that there is still a small but steady negative slope for 5-HT decrease. The caecum and colon are depleted of 5-HT at a fairly even rate to 25 per cent of control levels at 6 days for both tissues. It evident here again that a slight negative slope is occurring between the fourth and sixth day.

In situ *p*-chlorophenylalanine (PCPA) decreases the level of 5-hydroxytryptamine (5-HT) in the gastrointestinal tract in a manner similar to that of the brain. However, the degree of depletion and the rate of decrease of 5-HT in the gastrointestinal tract is different than that occurring in the brain In no portion of the intestine is the 5-HT decreased to as great an extent as that in the brain.

It is interesting that the slope of depletion of the antrum of the stomach and the brain is very similar. The rest of the gastrointestinal tract has a much shallower slope. The brain has the least amount of 5-HT per gram of tissue and the antrum the largest amount of 5-HT of the tissues studied here. This large difference in quantitative amounts, coincidental with similar depletion rates of 5-HT by PCPA, might indicate that the anabolism or catabolism of 5-HT is similar in these tissues.

The corpus of the stomach is perhaps the most interesting of the tissues studied, in that almost no depletion of 5-HT occurs even though it has a relatively high concentration of 5-HT. No explanation for this lack of depletion of 5-HT is evident from these experiments.

Acknowledgements—The author thanks Miss Catherine Busche and Miss Candace Jenkins for their help.

Department of Pharmacology,
School of Medicine,
University of Washington,
Seattle, Wash. 98105, U. S. A.

LAVERN J. WEBER*

* Present address: Department of Pharmacology, School of Pharmacy, Oregon State University, Corvallis, Ore. 97331.

REFERENCES

1. J. H. THOMPSON, *Ir. J. med. Sci.* **490**, 411 (1966).
2. A. PLETSCHER, K. F. GEY and W. P. BURKARD, in *Handbook of Experimental Pharmacology*, (Eds. O. EICHLER and A. FARAH), vol. 19, p. 649. Springer-Verlag, New York (1966).
3. W. LOVENBERG, E. JEQUIER and A. SJOERDSMA, in *Advances in Pharmacology*, (Eds. S. GARATTINI and P. A. SHORE), vol. 6A, p. 21. Academic Press, New York (1968).
4. M. D. GERSHON, *Gastroenterology* **54**, 453 (1968).
5. K. B. KOE and A. WEISSMAN, *J. Pharmac. exp. Ther.* **154**, 499 (1966).
6. R. V. GREGG, *J. Morphology* **119**, 81 (1966).
7. S. UDENFRIEND, H. WEISSBACH and B. B. BRODIE, in *Methods of Biochemical Analysis*, (Ed. DAVID GLICK), vol. 6, p. 96. Interscience, New York (1958).

Biochemical Pharmacology, Vol. 19, pp. 2172–2176. Pergamon Press. 1970. Printed in Great Britain

Reversal of the growth inhibitory effects of 6-methylthiopurine ribonucleoside

(*Received* 26 *September* 1969; *accepted* 10 *December* 1969)

MeMP*-RIBONUCLEOSIDE, an agent of interest because of its antitumor activity in experimental systems,[1] is metabolized in mammalian cells to the monophosphate derivative, but not to the di- or triphosphates.[1,2] A metabolite of MeMP-ribonucleoside strongly inhibits an early step of purine

* Abbreviations: MP, 6-mercaptopurine; MeMP, 6-methylthiopurine; AIC, 4-amino-5-imidazole-carboxamide; PRPP, 5-phosphoribosyl-1-pyrophosphate.

synthesis *de novo*.[1, 3] Apparently this blockade is a feedback inhibition and is produced by the monophosphate, because MeMP-ribonucleoside does not inhibit proliferation of cells that have lost the capacity to form the nucleotide.[4, 5] Additional evidence for this site of inhibition is the observation that the ribonucleotide of MeMP is the most potent known nucleotide inhibitor of PRPP amidotransferase (EC 2.4.2.14),[6] the enzyme catalyzing the first step of purine biosynthesis and the site of feedback control by the natural nucleotides.[6, 7] These facts suggest that inhibition of PRPP amidotransferase may be the action of MeMP-ribonucleoside (after its conversion to the nucleotide) that is responsible for its biological effects. If this is so, then inhibition by MeMP-ribonucleoside should be reversible by compounds that enter the purine pathway at points past this site of blockade; and such reversals would provide additional evidence for inhibition of purine biosynthesis as the metabolic area in which MeMP-ribonucleoside exerts its initial effects. We report here studies of the effectiveness of AIC and purines in preventing or reversing the inhibition by MeMP-ribonucleoside of the growth of cells in culture.

