ACKNOWLEDGMENTS

We wish to thank Dr. K. E. Van Holde for valuable discussions and suggestions on the subject; some exploratory experiments pertaining to this work were carried out by one of us (A.J.S.) in Dr. Van Holde's laboratory in 1960–61.

REFERENCES

Beychok, S., and Warner, R. C. (1959), *J. Am. Chem. Soc. 81*, 1892.
Donovan, J. W., Laskowski, M., Jr., and Scheraga, H. A. (1960), *J. Am. Chem. Soc. 82*, 2154.
Donovan, J. W., Laskowski, M., Jr., and Scheraga, H. A. (1961), *J. Am. Chem. Soc. 83*, 2686.
Edsall, J. T., and Wyman, J. (1958), Biophysical Chemistry, Vol. I, New York, Academic.
Foss, J. G. (1960), *Biochim. Biophys. Acta 43*, 300.
Harris, F. E., and Rice, S. A. (1954), *J. Phys. Chem. 58*, 725.
Hermans, J., Jr., and Scheraga, H. A. (1961a), *J. Am. Chem. Soc. 83*, 3283.
Hermans, J., Jr., and Scheraga, H. A. (1961b), *J. Am. Chem. Soc. 83*, 3293.
Holcomb, D. N., and Van Holde, K. E. (1962), *J. Phys. Chem. 66*, 1999.
Jirgensons, B. (1958), *Arch. Biochem. Biophys. 74*, 70.
Rice, S. A., and Nagasawa, M. (1961), Polyelectrolyte Solutions, New York, Academic, chap. 7.
Scheraga, H. A. (1963), *Proteins 1* (2nd ed.), 477.
Sophianopoulos, A. J., Rhodes, C. K., Holcomb, D. N., and Van Holde, K. E. (1962), *J. Biol. Chem. 237*, 1107.
Tanford, C. (1961a), *J. Am. Chem. Soc. 83*, 1628.
Tanford, C. (1961b), Physical Chemistry of Macromolecules, New York, Wiley.
Tanford, C., and Wagner, M. (1954), *J. Am. Chem. Soc. 76*, 3331.

On the Similarity of Dihydrofolate Reductases from Amethopterin-sensitive and Amethopterin-Resistant Mouse Leukemia (L1210) Cells*

EVA R. KASHKET,† ELIZABETH J. CRAWFORD, MORRIS FRIEDKIN, S. R. HUMPHREYS, AND ABRAHAM GOLDIN

From the Department of Pharmacology, Tufts University School of Medicine, Boston, Mass., and the Drug Evaluation Branch, National Cancer Institute, Bethesda, Md.

Received August 3, 1964

The dihydrofolate reductase activity of amethopterin-resistant mouse lymphoid-leukemia (L1210) cells increases manyfold over that of drug-sensitive cells. This has been attributed to the selection of resistant cells that produce the same kind of dihydrofolate reductase molecules as their sensitive ancestors but in greater numbers. This conclusion now appears to be justified in view of the identical behavior of the two enzyme proteins (one from amethopterin-sensitive cells, the other from the amethopterin-resistant subline) on ion-exchange columns, as well as the striking similarity in kinetic behavior with a variety of folate analogs used either as inhibitors or as substrates. Identical enzyme activations were observed with sodium chloride or *p*-mercuribenzoate.

Amethopterin treatment of mice given implants of lymphoid-leukemia (L1210) cells results in sublines of tumor cells that are resistant to the inhibitory action of the antifolate (Law, 1956). This resistance may be owing in part to a marked rise in dihydrofolate reductase, an enzyme known to be exquisitely sensitive to the inhibitory action of amethopterin. The enhanced enzymatic activity allows folic acid compounds to be reduced to the coenzymatically active tetrahydrofolates in quantities sufficient for cellular multiplication in the presence of the drug (Misra *et al.*, 1961). Selection of amethopterin-resistant mutant leukemic cells with elevated enzyme activity rather than induction is suggested by the persistence of high enzyme levels for at least ten transfers of the leukemia cells in drug-free animals (Friedkin *et al.*, 1962a). Mathematical models consistent with the foregoing concept have been developed which relate dihydrofolate reductase activities in resistant and sensitive cells, mutation rates, and the life spans of leukemic mice treated with amethopterin (Friedkin and Goldin, 1962).

