used in this study: Dr. Roland K. Robins for compounds III, V, VI, VIII–X, XVII, XVIII, XXIII–XXVI, XXVIII, XXIX, XXXVI, XXXVII, XLIV, and XLV; Drs. Daniel H. Murray and John Prokop for compounds IV, XV, XVI, XXVII, XXXII–XXXIV, and XXXVIII–XLIII; Drs. Leon Goodman and Elmer J. Reist for compounds XI–XIV, XXXI, and XXXV; Dr. Charles G. Smith for compound XXI; Dr. Robert E. Handschumacher for compound VII; and the CCNSC for compound XXII. It is also a pleasure to acknowledge Dr. Charles A. Nichol for his interest and encouragement of this work.

Differential Binding to the Hydrophobic Bonding Region of T_2 Phage Induced, *Escherichia coli* B, and Pigeon Liver Dihydrofolic Reductases[1,2]

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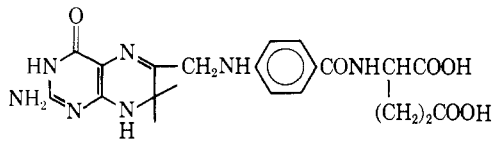
Forty-nine selected 2,4-diamino heterocycles have been compared for their relative inhibition of the dihydrofolic reductases from pigeon liver, *Escherichia coli* B, and T_2 phage induction; 44 of these compounds were 1-substituted 4,6-diamino-1,2-dihydro-*s*-triazines. The results clearly showed that parts of the hydrophobic bonding region on dihydrofolic reductase were distinctly different in the enzymes from the three species; some areas in the hydrophobic region were quite similar on the enzyme from pigeon liver and T_2 phage induction, but other areas showed a closer similarity in the enzymes from *E. coli* B and T_2 phage induction. The hydrophobic bonding region of the dihydrofolic reductases was sufficiently different from *E. coli* B and T_2 phage induction that similar studies on tumors induced by viruses may reveal differences in binding to the hydrophobic region; these may be anticipated to be chemotherapeutically exploitable, particularly with active-site-directed irreversible inhibitors of dihydrofolic reductase. The antibacterial agent trimethoprim (L), which complexes 40,000–70,000 times stronger to bacterial enzymes than vertebrate enzymes, complexed to the T_2 phage induced enzyme in the poor manner observed with vertebrate enzymes; this observation along with comparative "inhibitor profiles" with the other compounds gives support to the possibility that the T_2 phage DNA particle diverged from a higher form of life than bacteria.

Dihydrofolic reductase is one of the key enzymes in cellular reproduction; the enzyme reduces dihydrofolic acid (I) and usually folic acid to tetrahydrofolic acid, the cofactor form of the vitamin which is then involved in a spate of enzymatic reactions including purine and pyrimidine biosynthesis.[5] The discovery of a hydrophobic bonding region on dihydrofolic reductase[6] led to an intense study of the conformational aspects of binding to this region[7–9] and its relative location on the enzyme. Evidence has been found[10–12] that this hydrophobic bonding region is located near the 4 or 8 position of dihydrofolate (I) when it is complexed to the enzyme;[13] since the hydrophobic bonding region is outside of the active-site, evolutionary changes[14] of amino acids in this region might be expected to occur more easily, without destroying the function of the enzyme, than in the active site. That binding to the hydrophobic bonding region could differ among species was demonstrated with dihydrofolic reductases isolated from *Escherichia coli* B and pigeon liver;[15,16] furthermore, these differences in hydrophobic bonding could be considerably amplified[3] by use of active-site-directed irreversible inhibitors.[13,17]



I

When *E. coli* is infected by T_{even} bacteriophages, the creation or stimulation of at least 12 enzymatic activities occurs,[18] including dihydrofolic reductase;[19] the dihydrofolic reductase induced by the phage has been established to be a new enzyme genetically controlled by the viral DNA and not just higher production of enzyme coded by the bacterial DNA.[18,19] Furthermore, when mammalian cells in tissue culture are infected by such tumorigenic viruses as polyoma virus or SV40, the levels of some of the enzymes involved in DNA synthesis are increased, including dihydrofolic re-

(1) This work was generously supported by Grant CA-08695 from the National Cancer Institute, U. S. Public Health Service.

(2) Paper XCVII in the series on Irreversible Enzyme Inhibitors[3] and Paper X on Hydrophobic Bonding to Dihydrofolic Reductase.[4]

(3) For the previous paper of this series see B. R. Baker, *J. Chem. Educ.*, in press.

(4) For the previous paper of this series see B. R. Baker and G. J. Lourens, *J. Pharm. Sci.*, in press.

(5) T. H. Jukes and H. P. Broquist in "Metabolic Inhibitors," R. M. Hochster and J. H. Quastel, Ed., Academic Press Inc., New York, N. Y., 1963, pp 481–534.

