydrolysed further, but was

subjected to filter-paper electrophoresis as described by Matthews (1954), a component was present which had the electrophoretic mobility and ultraviolet-absorption spectrum of 8-azaguanic acid. It was found that under the conditions used for enzyme formation by washed suspensions of *Staph. aureus* there was a net synthesis of RNA of the order of 10 % in 3 hr. (Creaser, in preparation). Sufficient 8-azaguanine was incorporated into the RNA in the experiment above to make it highly probable that all the RNA synthesized in the presence of 8-azaguanine contained this analogue.

Carbon from [^{14}C]uracil was incorporated into *Staph. aureus* as uracil, thymine and cytosine and it is thus possible to measure the incorporation into the pyrimidines of both DNA and RNA in the same sample of cells. It is shown elsewhere that this incorporation can be used as a measure of the synthesis of RNA and DNA (Creaser, in preparation). It was found that 8-azaguanine, at concentrations which prevented enzyme formation, did not reduce the incorporation of radioactivity from [^{14}C]uracil into the nucleic acids of *Staph. aureus*.

Effect of glucose upon the inhibition of enzyme formation by 8-azaguanine

It has been shown previously (Creaser, 1955a) that glucose increases the stimulation of β -galactosidase formation by purines and pyrimidines. It

Fig. 2. Effect of addition of 8-azaguanine during β -galactosidase formation. Cells were forming enzyme in system A and 8-azaguanine was added at times indicated by arrows to a final concentration of 100 $\mu\text{g./ml.}$ ●, Enzyme formation in uninhibited systems; ○, enzyme formation after addition of 8-azaguanine.

Fig. 3. Effect of glucose upon inhibition of β -galactosidase formation by 8-azaguanine. ●, Effect of 8-azaguanine concentration upon β -galactosidase formation in system A in 3 hr.; ○, response when glucose was omitted from the system.

was not unreasonable to suppose therefore that glucose would enhance the effect of an unnatural purine upon β -galactosidase formation. Fig. 3 shows that a given concentration of 8-azaguanine produced a much larger inhibition of β -galactosidase formation in the presence of glucose than when glucose was omitted from the system. It was observed that the amount of 8-azaguanine incorporated into the RNA fraction was 60% less when glucose was absent.

Effect of age of culture upon inhibition of enzyme formation by 8-azaguanine

Fig. 4 shows that there is a difference in the sensitivity of β -galactosidase formation and glucozymase formation to inhibition by 8-azaguanine, in that the former process is prevented by the analogue at all stages of growth, whereas the latter synthesis is inhibited only at certain phases in the growth cycle. It seems probable that this variation in the sensitivity of glucozymase formation at different stages of growth is the reason why a constant degree of inhibition of glucozymase formation by 8-azaguanine was not observed. With *Staph. aureus* Dunn, catalase formation was always sensitive to inhibition by 8-azaguanine, thus resembling β -galactosidase formation. In the Duncan strain, however, catalase formation was similar to glucozymase formation, being inhibited by 8-azaguanine only at certain stages in the growth cycle. A further difference between the two strains with respect to catalase formation is shown in Table 1. This shows that, under identical conditions, there is a greater synthesis of catalase in strain Dunn than in strain

Fig. 4. Effect of cultural age of cells upon inhibition of enzyme formation by 8-azaguanine. Cells were grown in casein hydrolysate medium; growth is shown by the broken line, which is a logarithmic plot of optical density against time. At the times indicated samples of organism were removed, harvested and washed, and incubated in the appropriate system in the presence or absence of 300 μ g. of 8-azaguanine/ml. The percentage inhibition of enzyme formation by 8-azaguanine was calculated and the curves show the relationship between this inhibition and the cultural age of the cells. A: β -galactosidase formation; cells were grown aerobically and incubated in system A for 3 hr. B: Catalase formation in *Staph. aureus* Dunn; cells were grown anaerobically and incubated in system B for 2 hr. C: Glucozymase formation; cells were grown anaerobically and incubated in system B for 3 hr. D: Catalase formation in *Staph. aureus* Duncan; cells were grown anaerobically and incubated in system B for 2 hr.

