*Biochemical Pharmacology*, Vol. 33, No. 18, pp. 2863–2867, 1984.
Printed in Great Britain.

0006–2952/84 $3.00 + 0.00
Pergamon Press Ltd.

# MECHANISM OF INHIBITION OF PHOSPHORIBOSYLATION OF 5-FLUOROURACIL BY PURINES*

MITSUZI YOSHIDA† and AKIO HOSHI
Pharmacology Division, National Cancer Center Research Institute, Tsukiji 5-chome, Chuo-ku, Tokyo 104, Japan

(*Received* 30 *January* 1984; *accepted* 16 *April* 1984)

**Abstract**—The mechanism of the abolishment of the cytotoxicity of 5-flurouracil by purines in L5178Y cells was determined by using phosphoribosylation enzymes for both 5-fluorouracil and hypoxanthine. Hypoxanthine inhibited the phosphoribosylation of 5-fluorouracil in the presence of both enzymes, but no inhibition by hypoxanthine was found without hypoxanthine phosphoribosyltransferase or at a high concentration of 5-phosphoribosyl 1-pyrophosphate (PRPP). Hypoxanthine, adenine and inosine decreased the intracellular concentration of PRPP to less than one-tenth of that of the control. These results suggest that 5-fluorouracil is activated directly to its nucleotide, 5-fluorouridine 5′-monophosphate, by the phosphoribosylation enzyme and that the inhibition of activation by purines is due to depletion of PRPP.

Previously, we have reported that hypoxanthine, adenine and inosine completely reversed the cytotoxicity of 5-fluorouracil (FU) though there is no pyrimidine derivative to reverse it completely [1, 2]. In a cell-free system the phosphoribosylation of FU is inhibited by hypoxanthine, adenine and inosine at a low concentration of 5-phosphoribosyl 1-pyrophosphate (PRPP) [2]. In L5178Y cells, FU is not metabolized to 5-fluorouridine (FUR) and directly transformed to its nucleotide, 5-fluorouridine 5′-monophosphate (FUMP) by phosphoribosylation and the inhibition of its metabolism may be due to a deficiency of PRPP caused by the phosphoribosylation of hypoxanthine and adenine [1].

In this study, the mechanism of inhibition of FU phosphoribosylation by purines were examined with partially purified enzymes.

## MATERIALS AND METHODS

*Reagents.* 5-Fluorouracil was supplied by Mitsui Pharmaceuticals, Inc., Tokyo. 5-Fluoro[6-$^{14}$C]uracil (55 mCi/mmole) and [8-$^{14}$C]adenine (54 mCi/mmole) were obtained from Amersham International plc. (Amersham, U.K.). [8-$^{14}$C]Hypoxanthine (52.8 mCi/mmole), [8-$^{14}$C]guanine hydrochloride (44.7 mCi/mmole) and [2-$^{14}$C]uracil (46.1 mCi/mmole) were purchased from New England Nuclear (Boston, MA, U.S.A.); PRPP, 4-hydroxy(3,4-d)-pyrazolopyrimidine (allopurinol), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), and purine and pyrimidine derivatives from Sigma Chemical Co. (St. Louis, MO, U.S.A.); xanthine oxidase from Miles Laboratories, Inc. (Elkhart, IN, U.S.A.); EDTA from Tokyo Chemical Industry Ltd., (Tokyo, Japan); polyethyleneimine (PEI)-cellulose and Tris from E. Merck (Darmstadt, West Germany); and Toyopearl H-55F from Toyo Soda Mfg. Co. (Tokyo, Japan).

*Cell culture and reversal studies.* L5178Y cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated (56° for 30 min) calf serum in a humidified atmosphere of 5% $CO_2$ in air at 37°. Reversal was calculated as reported previously [3]. Reversal was graded as −, 10–9; +, 20–49; ++, 50–79; and +++, 80–100% of the control.

