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CHEMICAL AND BIOLOGICAL STUDIES ON 1:2-DIHYDRO-*s*-TRIAZINES—XIII

INHIBITION OF RESPONSE TO *p*-AMINOBENZOIC ACID AND MECHANISM OF ACTION IN *LACTOBACILLUS ARABINOSUS* BIOASSAY SYSTEMS*

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Abstract—A series of 1:2-dihydro-*s*-triazines has been studied in *Lactobacillus arabinosus* 17–5 (ATCC no. 8014)–*p*-aminobenzoic acid bioassay systems. Differences in biological activity within the series could be correlated with certain variations in structure and substitution in the molecule. Maximum activity was observed both with 2:2-dimethyl substitution in the triazine ring together with 3′-halogen substitution in the phenyl ring, and with *n*-hexyl to *n*-tridecyl substitution at the 2-position of the triazine ring, irrespective of *p*- or *m*-phenyl substitution.

The active derivatives exhibited synergism with both sulphadiazine and aminopterin. Similarly, synergism between sulphadiazine and aminopterin was observed in this bioassay system. Inhibition analyses indicate that these three classes of inhibitor differ with respect to loci of activity.

In conjunction with previous evidence, the data suggest that in this *L. arabinosus*—PABA system, the 1:2-dihydro-*s*-triazines interfere with a diphosphopyridine nucleotide-mediated reduction of the pteroylglutamic acid (or pteroylglutamic acid-like) intermediate synthesized from *p*-aminobenzoic acid in the conversion of *p*-aminobenzoic acid to structures exhibiting citrovorum factor activity.

INTRODUCTION

PREVIOUS reports from these laboratories have described the synthesis[1–3] and the microbiological[4–8] and anti-tumour[9, 10] activity of a series of 1:2-dihydro-*s*-triazines (D·HCl's). Various derivatives of this series also have been shown to exhibit significant biological activity in experimental *Plasmodium*[1, 11] and *Eimeria*[12] infections, mammalian liver[13] and botanical systems,[14] experimental murine toxoplasmosis,[15] and in tissue culture.[16]

Previous studies have indicated that the primary mechanism of action of these compounds in microbiological systems is the inhibition of a diphosphopryidine nucleotide (DPN)-dependent reduction of pteroylglutamic acid (PGA) in the synthesis of structures exhibiting citrovorum factor (CF) activity.[17, 18] During the course of these investigations, it was observed that certain dihydrotriazines also inhibited the

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response of *Lactobacillus arabinosus* to *p*-aminobenzoic acid (PABA).[7] The present report is concerned with the results of studies originating from this observation.

METHODS

A bioassay system based upon the response of *Lactobacillus arabinosus* (17–5) no. 8014* to PABA[19, 20] was used in these experiments. Past experience indicated that the usual media prepared from hydrolysed casein were not entirely satisfactory, as judged by the results of routine replicate PABA titrations done over periods of several weeks. Inherent differences in the hydrolysis and purification by charcoal adsorption of crude caseins[21] have undoubtedly been a source of difficulty, since the response of *Lactobacillus arabinosus* to PABA has been reported to vary with the type of casein hydrolysate used.[22] Similarly, the presence of purine bases in the medium results in the growth of *Lactobacillus arabinosus* in the absence of PABA.[23]

The medium described by Koft *et al.*[24], with minor modifications, yields reproducible optical densities ($\times$ 10) of from 4·0 to 6·0 from minimal inocula of the stock strain of *Lactobacillus arabinosus* in the presence of PABA, and no growth in its absence. Bacto-vitamin-free casamino acids (Difco), 200 g/l., treated at pH 3·5 with 20 g/l. of Darco no. S-51 for 5 min at 25°C and adjusted to pH 5·8 with 40% NaOH,[21] were substituted for the usual casein hydrolysate at 0·5% final concentration. The L-tryptophan was treated similarly. The proper purification by adsorption of these ingredients is critical; too little treatment results in growth in the absence of PABA while over-adsorption results in growth failure, even in the presence of PABA or PGA. Since the addition of purine bases to the modified medium obviated the need for PABA, purines (and pyrimidines) were omitted. The glucose and phosphates were autoclaved as a separate solution, and the modified medium was otherwise identical with that described by Koft *et al.*[24]

All dilutions were made with double glass-distilled water in chemically clean glassware. The various solutions constituting the basal medium were autoclaved for 5 min at 15 lb/in^2 pressure, combined aseptically and dispensed in 10 ml volumes in optically standardized 22 $\times$ 175 mm tubes, which were closed with loose-fitting aluminium caps.

