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MECHANISM OF INHIBITION OF *CRITHIDIA FASCICULATA* BY ADENOSINE AND ADENOSINE ANALOGS*

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Abstract—Both adenosine and 2'-deoxyadenosine are inhibitory to the growth of *Crithidia fasciculata*. Half-maximal inhibition is caused by 0.3 mM adenosine. Effective reversal of the inhibition is brought about by uridine or cytidine; uracil has little effect. This difference may be attributed to inhibition of 5-phosphoribosyl-1-pyrophosphate (PRPP) synthetase by adenosine nucleotides (especially ADP) formed inside the cell, thus interrupting pyrimidine nucleotide biosynthesis, either *de novo* or from exogenous uracil. Uridine, on the other hand, is converted to the ribonucleotide by uridine kinase and thus bypasses the block. The adenosine analogs tubercidin and cycloadenosine are the most potent inhibitors of the growth of *C. fasciculata* so far tested. This inhibition of growth is reversed by pyrimidine nucleosides and with adenosine.

Adenosine (or its nucleotides) has been found to be an inhibitor of the growth of a variety of cell types. In *Neurospora crassa*, deamination of cytidine to uridine is affected [1]. Inhibition of *Aerobacter aerogenes* [2] and *Micrococcus sodonensis* [3, 4] is brought about by interference with the biosynthesis of thiamine [5, 6], while in *Staphylococcus aureus* biosynthesis of thymidine is the site of action [7]. Interconversion of adenine nucleotides to guanine nucleotides was affected in *Bacillus subtilis*. Evidence for interference with the biosynthesis of pyrimidines has been found in fibroblasts, lymphoid cells [8, 9] and *Drosophila* cells [10]. Mouse L-cells can be made sensitive to adenosine in the presence of 2'-deoxycoformycin [11], an inhibitor of adenosine deaminase [12]. Other sites of action of adenosine are on gluconeogenesis [13], cyclic AMP metabolism [14–17] and protein synthesis [18]. Synthesis of 5-phosphoribosyl-1-pyrophosphate (PRPP) is inhibited by adenosine [19] as well as by 3'-deoxyadenosine [20]. 2'-Deoxyadenosine is also inhibitory to the growth [21, 22] of Ehrlich ascites tumor cells but the effect is reversed by adenosine.

Two analogs of adenosine, tubercidin (7-deazaadenosine, 4-aminopyrazolo-(2,3-*d*)-pyrimidine β-D-ribofuranoside) and cycloadenosine [9-(β-DL-2α, 3α-dihydroxy-4β-[hydroxymethyl]-cyclopentyl)-adenine] (Fig. 1), are highly toxic to a number of different types of cells. Tubercidin was found to be incorporated into both RNA and DNA of fibroblasts and nucleic acids of vaccinia and Mengo viruses [23]. Inside cells, tubercidin is present largely as the triphosphate [23–26] in erythrocytes or in *Schistosoma mansoni* and ATP levels are much reduced [25]. Its effects on *Streptococcus faecalis* are reversed by adenosine, deoxyadenosine, uridine, ribose-5-phosphate, pyruvate and certain amino acids [27]. In the malarial parasite *Plasmodium knowlesi*, tubercidin completely inhibits incorporation of adenosine or orotate into RNA [28].

In mammalian cells, tubercidin has a number of effects on RNA metabolism. As a result of its incorporation, ribosomal RNA maturation is inhibited [29] and methylation of tRNA is affected [30]. Methylation of histones is inhibited [31] possibly through the formation of *S*-tubercidinyl-homocysteine [32]. Tubercidin has also been tested in schistosome infections in monkeys [33].

Cycloadenosine has been studied chiefly in H: Ep. #2 cells and mutants of this cell line in culture. It is largely converted to nucleotides, but not incorporated into nucleic acids. It inhibits *de novo* biosynthesis of purines, but less effectively than adenosine [34]. The analog also interferes with guanine metabolism [35]. It may also have toxic effects as such, since mutant cells incapable of forming the nucleotide (lacking adenosine kinase) are still somewhat sensitive [36].

