Europ. J. Cancer Vol. 15, pp. 415–421.

0014-2964/79/0401-0415 $02.00/0

A Suicide Technique to Study Purine Antimetabolites on Bone Marrow and Tumor Colony-Forming Cells*

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Abstract—*A new technique was used to study the effects of purine antimetabolites on bone marrow colony forming cells (CFC), cells which are of critical importance in cancer chemotherapy. By extending the* ^{3}H-*thymidine suicide technique to include* ^{3}H-*purines, viz., hypoxanthine, deoxyadenosine, adenosine and adenine, the utilization of these purines for nucleic acid synthesis may be investigated. Antimetabolites may affect nucleic acid metabolism at several loci; to assess which of these loci were important to CFC, suicide studies were carried out with viable bone marrow cells. Primary cultures of mouse bone marrow were incubated alone and in the presence of 6-mercaptopurine (6-MP), with tritiated purines of sufficient radioactivity to inhibit colony formation (suicide). If any of the processes (e.g., transport, metabolism or incorporation of the* ^{3}H-*purines into nucleic acids) was inhibited, suicide by the* ^{3}H-*purines was prevented. Specific probes, viz., persantin, antimetabolites and nonradioactive purines, were used to localize pathways inhibited by 6-MP. Nonradioactive purines were able to reverse the suicide of all tritiated purines. 6-Mercaptopurine was able to reverse the suicide of* ^{3}H-*hypoxanthine but did not reverse the suicide of the other tritiated purines. We suggest that 6-MP affects the metabolism of hypoxanthine in CFC, but not of adenine, adenosine or deoxyadenosine.*

INTRODUCTION

THE COLONY forming cell (CFC) is a committed hemopoietic progenitor cell. An *in vitro* assay for the bone marrow CFC developed by Bradley and Metcalf [1], and concurrently by Pluznik and Sachs [2], made this cell available for investigation. There have been several reports on the effect of cytotoxic agents on hemic colony forming cells [3–5]. Since less than 1% of the bone marrow cells are CFC, direct biochemical studies are not experimentally feasible; whether or not the CFC can be identified morphologically is questionable [6–8]. No direct biochemical studies have been made because there is no available means of isolating CFC. Bone marrow cell fractions were enriched with respect to CFC by repeated density gradient separation, but populations of CFC of sufficient purity for biochemical analysis were not obtained [8].

Bone marrow is often the dose limiting tissue in the use of anti-neoplastic agents [9]. Knowledge of the metabolic processes and precursors that colony forming cells rely on for nucleic acid synthesis would be of value in designing chemotherapeutic regimens that spare the hematopoietic cell renewal system from toxicity.

A novel, indirect method to study nucleoside or base precursor requirements for nucleic acid synthesis in colony forming cells was developed by Uyeki *et al.* [10–12]. The technique extended the thymidine suicide technique reported by Dutton and Mishell [13] to include other tritiated nucleosides or bases. Their incorporation into nucleic acids and their subsequent lethal irradiation of the cell were quantitated by comparing decreased numbers of colonies in ^{3}H-purine treated cultures with untreated control cultures. Conversely, blocking the incorporation of the tritiated DNA precursor into nucleic acids resulted in the return of colonies tallied toward control values.

In this paper the biological effect, suicide, will be defined as the inhibition of colony

Accepted 22 August 1978.
*Supported by NIH Grants No. ES 01004 and GM 07044.

formation due to the incorporation of a lethal amount of tritium in the CFCs' nucleic acids. Purine metabolism was studied by incubating CFC cell cultures alone and in the presence of an antimetabolite with a tritiated purine of sufficient radioactivity to inhibit colony formation (hereafter referred to as suicide). If an antimetabolite inhibited any of the processes, such as transport, metabolism or the incorporation of the tritiated purine into nucleic acids, the suicide would be reversed.

