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THE INHIBITION OF PURINE BIOSYNTHESIS *DE NOVO* IN *BACILLUS SUBTILIS* BY CORDYCEPIN

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SUMMARY

1. Cordycepin has been shown to inhibit purine biosynthesis *de novo* as evidenced by a decreased incorporation of radioactive formate into the nucleic acid purines of *Bacillus subtilis*.

2. The incorporation of radioactive 5-amino-4-imidazolecarboxamide into the purine ring was not severely inhibited by cordycepin.

3. Radioautography of an alcohol-soluble extract of *B. subtilis* which were grown with cordycepin and either radioactive formate or glycine failed to show an accumulation of any intermediates.

4. The formation of formylglycinamide ribonucleotide, which was caused to accumulate by the addition of 6-diazo-5-oxo-L-norleucine, was suppressed in the presence of cordycepin.

These results indicate that in *B. subtilis* cordycepin inhibits a step in purine biosynthesis *de novo* which is prior to the formation of glycinamide ribonucleotide.

INTRODUCTION

Previous studies with *B. subtilis* ATCC No. 10783 demonstrated an inhibition of growth by cordycepin and the reversal of this inhibition by the addition of purines[1]. AICA also prevented growth inhibition caused by cordycepin although not as effectively as preformed purines. This suggested an inhibition of nucleic acid synthesis possibly by interference with the synthesis of purines *de novo*.

This report presents a continuation of this work in which the incorporation of radioactive precursors into the purine ring of the nucleic acids of *B. subtilis* was studied in the presence and absence of cordycepin. In agreement with our earlier findings[1] the results indicate that the synthesis of purines *de novo* is inhibited in the presence of cordycepin, and the specific enzymatic site appears to involve a reaction prior to the formation of glycinamide ribonucleotide.

Abbreviations: AICA, 5-amino-4-imidazolecarboxamide; FGAR, formylglycinamide ribonucleotide; DON, 6-diazo-5-oxo-L-norleucine; GAR, glycinamide ribonucleotide.

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MATERIALS AND METHODS

The chemically defined medium of HERRIOTT[2] was used for the growth of *B. subtilis*.

Cordycepin was isolated from culture filtrates of the mold *Cordyceps militaris* according to a published procedure[3]. The compound was recrystallized twice from water before use in these studies.

[8-^{14}C]Cordycepin was isolated from a culture of *C. militaris* to which radioactive adenine had been added. 5 μC of [8-^{14}C]adenine (8.5 μC/μmole) was added to a 10-day old culture of *C. militaris* and the organism was allowed to grow for an additional two weeks. The radioactive cordycepin was isolated and purified by a published procedure[4]. The specific activity of the final product was 8750 counts per min/μmole.

Adenine, adenosine, [8-^{14}C]adenine, [2-^{14}C]AICA, [^{14}C]sodium formate, and [1-^{14}C]glycine were purchased from Calbiochem. L-Glutamine was obtained from Sigma Chemical Co. 6-Diazo-5-oxo-L-norleucine was a gift from the Parke Davis Co. *B. subtilis* ATCC No. 10783 was obtained from the American Type Culture Collection and was used exclusively in the following studies.

RESULTS

The incorporation of [^{14}C]formate

Previous studies have demonstrated the ability of *B. subtilis* to cleave cordycepin into cordycepose and adenine[1]. The specific activity of nucleic acid purines synthesized from ^{14}C-labeled precursors *de novo* is lowered in the presence of intact adenine, because the latter compound can be incorporated intact into the purines of nucleic acids[5]. For this reason in the experiments to be reported below, free adenine was added to a control vessel at a concentration equal to that of cordycepin. Any lowering of specific activity of the nucleic acid purines in the presence of a ^{14}C-labeled precursor, below that of the control could then be interpreted as being due to a block in the synthesis of purines *de novo*, and not to a dilution caused by the exogenous source of adenine provided by cordycepin hydrolysis.