Both adenosine and deoxyadenosine are inhibitory to the growth of *Crithidia fasciculata*, but are capable of replacing adenine as a purine source required for growth, given sufficient time or provided a pyrimidine nucleoside is present. The adeno-

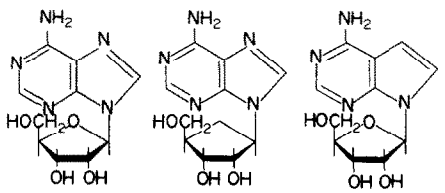

Fig. 1. Structures of adenosine, tubercidin and cycloadenosine.

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sine analogs are the most potent inhibitors of the growth of *C. fasciculata* so far studied. The effects of all of the compounds are reversed by pyrimidine nucleosides, but not by uracil. It is proposed that these compounds act, after phosphorylation, primarily by inhibiting synthesis of PRPP, although the analogs have other sites of action as well.

MATERIALS AND METHODS

C. fasciculata was grown in medium 2 of Kidder and Dutta [37] in which the high level of folate was replaced by biopterin plus folate, each at 0.01 μg/ml. Adenine was omitted and replaced by other purines. Pyrimidines and their derivatives were also added as indicated. After 4 days of incubation at 25°, growth was measured as turbidity in a Lumetron colorimeter with a 650-nm filter. All growth experiments were carried out in triplicate and repeated a number of times.

In other experiments cells were grown in a medium having a low purine content (purine-depleted cells). After harvesting by centrifugation and washing with Hanks' solution plus glucose (0.14 M), the cells were suspended in the same solution to give an absorbance of 0.688 (160×10^6 cells/ml). To 0.9 ml of cell suspension, radioactive substrates (adenosine[8-^{14}C], AMP[8-^{14}C] or AMP[^{32}P]) were added at the desired concentration and the volume was made up to 1.0 ml with H_2O. After incubation at 22° for various periods of time, the cells were removed by centrifugation and extracted for 20 min with 0.5 ml of 2 M perchloric acid, which was then neutralized with KOH. The uptake of substrates was determined by plating aliquots of the supernatant fluid on stainless steel planchets and counting in a Tracerlab ultrathin-window scaler; the results were corrected for self-absorption. A similar technique was used to study the effect of uridine or uracil on the transport of adenosine, except that incubation was for 5 sec.

Chromatography on Whatman No. 1 paper (descending) using either iso-butyric acid–ammonia–H_2O (66:1:33, v/v) or *n*-butanol–acetic acid–H_2O (20:3:7, v/v) was used to separate the compounds found in the cell extracts. They were identified by cochromatography with unlabeled standards, located by viewing under ultraviolet light. Labeled compounds were located by running strips in a Tracerlab 4π scanner and quantification was by planimetry.

Enzymes of the pyrimidine biosynthetic pathway were assayed as described previously [38] and tested for inhibition by adenosine.

To test for the effects of adenosine and tubercidin on the synthesis of PRPP, cells were grown in 400 ml of complete medium for 2 days, harvested by centrifugation, washed in 0.02 M phosphate buffer, pH 7, then divided and resuspended in three types of media (250 ml each). These were: A, complete medium; B, complete medium plus tubercidin at 0.1 mM; and C, complete medium plus adenosine at 3 mM. The cell concentrations in these media were about 60-fold greater than those at the initiation of growth experiments. After incubation at 25° for 5 hr, the cells were recovered by centrifugation, washed in 0.02 M phosphate buffer, pH 7, resuspended, each in 2 ml of buffer, and sonicated with a Wave Energy Ultra Systems tip two or three times, 1 min each, with cooling between. The sonicates were centrifuged for 1 hr at 40,000 *g* to eliminate particle-bound phosphatases and tested for PRPP in an assay mixture containing 40 nmoles of [8-^{14}C]guanine, 1 m-mole of phosphate buffer, pH 6.5, 0.2 m-mole Mg^{2+} and 0.03 ml of cell extract in a total volume of 0.13 ml. After incubation at 35° for 10 min, the reaction was stopped by the addition of a drop of glacial acetic acid. Controls for guanine phosphoribosyltransferase activity were run with 70 nmoles PRPP added. The reaction mixtures were streaked on strips of Whatman No. 1 paper and subjected to electrophoresis using formic acid–H_2O (8:300) as electrolyte for 1.5 hr at 400 V. Results were quantified as described above.