Table 1. *Effect of 8-azaguanine upon catalase formation at different stages in the growth cycle of two strains of Staphylococcus aureus*

Cells were grown anaerobically in casein hydrolysate medium for the time shown. They were then harvested, washed and incubated for 2 hr. in system B in the presence or absence of 300 μ g. of 8-azaguanine/ml. The initial and final catalase activities were measured and the percentage inhibition of enzyme formation by 8-azaguanine was calculated. Catalase activity is expressed as Q_{O_2} at 25° (μ l. of O_2 evolved/hr./mg. dry wt. of cells). Inhibition (%) calculated as

$$100 - \left(100 \times \frac{c-a}{b-a} \right).$$

Growth period (hr.)	Catalase activity (Q_{O_2} at 25°)			Ratio (b):(a)	Inhibition of catalase formation by 8-azaguanine (%)
	(a) Initial	(b) Final	(c) Final		
		no inhibitor	+ 8-azaguanine		
<i>Staph. aureus</i> Dunn					
1	21 500	495 000	56 000	23	93
2	23 600	840 000	56 500	35.5	96
4	21 000	760 000	87 500	36	91
6	11 000	575 000	39 000	52	95
12	2 500	445 000	20 000	118	96
<i>Staph. aureus</i> Duncan					
1	250 000	565 000	340 000	2.3	71
2	314 000	640 000	574 000	2.0	20
4	210 000	560 000	560 000	2.7	0
6	292 000	550 000	561 000	1.9	0
12	78 000	200 000	165 000	2.6	29

Table 2. *Effect of purines and pyrimidines upon inhibition of β -galactosidase formation by 8-azaguanine*

Cells were incubated in system A, in the presence or absence of 100 μ g. of 8-azaguanine/ml., with the addition of the purine or pyrimidine base detailed below. After incubation for 3 hr. the β -galactosidase activity was determined and the percentage inhibition of enzyme formation by 8-azaguanine calculated. β -Galactosidase activity is expressed as enzyme units: (1 unit is the amount of enzyme liberating 1 μ m-mole of *o*-nitrophenol from *o*-nitrophenyl- β -D-galactopyranoside in 1 hr. under the conditions described by Creaser, 1955*a*).

Purine or pyrimidine added (final concn. 10 μ g./ml.)	β -Galactosidase formation (units/mg. dry wt. of cells)		Inhibition of formation by 8-azaguanine (%)
	8-Azaguanine absent	8-Azaguanine present	
Nil	1520	16	99
Adenine	572	24	96
Guanine	1720	1880	0
Xanthine	2100	1240	41
Hypoxanthine	2060	1120	46
Uracil	2040	48	98
Thymine	1720	28	98

Duncan. Furthermore, the initial catalase activity of the cells, as harvested from the growth medium, is greater in the Duncan than in the Dunn strain.

Reversal of inhibition caused by 8-azaguanine

Table 2 shows that the inhibition of β -galactosidase formation by 8-azaguanine could be reversed by the further addition of guanine or xanthine or hypoxanthine to the system. Adenine normally inhibited β -galactosidase formation when it was added as the only purine supplement to the system. On the rare occasions when it did not inhibit, adenine was much less effective than the other purines tested in reversing the effects of 8-azaguanine. Results identical with those shown in Table 2 were obtained when catalase formation was studied. The addition of either uracil or thymine did not affect the inhibition of either catalase or β -galactosidase formation by 8-azaguanine. Reversal of inhibition was still possible when guanine was added after 8-azaguanine. Fig. 5 shows that, with β -galactosidase formation, such reversal was obtained when guanine was added up to 2 hr. after the analogue. The rate of 'reversed' synthesis decreased as the time of exposure was prolonged. This decrease in rate was more marked with catalase formation, where no reversal was observed after the cells had been exposed to the analogue for 90 min. or longer.

