Studies on Fluorinated Pyrimidines

X. *In Vivo* Studies on Tumor Resistance*

CHARLES HEIDELBERGER, ANNE GHOBAR, RALPH K. BAKER, AND K. L. MUKHERJEE

(*McArdle Memorial Laboratory, The Medical School, University of Wisconsin, Madison 6, Wis.*)

SUMMARY

The biochemical mechanism of resistance has been studied at chemotherapeutic doses in mice bearing 5-fluorouracil-sensitive and- resistant Ehrlich ascites carcinomas. The 5-fluorouracil-resistant tumor is also resistant to 5-fluorouridine and 5-fluoro-2′-deoxyuridine. The incorporation of formate-C^{14} into DNA thymine is inhibited to a much greater extent and for a longer duration in the susceptible than in the resistant tumor. Biochemical dose-response studies in mice treated with 5-fluorouracil, 5-fluorouridine, and 5-fluoro-2′-deoxyuridine show a much greater inhibition of the incorporation of formate-C^{14} into DNA thymine in the susceptible than in the resistant tumor. In the susceptible tumor, the incorporation of uracil-2-C^{14} into DNA thymine was inhibited by 5-fluorouracil and 5-fluoro-2′-deoxyuridine, but not by 5-fluorouridine, whereas none of the drugs inhibited this incorporation in the resistant cells. None of the drugs significantly affected the incorporation of uracil-2-C^{14} into RNA uracil in either tumor. There was a minor decrease in the conversion of 5-fluorouracil into 5-fluorouridylic acid and RNA in the resistant tumor, but the amount of 5-fluoro-2′-deoxyuridylic acid produced was about the same in both tumors. These results show that, administered in therapeutic doses to intact animals, the fluorinated pyrimidines inhibit the synthesis of DNA thymine in the susceptible but not in the resistant tumor.

A number of studies on the biochemistry (1, 8, 10) and metabolism (6, 7, 16) in mammalian tumor systems of the tumor-inhibitory (12, 13) fluorinated pyrimidines (9) have been reported from this laboratory. In the course of this research, a 5-fluorouracil-resistant line of the Ehrlich ascites carcinoma has been developed, and the present paper reports on metabolic studies *in vivo* aimed at elucidation of the biochemical mechanism of the resistance. In an accompanying report complementary studies *in vitro* are described (14).

As a result of the findings of Brockman and his associates it has been established that resistance to 8-azaguanine (3,4) and 6-mercaptopurine (2) in various bacteria and tumors is accompanied by a loss of the ability of the resistant cells to convert the fraudulent base into the corresponding ribonucleotide, a process which evidently is a "lethal synthesis." In the case of 5-fluorouracil (FU) resistance, Brockman, with bacteria (5), has demonstrated that in the resistant cells the conversion of FU into 5-fluorouridylic acid (FURP) does not occur. Reichard *et al.* (17) have shown that uracil is not converted into uridylate in resistant tumors and have inferred that, hence, FU is not converted into FURP. In our FU-resistant line of the Ehrlich ascites carcinoma, in contrast to the previously cited studies, the resistance cannot be attributed to a lack of conversion of FU into nucleotides.

* This work was supported in part by a grant-in-aid of the Wisconsin Division of the American Cancer Society; a grant, C-2832, from the National Cancer Institute, National Institutes of Health, U.S. Public Health Service; and by a grant-in-aid from Hoffmann-LaRoche, Inc. A preliminary account of part of this work appeared in *Fed. Proc.*, 18: 244, 1959. Paper IX is in *J. Biol. Chem.* (16).

Received for publication December 31, 1959.

MATERIALS AND METHODS

Female Swiss albino mice were obtained from Taconic Farms, Germantown, N.Y. The hypotet-

raploid Ehrlich ascites carcinoma was transplanted as described (13). The tumors in the mice used in the metabolic experiments were transplanted 7 days before the drugs, and labeled precursors were injected intraperitoneally. 5-Fluorouracil-2-C^{14} was synthesized in this laboratory (6). Sodium formate-C^{14} and uracil-2-C^{14} were obtained from New England Nuclear Corp. on allocation from the U.S. Atomic Energy Commission. The fluorinated pyrimidines were generously donated by Dr. Robert Duschinsky of Hoffmann-LaRoche, Inc.