Guanine was used in the assay for PRPP for three reasons. Adenine gives rise to both AMP and IMP in *C. fasciculata* extracts, the guanine phosphoribosyltransferase is the most active enzyme of this type in the cell, and GMP is less readily degraded than other nucleotides. Use of labeled orotate leads to the formation of OMP, UMP, uridine and uracil in cell extracts. The addition of PRPP to these cell extracts was used to demonstrate that there was no inhibition of GPRTase.

Growth experiments with tubercidin and cycloadenosine were carried out as described above for adenosine.

The purines and pyrimidines used were products of either Schwarz Laboratories, Orangeburg, NY or Sigma Chemical Co, St. Louis, MO. Adenosine[8-^{14}C], 48 mCi/m-mole, guanine[8-^{14}C], 58 mCi/m-mole, and AMP[8-^{14}C] (10 mCi/m-mole) were obtained from Schwarz/Mann, Orangeburg, NY and ATP[α-^{32}P], 10.3 mCi/m-mole, from Amersham/Searle, Arlington Heights, IL. To obtain AM[^{32}P], the ATP[α-^{32}P] was hydrolyzed by boiling for 8 min in 1 N HCl. The ^{32}P-labeled AMP was purified by chromatography in the two solvents mentioned above. Tubercidin was purchased from CalBiochem, La Jolla, CA and cycloadenosine (Fig. 1) was a gift of Dr. D. L. Hill.

RESULTS

Adenosine

As may be seen from Fig. 2, adenosine is inhibitory to the growth of *C. fasciculata* with 50 per cent inhibition occurring at 80 μg/ml (0.3 mM). Deoxyadenosine (Fig. 3) is two to three times more inhibitory than adenosine. AMP on the other hand produces only about 20 per cent inhibition at 0.77 mM. Neither inosine nor guanosine causes inhibition at similar concentrations. Also shown in Fig. 2 is the relative ineffectiveness of uracil and deoxycytidine as compared to cytidine or uridine. Although cytosine cannot be utilized by the flagellate, uracil is active metabolically as shown by its ability, in the presence of hypoxanthine, to counteract the inhibition produced by allopurinol [39]. Inhibition by deoxyadenosine is also relieved by uridine and cytidine in relatively high concentrations (Fig. 3). The pyrimidine deoxyribonucleosides are less effec-

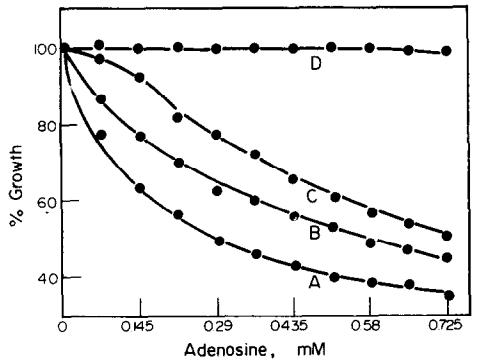

Fig. 2. Dose-responses of *C. fasciculata* to adenosine in the presence of adenine, 50 μg/ml. Key: (A) no additions; (B) uracil, 20 μg/ml; (C) deoxycytidine, 80 μg/ml; and (D) cytidine or uridine, 40 μg/ml. Absorbance of control cultures, 0.7 to 0.8.

tive. Pyrimidine ribonucleotides were less active than the ribonucleosides, probably because they are only slowly dephosphorylated before being taken up by the cells as ribonucleosides.

Growth studies show that the effect of adenosine is not to slow the growth rate, but to induce a prolonged lag phase proportional to the adenosine concentration. When growth starts the rate is approximately the same as that with guanine as a source of purine, a doubling time of about 8 hr.

