[Chem. Pharm. Bull.
30(3)1018—1023(1982)]

Mode of Action of 5-Fluorocytidine and 5-Fluoro-2′-deoxycytidine in L5178Y Cells *in Vitro*[1]

MITSUZI YOSHIDA,*[a] AKIO HOSHI,[a] KAZUO KURETANI,[a] and MINEO SANEYOSHI[b]

National Cancer Center Research Institute,[a] Tsukiji 5-1-1, Chuo-ku, Tokyo 104, Japan
and Faculty of Pharmaceutical Sciences, Hokkaido University,[b]
Kita-12, Nishi-6, Kita-ku, Sapporo 060, Japan

(Received August 10, 1981)

The mode of antiproliferative action of two 5-fluorocytosine nucleosides, 5-fluorocytidine (FCR) and 5-fluoro-2′-deoxycytidine (FCdR), was examined using mouse leukemia L5178Y cells *in vitro*. FCR and FCdR were markedly active against L5178Y cells, though the cells were deficient in cytidine deaminase activity. Both compounds increased the incorporation of ^{14}C-labeled thymidine into the acid-insoluble fraction of L5178Y cells and decreased labeled deoxycytidine incorporation. In reversal studies, the antiproliferative effects of both compounds were almost abolished by simultaneous addition of thymidine or deoxyuridine. Deoxycytidine completely reversed the growth inhibition caused by FCdR, but not that caused by FCR. These results demonstrate that the cytotoxicity of both compounds is due to inhibition of thymidylate synthetase, presumably through formation of 5-fluoro-2′-deoxyuridine monophosphate (FdUMP) after deamination by deoxycytidylate deaminase in the pyrimidine *de novo* pathway.

Keywords——leukemia L5178Y; precursors incorporation; reversal studies; fluorocytidine; fluorodeoxycytidine; fluorouridine; fluorodeoxyuridine; cytidine deaminase; fluorouracil; deoxycytidylate deaminase

In a previous report[2] on the mechanisms of antitumor activity of 5-fluorouracil (FU) and its nucleoside, it was suggested that the antiproliferative effect of 5-fluoro-2′-deoxyuridine (FUdR) is due to the inhibition of thymidylate synthetase, and that the sites of action of FU and 5-fluorouridine (FUR) are more complex, involving thymidylate synthetase and RNA synthesis in mouse leukemia L5178Y cells. Substitution at the 5-position of 2,2′-anhydro-1-β-D-arabinofuranosylcytosine (cyclocytidine) with fluorine was found to have no effect on its antitumor activity.[3]

5-Fluorocytosine nucleosides, 5-fluorocytidine (FCR) and 5-fluoro-2′-deoxycytidine (FCdR) were synthesized by Fox *et al.*[4] and antitumor activities of these compounds have been demonstrated against transplanted tumors.[4,5] Mouse leukemia cell line L5178Y was considered to show a decreased activity of cytidine deaminase (which transforms FCR to FUR), since 1-β-D-arabinofuranosylcytosine was markedly active against this line without deamination to form 1-β-D-arabinofuranosyluracil, an inactive metabolite.[6] In contrast, FCR and FCdR were markedly active against this line.

In this report, the role of cytidine deaminase in the activation of FCR and FCdR and the mode of action of both compounds were examined using L5178Y cells, and the results were compared with those for FUR and FUdR.[2]

Materials and Methods

Chemicals——5-Fluorocytidine (FCR) was synthesized as reported previously[7] and 5-fluoro-2′-deoxycytidine (FCdR) was synthesized from 5-fluorocytosine by the method of Duschinsky *et al.*[8] 5-Fluorouracil, 5-fluorouridine and 5-fluoro-2′-deoxyuridine were supplied by Mitsui Pharmaceuticals, Inc., Tokyo. Thymidine, uridine, deoxyuridine, cytidine and deoxycytidine were purchased from Sigma Chem. Co., U.S.A. ^{14}C-Labeled thymidine (61 Ci/mol), uridine (481 Ci/mol), L-leucine (324 Ci/mol) and ^{3}H-labeled deoxycytidine (2.0 Ci/mmol) were purchased from the Radiochemical Centre, Ltd., U.K.

Cell Culture——A mouse leukemia cell line, L5178Y, was incubated in RPMI 1640 medium supplemented with 10% heat-inactivated (at 56°C for 30 min) calf serum in a humidified atmosphere of 5% CO_2 in air at 37°C.

