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# CAFFEINE: ITS ACTION ON PURINE METABOLIZING ENZYMES*

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## SUMMARY

The many pharmacological and biochemical effects of caffeine may be explained in part by its inhibitory action in vivo and in vitro upon enzymes which metabolize purines. We have demonstrated that in Crithidia fasciculata this methylxanthine (as well as theophylline) is a rather weak competitive inhibitor of adenine aminohydrolase, ribonucleoside hydrolase, hypoxanthine and guanine phosphoribosyltransferases. Caffeine does not interfere with purine base transport in Crithidia, however in leucocytes purine uptake is reduced. While the methylxanthines are weak purine enzyme inhibitors, the large number of enzymes affected accounts for their physiological importance in these cells.

## INTRODUCTION

Caffeine (1,3,7-trimethylxanthine) and theophylline (1,7-dimethylxanthine) share in common several pharmacological actions of great interest. They stimulate the central nervous system, produce diuresis, stimulate cardiac muscle and relax smooth muscle, notably bronchial muscles (8). Caffeine at high concentrations, has been reported to be mutagenic in certain bacteria, fungi, plants and Drosophila melanogaster (9). It has been found to cause chromosomal breaks in HeLa cells (10) and in cultured lymphocytes (11) and to be teratogenic in mice (12). It inhibits DNA dark repair of UV induced damage in human cells (13). The exact mechanisms behind the effects of the methylxanthines are largely unknown.

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Following the finding by Butcher and Sutherland (14) that theophylline and caffeine are inhibitors of cyclic nucleotide phosphodiesterase, it was assumed that many, if not all, actions of methylxanthines were secondary to elevated cyclic AMP levels. However, this explanation is inadequate in explaining the many biochemical events caused by these compounds (8).

Since the methylxanthines are purines the possibility exists that, (i) they might compete with dietary purines for a protein carrier system in transport or, (ii) they might inhibit the enzymes in the purine interconversion pathway.

These possibilities were studied in the kinetoplastid flagellate Crithidia fasciculata and to a lesser degree in bovine and human leucocytes.

## MATERIALS AND METHODS

Culture of organism - Stock cultures of Crithidia were maintained in a medium consisting of 2% proteose-peptone (Difco), 5% glucose, 0.1% Liver Fraction L, supplemented with folic acid (2.5 µg/mL) and filter-sterilized hemin (25 µg/mL) added separately. Cells to be used in experiments were obtained by inoculating 10 mL of a stock culture into 200 mL of the defined medium of Kidder and Dutta (1) minus purine, in low profile flasks. These cultures were incubated at 25°, their growth being made possible by the purine carried over in the inoculum (depleted cells). Purine replete cells were obtained by growing the cells in the above defined medium containing adenine (40 µg/mL).

Three days after inoculation, cells were harvested by centrifugation at 40,000 g for five minutes, washed in Hanks' solution plus glucose (0.14 M) and resuspended in the same solution to a density of 10% transmission at 650 nm. This density represents $\sim 2 \times 10^8$ cells/mL.

Preparation of leucocytes - Leucocytes were isolated from whole blood by the method of Hullinger and Blazkovek (3). The plasma was centrifuged 800 x g for three minutes: the plasma containing the platelets was discarded and the white cells in the pellet were washed by resuspending in phosphate buffered saline. Most of the remaining erythrocytes were removed by resuspending the pellet in 5 ml of buffer, adding 5 ml of distilled water (23°C) and mixing the suspension gently for sixty seconds. Isotonicity was restored by the addition of 0.39 ml of 2N NaCl. The cells were washed in buffer, resuspended and used immediately.

Transport assays - All assays reported here were carried out at 22° in plastic microcentrifuge tubes (1.5 mL capacity) as

previously described (2). Aliquots (0.5 mL) of cell suspension (2- to 3-day culture) were pipeted into the tubes and 0.05 mL of radioactive substrate added with an Eppendorf pipet. After a brief vortexing the tubes were placed in an Eppendorf microcentrifuge When this type of centrifuge is turned on the cells are sedimented and removed from contact with the medium within a fraction of a second. For transport studies the cells were allowed to remain in contact with the medium for 5- or 10-second periods. Two separate aliquots of 0.05 mL of the supernatant fluid were placed on stainless steel planchets, spread with 50% ethanol and dried for radioactivity determinations. The cells were then re-suspended by vigorous vortexing and two further 0.05 mL samples plated to obtain a zero time (total) count. The presence of cells in the latter samples necessitated a correction for self absorption (which was determined to be 10%). The difference between the counts obtained from the supernatant fluid and the cell suspension represents the amount of substrate taken up by the cells. The usual method of determining rapid uptake by counting the radio-activity of the cells removed from the medium by collection on Milipore filter pads was found to be too slow to be useful with Crithidia. All radioactive determinations were made in a Tracerlab ultrathin window scaler.

