Studies with Tetrahydrohomofolate and Thymidylate Synthetase from Amethopterin-Resistant Mouse Leukemia Cells*

David Livingston,† Elizabeth J. Crawford, and Morris Friedkin‡

ABSTRACT: The inhibitory action of amethopterin and tetrahydrohomofolate on the utilization of [^{14}C]deoxyuridine for deoxyribonucleic acid synthesis has been studied with cell suspensions of amethopterin-resistant and amethopterin-sensitive mouse leukemia cells. The inhibition of deoxyuridine uptake with 10^{-4} M amethopterin was significantly higher in sensitive cells than in resistant cells. Under the same conditions the inhibition of deoxyuridine uptake with 10^{-4} M tetrahydrohomofolate was 14% in sensitive cells and 7.1% in resistant cells. The low level of inhibition with tetrahydrohomofolate was not consistent with the concept that this folate analog is an effective therapeutic agent in amethopterin-resistant mouse leukemia due to the inhibition of thymidylate synthetase as the target enzyme. It is concluded that tetrahydrohomofolate must act elsewhere, perhaps by inhibiting the uptake of folate by the leukemia cells. Thymidylate synthetase has been extracted from amethopterin-resistant mouse leukemia cells and partially purified by salt fractionation and gel filtration. The enzyme can be stabilized for storage by the addition of sucrose. Based on the spectrophotometric assay of thymidylate synthetase the enzyme from mouse leukemia cells is 25-fold more resistant to inhibition by tetrahydrohomofolate than the same enzyme from *Escherichia coli*.

Tetrahydrohomofolate, an analog of folic acid, inhibits thymidylate synthetase of *Escherichia coli* and is a potent growth inhibitor of *Streptococcus faecalis* (Goodman *et al.*, 1964). On the basis of these findings tetrahydrohomofolate was tested in mice carrying an amethopterin-resistant line of leukemia cells and was found to prolong significantly the survival of these animals at a daily dosage of 400 mg/kg (Mead *et al.*, 1966). Studies were then initiated to determine whether or not the chemotherapeutic effect of tetrahydrohomofolate in leukemic mice was due to the inhibition of leukemia cell thymidylate synthetase.

Since a convenient assay of thymidylate synthetase in leukemia cells was unavailable at the time this research was initiated, an indirect approach was used based on earlier observations that aminopterin blocks incorporation of deoxyuridine into DNA by intact cells (Friedkin and Roberts, 1956). It was reasoned that if tetrahydrohomofolate does indeed inhibit thymidylate synthetase of leukemia cells as the main target enzyme, a profound inhibition of deoxyuridine uptake into DNA should be demonstrable both in amethopterin-sensitive and amethopterin-resistant leukemia cells.

This report describes experiments which indicate that tetrahydrohomofolate has slight effect on incorporation of deoxyuridine into DNA of mouse leukemia cells. The question arose whether or not tetrahydrohomofolate has any direct effect on thymidylate synthetase of leukemia cell origin. This led to studies described herein with cell-free extracts and partially purified preparations of leukemia cell thymidylate synthetase.

Materials and Methods

Tyrode's ascorbate medium contained (g/100 ml) NaCl, 0.9; KCl, 0.02; $CaCl_2$, 0.02; $MgCl_2$, 0.01; glucose, 0.10; $NaHCO_3$, 0.10; NaH_2PO_4, 0.005; and ascorbic acid, 1.0. The mixture was adjusted to pH 8.2 with 5 N NaOH.

Buffer A was 0.05 M Tris, 10 mM mercaptoethanol, and 1 mM disodium EDTA, adjusted to pH 7.4 with HCl.

Buffer B was the same as buffer A but adjusted to pH 8.4 with HCl.

dl-Tetrahydrofolic acid and *dl*-tetrahydrohomofolic acid were prepared by catalytic reduction (O'Dell *et al.*, 1947; Kisliuk, 1957). Deoxyuridylic acid labeled with tritium at position 5 was kindly supplied by Dr. DeWayne Roberts (commercial source: Calbiochem, Los Angeles, Calif). [^{14}C]Deoxyuridine was obtained from the New England Nuclear Corp., Boston; Sephadex G-100 from Pharmacia, Uppsala, Sweden; and DBA/2JA mice from Jackson Memorial Laboratories, Bar Harbor, Maine.