When cells are incubated with labeled adenosine in order to determine its fate inside the cell, it is found that, at relatively low concentrations of adenosine (50 μM), the only labeled compounds in cell extracts were AMP, ADP and ATP and, although all the adenosine had disappeared from the medium, some hypoxanthine had been produced and appeared in the medium (Fig. 4). Free adenine was never found in cell extracts and appeared occasionally in the medium, in traces only. When higher concentrations of adenosine were administered (Fig. 5), again only the three nucleotides were present in cell extracts. The concentration of AMP rose gradually, that of ADP rose steeply, and ATP remained constant as the external adenosine concentration increased.

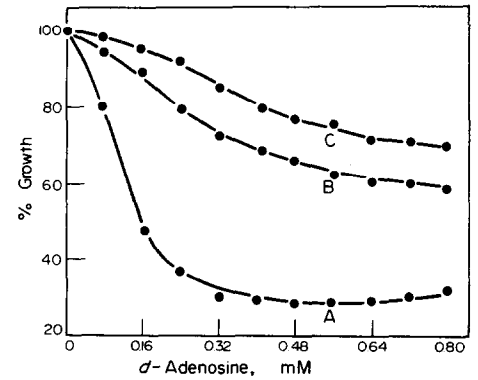

Fig. 3. Dose-responses of *C. fasciculata* to deoxyadenosine in the presence of adenine, 50 μg/ml. Key: (A) no addition; (B) uridine (or cytidine), 100 μg/ml; and (C) uridine (or cytidine), 200 μg/ml. Absorbance of control cultures, 0.7 to 0.8.

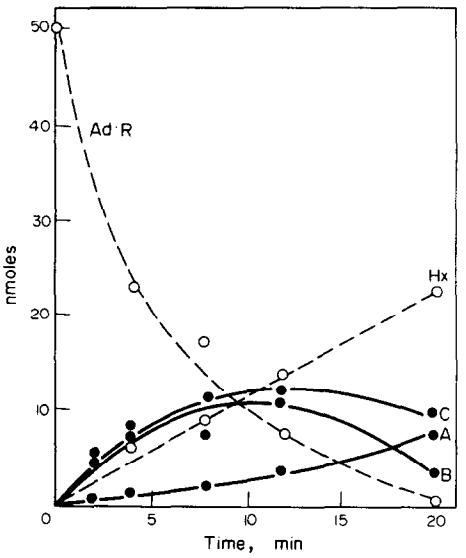

Fig. 4. Time course of adenosine[8-^{14}C] uptake and metabolism by *C. fasciculata* (see Materials and Methods). Broken lines and open circles indicate compounds found in the supernatant fluid. Solid lines and closed symbols indicate compounds in total cell extracts. Key: (A) AMP; (B) ATP; and (C) ADP.

When AMP[8-^{14}C] was supplied to the cells the radioactivity slowly disappeared from the medium and appeared in the cells as nucleotides. Remaining in the medium were AMP, adenosine, adenine and hypoxanthine, in varying amounts at different times. While it was clear that these degradation products of AMP were being taken up and converted to nucleotides in the cell, it was not certain that AMP itself was or was not entering the cells as such. Use of ^{32}P-labeled AMP showed that it was not.

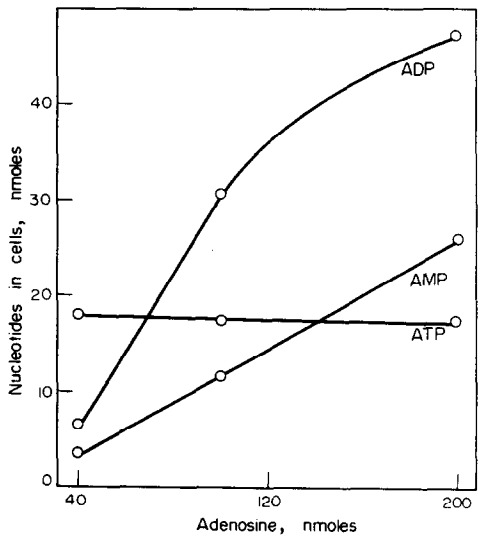

Fig. 5. Production of intracellular nucleotides in *C. fasciculata* (total cells) in response to increasing amounts of adenosine[8-^{14}C] in the incubation medium. Incubation was for 20 min; chromatography was done in iso-butyric acid–ammonia–H_2O. No guanine-containing compounds were observed.