Suicide of colony forming cells has been used as a probe to study inhibitors of the metabolic pathways required for the incorporation of tritiated pyrimidines into DNA [11, 12]. In the present work we investigated the effect of purine antimetabolites, 6-mercaptopurine and 6-thioguanine (6-TG) on the metabolism of tritiated purines: hypoxanthine (Hyp), adenine (Ade), adenosine (Ado) and deoxyadenosine (dAdo). Several enzymes and functions have been reported to be inhibited by 6-MP and its nucleotides. These include the inhibition of purine salvage enzymes, NAD formation, and acetylation by coenzyme A [14]. The most sensitive site seems to be the inhibition of *de novo* purine synthesis [15]. With the exception of lymphocytes [16, 17], there is little evidence of *de novo* purine synthesis in bone marrow cells [18]; nucleic acid synthesis is probably the result of utilization of preformed purines by salvage pathways [19]. This study was undertaken to investigate the effect of 6-MP on the metabolism of several purines in CFC.

MATERIALS AND METHODS

Materials

Female BDF_1 (C57B1/6 × DBA/2) and DBA/2 mice weighing approximately 20 g each were purchased from Jackson Laboratories, Bar Harbor, Maine, and were used when they were 8–16 weeks old.

Compounds were purchased as follows: adenine and guanine were purchased from Sigma Chemical Co., St. Louis, Mo.; tritiated hypoxanthine (G-^{3}H) Hyp (sp. Act. 9 Ci/mmole) was purchased from New England Nuclear, Boston, Mass.; (8-^{3}H) dAdo (sp. Act. 7 Ci/mmole), (2, 8) ^{3}H Ado (sp. Act. 60 Ci/mmole), (2, 8) ^{3}H Ade (sp. Act. 4 Ci/mmole) were purchased from ICN Pharmaceuticals Inc. (Irving, CA). 6-Mercaptopurine and 6-thioguanine were purchased from Burroughs-Wellcome. Calf and horse serum and Hanks' balanced salts solution were purchased from K. C. Biologicals, Lenexa, Kansas. Concentrates of minimum Eagle's medium and McCoy's 5A (modified) medium were purchased from Grand Island Biological Co., Grand Island, N.Y.

Unwashed studies on tissue cultures

Bone marrow cells (1×10^5 cells) were plated in 0.5 ml McCoy's 5A media (modified) with 10% fetal calf serum (FCS) and 5% horse serum in 35 mm tissue culture dishes. Then drugs and radioactive agents were added to these cell suspensions. An agar-media admixture was then added to make 1.0 ml semisolid media, such that the final concentration was 0.3% of Bactoagar. Bone marrow cells were cultured for CFC–GM according to our modification [3] of the technique of Metcalf and Moore [20]. As the source of colony stimulating factor (CSF), 0.05 ml of L-929 cell conditioned medium was added. Cultures were examined for clonal growth after 7 days incubation at 37°C. Microscopic examination of cells taken from clonal aggregates of control cultures revealed morphology characteristics of monocytic cell lineage; using L-929 cell conditioned media as the colony stimulating factor (CSF), no granulocytic colonies were detected in our cultures.

P815 mastocytoma cells were cloned with similar techniques except that 1×10^3 cells were plated and no CSF was added.

Washout studies on tissue cultures

Higher concentrations of the drugs and radioactive agents were used in washed-out studies than in unwashed experiments, due to the duration of drug exposure, i.e., several hours vs 7 days, respectively. Bone marrow cells were plated at 2×10^5 cells per well in 1.0 ml media + serum (as described for unwashed studies). These culture dishes consisted of six 35 mm wells per cluster dish (Costar 6 well). After a drug preincubation period, cells were pulsed with radioactive nucleic acid precursors for designated periods. A mixture of nonradioactive deoxynucleosides (10^{-5} M) was added to cultures to terminate the radioactive pulse (chase). After the chase cells were washed twice with Hanks' balanced salt solution + 5% calf serum, cells were centrifuged at 300 *g* in between washes, using a Cooke carrier to hold each Costar cluster dish in the centrifuge. After the final wash, cells were resuspended in 1.0 ml semisolid media and cultured as was described for unwashed cultures.