Crystalline PABA and other metabolites, the various D·HCl's, and the other inhibitors used were prepared in appropriate concentrations in sterile double glass-distilled water, and were added aseptically in volumes of 0·1 ml to the cooled, sterile medium to the desired final concentration.

Inocula were prepared daily from viable stock cultures of *Lactobacillus arabinosus* maintained on 5% horse blood agar plates. The surface colonies were emulsified in sterile, physiologic saline, and the suspension of bacteria was adjusted to an optical density of approximately 0·05 $\times$ 10; 0·1 ml of the suspension was added to each 10 ml tube of media. All titrations were incubated for 48 hr at 37 °C. Optical density was read directly in a Lumetron colorimeter adjusted so that medium blanks read zero vs. the 650 mμ filter.

The synthetic medium *C* described by Dunn *et al.*,[25] without purines, also was used for certain experiments on the growth response to PABA. It is of interest to note that unlike the results obtained by others,[26] L-methionine did not satisfy the PABA require-

*American type culture collection number.

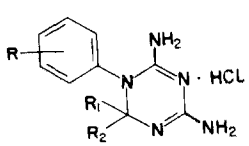
ment of the strain of *Lactobacillus arabinosus* used in these experiments. Apparently at least two varieties of *Lactobacillus arabinosus* 17–5 exist, a PABA-requiring and a PABA-independent strain, which are otherwise identical;[27, 28] however, it is evident that the strain used in the present studies possesses a clear-cut PABA requirement when cultured in an appropriate medium.

The D·HCl's and other inhibitors used were titrated from 1 mg/ml to the minimal inhibiting dose in the presence of 0·1, 0·01 or 0·001 μg of PABA per ml, the last concentration being the minimal one which supported maximal growth under the present experimental conditions. The 50 per cent minimal inhibiting dose[29] for excess and minimal concentrations of PABA was interpolated from semi-log plots of the data obtained from such titrations. Inhibition analyses[30] were done in systems containing 0·001 μg of PABA per ml, to which were added the minimal (or multiples of) concentrations of the respective D·HCl's that effected 50 per cent inhibition of growth, as determined by previous titration. Various metabolites were then titrated in these inhibited systems to determine their ability to reverse inhibition.

Structure–activity relationships

Halogen substitution. Chloro- or bromo-substitution at the 3′-position of the phenyl ring resulted in increased activity as compared with the unsubstituted phenyl derivatives (Table 1). Unlike the results obtained in *Streptococcus faecalis*–PGA bioassay systems,[4] di-halogen substitution did not result in a further increase in activity beyond that characteristic of the compounds bearing a halogen at the 3′-position of the phenyl ring, e.g. D-69·HCl and D-54·HCl, Table 1. Those derivatives with asymmetrical substitution at the 2-position of the triazine ring (Table 1) were generally less active than the corresponding 2:2-dimethyl-substituted compounds.

TABLE 1. INHIBITORY ACTIVITY OF 1:2-DIHYDRO-*s*-TRIAZINES IN *p*-AMINOBENZOIC ACID BIOASSAY SYSTEMS



Compound no.	Substitution			50% inhibition index ($\times 10^{-3}$) *vs.* *Lactobacillus arabinosus*, PABA, 0·001 μg/ml
	R	R_1	R_2	
D-23·HCl	H	CH_3	CH_3	2·7
D-69·HCl	3′-Cl	CH_3	CH_3	0·2
D-91·HCl	3′-Br	CH_3	CH_3	0·25
D-20·HCl	4′-Cl	CH_3	CH_3	2·7
D-65·HCl	4′-Br	CH_3	CH_3	2·5
D-54·HCl	3′ : 4′-Cl_2	CH_3	CH_3	0·24
D-66·HCl	H	CH_3	C_2H_5	100+
D-114·HCl	3′-Cl	H	C_2H_5	3·5
D-130·HCl	3′-Br	H	C_2H_5	3·2
D-43·HCl	4′-Cl	CH_3	C_2H_5	21·0
D-75·HCl	3′ : 4′-Cl_2	CH_3	C_2H_5	2·8
D-67·HCl	3′ : 4′-Cl_2	H	CH_3	2·0