Table 1. Analysis of cell-free extracts for 5-phosphoribosyl-1-pyrophosphate from cells incubated with either adenosine or tubercidin

Additions to incubation medium	Guanosine monophosphate formed* (nmoles) Expt. 1	Expt. 2
None	11.5	36.9
Adenosine, 3 mM	4.0 (66%)	12.8 (65%)
Tubercidin, 0.1 mM	2.7 (76%)	15.3 (59%)

* Numbers in parentheses equal the decrease in guanosine monophosphate formed.

Radioactivity did not disappear from the medium and none appeared in the cells.

It was found that uridine or uracil did not inhibit the transport of adenosine[8-^{14}C].

Experiments on the enzymes of the pyrimidine biosynthetic pathway which could be tested in *C. fasciculata* [38] showed that adenosine *per se* had no effect on their activity.

Incubation of cell suspensions with either adenosine or tubercidin greatly reduced the amount of PRPP for synthesis of GMP as compared to controls incubated in their absence (Table 1).

Tubercidin

Tubercidin causes 50 per cent inhibition of the growth of *C. fasciculata* at a concentration of 0.024 μg/ml (8.9×10^{-8} M, average of ten experiments) and is by far the most inhibitory substance so far tested. Similar inhibition by 6,7-diphenylpteridine was at 2.2×10^{-5} M [40], by 4-phenoxy-2,6-diaminopyridine at 2.8×10^{-5} M [41] and by 4-aminopyrazolo-(3,4-*d*)-pyrimidine at 1.4×10^{-4} M [42].

As shown in Fig. 6, the compounds which are most effective in the reversal of inhibition by tubercidin are uridine (or cytidine) and adenosine. Adenosine alone cannot be used above a concentration of 50 μg/ml (0.2 mM) because of its inhibitory effect. In the presence of pyrimidine ribonucleosides, however, this inhibition is reversed and

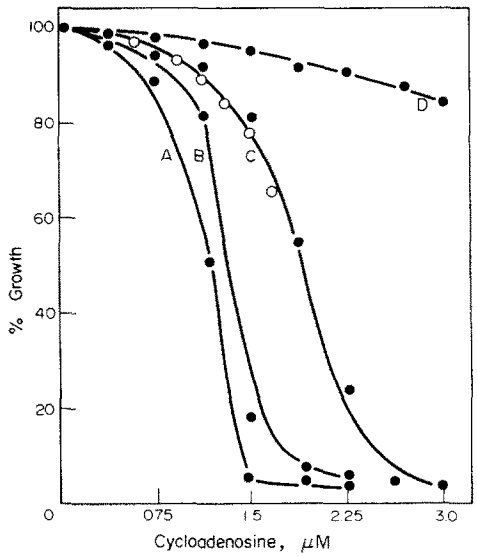

Fig. 7. Dose-response of *C. fasciculata* to cycloadenosine in the presence of adenine, 50 μg/ml. Key: (A) no additions; (B) adenine, 40 μg/ml; (C) guanine or hypoxanthine, 40 μg/ml (closed circles), or uridine, 10 μg/ml (open circles); and (D) guanine, 40 μg/ml, + uridine, 10 μg/ml.

