Biochemical Pharmacology, Vol. 21, pp. 761–770. Pergamon Press, 1972. Printed in Great Britain

# INHIBITION OF THYMIDYLIC ACID KINASE BY 5-SUBSTITUTED PYRIMIDINES*

CHI-BOM CHAE, M. CATHERINE DOBBINS, J. LOGAN IRVIN and CLAUDE PIANTADOSI

Departments of Biochemistry and Medicinal Chemistry, University of North Carolina, Chapel Hill, N.C. 27514, U.S.A.

(*Received* 23 *February* 1971; *accepted* 27 *September* 1971)

**Abstract**—2-Mercapto-5-(*p*-chlorobenzyl) orotic aldehyde (MCBOA) inhibits the incorporation of labeled thymidine into TTP and DNA in Ehrlich ascites carcinoma cells *in vitro*. MCBOA inhibits phosphorylation of TMP to TDP by high-speed supernatants of Ehrlich ascites carcinoma and regenerating rat liver, and the inhibition is of the competitive type. MCBOA does not affect thymidine kinase, TDP kinase or the phosphorylation of uridine to UTP in high-speed supernatants of Ehrlich ascites carcinoma. Pyrimidines with bulky groups at the C-5 position inhibit TMP kinase.

THE SYNTHESIS of DNA is regulated in part by the controlled synthesis of TTP; the levels of enzymes involved in the synthesis of TTP such as TdR† kinase,[1,2] TMP kinase,[3] TMP synthetase[4] and dCMP deaminase[4] are minimal in resting cells, but they begin to rise in the early synthetic period (S-phase) prior to DNA synthesis during the cell cycle.

TMP is synthesized mainly by two pathways, *de novo* and salvage (preformed) pathways, and thus the inhibition of one pathway may be overcome by the production of the metabolite by an alternate pathway. Therefore, the inhibition of TMP kinase should be very effective in blocking both *de novo* and salvage pathways for production of TTP and, ultimately, DNA-thymine in replicating cells.

In this paper, experimental evidence is presented on the inhibition of TMP kinase of Ehrlich ascites carcinoma by 2-mercapto-5-(*p*-chlorobenzyl) orotic aldehyde (MCBOA) and other 5-substituted pyrimidines in a cell-free system. A preliminary account of this work has appeared elsewhere.[5] In addition to its effect on TMP kinase, MCBOA inhibits pathways of both pyrimidine and purine synthesis and also inhibits synthesis of RNA, DNA and proteins.[5]

## EXPERIMENTAL

### *Preparation of soluble extract*

*From Ehrlich ascites carcinoma cells.* The Ehrlich ascites carcinoma cells were collected from Swiss mice inoculated 7 days previously, and the cells were washed with cold 0·154 M KCl. All steps were performed at 2°. To the packed cells, 2 vol. of

* This work was aided by Grant CA-06364 from the National Institutes of Health and an institutional grant (In-15L) from the American Cancer Society.

† Abbreviations used: DMSO, dimethylsulfoxide; MCBOA, 2-mercapto-5-(*p*-chlorobenzyl) orotic aldehyde; TdR, thymidine.

0·154 M KCl were added, and the cell suspension was sonically disrupted with a Branson sonifier, model S-110. The sonically disrupted suspension was centrifuged in a Spinco model L ultracentrifuge at 105,000 *g* in a No. 40 rotor for 60 min. The supernatant was stored in 1·2 ml vol. in 13 × 100 mm tubes in a liquid nitrogen refrigerator. TMP kinase activity was stable up to 2 months.

*From regenerating rat liver.* A two-thirds partial hepatectomy[6] was performed on albino, male, Sprague–Dawley rats. The remaining liver was removed after 30 hr and pressed through a tissue press. Two vol. of 0·154 M KCl were added to the pressed liver, and the tissue was homogenized in a Thomas tissue homogenizer (Potter–Elvehjem type) with a Teflon pestle. The homogenate was centrifuged in a No. 40 rotor in a Spinco model L ultracentrifuge for 60 min. The supernatant was stored in a liquid nitrogen refrigerator.