P815 mastocytoma cells were cloned with similar techniques except that 1×10^3 cells were plated and no CSF was added.

Statistical significance was evaluated using Duncan's new multiple range test in association with analysis of variance. Data presented are the mean ± standard error of triplicate samples and are representative of at least 9 samples.

RESULTS

6-Mercaptopurine was used to assess its ability to reverse ^{3}H-purine suicide. The doses and time of exposure were chosen in consideration of the dose used clinically as well as the half-life of 6-MP in the body. The half-life has been reported to be approximately 90 min [21].

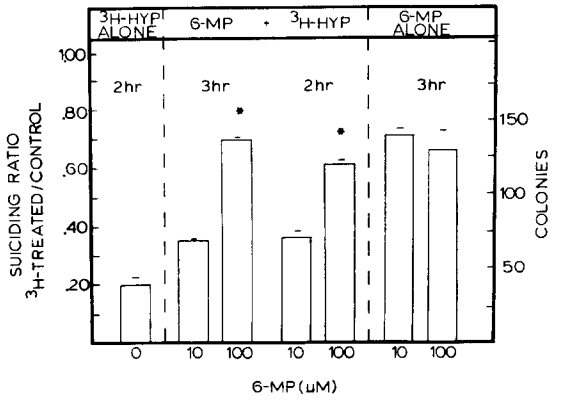

Fig. 1. The reversal of ^{3}H-Hyp suicide with 10 or 100 μM 6-mercaptopurine. Bone marrow cells (2×10^5) were pulsed with 2 μCi of ^{3}H-hypoxanthine in the presence of (2 hr) or after a 1 hr preincubation (3 hr) with 6-mercaptopurine.

The effect of 6-MP on ^{3}H-Hyp suicide is shown in Fig. 1. A 2 μCi pulse with Hyp for 2 hr reduced colony survival to 20% of untreated controls. When cell cultures were preincubated for 1 hr with 6-MP (3 hr), or when 6-MP was added at the same time as ^{3}H-Hyp (2 hr), ^{3}H-Hyp suicide was reversed. In the presence of 10 μM 6-MP, colony numbers were approximately 200% of the ^{3}H-Hyp control. This difference is not significant for 10 μM but does become significant for 100 μM 6-MP. Control values for 6-MP indicate that suicide reversal occurs at concentrations not particularly toxic to the cells.

Hypoxanthine and guanine are transported by the same carrier and are phosphorylated by the same enzyme in mammalian cells [22]. Preliminary experiments (not shown) indicated unlabeled purine concentrations less than 1 μM did not reverse ^{3}H-purine suicide. Unlabeled Hyp (Fig. 2) and Gua (Fig. 3) at 10 and 100 μM concentrations reversed ^{3}H-Hyp suicide. The magnitude of reversal was slightly greater than that seen with 6-MP. Unlabeled purine control cultures were not significantly different from untreated controls.

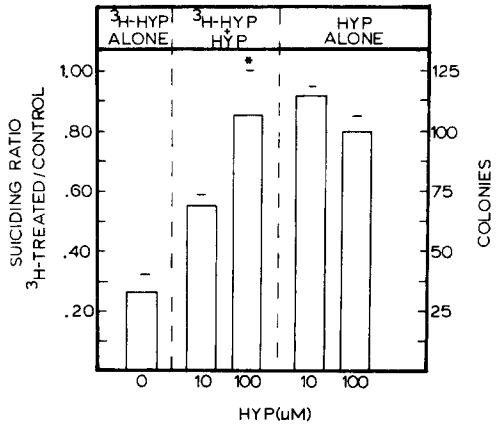

Fig. 2. Reversal of ^{3}H-Hyp suicide with 10 or 100 μM hypoxanthine. Bone marroe cells (2×10^5) were pulsed with 2 μCi of ^{3}H-hypoxanthine for 2 hr in the presence of unlabeled hypoxanthine.