Transplantation and Maintenance of FR8 and L1210 Leukemia Cells in Mice. DBA/2JA male mice weighing 20–25 g were injected subcutaneously in the inguinal area with 0.20 ml of either 1:10 or 1:100 (v/v) suspensions of spleen cells in sterile 0.9% NaCl from previously

* From the Department of Pharmacology, Tufts University School of Medicine, Boston, Massachusetts 02111. *Received March 25, 1968.* This study was supported in part by funds generously provided by the U. S. Public Health Service Grant CA 05997.

† Supported in part by funds from U. S. Public Health Service Training Grant 3 TO1 GM00765. Present address: Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare, Bethesda, Md. 20014.

‡ Present address: Department of Biochemistry, Tufts University School of Medicine, Boston, Mass. 02111.

inoculated mice (Goldin *et al.*, 1960a,b; Misra *et al.*, 1961). Both lines were obtained from Dr. A. Goldin, National Cancer Institute, Bethesda, Md. The FR8 strain is an amethopterin-resistant subline of L1210 with a high level of dihydrofolate reductase. Predictably, in 7 or 8 days, inguinal tumors and grossly enlarged spleens appeared. The animals were then sacrificed, and the spleens and inguinal tumors were removed. Some spleens were used for further transplantation.

Preparation of Leukemia Cell Suspensions. Spleens were removed from mice with fully developed leukemic tumors and dispersed in chilled Tyrode's ascorbate medium (1 g wet weight of tissue plus 2 ml of buffer) by repeated forced passage through a no. 21 hypodermic needle attached to a 5-ml sterile syringe. The suspension was stored in an ice bath and was used within 30 min.

Incubation of Leukemia Cell Suspensions with [^{14}C]Deoxyuridine. Each incubation mixture (total volume, 0.3 ml) consisted of 0.20 ml of cell suspension, 0.05 ml of a water solution of [^{14}C]deoxyuridine (32 μCi/μmole, 100 μmoles/ml), and amethopterin. The labeled compound was added last. This mixture was incubated with agitation at 37° as previously described (Friedkin and Wood, 1956). Following the assay period, the tubes were immediately immersed in an ice bath and the acid-insoluble material was precipitated with 3.0 ml of chilled 3% perchloric acid. The precipitate was washed once with 3.0 ml of perchloric acid and twice with 1 ml of distilled water and finally dissolved in 0.20 ml of 0.15 M NH_4OH in 50% ethanol. The solution was plated on aluminum planchets, dried under a heating lamp, and counted in a open-window gas-flow counter. The method was validated by hydrolysis of the DNA and isolation of thymine by paper chromatography. Essentially all of the radioactivity was found in DNA thymine.

Purification of Thymidylate Synthetase from Spleens of FR8-Injected Mice. Spleens from four FR8-injected animals were removed, placed in ice-cold 0.9% NaCl, and then homogenized in ten volumes of 0.9% NaCl–0.05 M Tris-Cl (pH 7.4) in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 4° at 17,000 rpm for 20 min. The clear red supernatant fluid thus obtained was subjected to salt fractionation as follows. $(NH_4)_2SO_4$ was added to a concentration of 1.4 M and the ensuing precipitate discarded. The further addition of $(NH_4)_2SO_4$ to the supernatant fluid to a final concentration of 2.4 M yielded a second precipitate containing thymidylate synthetase. This fraction was dissolved in 10 ml of buffer B and subjected to gel filtration on a column of Sephadex G-100 (67 × 7 cm). Buffer B was used throughout. The effluent fluid was collected in 20-ml fractions. Protein was determined by absorbancy at 280 mμ. Thymidylate synthetase (Wahba and Friedkin, 1961) and dihydrofolate reductase (Osborn and Huennekens, 1958) were assayed spectrophotometrically. The elution profile is shown in Figure 1. Values in the figure for thymidylate synthetase are expressed as absorbancy increase at 340 mμ/min for 0.9-ml aliquots; those for dihydrofolate reductase are expressed as absorbancy decrease at 340 mμ/min for 0.1-ml aliquots. Hemoglobin appeared in tubes centering at fraction 50. Fractions containing thymidylate synthetase were combined (tubes 40–44) and then poured through a DEAE-cellulose column (1.2 × 9 cm) previously washed with buffer A. After continued washing of the column with 200 ml of buffer A the enzyme was eluted with buffer A containing 0.4 M NaCl. The preparation showed a green fluorescence. The specific activity of the enzyme was 0.6 μmole/mg of protein per hr representing a 50-fold purification. The activity stabilized by addition of sucrose to a final concentration of 20%. The preparation was stored at 5°.