(6) B. R. Baker, B.-T. Ho, and D. V. Santi, *J. Pharm. Sci.*, **54**, 1415 (1965).

(7) B. R. Baker and J. H. Jordaan, *ibid.*, **54**, 1740 (1965).

(8) B. R. Baker and B.-T. Ho, *J. Heterocyclic Chem.*, **2**, 335 (1965).

(9) B. R. Baker and G. J. Lourens, *ibid.*, **2**, 344 (1965).

(10) B. R. Baker, T. J. Schwan, J. Novotny, and B.-T. Ho, *J. Pharm. Sci.*, **55**, 295 (1966).

(11) B. R. Baker, J. K. Coward, B.-T. Ho, and D. V. Santi, *ibid.*, **55**, 302 (1966).

(12) B. R. Baker and H. S. Shapiro, *ibid.*, **55**, 308 (1966).

(13) For a review on mode of binding to dehydrofolic reductase see B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active-Site," John Wiley and Sons, Inc., New York, N. Y., 1967, Chapter X.

(14) For reviews on evolution of enzymes see (a) V. Bryson and H. J. Vogel, "Evolving Genes and Proteins," Academic Press Inc., New York, N. Y., 1965, and (b) ref 13, Chapter IX.

(15) B. R. Baker and B.-T. Ho, *J. Pharm. Sci.*, **55**, 470 (1966).

(16) Further examples of species differences in the ability of dihydrofolic reductase to bind 2,4-diamino heterocycles that have varying hydrophobic groups have been collated by Hitchings and Burchall; see (a) G. H. Hitchings and J. J. Burchall, *Advan. Enzymol.*, **27**, 417 (1965), and (b) J. J. Burchall and G. H. Hitchings, *Mol. Pharmacol.*, **1**, 126 (1965).

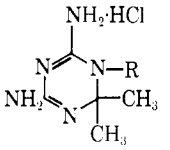
(17) B. R. Baker, *J. Pharm. Sci.*, **53**, 347 (1964).

(18) For leading references see C. K. Mathews and K. E. Sutherland, *J. Biol. Chem.*, **240**, 2142 (1965).

(19) C. K. Mathews and S. S. Cohen, *ibid.*, **238**, PC853 (1963).

TABLE I

INHIBITION OF DIHYDROFOLIC REDUCTASES BY 1-ALKYL- AND 1-PHENYLALKYL-*s*-TRIAZINES



Compd	R	μM concn for 50% inhib[a]			Pigeon liver/*E. coli* B	T_2 phage/*E. coli* B	T_2 phage/pigeon liver
		T_2 phage	*E. coli* B[b]	Pigeon liver[b]			
II	CH_3	65	48	74	1.5	1.4	1/1.1
III	C_2H_5	56	62	200	3.5	1/1.1	1/3.5
IV	n-C_3H_7	2.8	4.7	11	2.3	1/1.7	1/3.9
V	n-C_4H_9	0.93	2.1	0.36	1/5.8	1/2.3	2.6
VI	i-C_5H_{11}	0.28	0.69	0.058	1/12	1/2.5	4.8
VII	n-C_6H_{13}	0.42	0.92	0.32	1/2.9	1/2.2	1.3
VIII	n-C_8H_{17}	0.075	0.27	0.14	1/1.9	1/3.6	1/1.9
IX	C_6H_5	0.14	3.0	0.11	1/27	1/21	1.3
X	$CH_2C_6H_5$	1.1	34	3.3	1/10	1/31	1/3
XI	$(CH_2)_2C_6H_5$	1.3	3.8	0.71	1/5.3	1/2.9	1.8
XII	$(CH_2)_3C_6H_5$	0.089	0.063	0.028	1/2.2	1.4	3.2
XIII	$(CH_2)_4C_6H_5$	0.066	0.21	0.041	1/5.1	1/3.2	1.6

[a] Assayed with 6 μM dihydrofolate as described in the Experimental Section. [b] Data previously reported.[15]

ductase;[20] however, not all viruses stimulate the dihydrofolic reductase level, but thymidine kinase may be stimulated leading to increased use of the alternate route to thymidylate.[20]

By combining these two lines of research, we posed the question "what differences in binding to the hydrophobic bonding region of dihydrofolic reductases from *E. coli* B and the T_2 phage induced enzyme can be detected with 2,4-diamino heterocycles substituted with alkyl and aryl groups?" Answers to such a question could be useful for two problem areas, particularly if differences were found.

