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# SHORT COMMUNICATIONS

## The differential effects of 8-azaguanine, 5-fluorouracil, 6-mercaptopurine and 6-thioguanine on the activities of some Krebs cycle enzymes in cell-free extracts from *Bacillus cereus*

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THE MICROORGANISM, *Bacillus cereus*, has been a useful test system in attempting to elucidate the mode of action of carcinostatic drugs such as 8-azaguanine (AG),[1] 6-mercaptopurine (MP),[2] 6-thioguanine (TG)[2] and 5-fluorouracil (FU).[3]

In contrast to the potent inhibition of protein biosynthesis produced by AG in *B. cereus*,[4–6] in the presence of the thiopurines[2] proteins continue to be synthesized by these cells. However, in experiments which measured protein synthesis by following the incorporation of radioactive amino acids into protein, it was observed that TG and MP frequently produced small depressions in the incorporation of radioactivity as compared to control cells at equivalent turbidities. Moreover, both drugs inhibited flagella formation, possibly involving a decreased formation of the protein, flagellin. It was postulated therefore that an effect by these thiopurines on DNA synthesis or function without altering DNA content might account for the observed decrease in RNA synthesis. This in turn could result in alterations of the synthesis of specific proteins, although total protein content would not be drastically changed.[2]

As for FU, this analogue produced a marked inhibition of growth and DNA synthesis in *B. cereus* while RNA and protein synthesis, measured colorimetrically and by isotope incorporation, were not specifically inhibited and proceeded at the rate of turbidimetric increase of cells.[3] The RNA found in FU-inhibited cells, although present in normal amounts, is atypical in that FU has been extensively incorporated into it.[3]

Since precursor incorporation experiments involved only total bacterial protein, the effects of MP, $^{2}$TG[2] and FU[3] on the production of a single protein, penicillinase, in *B. cereus* were studied. None of these analogues was found to interfere with either the induction of, or the enzymatic activity of penicillinase. Nevertheless, based on the examination of a single enzyme, the possibility of a selective alteration in the synthesis of specific proteins could not be excluded. This study was undertaken to see if an examination of the effects of MP, TG and FU on the activities of a series of enzymes involved in a common metabolic pathway might reveal whether any of these analogues were causing alterations in the synthesis of specific proteins. For comparison, the purine analogue AG was included, since this drug markedly inhibits penicillinase production[7] and total protein biosynthesis[4–6] in *B. cereus*.

### MATERIALS AND METHODS

*B. cereus* 569H was grown on a casamino acids (Difco)–salts medium[8] in a Gyratory shaker at 37°. Additions of FU, MP and TG (final concentrations 160, 30 and 30 $\mu$M respectively) were made to 800 ml of bacterial cultures at $A_{540}$ of 0·150, as measured in a Beckman spectrophotometer model DU. Addition of AG (final concentration 160 $\mu$M) was made to 800 ml of a bacterial culture at $A_{540}$ of 0·200. Growth was measured turbidimetrically and control and drug-treated cultures were allowed to reach $A_{540}$ of 0·400, at which time the cells were harvested by centrifugation at 4° and washed with cold water. A control culture required about 65 min to grow from $A_{540}$ of 0·150 to 0·400. After the addition of MP, TG or FU, the observed times were about 90, 110 and 225 min respectively. A control culture required about 45 min to grow from $A_{540}$ of 0·200 to 0·400. After the addition of AG, the observed time was about 155 min. Washed cells were suspended in 5 ml of 0·02 M Tris buffer

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(pH 7·4) and disrupted by sonication (MSE model 60W) for 4 min at 2°. The broken suspension was centrifuged at 16,000 *g* for 30 min. The resulting supernatant fluid was divided into 1-ml portions which were frozen in a dry ice–acetone bath and stored at −20°. No noticeable differences in enzymatic activities could be detected between freshly prepared extracts and those frozen and stored at −20° for up to 3 days.