Mouse adenocarcinoma 755 cells were grown in suspension culture on a rotary shaker as described for H.Ep.-2 cells; the medium (SRI-14) contained no added purines or pyrimidines.[8] The indicated concentrations of MeMP-ribonucleoside and of reversal agents are those at the initiation of the experiment; no determinations were made of changes in concentrations during the period of the experiment. The results of the reversal studies are presented in Figs. 1 and 2, which also give details of the growth conditions. In Fig. 2 it should be noted that negative values indicate some degree of destruction of the cell inoculum. The control cultures increased about 20-fold in cell number over the 72-hr experimental period (Fig. 1). MeMP-ribonucleoside at a concentration of 3·5 μM was toxic to the cells; it either

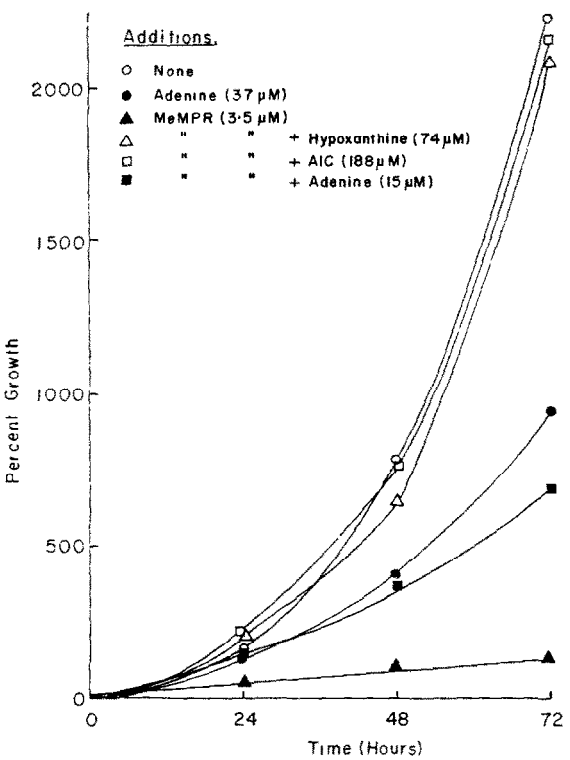

FIG. 1. Effects of 4-amino-5-imidazolecarboxamide (AIC) and purines in reversing or preventing inhibition of cell proliferation by 6-methylthiopurine-ribonucleoside (MeMPR). To 125-ml flasks containing growing cultures of adenocarcinoma 755 cells (9×10^5 cells in 30 ml of medium), MeMPR and candidate reversal agents were added simultaneously, and the cells were counted 24, 48 and 72 hr thereafter in a Coulter counter.