Determination of Antiproliferative Activity——After L5178Y cells (5×10^4 cells/ml) had been cultured for 48 h with the test compound at various concentrations, the number of cells was counted with a micro cell counter (Model CC-108, Toa Medical Electronic Co., Kobe, Japan). Antiproliferative activity of the test compound was measured in terms of the ratio of cell numbers in treated and control groups (T/C%), and IC_{50} (the concentration in μg/ml required for 50% inhibition of growth) was calculated from the log-probability graphs as reported previously.[9)]

Purification of Cytidine Deaminase——Purification and enzyme assay of cytidine deaminase were done according to the method of Tomchick *et al.*[10)] Kidneys were removed from female ddN mice and the capsules were excised. The 30% tissue homogenate was prepared in cold 0.154 M KCl with a Potter Elvehjem homogenizer fitted with a Teflon pestle, and centrifuged for 60 min at 78000×*g*. The supernatant was heated at 60°C for 7 to 10 min. The heat-denatured mixture was cooled, then centrifuged for 15 min at 17300×*g*, and the supernatant was treated with solid ammonium sulfate from 40 to 50% saturation. The mixture was centrifuged and the resulting pellet was dispersed in 0.02 M potassium phosphate buffer (pH 7.5), divided into small aliquots and stored in the frozen state. L5178Y cells were harvested from female BDF_1 mice inoculated with 10^7 cells intraperitoneally 12 d previously. The cells were washed twice with 0.9% NaCl solution and the subsequent procedure was as described above.

Cytidine Deaminase Assay——The incubation mixture contained 0.3 μmol of cytidine, 0.6 ml of 0.25 M glycylglycine buffer (pH 6.7), 0.1 ml of enzyme and water to give a final volume of 3.0 ml. The buffer, enzyme and water were preincubated for 10 min prior to the addition of the substrate, after which the incubation was continued for 10 min at 37°C. The reaction was then terminated by the addition of 0.2 ml of 26% perchloric acid. The precipitated protein was removed by centrifugation. The absorbance of the clear solution was measured at 290 nm with a Hitachi Perkin-Elmer model 139 spectrophotometer. A unit of enzyme was defined as that amount capable of deaminating 1 micromol of cytidine in 10 min at 37°C, and the specific activity as units per mg of protein. Protein content was determined, with bovine serum albumin as the standard, by the method of Lowry *et al.*[11)]

Incorporation of Labeled Precursors into Acid-Insoluble Fraction——As a precursor, 7.4 kBq (0.2 μCi) of labeled thymidine, 18.5 kBq (0.5 μCi) of uridine, L-leucine or 37 kBq (1 μCi) of deoxycytidine was used. L5178Y cells (2.5×10^5 cells) were incubated for 30 min with a precursor in the presence of the test compound at various concentrations in a volume of 1 ml. A 0.4 ml aliquot of the cell suspension was collected on a Whatman GF/C filter paper after being chilled to 0°C and washed with ice-cold 5% trichloroacetic acid. The radioactivity was counted with a Packard Tri-Carb 3320 liquid scintillation spectrometer, using a toluene scintillator.[2)]

Reversal Studies——5-Fluorocytosine nucleosides, FCR and FCdR, were used at equieffective concentrations (IC_{90}) to compare the mode of action of the two nucleosides, as reported previously.[2)] L5178Y cells (5×10^4 cells/ml) were exposed to FCR or FCdR in the presence of a selected pyrimidine nucleoside at various concentrations for 48 h, and then the effect of the pyrimidine nucleoside on the 90% inhibition of growth caused by FCR or FCdR was examined by measuring the cell growth.

Results

Antiproliferative Activity of FCR and FCdR

Dose-response plots for FCR, FCdR, FUR and FUdR are shown in Fig. 1. FCR and FCdR, as well as FUR and FUdR, were markedly active against L5178Y cells *in vitro* and the IC_{50}s of FCR and FCdR were 0.0054 μg/ml (2.1×10^{-8} M) and 0.0012 μg/ml (4.9×10^{-9} M), respectively. The antiproliferative activity of 5-fluorocytosine (FC) was very weak and its IC_{50} was over 3000 times that of FCR. The activity of FCR was equal to that of FUR (1.8×10^{-8} M), while FCdR had half the activity of FUdR (2.8×10^{-9} M).

Cytidine Deaminase Activity of Mouse Kidney and L5178Y Cells

The activity of cytidine deaminase was measured in mouse kidney, and the specific activities were 0.082 and 1.6 units/mg protein in 78000×*g* supernatant and 40—50% ammonium sulfate fraction, respectively, However, that enzyme activity was not found in any fraction from L5178Y cells.