Aconitase, fumarase, malate dehydrogenase, succinic dehydrogenase and NADH oxidase were determined spectrophotometrically with a Cary 15 recording spectrophotometer in a 1-cm light path in a total volume of 1·0 ml at 30°. The amount of cell-free extract was adjusted so that the change in optical density was directly proportional to the amount of enzyme. In each experiment the assays were done in duplicate and the average was taken.

Aconitase (EC 4.2.1.3) was measured by observing the increase in absorbance at 240 mμ in the presence of citrate.[9] Because of its lability and loss of activity upon dilution, a small quantity of the cell-free extract was added directly to the test system. Fumarase (EC 4.2.1.2) was measured by observing the increase in absorbance at 300 mμ in the presence of fumarate.[10] Malate dehydrogenase (EC 1.1.1.37) was determined by measuring the oxidation of NADH at 340 mμ in the presence of oxalacetate.[11] Succinic dehydrogenase (EC 1.3.99.1) was measured by following the decrease in absorbance at 600 mμ with 2,6-dichlorophenolindophenol as an electron acceptor.[12] NADH oxidase was measured by following the decrease in absorbance at 340 mμ.[13] A unit of enzymatic activity is defined as an optical density change at the indicated wavelength of 0·001 per min. Specific activities are expressed as units per min per mg of protein. Protein was determined by the method of Lowry *et al.*[14] with bovine serum albumin as standard.

## RESULTS AND DISCUSSION

8-*Azaguanine*. As can be seen (Table 1), AG decreased the activities of aconitase, fumarase and malate dehydrogenase, whereas the activity of succinic dehydrogenase was unaffected. In addition, NADH oxidase, whose activity reflects in part the functioning of the electron carriers of the terminal respiratory chain,[15] was unchanged.

TABLE 1. EFFECTS OF ANTIMETABOLITES ON THE ACTIVITIES OF SOME KREBS CYCLE ENZYMES IN CELL-FREE EXTRACTS FROM *B. cereus**

| Preparation | Enzyme | | | | |
|---|---|---|---|---|---|
| | Aconitase | Malate dehydrogenase | Fumarase | Succinic dehydrogenase | NADH oxidase |
| Control (7) | 342 ± 51 | 3241 ± 158 | 33 ± 2·2 | 371 ± 43 | 175 ± 15 |
| 8-Azaguanine (4) | 126 ± 29[a]† | 1712 ± 158[d] | 8·4 ± 1·0[d] | 501 ± 97 | 146 ± 11 |
| 5-Fluorouracil (4) | 142 ± 26[b] | 1749 ± 39[d] | 8·1 ± 0·8[d] | 321 ± 27 | 216 ± 14 |
| 6-Mercaptopurine (5) | 406 ± 60 | 4451 ± 600[b] | 38 ± 1·7 | 400 ± 31 | 127 ± 5[b] |
| 6-Thioguanine (5) | 378 ± 33 | 5832 ± 306[d] | 37 ± 1·9 | 440 ± 41 | 102 ± 16[c] |

* Cell-free extracts were prepared from control and drug-treated cells as described in the text. The number of experiments is indicated in the parentheses. The values represent the average ± S.E.M. of the specific activities which are expressed as changes in optical density units per min per mg of protein, as defined in the text.

† Lettered superscript indicates statistical probability of no difference when compared with corresponding control activity: a = $P < 0.05$; b = $P < 0.025$; c = $P < 0.01$; d = $P < 0.005$.

It is of interest that when a cell-free extract from control cells was fractionated by differential centrifugation (105,000 *g* for 90 min) aconitase, fumarase and malate dehydrogenase were found almost exclusively in the soluble fraction, whereas succinic dehydrogenase and NADH oxidase (to a lesser extent) were localized predominantly in the particulate fraction.* AG inhibits protein synthesis in exponentially growing cells of *B. cereus*.[4–6] It may be that the synthesis of soluble enzymes of *B. cereus* is particularly sensitive to inhibition by AG compared to membrane-bound

* P. Klubes and K. L. Hartmann, unpublished results.