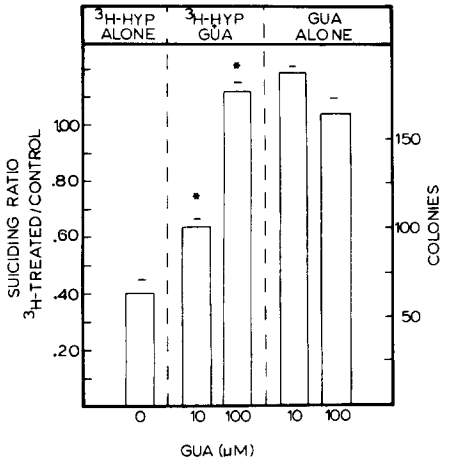

Fig. 3. Reversal of ^{3}H-Hyp suicide with 10 or 100 μM guanine. Bone marrow cells were pulsed with 2 μCi of ^{3}H-hypoxanthine for 2 hr in the presence of unlabeled guanine.

In an attempt to distinguish drug effects on transport from effects on metabolism or incorporation of the ^{3}H-purine into nucleic acids, a transport inhibitor was investigated for its ability to reverse the suicide effect. Persantin (Fig. 4) (2, 6,-bis(diethanolamine)-4, 8-dipiperidino-pyrimido(5, 4-d)pyrimidine), a specific inhibitor of nucleoside transport in mammalian cells, reversed the suicide of 3 μCi of ^{3}H-adenosine when added to cultures of P815 cells 1.5 hr prior to and during a 3 hr ^{3}H-Ado pulse.

The thio-substituted analogs of Hyp and Gua (6-MP and 6-TG) are probably transported into cells by the same transport site. 6-Thioguanine should compete with ^{3}H-Hyp as

well as 6-MP for transport into the cell but should not inhibit the same purine salvage enzymes. The effect of 6-TG on ^{3}H-Hyp suicide is shown in Fig. 5. 6-Thioguanine did not reverse ^{3}H-Hyp suicide when used in concentrations in which 6-MP was effective. 6-Thioguanine treated cultures were consistently, but not significantly, greater than the ^{3}H-Hyp controls, indicating some inhibition of transport and suicide might have occurred.

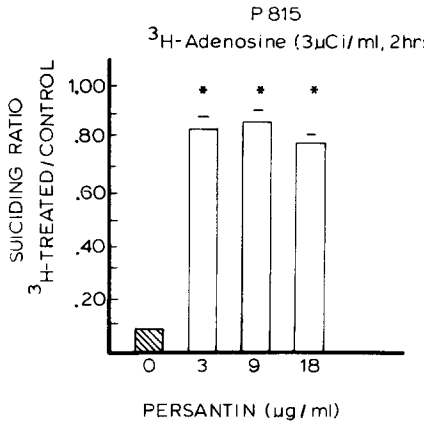

Fig. 4. The reversal of ^{3}H-adenosine suicide by persantin. 1 × 10^3 *P815 mastocytoma cells were incubated with* 3, 9 *or* 18 μg/ml *of persantin for* 1.5 hr, *followed by a* 2-hr *pulse with* ^{3}H-*adenosine* (3 μCi/ml).

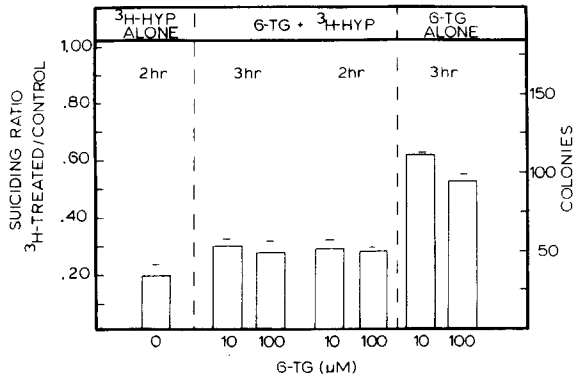

Fig. 5. The effect of 6-thioguanine ^{3}H-Hyp suicide. Bone marrow cells (2 × 10^5) *were pulsed for* 2 hr *with* 2 μCi *of* ^{3}H-*hypoxanthine in the presence of* (2 hr) *or after a* 1-hr *preincubation* (3 hr) *with* 6-*thioguanine.*