*Assay for phosphorylation of thymidine*

To 0·2 ml of reaction mixture containing 3 μmoles ATP, 3 μmoles $MgCl_2$, 3 μmoles 3-phosphoglycerate, and 30 μmoles tris buffer, pH 8·0, were added 30 μl drug dissolved in dimethylsulforide (DMSO) or 30 μl DMSO for a control and 10 μl $^3$H-TdR (1 μc, 10 mμmoles) in 50% ethanol. The tubes were preincubated for 30 min at 37°, and the reaction was started by the addition of 0·1 ml of soluble extract. The tubes were then incubated for the appropriate length of time. At the end of incubation, the tubes were heated for 2 min in boiling water to stop the reaction. The tubes were then centrifuged. A sample (20 μl) of the supernatant was applied 2 cm from the bottom of a 2·5 × 9 in. strip of DEAE, DE-81 paper with a Drummond disposable micropipette and allowed to dry. A standard mixture of TdR, TMP, TDP and TTP was applied as a marker at a point on the line loaded with the sample, and again allowed to dry. The paper strips were hung in a Colab chromatography tank and developed with a solvent mixture of acetic acid (1 M) and sodium citrate buffer,[7] pH 3·2 or 3·5 depending on the degree of resolution desired, until the solvent front reached approximately 12 cm from the bottom. $R_f$ values were 0·07 for TTP, 0·2 for TDP, 0·49 for TMP, and 0·87 for TdR when pH 3·5 buffer was used. With the pH 3·2 buffer, we found $R_f$ values of 0·03 for TTP, 0·045 for TDP, 0·35 for TMP, and 0·9 for TdR. The paper was allowed to dry, and the positions of the nucleotides were identified under an ultraviolet lamp. These areas were cut out and placed in scintillation vials containing 16 ml of a PPO–POPOP scintillation mixture,* and the radioactivity was determined in a Packard scintillation spectrometer, model 3003. Counting efficiency was approximately 6·5 per cent for the tritium.

*Assay for TMP kinase*

The assay for TMP kinase was identical with that described above for thymidine phosphorylation except that $^3$H-TMP (1 μc/tube) was used as substrate. In Fig. 1, the phosphorylation of TMP by different amounts of the soluble extract is shown. In assays where $K_m$ was determined, the concentration of TMP varied from 5 to 50 mμmoles, and to each tube was added 0·1 ml of soluble extract which had been diluted 2:1 with 0·154 M KCl. The substrates were added in 10 μl of 50% ethanol.

* In 1 l. of toluene, 5 g of 2,5-diphenyloxazole (PPO) and 0·3 g of 1,4-bis-(4-methyl-5-phenyloxazolyl) benzene (dimethyl-POPOP) were dissolved.

The radiopurity of the $^3$H-TMP was found by paper chromatography to be approximately 60 per cent (the impurity being principally TdR) and the specific activities of $^3$H-TMP of different concentrations were corrected for the radiopurity of $^3$H-TMP. The total counts per minute in TMP, TDP and TTP after the reaction was about 90 per cent of the radioactivity of $^3$H-TMP added. No attempt was made to correct for the partial loss of the radioactivity.

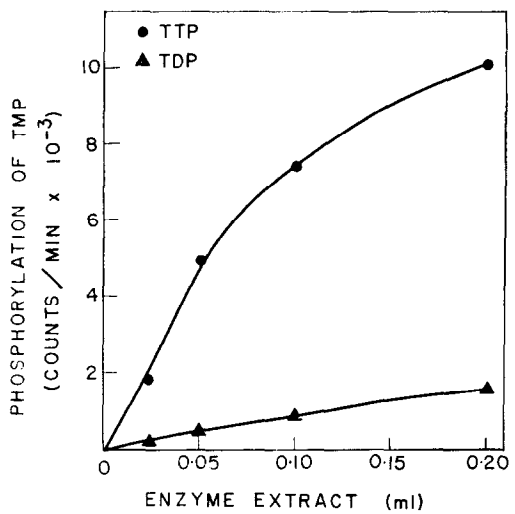



FIG. 1. Effect of enzyme concentration on the phosphorylation of TMP. The precursor, $^3$H-TMP (1 $\mu$c, 0·1 $\mu$c/m$\mu$mole) was added in 10 $\mu$l of a 50% ethanol solution. The experimental conditions are described in Experimental. The incubation time was 30 min.