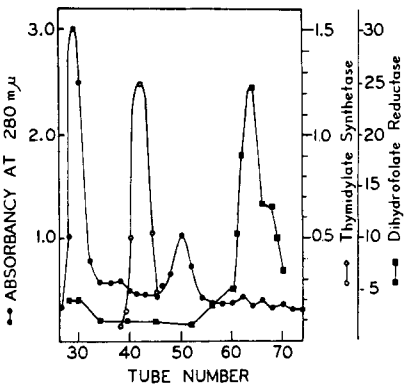

FIGURE 1: Elution profile of thymidylate synthetase and dihydrofolate reductase from spleens of FR8-injected mice. See section on Materials and Methods for experimental details.

Results

The Effect of Amethopterin on [^{14}C]Deoxyuridine Uptake by Sensitive and Resistant Leukemia Cells. Two leukemia cell lines (one sensitive and the other resistant to amethopterin as shown by survival of drug-treated mice carrying these lines (Goldin *et al.*, 1960a,b; Misra *et al.*, 1961)) were tested for relative sensitivity and resistance by an *in vitro* assay (Friedkin and Wood, 1956). The assay was based on the concept that amethopterin would be less likely to inhibit [^{14}C]deoxyuridine incorporation into DNA of resistant cells than into DNA of sensitive cells. Adequate amounts of tetrahydrofolate are required for conversion of deoxyuridylate into thymidylate. Tetrahydrofolate is the product of the dihydrofolate reductase reaction. Diminished generation of this cofactor due to the inhibitory action of amethopterin should result in decreased deoxyuridine utilization for DNA synthesis.

Although the incorporation of [^{14}C]deoxyuridine into DNA was not linear (*i.e.*, the rate fell off after the first 10-min incubation (Figure 2)) it is quite clear that a distinct difference in sensitivity to amethopterin exists between the sensitive and resistant cells used in these experiments. The amethopterin-resistant cells showed negligible inhibition by amethopterin at 10 min. This inhibition increased with time whereas with drug-sensitive cells inhibition at 10 min was already 62%.

Kessel *et al.* (1965) have shown that in many cell types amethopterin resistance is closely related to the degree of permeability to the drug. It is possible that the entry of amethopterin into resistant cells during the first 10-min incubation is limited and is therefore insufficient

TABLE I: Effects of Amethopterin and Tetrahydrohomofolate on [^{14}C]Deoxyuridine Uptake by Sensitive and Resistant Leukemia Cells.[a]

Exptl Conditions	Amethopterin-Sensitive L1210		Amethopterin-Resistant FR8	
	Cpm in Acid-Insoluble Fraction	% Inhibn	Cpm in Acid-Insoluble Fraction	% Inhibn
Zero-time incubation	57		40	
1-hr incubation at 37°	1763	None	2484	None
1-hr plus amethopterin (10^{-4} M)	629	65	2038	18
1-hr plus tetrahydrohomofolate (10^{-4} M)	1522	14	2307	7,1

[a] See Materials and Methods section for experimental details.

to produce inhibition, whereas in sensitive cells enough amethopterin enters within 10 min to produce a significant degree of inhibition. If the inhibition by amethopterin is calculated for the 50-min period between 10 and 60 min (Figure 2), 44% inhibition was obtained with the resistant cells compared with a value of 75% for the sensitive cells.

A somewhat surprising finding was the relatively high level of extracellular amethopterin (10^{-4} M) required to produce a significant inhibition of [^{14}C]deoxyuridine uptake into DNA in sensitive cells. In experiments of a similar nature, during a 1-hr incubation, 4% inhibition occurred in the resistant cells and 20% inhibition in the sensitive cells in the presence of 10^{-5} M amethopterin. It is possible that relatively long periods of time are required to completely deplete the intracellular stores of tetrahydrofolate once the generation of this cofactor is cut off by amethopterin.