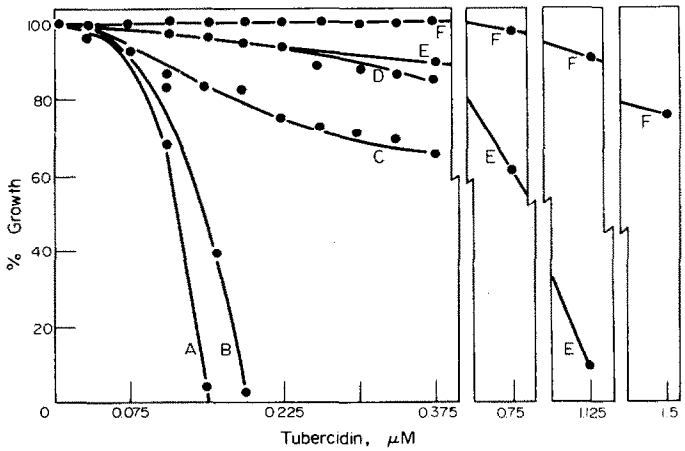

Fig. 6. Dose-response of *C. fasciculata* to tubercidin in the presence of adenine, 50 μg/ml. Key: (A) no additions; (B) uracil, 40 μg/ml; (C) adenosine, 50 μg/ml; (D) cytidine, 80 μg/ml; (E) uridine, 80 μg/ml; and (F) uridine, 80 μg/ml, + adenosine, 100 μg/ml.

in the presence of both, inhibition by tubercidin is overcome. Neither guanosine nor inosine was active in reversal.

Cycloadenosine

Cycloadenosine is somewhat less inhibitory than tubercidin. Half-maximal inhibition occurs at a concentration of 0.31 μg/ml (1.2×10^{-6} M, average of eight experiments). If only one isomer of DL-cycloadenosine were active, this value would be 6×10^{-7} M. Figure 7 illustrates that, while adenine has little effect on the inhibition, both guanine and uridine are effective in reversal. Both hypoxanthine and xanthine and their ribonucleosides are as active as guanine, and cytidine is as active as uridine (not shown). Adenosine (50 μg/ml) gives complete reversal. A combination of guanine and uridine gives nearly complete reversal (Fig. 7).

DISCUSSION

The mechanisms by which adenosine produces inhibition can be seen to depend upon the organism used to study it. Those mechanisms which are applicable in the case of bacteria autotrophic for purines and thiamine [2–6] do not apply to *C. fasciculata*, which requires an exogenous supply of both. Interference with interconversion of purines [43] can also be discarded, since guanine and hypoxanthine had no effect on the inhibition. Implications of interference with pyrimidine metabolism have been found in a bacterium [7], a fungus [1] and mammalian cells [8, 9, 19] as well as in *C. fasciculata*.

In the case of a thymineless mutant [7], both adenosine and uridine were inhibitory to growth and the site of action was on conversion of thymine to thymidine or thymidyate. Adenosine inhibition in *Drosophila* also appears to involve deoxyribonucleotide metabolism [10]. The inferior activity of deoxyribonucleosides as compared to ribonucleosides in reversing inhibition of *C. fasciculata* (Fig. 2) would seem to eliminate direct involvement of deoxyribonucleotide metabolism in the flagellate. The ability of either uridine or cytidine to bring about complete reversal removes the possibility that adenosine interferes in their interconversion as it does in *Neurospora* [1]. This fact also appears to eliminate an effect of adenosine on cAMP levels.

Green and Chan [8] attribute the toxicity of adenosine in mammalian cells to the fact that these cells are relatively poor in adenosine deaminase. This is borne out by the increased sensitivity of L-cells [11] to adenosine in the presence of an inhibitor of adenosine deaminase. *C. fasciculata* lacks this enzyme entirely [44]. Degradation of adenosine to non-inhibitory products would have to proceed by means of a ribonucleoside hydrolase, a relatively weak activity [45], and further conversion of the adenine to hypoxanthine by adenine deaminase [44], a much more active enzyme. Neither adenine nor hypoxanthine is inhibitory at concentrations comparable to those at which adenosine inhibits.

Adenosine *per se* was not inhibitory to the enzymes of the early pyrimidine biosynthetic pathway studied previously [38]. Interruption of this pathway should be bypassed by uracil as is the inhibition by allopurinol [39]. Furthermore, Ishii and Green [9] found that orotate accumulated in the presence of adenosine, that the conversion of aspartate to UMP was inhibited 80–85 per cent, and that there was inhibition of orotate phosphoribosyl-transferase. They likewise found that uridine was the most effective compound for reversal of inhibition. Allison *et al.* [19] found PRPP levels in lymphoblasts reduced in the presence of adenosine.