*Separation of purine and pyrimidine phosphoribosyltransferase.* L5178Y cells were harvested from female $BDF_1$ mice inoculated with $10^7$ cells intraperitoneally 12 days before. A crude cell-free extract was prepared by repeated freezing and thawing of L5178Y cells suspended in 50 mM Tris–HCl buffer at pH 7.4 and centrifugation at 100,000 *g* for 60 min. The crude extract was treated with ammonium sulfate by two steps (40 and 65% saturation). The precipitate, collected by centrifugation (10,000 *g*, 20 min), was dissolved in 50 mM Tris–HCl buffer (pH 7.4) containing 1 mM DTT, and stored in the frozen state (−30°).

Fractions containing FU and hypoxanthine phosphoribosyltransferase activities were separated by gel filtration (Toyopearl H-55F) of the ammonium sulfate fraction and subsequent DEAE-Sepharose column chromatography (1.5 × 7 cm).

*Enzyme assay.* Purine nucleoside phosphorylase activity was determined by the method of Hopkinson *et al.* [4] and Ogawa *et al.* [5]. The reaction at 37° was started by adding inosine and the increase in absorbance was followed at 293 nm with a Carl Zeiss PMQ 3 spectrophotometer (Oberkochen, West Germany). An absorbancy of 1.000 corresponded to 85 nmoles of uric acid. The specific activity is expressed as nmoles of uric acid produced at 37°/min/mg of protein. The protein content was determined by the Bio-Rad Laboratories (Rich-

* Part of this work was presented at the 101st Annual Meeting of the Pharmaceutical Society of Japan, Kumamoto, April 1981.

† Author to whom correspondence should be addressed.

mond, CA, U.S.A.) assay procedure based on the method of Bradford [6].

FU, uracil and purine phosphoribosyltransferase activities were determined by the filter technique of Schmidt *et al.* [7] by using a glass filter disc. The reaction mixture in a final volume of 0.2 ml contained 1 mM $MgCl_2$, 2.5 mM PRPP, 0.25 M Tris–HCl or glycine buffer, 0.1 ml of enzyme solution, 1 mM DTT and 0.4 $\mu$Ci of $^{14}$C-labeled FU, uracil or purine. The radioactivity of the nucleotide binding to PEI-cellulose was assayed with a Packard Tri-Carb 3320 liquid scintillation spectrometer, by using a toluene scintillator.

*Determination of intracellular PRPP concentration.* The intracellular PRPP concentration was determined by a method using the enzyme reaction of Wood *et al.* [8]. The enzymes used in these reaction were adenine (EC2.4.2.7) and hypoxanthine (EC2.4.2.8) phosphoribosyltransferase, which were separated by Sephadex G-100 (Particle size 40–120 $\mu$m) column chromatography (2.5 × 45 cm). The phosphoribosylation of adenine and hypoxanthine was carried out under the conditions described above except for the use of 5 mM $MgCl_2$, 50 mM Tris–HCl (pH 7.5) or glycine buffer (pH 9.0), 2 mM DTT and the supernatant instead of PRPP.

## RESULTS

*Reversal of FU cytotoxicity by purines.* We attempted to find purine derivatives to reverse the growth inhibition caused by FU. FU was used at $IC_{90}$ ($3.8 \times 10^{-6}$ M) which caused 90% inhibition of growth, throughout these studies. Hypoxanthine and adenine showed high reversal activity (+ + +) against FU cytotoxicity as reported previously [1]. Inosine also showed such high activity (+ + +) as to completely abolish the FU cytotoxicity. Allopurinol, a xanthine oxidase inhibitor, showed high reversal activity (+ +), but the maximum growth was less than 80% of that of the control because of the cytotoxicity of allopurinol itself at high concentration. Xanthine and adenosine also showed weak reversal activity (+). Other purine derivatives, guanine, purine, 6-methylpurine, 6-chloropurine, azathiopurine, uric acid, guanosine, xanthosine, deoxyadenosine, and deoxyguanosine, did not affect the FU cytotoxicity (−). The effects of allopurinol and xanthine may be due to an increase in intracellular concentration of hypoxanthine as a result of the enzyme inhibition and the product inhibition of the xanthine oxidase reaction, respectively.