The Effect of Tetrahydrohomofolate on [^{14}C]Deoxyuridine Uptake by Leukemia Cells. Having ascertained that the leukemia cell lines employed in these studies were indeed amethopterin-sensitive and resistant as shown by the above studies, we then tested tetrahydrohomofolate under the same conditions fully expecting to observe an inhibition of deoxyuridine uptake. Much to our surprise, tetrahydrohomofolate was only poorly inhibitory (Table I).

The stability of tetrahydrohomofolate during the incubation period was checked by removing aliquots for

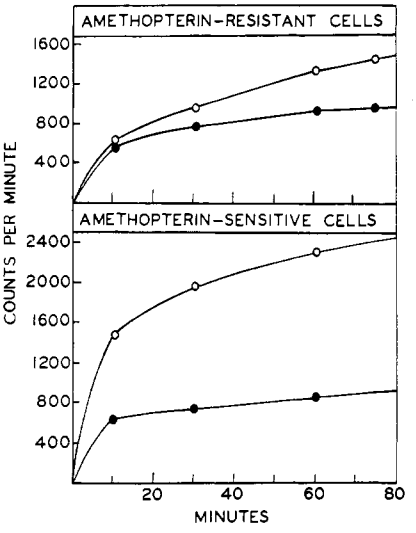

FIGURE 2: Inhibition of [^{14}C]deoxyuridine uptake by amethopterin-sensitive and amethopterin-resistant leukemia cells. See section on Materials and Methods for experimental details. (O—O) No inhibitor; (●—●) 10^{-4} M amethopterin.

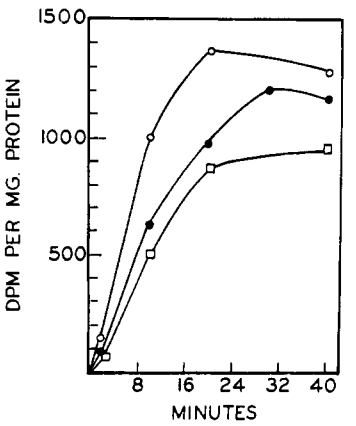

FIGURE 3: Inhibition of thymidylate synthetase in crude extracts of leukemia cells by tetrahydrohomofolate. Extracts were prepared as follows. Spleens from leukemic mice (injected with FR8 amethopterin-resistant subline) were homogenized in chilled buffer A (2.5 ml of buffer/g wet weight of tissue; six passes at 500–600 rpm in a Potter-Elvehjem homogenizer). A clear extract was obtained by centrifugation of the homogenate for 12 min at 12,000 rpm at 4°. Protein concentration of each preparation was determined by the method of Lowry *et al.* (1951) and fresh enzyme was prepared for each experiment. The enzyme did not lose activity on storage overnight at −15°. Thymidylate synthetase was assayed by Roberts' modification of Greenberg's procedure (Roberts, 1966). In this method, tritium at position 5 of dUMP is labilized during the synthesis of dTMP and is counted in the supernatant fluid after charcoal treatment of the reaction mixture. Incubations were carried at 37° for varying periods of time with and without tetrahydrohomofolate. (O—O) No inhibitor added; (●—●) 10^{-5} M tetrahydrohomofolate; (□—□) 2×10^{-5} M tetrahydrohomofolate.

TABLE II: Inhibition of Mixtures of Thymidylate Synthetase from *E. coli* and FR8 Leukemia Cells by Tetrahydrohomofolate.[a]

Amt of Tetrahydrohomofolate Present (M)	Thymidylate Synthetase Act.[b]			
	FR8 Enzyme Alone	*E. coli* Enzyme Alone	Mixture of FR8 and *E. coli* Enzyme	Calcd Act. of Mixture
3×10^{-6}	98	38	71	68
13×10^{-6}	77	15	44	46
28×10^{-6}	68	5	37	37

[a] The conditions in this experiment are as described in Figure 4. Both enzymes were diluted in buffer B containing 20% sucrose (w/v) in order to give the same activity in the absence of inhibitor. Enzyme was added last in each instance. [b] Per cent of uninhibited rate.

enzymatic assay with *E. coli* thymidylate synthetase. The assay for tetrahydrohomofolate was based on inhibition of the enzyme. It was concluded that the poor inhibition of [^{14}C]deoxyuridine uptake by tetrahydrohomofolate could not be due to destruction of tetrahydrohomofolate by oxidation.