Accumulation studies - In a manner similar to that described above, but at longer times of incubation, the amounts of substrate taken up, as well as their matabolic fate inside the cell were determined. Cell pellets were washed three times with Hank's solution and extracted with 0.5 mL of 4 M perchloric acid for 20 minutes. The extract was neutralized with saturated KOH and the $KClO_4$ removed by centrifugation. The cell extract and the remainder of the incubation medium were reduced in volume *in vacuo* over solid NaOH and streaked on Whatman #1 paper for descending chromatography in one or more of the following solvents: (1) *iso*-butyric acid:ammonia:water (66:1:33, v/v/v); (2) *n*-propanol:tetrahydrofurfurol:0.08 M sodium citrate, pH 3.2 (2:1:1, v/v/v); (3) *iso*-propanol:ammonia:water (73:10:17, v/v/v); (4) *n*-butanol:acetic acid:water (20:3:7, v/v/v). Radioactive areas were located in a Tracerlab 4π scanner and identified by comparison with authentic compounds which were co-chromatographed and located by ultra violet absorbance. Areas under the peaks were quantified by planimetry.

In experiments testing for competition for transport, the radioactive substrate plus the unlabeled base to be tested were added to the centrifuge tubes. Cell suspensions were added last, the mixture vortexed and sampled as described above.

Enzyme assays - Cells were harvested, washed and suspended in 2 mL 0.05 M phosphate buffer at pH 7.2 and sonicated two times, with cooling between sonications, using a Wave Energy Ultra Systems Tips. The cell sonicate was centrifuged one hour at 40,000 g and the supernatant fluid either used without further treatment or it was subjected to further centrifugation at 80,000 g for two hours.

Phosphoribosyltransferases for adenine, guanine or hypoxanthine were assayed as previously described (4). The assays for adenine deaminase and nucleoside hydrolase were carried out as previously described (5,6).

Materials - The following labeled compounds were obtained from Schwarz/Mann: [8-$^{14}$C]adenine, 52 Ci/mol; [8-$^{14}$C]guanine, 48 Ci/mol;

[8-$^{14}$C]hypoxanthine, 53 Ci/mol; [8-$^{14}$C]inosine, 42 Ci/mol. Prior to use all radioactive substrates were diluted 1:10 with the unlabeled compound to reduce the cpm to a useful level.

[2-$^{14}$C]caffeine was not commercially available. It was prepared by the methylation of [2-$^{14}$C]xanthine with dimethylsulfate at alkaline pH, according to the method of Bredereck, et al, (7). [2-$^{14}$C]caffeine had a specific activity of 5.68 Ci/mol.

Solvents were J. T. Baker Reagent Grade while other chemicals and ingredients of the media were purchased from Sigma Chemical Co.

## RESULTS AND DISCUSSION

Caffeine causes growth inhibition in Crithidia, which is most effectively reversed by adenine. Caffeine loses the ability to inhibit growth when the ratio of caffeine to adenine in the growth medium is roughly 10 to 1. In neither of the cell types studied (Crithidia or leucocytes) was caffeine changed (demethylated). This was shown by incubating whole cells and cell sonicates with [2-$^{14}$C] caffeine (3) which never resulted in the production of other labelled compounds. The conclusion was that the growth inhibiting effect as well as the enzyme inhibitions, described below, were caused by the unmodified molecule.

It was consistently noted that when purine-depleted Crithidia cells (2) were exposed to caffeine (5-10 mM) they rapidly (10 min) changed from a slim cylindrical shape to spherical. Adenine (2 mM), added before the caffeine, prevented this alteration. Theophylline at 10 mM did not cause such a change. Dibutyryl cyclic AMP did not mimic caffeine regarding cell morphology or caffeine and theophylline regarding purine accumulation (Table 1).

When Crithidia cells were exposed to various concentrations of [$^{14}$C] labelled methylxanthines for 5 sec and the amount of radioactivity within the cells determined (2) it was found that the rate of transport was a function of its concentration in the medium, suggesting that a facilitated process for rapid transport of the methylxanthines exist (diffusion is undetectable in 5 sec with low substrate concentrations). The rate of transport

Table 1. Effects of caffeine, theophylline and c-AMP on the accumulation of purines.

| [$^{14}$C]purine 0.1 mM | % of Control in the Presence of: Bovine Leucocytes Caffeine 10 mM | C. fasciculata cells Caffeine 10 mM | Theophylline 10 mM | Dibutyryl c-AMP 0.1 mM |
|---|---|---|---|---|
| Guanine | 2.9 | 40.3 | 59.4 | 115.0 |
| Adenosine | 57.9 | 38.4 | -- | 113.2 |
| Guanosine | 71.4 | 79.5 | 82.4 | 198.3 |
| Hypoxanthine | -- | 36.3 | -- | -- |
| Adenine | 63.7 | 47.2 | 44.0 | 121.0 |

Values are means of duplicate assays. Incubation was at 25° for 30 min. Each assay tube contained ∿ 2 x $10^8$ cells/ml. Each assay tube contained ∿ 2 x $10^5$ leucocytes per ml.

varied with pH and with temperature further suggesting a protein carrier system for these compounds. Neither adenine, guanine nor hypoxanthine interfered with the 5 sec transport of the methylxanthines suggesting that the latter compounds use different carried systems than the natural purines for entry into the cell.

