MOLECULAR PATHOLOGY, 9, 297–303 (1973)

The Effect of 5-Fluorodeoxyuridine on the Synthesis of Deoxyribonucleic Acid Pyrimidines in Precancerous Rat Liver

JAY I. GOODMAN[1] AND VAN R. POTTER

McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706

(Received October 24, 1972)

SUMMARY

GOODMAN, JAY I., AND POTTER, VAN R.: The effect of 5-fluorodeoxyuridine on the synthesis of deoxyribonucleic acid pyrimidines in precancerous rat liver. *Mol. Pharmacol.* **9**, 297–303 (1973).

Precancerous livers were induced in rats by feeding the animals a diet containing 0.05% (w/w) of the hepatocarcinogenic azo dye 3′-methyl-4-dimethylaminoazobenzene for 3 weeks. The contribution of *de novo* gnd salvage pathways to the synthesis of DNA pyrimidines in precancerous liver *in vivo* was monitored by simultaneously measuring the incorporation of [6-^{14}C]orotic acid into DNA cytosine and DNA thymine and of [*methyl*-^{3}H]-thymidine into DNA thymine, respectively. The incorporation of these precursors into total liver DNA, the DNA of hepatic cell suspensions, and the DNA of different classes of hepatic nuclei was determined. In addition, the activities of deoxycytidine monophosphate deaminase (dCMP aminohydrolase, EC 3.5.4.12), thymidine kinase (ATP:thymidine 5′-phosphotransferase, EC 2.7.1.21) and thymidylate synthetase (EC 2.1.1b) were measured in hepatic cell suspensions and in whole homogenates prepared from precancerous liver. These studies emphasize that, with regard to several aspects of pyrimidine nucleotide metabolism, precancerous liver is composed of a heterogeneous population of cells. The use of thymidine incorporation as the sole measure of the rate of DNA synthesis is shown to be questionable, and it is suggested that simultaneous assessment of both *de novo* and salvage pathways for the synthesis of DNA precursors is required for an accurate estimate of the extent of DNA synthesis.

INTRODUCTION

Progressive alterations in liver histology (1–3) with bile duct proliferation being one of the most prominent changes (1), and in liver biochemistry (4, 5) occur when rats are placed on a diet containing the hepatocarcinogenic azo dye 3′-methyl-4-dimethylaminoazobenzene. While studies on normal liver biochemistry are hampered by the fact that this organ is composed of several different cell types, the problem is compounded when examining precancerous livers, which are both morphologically and biochemically changing as the animals continue on the carcinogen diet. Therefore it is desirable to develop experimental approaches which attempt to assess the biochemical alterations in

Financial support was provided in part by Grants CA-07175 and T01-CA 5002 from the National Cancer Institute.

[1] This study was performed during the tenure of United States Public Health Service Postdoctoral Fellowship (1-F02-CA-43985) awarded by the National Cancer Institute. Present address, Department of Pharmacology, Michigan State University, East Lansing, Michigan 48823.

different cell types rather than examining only whole liver. Kaufmann *et al.* (6) developed a method for the isolation of hepatic nucleoli of different sizes and studied the effect of partial hepatectomy on the incorporation of [^{14}C]orotic acid into the RNA of hepatic nucleoli of different sizes (7). Haines *et al.* (8) examined the distribution of DNA polymerase (deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7) in different classes of adult rat liver nuclei separated by rate zonal centrifugation. This procedure separates rat liver nuclei into two main classes, diploid and tetraploid (9). Naora (10) has shown that approximately 90% of the hepatocyte (parenchymal) cells in adult rat liver are tetraploid, and therefore the separation into diploid and tetraploid classes represents nuclei derived from nonhepatocyte cells and hepatocyte cells, respectively. Bushnell *et al.* (11) employed centrifugation in discontinuous sucrose gradients for the rapid and convenient separation of liver nuclei into two classes, N-1 and N-2. Based on the differential incorporation of orotic acid and cytidine into the RNA of these nuclei, it was concluded that class N-1 nuclei were derived mainly from hepatocytes and class N-2 nuclei were derived mainly from nonhepatocytes (11). The marked changes in the distribution of DNA between N-1 and N-2 nuclei as well as the changes in the incorporation of [^{3}H]TdR[2] into the DNA of these two classes of nuclei during the first 42 days of 3'-MeDAB hepatocarcinogenesis were documented by Sneider *et al.* (12). Samples of the same livers used by Sneider *et al.* (12) were subjected to histological and radioautographic analysis by Rabes *et al.* (13). These studies (13) confirmed the usefulness of the method of Bushnell *et al.* (11) for separating liver nuclei into hepatocyte and nonhepatocyte populations, although absolute separation is not achieved. The changes in the number of hepatocytes and nonhepatocytes, expressed as a percentage of total cell number (13) were very similar to the changes in the percentage of hepatic DNA in the N-1 and N-2 nuclear fractions, respectively (12), and the changes in [^{3}H]TdR-labeled hepatocytes and nonhepatocytes as a percentage of total cell number (13) were very similar to the changes in the distribution of [^{3}H]TdR-labeled DNA in the N-1 and N-2 nuclear fractions, respectively (12).