The Effect of Tetrahydrohomofolate on Thymidylate Synthetase from Extracts of Leukemia Cells. In view of the poor inhibition of deoxyuridine uptake by tetrahydrohomofolate it appeared possible that the chemotherapeutic effects of tetrahydrohomofolate in leukemic mice might be unrelated to thymidylate synthetase as a target enzyme. In fact leukemia cell thymidylate synthetase might be completely unaffected by tetrahydrohomofolate in contrast to the known sensitivity of the enzyme from *E. coli*. It became important to test this possibility directly with isolated enzymes. Fortunately, at about this time, the simple isotopic method of Greenberg (modified by Roberts) became available for the assay of thymidylate synthetase. This involved the release of tritium from the 5 position of doxyuridylate during thymidylate synthesis (Roberts, 1966).

With crude extracts prepared from FR8 leukemia cells, the isotopic assay (Figure 3) showed that the inhibition of thymidylate synthetase by *dl*-tetrahydrohomofolate was 37% at 10^{-5} M and 50% at 2×10^{-5} M in 10 min. This is a relatively poor amount of inhibition when compared with a value of 50% at 2×10^{-6} M in the spectrophotometric assay of *E. coli* thymidylate synthetase (Plante *et al.*, 1966).

The leukemia cell thymidylate synthetase was further purified by ammonium sulfate fractionation, gel filtration (Figure 1), and DEAE-cellulose column chromatography, making possible the use of the direct spectrophotometric assay of thymidylate synthetase. The spectrophotometric assay confirmed results obtained earlier with the isotopic method. Thymidylate synthetase from leukemia cells was much less sensitive to inhibition by tetrahydrohomofolate than the enzyme from *E. coli* (Figure 4).

In order to rule out the possibility that a soluble compound in the purified preparation of thymidylate synthetase from leukemia cells might be interfering with the inhibition of thymidylate synthetase, mixtures of enzyme from *E. coli* and leukemia cells were assayed in the presence and absence of tetrahydrohomofolate. Since the inhibition found for the mixture of enzymes (Table II) was almost exactly that predicted from results with the enzymes tested separately, it was concluded

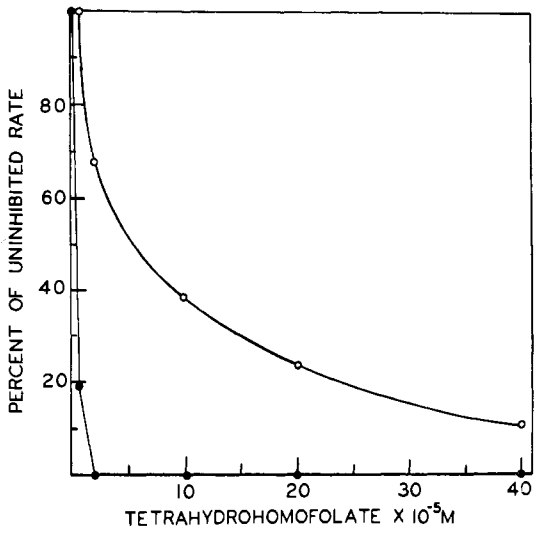

FIGURE 4: Comparison of the inhibition by tetrahydrohomofolate of thymidylate synthetase from FR8 leukemia cells and from *E. coli*. Thymidylate synthetase from spleens of mice injected with FR8 leukemia was prepared as described in the section on Materials and Methods. Thymidylate synthetase from *E. coli* was prepared as previously described (Friedkin *et al.*, 1962). The enzymes were assayed spectrophotometrically in a modified low Mg^{2+} medium consisting of 0.16 mM *dl*-tetrahydrofolate, 12 mM HCHO, 1 mM $MgCl_2$, 0.04 mM dUMP, 50 mM Tris-Cl (pH 8.4), 80 mM mercaptoethanol, and 1 mM EDTA (Wahba and Friedkin, 1961). The Mg^{2+} concentration was decreased to $^1/_{20}$ of the original level to eliminate turbidity in the reaction mixture. The leukemia enzyme is not significantly stimulated by Mg^{2+} unlike the bacterial enzyme (Wahba and Friedkin, 1962). This lack of Mg^{2+} requirement has been described for thymidylate synthetase from ascites cells (Reyes and Heidelberger, 1965) and from chick embryos (Lorenson *et al.*, 1967). (O—O) Enzyme from leukemia cells; (●—●) enzyme from *E. coli*.

that the leukemia enzyme preparation did not contain an antitetrahydrohomofolate substance and that indeed the thymidylate synthetase of leukemia cells was intrinsically less sensitive to inhibition by tetrahydrohomofolate than the *E. coli* enzyme by a factor of 20–30.