The dihydrofolate reductases from amethopterin-sensitive and -resistant leukemia cells have now been compared in order to determine whether there has been a selection of mutant cells in which a new molecular species of a more active reductase is produced, or whether the selection pressure is toward a cell producing a greater number of the original enzyme molecules. The striking similarity of the reductases of amethopterin-resistant and -sensitive cells reported in this communication strongly supports the latter possibility. A preliminary report has been presented elsewhere (Kashket *et al.*, 1964).

* This investigation was supported in part by research funds generously given by the National Cancer Institute (grant CA-05997).

† Postdoctoral Trainee in Biochemical Pharmacology (USPH 5T1 GM-765).

EXPERIMENTAL PROCEDURES

Materials.—Folic acid as well as the various analogs were reduced to the dihydro form with dithionite in 1 M mercaptoethanol (Friedkin *et al.*, 1962b). The folic acid analogs 10-nitrosofolic acid, 3′-iodo-10-nitrosofolic acid, 3′-bromofolic acid, and 3′,5′-dibromofolic acid were prepared by Dr. L. Plante at Tufts University. Homofolic acid was prepared by Drs. L. Goodman and J. DeGraw, Stanford Research Institute (Goodman *et al.*, 1964). Amethopterin (Lederle Laboratories) was purified by the method of Noble (1961). Hydroxylapatite was prepared by the method of Tiselius *et al.* (1956). NADPH was purchased from Pabst Laboratories.

Preparation of Dihydrofolate Reductase.—Acetone powders were prepared from tumors formed by the implantation of male mice (CDBA, (National Institutes of Health animal farm)) with amethopter-in-sensi-

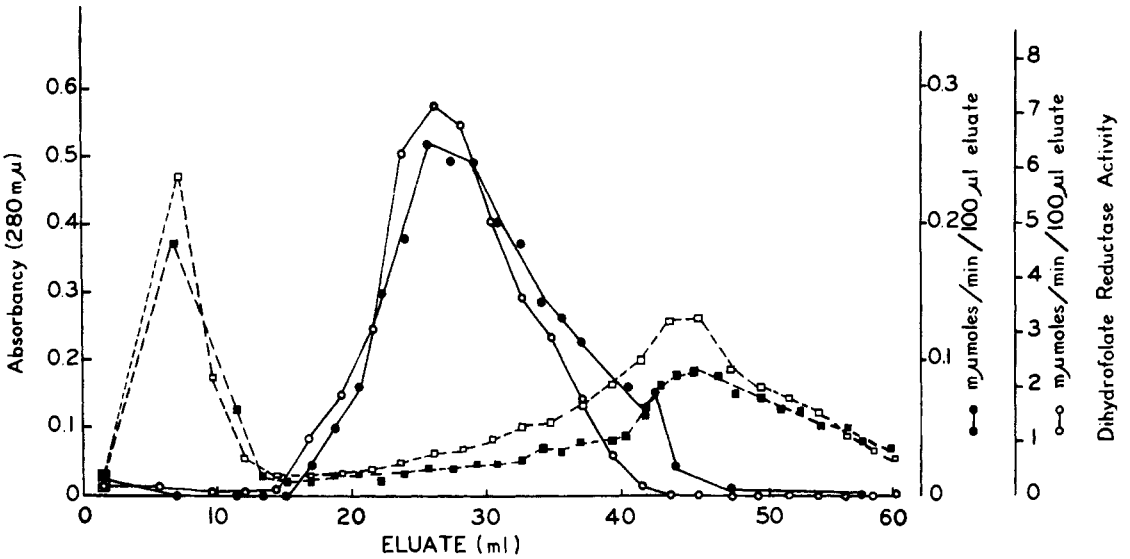

FIG. 1.—Chromatography of dihydrofolate reductase from amethopterin-sensitive (solid circles and and squares) and -resistant (open circles and squares) mouse L1210 leukemia cells on hydroxylapatite columns. The enzymatic activity (circles) was assayed as in Table I, and is expressed as mμmoles 7,8-dihydrofolate reduced/minute/aliquot of eluate. The absorbancy values at 280 mμ (squares) reflect the protein concentration.