The studies to be reported here represent an attempt to develop a protocol for estimation *in vivo* of the extent of DNA synthesis and the effectiveness of a DNA synthesis inhibitor, FUdR, in a heterogeneous cell population. Precancerous liver was chosen as the model system because of the interest in the applicability of these methods to the study of carcinogenesis and the development of cancer-chemotherapeutic agents.

The effect of 5-fluorouracil on DNA thymine synthesis from [^{3}H]TdR and [^{14}C]formate in human tumors has been studied (14), and it was shown that a decrease in formate incorporation was usually but not always associated with a reciprocal increase in TdR incorporation. The literature on the action of fluorinated pyrimidines has been thoroughly reviewed (15). Because of the existence of alternative *de novo* and salvage pathways to the synthesis of pyrimidine nucleotides, a priori predictions concerning the route of synthesis of these metabolites in a given tissue cannot be made with certainty. The importance of this statement in regard to chemotherapeutic attempts at inhibiting the synthesis of dTTP in cancerous tissue has been discussed (16).

In these experiments the contribution of *de novo* and salvage pathways to the synthesis of DNA pyrimidines in precancerous rat liver was monitored by simultaneously measuring the incorporation of [6-^{14}C]orotic acid into DNA cytosine and DNA thymine and of [^{3}H]TdR into DNA thymine, respectively. Hepatic cell suspensions were prepared and examined. Different classes of nuclei were isolated from whole liver homogenates and from homogenates of hepatic cell preparations. In addition, the use of [^{3}H]TdR incorporation as the sole measure of the rate of DNA synthesis is shown to be questionable, and it is suggested that simultaneous assessment of both *de novo* and salvage pathways to the synthesis of DNA precursors is needed

[2] The abbreviations used are: TdR, thymidine; [^{3}H]TdR, [*methyl*-^{3}H]thymidine; FUdR, 5-fluoro-2'-deoxyuridine; 3'-MeDAB, 3'-methyl-4-dimethylaminoazobenzene.

for an accurate estimate of the extent of DNA synthesis in mammalian tissues.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing approximately 200 g were maintained on a diet containing 0.05% of the hepatic carcinogen 3'-MeDAB for 21 days. Experiments were begun at the beginning of the 22nd day. Feeding conditions and the composition of the carcinogen diet were described previously (12). [^{3}H]TdR and [6-^{14}C]orotic acid were purchased from New England Nuclear Corporation and had specific radioactivities of 6.7 Ci/mmole and 42.5 mCi/mmole, respectively. The labeled precursors were diluted with sterile distilled water. At 0.5-hr intervals each rat received an intraperitoneal injection containing [^{3}H]TdR (10 μCi/100 g of body weight and [6-^{14}C]orotic acid (5 μCi/100 g of body weight). Each animal received a total of five injections and was killed 90 min after the last injection.

Livers were minced, and a homogenate was prepared from one portion of the mince while a hepatic cell suspension was prepared from the remaining portion. In this manner we made comparisons between hepatic cell preparations and a sample of the same liver from which they were derived. Hepatic cell suspensions were prepared essentially by a combination of the procedure of Jacob and Bhargava (17) and Howard and Pesch (18). The hepatic mince was added to an enzyme solution (0.05% collagenase and 0.10% hyaluronidase) and incubated, with shaking, for 20 min at 37°. The preparation was then gently homogenized in a Potter-Elvehjem homogenizer with three strokes from a loosely fitting, soft rubber pestle made from a rubber stopper attached to a glass rod. The suspension was filtered once through a layer of cheesecloth, and the cells were sedimented by centrifugation at 50 × g for 1.5 min. Nuclei from homogenates of livers, and homogenates of hepatic cell suspension, were separated into class N-1 (hepatocyte) nuclei and class N-2 (nonhepatocyte, "stromal") nuclei as previously described (11, 12).