In the metabolism experiments, groups of three mice were used for each point. The tumor cells from each group were pooled, centrifuged, washed rapidly with saline, and acid-soluble extracts, nucleic acids, and nucleic acid pyrimidines were isolated as described previously (8). DNA thymine and RNA uracil were located on paper chromatographs with a Mineralite, and the spots were punched out and the radioactivity measured in a gas-flow counter to at least 10 per cent statistical accuracy. Since the papers were of equal thickness, no corrections were made for self-absorption. After determination of the radioactivity, the papers were eluted with 0.05 N HCl, the quantity and purity of the bases were determined spectrophotometrically, and the specific activities calculated.

The radioactivity of acid-soluble fractions was determined, and they were fractionated when necessary on columns of Dowex-1-formate. Ten-ml. fractions were collected and radioactivity measured to locate the peaks of labeled compounds. The peak tubes were combined, and the total radioactivity was determined. The columns were washed with water and 0.05 M formic acid to remove free bases and nucleosides. From this fraction FU, 5-fluorouridine (FUR), and 5-fluoro-2′-deoxyuridine (FUDR) were separated by paper chromatography following acetylation as described by Kaldor and Heidelberger (15). The columns were eluted with 1.5 M formic acid, followed by 6 M formic acid, which eluted the 5-fluorouridylic acid (FURP) and 5-fluoro-2′-deoxyuridylic acid (FUDRP); the ribonucleoside di- and triphosphates were removed with 0.5 M HCl. The combined FURP and FUDRP fraction was dephosphorylated with prostatic phosphomonoesterase, and the resulting FUR and FUDR were separated by ion-exchange chromatography with borate buffer as described previously (1).

The description of the method for the development of a FU-resistant line of the Ehrlich ascites carcinoma has been given previously (12). In this tumor, once resistance had been established, transplantation was carried out without FU-treatment. However, between the 40 and 45th transplant-generations the resistance was partially lost. This line was then transplanted in mice treated with 5-fluorouracil at 25 mg/kg for 1 week starting 1 day after transplantation, and by the 60th generation resistance was again achieved. Subsequent transplantations have always been maintained in mice treated daily with 25 mg/kg of FU.

TABLE 1

SURVIVAL TIMES OF MICE TREATED WITH FLUORINATED PYRIMIDINES AND BEARING SUSCEPTIBLE AND RESISTANT LINES OF THE EHRLICH ASCITES CARCINOMA

Ten mice per group received the drugs intraperitoneally, at the doses indicated, daily for 1 week starting 1 day after transplantation.

Group	Dose (mg/kg/day)	Susceptible		Resistant	
		Mean survival (days)	Range	Mean survival (days)	Range
Controls	0	13.2	9–18	10.0	8–11
5-Fluorouracil	25	22.1	11–35	11.7	9–15
5-Fluorouridine	4.5	26.3	11–39	10.1	8–16
5-Fluoro-2′-deoxyuridine	50	65.7	29–100	10.5	8–16

RESULTS

Unfortunately the 5-fluorouracil-resistant line of the Ehrlich ascites carcinoma that we had previously reported to be susceptible to 5-fluorouridine (12) was not stable and reverted to sensitivity before any biochemical experiments could be carried out. The present resistant line was obtained by the same method as described for the other line, and is invariably carried in FU-treated mice to prevent reversion. As can be seen in Table 1 this tumor is cross-resistant to all the fluoropyrimidines tested and thus differs from our earlier line. No indication of FU-dependence has been observed.

The results of the biochemical dose-response experiments are shown in the charts. The values

shown at each point represent the averages of duplicate analyses on the pooled cells from three mice. These data are in general less consistent than those obtained *in vitro*. This is a result of the fact that in each experiment *in vivo* large numbers of mice were used in which considerable variation was encountered in the volumes of ascites and cell concentrations, resulting in different dilutions of the drugs and nucleic acid precursors and doubtless different states of metabolic activity. In contrast, a single pool of cells from many mice was used in the experiments *in vitro* (14). In every case, side-by-side comparisons were made of susceptible and resistant tumors within each experiment.