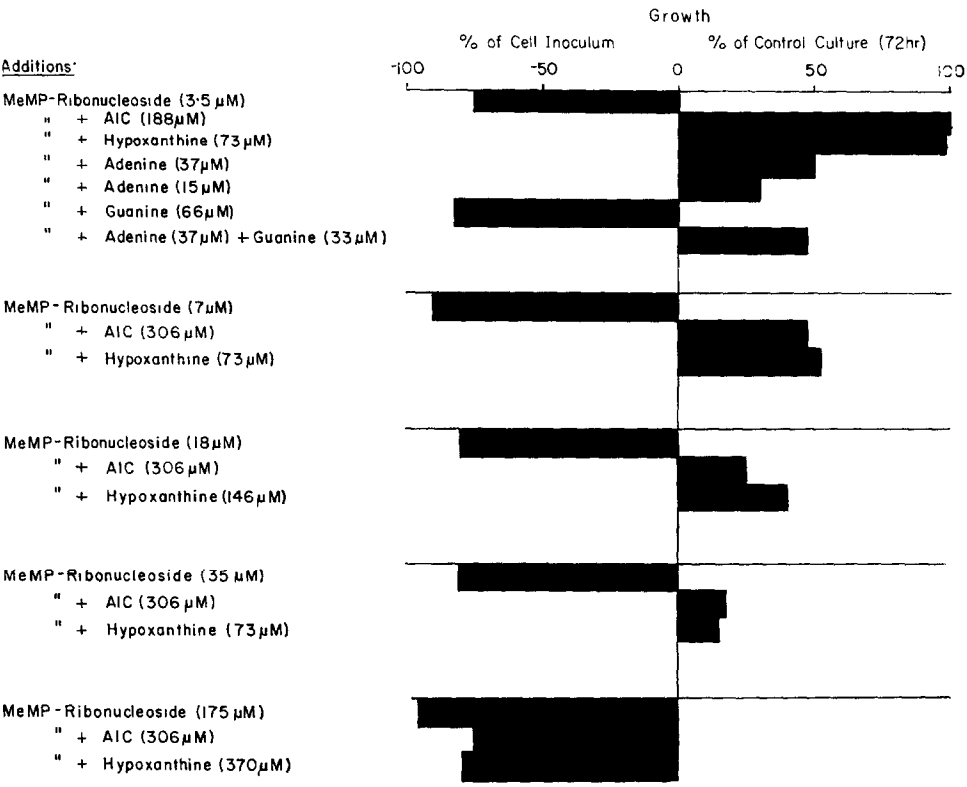

FIG. 2. Effects of 4-amino-5-imidazolecarboxamide (AIC) and purines on the inhibition by 6-methylthiopurine (MeMP)-ribonucleoside of the proliferation of adenocarcinoma 755 cells. Cells were grown in suspension culture as described in Fig. 1. Each section of the chart shows first the growth of a culture for 72 hr in the presence of a certain concentration of MeMP-ribonucleoside and then the growth of similar cultures in the presence of the same concentration of MeMP-ribonucleoside plus the candidate reversal agents. The positive numbers on the right-hand side of the chart are the growth of the various cultures at 72 hr calculated as per cent of the growth of control cultures. (The control cultures increased in number about 20-fold.) The negative values on the left-hand side of the chart are for experiments in which the cell count at 72 hr was below that of the inoculum. These negative values are calculated as per cent of the cell inoculum and indicate destruction of cells of the inoculum. For example, "-50" means that at 72 hr the cell count was 50 per cent of that at the beginning of the experiment.

inhibited cell proliferation almost completely (Fig. 1), or in some experiments (Fig. 2) actually decreased the number of cells below the initial inoculum. Hypoxanthine (73 μM) or AIC (188 μM) completely reversed inhibition by this concentration of MeMP-ribonucleoside. Adenine at 15 μM concentration gave only partial reversal (Figs. 1 and 2). A concentration of 37 μM adenine was slightly more effective (Fig. 2), but this concentration was itself inhibitory (Fig. 1). Guanine was not a reversal agent, and combinations of adenine and guanine were no more effective than adenine alone (Fig. 2). When the concentration of MeMP-ribonucleoside was increased above 3·5 μM, the initial cells were almost entirely destroyed. Complete reversal of the effects of these higher concentrations was not obtained with either hypoxanthine or AIC, but both of these compounds permitted some cell proliferation at concentrations of MeMP-ribonucleoside up to 35 μM. When the concentration of inhibitor was raised to 175 μM, neither hypoxanthine nor AIC afforded any protection against the destructive effects of the inhibitor (Fig. 2). The protection afforded by AIC and hypoxanthine at low