*Enzyme activities in the crude extract of L5178Y cells.* To obtain information concerning the abolishment of FU cytotoxicity by hypoxanthine, adenine and inosine, the activities of purine nucleoside phosphorylase and phosphoribosyl-transferase for formation of purine and pyrimidine in a crude extract of L5178Y cells were determined.

As shown in Fig. 1, the activity of purine nucleoside phosphorylase was high and the specific activity was estimated to be 50 nmoles/min/mg of protein. Therefore, the reversal of FU cytotoxicity by inosine may have resulted from the production of hypoxanthine by intracellular purine nucleoside phosphorylase.

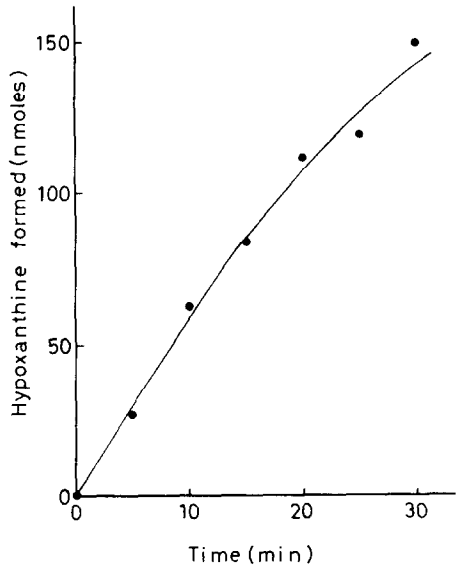


Fig. 1. Enzymatic formation of hypoxanthine from inosine by a crude extract of L5178Y cells. A reaction mixture containing 0.005 ml of xanthine oxidase, 0.8 ml of the cell-free extract (0.13 mg of protein) diluted with 50 mM sodium phosphate buffer (pH 7.4), 1.3 $\mu$moles of inosine and distilled water in a volume of 1 ml was incubated at 37°. Hypoxanthine formed was determined photometrically as uric acid after the enzymatic conversion with xanthine oxidase.

The ability of the enzymes to phosphoribosylate FU was as shown in Fig. 2. The enzyme activities were assayed at the optimal pH for each purine or pyrimidine. The phosphoribosyltransferase activities for hypoxanthine (H-PRT) and adenine (A-PRT) were 25 and 20 times that for FU (FU-PRT) during 10 min of incubation, respectively. The enzyme activities for guanine and uracil were less than one-fifth of that for FU.

*Inhibition of FU phosphoribosylation by purines.* Previously we found that the abolishment of FU cytotoxicity by hypoxanthine occurs only in a state of co-existence with FU [1] and that the abolishment by purines results from the inhibition of FU phosphoribosylation, the first reaction in the activation pathway of FU, whereas the enzymes catalyzing the phosphoribosylation of hypoxanthine and adenine are generally considered to be hypoxanthine–guanine phosphoribosyltransferase (EC 2.4.2.8) and adenine phosphoribosyltransferase (EC 2.4.2.7), respectively. The phosphoribosylation enzyme for FU is considered to be orotic acid phosphoribosyltransferase (EC 2.4.2.10) [9, 10] or uracil phosphoribosyltransferase [11–13]. Thus, these enzymes are distinguishable from each other. Therefore, we investigated to see if these purines directly inhibit the enzyme involved in FU phosphoribosylation or indirectly affect the PRPP concentration. H-PRT and FU-PRT were separated by gel filtration and subsequent ion exchange column chromatography as shown in Fig. 3. In the F-II fraction containing high FU-PRT activity, hypoxanthine did not affect the FUMP formation as shown in Fig. 4