Experiments with other tritiated purines were done in order to investigate the specificity of the 6-MP reversal of ^{3}H-purine suicide. Ten and 100 μM 6-MP did not significantly alter the fraction surviving a suicide pulse with ^{3}H-Ade, ^{3}H-Ado or ^{3}H-dAdo (Fig. 6). 6-Mercaptopurine inhibited either the metabolism or incorporation of ^{3}H-Hyp into nucleic acids but failed to inhibit the corresponding pathways for adenine, adenosine and deoxyadenosine.

The effect of incubating the bone marrow cells with increasing concentrations of ^{3}H-Hyp is shown in Fig. 7. Unlike the previous experiments where both the drug and the tritiated purine were washed out after 3 hr, in this experiment ^{3}H-Hyp was added to the cultures and was in contact with the colony forming cells for the remainder of the culture period. The dose–response curve was very steep; there seems to be a threshold for an inhibitory effect below 0.05 μCi/ml. Complete inhibition of colony formation occurred at 0.3 μCi/ml.

DISCUSSION

6-Mercaptopurine is a hypoxanthine analog. It is a substrate for and a competitive inhibitor of the enzymes that act on hypoxanthine and inosinate (see Fig. 8). 6-Mercaptopurine is a substrate for and a competitive inhibitor of hypoxanthine-guanine phosphoribosyltransferase [23]. This step is

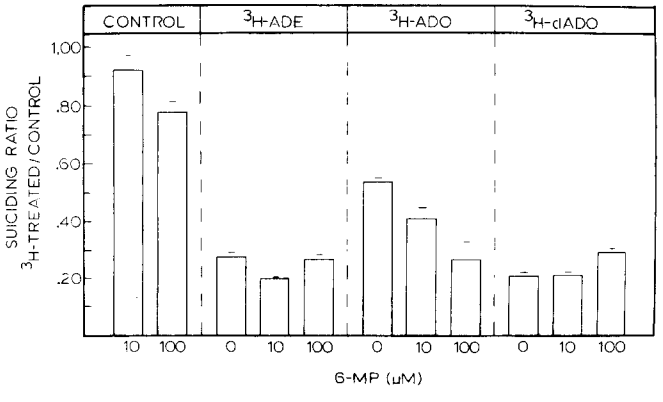

Fig. 6. The effect of 10 *or* 100 μM 6-*MP on suicide with* ^{3}H-*adenine* (1 μCi, 2 hr), ^{3}H-*adenosine* (1 μCi, 2 hr) *and* ^{3}H-*deoxyadenosine* (3 μCi, 2 hr). *Bone marrow cells* (2 × 10^5) *were pulsed with the indicated* ^{3}H-*purine either alone or in the presence of* 10 *or* 100 μM 6-*mercaptopurine.*

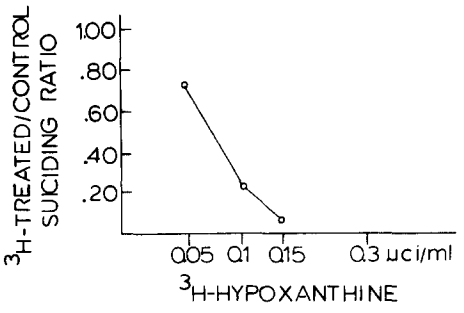

Fig. 7. Suicide of bone marrow CFC with increasing concentrations of ^{3}H-Hyp. The indicated concentrations of ^{3}H-hypoxanthine were added to the cultures and were in contact with the cells for 1 week.