levels of MeMP-ribonucleoside cannot be interpreted as direct competition with this analog for the enzymes catalyzing nucleotide formation, since AIC is converted to the nucleotide by adenine phosphoribosyltransferase (EC 2.4.2.7)[9, 10] and hypoxanthine by hypoxanthine phosphoribosyltransferase (EC 2.4.2.8),[9] whereas MeMP-ribonucleoside is phosphorylated by adenosine kinase (EC 2.7.1.20).[2, 11] The fact that combinations of adenine and guanine were no better than adenine alone cannot be explained readily but would appear to preclude an inadequate rate of GMP synthesis from adenine as an explanation for the failure of adenine alone to give complete reversal. A possible explanation for the failure of the adenine–guanine combination is that IMP has some function other than as an intermediate in purine biosynthesis and that the conversion of AMP to IMP is not fast enough to satisfy this requirement. Carey and Mandel[12] advanced a similar explanation to explain the failure of adenine and guanine to reverse inhibition by 6-mercaptopurine.

The ability of AIC or hypoxanthine to completely reverse inhibition produced by the lower concentrations of MeMP-ribonucleoside indicates that the initial action of this inhibitor is on an early step of purine biosynthesis prior to the point of entry of AIC. The failure of AIC and hypoxanthine to protect completely against the higher concentrations of MeMP-ribonucleoside suggests that another site, or other sites, of inhibition become significant once the cell has accumulated MeMP-ribonucleotide in excess of the amount required to inhibit PRPP amidotransferase. The relative importance of the blockade of purine synthesis and the other site(s) of inhibition to cytotoxicity and antitumor activity cannot be assessed from the data at hand. However, from the data of Fig. 2 it is apparent that AIC or hypoxanthine protected completely against a concentration of MeMP-ribonucleoside (3·5 μM) that not only prevented cell proliferation but destroyed 75 per cent of the cells initially present. These facts indicate that blockade of purine synthesis *de novo* does produce cytotoxicity. On the other hand, the failure of AIC and hypoxanthine to provide any protection against a 175 μM concentration of MeMP-ribonucleoside indicates that at this concentration the analog produces a cytotoxicity independent of that resulting from blockade of purine synthesis and sufficient to kill essentially all of the cells, so that reversal of the blockade of purine synthesis affords no relief of cytotoxicity. At intermediate concentrations of MeMP-ribonucleoside (up to 35 μM), AIC and hypoxanthine gave partial reversal; this result suggests that at these concentrations the cytotoxicity is due in part to the blockade of purine synthesis *de novo* and in part to inhibition at the other site(s).

In experiments similar to those reported here, Hakala and Nichol[13] found that AIC effectively reversed the growth-inhibitory action of MP in Sarcoma 180 cells in culture. Thus, the primary effects of both MP and MeMP-ribonucleoside appear to be on an early step of purine synthesis *de novo*. Since MeMP-ribonucleotide is a metabolite of MP[14, 15] and is more effective than MP-ribonucleotide as an inhibitor of PRPP amidotransferase,[6] it is probable that reversal of the effects of both MP and MeMP-ribonucleoside by AIC is the result of circumvention of blockade of this enzyme produced by MeMP-ribonucleotide.

Acknowledgement—This work was supported by Grant No. T-13K from the American Cancer Society and by Contract PH43-66-29 with Chemotherapy, National Cancer Institute, National Institutes of Health.

*Kettering–Meyer Laboratory,** L. Lee Bennett, Jr.
Southern Research Institute, Doris J. Adamson
Birmingham, Ala. 35205, U. S. A.

* Affiliated with the Sloan–Kettering Institute for Cancer Research, New York, N. Y.

REFERENCES

1. L. L. Bennett, Jr., R. W. Brockman, H. P. Schnebli, S. Chumley, G. J. Dixon, F. M. Schabel, Jr., E. A. Dulmadge, H. E. Skipper, J. A. Montgomery and H. J. Thomas, *Nature, Lond.* **205**, 1276 (1965).
2. I. C. Caldwell, J. F. Henderson and A. R. P. Paterson, *Can. J. Biochem.* **44**, 229 (1966).
3. J. F. Henderson, *Biochem. Pharmac.* **12**, 551 (1963).
4. L. L. Bennett, Jr., H. P. Schnebli, M. H. Vail, P. W. Allan and J. A. Montgomery, *Molec. Pharmac.* **2**, 432 (1966).