Radioactivity in DNA pyrimidines of liver homogenates, homogenates of hepatic cell suspensions, and N-1 and N-2 nuclei was determined in the following manner. The preparations were treated with 10% trichloracetic acid (final concentration), and the precipitate was washed once with 10% trichloracetic acid and extracted successively with 95% ethanol containing 10% potassium acetate (19), 100% ethanol, chloroform–methanol (1:2), and ether. DNA was extracted from the lipid-extracted nucleic acid–protein precipitates, dissolved in 91% formic acid, and hydrolyzed to purine and pyrimidine bases as previously described (20). A sample of the formic acid digest was spotted onto a 46 × 57 cm sheet of Whatman No. 3MM chromatography paper and developed by descending chromatography in the first dimension with 2-propanol–concentrated HCl–water (65:17.2:17.8, v/v/v) (20). Descending chromatography in the second dimension was performed with 1-butanol (saturated with water) in an NH_3 atmosphere (21). The bases were localized under ultraviolet light. Typical R_F values of various bases in the first dimension were: thymine, 0.78; cytosine, 0.47; uracil, 0.67; adenine, 0.32; guanine, 0.22. In the second dimension R_F values, in the same order, were 0.43, 0.27, 0.20, 0.30, and 0.06. The ultraviolet-absorbing regions corresponding to thymine and cytosine were cut out of the chromatogram, and the bases were eluted with water. The concentration of pyrimidine bases was determined by ultraviolet absorption (22), and the radioactivity in the bases was determined by liquid scintillation techniques. Scintisol—Complete, purchased from Isolab, Inc., Akron, Ohio, was employed as the liquid scintillation counting medium.

Enzyme assays were performed on supernatant fractions obtained by centrifuging whole liver homogenates (50% w/v) and homogenates of hepatic cell suspensions (cells from 1 g of liver in 1 ml of buffer) at 105,000 × g for 60 min. The homogenates were prepared in 0.01 M Tris, pH 8.0. Protein was determined by the biuret method (23). Enzyme activities were linear with time over the period of measurement and were directly proportional to the amount of supernatant protein added to the reaction mixtures. The activity was stable for 48 hr if the preparations were stored at −20°.

dCMP deaminase (EC 3.5.4.12). When this enzyme was to be assayed, the homogenizing

buffer contained 20% (v/v) ethylene glycol (24). Reaction mixtures for determining the activity of dCMP deaminase were described previously (24). [2-^{14}C]dCMP (purchased from New England Nuclear Corporation) was employed as substrate. Reactions were terminated by making the mixtures 5 N with respect to HCl and placing them in an oven at 100° for several hours. The dried HCl digest was dissolved in water, and an aliquot was spotted on a strip of Whatman No. 1 chromatography paper and developed by descending chromatography with 2-propanol–concentrated HCl–water (65:17.2:17.8, v/v/v). Only two radioactive spots were detectable: uracil (R_f 0.74) and cytosine (R_f 0.56). The radioactivities of uracil and cytosine were determined by cutting out the appropriate regions of the chromatogram, placing the paper in a scintillation vial, and eluting with 1 ml of water prior to adding the scintillation counting medium described above; the resulting data were used to calculate the percentage of radioactivity in uracil.

Thymidine kinase (*EC 2.7.1.21*). Thymidine kinase activity was determined as previously described (25).

Thymidylate synthetase (*EC 2.1.1b*). Reaction mixtures for determining thymidylate synthetase activity were prepared as previously described (26). Reactions were terminated and the nucleotides were hydrolyzed to their corresponding bases as described for dCMP deaminase. The dried HCl digest was dissolved in water, and an aliquot was spotted on a strip of Whatman No. 1 chromatography paper and developed by descending chromatography with 1-butanol (saturated with water) in and NH_3 atmosphere (21). Only two radioactive spots were detectable: thymine (R_f 0.43) and uracil (R_f 0.20). The percentage of radioactivity in thymine was calculated as described above for the dCMP deaminase assay.