Structural alterations resulting in loss of activity. The increased activity resulting from *m*-substitution in the phenyl ring is further emphasized by comparison with the corresponding *p*- and *o*-derivatives of the halogen-substituted series (Table 2). Substi-

TABLE 2. EFFECT OF PHENYL SUBSTITUTION ON ACTIVITY OF 1:2-DIHYDRO-2:2-DIMETHYL-*s*-TRIAZINES* IN *p*-AMINOBENZOIC ACID BIOASSAY SYSTEMS

Compound no.	Substitution*† R	50% inhibition index ($\times 10^{-3}$) *vs.* *Lactobacillus arabinosus*, PABA, 0·001 μg/ml
D-102·HCl	4′-F	3·0
D-103·HCl	2′-F	100·0+
D-83·HCl	4′-CH_3	1·8
D-93·HCl	3′-CH_3	0·3
D-38·HCl	2′-CH_3	100·0+
D-92·HCl	2′ : 6′-$(CH_3)_2$	100·0+
D-20·HCl	4′-Cl	2·7
D-69·HCl	3′-Cl	0·2
D-68·HCl	2′-Cl	15·0
D-96·HCl	4′-OCH_3	3·5
D-98·HCl	2′-OCH_3	100·0+
D-65·HCl	4′-Br	2·5
D-91·HCl	3′-Br	0·25
D-82·HCl	2′-Br	100·0+
D-100·HCl	4′-I	4·5
D-128·HCl	3′-I	0·52

*See Table 1 for structure.
†In this series, $R_1 = R_2 = CH_3$.

tution at the *o*-position of the phenyl ring or the presence of a large blocking group at the 2-position of the triazine ring resulted in a marked decrease in activity (Table 2), as observed in other bioassay systems.[4–7] Similarly, the anilinodihydrotriazines prepared by isomerization of the corresponding D·HCl derivatives[1, 2] were relatively inactive, as was the case in other bioassay systems.[4–7]

Effect of 2-(n-*alkyl*) *substitution.* As illustrated in Table 3, activity increases markedly as the number of carbon atoms in *n*-alkyl configuration at the 2-position of the triazine ring increases beyond *n*-butyl or *n*-pentyl. Maximum activity is obtained when the substituent at the 2-position is between *n*-hexyl and *n*-tridecyl, irrespective of substitution in the phenyl ring (Table 3). This increase in activity with increasing length of the carbon chain at the 2-position of the triazine ring differs from that observed with *Streptococcus faecalis* and *Pediococcus cerevisiae*[31] (formerly termed *Leuconostoc citrovorum*) only in that, in these latter bioassay systems, maximum activity was more sharply defined between the *n*-hexyl and *n*-decyl derivatives.

The inhibition induced by this series of compounds is essentially non-competitive with respect to PABA, as well as to PGA and its derivatives in this, as in other[4–7] bioassay systems.

TABLE 3. EFFECT OF 2-(*n*-ALKYL) SUBSTITUTION AT 2-POSITION OF TRIAZINE RING ON ACTIVITY OF 1:2-DIHYDRO-*s*-TRIAZINES IN *p*-AMINOBENZOIC ACID BIOASSAY SYSTEMS