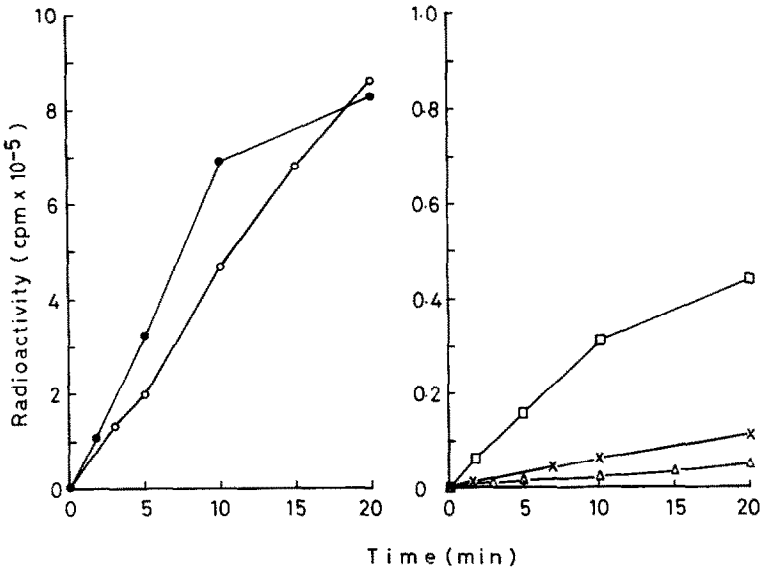


Fig. 2. Comparison of FU, uracil and purine phosphoribosyltransferase activities in the crude extract. Enzyme activity was assayed of optimal pH; ○——○, adenine (pH 7.5); ●——●, hypoxanthine (pH 9.0); □——□; FU (pH 8.5); ×——×, uracil (pH 9.5); △——△, guanine (pH 9.5). A mixture containing 1 mM $MgCl_2$, 2.5 mM PRPP, 0.25 M buffer, the crude extract (12 mg of protein) and 2 $\mu$Ci of $^{14}$C-labeled substrate (sp. act., *ca* 50 mCi/mmole) in a volume of 1 ml was incubated at 37°. Samples were removed from the reaction mixture at the indicated times and assayed as described in Materials and Methods.

(left). When the F-I fraction containing H-PRT was added to this system, FUMP formation was inhibited by even 0.2 mM hypoxanthine as shown in Fig. 4 (right). However, in the presence of a higher concentration of PRPP (2.5 mM), inhibition was not obtained by even 2.0 mM hypoxanthine (Fig. 5). These results clearly show that the inhibition of FUMP formation was the result not of enzyme inhibition by hypoxanthine, but of a deficiency of PRPP accompanied by phosphoribosylation of hypoxanthine.

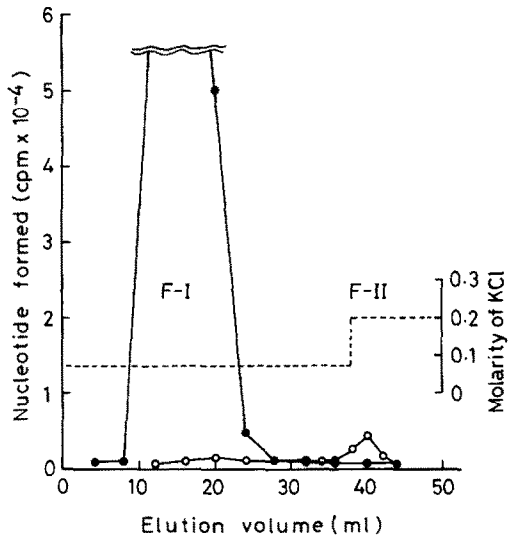


Fig. 3. DEAE-Sepharose elution profile of hypoxanthine and FU phosphoribosyltransferase activities. After gel filtration of the ammonium sulfate fraction, the fractions containing FU phosphoribosyltransferase activity were applied to a DEAE-Sepharose column (1.5 × 7 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.4) and 1 mM DTT. Elution was performed at a flow rate of 36 ml per hr with increasing concentrations of KCl from 0.07 to 0.2 M. Fractions of 2.0 ml each were collected and assayed for FU (○——○) and hypoxanthine (●——●) phosphoribosyltransferase activities.