Examination of the metabolism of labeled adenosine by *C. fasciculata* showed that no detectable amounts of adenosine accumulate in the cell, even at extracellular concentrations capable of causing inhibition (Fig. 5). The only compounds present in cell extracts were the mono-, di- and triphosphates of adenosine. This indicates that it is these compounds which are the actual cause of inhibition of growth. This is in accord with the observation of Ishii and Green [9] that mutant cells lacking adenosine kinase are not inhibited by adenosine.

It will be noted (Fig. 5) that in the presence of inhibitory concentrations of adenosine large amounts of ADP are accumulated as well as substantial amounts of AMP, while ATP remains constant. Under these conditions, the adenylate energy charge of the cell is low, 0.45 at the 0.2 mM level of adenosine. This situation tends to slow down ATP-utilizing biosynthetic reactions [46]. More specifically, ADP has been shown to be a potent inhibitor of PRPP synthetase [47–49]. The other nucleotides are less inhibitory, but appear to have an additive affect [49].

Cells of *C. fasciculata* have been found to contain 0.20 to 0.35 μmole ATP/10^9 cells [50]. The cell suspension used contained 0.24×10^9 cells or 48–84 nmoles ATP. Since at 20 min the cells had taken up 76 nmoles adenosine, it would require approximately 100 nmoles ATP to produce the amounts of AMP and ADP present. This would have used up all of the ATP present as well as some newly formed during the incubation. It would appear that the rate of energy-linked production of ATP cannot keep up with its utilization by adenosine kinase. While some of the labeled ATP could arise through the nucleoside diphosphokinase reaction, some could have been formed by energy-linked phosphorylation of the labeled ADP. In addition to the relatively large amounts of labeled ADP, it is clear that even larger amounts of unlabeled ADP must also be present, formed from the unlabeled ATP already in the cells before incubation with adenosine. The inhibition of orotate phosphoribosyl-transferase [9], therefore, could be due to a deprivation of one of its substrates (PRPP) allowing the other (orotate) to accumulate. This would explain why uridine or cytidine is so much superior to uracil in reversing inhibition; their utilization by the cell would not require PRPP. Direct evidence of the inhibition of PRPP synthesis by adenosine and tubercidin was obtained by analyzing cell-free extracts for PRPP after incubation in the presence of these compounds. As may be seen from Table 1, this treatment drastically reduced the amount of PRPP available for conversion of guanine to GMP. These values may be too low inasmuch as unlabeled purine and pyrimidine bases may have

been present in the cell-free extracts thus using up some of the PRPP present.

Escape of *C. fasciculata* from inhibition at the end of the lag phase in the presence of adenosine must represent a conversion of the excess nucleotides to noninhibitory products by phosphatases, ribonucleoside hydrolases and by adenine deaminase. With the small inoculum of cells used in growth experiments, this could take a much longer time than with the relatively dense cell suspensions used with radioactive adenosine.

Experiments with the two types of labeled AMP were carried out to determine why this compound is so much less inhibitory than is adenosine. It was found that ^{32}P-labeled AMP did not transfer radioactivity to the cells, although radioactivity from AMP[8-^{14}C] was readily detected. Since much of the AMP[8-^{14}C] remained as such in the suspending medium, it appears that its degradation by phosphatases or 5'-nucleotidase is relatively slow and probably rate limiting in the series phosphatase, hydrolase, deaminase.

Deoxyadenosine appears to have inhibitory effects, in addition to that exhibited by adenosine, which are not reversed by uridine. A similar difference is found with mouse L-cells in the presence of 2'-deoxycoformycin; adenosine is cytostatic while deoxyadenosine is cytocidal [11]. This could be a result of the inhibition of DNA synthesis as observed by Klenow [21, 22].