In Chart 1 is shown the effect of the usual chemotherapeutic dose of 5-fluorouracil, 25 mg/kg, on the incorporation of formate-C^{14} into DNA thymine in mice bearing the susceptible and resistant ascites tumors. The time indicated on the abscissa represents the interval between the administration of the drug intraperitoneally and the injection of the labeled formate. Four hours were then allowed between the administration of formate and the sacrifice of the mice. It is evident that at all time intervals there was a great difference in the degree of inhibition found in the two tumors, such that the inhibition was almost complete in the susceptible tumor and negligible in the resistant tumor except at ½ hour. Thus the duration of effect of the FU on the inhibition of this reaction is much greater in the susceptible tumor. In all subsequent experiments the interval between the administration of the drug and the labeled precursor was 10 hours, and the mice were sacrificed 12 hours after the precursor was given.

The biochemical dose-response curves of the fluoropyrimidines on the incorporation of formate into DNA thymine in the susceptible and resistant tumors are shown in Chart 2. It will be noted that in two of the three experiments the specific activities of the control thymines were the same in the two tumors, indicating that the thymidylate synthetase activity and DNA biosynthesis were about the same. With FU, FUDR, and FUR the formate incorporation into DNA thymine was significantly inhibited in the susceptible tumors. In the resistant tumors, inhibition was found only at 50 mg/kg of FU, a toxic dose, double the chemotherapeutic level. As had been found previously (8) very small doses of FUR effectively inhibited the formate incorporation, and the reason for this, as well as the stimulation produced by FUR at the lowest dose, is not understood. Nevertheless, it is clear that the fluorinated pyrimidines do not effectively inhibit the incorporation of formate into DNA thymine in the resistant tumors.

In view of the findings of Brockman *et al.* (5) in FU-resistant bacteria and Reichard *et al.* (17) with FU-resistant tumors that FU is not converted into its ribonucleoside and ribonucleotide, we have investigated the effect of fluoropyrimidines in our tumor lines on the incorporation of uracil-2-C^{14} into DNA thymine and RNA uracil. The results are shown in Chart 3. Although the control values show that there is less utilization of uracil-2-C^{14} for both thymine and uracil in the resistant tumors, its incorporation into RNA uracil is not markedly inhibited by chemotherapeutically ef-

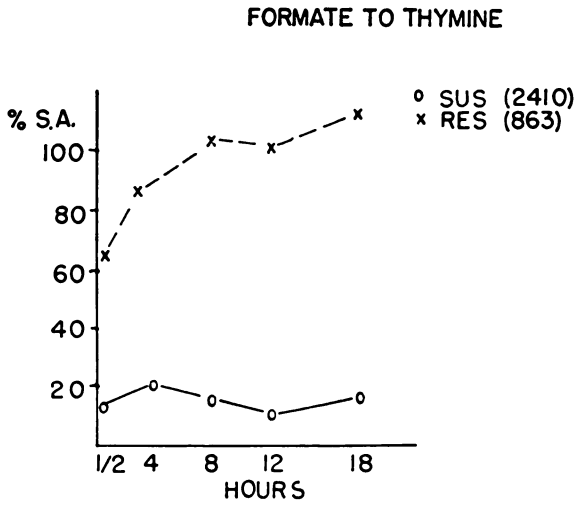

CHART 1.—Duration of the effect of 5-fluorouracil, 25 mg/kg, on the incorporation of formate-C^{14} into DNA thymine in susceptible and resistant Ehrlich ascites tumors. Per cent of specific activity of control thymine against time between administration of FU and formate. The mice were killed 4 hours after the formate administration. The specific activities of the control thymines are given in parentheses.

fective doses of FU in either tumor. Thus, this biosynthetic pathway does not appear to be involved in the mechanism of resistance. On the other hand, the incorporation of uracil-2-C^{14} into DNA thymine is inhibited in the sensitive and not in the resistant tumors, thus further supporting the concept that the methylation reaction leading to the formation of thymine is intimately involved in the mechanism of action and of resistance. The considerable stimulation by FU of the incorporation of uracil into thymine in the resistant tumors was not found when formate was the labeled precursor. Hence, this effect cannot involve thymidylate synthetase or a subsequent reaction and must reflect a metabolic change between uracil and deoxyuridylic acid affected by the drug in the resistant tumor. Since the time intervals are long,

this effect might involve a FU-induced change, such that the pool size of uracil would somehow be reduced. However, no information on this point is now available.

Somewhat similar results were obtained with FUDR and FUR (Chart 4). Neither drug affected the incorporation of uracil-2-C^{14} into RNA uracil in either tumor. However, FUDR did, and FUR did not, inhibit the incorporation of uracil into DNA thymine in the susceptible tumors, and both compounds stimulated this incorporation in the resistant cells.