tive and amethopterin-resistant (FR 8) lymphoid leukemia (L1210) cells as decribed previously (Misra *et al.*, 1961; Friedkin *et al.*, 1962a). The powders (1.6 g) were extracted with 16 ml of 0.1 M potassium phosphate buffer, *p*H 7.5, and nucleic acids were removed by precipitation with 0.3 volume of 2% protamine sulfate. The supernatant fluids were applied to hydroxylapatite columns (6.0 × 0.8 cm) previously equilibrated with 200 ml of 0.05 M potassium phosphate buffer, *p*H 6.5. As previously shown by Mathews and Huennekens (1963), dihydrofolate reductase is adsorbed to hydroxylapatite under the foregoing conditions and can be desorbed with purification by gradient elution with 100 ml of potassium phosphate buffer, *p*H 6.5, from 0.05 M to 1.0 M.

Active fractions were pooled, diluted with water to reduce the buffer concentration to 0.05 M, and then concentrated by adsorption on hydroxylapatite columns and elution with 1.0 M potassium phosphate buffer, *p*H 6.5. All manipulations were carried out at 4°.

The assays of dihydrofolate reductase activity were carried out essentially as described by Misra *et al.* (1961), with the addition of NaCl to a final concentration of 0.6 M. (This concentration of NaCl activates the enzyme maximally.) Protein concentrations were estimated turbidimetrically with perchloric acid (M. Friedkin and E. J. Crawford, unpublished procedure) or by the method of Lowry *et al.* (1951).

RESULTS

Column Chromatography.—Although the specific activities of dihydrofolate reductase from extracts of amethopterin-sensitive and amethopterin-resistant mouse leukemia (L1210) cells differ by a factor of 30, the elution patterns obtained by chromatography of the two enzymes on hydroxylapatite columns closely resemble each other (Fig. 1). Upon gradient elution both enzymes were desorbed maximally at a phosphate buffer concentration of 0.19 M (the phosphate concentration was determined by the method of Fiske and Subbarow, 1925).

The two enzymes also behaved similarly when concentrated by adsorption on and elution from hydroxylapatite columns. The purification achieved after chromatography and concentration with either enzyme was 25- to 30-fold, with recoveries of 85–100%, based on the specific activity of the initial extracts of the acetone powders.

Kinetic Studies with Various Dihydrofolate Analogs.—A number of dihydro analogs of folic acid act as substrates for dihydrofolate reductase (M. Friedkin, E. J. Crawford, and L. T. Plante, unpublished data). Maximum velocities observed with five different folate analogs were similar for both enzymes (Table I).

Activation by Sodium Chloride.—Various ions have been found to stimulate the activity of dihydrofolate reductases (Bertino, 1962; M. Friedkin and E. J. Crawford, unpublished data). The dihydrofolate reductases from drug-sensitive and -resistant cells were equally stimulated by sodium chloride (Fig. 2). Maximal stimulation (370% of that in 0.05 M Tris-HCl buffer) occurred at 0.6 M NaCl with both enzymes.

Sodium chloride also had a striking effect on the enzyme kinetics obtained with various dihydrofolate analogs. Under the standard conditions of assay in which NaCl was present at a final concentration of 0.6 M, 3′-bromo-7,8-dihydrofolate showed initial reactions velocities 68 and 73% of that with 7,8-dihydrofolate for the sensitive and resistant enzymes, respectively (Table I). In the absence of added sodium chloride, the analogs exhibited initial velocities 440 and 450% of that with 7,8-dihydrofolate, for the two enzymes. The analog 3′,5′-dibromo-7,8-dihydrofolate behaved similarly.

