Chemical and Biological Studies on *1,2*-Dihydro-*s*-triazines. V. Inhibition of Derivatives of Pteroylglutamic Acid. (20478)

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Previous reports have described the non-competitive inhibition of pteroylglutamic acid (PGA) in *Streptococcus faecalis* No. 8043* systems by the dihydrotriazine hydrochlorides (1,2) and the reversal of this inhibition by dihydropteroylglutamic acid† (dihydro-PGA), N^{10}-formylpteroylglutamic acid† (N^{10}-formyl-PGA), leucovorin† and citrovorum factor† (CF)(3). The present report concerns the inhibition of these latter metabolites by appropriate concentrations of the dihydrotriazine hydrochlorides.

Methods. The media, methods of preparation and assay employed with *S. faecalis* were as described previously(2,3) except that minimal and excess concentrations of dihydro-PGA, N^{10}-formyl-PGA, leucovorin and CF were substituted for PGA in the respective assays. The medium described by Sauberlich and Bauman(4) was used for *Leuconostoc citrovorum* No. 8081‡ assays; otherwise methods were the same except that titrations were incubated for 48 hours. The dihydrotriazine hydrochlorides characterized by a 50% inhibition index of less than 100 vs PGA(3) were titrated in these systems and the 50% inhibition indices(5) computed.

Results. The calculated 50% inhibition indices of representative compounds vs these metabolites are compared with those obtained vs PGA in *S. faecalis* and vs CF in *L. citrovorum* assay systems in Table I. The inhibition of reduced or formylated PGA as well as CF could be accomplished in *S. faecalis* systems only by considerably higher inhibitor: metabolite ratios than those which were effective vs PGA. The 2,2-dimethyl-substituted derivatives in general were more active than the analogous 2-mono-substituted compounds. The unsubstituted phenyl (D-23 • HCl) and 3′-chlorophenyl (D-69 • HCl) compounds exhibited greatest activity vs CF in both microbiological systems. Halogen substitution at the 4′- and 3′,4′-positions of the phenyl ring had little effect on activity vs dihydro-PGA or N^{10}-formyl-PGA but resulted in decreased activity vs CF in either system as illustrated by a comparison of the activity of compounds D-23 • HCl, D-20 • HCl, and D-54 • HCl§ while methyl substitution at the *4′*-position of the phenyl ring (D-83 • HCl) resulted in a marked decrease in activity vs dihydro-PGA as well as CF (Table I). These relationships between structure and biological activity vs these metabolites will be considered elsewhere in greater detail.

The inhibition indices obtained with a number of derivatives in preliminary titrations suggested competitive inhibition. However, when such compounds were titrated vs a wider range of concentration the inhibition indices shifted with increasing concentrations of metabolites, indicating that competitive inhibition obtains only over a relatively limited range of metabolite concentrations (Table II). This range varied with different derivatives, but in no instance did it exceed a 5-10 fold difference in concentration. The inhibition of CF in *L. citrovorum* assay systems appeared to be non-competitive over the range of metabolite concentrations noted in Table II.

Discussion. The inhibitory activity of this series of dihydrotriazines vs dihydro-PGA, N^{10}-formyl-PGA and CF resembles that exhibited vs PGA(3) in that a competitive in-

* A.T.C.C. No. 8043.

† We wish to thank the Lederle Laboratories, Pearl River, N. Y., for generous samples of these compounds.

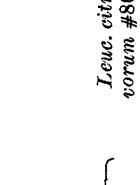
Another sample of dihydro-PGA synthesized in these laboratories by Dr. E. J. Modest also was used in these studies.

‡ A.T.C.C. No. 8081.

§ Two samples of this compound were used in these studies, one synthesized in these laboratories by Dr. E. J. Modest and another supplied through the courtesy of Imperial Chemical Industries, Ltd., Manchester, England.

TABLE I. Comparative Activity of Dihydrotriazine Hydrochlorides vs. PGA, Dihydro-PGA, N^{10}-Formyl-PGA and CF.

Structure



Compound	R_1	R_2	R_3	50% Inhibition index *Strep. faecalis* #8043 PGA* .01	Dihydro-PGA .001	N^{10}-formyl-PGA .001	CF† .001	*Leuc. citrovorum* #8081 CF† .001
D-23 • HCl	phenyl	methyl	methyl	10	800	820	2500	9200
D-69 • HCl	3'-chlorophenyl	"	"	32.5	3000	860	2000	9370
D-83 • HCl	4'-methylphenyl	"	"	40	22000	860	114000	180000
D-20 • HCl	4'-chlorophenyl	"	"	9.5	800	740	10500	20000
D-84 • HCl	"	H	*n*-hexyl	45	15000	3600	48000	116000
D-54 • HCl‡	3',4'-chlorophenyl	methyl	methyl	10	760	390	11600	22000
D-75 • HCl	"	"	ethyl	37.5	2000	1800	200000	187000
D-76 • HCl	"	H	*n*-propyl	95.0	60000	6200	200000	184000

Concentration of metabolites in γ/ml. * See bibliography #1 and 2. † Similar results with leucovorin. ‡ Similar results with sample supplied by Imperial Chemical Industries, Ltd.