*Rate of phosphorylation of thymidine*

Before studying the effects of MCBOA and other pyrimidines on the phosphorylation of TdR to TTP, we first conducted assays with the soluble extract from Ehrlich ascites carcinoma cells to determine the rate of phosphorylation of $^3$H-TdR and to determine the effect of KCl and DMSO (which was used in later assays as the drug solvent) on the phosphorylation pattern. The rate of phosphorylation of TdR to TTP was essentially the same when the incubation was done with the addition of 30 $\mu$l water or 30 $\mu$l of 0·154 M KCl, but 30 $\mu$l DMSO (1·2 mM final concentration) produced an inhibition of TMP kinase which was especially apparent after 15 min of incubation (Figs. 2a and 2b). Incubation in the presence of water, KCl or DMSO for 15 min resulted in amounts of labeled products such that $^3$H-TTP > $^3$H-TMP > $^3$H-TDP. In subsequent kinetic assays, the incubation time was 15 min, during which time the rate of phosphorylation of TMP to TTP in the presence of DMSO was linear. Although DMSO was shown to be an inhibitor of TMP kinase, its use in the inhibition and kinetic studies was necessary because of the poor solubility of MCBOA in aqueous solvents.

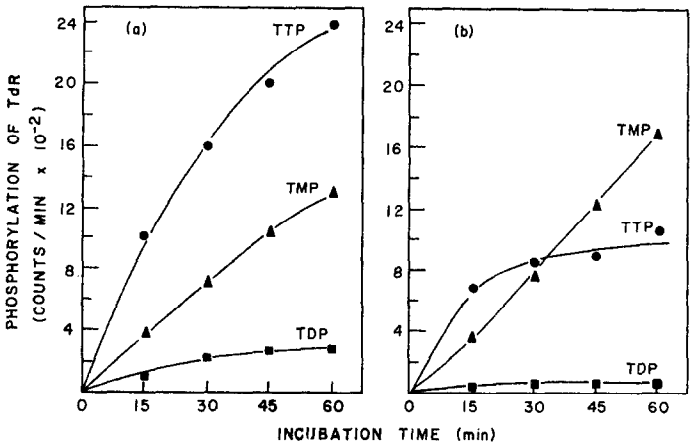



FIG. 2. Rate of phosphorylation of TdR in the presence of: (a) 30 $\mu$l $H_2O$ or 0·154 M KCl, and (b) 30 $\mu$l DMSO (1·2 mM final). The precursor, $^3$H-TdR (1 $\mu$c, 0·1 $\mu$c/m$\mu$mole), was added in 10 $\mu$l of a 50% ethanol solution. The experimental conditions are described in Experimental.

*Chemicals*

TMP-methyl-$^3$H, TdR-methyl-$^3$H, and uridine-5-$^3$H were purchased from the New England Nuclear Corp., and D(−) 3-phosphoglycerate (sodium salt), ATP and all other nucleotides were obtained from Sigma Chemical company.