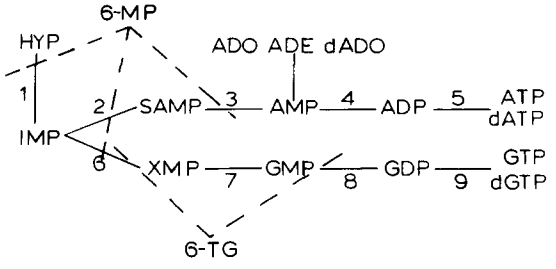

Fig. 8. Diagram of pathways of purine metabolism prior to their incorporation into nucleic acids in bone marrow CFC. Dotted lines indicate putative sites of inhibition by the indicated antimetabolites. The names of the enzymes are: (1) *hypoxanthine–guanine phosphoribosyl-transferase,* (2) *adenylosuccinate synthetase,* (3) *adenylosuccinate lyase,* (4) *and* (5) *kinases,* (6) *inosinate dehydrogenase,* (7) *guanylate synthetase and* (8) *and* (9) *kinases.*

often called a lethal synthesis since the product formed is the most active metabolic form, thio-inosinate (T-IMP). T-IMP competitively inhibits IMP dehydrogenase isolated from Erlich ascites cells, thus, blocking the formation of guanylate (GMP) from IMP [24]. Biosynthesis of adenylate (AMP) is also inhibited by 6-MP because of the inhibition of adenylosuccinate synthetase (EC 6.3.4.4) and adenylosuccinate lyase (EC 4.3.2.2) [25]. Only purine salvage pathways are considered because bone marrow probably does not have a *de novo* purine synthetic route.

Cited evidence [14, 23–25] for the involvement of hypoxanthine metabolism in the mechanism of action of 6-MP led us to test our reversal hypothesis with this compound. We expected that a 6-MP induced block in hypoxanthine metabolism would result in a decrease in the amount of hypoxanthine incorporated into the cells' nucleic acid and should reverse the suicide of ^{3}H-Hyp.

Our results showed that 6-MP inhibited the incorporation of ^{3}H-Hyp to the extent that colony forming cells were able to survive an otherwise lethal exposure to ^{3}H-Hyp. A tritiated nucleoside or base will suicide if it is transported into the cell, metabolized and incorporated into nucleic acids (as the triphosphate, or deoxynucleoside triphosphate) in sufficient amounts to lethally irradiate the cell. Since 6-MP reversed the suicide of ^{3}H-Hyp, one or more of the above processes were inhibited.

The same degree of suicide reduction occurred with the addition of 10–100 μM unlabeled Gua and Hyp. These two purines share a common pathway for transport. Both are subsequently phosphorylated by Hyp–Gua phosphoribosyltransferase. We cannot presently distinguish which of the processes are affected in the reversal of ^{3}H-Hyp suicide.

From the persantin experiment (Fig. 4) we concluded that inhibition of transport of the tritiated purine into the cell will result in the reversal of the suicide effect. Hypoxanthine and guanine are transported into mammalian cells by the same transport site [22]. Thio-substituted analogs, such as 6-MP, are probably transported by the same carrier, although with reduced affinity. For this reason we investigated the effect of 6-thioguanine (6-TG) on ^{3}H-Hyp suicide. 6-Thioguanine should compete as well as 6-MP with ^{3}H-Hyp for transport into the cell; however, 6-TG did not reverse ^{3}H-Hyp suicide, indicating that competition for a transport carrier was not the mechanism by which 6-MP reversed ^{3}H-Hyp suicide. 6-Thioguanine inhibits enzymes along the path from IMP to GMP (Fig. 8) and, therefore, should not inhibit the incorporation of ^{3}H-Hyp into nucleic acids via AMP [25]. The inability of 6-TG to reverse ^{3}H-Hyp suicide indicated that, under our conditions, very little hypoxanthine was metabolized to GMP or other guanine nucleotides. Nucleic acid incorporation might well explain the toxicity of 6-TG in view of the absence of an effect on purine salvage.