A study was then carried out to compare the incorporation of 5-fluorouracil into acid-soluble

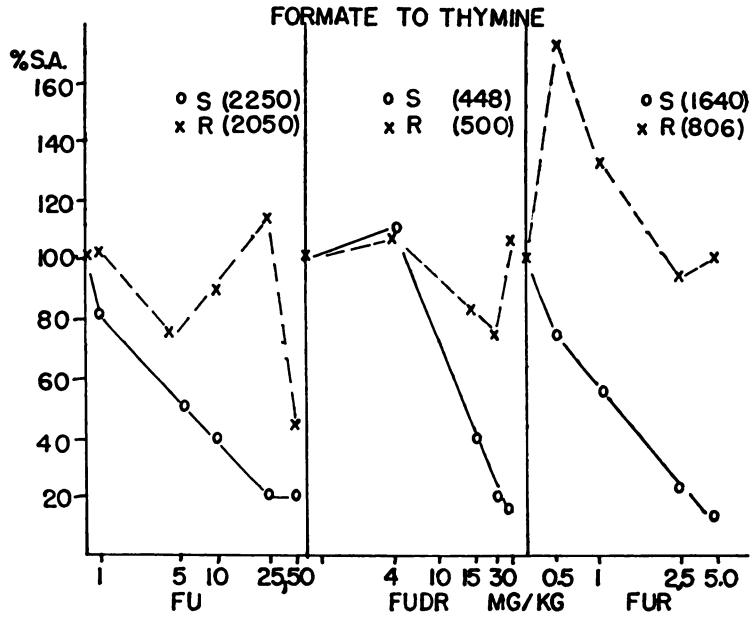

CHART 2.—Dose-response curve on the effects of 5-fluorouracil, 5-fluorouridine, and 5-fluoro-2′-deoxyuridine on the incorporation of formate-C^{14} into DNA thymine in susceptible and resistant tumors. The drug was given, 10 hours later the formate was injected, and 12 hours afterwards the mice were killed. The specific activities of the untreated controls are given in parentheses. Per cent of specific activity of control thymine against dose.

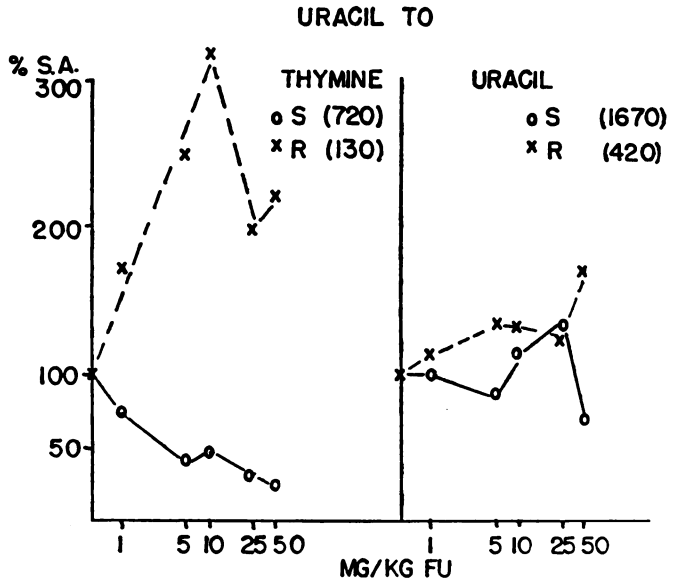

CHART 3.—Dose-response curve on the effect of 5-fluorouracil on the incorporation of uracil-2-C^{14} into DNA thymine and RNA uracil in susceptible and resistant tumors. The same time intervals as in Chart 2. Per cent of specific activity of control thymine against dose.

nucleotides and into RNA in the two tumors. In Table 2 it is shown that there is some decrease in the conversion of FU into FURP and into RNA in the resistant tumor. However, this decrease is not considered to be sufficient to explain the degree of resistance of the tumor. Of considerable interest was the finding that the conversion of FU into FUDRP, the inhibitor of thymidylate synthetase (14), was reduced in the resistant cells to a level about one-half that found in the susceptible cells. Thus, although the *in vivo* reactions, which give an indirect measure of thymidylate synthetase, show inhibition by fluorinated pyrimidines in the susceptible but not the resistant tumors, nevertheless the inhibitor of this reaction, FUDRP, was produced to the same order of magnitude in both tumors. Results showing even less difference in FUDRP production between the tumors will be given in the following paper on studies *in vitro* (14).