A typical experiment was carried out as follows: Each of three flasks containing 1.5 liters of media were inoculated with 40 ml of a 10 h culture of *B. subtilis*. After the cultures had reached the log phase of growth, 20 μC of [^{14}C]formate (specific activity 7–12 mC/mmole) was added to each flask. No further additions were made to the first culture, while 0.12 mmoles of adenine was added to the second culture and 0.12 mmoles of cordycepin was added to the third. After incubating for 3 h the bacteria were harvested by centrifugation and washed four times with cold 5 % trichloroacetic acid. The washed residue was suspended in 1 NaOH and incubated at 30° for 15 h to hydrolyze the RNA. Neutralization with HCl was followed by precipitation with 5 % trichloroacetic acid. The precipitate was solubilized in 1 N NaOH and reprecipitated with 5 % trichloroacetic acid. This precipitate, which contained DNA, was saved for later treatment.

The supernatant fluid resulting from both trichloroacetic acid precipitations was treated with charcoal to adsorb the nucleotides and eliminate material which would interfere with subsequent paper chromatography. The solution which had been treated with charcoal was centrifuged and the supernatant fluid discarded after determining that greater than 90 % of the ultraviolet (260 mμ) absorbing material

had been adsorbed. Stirring the charcoal in a mixture of 95 % ethanol–conc. ammonia–water (5:1:24, v/v) eluted the nucleotides originally present in RNA. This eluate was evaporated to dryness and treated with 70 % perchloric acid at 100° for 1 h. The solution was adjusted to pH 7 with KOH and the resulting $KClO_4$ was removed by centrifugation after chilling in ice. Purine and pyrimidine bases were separated by descending paper chromatography on Whatman No. 3 filter paper using an isopropanol–HCl–H_2O solvent system[6]. The ultraviolet-absorbing bands corresponding to the four bases of RNA were cut from the dried chromatograms and eluted with hot water.

The precipitate obtained from the trichloroacetic acid treatment described above was heated with 70 % perchloric acid at 100° for 1 h. Potassium perchlorate formed by the addition of KOH, was removed by centrifugation after chilling in ice. The supernatant fluid, which contained the purines and pyrimidines of DNA, was chromatographed on paper as previously described.

Radioactivity was determined by duplicate plating of aliquots of the purine and pyrimidine bases eluted from the paper chromatograms. The planchets were counted in a Nuclear Chicago scaler.

Concentration of the free bases was determined by using their respective molecular extinction coefficients[7].

TABLE I

EFFECT OF CORDYCEPIN ON THE INCORPORATION OF [^{14}C]FORMATE INTO NUCLEIC ACID PURINES AND PYRIMIDINES

20 μC of [^{14}C]formate was added to each culture after reaching the log phase of growth. The addition of either adenine or cordycepin was also made at this time and the cultures were incubated for 3 h at 37°. Purine and pyrimidine bases were isolated and their specific activities determined as described in the text.

	Counts/min/μmole		
	Control	*Plus 0.12 mmoles adenine*	*Plus 0.12 mmoles cordycepin*
RNA			
Adenine	15 400	2380	640
Guanine	14 200	8050	1900
Cytosine	755	605	640
Uracil	685	690	650
DNA			
Adenine	16 300	7200	1020
Guanine	13 000	6550	2060

The specific activities of the isolated bases are given in Table I. The flask containing adenine represents the maximal amount of adenine which could arise had all the cordycepin been hydrolyzed. Although the concentration of adenine achieved in these vessels was not experimentally determined, it could not have been very great, because growth inhibition would not have been achieved under these conditions. However, cordycepin causes suppression of formate incorporation which is greater than the adenine control. This decrease in incorporation is consistent with the hypothesis that cordycepin blocks purine biosynthesis *de novo*. The effect of cordycepin

on the labeling of purines in both RNA and DNA was similar, so in subsequent experiments the purines from both nucleic acids were treated collectively.

The labeling of pyrimidines, presumably by $^{14}CO_2$ formed from formate, was not affected by cordycepin, indicating a lack of interference in the biosynthesis of pyrimidines *de novo* (Table I).

Incorporation of [8-^{14}C]adenine and [8-^{14}C]cordycepin

The results of [^{14}C]formate incorporation could be interpreted as a dilution of nucleic acid purines by either free adenine or the adenine arising from cordycepin cleavage, rather than an inhibition of purine biosynthesis *de novo*. If this were true, cordycepin would have to be a better source of purine for nucleic acids than free adenine to account for the results of formate incorporation. To test this hypothesis the incorporation of [8-^{14}C]adenine and [8-^{14}C]cordycepin into nucleic acid purines was examined.