Incubating bovine leucocytes with caffeine for 1-10 min and then adding [$^{14}$C] adenine resulted in a decrease in purine ribonucleotide accumulation corresponding to increase in caffeine concentration (1-10 mM) which was used for the preincubation. This corroborates the finding of Kübert, et al (15) that caffeine-treated leucocytes exhibited decreased conversion of adenine to ATP. Caffeine also inhibits the accumulation of other purine bases and nucleosides (Table 1).

When the various purine bases were added to caffeine-treated Crithidia cells, they were transported (5 sec) readily, but due to impairment of the trapping mechanisms (phosphoribosyltransferases) by caffeine they flow out of the cell by diffusion or by backward flux on the transport system. We then determined the fate of the purine precursors. We previously reported that

Table 2. Intracellular accumulation of nucleotides in Crithidia in the presence of caffeine

| Purine 100 µM | Purine Compounds found within cells (n mol) | nucleotides in cell (n mol) | | | % of nucleotides in cell | | |
|---|---|---|---|---|---|---|---|
| | | AMP | ADP | ATP | AMP | ADP | ATP |
| Adenine | 51.0 | <1 | 19.4 | 31.6 | <1 | 37.3 | 62 |
| + Caffeine | 16.4 | <1 | 6.4 | 9.9 | <1 | 39.0 | 60.3 |
| Adenosine | 43.2 | <1 | 15.1 | 28.1 | <1 | 34.8 | 65.0 |
| + Caffeine | 16.4 | <1 | 6.4 | 9.9 | <1 | 39.0 | 60.3 |
| Hypoxanthine | 39.2 | 9.8 | 19.6 | 9.8 | <1 | 50.3 | 49.7 |
| + Caffeine | 8.6 | 0 | 3.4 | 5.2 | <1 | 39.4 | 60 |
| | | GMP | GDP | GTP | GMP | GDP | GTP |
| Guanine | 86.0 | 1 | 48.1 | 36.9 | 1.2 | 55.9 | 42.9 |
| + Caffeine | 21.3 | 1 | 12.2 | 9.03 | 1 | 57.3 | 42.3 |

Nucleotide concentration was determined at the end of 20 minutes incubation at 22°. Perchloric acid extracts of cells were analyzed as previously described (2). Results are averages of duplicate assays.

adenine (2) and adenosine (16) rapidly appear in the cell as adenine nucleotides. Inosine and hypoxanthine appear in the cell also as adenine nucleotides with only traces of IMP (2). Crithidia cells were preincubated with caffeine for 30 min at 22°. Labelled purine was then added and the cells were incubated again for 20 min. The amount of label incorporated into the cell was determined. Caffeine appears to alter the incorporation into the cell, but has little influence on the proportion of nucleotides formed (Table 2). This again indicates that caffeine inhibits the phosphoribosyltransferases and also shows that it has no significant effect upon ribonucleotide kinases.

In vitro studies, using extracts of Crithidia cells subjected to gel filtration (Sephadex G-200) substantiated the fact that several enzymes (adenine deaminase, guanine and hypoxanthine

Table 3. The degree of inhibition of purine metabolizing enzymes of Crithidia by two methylxanthines

| Enzyme | No inhibitor $K_m^*$, mM | Caffeine 10 mM | Caffeine $K_m^*$, mM | Theophylline 10 mM | Theophylline $K_m^*$, mM |
|---|---|---|---|---|---|
| | | % inhibition | | % inhibition | |
| Adenine deaminase | 3.5 | 60.8 | 20 | 67.6 | 22 |
| HPRTase | 0.9 | 41.4 | 4 | 34.5 | 4.8 |
| GPRTase | 1.6 | 50.0 | 8.3 | 58.5 | 6.6 |
| Nucleoside hydrolase | 2.5 | 68.3 | 16.7 | 83.3 | - |

The assays were carried out as previously described for adenine deaminase (5), nucleoside hydrolase, (6) (inosine was used as substrate) and the phosphoribosyltransferases (4).