Effects of FCR and FCdR on Incorporation of Labeled Precursors into the Acid-Insoluble Fraction

As shown in Fig. 2, FCR and FCdR inhibited the incorporation of labeled deoxycytidine into the cold trichloroacetic acid insoluble fraction, but did not affect that of L-leucine at concentrations up to 100 μg/ml. Thymidine incorporation was enhanced by both compounds

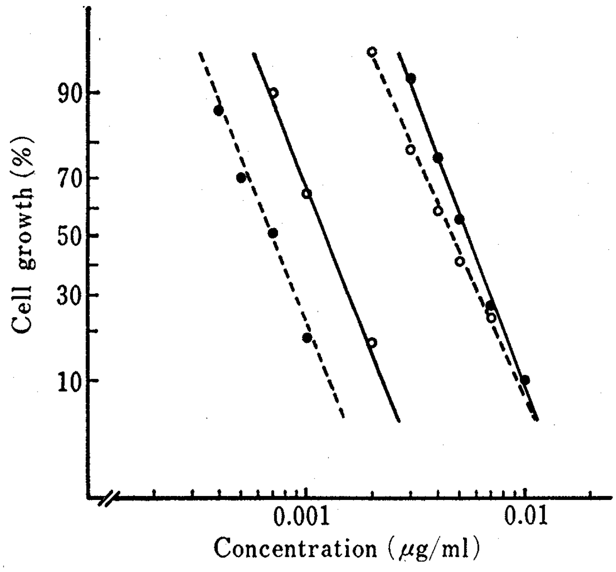

Fig. 1. Cell Growth Inhibition by FCR, FCdR, FUR and FUdR

——○—— : FCdR, ----●---- : FUdR,
——●—— : FCR, ----○---- : FUR.

L5178Y cells $(5\times10^4$ cells/ml) were cultured for 48 h in RPMI 1640 medium containing various concentrations of fluorinated pyrimidine. The results are plotted on a probability scale against the concentration on a log scale.

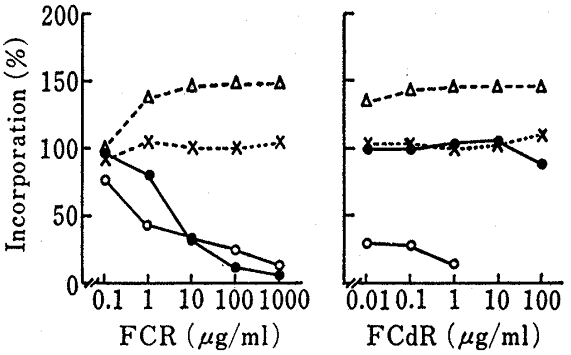

Fig. 2. Effects of FCR and FCdR on Incorporation of Labeled Nucleic Acid Precursor into the Acid-Insoluble Fraction of Cells

△----△ : ^{14}C-thymidine, ●——● : ^{14}C-uridne,
×------× : ^{14}C-L-leucine, ○——○ : ^{3}H-deoxycytidine.

L5168Y cells $(2.5\times10^5$ cells/ml) were incubated for 30 min at 37°C in RPMI 1640 medium containing labeled precursor plus various concentrations of FCR or FCdR.

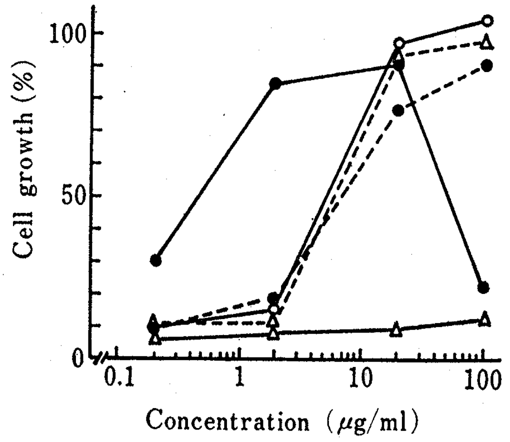

Fig. 3. Effects of Selected Pyrimidine Nucleosides on the Growth Inhibition caused by FCR

●——● : + thymidine, ○——○ : + uridine,
●----● : deoxyuridine, △----△ : + cytidine,
△——△ : + deoxycytidine.