(1) The T_2 phage–*E. coli* B system could serve as a model on whether or not enzymes present in a viral-induced cancer cell might be different from the enzymes present in the uninfected normal cell; such differences could indicate whether cancer chemotherapy would be feasible by selective inhibition of dihydrofolic reductase by differential binding to the hydrophobic region. (2) Depending upon the type[16b] of "inhibition profile" observed, it might be possible to relate the T_2 phage to lower or higher forms of life on the evolutionary scale.[14a]

Experimental Section[21]

Buffers.—Buffer A was 0.05 *M* Tris, pH 7.4. Buffer B was the same with 10 m*M* mercaptoethanol and 1 m*M* tetrasodium ethylenediaminetetraacetate added.

Dihydrofolic Reductases. A. *Escherichia coli* **B.**—A suspension of *E. coli* B in buffer A was passed through a French press at 1406 kg/cm². After removal of nucleic acid with streptomycin, the enzyme was precipitated between 45 and 90% saturated $(NH_4)_2SO_4$. This fraction was dissolved in buffer A, stored at 3°, and used without further purification. This isolation method also gives a preparation of thymidylate synthetase, which has been previously described.[22]

B. T_2 Phage Induced.—We wish to thank Professor Seymour S. Cohen for *E. coli* B cells infected with T_2 phage.[23] The method of Mathews and Sutherland[18] for isolation and separation of the T_6 phage induced enzyme from the *E. coli* B enzyme was used. A smooth suspension of 7.8 g of T_2-infected cells in 20 ml of buffer A was passed through a prechilled French pressure cell at 1406 kg/cm². The exudate was diluted with 10 ml of buffer A, then blended 30 sec in a small Waring blendor head. The mixture was centrifuged at 20,000 rpm for 10 min in a Spinco L No. 40 rotor. The supernatant (31 ml) was decanted, then 10.3 ml of 5% aqueous streptomycin was added with stirring and ice cooling. After 10 min, the separated nucleic acids were removed by a similar centrifugation at 20,000 rpm to give 43 ml of supernatant. To the stirred solution cooled in an ice bath was added 13.5 g of $(NH_4)_2SO_4$ (50% of saturation) over a period of 3 min. After 20 min more, the precipitate was collected by centrifugation for 10 min at 20,000 rpm. The supernatant containing the *E. coli* B dihydrofolic reductase was rejected. The precipitate containing T_2 phage induced dihydrofolic reductase was dissolved in 20 ml of buffer A to give 24 ml of solution which was stored at 3°. For assay, the solution was diluted tenfold; 50 μl of the diluted enzyme in a total volume of 3.1 ml containing 6 μM dihydrofolate and 30 μM TPNH gave an optical density change at 340 mμ of 0.0084 unit/min when assayed by the previously described method.[22]

C. **Pigeon Liver.**—The enzyme from this source was isolated and assayed as previously described.[22]

Assay Method.—The dihydrofolic reductases were assayed with 6 μM dihydrofolate and TPNH in a total of 3.10 ml of solution containing buffer B as previously described[22] by observing the rate of optical density decrease at 340 mμ. The T_2 phage and the *E. coli* B enzymes were assayed with 30 μM TPNH and the pigeon liver enzyme with 12 μM TPNH.

Inhibitors.—The inhibitors were dissolved in 1 m*M* HCl and dilutions were made with the same solvent; up to 0.3 ml of these solutions could be employed in the assay by replacing a corresponding amount of buffer B.

Compounds XIV and XXXVI–XL were a gift from Dr. Harry B. Wood, Jr., of the CCNSC. Compounds XLVI–L were generously supplied by Dr. George H. Hitchings, Burroughs Wellcome & Co. The remainder of the compounds have been previously prepared in this laboratory and references are cited in Tables I–V.

Results

The inhibition results of the dihydrofolic reductases from T_2 phage, *E. coli* B, and pigeon liver with 49 inhibitors are presented in Tables I–V; these inhibitors can be divided into five discreet classes.

Series A. 1-Alkyl and 1-Phenylalkyl Variants.—It is noteworthy that the 1-methyl-*s*-triazine (II) binds

(20) P. M. Frearson, S. Kit, and D. R. Dubbs, *Cancer Res.*, **26**, 1653 (1966).

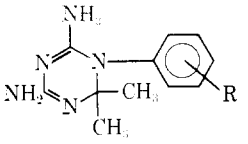
(21) The technical assistance of Maureen Baker and Ann Jaqua with the enzyme assays is acknowledged.

(22) B. R. Baker, B.-T. Ho, and T. Neilson, *J. Heterocyclic Chem.*, **1**, 79 (1964).

(23) J. G. Flaks and S. S. Cohen, *Biochim. Biophys. Acta*, **25**, 667 (1957).