Compound no.	Substitution*		50% inhibition index ($\times 10^{-3}$) *vs.* *Lactobacillus arabinosus*, PABA, 0·001 μg/ml
	R_1	R_2	
R=H			
D-133·HCl	H	C_2H_5	3·2
D-136·HCl	H	n-C_4H_9	3·8
D-134·HCl	H	n-C_5H_{11}	0·35
D-113·HCl	H	n-C_6H_{13}	0·35
D-111·HCl	H	n-$C_{11}H_{23}$	0·01
D-121·HCl	H	n-$C_{13}H_{27}$	0·03
D-112·HCl	H	n-$C_{17}H_{35}$	3.0
R=3′-Br			
D-130·HCl	H	C_2H_5	3.2
D-131·HCl	H	n-C_5H_{11}	0·35
D-124·HCl	H	n-C_6H_{13}	0·3
D-122·HCl	H	n-$C_{11}H_{23}$	0·025
D-139·HCl	H	n-$C_{13}H_{27}$	0·03
R=3′-Cl			
D-114·HCl	H	C_2H_5	3·5
D-126·HCl	H	n-C_5H_{11}	0·23
D-110·HCl	H	n-C_6H_{13}	0·23
D-109·HCl	H	n-$C_{11}H_{23}$	0·031
R=4′-Cl			
D-45·HCl	H	CH_3	10·0
D-60·HCl	H	C_2H_5	12·1
D-48·HCl	H	n-C_3H_7	7·5
D-87·HCl	CH_3	n-C_3H_7	3.7
D-84·HCl	H	n-C_6H_{13}	2·5
D-127·HCl	H	n-C_9H_{19}	0·023
D-106·HCl	H	n-$C_{11}H_{23}$	0·016
R=3′ : 4′-Cl_2			
D-67·HCl	H	CH_3	2·0
D-63·HCl	H	C_2H_5	3·2
D-76·HCl	H	n-C_3H_7	3·1
D-123·HCl	H	n-C_4H_9	2·9
D-78·HCl	H	n-C_5H_{11}	3·0
D-95·HCl	H	n-C_6H_{13}	2·8
D-117·HCl	H	nC_7H_{15}	0·3
D-118·HCl	H	n-C_8H_{17}	0·031
D-115·HCl	H	n-C_9H_{19}	0·029
D-116·HCl	H	n-$C_{10}H_{21}$	0·025
D-101·HCl	H	n-$C_{11}H_{23}$	0·035
D-108·HCl	H	n-$C_{13}H_{27}$	0·02

*See Table 1 for structure.

Mechanism of action

Reversal. The minimal concentrations of PGA and of certain of its derivatives which support maximal growth of *Lactobacillus arabinosus* under the present experimental conditions are compared with PABA in Table 4. The effect of the addition of these and other metabolites to systems inhibited by the D·HCl's is also illustrated in Table 4.

TABLE 4. REVERSAL OF INHIBITION IN *Lactobacillus arabinosus–p*-AMINOBENZOIC ACID BIOASSAY SYSTEMS: COMPARISON OF 1:2-DIHYDRO *s*-TRIAZINES, SULPHADIAZINE AND AMINOPTERIN

Metabolite*	Minimal effective concentration for maximal growth (μg/ml)	Reversal of inhibition in the presence of PABA (0·001 μg/ml)			
		Conc. of reversing agent (μg/ml)	Inhibitor†		
			D-54·HCl (0·24 μg/ml)	Sulphadiazine (0·6–1·0 μg/ml)	Aminopterin (0·02–0·03 μg/ml)
7:8-Dihydropteroylglutamic acid	0·001	0·1	Complete‡	Complete‡	Complete‡
Natural citrovorum factor	0·001	0·005	Complete	Complete	Complete
Thymidine	0·1	0·1	Complete	Complete	Complete
Diphosphopyridine nucleotide	§	0·1	Complete	None	None
Nicotinic acid	§	1·0	Complete	None	None
Nicotinamide	§	1·0	Complete	None	None
p-Aminobenzoic acid	0·001	1·0–10·0	Partial**	Complete	Complete
Pteroylglutamic acid	0·1	10·0+	Partial	Complete	Complete
^{10}N-Formylpteroylglutamic acid	0·01	10·0+	Partial	Complete	Complete
Ascorbic acid	§	2500	Complete	Partial**††	None
Vitamin B_{12}	§	100	Complete	None	None

*Other growth factors without effect as reversing agents except purines, which eliminate PABA requirement of *L. arabinosus*.
†All inhibitors employed at the 50 per cent minimal inhibiting dose (50 per cent MID) and multiples thereof. Results similar to those reported with D-54·HCl obtained with several other dihydrotriazines at their 50 per cent MID and multiples thereof (cf. Tables 1–3).
‡Effective against as much as 4-10 × 50 per cent MID of inhibitor.
§Employed as reversing agent only; will not support growth in the absence of purines or the other metabolites listed.
**Effective against only 1-3 × 50 per cent MID of inhibitor.
††L-Cysteine exhibits similar activity; other biological reducing agents ineffective.