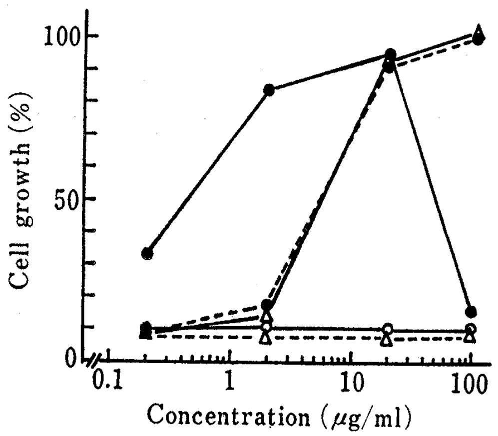

Fig. 4. Effects of Selected Pyrimidine Nucleosides on the Growth Inhibition caused by FCdR

●——● : +thymidine, ○——○ : +uridine,
●----● : +deoxyuridine, △------△ : +cytidine,
△——△ : +deoxycytidine.

by 50% of the control. FCR inhibited uridine incorporation significantly, while FCdR affected it only slightly even at 100 μg/ml. The effect of FCdR on precursor incorporation was more significant than that of FCR, except for uridine incorporation.

Reversal Studies

Reversal of growth inhibition in the presence of selected pyrimidine nucleoside was studied to obtain information concerning the possible metabolism of 5-fluorocytosine nucleosides and the mechanism of growth inhibition. FCR and FCdR were used at equieffective concentrations, IC$_{90}$, which were estimated to be 0.02 μg/ml $(7.7\times10^{-8}\,\text{M})$ and 0.003 μg/ml $(1.2\times10^{-8}\,\text{M})$, respectively, from Fig. 1. Fig. 3 showed that cytidine and uridine at 100 μg/ml completely reversed the 90% inhibition of growth caused by FCR. Further, 2 μg/ml of thymidine and 100 μg/ml of deoxyuridine restored the growth to 90% of the control, but 100 μg/ml of deoxycytidine had no effect. Similarly, thymidine and deoxyuridine completely reversed the growth inhibition by FCdR as shown in Fig. 4. Moreover, deoxycytidine also reversed FCdR cytotoxicity completely, whereas cytidine and uridine did not have any effect on FCdR cytotoxicity.

Discussion

5-Fluorocytosine nucleosides, FCR and FCdR, were found to be potent growth inhibitors of mouse leukemia L5178Y cells, but the antitumor activity of 5-fluorocytosine (FC) itself was very weak and its IC_{50} was over 3000 times those of FCR and FUR. This result is consistent with the fact that FC is not active in experimental tumor systems,[12,13)] and suggests that FC cannot be utilized in animal cells because they have no cytosine phosphoribosyl transferase or cytosine deaminase activity by which the drug can be converted to 5-fluorouracil (as occurs in some fungi[14)]).

Among FCR, FCdR, FUR and FUdR activities, the antiproliferative effects of deoxyribose derivatives such as FCdR and FUdR were stronger than those of the corresponding ribose derivatives, and the uridine derivatives of FUR and FUdR were more potent inhibitors than the corresponding cytidine derivatives. The active forms of these 5-fluorocytosine nucleosides seem to arise during metabolic transformations of the drugs in L5178Y cells. However, cytidine deaminase activity was not found in this cell line; thus, the conversion of these fluorocytosine nucleosides to fluorouridine derivatives should not occur at the nucleoside level.

During a short exposure (30 min) of L5178Y cells to 5-fluorinated pyrimidine and labeled nucleic acid precursors, the effects of FCR and FCdR on precursor incorporation were similar to those of FUR and FUdR given in a previous report,[2)] respectively. Suppression of deoxycytidine incorporation by these compounds indicates an inhibition of DNA synthesis and