Table II

Inhibition of Dihydrofolic Reductases by Polar-Substituted 1-Aryl-s-triazines

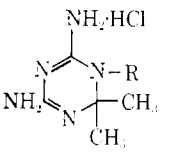


Compd	R	μM concn for 50% inhib[a] T₂ phage	*E. coli* B	Pigeon liver[b]	Pigeon liver/*E. coli* B	T₂ phage/*E. coli* B	T₂ phage/pigeon liver
IX	H	0.14	3.0	0.11	1/27	1/21	1.3
XIV	*p*-COOH	750	1900	1100	1/1.7	1/2.5	1/1.5
XV	*m*-COOH	79	130	110	1/1.2	1/1.4	1/1.4
XVI	*p*-$COOC_2H_5$	35	38	42	1.0	1/1.1	1/1.2
XVII	*p*-CN	9.5	91	8.0	1/11	1/9.6	1.2
XVIII	*p*-$COCH_2Cl$	1.6	26	1.2[c]	1/22	1/16	1.3
XIX	*m*-$COCH_2Cl$	1.3	4.3	1.7[c]	1/2.5	1/3.3	1/1.3
XX	*p*-$CH_2NH_3^+$	7.9	130	10	1/13	1/16	1/1.3
XXI	*p*-CH_2NHAc	1.2	30	0.11	1/270	1/25	11
XXII	*m*-CH_2OH	0.70	4.1	0.43[c]	1/9.6	1/5.8	1.6
XXIII	*m*-OCH_3	0.27	3.9	0.54[d]	1/7.2	1/14	1/2.0
XXIV	*o*-CF_3	0.030	2.3	0.080	1/29	1/77	1/2.7
XXV	*m*-NO_2	0.080	3.1	0.072	1/43	1/39	1.1

[a] All assays were performed with 6 μM dihydrofolate as described in the Experimental Section. [b] Data previously reported,[8] unless otherwise indicated. [c] Data previously reported by B. R. Baker and B.-T. Ho, *J. Pharm. Sci.*, **58**, 28 (1967); paper LXX of this series. [d] Data previously reported.[4]

Table III

Inhibition of Dihydrofolic Reductase by Nonpolar-Substituted 1-Aryl-s-triazines



Compd	R	μM concn for 50% inhib[a] T₂ phage	*E. coli* B[b]	Pigeon liver	Pigeon liver/*E. coli* B	T₂ phage/*E. coli* B	T₂ phage/pigeon liver
IX[c]	C_6H_5	0.14	3.0	0.11	1/27	1/21	1.3
XXVI	$C_6H_4C_6H_5$-*p*	11	5.8	160	28	1.9	1/15
XXVII	$C_6H_4C_6H_5$-*m*	0.011	1.0	1.3	1.3	1/91	1/120
XXVIII	$C_6H_4COC_6H_5$-*m*	0.053	0.61	1.1	1.8	1/11	1/21
XXIX	9-Fluorenon-2-yl	8.1	1.5	85	57	5.4	1/11
XXX	$C_6H_4CH_2C_6H_5$-*o*	0.016	0.28	0.019	1/15	1/17	1/1.2
XXXI	$C_6H_4CH_2C_6H_5$-*p*	0.22	4.8	0.062	1/77	1/22	3.5
XXXII	$C_6H_4(CH_2)_2C_6H_5$-*o*	0.015	0.092	0.024	1/3.8	1/6.2	1/1.6
XXXIII	$C_6H_4(C_3H_7\text{-}n)$-*p*	0.13	7.2	0.064	1/110	1/55	2.0
XXXIV	*p*-$C_6H_4(CH_2)_3C_6H_3Cl_2$-2,4	0.047	1.2[e]	0.0053[e]	1/230	1/25	8.9
XXV	*o*-$C_6H_4(CH_2)_4C_6H_3Cl_2$-2,4	0.0078	0.18[e]	0.0080[e]	1/22	1/23	1.0
XXXVI	C_6H_3Cl-3	0.019	0.60	0.0085	1/71	1/32	2.2
XXXVII	C_6H_4Cl-4	0.073	0.40[d]	0.44[e]	1.1	1/5.5	1/6.1
XXXVIII	$C_6H_3Cl_2$-3,4	0.019	0.16[d]	0.015[d]	1/11	1/8.5	1.3
XXXIX	C_6H_4Cl-2	25	200[d]	160[d]	1/1.2	1/8.0	1/6.4
XL	$C_6H_3(CH_3)_2$-2,6	31	96[e]	150	1.6	1/3.1	1/4.8

[a] All assays were performed with 6 μM dihydrofolate as described in the Experimental Section. [b] Data previously reported[15] unless otherwise indicated. [c] Data previously reported.[25] [d] Data previously reported.[26] [e] New data.

the same to all three enzymes within experimental error[24] (Table I). The 4,6-diamino-1,2-dihydro-*s*-triazine is believed[15] to complex within the active site in the region that normally complexes the pteridine ring of dihydrofolate; furthermore, the hydrophobic bonding region probably does not start until the third carbon of the side chain (note II and III have similar binding).