5. I. C. CALDWELL, J. F. HENDERSON and A. R. P. PATERSON, *Can. J. Biochem.* **45**, 735 (1967).
6. D. L. HILL and L. L. BENNETT, JR., *Biochemistry, N.Y.* **8**, 122 (1969).
7. J. B. WYNGAARDEN and D. M. ASHTON, *J. biol. Chem.* **234**, 1492 (1959).
8. G. G. KELLEY, M. H. VAIL, D. J. ADAMSON and E. A. PALMER, *Am. J. Hyg.* **73**, 231 (1961).
9. R. W. BROCKMAN, G. G. KELLEY, P. STUTTS and V. COPELAND, *Nature, Lond.* **191**, 469 (1961).
10. J. G. FLAKS, M. J. ERWIN and J. M. BUCHANAN, *J. biol. Chem.* **228**, 201 (1957).
11. H. P. SCHNEBLI, D. L. HILL and L. L. BENNETT, JR., *J. biol. Chem.* **242**, 1997 (1967).
12. N. H. CAREY and H. G. MANDEL, *J. biol. Chem.* **236**, 520 (1961).
13. M. T. HAKALA and C. A. NICHOL, *Biochim. biophys. Acta* **80**, 665 (1964).
14. P. W. ALLAN, H. P. SCHNEBLI and L. L. BENNETT, JR., *Biochim. biophys. Acta* **114**, 647 (1966).
15. P. W. ALLAN and L. L. BENNETT, JR., *Proc. Am. Ass. Cancer Res.* **9**, 2 (1968).

Biochemical Pharmacology, Vol. 19, pp. 2176–2179. Pergamon Press. 1970. Printed in Great Britain

Sodium salicylate and L-glutamic dehydrogenase activity of the brain

(*Received* 26 *November* 1969; *accepted* 9 *January* 1970)

IT HAS been shown by Gould *et al.*[1] that sodium salicylate inhibits the activity of L-glutamic dehydrogenase (L-glutamate NAD (P) oxido reductase EC 1.4.1.2)* when added to purified enzyme preparations of bovine liver. It also inhibits glutamate–pyruvate transaminase in rat serum and tissue extracts and the drug is active against xanthine dehydrogenase of the liver.[2, 3]

The important role played by GDH in brain metabolism suggested to us to study the effect of salicylate on the enzymes involved in the formation and breakdown of glutamic acid in the brain.

Normal white mice of known pedigree were used throughout this study. The animals were killed by cervical dislocation and the brain immediately removed, blotted, weighed and immersed in 1 ml of ice-cold distilled water. The homogenates were prepared in a glass Potter–Elvehjem apparatus with a Teflon pestle for 3 min and sufficient cold distilled water to give a 10 per cent homogenate and then centrifuged at 0° in an International apparatus at −2° for 20 min and the cell-free supernatant used for the assays according to the method of Olsen and Anfinsen.[4] GDH being confined to the mitochondria, the homogenates were prepared with distilled water instead of buffer and incubated 1 hr at 0° in the way to obtain full activity by disruption of the mitochondria membrane.[5] The results are expressed in micromoles of NADH oxidized per milligram protein per minute.[6] The values were recorded by observing the changes in absorption at 340 nm in a Zeiss spectrophotometer PQMII. Protein was determined spectrophotometrically.[7]

Cell-free extracts were incubated for 60 min with different concentrations of sodium salicylate and the enzymatic activity measured as shown in Fig. 1. The percentages of inhibition increased with the concentration of salicylate and the time of incubation at 0°. After 24 hr there is a 100 per cent inhibition even with 0·1 ml of the 0·3 M solution (Table 1).

It has been shown by Tomkins *et al.*[8] that crystalline GDH of bovine liver (molecular weight 1,000,000) can be dissociated in four subunits when inactivated by diethylstilbestrol and other compounds. These subunits are devoid of GDH activity but showed ADH activity. We were therefore interested to know if the *in vitro* inactivation of this enzyme in brain extracts could be explained by the disaggregation of the molecule. We found that when the activity decreased there was a marked

* Abbreviations used: GDH = L-glutamic dehydrogenase; ADH = alanine dehydrogenase; ADP = adenosine 5-diphosphate.