The pathway from hypoxanthine to AMP is the pathway most affected by antimetabolite intervention; hence, we conclude that the major pathway for hypoxanthine incorporation into nucleic acids in bone marrow CFC is via adenine nucleotides. These results agree with those of Henderson *et al.* [26], who showed that 85% of the radioactivity was associated with adenine nucleotides after incubating Ehrlich ascites cells with ^{14}C-Hyp.

The degree to which 6-MP inhibited hypoxanthine metabolism was estimated in the following manner: the suicide ratio of ^{3}H-treated to control of 2 μCi of Hyp was 0.2 (Fig. 1). This corresponded to a "no-

washout" suicide curve (Fig. 7) concentration of ^{3}H-Hyp of 0.10 μCi/ml. In the presence of 100 μM 6-MP, the suicide ratio (3 hr) was reversed to 0.80. This increment of reversal corresponded to a no-washout ^{3}H-Hyp concentration of 0.05 μCi/ml. According to our standard suicide curve, 6-MP inhibited hypoxanthine incorporation into nucleic acids by about 50%; this agrees with other authors who measured the incorporation of Hyp into nucleic acids after similar exposures to 6-MP [27, 28]. This finding was further substantiated in our unpublished study with P815 tumor cells. Under conditions identical to the bone marrow experiments, ^{3}H-Hyp incorporation into acid precipitable material (10% TCA) was inhibited by 52% when 100 μM 6-MP was added to cultures with the ^{3}H-Hyp pulse.

6-Mercaptopurine failed to reverse the suicide of tritiated Ado, dAdo and Ade. Hence, we suggest that 6-MP failed to inhibit reactions necessary for the incorporation of these purines into nucleic acids. Figure 8 depicts the probable pathways in CFC for Hyp metabolism to AMP and GMP. Inhibition of one or all three enzymes, hypoxanthine–guanine phosphoribosyltransferase, adenylosuccinate synthetase and adenylosuccinate lyase (Fig. 8, Nos. 1, 2 and 3), could account for the reversal of ^{3}H-Hyp suicide and the failure to reverse the suicide of ^{3}H-Ade, Ado, and dAdo. The diagram was drawn to indicate that these purines incorporate into nucleic acids by pathways not inhibited by 6-MP and does not imply that these purines are metabolized directly to AMP.

Unavailability of labeled intermediates or specific inhibitors for the three enzymes prevented further localization of a single enzyme as being the most important site of action for 6-MP, if this is, indeed, the case. These enzymes have been previously shown to be inhibited by 6-MP [14], and our results with CFC were consistent with these findings. IMP dehydrogenase (Fig. 8, No. 4), an enzyme which has also been reported to be inhibited by 6-MP, cannot be evaluated here because the predominant pathway in the CFC for hypoxanthine metabolism prior to nucleic acid incorporation is via adenine nucleotides. Inhibiting inosinate dehydrogenase may therefore be of little consequence to ^{3}H-Hyp suicide.

There are multiple mechanisms of action assigned to 6-MP and its nucleotides [14, 16, 24]. Of the 6–8 loci of action, one must decide which are really important to the economy of the cell. Elion [14] suggested that this depends on the importance of individual enzymatic reactions, enzyme concentrations in the cell, relative pool sizes of metabolites and inhibitors, and binding constants of substrates and inhibitors. Suicide as a means of biochemical investigation has the advantage of using viable target cells for study rather than cell extracts that may not reflect the *in vivo* situation [29]; moreover, the heterogeneity of cells making up the bone marrow precludes any direct biochemical analyses.

Our ^{3}H-Hyp pulse concentration (10^{-8} M) is well below the normal serum concentration of Hyp. With minimal perturbation of *in vivo* enzyme and substrate concentrations, we hope to obtain *in situ* indications of purine utilization and anti-tumor drug effects on single target cells. These studies begin to bridge the gap between enzymatic assays in test tubes and the microenvironment that is the milieu of the cell.

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