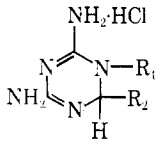
The increment in hydrophobic binding between methyl and *n*-butyl (V) with the T_2 phage enzyme is 70-fold, which is stronger than the 23-fold observed with *E. coli* enzyme, but not as strong as the 210-fold increment observed with the pigeon liver enzyme; a 460-fold increment in binding to the T_2 phage enzyme is observed with the 1-phenyl group (IX) which is much stronger than the 16-fold increment observed with the *E. coli* enzyme and is similar to the 670-fold increment observed with the pigeon liver enzyme.

With the T_2-enzyme an additional 12-fold increment is observed by increasing the *n*-butyl group (V) to *n*-octyl (VIII); an eightfold increment is observed with *E. coli* enzyme, but only a 2.6-fold increment with the pigeon liver enzyme. In contrast, terminal substitution of the *n*-butyl group with a phenyl to give phenylbutyl (XIII) results in a 9–14-fold increment

[24] The experimental error in binding increments is less than a factor of 1.5-fold.

TABLE IV

INHIBITION OF DIHYDROFOLIC REDUCTASE BY 1-ARYL-*s*-TRIAZINES WITH LARGE 2 SUBSTITUENTS

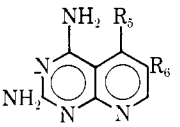


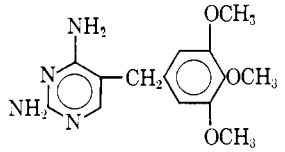
Compd	R_1	R_2	μM concn for 50% inhib[a] T_2 phage	*E. coli* B[b]	Pigeon liver[b]	Pigeon liver/*E. coli* B	T_2 phage/*E. coli* B	T_2 phage/pigeon liver
XLI	C_6H_4Cl-3	C_6H_5	0.18	1.0[c]	5.5[c]	5.5	1/5.6	1/31
XLII	C_6H_4Cl-3	$CH_2C_6H_5$	0.12	0.62[d]	1.1	1.8	1/5.2	1/9.2
XLIII	C_6H_4Cl-3	C_6H_4NHAc-*p*	64	180	190	1.0	1/2.8	1/3.0
XLIV	$(CH_2)_4C_6H_5$	C_6H_4NHAc-*p*	4.4	0.41	0.62	1.5	11	7.1
XLV	C_2H_5	C_6H_5	1300	310	15,000	48	4.2	1/12

[a] All assays were performed with 6 μM dihydrofolate as described in the Experimental Section. [b] Data previously reported,[15] unless otherwise indicated. [c] Data previously reported.[26] [d] Data not previously reported.

TABLE V

INHIBITION OF DIHYDROFOLIC REDUCTASE BY TRIMETHOPRIM AND 5-DEAZAPTERIDINES



Compd	R_5	R_6	μM concn for 50% inhib T_2 phage	*E. coli* B	Pigeon liver	Pigeon liver/*E. coli* B	T_2 phage/*E. coli* B	T_2 phage/pigeon liver
XLVI	H	$CH_2C_6H_5$	0.32	0.0095	0.43	45	34	1/1.3
XLVII	H	*n*-C_4H_9	0.12	0.032	0.16	5.0	3.7	1/1.3
XLVIII	CH_3	*n*-C_4H_9	0.047	0.032	0.075	2.3	1.5	1/1.6
XLIX	CH_3	*s*-C_4H_9	0.0090	0.0009	0.027	30	10	1/3.0
L	 trimethoprim		0.68	0.0003	16	53,000	2300	1/23

with all three enzymes. Thus, since the T_2 phage and *E. coli* B enzymes can give a near equal increment in binding with a terminal phenyl (as in XIII) or a terminal butyl (as in VIII) on the *n*-butyl group, it appears that this part of the hydrophobic bonding region can complex the staggered butyl group or a flat phenyl group with equal efficiency; in contrast, the pigeon liver enzyme has a greater affinity for the flat phenyl group of XIII by a factor of 3 compared to the terminal butyl group of VIII.

The branching of butyl (V) to isoamyl (VI) gives a 3–6-fold increment in binding to all three enzymes.

The two most potent compounds in this series for inhibition of the T_2 phage enzyme are the *n*-octyl- (VIII) and phenylbutyl-*s*-triazine (XIII). The *n*-octyl compound (VIII) is about fourfold more effective on the T_2 phage enzyme than the *E. coli* B enzyme and about twice as effective as on the pigeon liver enzyme. The phenylbutyl-*s*-triazine (XIII) is about three times more effective on the T_2 phage enzyme than the *E. coli* enzyme, but about the same with the pigeon liver enzyme.