7:8-Dihydro-PGA,*[32] natural CF,†[33] thymidine or DPN‡ are more effective in the reversal of inhibition than is either PABA or unaltered PGA, even when the latter are provided in excess of the concentrations required for maximal growth in uninhibited systems. Similarly, ^{10}N-formyl-PGA†[34] is no more effective than unaltered PGA. Reversal by reduced, but not unaltered or formylated PGA, together with reversal by DPN or its precursors, suggests that in this system the D·HCl's interfere with the DPN-mediated reduction of PGA, as has been described in other microbiological systems.[8, 17, 18]

The effects of ascorbic acid and vitamin B_{12} (Table 4) also are similar to those observed in other microbiological systems inhibited by the D·HCl's.[8, 17, 18] The effectiveness of ascorbic acid in reversing inhibition is probably the result of physicochemical alteration of the redox potential, as appeared to be the case previously,[8, 17, 18] rather than a specific metabolite response. Neither ascorbic acid nor other biological reducing agents satisfy the nicotinic acid requirement of *Lactobacillus arabinosus*. The observation that vitamin B_{12} exhibits some activity, particularly when the medium contains either reduced PGA or CF, again suggests[8, 17, 18] an intimate relationship between the metabolism of vitamin B_{12} and either the synthesis or the utilization of structures exhibiting CF activity, or both. That DPN (or a suitable precursor) is essential to the cell for purposes other than the synthesis of CF is evident from the inability of reduced PGA or preformed CF to support the growth of *Lactobacillus arabinosus* in the absence of DPN.

Inhibition of *Hemophilus parainfluenzae* in the DPN-bioassay systems by the D·HCl's and reversal of inhibition by DPN have been reported.[17] This inhibition has been shown to be competitive over at least a 100-fold range of concentration of DPN.§ The results of other experiments with *Lactobacillus arabinosus*, wherein DPN and its precursors were titrated in the presence of 0·001 μg of PABA per ml, indicate a competitive relationship between the D·HCl's and DPN over at least a tenfold range of concentration, whereas the inhibition of nicotinic acid, nicotinamide, PABA and PGA is noncompetitive (Table 5). Inhibition of DPN by the D·HCl's in either bioassay system was readily reversed by excess DPN or its precursors, whereas inhibition induced by 4-APGA or sulpha in similar experiments was not so reversed (Table 4).

The possible argument that the adenine component of DPN might be released and utilized under the experimental conditions of *Lactobacillus arabinosus* assay is considered to be invalid. Reversal of D-54·HCl inhibition by DPN (0·1 μg/ml) (Table 4) cannot be due to liberated adenine inasmuch as there is insufficient purine present in that quantity of DPN to support growth, even if there were complete cleavage of the adenine component. In addition, neither DPN nor its precursors, nicotinic acid or

*Synthesized by one of us (E.J.M.), after O'Dell *et al.*[32]

†We wish to express our appreciation to the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, for generous samples of these compounds. Although none of the 4-hydroxypteridines used in the reversal studies was tested for chromatographic purity, each was shown to be 95-100 per cent pure by ultra-violet spectral analysis. This evidence, together with a careful analysis of the reversal data in Table 4, precludes erroneous conclusions due to the possible presence of small amounts of PABA or *p*-aminobenzoylglutamic acid in the pteridine samples used.

‡Pabst Laboratories, Milwaukee, Wisconsin, purity 96 per cent. Pabst TPN (purity 100 per cent) was but slightly effective (less than 10 per cent) in reversing D·HCl inhibition in these systems.

§In *Hemophilus parainfluenzae*–DPN dependent systems, full growth is obtained at concentrations of 0·001, 0·01 and 0·1 μg/ml of DPN. At these concentrations, for example, inhibition indices of 1000, 1000 and 900, respectively, were obtained with D-95·HCl. For experimental details, cf. ref. 17. These studies will be published in detail in another paper.

nicotinamide, will support the growth of *Lactobacillus arabinosus* in the absence of purines, PABA, PGA or its derivatives (Table 4). Although purines obviate the requirement for PABA in the present bioassay system, they do not replace the specific requirement of *Lactobacillus arabinosus* for either nicotinic acid, nicotinamide or DPN, even in the presence of PABA, PGA or its derivatives. Furthermore, other assay media (e.g. Difco) designed specifically for the assay of nicotinic acid or its derivatives contain both purines and PABA. Neither excess adenine nor guanine reverses D·HCl inhibition in any of the microbiological systems studied in these laboratories,[4–8, 17] whereas D·HCl inhibition is readily reversed by nicotinic acid, nicotinamide or DPN in other microbiological systems[7, 8, 17, 18] as well as in *Lactobacillus arabinosus*–PABA assay systems (Table 4).