*Determined from Lineweaver-Burk plots with the natural substrates of the enzymes.

phosphoribosyltransferases and ribonucleoside hydrolase) in the purine salvage pathway were competitively inhibited by caffeine and theophylline (Table 3). In vivo experiments strongly indicate that adenine phosphoribosyltransferase is also inhibited but because of so many competing reactions for adenine as substrate, it is difficult to ascertain the exact effects the methylxanthines have on this enzyme. Fredholm, et al (17) recently reported that theophylline inhibits 5'-nucleotidase and alkaline phosphatase in rabbit kidney. We have observed the same effect in Crithidia, however this enzyme is very weak and quite unstable in the flagellate.

In light of the present evidence and from reports in the literature, the following hypothesis is offered to explain growth inhibition of Crithidia caused by the methylxanthines, and their mutagenicity for certain organisms and certain kinds of cells, which may help to account for the ambiguities which have appeared in the literature.

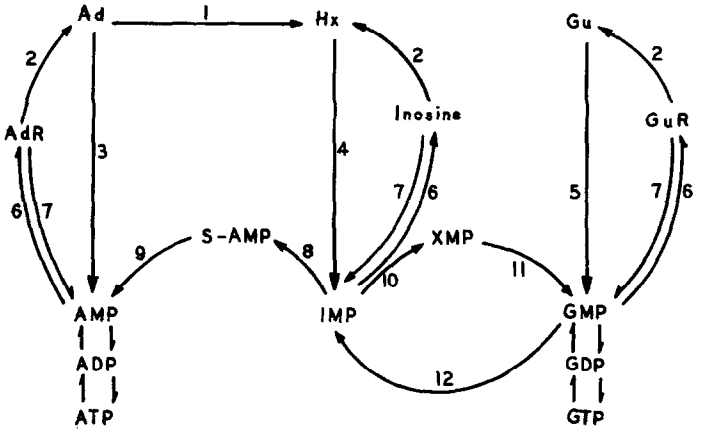


Figure 1: Pathways of purine metabolism in purine-dependent cells. Numbers represent the following enzymes: 1-adenine deaminase (Crithidia); 2-ribonucleoside hydrolase (Crithidia) or ribonucleoside phosphorylase (blood cells); 3-5=adenine, hypoxanthine and guanine phosphoribosyltransferases; 6=mononucleotide phosphatases; 7=ribonucleoside kinases; 8=adenylosuccinate synthetase; 9=adenylosuccinate lyase; 10=IMP dehydrogenase; 11=GMP synthetase; 12=GMP reductase.

Crithidia possesses purine-metabolizing enzymes similar in many respects to those of the lower invertebrates and also to those of certain mammalian cells. Adenine deaminase (reaction 1, Fig. 1) was found to be inhibited by the methylxanthines. This enzyme converts adenine to hypoxanthine which is then phosphoribosylated to IMP to be shunted to whichever final ribonucleotide (ATP or GTP) the cell needs. In the presence of purine bases and/or their ribonucleosides and methylxanthine, the cell is confronted with five partial blocks; adenine → hypoxanthine (1,Fig.1); ribonucleoside → base (2,Fig.1); adenine → AMP (3,Fig.1); hypoxanthine → IMP (4,Fig.1) and guanine → GMP (5,Fig.1) in the eventual production of ATP and GTP for energy and nucleic acid synthesis.

Interest has long focused on the nature of the primary lesions leading to the loss of the capacity for self replication and to mutagenicity. Purine-requiring cells which possess adenine deaminase and ribonucleoside hydrolase, when in the presence of methylxanthines and, therefore, having a diminished

capacity to accumulate purine nucleotides, would presumably have a higher priority to produce energy yielding compounds rather than nucleic acids. Any synthesis of DNA under these conditions would be taking place in a precursor pool of unbalanced base ratios. This situation has been postulated to cause errors of base pairing and to cause mutagenesis in bacteria (18), and may also account for the fact that DNA synthesized in the presence of caffeine has a lighter than normal molecular weight and more single stranded regions (13).

Mammalian cells which are incapable of purine synthesis (i.e., bone marrow, red blood cells, leucocytes, etc.) would be expected to be most susceptible to the inhibitory actions of the methylxanthines. Several tissues show high rates of purine turnover and yet are incapable of purine synthesis, relying upon purine salvage (19, 20, 21, 22). In such cells (not in Crithidia, however) methylxanthines may inhibit transport as well as the other purine metabolizing enzymes discussed above. In preliminary experiments with leucocytes we have found purine transport inhibition by caffeine. Cells which have a de novo purine pathway are able to bypass the purine phosphoribosyl-transferase reactions by interconversions from IMP (Fig. 1).

As methylxanthines have many sites of action, inhibition of one enzyme may result in a cascade of effects (i.e., disturbance of ion gradients, of membrane structure resulting in cell shape, etc). We believe that we have demonstrated some of the sites of action in certain cell types. Whether these observations prove valid for other cell types remains to be determined.

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