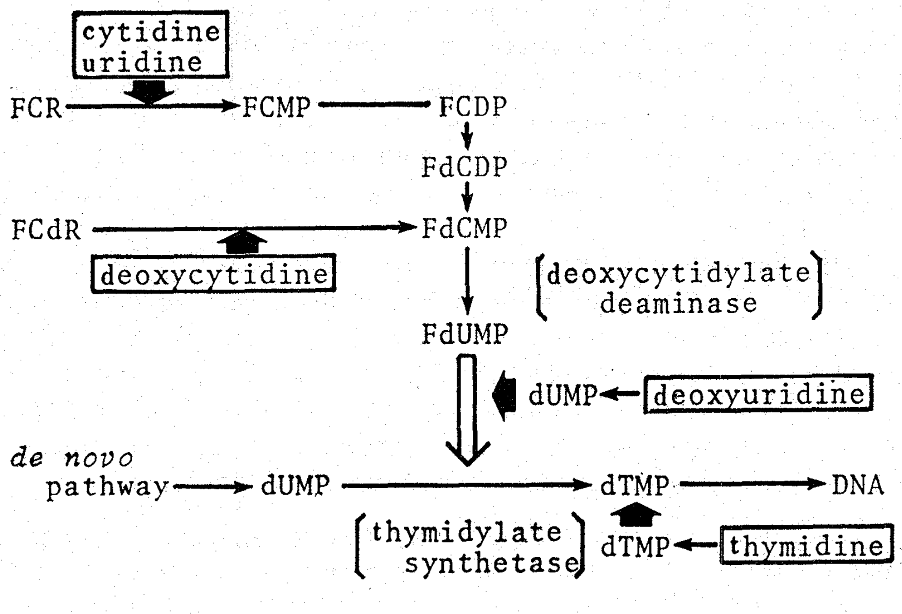

Fig. 5. Proposed Inhibition Sites of FCR and FCdR and the Sites of Reversal by Pyrimidine Nucleosides of Their Inhibitions

⇨, site of inhibition; ➡, site of reversal;
FCMP, 5-fluorocytidine 5′-monophosphate; FCDP, 5-fluorocytidine 5′-diphosphate; FdCMP, 5-fluoro-2′-deoxycytidine 5′-monophosphate; FdCDP, 5-fluoro-2′-deoxycytidine 5′-diphosphate; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; dUMP, 2′-deoxyuridine 5′-monophosphate; dTMP, 2′-deoxythymidine 5′-monophosphate.

increased thymidine incorporation suggests an enhanced utilization of exogenous thymidine under conditions where FCR and FCdR inhibit thymidylate synthesis leading to the reduction of the thymidylate pool in the cells. Therefore, FCR and FCdR, as well as FUR and FUdR, were considered to inhibit DNA synthesis by blocking thymidylate synthesis.

Reversal studies offered further evidence of activation of fluorocytosine nucleosides and for the above mechanisms of growth inhibition. The abolishment of FCR cytotoxicity by both cytidine. and uridine suggests that both compounds and FCR (also FUR) may be phosphorylated to the corresponding nucleotides by the same enzyme, uridine kinase, as described by Reichard and Sköld,[15] so that both compounds would inhibit the phosphorylation of FCR in a competitive manner as shown in Fig. 5. This would also explain the inhibition of labeled uridine incorporation caused by FCR and the complete reversal by uridine when FUR was used as an inhibitor.[2] Similarly, deoxycytidine competes with FCdR for the other enzyme, deoxycytidine kinase, because cytidine and deoxycytidine at less than 100 μg/ml did not reverse the FCdR and FCR cytotoxicities, respectively.

In a previous report,[2] the antiproliferative effect of FUdR was found to be completely abolished by thymidine and deoxyuridine. However, the growth inhibition caused by FU or FUR was reversed only to 20—40% of the control by both thymidine and deoxyuridine. It is considered that FUdR cytoxicity is due to the inhibition of thymidylate synthetase, and that the sites of action of FU and FUR are more complex, involving thymidylate synthetase and RNA synthesis. In the present report, both thymidine and deoxyuridine completely overcame the growth inhibition caused by FCR and FCdR. Reversal by thymidine occurred even at 0.2 μg/ml and was much stronger than that by deoxyuridine and other pyrimidines. Deoxycytidine did not show any effect on the inhibition caused by FCR, though partial reversal was seen at concentrations higher than 500 μg/ml. These results demonstrate that the growth inhibition caused by FCR or FCdR is due only to the inhibition of thymidylate synthetase, presumably through formation of an active inhibitor, 5-fluoro-2′-deoxyuridine monophosphate (FdUMP). Therefore, thymidine would seem to avert the reduction of the thymidylate pool after its conversion to thymidine monophosphate (dTMP), and deoxyuridine (also after conversion to deoxyuridine monophosphate, dUMP) competes with FdUMP for the active site of thymidylate synthetase as shown in Fig. 5.

The mechanism of antitumor activities of FCR and FCdR has been studied *in vitro*.[12,16] Incorporation of FCR and FCdR into *t*RNA and DNA as cytidine analogs was reported by Lu *et al*.[16a] and Tanaka *et al*.,[16b] respectively. The ready deamination of FCR and FCdR to FUR and FUdR was also demonstrated in human lymphoblastic cells by Tattersall *et al*.[16c] and Brox *et al*.,[16d] respectively. However, systematic analyses of these compounds have not been carried out so far.

