BIOCHEMICAL TRANSFORMATIONS AS DETERMINED BY COMPETITIVE ANALOGUE-METABOLITE GROWTH INHIBITIONS

I. SOME TRANSFORMATIONS INVOLVING ASPARTIC ACID

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Preparation of analogues of metabolites to test their ability to inhibit growth of microorganisms has been recognized in recent years as an approach to the problem of seeking out new chemotherapeutic agents (1–3). Amino acids are among the bacterial growth factors for which antagonistic analogues have been prepared. Antagonisms between ethionine and methionine (4, 5), between α-amino-γ-methoxybutyric acid and methionine (6), between β-2-thienylalanine and phenylalanine (7), and between either β-aminobutyric acid or isoserine and β-alanine (8) have been reported.

In the present contribution, dealing with the mechanism of competitive inhibition and its utilization in elucidation of biochemical transformations in growing organisms, it is proposed that (*a*) the antibacterial index (see Table I) is a function of the inhibited enzyme system which is the limiting factor for growth of the organism; (*b*) upon supplying the product of this specific limiting enzyme system to the organism in excess of growth requirements, the analogue either becomes ineffective as a growth inhibitor or affects at higher concentrations another enzyme system, in which case a higher antibacterial index, corresponding to this second enzyme system, is obtained; (*c*) precursors of the metabolite may be effective in preventing inhibition of growth by the analogue over wide ranges of concentration.

Applying these proposals to the investigation of the competitive inhibition of aspartic acid utilization by *dl-"para"*-hydroxyaspartic acid has indicated that for *Escherichia coli* glutamic acid is a limiting precursor of aspartic acid (by transamination) and that the β-alanine moiety of pantothenic acid is a product of aspartic acid metabolism.

The fundamental new observations upon which the present deductions and interpretations rest are as follows: (*a*) *dl-para*-Hydroxyaspartic acid inhibits the growth of *Escherichia coli*. This inhibition is completely prevented by aspartic acid and is competitive in nature; *i.e.*, a defined inhibition occurs at a definite ratio of analogue to aspartic acid. (*b*) The competitive inhibition of growth of *Escherichia coli* by hydroxyaspartic acid is also prevented by glutamic acid over wide ranges of concentration.

(*c*) Pantothenic acid alters the inhibition in a characteristic manner namely, that of changing the antibacterial index (9) to a higher value. Details of the experiments and interpretations concerning these transformations and of additional competitive inhibitions are presented below.

EXPERIMENTAL

dl-para-Hydroxyaspartic Acid—*dl-para*-Hydroxyaspartic acid was prepared by the method of Dakin (10). Only this isomer was used in the tests described in this investigation.

meso-Diaminosuccinic Acid—The less soluble of the two optically inactive forms of diaminosuccinic acid was prepared as described by Lehrfeld (11). Work of Farchy and Tafel (12) has indicated that this isomer is *meso*-diaminosuccinic acid.

Testing Methods—Tests with *Leuconostoc mesenteroides* were carried out as described by Hac and Snell (13) for aspartic acid assay.

For tests with *Escherichia coli*, an inorganic salts-glucose medium was prepared as follows: Na_2SO_4, anhydrous, 1 gm., NH_4Cl 1 gm., K_2HPO_4 0.8 gm., $MgSO_4 \cdot 7H_2O$ 80 mg., glucose 2 gm., $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ 20 mg., and casein, digested with trypsin, 200 γ were dissolved in water, diluted to 100 cc., and filtered. For assays, 5 cc. of this double strength medium were added to test materials in 5 cc. of water; the tubes were capped, autoclaved 10 minutes at 15 pounds steam pressure, and inoculated. Time and temperature of incubation are given with Tables I to VI.

The organism was a strain of *Escherichia coli* carried in this laboratory on yeast extract-glucose agar (1 per cent glucose, 1 per cent yeast extract, and 1.5 per cent agar). Although this strain would not grow initially on the inorganic salts-glucose medium, it grew very rapidly on a previously described complete medium (14). The organism was adapted to the simple medium gradually over a period of several weeks by decreasing the proportion of this complex medium and increasing the inorganic salts-glucose medium which was supplemented with 10 mg. of aspartic acid per 10 cc. The organism was then carried by daily transfers on the latter medium supplemented with 0.15 cc. of the complete medium per 10 cc. After 16 to 24 hours growth, the cells were centrifuged, washed once with 10 cc. of 0.9 per cent sodium chloride, and resuspended in 10 cc. of the saline. One drop of a 1:20 dilution of this saline suspension was used to inoculate each culture tube.