*Change in intracellular PRPP concentration by purine derivatives.* To confirm experimentally that the complete reversal of FU cytotoxicity is due to PRPP deficiency caused by hypoxanthine, adenine or inosine, the effect of these purine derivatives on the intracellular concentration of PRPP in L5178Y cells was determined by a method using the enzyme reaction. When hypoxanthine or inosine was added to the medium, A-PRT and [8-$^{14}$C]adenine were used to eliminate interference by the additive, while in the case of the addition of adenine, H-PRT and [8-$^{14}$C]hypoxanthine were used for PRPP estimation for the same reason. The intracellular PRPP concentration was decreased to less than one-tenth of that of the control by 20 $\mu$g of hypoxanthine or adenine per ml and to 15% by 20 $\mu$g of inosine per ml during 30 min as shown in Fig. 6. As reported previously, hypoxanthine, adenine or inosine at the concentration of 20 $\mu$g/ml completely reverse FU cytotoxicity at cellular levels [1, 2]. These results suggest that the intracellular PRPP was rapidly consumed by phosphoribosylation of both purine bases and that this PRPP-deficiency state prevented the phosphoribosylation of FU.

## DISCUSSION

We have been concerned with the mechanism of action of the 5-fluorinated pyrimidines, FU, 5-

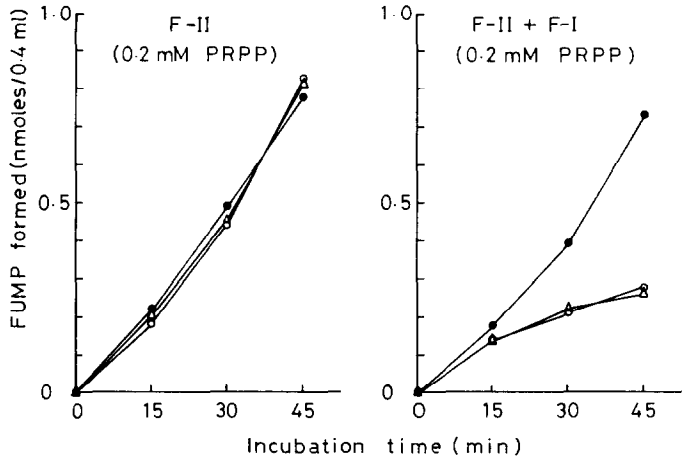


Fig. 4. Effects of hypoxanthine and F-I fraction on phosphoribosylation of FU at a low PRPP concentration. Hypoxanthine (△——△, 0.2 mM; ○——○, 2.0 mM) was added to a reaction mixture containing $^{14}$C-FU, 0.2 mM PRPP and separated F-II (FU-PRT fractions) in the presence or absence of F-I (H-PRT fractions). Without hypoxanthine (●——●). The radioactivity of the FUMP formed was counted as described in Materials and Methods at 40% efficiency. This experiment was repeated three times with the same results.

fluorouridine (FUR), 5-fluoro-2′-deoxyuridine. (FUdR) and 1-hexylcarbamoyl-5-fluoro-uracil (HCFU). Though FU and FUR have similar antiproliferating action against mouse leukemia L5178Y cells, the effects of the simultaneous addition of uridine on FU and FUR cytotoxicity are different [3]. To determine the activation pathway of FU, it is very important to find compounds which reverse FU cytotoxicity, like uridine for FUR and deoxyuridine for FUdR. As reported previously [1], we have found that FU cytotoxicity is completely reversed by hypoxanthine and adenine but not by guanine and uracil in L5178Y cells [1]. Furthermore, this reversal is caused by simultaneous addition of hypoxanthine and FU, but not by addition of hypoxanthine after exposing the cells to FU for 3 hr. As concerns FU metabolism, it has been thought that FU is converted to its ribonucleotide (5-fluorouridine 5′-monophosphate, FUMP) via FUR by uridine phosphorylase and kinase or directly to FUMP by phosphoribosyltransferase [14]. Our findings suggest that FU is not converted to FUR, but may be directly metabolized to FUMP by phosphoribosyltransferase similar to nucleotidation in purine bases.