RESULTS

The incorporation of [^{3}H]TdR and [6-^{14}C]-orotic acid into hepatic DNA pyrmidines was determined (Table 1). These results indicate the presence of markedly different cell populations in the livers of these animals. The specific radioactivity of the pyrimidine bases and the ratio of ^{3}H to ^{14}C in DNA thymidine were different in the various DNA preparations, indicating substantial differences in the ratio of *de novo* to "salvage" pathways to dTMP (see ref. 16).

TABLE 1

Incorporation of [^{3}H]TdR and [6-^{14}C]orotic acid into DNA pyrimidines of rats fed 3'-MeDAB for 21 days

Each value is the mean from three or four experiments. For each experiment the bases hydrolyzed from approximately 2.5 mg of DNA were isolated and analyzed as described under MATERIALS AND METHODS.

Fraction	Thymine			Cytosine ^{14}C
	^{3}H	^{14}C	^{3}H:^{14}C	
	dpm/μmole			*dpm/μmole*
Homogenate				
Total DNA[a]	292,000	4,200	69	5,400
N-1	322,000	5,500	59	6,600
N-2	251,000	2,500	101	3,400
Hepatic cells				
Total DNA	365,000	3,400	106	9,500
N-1	481,000	5,000	96	12,800
N-2	178,000	1,100	157	3,700

[a] After 21 days of 3'-MeDAB feeding the distribution of the DNA recovered was as follows: approximately 62% of the homogenate DNA was in the N-1 nuclei and 38% in the N-2 nuclei; approximately 75% of the DNA in the hepatic cell preparation was in the N-1 nulcei and 25% in the N-2 nuclei. Between 63% and 70% of the homogenate DNA was recovered in the N-1 plus N-2 nuclear fractions, and 12–15% of the liver DNA was recovered in the hepatic cell preparations.

The effect of FUdR, a direct precursor of the potent thymidylate synthetase inhibitor 5-fluoro-2'-deoxyuridylate (27), on the incorporation of [^{3}H]TdR and [6-^{14}C]orotic acid into hepatic DNA pyrimidines is shown in Table 2. The marked decreases in the incorporation of orotic acid into DNA thymine indicates that thymidylate synthetase was almost completely inhibited in all cases. This decrease in the incorporation of orotic acid into DNA-thymine along with the decrease in incorporation of orotic acid into DNA cytosine (Table 2) indicates that the synthesis of DNA was inhibited in these

TABLE 2

Relative effects of FUdR on incorporation of [^3H]-TdR and [6-^{14}C]orotic acid into DNA pyrimidines of rats fed 3'-MeDAB for 21 days

Animals received one intraperitoneal injection of FUdR (50 mg/kg) 30 min before the first injection of labeled precursors. The results are expressed as percentages of control activity as given in Table 1.

Fraction	Thymine			Cytosine
	^3H	^{14}C	^3H:^{14}C[a]	^{14}C
	% control			*% control*
Homogenate				
Total DNA	116	10	806	60
N-1	143	8	1047	64
N-2	64	12	535	55
Hepatic cells				
Total DNA	67	5	1439	36
N-1	58	0		27
N-2	93	24	6627	71

[a] Both components of the ratio are in disintegrations per minute per micromole.

TABLE 3

Activities of thymidylate synthetase, thymidine kinase, and dCMP deaminase in liver homogenates and hepatic cell suspensions prepared from rats fed 3'-MeDAB for 21 days

Reaction mixtures, containing 0.1–0.2 mg of protein, were incubated at 37° for 30 min. Enzyme activity was assayed as described under MATERIALS AND METHODS. Each value is the mean ± standard error from four experiments.

Enzyme	Homogenate	Hepatic cells
	pmoles/min/mg protein	
Thymidylate synthetase	1.4 ± 0.4	2.4 ± 0.4
Thymidine kinase	6.3 ± 0.9	3.4 ± 1.3
dCMP deaminase	97 ± 21	54 ± 12

livers, although to a variable extent in the different hepatic cell populations. The conclusion that DNA synthesis was inhibited would have been quite different if one had examined only [^3H]TdR incorporation.