Kinetic Studies with Inhibitors.—The effect of two potent inhibitors of dihydrofolate reductase, amethopterin and its 3′-bromo-5′-chloro derivative, was studied with the enzyme from the two sources. Amethopterin and its halogenated derivative were found in both cases to exhibit "pseudo-irreversible" enzyme kinetics, like the rat liver dihydrofolate reductase studied by Werkheiser (1961) and the chicken liver enzyme studied by Mathews and Huennekens (1963); *i.e.*, the inhibition exerted by amethopterin is of a competitive nature, but since the affinity for the enzyme is great, at high inhibitor concentrations the kinetics appears to be noncompetitive. When the enzymatic activities were analyzed by the method of

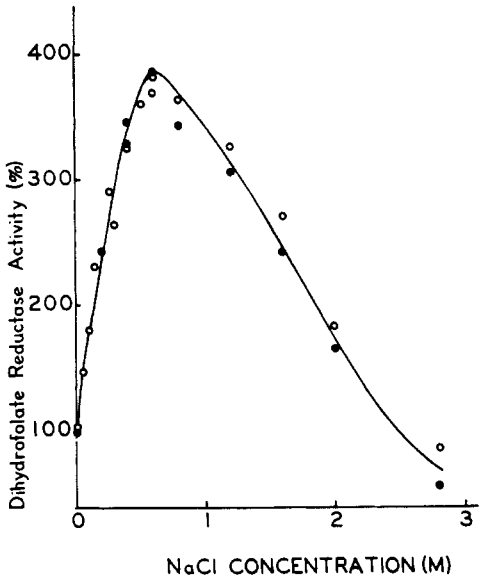

FIG. 2.—The stimulatory effect of sodium chloride on dihydrofolate reductase activity from amethopterin-sensitive (open circles) and resistant (solid circles) mouse leukemia L1210 cells. The conditions of assay were similar to those described in Table I. NaCl was present in final concentrations as indicated. The activities are expressed as percentages, relative to assays in the absence of NaCl.

Ackerman and Potter (1949), the tumor enzymes were equally inhibited by amethopterin and 3′-bromo-5′-chloroamethopterin (Table II).

Miscellaneous Comparisons.—When the two enzymes were compared with respect to their affinity for NADPH (one of the participants of the dihydrofolate reductase reaction) the K_m for this hydrogen donor was found to be the same within experimental error (Table II).

The dihydrofolate reductases extracted from various sources (Kaufman, 1964; Perkins and Bertino, 1964) have been found to be markedly stimulated by mercurials. *p*-Mercuribenzoate stimulated the enzymes from sensitive and resistant leukemia cells equally (Table II).

DISCUSSION

The present study with mouse lymphoid-leukemia cells was designed to make a choice between two possibilities: drug-resistant cells produce a greater number of enzyme molecules identical to those in drug-sensitive cells, or form an intrinsically more active dihydrofolate reductase protein. The first was found to be the more likely case.

The enzymes from the two types of tumor cells were compared with respect to activity with various analogs of folic acid as substrates, affinity for the hydrogen donor (NADPH), and kinetics of inhibition by various inhibitors of reductase activity. In addition, catalytic activity was studied under various salt concentrations. By these criteria the enzymes behaved similarly.

Although the specific activities differed in extracts from drug-sensitive and -resistant cells, the two enzymes behaved similarly during purification. The enzymes showed the same elution patterns on chromatography with hydroxylapatite. This is consistent with the enzymes' being identical protein molecules.

The mechanism of dihydrofolate reductase increase during development of amethopterin resistance remains to be elucidated. Amethopterin-sensitive cells do not contain an endogenous inhibitor of dihydrofolate reductase since the activities of extracts of amethopterin-sensitive and amethopterin-resistant cells were strictly additive. Furthermore, enzyme from sensitive cells was not more labile than enzyme from resistant cells. Although Bertino *et al.* (1963) have evidence for a direct drug-induced rise of dihydrofolate reductase in human leukocytes, the persistence of the elevated

TABLE I
COMPARISON OF RATES OF REDUCTION OF VARIOUS DIHYDROFOLATE ANALOGS WITH REDUCTASE FROM AMETHOPTERIN-SENSITIVE AND AMETHOPTERIN-RESISTANT L1210 MOUSE LEUKEMIA CELLS[a]

Substrate	Maximum Velocity (%)	
	Enzyme from Sensitive Cells	Enzyme from Resistant Cells
7,8-Dihydrofolate	100	100
7,8-Dihydrohomofolate	130	134
3′-Bromo-7,8-dihydrofolate	73	68
10-Nitroso-7,8-dihydrofolate	4	5
3′-Iodo-10-nitroso-7,8-dihydrofolate	12	13
7,8-Dihydropteroyl-D-glutamate	43	44