In L5178Y cells, the 5-fluorocytosine nucleosides, FCR and FCdR, seem to be deaminated to FdUMP, an active inhibitor, by deoxycytidylate deaminase (involved in pyrimidine *de novo* synthesis) after formation of fluorodeoxycytidylate by phosphorylation and subsequent reduction as shown in Fig. 5, since the cells are defective in cytidine deaminase activity. This finding supports the view that the antiproliferative activity of FCR, as well as that of FCdR, was simply due to the inhibition of DNA synthesis because its activation pathway is different from that of FUR, whose cytoxicity is due to composite inhibitions.

Acknowledgement The authors are indebted to Mitsui Pharmaceuticals, Inc., Tokyo, for kindly supplying 5-fluorouracil nucleosides. This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare.

References and Notes

1) Part of this work was presented at the 37th Annual Meeting of the Japanese Cancer Association, Tokyo, August, 1978.
2) M. Yoshida, A. Hoshi, and K. Kuretani, *J. Pharm. Dyn.*, 3, 374 (1980).

3) M. Yoshida, A. Hoshi, K. Kuretani, T. Kanai, and M. Ichino, *Gann*, **66**, 561 (1975).
4) J.J. Fox, I. Wempen, and R. Duschinsky, Proceedings of the 4th "International Congress of Biochemistry," Vol. XV, Vienna, September, 1958, p. 6.
5) J.H. Burchenal, E.A.D. Holmberg, and J.J. Fox, Proceedings of the 4th "International Congress of Biochemistry," Vol. XV, Vienna, September, 1958, p. 185; J.H. Burchenal, E.A.D. Holmberg, J.J. Fox, S.C. Hemphill, and J.A. Reppert, *Cancer Res.*, **19**, 494 (1959).
6) A. Hoshi, M. Yoshida, F. Kanzawa, K. Kuretani, T. Kanai, and M. Ichino, *Chem. Pharm. Bull.*, **21**, 1446 (1973).
7) M. Saneyoshi, M. Inomata, and F. Fukuoka, *Chem. Pharm. Bull.*, **26**, 2990 (1978).
8) R. Duschinsky, J. Gabriel, M. Hoffer, J. Berger, E. Titsworth, E. Grunberg, J.H. Burchenal, and J.J. Fox, *J. Med. Chem.*, **9**, 566 (1966).
9) M. Yoshida, K. Kuretani, and A. Hoshi, *Chem. Pharm. Bull.*, **26**, 429 (1978).
10) R. Tomchick, L.D. Saslaw, and V.S. Waravdekar, *J. Biol. Chem.*, **243**, 2534 (1968).
11) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
12) M.L. Eidinoff, M.A. Rich, and A.G. Perez, *Cancer Res.*, **19**, 638 (1959).
13) C. Heidelberger, N.K. Chaudhuri, P. Danneberg, D. Mooren, L. Griesbach, R. Duschinsky, R.J. Schnitzer, E. Pleven, and J. Scheiner, *Nature*, **179**, 663 (1957); C. Heidelberger, L. Griesbach, B.J. Montag, D. Mooren, O. Cruz, R.J. Schnitzer, and E. Grunberg, *Cancer Res.*, **18**, 305 (1958); L.W. Law, *Proc. Am. Assoc. Cancer Res.*, **2**, 318 (1958).
14) B.A. Koechlin, F. Rubio, S. Palmer, T. Gabriel, and R. Duschinsky, *Biochem. Pharmacol.*, **15**, 435 (1966); R.J. Fass and R.L. Perkins, *Ann. Intern. Med.*, **74**, 535 (1971).
15) P. Reichard and O. Sköld, "Methods in Enzymology," Vol. VI, ed. by S.P. Colowick, and N.O. Kaplan, Academic Press Inc., New York, 1963, pp. 194—197.
16) *a*) L.W. Lu, W. Tseng, and K. Randerath, *Biochem. Pharmacol.*, **28**, 489 (1979); *b*) M. Tanaka, S. Yoshida, M. Saneyoshi, and T. Yamaguchi, *Cancer Res.*, 41, 4132 (1981); *c*) M.H.N. Tattersall, K. Ganeshaguru, and A.V. Hoffbrand, *Biochem. Pharmacol.*, **23**, 2533 (1974); *d*) L.W. Brox, G.A. LePage, S.S. Hendler, and D.H. Shannahoff, *Cancer Res.*, **34**, 1838 (1974).