Matthews (1954) found that the inhibition of the formation of tobacco mosaic virus by 8-azaguanine could be reversed by guanosine. When plants were treated with guanosine plus 8-azaguanine, the amount of 8-azaguanine incorporated into the virus nucleic acid was less than when 8-azaguanine was used alone. This type of effect was not found in the present work. Addition of guanine to a system, in concentration sufficient to reverse the effects of

Fig. 5. β -Galactosidase formation after addition of guanine to a system inhibited by 8-azaguanine. Cells were incubated in system A with the addition of 100 μ g. of 8-azaguanine/ml.; β -galactosidase formation in this system is shown by curve C. At times indicated by arrows guanine was added to a final concentration of 10 μ g./ml. Subsequent enzyme formation curves are described by figures showing time (hr.) of addition of guanine.

8-azaguanine, did not reduce the amount of 8-azaguanine incorporated into RNA. However, upon such reversal there was an increase in the amount of carbon from [14 C]uracil incorporated into the nucleic acid, this increase being more marked in the RNA fraction (Table 3).

It was also found that there was an optimum concentration of glucose for the reversal of 8-azaguanine inhibition by guanine, this concentration being different from that which was optimum

Table 3. *Reversal of 8-azaguanine inhibition of enzyme formation by guanine*

Cells were incubated in system *A*, in the presence of 100 μ g. of 8-azaguanine/ml. and in the absence or presence of 10 μ g. of guanine/ml. In both cases 10 μ g. of [14 C]uracil/ml. was present. After 3 hr., enzyme formation and incorporation of radioactivity were measured.

Process	In the presence of 8-azaguanine	In the presence of 8-azaguanine + guanine
Catalase formation (increase in Q_{O_2} at 25°)	30 000	428 000
β -Galactosidase formation (increase in units/mg. of cells)	22	3 132
Incorporation of carbon from [14 C]uracil into RNA (μ mg. of uracil/mg. of cells)	204	1 180
Incorporation of carbon from [14 C]uracil into DNA (μ mg. of uracil/mg. of cells)	43	137

for enzyme formation. Thus catalase formation in system *B* was greatest when the glucose concentration was 3 mg./ml. or more, whereas the reversal, by guanine, of the inhibition of catalase formation by 8-azaguanine was maximal when the glucose concentration was 1 mg./ml. It has been shown that stimulation of β -galactosidase formation by glucose is optimum when the glucose concentration in the system is 1 mg./ml. (Creaser, 1955*a*). In Fig. 5 it can be seen that the effect of guanine was greatest when the natural purine was added 30 min. after the analogue. It seems probable that in this time the metabolic activities of the bacteria had reduced the glucose concentration in the medium to a level which was optimum for the reversal, by guanine, of the inhibition of β -galactosidase formation caused by 8-azaguanine.

DISCUSSION

The phrase 'enzyme formation' has been used throughout this work because rigid proof that increases in enzymic activity correspond quantitatively to synthesis of apoenzyme protein in *Staph. aureus* is not available. However, it can be shown that enzyme formation occurs only under conditions in which protein synthesis takes place. Gale & Folkes (1953) showed that washed suspensions of *Staph. aureus* would synthesize protein only when supplied with a complete mixture of amino acids and that protein synthesis was abolished by 10 μ g. of chloramphenicol/ml.; these observations are equally true for the three enzymes whose formation has been studied in this work. Furthermore, the similarity between the degree of enzyme formation in intact cells and in the disrupted-cell system of Gale & Folkes indicates that the relationship between enzyme activity and enzyme content is not grossly disproportionate.

Matthews (1954) found that the incorporation of 8-azaguanine into the nucleic acid of tobacco mosaic virus was accompanied by a decrease in infectivity of the virus. This implies that the ability of the virus nucleic acid to promote the

synthesis of protein had been impaired by such incorporation, and it is suggested that, in the case of *Staph. aureus*, incorporation of 8-azaguanine into the RNA likewise renders it incapable of catalysing the synthesis of enzyme proteins. It has been found that the incorporation of guanine into the nucleic acid of *Staph. aureus* is not a reversible process (Creaser, unpublished work), and thus 8-azaguanine could only become incorporated into RNA when synthesis of this material was occurring. Hence inhibition of enzyme formation by 8-azaguanine in *Staph. aureus* indicates that such formation is dependent upon RNA synthesis. The observation that addition of 8-azaguanine inhibits further synthesis of catalase and β -galactosidase when added during the course of enzyme formation shows that a continuous synthesis of RNA is essential for induced enzyme formation to occur. This conclusion was arrived at previously by Pardee (1954) and Spiegelman *et al.* (1955), using different methods of approach.