[8-^{14}C]adenine was diluted with cold adenine to give the same specific activity as the previously prepared [8-^{14}C]cordycepin. Equal molar amounts of either compound were added to separate cultures of *B. subtilis* which were in the log phase of growth. After 3 h incubation the bacteria were harvested and washed five times with cold 5 % trichloroacetic acid (final concentration). The washed debris was heated with 50 % trichloroacetic acid for 30 min at 100 ° to solubilize the nucleic acids and precipitate protein[8]. Trichloroacetic acid was removed by several extractions with ether. An equal volume of 2 N HCl was added to the supernatant fluid and the solution was heated for 1 h at 100°. The liberated purines were separated by descending paper chromatography on Whatman No. 3 filter paper using a *n*-butanol–water–formic acid solvent (77:13:10, v/v). The specific activity was determined as described previously.

The adenine moiety of cordycepin is no better than free adenine as a source of nucleic acid purines (Table II). Therefore it seems likely that in addition to acting as a source of preformed purines, cordycepin must also be inhibiting the synthesis of the purine ring *de novo*.

Incorporation of 5-amino-4-[2-^{14}C]imidazolecarboxamide

AICA has previously been shown to reverse cordycepin inhibition[1]. The incorporation of [2-^{14}C]AICA was studied in the presence of cordycepin. If cordycepin

TABLE II

THE INCORPORATION OF [^{14}C]ADENINE AND [^{14}C]CORDYCEPIN INTO NUCLEIC ACID PURINES

The addition of [^{14}C]adenine and [^{14}C]cordycepin was made to separate cultures of *B. subtilis* which were in the log phase of growth. The cells were harvested after a 3-hour incubation period and the purine bases were liberated. The specific activities of the added [^{14}C]adenine and [^{14}C]-cordycepin were 4030 counts/min/μmole and 4200 counts/min/μmole respectively.

	Counts/min/μmole	
Purine from nucleic acids	*Plus 120 μmoles [^{14}C]adenine*	*Plus 120 μmoles [^{14}C]cordycepin*
Adenine	1010	915
Guanine	660	540

influences some reaction prior to the formation of AICA-ribonucleotide, the incorporation of [2-^{14}C]AICA into nucleic acid purine should not be effected by cordycepin.

[2-^{14}C]AICA was added to each of three 500 ml cultures of *B. subtilis* which were in the log phase of growth. No further additions were made to the first culture, while 40 μmoles of adenine and 40 μmoles of cordycepin were added to the second and third cultures respectively. After a 3 h incubation period the cells were harvested by centrifugation and washed four times with 5 % trichloroacetic acid. Purines were liberated by treating the cells with 1 N HCl at 100° for 1 h. Paper chromatography, utilizing the *n*-butanol–water–formic acid solvent previously described, was used to separate adenine and guanine.

The specific activities of the isolated purines are given in Table III.

TABLE III

THE EFFECT OF CORDYCEPIN ON THE INCORPORATION OF 5-AMINO-4-[2-^{14}C]IMIDAZOLECARBOXAMIDE

2 μC of [2-^{14}C]AICA (3.5 μC/μmole) was added to log phase cultures of *B. subtilis*. Adenine and cordycepin were also added and the cultures were grown for 3 h. Details of the experiment are given in the text.

	Counts/min/μmole		
	Control	*Plus 40 μmoles adenine*	*Plus 40 μmoles cordycepin*
Adenine	615	18	162
Guanine	556	24	152

The difference in specific activities of purines derived from cells grown in the presence of cordycepin and the control is assumed to be a reflection of the cleavage of some cordycepin to free adenine. However, these results are the inverse of those obtained when [^{14}C]formate was used as a purine precursor. The incorporation of [^{14}C]formate was decreased by the addition of cordycepin in excess of the adenine control whereas the incorporation of [2-^{14}C]AICA is greater in the presence of cordycepin than in the adenine control. This evidence is consistent with a site of action of cordycepin inhibition which is prior to the formation of AICA-ribonucleotide.

Radioautography of radioactive intermediates

In earlier work an attempt was made to detect ultraviolet-absorbing intermediates which might have accumulated in the presence of cordycepin. One approach for identifying early intermediates which do not absorb ultraviolet light involves growing the organism in the presence of both the inhibitor and a radioactive purine precursor such as [^{14}C]formate. Any accumulated intermediate resulting from inhibition can subsequently be identified by radioautography. Preliminary evidence for the site of action of a number of inhibitors has been obtained by using such a technique[9,10].