[a] For the measurement of enzymatic activity each cuvet contained 1.0 ml of 0.05 M Tris-HCl buffer, *p*H 7.3, containing 0.01 M 2-mercaptoethanol, 0.001 M EDTA, and 0.6 M NaCl. In addition, NADPH (0.5 μmole) and 10 μl (15–80 μg of protein) of the purified enzyme preparations were preincubated for 3 minutes at 37°. The total volume was 1.025 ml. The reaction was started by the addition of 0.1 μmole of dihydrofolate or one of its analogs. As described previously (Misra *et al.*, 1961), the enzymatic activity was measured by observing the decrease in absorbancy at 340 mμ for 5 minutes at 37° with a Beckman DU spectrophotometer with a Gilford multiple-sample absorbance recorder. This decrease ranged from 0.005 to 0.050 absorbancy units/minute under the conditions used. As these enzyme preparations were free of NADPH-oxidase activity, the reference cell contained the Tris buffer–mercaptoethanol–EDTA mixture. The specific activities of the dihydrofolate reductase preparations used were 2.3 μmoles of dihydrofolate reduced/mg protein/hour for enzyme from amethopterin-sensitive cells and 18.8 μmoles/mg protein/hour for that of amethopterin-resistant cells. The amounts of enzyme used were adjusted to yield approximately equal activities in each assay cuvet.

TABLE II
COMPARISON OF VARIOUS PROPERTIES OF DIHYDROFOLATE REDUCTASE FROM AMETHOPTERIN-SENSITIVE AND AMETHOPTERIN-RESISTANT L1210 MOUSE LEUKEMIA CELLS[a]

	Source of Enzyme	
	Sensitive Cells	Resistant Cells
K_I of amethopterin	1.3×10^{-10} M	1.7×10^{-10} M
K_I of 3′-bromo-5′-chloroamethopterin	1.1×10^{-10} M	1.1×10^{-10} M
Activation by *p*-mercuribenzoate (1×10^{-4} M)	133%	128%
K_m of NADPH	1.95×10^{-5} M	1.95×10^{-5} M

[a] The conditions of assay were similar to those described in Table I. The activation by *p*-mercuribenzoate was determined in the absence of salt and with Tris buffer lacking mercaptoethanol and EDTA. For the determination of the K_m of NADPH, the concentration of the hydrogen donor was varied from 7×10^{-6} M to 7×10^{-5} M. For the determination of the K_I of amethopterin and 3′-chloro-5′-bromoamethopterin, the reactions were carried out by varying the enzyme concentration at several inhibitor concentrations. The results obtained were analyzed by the method of Ackerman and Potter (1949).

levels in drug-free leukemic mice (Friedkin *et al.*, 1962a) suggests that selection of a new population of leukemia cells occurs during drug treatment. Mutation to a nonrepressible gene could account for the increased synthesis of the dihydrofolate reductase molecules.

It should be noted in conclusion that altered enzyme formation correlated with the development of amethopterin resistance has been reported by Sirotnak *et al.* (1964) to occur in *Diplococcus pneumoniae*. The dihydrofolate reductase from the mutant cells was strikingly less sensitive to the drug than that of the wild type, as well as differing in heat sensitivity and optimum *p*H.

References

Ackerman, W. W., and Potter, V. R. (1949), *Proc. Soc. Exptl. Biol. Med. 72*, 1.

Bertino, J. R. (1962), *Biochim. Biophys. Acta 58*, 377.

Bertino, J. R., Donohue, D. M., Simmons, B., Gabrio, B. W., Silber, R., and Huennekens, F. M. (1963), *J. Clin. Invest. 42*, 466.

Fiske, C. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66*, 375.

Friedkin, M., Crawford, E. J., Humphreys, S. R., and Goldin, A. (1962a), *Cancer Res. 22*, 600.

Friedkin, M., Crawford, E. J., and Misra, D. (1962b), *Federation Proc. 21*, 176.