enzymes. Differential effects by AG on the inhibition of synthesis of the enzymes penicillinase and catalase in *B. cereus* have been shown to occur.[7]

*5-Fluorouracil.* FU decreased the activities of aconitase, fumarase and malate dehydrogenase, whereas the activities of succinic dehydrogenase and NADH oxidase were unaffected (Table 1). These results are identical to those produced by AG. This is noteworthy since FU does not produce inhibition in total protein synthesis in *B. cereus*[3] as does AG.[4-6]

Both FU[3] and AG[16, 17] are readily incorporated into the RNA of *B. cereus*. Although the inhibition in protein synthesis by AG in *B. cereus* is related to its incorporation into RNA,[4-6] the extensive incorporation of FU into RNA of *B. cereus* does not inhibit total protein synthesis.[3] Nevertheless, it is possible that the presence of FU in the RNA of *B. cereus* could interfere with the normal production of specific proteins without depressing overall protein synthesis. For example, such an effect may be related to the observations that FU prevents formation of mature ribosomes in *B. cereus*[18] and *Escherichia coli*,[19] is incorporated into messenger RNA,[20] and produces altered secondary structure in transfer RNA containing FU.[21] In addition, among the apparent consequences of the incorporation of FU into RNA are the production of altered or inactive enzymes,[22] decreased enzyme synthesis in *E. coli*,[23] and alterations in the coat protein of TMV virus mutants[24] and amber mutants of phage *R*17[25] (see Mandel[26] for a recent review).

From the available data, it is impossible to determine whether the decrease in the activities of aconitase, fumarase and malate dehydrogenase produced by FU is due to a selective inhibition in the synthesis of these enzymes, to the production of inactive or altered enzymes, or to a combination of these or other effects. Nevertheless, it seems clear that FU, which does not inhibit total protein synthesis in exponentially growing cells of *B. cereus*, can produce selective alterations in the activities of several enzymes comparable to those produced by AG. Presumably this is a consequence of the incorporation of FU into RNA which resulted in the interference, in some way, with the normal biosynthesis of specific proteins.

*6-Mercaptopurine and 6-thioguanine.* As can be seen (Table 1), the thiopurine analogues, TG and MP, produced identical alterations in enzymatic activities. Malate dehydrogenase was increased, whereas the activities of aconitase, fumarase and succinic dehydrogenase were unchanged. Furthermore, both drugs decreased the activity of NADH oxidase.

The similarity in the changes in enzymatic activities produced by the two drugs is interesting in that they produce very similar biochemical actions on nucleic acid and protein biosynthesis in *B. cereus*.[2] Both drugs selectively inhibit RNA synthesis, whereas protein and DNA synthesis are depressed in accordance with a decreased formation of cell mass. In addition, MP is apparently not incorporated into RNA or DNA of *B. cereus*,[27] while at most there is only a minute incorporation of TG into polynucleotides.[2] Therefore, any effect that these analogues might have on the synthesis of specific proteins in *B. cereus* is apparently not the result of their incorporation into DNA or RNA, although such an effect cannot be excluded absolutely.

It is of interest that TG and MP increased the activity of malate dehydrogenase, although it is not clear that the change is due to increased synthesis of the enzyme. It may be that the thiopurines are producing alterations in the synthesis of proteins so that the synthesis of some proteins is increased while that of others is decreased. This may explain the observation that when the effect of the thiopurines on protein biosynthesis in whole cells of *B. cereus* was measured by using radioactive amino acids as precursors, small drug-induced deviations from control values were frequently produced.[2] There are also indications that in other systems the analogues can exert specific inhibitory effects on the formation of certain proteins. For example, they can selectively alter the protein composition of rabbit serum[28] and the enzyme composition of tumor homogenates.[29]

Effects of MP and TG on the nucleotide coenzymes, NAD and CoA, have been reported in mammalian cells,[30, 31] but in *B. cereus* MP has no effect on the synthesis of NAD or CoA.[32] Therefore, the possibility that the increased activity of malate dehydrogenase is due to an effect by the thiopurines on the NAD coenzyme of malate dehydrogenase[33] seems unlikely.