TABLE 5. FIFTY PER CENT INHIBITION INDICES FOR D-69·HCl* IN *Lactobacillus arabinosus*–PABA ASSAY SYSTEMS

Metabolite	Concentration (μg/ml)					
	0·001	0·01	0·1	1·0	10·0	100·0
DPN†			3·5	0·65	0·08§	0·08§
Nicotinic acid†	150	30§	2·9§	0·29§	0·02§	toxic
Nicotinamide†	150§	22§	2·5§	0·30§	0·032	toxic
PABA‡	180§	55§	31§	3·0§	0·6§	toxic
PGA‡	200§	63§	29§	4·2§	1·5§	toxic

*Similar results obtained with several other active D·HCl compounds (cf. Tables 1–3).

†In presence of 0·001 μg of PABA per ml. Does not support growth in absence of PABA, or PGA or its derivatives.

‡In presence of 2·0 μg of nicotinic acid per ml. Does not support growth in absence of nicotinic acid, nicotinamide or DPN.

§Maximum growth obtained at these concentrations of metabolite in absence of inhibitor.

Comparison with 4-APGA and sulpha. The pattern of reversal of 4-APGA differs from that of D·HCl inhibition, as in other bioassay systems.[5, 6, 17, 18] 4-APGA is an effective inhibitor in this *Lactobacillus arabinosus*–PABA bioassay system, 0·02–0·03 μg/ml inducing 50 per cent inhibition. Inhibition is completely reversed by PABA, PGA or its derivatives, adenine, guanine or thymidine, poorly by thymine, and not at all by various biological reducing agents, vitamin B_{12}, DPN or its precursors (Table 4).

Sulpha is a less potent inhibitor than 4-APGA, about 0·6–1·0 μg/ml being needed for 50 per cent inhibition. The pattern of reversal of sulpha inhibition resembles that of 4-APGA (Table 4), except that high concentrations of ascorbic acid or L-cysteine are partially effective. DPN and its precursors, however, were ineffective, in contrast to the reversal of the D·HCl's by these metabolites.

Considerable experimental evidence indicates that PABA serves as a precursor of a substance (or substances) exhibiting CF activity[35] which may differ in certain respects from natural CF,[33] and that PGA or PGA-like substances may be intermediate in the biosynthesis of CF activity by *Lactobacillus arabinosus*.[36, 37] Differences in the inhibitory effects of sulphanilamide and 4-APGA upon this biosynthesis have been reported by Mitbander and Sreenivasin,[35] who observed that sulphanilamide was the more effective inhibitor when PABA was the precursor, whereas 4-APGA was more

effective when PGA was the precursor of CF activity in resting cell preparations of *Lactobacillus arabinosus*. These differences in the effectiveness of sulpha and 4-APGA also have been observed in the present studies.

Similarly, sulpha and D·HCl inhibition differ when PABA and PGA are utilized as precursors of CF activity (Fig. 1). Sulpha is most effective against PABA, while the

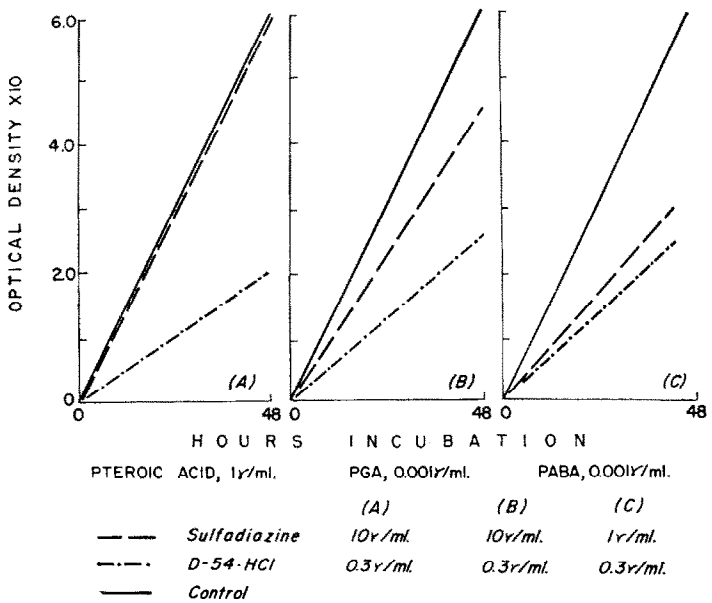


FIG. 1. Relative effectiveness of sulphadiazine and 1:2-dihydro-*s*-triazine (D-54·HCl)* *vs.* different precursors of citrovorum factor activity in *Lactobacillus arabinosus* systems.