## RESULTS

*Effect of MCBOA on the phosphorylation of TdR and DNA synthesis* in vitro

We have reported[5] that MCBOA inhibits incorporation of TdR into DNA in Ehrlich ascites carcinoma *in vitro*. In order to locate the site of inhibition, the Ehrlich ascites carcinoma cells were incubated with MCBOA and $^{14}$C-TdR, and then thymidine nucleotides were isolated from the cells by the method described in a previous paper.[8] At a concentration of 0·5 mM, MCBOA inhibited incorporation of TdR-$^{14}$C into DNA by 40–60 per cent, but the label accumulated in TTP (200 per cent of control) as shown in Table 1. This result suggests that the conversion of TTP to DNA was blocked by MCBOA. However, at a higher concentration of MCBOA (1 mM) the labels in both TTP and DNA are lower than the control values; 30–70 per cent inhibition for TTP and 90 per cent for DNA. This experiment suggests that MCBOA inhibits phosphorylation of TdR (or TMP) and the DNA polymerase reaction, the later reaction being more sensitive to the drug than the former. It was shown previously[8] that 5-fluoroorotic aldehyde inhibits the DNA polymerase reaction, while 5-fluorouracil showed no effect on the synthesis of either TTP or DNA when TdR was used as a precursor. This seems to suggest that the 6-carboxaldehyde group of MCBOA could be responsible for the inhibition of DNA polymerase in whole cells.

*Inhibition of TMP kinase by MCBOA in a cell-free system*

The effect of MCBOA on the phosphorylation of $^3$H-TdR was then tested in a cell-free system of Ehrlich ascites carcinoma, and it was found that MCBOA inhibits

TABLE 1. EFFECT OF MCBOA ON THE SYNTHESIS OF TTP AND DNA IN EHRLICH ASCITES CARCINOMA CELLS *IN VITRO**

| Concn of MCBOA (mM) | Expt | $^{14}C/^{3}H$ as % of control† TTP | DNA |
|---|---|---|---|
| 0·5 | 1 | 222 | 52 |
| | 2 | 204 | 38 |
| 1·0 | 1 | 69 | 8 |
| | 2 | 27 | 11 |

* Cells (0·5 ml packed volume) in 3 ml KRP were labeled with 2·5 $\mu$c, 0·08 $\mu$mole, of TdR-2-$^{14}C$ for 30 min at 37° in the presence of MCBOA or 0·1 ml DMSO (control) and mixed with the cells (0·5 ml packed volume) labeled with 12·5 $\mu$c TdR-$^{3}H$ for 30 min in the absence of MCBOA. The doubly labeled nucleotides were separated from the mixed cells by ion-exchange thin-layer chromatography.[8] The data are averages of duplicates in each experiment.

† $^{14}C/^{3}H$ in disintegrations per minute: TTP, 0·22; DNA, 0·21.

phosphorylation of TdR to TTP by 50 per cent at 0·5 mM (Fig. 3). However, MCBOA apparently does not affect phosphorylation of TdR to TMP (TdR kinase), but inhibits phosphorylation of TMP to TDP (TMP kinase). MCBOA does not affect the conversion of TDP to TTP as shown by the similar "per cent of control" values for TDP and TTP (Table 2). The fact that MCBOA does not affect the TdR kinase reaction supports the conclusions by Baker *et al.*[9] that TdR kinase is not affected by a bulky group at the C-5 position of pyrimidines or pyrimidine nucleosides. This observation, however, is not surprising, since there is strong evidence that the TdR kinase is actually

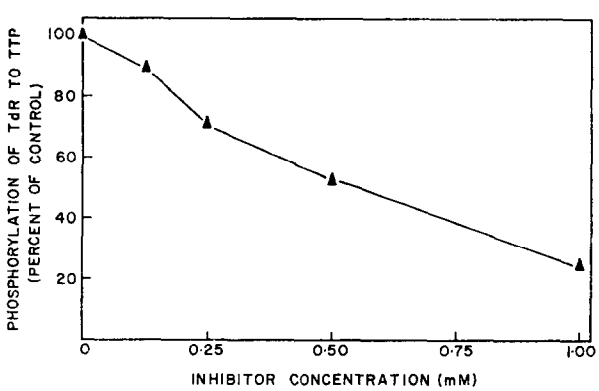



FIG. 3. Effect of MCBOA on the phosphorylation of TdR to TTP. The precursor, $^{3}H$-TdR (1 $\mu$c/m$\mu$mole), was added in 10 $\mu$l of a 50% ethanol solution. The experimental conditions are described in Experimental.