DISCUSSION

These experiments on biochemical dose-response and duration of effect show that the incorporation of formate into DNA thymine is inhibited by fluoropyrimidines in the susceptible, but not in the resistant tumor. Furthermore, the lack of effect of the compounds on the incorporation of uracil-2-C^{14} into RNA uracil, even in the susceptible tumor, eliminates the possibility that

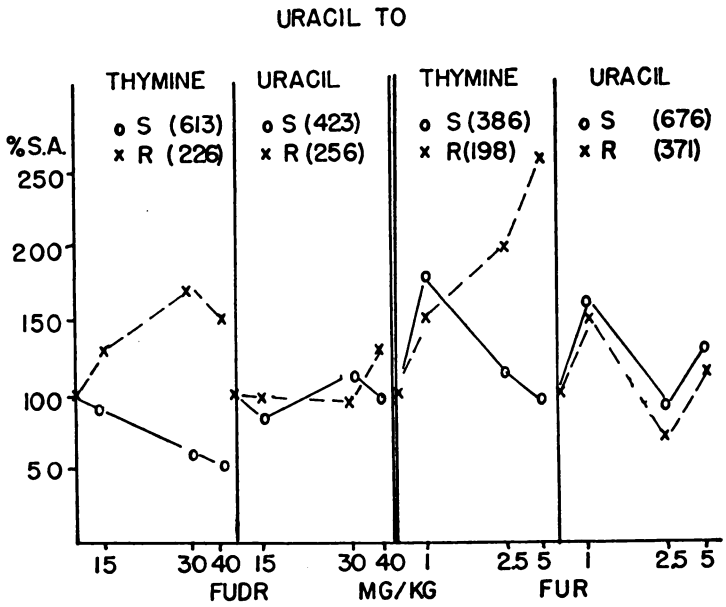

CHART 4.—Dose-response curves on the effects of 5-fluorouridine and 5-fluoro-2′-deoxyuridine on the incorporation of uracil-2-C^{14} into DNA thymine and RNA uracil in susceptible and resistant tumors. The time intervals were the same as in Chart 2. Per cent of specific activity of control thymine against dose.

TABLE 2

CONVERSION OF 5-FLUOROURACIL-2-C^{14} INTO THE ACID-SOLUBLE FRACTION AND INTO RIBONUCLEIC ACID

5-Fluorouracil-2-C^{14}, 25 mg/kg, was injected intraperitoneally into mice bearing susceptible and resistant Ehrlich ascites carcinoma. The acid-soluble fraction was measured 1 hour, and RNA 12 hours, after the injection.

	Susceptible	Resistant
Total counts/min injected	50,000,000	50,000,000
Total counts/min in acid-soluble fraction	270,000	96,000
Total counts/min in nucleosides	30,000	31,000
Total counts/min in FURP, FUDRP fraction	90,000	52,000
After dephosphorylation, total counts/min in FUR	75,000	44,000
" " " counts/min " FUDR	3,600	1,660
FUDRP as per cent of recovered FURP+FUDRP	4.6	3.6
Specific activity of RNA (counts/min/μg by orcinol)	23	8.6 (37%)

the acquisition of resistance is accompanied by a deletion of the capacity to utilize uracil and 5-fluorouracil, as reported by others in resistant bacteria and other resistant tumor lines (5, 17). These experiments led to the proper experimental conditions for the determination of the mechanism of resistance in the *in vitro* experiments to be reported in the next paper (14). Conversely, these experiments show that the mechanism worked out *in vitro* obtains in the living animals at chemotherapeutic doses. The importance of this cannot be overemphasized, because the literature is replete with reports of biochemical effects of drugs, which are inferred to be relevant to mechanism of action without any information as to the relationship of the biochemical effect to the chemotherapeutic result obtained in the intact animal. It is incumbent upon those working in these fields to demonstrate unequivocally that the effects of drugs on biochemical systems, from which mechanistic significance is drawn, obtain in the whole animal at normal chemotherapeutic doses.