**cf.* Table 1 for structure.

D·HCl's are equally effective against PABA or PGA. This difference in relative activity is also observed when pteroic acid* is used as a precursor. The response of *Lactobacillus arabinosus* to pteroic acid is unaffected by sulphonamides,[38] while the D·HCl's are as active as when either PABA or PGA is the precursor (Fig. 1). Similar results have been obtained with certain other synthetic substances which exhibit PGA-like activity in appropriate microbiological systems.[39]

Synergism with sulpha and 4-APGA. Different loci of inhibition by sulpha and the D·HCl's are further evidenced by synergistic activity, as illustrated in Fig. 2 (A). In this method of graphic analysis,[40, 41] fractions of the 50 per cent minimal inhibiting concentration of one inhibitor are titrated in the presence of several different fractions of the 50 per cent minimal inhibiting concentration of the second inhibitor and the various fractional combinations required for 50 per cent inhibition determined. The concentrations so obtained are then plotted as fractional values of unity as represented by the 50 per cent minimal inhibiting concentration of each inhibitor on one axis. In the case of potentiation, the resulting figure is displaced to the left, the extent of deviation representing a measure of synergism, as illustrated in Fig. 2 (A). It is of

*See second footnote on p. 24.

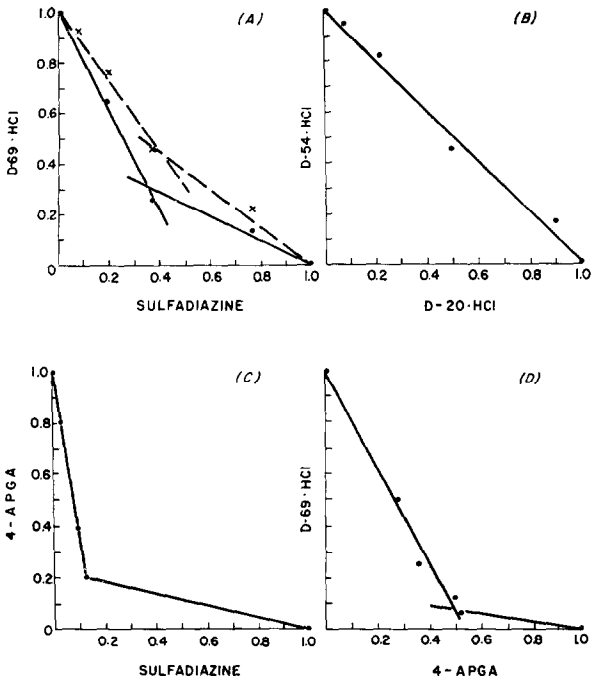
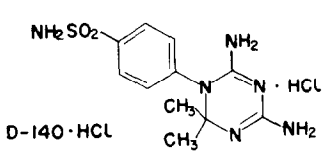


FIG. 2. Effect of combinations of inhibitors in *Lactobacillus arabinosus*—*p*-aminobenzoic acid systems (A) 1:2-dihydro-*s*-triazine (D-69·HCl)* and sulphadiazine (————); 1:2-dihydro-*s*-triazine (D-122·HCl)* and sulphadiazine (— — — —). (B) Two 1:2-dihydro-*s*-triazines (D-54·HCl, D-20·HCl)*. (C) 4-Aminopteroylglutamic acid and sulphadiazine. (D) 4-Aminopteroylglutamic acid and 1:2-dihydro-*s*-triazine (D-69·HCl)*.

**cf.* Table 1 for structures.

interest to note that despite the synergism exhibited by combinations of D·HCl and sulpha, a compound (D-140·HCl)* incorporating the structural features of sulphanilamide into the D·HCl molecule:



was inactive in *Lactobacillus arabinosus*–PABA systems. On the other hand, additive effects of combinations representing activity at a single locus result in a straight line with a slope of 45°, as illustrated in Fig. 2 (B). Similar analyses of combinations of sulpha with 4-APGA (Fig. 2 (C)) and D·HCl with 4-APGA (Fig. 2 (D)) also indicate

*The synthesis of this and the other previously undescribed dihydrotriazines considered here will be reported elsewhere.