TABLE 2. EFFECT OF MCBOA ON THE PHOSPHORYLATION OF THYMIDINE AND URIDINE BY AN EXTRACT OF EHRLICH ASCITES CARCINOMA CELLS*

| Inhibitor | Concn (mM) | Percent of control MP | DP | TP | MP+DP+TP |
|---|---|---|---|---|---|
| Expt 1 Phosphorylation of $^3$H-TdR | | | | | |
| None† | | 100 | 100 | 100 | 100 |
| MCBOA | 0·5 | 112 | 66 | 51 | 113 |
| | 1·0 | 110 | 16 | 19 | 104 |
| Expt 2 Phosphorylation of $^3$H-UR | | | | | |
| None‡ | | 100 | 100 | | 100 |
| MCBOA | 0·5 | 112 | 116 | | 110 |
| | 1·0 | 115 | 112 | | 108 |

* MP, DP and TP, nucleoside mono-, di- and tri-phosphates respectively. The precursor, $^3$H-TdR (1 $\mu$c, 0·1 $\mu$c/m$\mu$mole) or $^3$H-UR (1 $\mu$c, 0·1 $\mu$c/m$\mu$mole) was added in 10 $\mu$l of a 50% ethanol solution. The experimental conditions are described in Experimental. The data are averages of duplicates in each experiment.

† Counts per minute: TMP, 600; TDP, 290; TTP, 3470.

‡ Counts per minute: UMP, 220; UDP + UTP, 540.

nonspecific for pyrimidine deoxynucleosides—for example, both deoxyuridine and TdR are equally effective as substrate for the enzyme.[10,11]

The specificity of the inhibition of TMP kinase by MCBOA is shown by the absence of inhibition of phosphorylation of uridine to UTP by MCBOA (Table 2). This result also suggests that TMP kinase has a site for the methyl group of thymine. The TMP kinases purified from *Escherichia coli*[12] and mouse hepatoma[13] are specific for TMP and use dUMP as substrate only to a limited extent.

The inhibition of TMP kinase of Ehrlich ascites carcinoma cells was of the competitive type (Fig. 4). The $K_m$ for TMP with the Ehrlich ascites TMP kinase in an

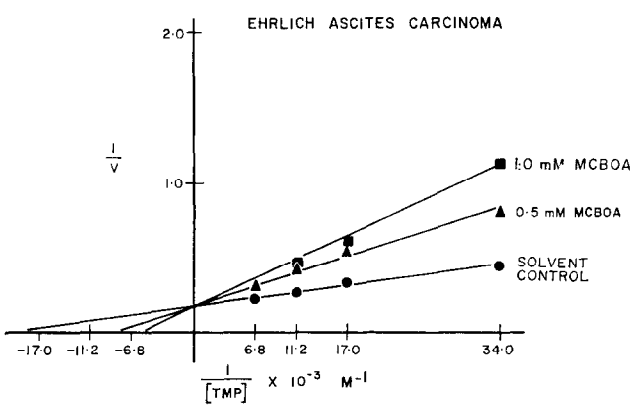



FIG. 4. Lineweaver–Burk plots of the inhibition by MCBOA of the phosphorylation of TMP to TDP and TTP by Ehrlich ascites TMP kinase. The experimental conditions are described in Experimental. V = millimicromoles TDP + TTP formed per milligram of protein per 15 min.