Friedkin, M., and Goldin, A. (1962), *Cancer Res. 22*, 607.

Futterman, S. (1957), *J. Biol. Chem. 228*, 1031.

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.

Kashket, E. R., Friedkin, M., Humphreys, S. R., and Goldin, A. (1964), *Bacteriological Proceedings*, p. 134.

Kaufman, B. T. (1964), *J. Biol. Chem. 239*, PC669.

Law, L. W. (1956), *Cancer Res. 16*, 698.

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

Mathews, C. K., and Huennekens, F. M. (1963), *J. Biol. Chem. 238*, 3436.

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

Noble, E. P. (1961), *Biochem. Prepn. 8*, 20.

Perkins, J., and Bertino, J. R. (1964), *Biochem. Biophys. Res. Commun. 15*, 121.

Sirotnak, F., Donati, G. J., and Hutchison, D. J. (1964), *Biochem. Biophys. Res. Commun. 14*, 292; *J. Biol. Chem. 239*, 2677.

Tiselius, A., Hjerten, S., and Levin, O. (1956), *Arch. Biochem. Biophys. 65*, 132.

Werkheiser, W. C. (1961), *J. Biol. Chem. 236*, 888.

Purification and Properties of Bovine Factor X: Molecular Changes During Activation*

Demetrios Papahadjopoulos, E. T. Yin, and Donald J. Hanahan†

From the Department of Biochemistry, University of Washington, Seattle

Received August 17, 1964

Factor X of bovine plasma was purified nearly 10,000-fold by combined $BaSO_4$ treatment and DEAE chromatography. Activation of the purified factor X by trypsin or Russell's viper venom resulted in an apparent reduction in molecular size or shape as revealed by chromatography on Sephadex G-100. In the presence of Ca^{2+} a further, pronounced decrease in molecular weight of activated factor X was evident from behavior of the latter component on Sephadex G-100. An examination of the various species of factor X on electrophoresis showed that activation of factor X resulted in a sharp decrease in the net negative charge on the molecule. Activated factor X was capable of activating plasma factor X, whereas 25% citrate, reportedly an activator, did not have any effect on plasma factor X. The affinity of plasma factor X and activated factor X for purified phospholipids was followed through use of gel filtration on Sephadex G-200. In this manner, it was observed that formation of a stable complex between the factor X activity and phospholipids (phosphatidyl serine/phosphatidyl choline, equimolar mixture) was effected only when factor X was activated (by trypsin or Russell's viper venom) and then only in the presence of Ca^{2+}. The net charge on the protein and the phospholipid molecules appear to be of considerable import in these interactions.

Factor X was described by Hougie *et al.* (1957) as a plasma component necessary for the conversion of prothrombin to thrombin. Subsequently, several investigators (Macfarlane, 1961; Esnouf and Williams, 1962; Straub and Duckert, 1961; Williams, 1964; Nemerson and Spaet, 1964; Ferguson *et al.*, 1960; Pechet and Alexander, 1960) have established that factor X can be activated by several physiological and nonphysiological agents prior to its participation in the conversion of prothrombin to thrombin. Interestingly, even though different types of activation systems and sources of factor X were employed in the above studies, it appeared to be general that factor X was changed enzymatically to a more active form. In addition, this activated factor X required the presence of factor V, phospholipids, and Ca^{2+} for its participation in the prothrombin-activating system.

Recently, Spaet and Cintron (1963) and Macfarlane and Ash (1964) have attempted to show that the "intrinsic system" of prothrombin activation also involves an intermediate which could be similar if not identical to activated factor X. This intermediate would appear to be equivalent to the "intermediate"

* This investigation was supported by grants from the American Cancer Society (G-22078) and the Life Insurance Medical Research Fund (G-63-48).

† Inquiries should be addressed to this author. Abbreviations used in this study are: pX, non-activated plasma factor X; X^{Tr}, trypsin activated factor X; X^{R}, Russell's viper venom activated factor X; X^{S}, "spontaneously" activated factor X; II, prothrombin; PS, phosphatidyl serine; PC, phosphatidyl choline; DEAE, diethyl aminoethyl; DFP, diisopropyl phosphofluoridate.