The methods employed in this experiment are basically those outlined by TOMISEK *et al.*[9]. A 1 liter culture of *B. subtilis* which was in the log phase of growth was subdivided into three smaller cultures, each containing 200 ml. No addition was made

to the first culture while 80 μmoles of adenine was added to the second and 80 μmoles of cordycepin to the third. After incubation at 37° for 15 min, 20 μC of [^{14}C]formate was added to each flask. The cultures were incubated for an additional 20 min, chilled by the addition of ice, and rapidly harvested by centrifugation. The packed cells were extracted with boiling 70 % alcohol for 5 min. After cooling, the debris was removed by centrifugation and the supernatant fluid evaporated to dryness. The small residue remaining after evaporation was dissolved in 0.5 ml of water and 0.02 ml placed on Whatman No. 1 filter paper for chromatography in two dimensions. Following descending chromatography in water saturated with phenol, the paper was chromatographed in the second direction with butanol–propionic acid–water[11]. Radioautography was carried out on these chromatograms using Kodak non-screen x-ray film with exposure time varying from five days to two weeks.

The individual areas of radioactivity on the chromatogram were not identified. However, there was no detectable difference between radioautograms of control cultures and those grown with either cordycepin or adenine, other than a general lowering of intensity in all radioactive areas of the latter two. The accumulation of a specific area of radioactivity on the radioautogram resulting from the presence of cordycepin was not observed. Similar results were obtained when [1-^{14}C]glycine was used in place of [^{14}C]formate.

The total amount of radioactivity in the alcohol-soluble extract of the control was 337 000 counts/min while the radioactivity in the presence of adenine and cordycepin was only 84 000 and 96 500 counts/min respectively. In spite of the fact that cordycepin appears to be acting in a manner similar to adenine, it should be recalled that cordycepin was exposed to *B. subtilis* for only 35 min. In the light of previous experiments, the conversion to free adenine during that period must have been extremely small.

An inhibition of any reaction following the incorporation of either formate or glycine should have caused an accumulation of a radioactive intermediate. Since this was not observed it was assumed that cordycepin might be inhibiting a reaction prior to the incorporation of either of these precursors. This assumption is consistent with the decreased amount of radioactivity in the alcohol-soluble extract of cells grown with cordycepin. Further evidence that cordycepin was inhibiting one of the first three reactions in purine biosynthesis is given in the following experiment.

Inhibition of formylglycinamide ribonucleotide formation

The amination of FGAR has been shown to be strongly inhibited by DON (ref. 12). This compound is a potent growth inhibitor of *E. coli* and it has been shown to cause a large intracellular accumulation of FGAR[10]. When bacteria are incubated in the presence of this inhibitor and [^{14}C]glycine the FGAR which accumulates is radioactive. Inhibition of any reaction prior to FGAR formation will result in a decrease in the amount of radioactivity incorporated into FGAR. HENDERSON[13] used a similar technique to examine feed-back inhibition of purine biosynthesis in ascites tumor cells.

Preliminary studies were made to determine the concentration of DON to be used in these experiments. It was found that the lowest level of DON which still caused complete inhibition of *B. subtilis* growth was 5 μg/ml.

B. subtilis cells were obtained by harvesting 3 liters of a culture which was in the

log phase of growth. These cells were resuspended in 200 ml of fresh medium containing 20 mg of glutamine. The later compound was added because it was shown to stimulate the production of FGAR[13]. Following 2 h growth, 35 ml aliquots of the culture were placed in three separate 125 ml erlenmeyer flasks. Nothing was added to the first culture while 14 μmoles of cordycepin was added to the second and 14 μmoles of adenosine to the third. After shaking for 5 min at 37°, 175 μg of DON and 3 μC of [1-^{14}C]glycine was added to each flask. The cultures were incubated for one hour after which time the cells were harvested by centrifugation and extracted with 2 ml of 2.5 N $HClO_4$. This extract was neutralized with KOH and after chilling in ice and centrifuging, the clear supernatant fluid was placed on a Dowex-1–formate column 40 mm high $\times$ 8 mm^2. 30 ml of 0.5 M formic acid was used to elute radioactive glycine and any trace of glycinamide ribonucleotide which might have been present. FGAR was eluted with 15 ml of 4.0 M formic acid. Aliquots of this eluate were plated and counted to obtain the total amount of radioactivity in the FGAR fraction.