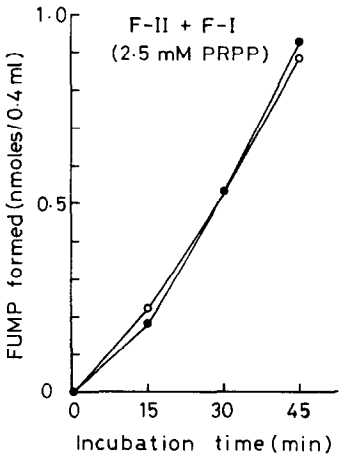


Fig. 5. Effects of hypoxanthine and F-I fraction on phosphoribosylation of FU at a higher PRPP concentration. Without hypoxanthine (●——●); 2.0 mM hypoxanthine (○——○). The assay described in Fig. 4 was used, changing only the PRPP concentration from 0.2 to 2.5 mM. This experiment was repeated three times with the same results.

In reversal studies, adenosine also showed weak reversal activity. With the crude extract, conversion of adenosine to hypoxanthine was obtained, but that of adenine was not. This result suggests that the slight reversal by adenosine is due to hypoxanthine to which adenosine is metabolized by adenosine deaminase and purine nucleoside phosphorylase and that the complete reversal by adenine is due to adenine itself. However, since the phosphoribosylation enzyme for hypoxanthine (hypoxanthine–guanine phosphoribosyltransferase) is different from that for adenine (adenine phosphoribosyltransferase) in mammalian cells, the inhibition of FU phosphoribosylation by hypoxanthine and adenine appears to be due not to their direct effect on the enzyme, but to their indirect effect on PRPP which is required for phosphoribosylation.

To confirm our hypothesis, we carried out investigation in two directions by determining (1) the effect of purines on the FU phosphoribosylation enzyme which was separated from H-PRT and A-PRT in the presence of various concentration of PRPP, (2) the effect of the purines on the intracellular PRPP concentration in cultured cells. In the present paper, we showed that hypoxanthine did not affect FU phosphoribosylation without H-PRT or at a high concentration of PRPP, and that the intracellular PRPP concentration was decreased within 30 min of incubation by exogenous hypoxanthine.

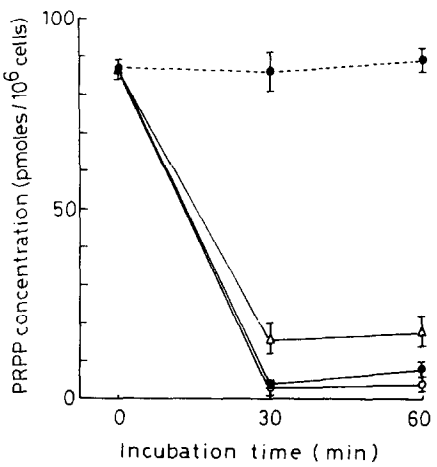


Fig. 6. Effects of hypoxanthine, adenine and inosine on intracellular concentration of PRPP. L5178Y cells ($10^6$/ml) were incubated and then exposed to 20 μg of hypoxanthine (●——●), adenine (○——○) or inosine (△——△) per ml at 37° for up to 60 min. No addition (●- - -●). Intracellular PRPP concentration was determined as described in Materials and Methods. Results are expressed as the mean (±S.E.) concentration per $10^6$ cells.

adenine or inosine at 20 μg/ml, which is the concentration at which complete reversal of FU cytotoxicity was found. These results suggest that in L5178Y cells FU is converted to its nucleotide, FUMP, by phosphoribosylation. This suggestion is consistent with the finding that the inhibitory effect of FU is not reversed by uridine which completely overcomes FUR cytotoxicity.

In general, FU, uracil and orotate phosphoribosylation is thought to be catalyzed by the same enzyme, pyrimidine phosphoribosyltransferase [9, 10]. The activity of FU phosphoribosyltransferase of L5178Y cells was found at the site of uracil phosphoribosyltransferase activity by isoelectric focusing in a granulated gel as previously reported [2]. However, the phosphoribosyltransferase enzyme for FU cannot be further purified and its substrate specificity cannot be determined because this enzyme is unstable.