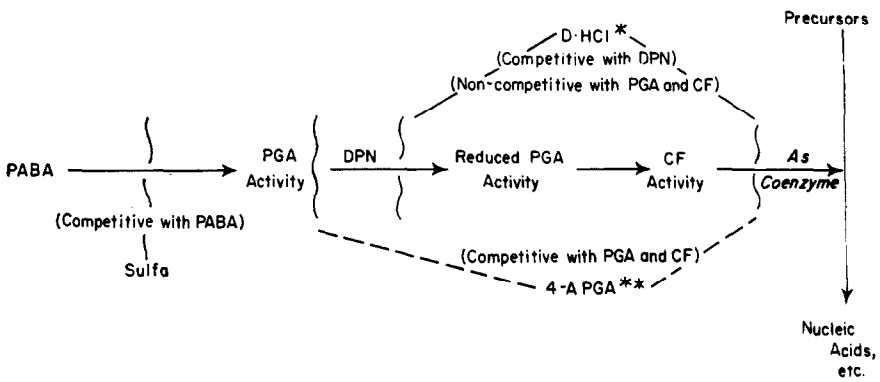
potentiation, and, accordingly, different loci of activity for 4-APGA, sulpha and D·HCl inhibition.

DISCUSSION

The results of inhibition analyses presented here indicate that in *Lactobacillus arabinosus*–PABA assay systems, the D·HCl's interfere with the biosynthesis of metabolic derivatives of PGA which are determinable as CF. The relatively poor reversal obtained with PGA or PABA, as compared with the effectiveness of reduced PGA, CF or DPN in reversing D·HCl inhibition, suggests that inhibition is the result of interference with a DPN-mediated reduction of PGA, or PGA-like structures synthesized from PABA, rather than of interference with the synthesis of the intermediate from PABA. The results presented in this communication and the accompanying paper[42] further support previous evidence that the primary mechanism of action of the D·HCl's in several microbiological systems[17, 18] appears to be interference with a DPN-mediated reduction concerned with PGA metabolism.

Conclusive evidence that the D·HCl's, in contrast to the analogues of PGA, block the reduction of PGA in certain microbiological systems has been presented.[5, 8, 17, 18] The competitive inhibition of DPN in *Lactobacillus arabinosus*–PABA assay systems is consistent with the inhibition of DPN by the D·HCl's in DPN-dependent *Hemophilus parainfluenzae* assay systems.*

The possible loci of D·HCl inhibition in this *Lactobacillus arabinosus*–PABA assay system are illustrated in Fig. 3. Although there is evidence that 4-APGA blocks



* D·HCls are relatively weak inhibitors of CF activity.

** 4-APGA blocks conversion of PGA activity to CF activity but not at DPN step.

FIG. 3. Postulated locus of 1:2-dihydro-*s*-triazine inhibition in conversion of *p*-aminobenzoic acid to citrovorum factor activity* by *Lactobacillus arabinosus*.
*All metabolic derivatives of PGA determinable as CF.

a reductive step in the conversion of PGA to CF activity in avian and mammalian liver systems *in vitro*,[43–46] there is no indication in this *Lactobacillus arabinosus*–PABA assay system (or in the other microbiological systems studied[4–6, 17, 18]) that 4-APGA inhibits a DPN-linked reductive step. Accordingly, the locus of inhibition of 4-APGA

*See fourth footnote on p. 24.

has been separated from that of D·HCl inhibition in Fig. 3. An interesting hypothesis is that the dihydrotriazines may be selective inhibitors of the primary, DPN-linked reduction of PGA to dihydro-PGA in certain microbiological systems.

Early studies on the mechanism of sulphapyridine inhibition in bacterial systems suggested that it may be concerned with interference with DPN-mediated reactions.[47, 48] However, other studies[49, 50] with a number of sulphonamides, as well as the present observations in *Lactobacillus arabinosus*–PABA assay systems, do not support this hypothesis but rather indicate that the locus of sulphonamide inhibition differs from either that of 4-APGA or the D·HCl's.

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