The effect of cordycepin and adenosine is shown in Table IV. Cordycepin inhibited the formation of FGAR to a greater extent than adenosine, a compound known to act as a feedback inhibitor in a similar system[13].

TABLE IV

THE EFFECT OF CORDYCEPIN ON FGAR ACCUMULATION IN THE PRESENCE OF DIAZO-OXO-NORLEUCINE

Freshly harvested *B. subtilis* cells were resuspended in media containing glutamine (0.1 mg/ml) and allowed to grow for 2 h. Adenosine and cordycepin were added and the cultures were incubated for an additional 5 min. 3 μC of [1-^{14}C]glycine and 175 μg of diazo-oxo-L-norleucine (5 μg/ml) were added and the cultures were further incubated for 1 h. The packed cells, which were harvested by centrifugation, were extracted with 2.5 N $HClO_4$ and FGAR was isolated as described in the text.

	Total counts in FGAR fraction
Control	42 000
Plus 14 μmoles adenosine	9600
Plus 14 μmoles cordycepin	4600

The elution of FGAR from Dowex-1–formate X8 was carried out according to a procedure developed with known compounds[13]. Authentic FGAR was not available for chromatography but portions of the FGAR fractions obtained in these experiments were chromatographed on paper after adsorption on charcoal. The chromatograms, which were subjected to radioautography, revealed that the radioactive material was homogeneous in three different solvent systems. Furthermore, the radioactivity obtained from FGAR fractions of the control, the adenosine culture, and cordycepin culture, migrated with the same R_F in each solvent system and was separate from any ultraviolet-absorbing material.

The decreased incorporation of [^{14}C]glycine into FGAR reflects an inhibition of one of the first three reactions in purine biosynthesis.

If the conversion of GAR to FGAR was blocked by cordycepin, GAR should accumulate. This compound would have been detected in radioautograms of the alcohol-soluble extract of bacteria grown in the presence of [1-^{14}C]glycine and cordy-

cepin. Since this was not observed, it is assumed that cordycepin exerts its growth-inhibiting effect on either the first or second reaction in purine biosynthesis. An examination of the effect of cordycepin on these two reactions is now being carried out at an enzymatic level.

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REFERENCES

1 F. ROTTMAN AND A. J. GUARINO, *Biochim. Biophys. Acta*, 80 (1964) 632.
2 R. M. HERRIOTT AND J. L. BARLOW, *J. Gen. Physiol.*, 36 (1952) 17.
3 N. M. KREDICH AND A. J. GUARINO, *Biochim. Biophys. Acta*, 41 (1960) 363.
4 N. M. KREDICH AND A. J. GUARINO, *Biochim. Biophys. Acta*, 47 (1961) 529.
5 M. E. BALIS, D. H. LEVIN, G. B. BROWN, G. B. ELION, H. VANDERWERFF AND G. H. HITCHINGS, *J. Biol. Chem.*, 196 (1952) 729.
6 G. R. WYATT, *Biochem. J.*, 48 (1951) 584.
7 G. R. WYATT, in E. CHARGAFF AND J. N. DAVIDSON, *The Nucleic Acids*, Vol. 1, Academic Press, New York, 1955, p. 243.
8 R. C. WOOD AND E. STEERS, *J. Bacteriol.*, 77 (1959) 760.
9 A. J. TOMISEK, H. J. KELLY AND H. E. SKIPPER, *Arch. Biochem. Biophys.*, 64 (1956) 437.
10 A. J. TOMISEK AND M. R. REID, *J. Biol. Chem.*, 237 (1962) 807.
11 A. A. BENSON, J. A. BASSHAM, M. CALVIN, T. C. GOODALE, V. A. HAAS AND W. STEPKA, *J. Am. Chem. Soc.*, 72 (1950) 1710.
12 B. LEVENBERG, I. MELNICK AND J. M. BUCHANAN, *J. Biol. Chem.*, 225 (1957) 163.
13 J. F. HENDERSON, *J. Biol. Chem.*, 237 (1962) 2631.