Many investigators have studied the use of methotrexate and FU in combination [15–19]. Cadman *et al.* [20] and Buesa-Perz *et al.* [21] found that pretreatment with methotrexate increased the intracellular PRPP concentration, leading to enhanced FU cytotoxicity. Thus, the available PRPP in the cells is very important for expressing the antitumor activity of FU, and this fact must be fully considered in the clinical use of FU with other drugs. The alternative nucleotidation of FU by uridine phosphorylase and subsequent kinase is found in other tumors [22, 23]. With the expectation of finding a difference in the activation pathways of FU in host and tumor, the effect of FU in combination with hypoxanthine and allopurinol was studied by Houghton and Houghton [22]. As to reduction of toxicity, further investigation of the pathways of FU activation in human tumors is needed [24–25].

We believe that inosine is an important reagent for determining FU activation pathways *in vitro*, because inosine is metabolized to hypoxanthine, which prevents FU nucleotidation, and to ribose-1-phosphate, which acts as ribose donor in the uridine phosphorylase reaction, by intracellular purine nucleoside phosphorylase. When inosine decreases FU cytotoxicity, the activation pathway of FU in the tumor is considered to be a phosphoribosylation reaction, whereas when enhancement by inosine is observed, the pathway is considered to be a uridine phosphorylase reaction.

## REFERENCES

1. M. Yoshida, A. Hoshi and K. Kuretani, *Biochem. Pharmac.* **27**, 2979 (1978).
2. M. Yoshida, A. Hoshi and K. Kuretani, *Nucleic Acids Symposium Series* No. 8 s175 (1980).
3. M. Yoshida, A. Hoshi and K. Kuretani, *J. Pharm. Dyn.* **3**, 374 (1980).
4. D. A. Hopkinson, P. J. L. Cook and H. Harris, *Ann. Human Genet.* **32**, 361 (1969).
5. K. Ogawa, T. Tominaga, S. Taoka, K. Yata and E. Tsubura, *Gann* **69**, 471 (1978).
6. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
7. R. Schmidt, M. Foret and U. Reichert, *Clin. Chem.* **22**, 67 (1976).
8. A. W. Wood, M. A. Becker and J. E. Seegmiller, *Biochem. Genet.* **9**, 261 (1973).
9. D. Kessel, J. Deacon, B. Coffey and A. Bakamjian, *Molec. Pharmac.* **8**, 731 (1972).
10. P. Reyes and M. E. Guganig, *J. biol. Chem.* **250**, 5097 (1975).
11. R. W. Brockman, J. M. Davis and P. Stutts, *Biochim. biophys. Acta* **40**, 22 (1960).
12. D. Kessel, T. C. Hall and P. Reyes, *Molec. Pharmac.* **5**, 481 (1969).
13. A. Nahas, E. D. Savlov and T. C. Hall, *Cancer Chemother. Rep.* Part I, **58**, 909 (1974).
14. P. Roy-Burman, in *Recent Results in Cancer Research* (Eds. P. Rentchnick *et al.*), Vol. 25, p. 49. Springer, New York (1970).
15. H. W. Bruckner, C. Schreiber and S. Waxman, *Cancer Res.* **35**, 801 (1975).
16. T. H. Maugh II, *Science* **194**, 310 (1976).
17. S. Waxman and H. Bruckner, *Science* **194**, 1112 (1976).
18. J. R. Bertino, *Science* **194**, 1113 (1976).
19. A. Goldin, *Science* **194**, 1115 (1976).
20. E. Cadman, R. Heimer and L. Davis, *Science* **205**, 1135 (1979).
21. J. M. Buesa-Perez, A. Leyva and H. M. Pinedo *Cancer Res.* **40**, 139 (1980).
22. J. A. Houghton and P. J. Houghton, *Biochem. Pharmac.* **29**, 2077 (1980).
23. P. M. Schwartz and R. E. Handschmacher, *Proc. Am. Ass. Cancer Res.* **19**, 111 (1978).
24. B. A. Chabner, in *Cancer Chemotherapy* 1980 (Ed. H. M. Pinedo), p. 9. Excerpta Medica, Amsterdam (1980).
25. J. A. Houghton and P. J. Houghton, *Eur. J. Cancer Clin. Oncol.* **19**, 807 (1983).