The two compounds in this series showing greatest specificity toward inhibition of T_2 phage enzyme over *E. coli* enzyme are the 1-phenyl (IX) and 1-benzyl (X) derivatives where 23–33-fold differences in binding are noted (Table I).

Series B. Polar-Substituted 1-Phenyl Variants.—Strong evidence was previously presented from this laboratory[8] that the 1-phenyl group of the *s*-triazine IX (Table II) was complexed to pigeon liver dihydrofolic reductase by hydrophobic bonding. The main line of evidence for hydrophobic bonding was the large decrease in binding observed when the phenyl group was substituted by either a cationic group (XX) or an anionic group (XIV, XV); that this decrease was not due to steric reasons was shown by acetylation of XX to XXI and esterification of XIV to XVI which allowed increased binding by blocking the polar group. Other polar groups such as CN (XVII), C=O (XVIII, XIX), CH_2OH (XXII), and OCH_3 (XXIII) also led to loss in binding; no correlation with the Hammet σ constant was observed. As can be noted in Table II, similar results were obtained from the dihydrofolic reductases from *E. coli* B and induced by T_2 phage; thus the 1-phenyl group of IX is most probably complexed by hydrophobic bonding to dihydrofolic reductase from all three sources.

In no case was one of the compounds in Table II more effective on the *E. coli* B enzyme than the T_2 phage induced enzyme. The greatest spread in binding with T_2 enzyme and the *E. coli* B enzyme was 77-fold observed with the trifluoromethyl group (XXIV); most of this difference is due to the 21-fold stronger

hydrophobic bonding of the parent 1-phenyl-*s*-triazine (IX) to the T_2 enzyme. The largest spread in binding between the three enzymes was noted with the *p*-acetamidomethyl derivative (XXI) where 270-fold stronger bonding to the pigeon liver enzyme than the *E. coli* B enzyme was observed.

Series C. Nonpolar-Substituted 1-Phenyl Variants.—It was previously noted (Table III) that the *E. coli* B enzyme was relatively insensitive to substitution on the *meta* or *para* position of the phenyl group, the greatest spread being about 12-fold between XXXIII and XXVIII; in contrast, the pigeon liver enzyme was highly sensitive to substitution on the inhibitor in these positions, a 19,000-fold difference being observed between XXVI and XXXVI.[15] Larger spreads have now been observed with these two enzymes; the largest spread in binding on the *E. coli* B enzyme in Table III is 78-fold between XXXIII and XXXII, but the largest spread on the pigeon liver enzyme is 30,000-fold between XXXIV and XXVI. The largest spread in Table III with T_2 phage induced enzyme is 1400 between XXVI and XXXV (excepting XXXIX and XL which are special cases to be discussed below); the spread on T_2 phage induced enzyme is somewhat more similar to the *E. coli* B enzyme than the pigeon liver enzyme, thus indicating less sensitivity to substitutions on the 1-aryl group with the T_2 phage enzyme than the pigeon liver enzyme.

Substitution of the 1-phenyl group of IX with an in-plane *p*-phenyl group as in XXVI has practically a negligible effect on the *E. coli* B enzyme; in contrast, inhibition is decreased 1500-fold with the pigeon liver enzyme and 80-fold with the T_2 phage induced enzyme. Similar results were observed with the large, flat 9-fluorenone-2-yl group of XXIX. An interesting contrast is seen in binding with the in-plane *m*-phenyl group of XXVII; a 12-fold loss in binding compared to IX occurs with the pigeon liver enzyme, but a small (threefold) gain in binding with the *E. coli* B enzyme and a larger (13-fold) increase in binding with the T_2 phage enzyme is observed; a similar pattern was observed with the *m*-benzoyl substituent (XXVIII).

It was previously observed[15] that the *m*-benzyl (XXX) and *m*-phenethyl (XXXII) substituents could give additional bonding to the enzymes from both *E. coli* B and pigeon liver, but the *p*-benzyl gave an increment in binding only with the pigeon liver enzyme. Longer aralkyl substituents such as phenylpropyl and substituted phenylbutyls (XXXIV, XXXV) were observed to give even greater enhancement in binding to the pigeon liver enzyme.[25] The pattern with the *E. coli* B enzyme observed with XXXI and XXXII was followed with XXXIV and XXXV, that is, the *m*-phenylbutyl (XXXV) gave a good increment in binding, but the *para* substituent (XXXIV) did not. The T_2 phage induced enzyme gave the same pattern as the *E. coli* B enzyme, that is, good enhancement in binding by *meta* substituents (XXX, XXXII, XXXV), but poor enhancement by *para* substituents (XXXI, XXXIV). The main difference in binding between the *E. coli* B enzyme and the T_2 phage induced enzyme can again be accounted for by the 21-fold stronger hydrophobic bonding by the 1-phenyl group of IX with the latter enzyme.