It is, however, necessary to ask whether continuous RNA synthesis is essential for the formation of all enzymes. The experiments shown in Fig. 3 indicate that this is not so. Formation of β -galactosidase, and the catalase of *Staph. aureus* Dunn, is completely inhibited by 8-azaguanine at all stages of growth, and thus it seems that formation of these enzymes is always dependent upon co-synthesis of RNA. The formation of glucozymase, and the catalase of *Staph. aureus* Duncan, is resistant to inhibition by 8-azaguanine at most stages in the growth cycle, and it is therefore suggested that formation of these two enzymes is not normally dependent upon RNA synthesis, such dependence occurring only at certain stages in the growth cycle and never being complete.

It is possible that when RNA synthesis appears to be essential for enzyme formation to occur, it is because the RNA which promotes the synthesis of these particular enzymes is of short active life, and continuous new synthesis is needed for uninterrupted enzyme formation. In the work described above such a concept would mean that the RNA

responsible for the formation of β -galactosidase, and the catalase of the Dunn strain, is of short active life, whereas the RNA responsible for the synthesis of glucozymase, and the catalase of the Duncan strain, is of long active life. Such a hypothesis would accord with the results of Gale & Folkes (1955) who studied enzyme formation in disrupted *Staph. aureus* Duncan. These authors harvested their cells during the period of active growth and found that, whereas the formation of catalase and glucozymase was stimulated by staphylococcal RNA, formation of β -galactosidase was not affected by RNA but required the presence of RNA precursors.

In *Staph. aureus* the enzyme β -galactosidase is adaptive (Creaser, 1955a) and glucozymase is constitutive (Gale & Folkes, 1955). With catalase formation, the results shown in Table 1 indicate that it is adaptive in strain Dunn and constitutive in strain Duncan. Thus in cases of enzyme formation where the involvement of an RNA of short active life has been postulated the enzyme is adaptive. Where an RNA of long active life appears to promote enzyme formation the enzyme is a constitutive one. The elucidation of whether or not this apparent correlation is fortuitous or is a general phenomenon in enzyme formation must await further investigation.

SUMMARY

1. The formation of β -galactosidase and catalase by washed suspensions of *Staphylococcus aureus* Dunn is inhibited by the purine analogue 8-azaguanine. Inhibition can be observed when the analogue is added during the course of enzyme formation subsequent to induction.

2. Addition of 8-azaguanine does not diminish nucleic acid synthesis by washed suspensions of *Staph. aureus*, as measured by the incorporation of [14 C]uracil, but the analogue becomes incorporated into the cellular ribonucleic acid (RNA).

3. The formation of β -galactosidase, and of catalase in *Staph. aureus* Dunn, is almost completely inhibited by 8-azaguanine, no matter at which

stage of growth the cells used in the experiment are harvested. Formation of glucozymase and of catalase in *Staph. aureus* Duncan is much less sensitive to inhibition by 8-azaguanine, and completely insensitive at certain stages of growth.

4. Inhibition of enzyme formation by 8-azaguanine can be reversed by the further addition of guanine or xanthine or hypoxanthine to the system. When inhibition of enzyme formation by 8-azaguanine is reversed by guanine there is no diminution in the amount of 8-azaguanine incorporated into RNA, but nucleic acid synthesis, as measured by incorporation of [14 C]uracil, is increased.

5. The implications of these findings in relation to involvement of RNA synthesis in enzyme formation are discussed, and it is suggested that the formation of different enzymes is promoted by types of RNA with different lengths of active life.

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