incubation mixture containing no DMSO was found to be $2{\cdot}1 \pm 0{\cdot}3 \times 10^{-5}$ M. The $K_m$ for TMP determined in the presence of 1·2 mM DMSO (30 $\mu$l) was $4{\cdot}6 \pm 0{\cdot}7 \times 10^{-5}$ M, while the $K_i$ with MCBOA was found to be $3{\cdot}9 \pm 0{\cdot}2 \times 10^{-4}$ M. The ratio of $K_i/K_m$ (DMSO) suggests that the binding of MCBOA to the enzyme is approximately 8·5-fold less than that of TMP, but this interpretation is subject to the uncertainties of considering these constants as true measures of binding. The smaller binding for MCBOA in comparison with TMP may be due to the absence of the deoxyribophosphate group on the MCBOA or to the possibility that the *p*-chlorobenzyl group may be too large for the site of the methyl group. MCBOA also inhibits TMP kinase from regenerating rat liver (Fig. 5). The $K_m$ for TMP determined in the presence of DMSO with the liver TMP kinase was found to be $5{\cdot}9 \pm 0{\cdot}5 \times 10^{-4}$ M while the $K_i$ for MCBOA in this system was found to be $1{\cdot}1 \pm 0{\cdot}1 \times 10^{-3}$ M. The inhibition by MCBOA also was of the competitive type with this enzyme.

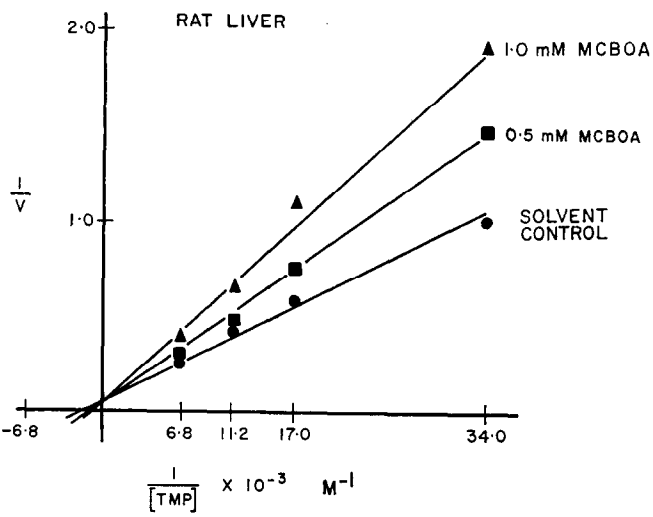
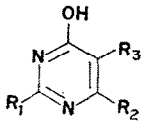



FIG. 5. Lineweaver–Burk plots of the inhibition by MCBOA of the phosphorylation of TMP to TDP and TTP by regenerating rat liver TMP kinase. The experimental conditions are described in Experimental. V = millimicromoles TDP + TTP formed per milligram of protein per 15 min.

*Effects of various 5-substituted pyrimidines on TMP kinase*

We have tested several 5-substituted pyrimidine analogs for the inhibition of Ehrlich ascites TMP kinase (Table 3). Although the compounds listed in Table 3 do not constitute an ideal group for a study of the specificity of the enzyme site for the methyl group of thymine, this series does show some interesting trends in inhibitory effects with respect to substituents at C-5, although the concentrations required are rather high. The groups which show inhibition are amino (compound 3), *p*-chlorobenzyl (compound 4), *p,m*-dimethylbenzyl (compound 6), phenylpropyl (compound 7), phenylethyl (compound 9), and naphthylmethyl (compound 10). These pyrimidines, except 5-aminouracil and compound 9, have 2-mercapto and 6-carboxaldehyde groups, but these groups alone have no effect on the TMP kinase activity as shown for

TABLE 3. EFFECTS OF 5-SUBSTITUTED PYRIMIDINES ON PHOSPHORYLATION OF [$^3$H] THYMIDINE BY AN EXTRACT OF EHRLICH ASCITES CARCINOMA CELLS*