Tubercidin has been found to be incorporated into nucleic acids by mouse L-cells and Mengo virus [23] by *S. faecalis* [27] and by *Sch. mansoni* [25]. It causes inhibition of DNA, RNA and protein synthesis in L-cells [23] and in the malarial parasite, *P. knowlesi* [28] and inhibition of ATP formation in *Sch. mansoni* [25]. It is not a substrate for adenosine phosphohydrolase [25, 51], the ribonucleoside hydrolase of *C. fasciculata* [45] or adenosine deaminase [12]. It has also been found to inhibit methylation of uracil residues in tRNA [52]. The efficacy of adenosine and uridine in reversal of the inhibition of *S. faecalis* is attributed to their contribution of ribose-1-phosphate [27]. In the presence of tubercidin the cells are unable to utilize glucose, but can use pyruvate or ribose-5-phosphate. While it is possible that the ribose-1-phosphate is used as a source of energy, it is also possible that it is isomerized to ribose-5-phosphate and used for PRPP synthesis. In *C. fasciculata*, ribonucleosides are converted to base plus ribose [45], the latter being poorly utilized (J. D. Shields III, *Honors Thesis*, Amherst College, 1959). Thus inosine, for example, would not be expected to contribute to PRPP formation. In *Sch. mansoni*, tubercidin causes no decrease in consumption of glucose or lactate [26].

From a plot of the data in Table 3 of Bloch *et al.* [27], an inhibition index for tubercidin of 0.06 can be calculated in the presence of ribose-5-phosphate at 10^{-6} M. This is about the same index as with added guanosine and inosine. With adenosine, deoxyadenosine and uridine the index is considerably higher. It appears that these latter nucleosides have some added effects other than sources of energy. The presence of 0.1 mM adenosine increased more than 1000-fold the amount of the analog required to cause 50 per cent inhibition. In *Crithidia* the increase was only 2.5-fold at 0.18 mM adenosine and because of the toxicity of adenosine higher concentrations could not be tested, except in the presence of uridine. On the other hand adenosine appears to be tremendously stimulatory to the growth of *S. faecalis* [27]. The effect of adenosine in the case of *C. fasciculata* may be attributed to competition with the analog for phosphorylation by adenosine kinase.

That tubercidin is so much more toxic than adenosine to *C. fasciculata* may be in part due to the fact that it cannot be degraded to noninhibitory products as can adenosine. Both are converted to mono-, di- and triphosphates [23–26] of which ADP (and probably tubercidin diphosphate) is a potent inhibitor of PRPP synthetase [49]. This would explain why pyrimidine nucleosides, but not the free bases, are effective in reversal of inhibition. Failure to obtain complete reversal of the effects of tubercidin even with high levels of pyrimidine nucleosides may be a reflection of incorporation of the analog into nucleic acids, as is seen in other organisms [23, 25, 27].

Cycloadenosine is also converted to phosphorylated compounds, largely di- and triphosphates, but little if any is incorporated into nucleic acids in H. Ep. #2 cells [34]. Mutant cells lacking adenosine kinase were somewhat resistant to the analog [34, 36].

Interference with *de novo* purine biosynthesis was found not to be a major effect of the analog [35]. This would be of no consequence in an organism such as *C. fasciculata*, which requires an exogenous source of purine. Hill *et al.* [35] suggest that its main effect is on conversion of XMP to GMP. This appears to be one site of inhibition in *C. fasciculata*, since guanine and guanosine give some relief of inhibition. However, the site of action may be farther back in the sequence of reactions leading from adenine (the dietary purine) to GMP. Hypoxanthine, inosine, xanthine and xanthosine appear to have activities comparable to that of guanine or guanosine. These data implicate adenylate deaminase as a possible site of inhibition.

It is unlikely that cycloadenosine is a substrate for the ribonucleoside hydrolase; it is not cleaved by the ribonucleoside phosphohydrolase of H. Ep. #2 cells [35]. It would, therefore, like tubercidin, not be degradable to less toxic substances and, like both adenosine and tubercidin, would interfere with pyrimidine metabolism.

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