The activities of thymidylate synthetase, thymidine kinase, and dCMP deaminase in homogenates and in hepatic cell suspensions, prepared from a portion of the same liver used for the homogenate, were determined (Table 3). While the absolute values of these enzymes varied up to 4-fold from one experiment to another, the ratios of the specific activities in the cells vs. the homogenate remained quite constant. In the hepatic cell preparations the activity of thymidylate synthetase was about double, and the activities of thymidine kinase and dCMP deaminase were about half, of the activities seen in the homogenate.

DISCUSSION

These studies indicate the presence of markedly different populations of cells in precancerous liver and demonstrate some of the problems associated with attempts at measuring DNA synthesis under these circumstances. A comparison was not made with normal adult liver because the degree of cell division in this organ is negligible, the extent of [^3H]TdR incorporation into DNA is very low (12), and the activities of enzymes involved in thymidine nucleotide synthesis, such as thymidylate synthetase and thymidine kinase (26), are barely detectable. The validity of estimates of the extent of DNA synthesis based entirely on [^3H]TdR incorporation were shown to be dubious. The radioactivity in the thymidine nucleotide pool that results from injected [^3H]TdR will be diluted by thymidine nucleotides produced by the *de novo* pathways. This possibility has been discussed previously (28, 29). The need to consider alternative pathways has been pointed out in a recent study (30), in which the responsiveness to FUdR of different transplatable murine leukemias was *inversely* correlated with tumor cell levels of thymidine kinase. However, resistance to FUdR may occur for just the opposite reason. A strain of Novikoff hepatoma cells in tissue culture is resistant to FUdR and has been shown to lack thymidine kinase activity (31).[3] Therefore a change in the relative activities of the alternative *de novo* and salvage pathways to the synthesis of pyrimidine nucleotides, as well as changes in pyrimidine deoxyribonucleotide pool sizes, can markedly alter the amount of radioactivity from [^3H]TdR that is incorporated into DNA. Under these cir-

[3] H. A. Campbell, unpublished observations.

cumstances one can not equate radioactivity incorporated into DNA with moles of DNA thymine synthesized. As seen in Table 2, when thymidylate synthetase is inhibited, more radioactivity form [^{3}H]TdR is incorporated into total DNA and the DNA in N-1 nuclei isolated from homogenates, at the same time that DNA synthesis is inhibited. These are similar to results obtained from whole homogenates, reported by Danneberg *et al.* (29). A possible explanation for the decreases in [^{3}H]TdR incorporation following FUdR administration (Table 2) may be that some cells were dying.

The data presented in Table 2 demonstrate the importance of monitoring the *de novo* and salvage pathways to both types of DNA pyrimidines. Here the question is whether or not FUdR administration has inhibited thymidylate synthetase and, if so, whether the salvage pathways can provide a thymidine nucleotide level sufficient to support DNA synthesis. The decrease in the incorporation of orotic acid into DNA thymine is a measure of the degree of inhibition of thymidylate synthetase. In this experiment (Table 2) the incorporation of orotic acid into DNA cytosine provides the best estimate of the degree of inhibition of DNA synthesis.

The method for preparing the hepatic cell suspensions yielded a population of cells that was not representative of the liver as a whole but rather a distinct, though not necessarily homogeneous, subpopulation. This is indicated by the differences in the incorporation of precursors into the DNA of the hepatic cell preparations as compared to that of the homogenate (Table 1) and the differences in the effect of FUdR on the labeling of the DNA of the cell preparations as compared to that of the homogenate (Table 2). In addition, the specific activities of several enzymes involved in thymidine nucleotide metabolism were not the same in the soluble fraction of the hepatic cell preparations as they were in the soluble fraction derived from a whole liver homogenate (Table 3). It is possible that some enzyme protein leaked out of the cells during preparation. However, one additional wash of the cells did not alter the activity. It is not surprising that the combination of enzymatic and mechanical means employed to prepare the cell suspensions showed some degree of selectivity in disaggregating the cells of the precancerous livers. The ability to obtain cells by this procedure depends on a balance between the firmness of attachement of neighboring cells and the susceptibility of the cells to lysis, either of which may be influenced by the stage in the cell cycle and the degree of 3′-MeDAB-induced toxicity.

These studies emphasize the need to consider the possibility of heterogeneous cell populations and suggest that assessment of both *de novo* and salvage pathways to the synthesis of DNA precursors must be made to obtain an accurate estimate of the extent of DNA synthesis in mammalian tissues. The implications of this type of experimental approach for cancer chemotherapy studies are evident.