| No. | Pyrimidines | Concn (mM) | Average exptl values (% of control)† TMP | TDP | TTP |
|---|---|---|---|---|---|
| | $R_1$ = OH | 0·5 | 100 | 100 | 100 |
| 1 | $R_2$ = CHO | 1·0 | 92 | 88 | 97 |
| | $R_3$ = H | | | | |
| | $R_1$ = OH | | | | |
| 2 | $R_2$ = H | 0·5 | 109 | 67 | 105 |
| | $R_3$ = I | 1·0 | 110 | 91 | 105 |
| | $R_1$ = OH | | | | |
| 3 | $R_2$ = H | 0·5 | 139 | 126 | 93 |
| | $R_3$ = $NH_2$ | 1·0 | 87 | 38 | 29 |
| | $R_1$ = SH | | | | |
| 4 | $R_2$ = CHO | 0·5 | 112 | 35 | 37 |
| | $R_3$ = $CH_2\phi$-Cl (p) | 1·0 | 110 | 16 | 19 |
| | $R_1$ = SH | | | | |
| 5 | $R_2$ = $CH_3$ | 1·0 | 100 | | 89 |
| | $R_3$ = $CH_2\phi$-Cl (p) | | | | |
| | $R_1$ = SH | | | | |
| 6 | $R_2$ = CHO | 0·5 | 104 | | 62 |
| | $R_3$ = $CH\phi$-$CH_3$ (m) | 1·0 | 100 | 76 | 53 |
| | $CH_3$ (p) | | | | |
| | $R_1$ = SH | | | | |
| 7 | $R_2$ = CHO | 1·0 | 112 | 67 | 52 |
| | $R_3$ = $(CH_2)_3$-$\phi$ | | | | |
| | $R_1$ = SH | | | | |
| 8 | $R_2$ = CHO | 1·0 | 96 | 78 | 110 |
| | $R_3$ = $(CH_2)_{11}$-$CH_3$ | | | | |
| | $R_1$ = $NH_2$ | | | | |
| 9 | $R_2$ = CHO | 0·5 | 110 | 42 | 43 |
| | $R_3$ = $(CH_2)_2\emptyset$ | 1·0 | 104 | 36 | 18 |
| | $R_1$ = SH | | | | |
| 10 | $R_2$ = CHO | 1·0 | 173 | 33 | 43 |
| | $R_3$ = $CH_2$  | | | | |
| | 24-Diamino | | | | |
| 11 | $R_2$ = $CH_2CH_3$ | 1·0 | | | 85 |
| | $R_3$ = $\phi$-Cl (p) | | | | |
| | 2,4-Diamino | | | | |
| 12 | $R_2$ = H | 0·5 | | | 93 |
| | $R_3$ = $CH_2$-$\phi$ $(CH_3)_3$ | 1·0 | | | 85 |
| 13 | 5-Iodo-deoxy- | 0·5 | 25 | 15 | 8 |
| | uridine | 1·0 | 20 | 3 | 4 |

* The precursor, $^3$H-TdR (1 $\mu$c, 0·1 $\mu$c/m$\mu$mole), was added in 10 $\mu$l of a 50% ethanol solution. The experimental conditions are described in Experimental. The data are averages of duplicate experiments in each case. Averages of duplicate experiments agree within 10 per cent. In the case of compound 4, five similar experiments were performed, and the standard deviation was $\pm$7 per cent. The probability of deviation of a single experiment from the mean by more than 10 per cent was 0·1.

† Counts per minute: TMP, 600; TDP, 290; TTP, 3470.

the carboxaldehyde group with compound 1. However, the C-6 carboxaldehyde group seems to enhance the inhibitory effect on TMP kinse (compare compounds 4 and 5).

Antifolic compounds with C-5 bulky groups, pyrimethamine (compound 11) and trimethoprim (compound 12) showed no effect on TMP kinase. This lack of effect on TMP kinase is probably due to the C-4 amino group which gives these compounds a resemblance to cytosine.

Iodouracil (compound 2) which has iodine at C-5 showed no effect on TMP kinase. Iodine is about the same size as methyl, and iodouracil is actually incorporated into DNA in place of thymine when injected into the animal.[14] Iododeoxyuridine (compound 13) inhibited both thymidine kinase and TMP kinase. It is probable that iododeoxyuridylic acid was formed by TdR kinase in our system, and this product would compete with TMP for TMP kinase. Iododeoxyuridylic acid is a competitive inhibitor of TMP kinase purified from *E. coli*.[12] Consequently, the inhibition of TMP kinase by iododeoxyuridine cannot be compared with the inhibitory effects of the pyrimidines of Table 3.