Substitution of *p*-chloro group (XXXVII) on the 1-phenyl-*s*-triazine (IX) was previously noted to give a 2.4-fold decrease in binding to the pigeon liver enzyme[7,26] but a 7.5-fold increase in binding to the *E. coli* B enzyme.[26] The 4-chloro group (XXXVII) gave only a twofold increase in binding to the T_2 phage induced enzyme, thus being intermediary between the other two species.

Substitution of a 3-chloro group (XXXVI) on IX gave a fivefold enhancement in binding to the *E. coli* B enzyme and a 13-fold enhancement in binding to the pigeon liver enzyme; similarly, XXXVI was a sevenfold better inhibitor of the T_2 enzyme than IX. A similar pattern was observed in the comparison of the 3,4-dichlorophenyl (XXXVIII) with phenyl (IX). Again the main difference in binding to the *E. coli* B and T_2 enzymes was the stronger binding to the latter by the 1-phenyl group.

The placement of substituents on the *ortho* position of the 1-phenyl group (IX) is known to shift the phenyl group out of coplanarity with the triazine ring,[27] thus leading to a decrease in binding in the cases previously studied. For example, the 2-chloro group (XXXIX) causes a 1400-fold loss in binding to the pigeon liver enzyme compared to IX and a 67-fold loss in binding to the *E. coli* B enzyme. The T_2 phage induced enzyme was inhibited 180-fold less by the 2-chlorophenyl-*s*-triazine (XXIX) than by IX. Similar results were observed with the 2,6-dimethylphenyl-*s*-triazine (XL). Thus, little selectivity was shown by these *ortho*-substituted phenyl-*s*-triazines. The total binding is little different than the 1-methyl-*s*-triazine (II) (Table I), indicating that these noncoplanar phenyl groups make no contribution to binding to the enzyme.

The biggest spread in specificity between the three enzymes with the compounds in Table III is observed with compounds XXVII, XXXIII, and XXXIV. The *m*-biphenyl-*s*-triazine (XXVII) is uniquely effective on the T_2 phage induced enzyme, being 90–120-fold more effective than on the *E. coli* B or pigeon liver enzyme. Compounds XXXIII and XXXIV are 110–250-fold more effective on the pigeon liver enzyme than the *E. coli* B enzyme.

Series D. Variants at the 2 Position of the *s*-Triazine.—We have previously observed[15] that introduction of larger groups at the 2 position of the *s*-triazine was more detrimental to binding to the pigeon liver enzyme than the *E. coli* B enzyme (Table IV). For example, the 2-phenyl substituent of XLI (Table IV) compared to XXXVI (Table III) caused little change in binding to the *E. coli* B enzyme, but a 650-fold loss in binding to the pigeon liver enzyme. When the 2-phenyl group (XLI) was increased in size to *p*-acetamidophenyl (XLIII), a large loss in binding occurred with both enzymes. With the T_2 phage enzyme, introduction of the 2-phenyl group (XLI) gave a ninefold loss in binding compared to XXXVI (Table III) which is intermediary between the effect on the other two enzymes; again, the larger *p*-acetamidophenyl (XLIII) gave a large loss in binding. The

(25) B. R. Baker, B.-T. Ho, and G. J. Lourens, *J. Pharm. Sci.*, in press; paper LXXXVI of this series.

(26) B. R. Baker and B.-T. Ho, *ibid.*, **53**, 1137 (1964).

(27) E. J. Modest, *J. Org. Chem.*, **21**, 1 (1956).

2-benzyl substituent (XLII) gave a pattern similar to 2-phenyl (XLI).

When the 2-(*p*-acetamidophenyl) group (XLIV) was introduced on the 1-phenylbutyl-*s*-triazine (XIII, Table I), little change in binding was observed with the *E. coli* B enzyme, but a 15- and 73-fold loss, respectively, occurred with the pigeon liver enzyme and the T_2 enzyme; thus, the T_2 phage induced enzyme was affected the most by this structural change.

The considerable variation in patterns with 2-substituents suggests that synthesis of further analogs in this area followed by enzymic evaluation might be rewarding.

Series E. Trimethoprim and Deazapteridines.—In an elegant study, Burchall and Hitchings[16] compared trimethoprim (L) (Table V) and some alkyl-substituted 2,4-diamino-5-deazapteridines as inhibitors of dihydrofolic reductases from four species of mammalian liver and three species of bacteria; clear "inhibition profiles" were obtained that distinguished the mammalian enzymes from the bacterial enzymes. Through the generosity of Dr. Hitchings in supplying the compounds to us, we were able to obtain comparative "inhibition profiles" for the T_2 phage induced, *E. coli* B, and pigeon liver enzymes; the results are presented in Table V.