ACKNOWLEDGMENTS

The authors wish to thank Drs. J. A. and E. C. Miller for providing the special diets used in these studies, and Dr. C. Heidelberger for providing the FUdR.

REFERENCES

1. J. M. Price, J. W. Harman, E. C. Miller, and J. A. Miller, *Cancer Res.* **12,** 192–200 (1952).
2. E. Farber, *Cancer Res.* **16,** 142–148 (1956).
3. R. Daoust and F. Molnar, *Cancer Res.* **24,** 1898–1909 (1964).
4. V. R. Potter and M. Watanabe, *in* "Proceedings of the International Conference on Leukemia-Lymphoma" (C. J. D. Zarafonetis, ed.), pp. 33–45. Lea and Febiger, Philadelphia, 1968.
5. P. R. Walker and V. R. Potter, *Advan. Enzyme Regul.* **10,** 339–364 (1972).
6. E. Kaufmann, A. Traub, and Y. Teitz, *Exp. Cell Res.* **49,** 215–218 (1968).
7. E. Kaufmann, N. Sidransky, and H. Shinozuka, *Exp. Cell Res.* **55,** 130–132 (1969).
8. M. E. Haines, I. R. Johnston, and A. P. Mathias, *FEBS Lett.* **10,** 113–116 (1970).
9. I. R. Johnston, A. P. Mathias, F. Pennington, and D. Ridge, *Biochem. J.* **109,** 127–135 (1968).
10. H. Naora, *J. Biochem. Biophys. Cytol.* **3,** 949–975 (1957).
11. D. E. Bushnell, E. D. Whittle, and V. R. Potter, *Biochim. Biophys. Acta* **179,** 497–499 (1969).
12. T. W. Sneider, D. E. Bushnell, and V. R. Potter, *Cancer Res.* **30,** 1867–1873 (1970).

13. H. M. Rabes, R. Hartenstein, and W. Ringelmann, *Cancer Res.* **32,** 83–89 (1972).
14. W. H. Wolberg, *Cancer Res.* **29,** 2137–2144 (1969).
15. C. Heidelberg, *Progr. Nucl. Acid Res. Mol. Biol.* **4,** 1–50 (1965).
16. T. W. Sneider and V. R. Potter, *Cancer Res.* **29,** 2398–2403 (1969).
17. S. T. Jacob and P. M. Bhargava, *Exp. Cell Res.* **27,** 453–467 (1962).
18. R. B. Howard and L. A. Pesch, *J. Biol. Chem.* **243,** 3105–3109 (1968).
19. W. J. Steele, N. Okamura, and H. Busch, *Biochim. Biophys. Acta* **87,** 490–492 (1964).
20. T. W. Sneider and V. R. Potter, *J. Mol. Biol.* **42,** 271–284 (1969).
21. R. D. Hotchkiss, *J. Biol. Chem.* **175,** 315–332 (1948).
22. H. A. Sober (ed.), "Handbook of Biochemistry with Selected Data for Molecular Biology," pp. G-12, G-18. Chemical Rubber Co., Cleveland, 1968.
23. A. G. Gornall, C. J. Bardawill, and M. M. David, *J. Biol. Chem.* **177,** 751–766 (1949).
24. S. Sonoda and H. Endo, *Biochim. Biophys. Acta* **191,** 425–429 (1969).
25. D. H. Ives, J. P. Durham, and V. S. Tucker, *Anal. Biochem.* **28,** 192–205 (1969).
26. T. W. Sneider, V. R. Potter, and H. P. Morris, *Cancer Res.* **29,** 40–54 (1969).
27. P. Reyes and C. Heidelberger, *Mol. Pharmacol.* **1,** 14–30 (1965).
28. Y. Sugino, E. P. Frenkel, and R. L. Potter, *Radiat. Res.* **19,** 682–700 (1963).
29. P. B. Danneberg, B. J. Montag, and C. Heidelberger, *Cancer Res.* **18,** 329–334 (1958).
30. D. Kessel and I. Wodinsky, *Mol. Pharmacol.* **6,** 251–254 (1970).
31. P. A. Morse, Jr., and V. R. Potter, *Cancer Res.* **25,** 499–508 (1965).