MCBOA seems to be the most active pyrimidine derivative of the series listed in Table 3, but the strength of inhibition of TMP kinase by this compound leaves much to be desired. The synthesis of the deoxynucleoside and deoxynucleotide derivatives of MCBOA might increase the binding of the drug to TMP kinase and enhance the inhibitory activity.

## DISCUSSION

The inhibition of TMP kinase of Ehrlich ascites carcinoma by pyrimidines with bulky groups at C-5 indicates that the enzyme site for the methyl of TMP might be relatively nonspecific and capable of accepting a variety of groups larger in effective volume than the methyl group. The experiments with a representative compound, MCBOA, show that TdR kinase, uridine kinase, and UMP kinase are not affected. Baker *et al.*[9] tested bulk tolerance of TdR kinase with 5-substituted pyrimidine nucleosides, and they concluded that TdR kinase is not affected by bulky groups at C-5 of the pyrimidine ring. This observation is not surprising, since there is convincing evidence that TdR kinase and deoxyuridine kinase are in fact the same enzyme.[10,11]

The inhibition of TMP kinase by 5-substituted pyrimidines suggests the possibility of a useful experimental system for study of the specificity of the enzymatic site for binding the methyl group. Systematic synthesis of 5-substituted pyrimidines with maximum inhibitory activity for TMP kinase and the synthesis of deoxynucleosides of these pyrimidines to potentiate the inhibitory activity and the permeability through cell membranes might lead to particularly effective inhibitors of TMP kinase in rapidly growing cells. MCBOA is obviously not an ideal inhibitor of TMP kinase, and its inhibitory effect on DNA polymerase *in vivo*[5] may be more important in explaining the inhibition of growth of the Ehrlich carcinoma by this compound.

*Acknowledgement*—We wish to thank Miss Sarah Jo Lohr and Mr. Donald Goodman for technical assistance.

## REFERENCES

1. F. J. Bollum and V. R. Potter, *Cancer Res.* **19,** 561 (1959).
2. E. Bresnick, S. S. Williams and H. Mosse, *Cancer Res.* **27,** 469 (1967).
3. S. M. Weissman, R. M. S. Smellie and J. Paul, *Biochim. biophys. Acta* **45,** 101 (1960).

4. F. MALEY and G. F. MALEY, *J. biol. Chem.* **235,** 2968 (1960).
5. C-B. CHAE, A. WILLIAMS, H. KRASNY, C. DOBBINS and J. L. IRVIN, *Proc. Am. Ass. Cancer Res.* **11,** 15 (1970).
6. G. M. HIGGINS and R. M. ANDERSON, *Arch. Path.* **12,** 186 (1931).
7. G. SERLUPI-CRESCENZI, C. PAOLINI and T. LEGGIO, *Analyt. Biochem.* **23,** 263 (1968).
8. C-B. CHAE, A. WILLIAMS, H. KRASNY, J. L. IRVIN and C. PIANTADOSI, *Cancer Res.* **30,** 2652 (1970).
9. B. R. BAKER, T. J. SCHWAN and D. V. SANTI, *J. med. Chem.* **9,** 66 (1966).
10. R. OKAZAKI and A. KORNBERG, *J. biol. Chem.* **239,** 269 (1964).
11. R. OKAZAKI and A. KORNBERG, *J. biol. Chem.* **239,** 275 (1964).
12. D. J. NELSON and C. E. CARTER, *J. biol. Chem.* **244,** 5254 (1969).
13. R. K. KIELLEY, *J. biol. Chem.* **245,** 4204 (1970).
14. M. L. EIDINOFF, L. CHEONG and M. A. RICH, *Science, N.Y.* **129,** 1550 (1959).