Discussion

Since tetrahydrohomofolate is a potent inhibitor of *E. coli* thymidylate synthetase and also prolongs the survival of mice inoculated with amethopterin-resistant FR8 leukemia cells (Goodman *et al.*, 1964; Mead *et al.*, 1966), it appeared quite likely that tetrahydrohomofolate would also inhibit the thymidylate synthetase of leukemia cells. This enzyme inhibition could then explain the antileukemic activity of tetrahydrohomofolate in mice.

dl-Tetrahydrohomofolate does inhibit partially purified thymidylate synthetase from leukemia cells to the extent of 50% at 5×10^{-5} M; however, tetrahydrohomofolate at 10^{-4} M did not substantially inhibit the incorporation of [^{14}C]deoxyuridine into DNA of intact cells. This low degree of inhibition may be due to the limited permeability that exists for entry of tetrahydrohomofolate into leukemia cells (Nahas, 1967). It is possible that tetrahydrohomofolate may have another site of action unrelated to thymidylate synthetase inhibition. Despite the failure to pin down the target site of tetrahydrohomofolate in leukemic mice, these studies have demonstrated a marked difference between thymidylate synthetase of bacterial and leukemia cell origin as evidenced by the 25-fold difference in sensitivity to the inhibitory action of tetrahydrohomofolate. R. L. Kisliuk (unpublished data) has recently found that tetrahydrohomofolate can act to some extent as a substrate of thymidylate synthetase from *S. faecalis*. This possibility has yet to be tested with the FR8 leukemia enzyme.

References

Friedkin, M., Crawford, E. J., Donovan, E., and Pastore, E. J. (1962), *J. Biol. Chem. 237*, 3811.

Friedkin, M., and Roberts, D. (1956), *J. Biol. Chem. 220*, 653.

Friedkin, M., and Wood, H., IV (1956), *J. Biol. Chem. 220*, 639.

Goldin, A., Humphreys, S. R., Chapman, G. O., Chirigos, M. A., and Venditti, J. M. (1960a), *Nature 185*, 219.

Goldin, A., Humphreys, S. R., Chapman, G. O., Venditti, J. M., and Chirigos, M. A. (1960b), *Cancer Res. 20*, 1066.

Goodman, L., DeGraw, J., Kisliuk, R. L., Friedkin, M., Pastore, E. J., Crawford, E. J., Plante, L. T., Al-Nahas, A., Morningstar, J. F., Jr., Kwok, G., Wilson, L., Donovan, E. F., and Ratzan, J. (1964), *J. Am. Chem. Soc. 86*, 308.

Kessel, D., Hall, T. C., and Roberts, D. (1965), *Science 150*, 752.

Kisliuk, R. L. (1957), *J. Biol. Chem. 227*, 805.

Lorenson, M. Y., Maley, G. F., and Maley, F. (1967), *J. Biol. Chem. 242*, 3332.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Mead, J. A. R., Goldin, A., Kisliuk, R. L., Friedkin, M., Plante, L. T., Crawford, E. J., and Kwok, G. (1966), *Cancer Res. 26*, 2374.

Misra, D. K., Humphreys, S. R., Friedkin, M., Goldin, A., and Crawford, E. J. (1961), *Nature 189*, 39.

Nahas, A. (1967), Ph.D. Thesis, Tufts University, Boston, Mass.

O'Dell, B. L., Vandenbelt, J. M., Bloom, E. S., and Pfiffner, J. J. (1947), *J. Am. Chem. Soc. 69*, 250.

Osborn, M. J., and Huennekens, F. M. (1958), *J. Biol. Chem. 233*, 969.

Plante, L. T., Crawford, E. J., and Friedkin, M. (1966), *J. Biol. Chem. 242*, 1466.

Reyes, P., and Heidelberger, C. (1965), *Mol. Pharmacol. 1*, 14.

Roberts, D. (1966), *Biochemistry 5*, 3546.

Wahba, A. J., and Friedkin, M. (1961), *J. Biol. Chem. 236*, PC11.

Wahba, A. J., and Friedkin, M. (1962), *J. Biol. Chem. 237*, 3794.