REFERENCES

1. Bosch, L.; Harbers, E.; and Heidelberger, C. Studies on Fluorinated Pyrimidines. V. Effects on Nucleic Acid Metabolism *in Vitro*. Cancer Research, **18**:335–43, 1958.
2. Brockman, R. W. A Mechanism of Resistance to 6-Mercaptopurine: Metabolism of Hypoxanthine and 6-Mercaptopurine by Sensitive and Resistant Neoplasms. Cancer Research, **20**:643–53, 1960.
3. Brockman, R. W.; Bennett, L. L.; Simpson, M. S.; Wilson, A. R.; Thomson, R. J.; and Skipper, H. E. A Mechanism of Resistance to 8-Azaguanine. II. Studies with Experimental Neoplasms. Cancer Research, **19**:856–69, 1959.
4. Brockman, R. W.; Sparks, C.; Hutchison, D. J.; and Skipper, H. E. A Mechanism of Resistance to 8-Azaguanine. I. Microbiological Studies on the Metabolism of Purines and 8-Azapurines. Cancer Research, **19**:177–88, 1959.
5. Brockman, R. W.; Davis, J. M.; and Stutts, P. Metabolism of Uracil and 5-Fluorouracil by Drug Sensitive and by Drug-resistant Bacteria. Biochim. et Biophys. acta, **40**:22–32, 1960.
6. Chaudhuri, N. K.; Montag, B. J.; and Heidelberger, C. Studies on Fluorinated Pyrimidines. III. The Metabolism of 5-Fluorouracil-2-C^{14} and 5-Fluoroorotic-2-C^{14} acid *in Vivo*. Cancer Research, **18**:318–28, 1958.
7. Chaudhuri, N. K.; Mukherjee, K. L.; and Heidelberger, C. Studies on Fluorinated Pyrimidines. VII. The Degradative Pathway. Biochem. Pharmacol., **1**:328–41, 1959.
8. Danneberg, P. B.; Montag, B. J.; and Heidelberger, C. Studies on Fluorinated Pyrimidines. IV. Effects on Nucleic Acid Metabolism *in Vivo*. Cancer Research, **18**:329–34, 1958.
9. Duschinksy, R.; Pleven, E.; and Heidelberger, C. The Synthesis of 5-Fluoropyrimidines. J. Am. Chem. Soc., **79**:4559–60, 1957.
10. Harbers, E.; Chaudhuri, N. K.; and Heidelberger, C. Studies on Fluorinated Pyrimidines. VIII. Further Biochemical and Metabolic Studies. J. Biol. Chem., **234**:1255–62, 1959.
11. Heidelberger, C.; Chaudhuri, N. K.; Danneberg, P.; Mooren, D.; Griesbach. L.; Duschinsky, R.; Schnitzer, R. J.; Pleven, E.; and Scheiner, J. Fluorinated Pyrimidines, A New Class of Tumor-Inhibitory Compounds. Nature, **179**:663–66, 1957.
12. Heidelberger, C.; Griesbach, L.; Cruz, O.; Schnitzer, R. J.; and Grunberg, E. Studies on Fluorinated Pyrimidines. VI. Effects of 5-Fluorouridine and 5-Fluoro-2′-deoxyuridine on Transplanted Tumors. Proc. Soc. Exper. Biol. & Med., **97**:470–75, 1958.
13. Heidelberger, C.; Griesbach, L.; Montag, B. J.; Mooren, D.; Cruz, O.; Schnitzer, R. J.; and Grunberg, E. Studies on Fluorinated Pyrimidines. II. Effects on Transplanted Tumors. Cancer Research, **18**:305–17, 1958.
14. Heidelberger, C.; Kaldor, G.; Mukherjee, K. L.; and Danneberg, P. B. Studies on Fluorinated Pyrimidines. XI. *In Vitro* Studies on Tumor Resistance. Cancer Research, **20**:903–9, 1960.
15. Kaldor, G., and Heidelberger, C. A New Paper Chromatographic Method of Separation of Various Pyrimidines and their Deoxyribonucleosides. Biochim. et Biophys. acta, **36**:249–50, 1959.
16. Mukherjee, K. L., and Heidelberger, C. Studies on Fluorinated Pyrimidines. IX. The Degradation of 5-Fluorouracil-6-C^{14}. J. Biol. Chem., **235**:433–37, 1960.
17. Reichard, P.; Sköld, O.; and Klein, G. Possible Enzymic Mechanism for the Development of Resistance against Fluorouracil in Ascites Tumors. Nature, **183**:939–41, 1959.

Studies on Fluorinated Pyrimidines X. *In Vivo* Studies on Tumor Resistance

Charles Heidelberger, Anne Ghobar, Ralph K. Baker, et al.

Cancer Res 1960;20:897-902.