One of the most striking contrasts observed by Burchall and Hitchings[16b] was the wide spread of affinity of trimethoprim (L) for bacterial enzymes *vs.* mammalian enzymes; L was found to be 50,000–70,000 times more effective on *E. coli* dihydrofolic reductase than the dihydrofolic reductase from rabbit, rat, or human liver. Note that in our test system, trimethoprim (L) was 53,000 times more effective on *E. coli* B enzyme than the pigeon liver enzyme. Of high significance is the closer similarity in the binding of trimethoprim (L) to T_2 phage induced enzyme and pigeon liver enzyme; only a 23-fold difference in binding between these two enzymes was observed. In contrast, *E. coli* B enzyme was affected 2300 times more strongly than the T_2 phage enzyme. Thus, toward trimethoprim (L), the T_2 phage induced enzyme is much more like a vertebrate enzyme than a bacterial enzyme.

With the other four compounds in Table V, the biggest difference in binding to the *E. coli* B and T_2 enzymes is with the 6-benzyl-5-deazapteridine (XLVI); the latter was 34 times more effective on the *E. coli* B enzyme. None of the compounds in Table V was more effective on the T_2 phage induced enzyme than the *E. coli* B enzyme; similarly, none of the compounds in Table V was more effective on the pigeon liver enzyme than the *E. coli* B enzyme. Thus, with the compounds in Table V, T_2 phage induced and pigeon liver dihydrofolic reductases have closely similar "inhibition profiles," but the T_2 phage induced and *E. coli* B enzymes have different "profiles," particularly with trimethoprim.

Discussion

From the above results, it is clear that certain parts of the hydrophobic bonding region of T_2 phage induced dihydrofolic reductase is different from the hydrophobic bonding region of the dihydrofolic reductase from *E. coli* B. There are some close similarities of the T_2 phage enzyme to the *E. coli* B in some areas, but also close similarities to the pigeon liver in other areas of the hydrophobic bonding region. The greatest difference in selectivity of inhibition of the T_2 phage enzyme over the *E. coli* B enzyme was 70–90-fold with the 1-(*m*-trifluoromethylphenyl)-*s*-triazine (XXIV, Table II) and the 1-(*m*-benzylphenyl)-*s*-triazine (XXVII, Table III).

Now consider the T_2 phage infection of *E. coli* B as a model system for infection of a mammalian cell by a tumorigenic virus. The 70–90-fold difference in binding to the virus-induced enzyme with XXIV and XXVII would most probably not be a big enough spread for effective chemotherapy. Note that the effective antibacterial, trimethoprim (L) (Table V), has a 50,000–70,000-fold difference in binding between bacterial and mammalian enzymes;[16] also note that the analogous 1-benzyl-*s*-triazine (X, Table I) without the three methoxyl groups is actually complexed tenfold better to the liver enzyme than the *E. coli* B enzyme. Thus, it is in the realm of possibility that continued study of 1-substituted *s*-triazines or 5-substituted pyrimidines could lead to reversible inhibitors several thousand fold more effective on a virus-induced enzyme than the host cell enzyme. Furthermore, even small differences in binding to the hydrophobic bonding region of virus-induced and host cell enzymes should be greatly magnified with active-site-directed irreversible inhibitors.[3,13]

The answer to the first question posed at the beginning of this paper is therefore a promising "yes;" there are sufficient differences in the hydrophobic bonding region of the two enzymes in the model T_2 phage–*E. coli* B system to warrant extensive study with tumor enzymes, particularly if induced by tumorigenic viruses.

The possible origin of viral genetic particles has been a frequent topic for speculation at coffee breaks and cocktail hours. On the paleontological time scale it is estimated that vertebrates and yeast evolved from a common ancestoral form about 1200 million years ago;[28] since bacteria were initially anaerobic they probably evolved even earlier than yeast. The poor inhibition of T_2 phage induced enzyme, mammalian, and pigeon liver enzymes by trimethoprim (L) compared to bacterial enzymes gives strong evidence that the viral particle arose in a period of time when higher forms of life such as vertebrates had already arisen; the "inhibition profile" of the T_2 phage enzyme with the other compounds in Tables I–V is intermediate between *E. coli* B and pigeon liver "inhibition profiles," although there are distinctive differences in the T_2 enzyme from the other two species. Of an even more speculative nature at this stage of knowledge in molecular paleontology[14] is that the T_2 phage may have been produced by a higher form of life as a protective mechanism against *E. coli* infection. Studies on the linear sequence of dihydrofolic reductases from bacteria, vertebrates, and virus induction will someday be done as is being done on cytochrome *c*;[28] then one should be able to make better judgment as to when on the time scale a virus particle diverged from some particular form of life.

(28) E. Margoliash and A. Schejter, *Advan. Protein Chem.*, **21**, 191 (1966).