TABLE II. Inhibition Indices of Dihydrotriazine Hydrochlorides vs. Increasing Concentrations of Metabolites.

Compound	50% Inhibition index *Streptococcus faecalis* #8043			
	Dihydro-PGA, γ/ml			
	.001	.01	.1	1.0
D-20 • HCl	800	1200	100	12
D-84 • HCl	1100	460	55	5
	N^{10}-formyl-PGA, γ/ml			
	.0001	.001	.01	.1
D-23 • HCl	920	820	340	42
D-69 • HCl	550	860	400	42
	Citrovorum factor, γ/ml*			
	.00005	.0005	.001	.002
D-69 • HCl	2000	10800	9370	18750
D-84 • HCl	76000	52000	48000	25000

* Similar results with leucovorin.

hibitor: metabolite relationship obtained only over a relatively narrow range of metabolite concentrations. Although this range was broader than that observed vs PGA, increasing concentrations of dihydro-PGA, N^{10}-formyl-PGA, and CF resulted in a marked shift in the inhibition index. Similar changes in the inhibition index with increasing concentrations of metabolite have been observed with 4-aminopteroylglutamic acid(6). These dihydrotriazines then are not typical examples of competitive inhibitors according to classical definition(7); thus the present results are interpreted to indicate that the inhibition of dihydro-PGA, N^{10}-formyl-PGA and CF by these dihydrotriazines is essentially non-competitive.

The decreased effectiveness of the inhibition of dihydro-PGA and N^{10}-formyl-PGA as compared to PGA is further indication that these compounds interfere with the biological conversion of PGA to CF in *S. faecalis* systems. The similarity in the response of either CF system to the compounds in this series suggests that the mechanism of utilization and inhibition of CF may the same in *S. faecalis* and *L. citrovorum*. However, the marked increase in the inhibition indices vs CF as compared to PGA, dihydro-PGA and N^{10}-formyl-PGA suggests that the inhibition of CF systems may involve a different mechanism of action, *i.e.*, interference with the utilization of CF rather than inhibition of the synthesis of

CF in PGA systems. This possible difference in mechanism of action may be of significance in the interpretation of the effects of the dihydrotriazines in other biological systems.

Studies with several of the early compounds in this series failed to demonstrate microbiological activity in systems in which thymine was substituted for PGA(1). However, experiments now in progress indicate that a number of derivatives synthesized more recently in these laboratories may exhibit significant anti-thymine activity. Details must await completion of current experiments.

Summary. Dihydropteroylglutamic acid, N^{10}-formylpteroylglutamic acid and citrovorum factor are inhibited non-competitively by a number of *1,2*-dihydro-*s*-triazines when substituted for pteroylglutamic acid in *S. faecalis* No. 8043 assay systems, but only by inhibitor:metabolite ratios considerably higher than those required for the inhibition of pteroylglutamic acid in the same microbiological system. Citrovorum factor also is similarly inhibited in *L. citrovorum* No. 8081 assay systems. The phenyl- and 3'- and 4'-halophenyl-2,2-dimethyl derivatives were the most active compounds of the series and certain correlations can be made between structure and microbiological activity vs these metabolites.

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Chemical and Biological Studies on *1,2*-Dihydro-*s*-Triazines. VI. Development of Resistance by *Streptococcus faecalis* No. 8043. (20479)

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The development of resistance to various analogs of pteroylglutamic acid (PGA) has been described in human leukemia(1) and in experimental mouse leukemia(2-8). Resistance to these chemotherapeutic agents also has been reported as occurring in *Tetrahymena geleii* by Kidder, Dewey, and Parks(9), in *Streptococcus faecalis* No. 8043 by Foley(10), and more recently by Burchenal, Waring, and Hutchison(11).

Previous publications from these laboratories have described the non-competitive inhibition of *S. faecalis* No. 8043* in PGA assay systems by another class of antagonists, the *1,2*-dihydro-*s*-triazines(12,13). The purpose of the present report is to describe experiments dealing with the isolation and origin of a number of strains of *S. faecalis* No. 8043 which are resistant to inhibition in PGA assay systems by these compounds. Media, methods of preparation and assay were identical with those previously described(12,13) unless otherwise stated.

* A.T.T.C. No. 8043.

Isolation of resistant strains. When *S. faecalis*-PGA assay systems containing inhibiting concentrations of the dihydrotriazine hydrochlorides were incubated beyond the usual 18-24 hour period, growth occurred after a prolonged lag phase. Thus the minimal inhibiting concentration of 4,6-diamino-1-(3',4'-dichlorophenyl)-1,2-dihydro-2,2-dimeth-