The reason for the decreased activity of NADH oxidase produced by TG and MP is obscure. The oxidation of NADH is dependent, in part, upon the integrity of the electron carriers of the terminal respiratory chain.[15] Any alteration in the activity or concentration (or in both) of any of the enzymes or coenzymes of the chain could conceivably decrease NADH oxidation. Therefore, the observed decrease may not necessarily be due to an action of these drugs on the synthesis of a specific protein or proteins. Further studies on the effects of the thiopurines on the composition of the terminal

respiratory chain of *B. cereus* may provide an explanation as to how these drugs affect the activity of NADH oxidase.

The possibility cannot be excluded that one or more of the alterations in enzymatic activities which were observed was due to a direct effect on an enzyme by an analogue or its derivatives still present in the cell-free extract. However, in cell-free extracts from control cells, no differences were detected in the activities of aconitase, fumarase or malate dehydrogenase when AG (100 $\mu$M) or FU (100 $\mu$M) was present in the assay mixture. Similarly, no changes in the activities of malate dehydrogenase or NADH oxidase were observed when MP (100 $\mu$M) or TG (20 $\mu$M) was present in the assay mixture.

In summary, three carcinostatic drugs, TG, MP and FU, which apparently do not inhibit total protein synthesis in exponentially growing cells of *B. cereus*, have produced specific alterations in the activities of several Krebs cycle enzymes in cell-free extracts obtained from drug-treated cells. Another drug, AG, which inhibits total protein synthesis, produced changes like those seen after the administration of FU. Further studies are necessary to determine the causes of these selective alterations in enzymatic activities.

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## Effect of melittin upon cellular and lysosomal membranes*

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MELITTIN is a basic polypeptide which constitutes over 50 per cent of the dry weight of crude bee venom. The toxin is of interest because its primary structure (Fig. 1) has been directly related to its activity

Gly-ile-gly-ala-val-leu-lys-val-leu-thr-thr-gly-leu-pro-ala-leu-ile-ser-trp-lys-arg-lys-arg-gluNH$_2$-glu(NH$_2$)$_2$

p

FIG. 1. Primary structure of melittin.[2]

upon biomembranes.[1–4] Since one end of the molecule contains amino acids which are relatively hydrophilic (positions 21–26) while the rest are relatively hydrophobic (positions 1–20), melittin may be considered a biological amphipath. Indeed, we have found that melittin, like other amphipathic molecules, is capable of interacting with artificial lipid membranes arranged either as monolayers or as lamellar bilayers (liposomes).[5] The direct disruption of lipid structures by melittin may account for its well established hemolytic and inflammatory properties, particularly melittin's capacity to induce cutaneous necrosis after intradermal injection (reviewed by Habermann[1]). Since lysosomes appear to mediate inflammation induced by a number of membrane-active agents such as bacterial toxins,[6] polyene antibiotics,[7] croton oil, etc.,[8] we studied the effects of melittin upon leukocytes and lysosomes.

Rabbit polymorphonuclear leukocytes were obtained from glycogen-induced peritoneal exudates and studied by methods previously described in detail.[9] Cells were suspended in Eagle's minimum essential medium (Grand Island Biologicals) and 20% fetal calf serum, permitted to settle for 30 min on glass slides, and gently washed free of unattached cells and debris. Thereafter, either melittin (Prof. E. Habermann, Giessen) in isotonic (0·9%) saline, or saline alone, was added in equal volumes (0·15 ml). Cells and additives were incubated for 15–20 min, air-dried, and stained with tetrachrome. After 15 min of incubation, control leukocytes (Fig. 2A) appeared relatively normal with intact, multilobulate nuclei, prominent heterophile granules and sharp cell borders. When exposed to melittin ($10^{-6}$ M) for 15 min, the cells maintained their sharp cell boundaries (Fig. 2B). However, the distinct leukocyte granules could no longer be discerned and vacuoles appeared in many cells. The

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