Results

From Table I it is evident that *dl-para*-hydroxyaspartic acid possessed no growth-stimulating properties, but on the contrary was toxic for *Escherichia coli*. The inhibition of growth was prevented by added aspartic acid,

and as the amount of aspartic acid was increased, the level of hydroxyaspartic acid necessary to inhibit growth increased. Even large amounts, more than 1 mg. per cc. of the analogue, were not inhibitory in the presence of sufficient amounts of aspartic acid. The molar ratio of hydroxyaspartic acid to aspartic acid necessary for maximum inhibition (the antibacterial index (9)) remained constant at a value of 10 to 15 over a wide range of concentrations. As judged from the amount of hydroxyaspartic acid required to inhibit growth in the absence of added aspartic acid, the maximum concentration of aspartic acid which *Escherichia coli* produced in the cell was approximately equivalent to an outside concentration of 10 γ per 10 cc. of medium.

TABLE I

Growth Inhibition by Hydroxyaspartic Acid and Its Reversal by l(+)-Aspartic Acid*

Test organism, *Escherichia coli;* incubated 16 hours at 37–38°.

(+)-Aspartic acid	Hydroxyaspartic acid*	Galvanometer reading†	*l*(+)-Aspartic acid	Hydroxyaspartic acid*	Galvanometer reading†
γ *per 10 cc.*	γ *per 10 cc.*		γ *per 10 cc.*	γ *per 10 cc.*	
0	0	43.3	100	0	44.0
0	10	42.5	100	100	37.5
0	30	36.5	100	300	21.0
0	50	13.0	100	1,000	10.0
0	100	4.0	100	2,000	3.5
10	0	42.5	300	0	44.0
10	30	41.0	300	300	40.0
10	50	40.0	300	1,000	20.5
10	100	13.9	300	3,000	10.0
10	200	4.0	300	5,000	2.0
30	0	43.7	1000	0	45.0
30	50	41.0	3000	0	47.7
30	100	39.0	300	10,000	2.8
30	200	13.0	1000	10,000	8.5
30	300	11.5	3000	10,000	51.6
30	500	4.5			

Antibacterial index (C_I/C_M) = 10 to 15. The antibacterial index (9) is the ratio of the concentration of inhibitor (C_I) to that of the essential metabolite (C_M) at which complete inhibition of the test organisms results.

* Less soluble racemic isomer designated as *dl-para*-hydroxyaspartic acid by Dakin (10).

† A measure of culture turbidity; distilled water reads 0, an opaque object 100.

Table II shows that *Leuconostoc mesenteroides*, which requires added aspartic acid for growth, not only did not utilize hydroxyaspartic acid in place of aspartic acid, but the analogue inhibited competitively the response to aspartic acid. The molar ratio for maximum inhibition was 60 to 200 for this organism.

meso-Diaminosuccinic acid also inhibited growth of *Escherichia coli*, as shown in Table III. The inhibition was similar to that obtained with

TABLE II

Growth Inhibition by Hydroxyaspartic Acid and Its Reversal by l(+)-Aspartic Acid

Test organism, *Leuconostoc mesenteroides;* incubated 72 hours at 30°.

l(+)-Aspartic acid	Hydroxyaspartic acid	Galvanometer reading
γ per 5 cc.	*γ per 5 cc.*	
0	0	5.0
0	50	5.5
0	3,000	4.0
50	0	51.2
50	100	50.5
50	300	43.0
50	1,000	25.0
50	3,000	10.5
50	10,000	6.0
500	0	52.9
500	1,000	50.0
500	3,000	49.0
500	10,000	26.2

Antibacterial index (C_I/C_M) = 60 to 200.

TABLE III

Growth Inhibition by meso-Diaminosuccinic Acid and Its Reversal by l(+)-Aspartic Acid

Test organism, *Escherichia coli;* incubated 18 hours at 38–39°.

l(+)-Aspartic acid	Diaminosuccinic acid	Galvanometer reading
γ per 10 cc.	*γ per 10 cc.*	
0	0	49.8
0	300	51.0
0	1000	1.0
10	0	43.0
10	300	46.0
10	1000	37.0
10	3000	1.0
30	0	45.0
30	1000	44.0
30	3000	12.0
30	5000	4.0
100	5000	17.0
300	5000	49.0

Antibacterial index = 100 to 200.

hydroxyaspartic acid, but the inhibitor was somewhat less effective (antibacterial index, 100 to 200).

Partial prevention of hydroxyaspartic acid toxicity by pantothenic acid, β-alanine, and asparagine was obtained, as shown in Table IV. At low levels of hydroxyaspartic acid, the effectiveness of 1 γ of pantothenic acid was approximately equivalent to 10 γ of aspartic acid in preventing the toxicity, but at concentrations above 200 to 300 γ of hydroxyaspartic acid, pantothenic acid was completely ineffective. β-Alanine was at least as active as aspartic acid in preventing the inhibitory action of hydroxy-

TABLE IV

Comparative Effects of l(+)-Aspartic Acid, Pantothenic Acid, β-Alanine, and Asparagine on Prevention of Hydroxyaspartic Acid Toxicity

Test organism, *Escherichia coli;* incubated 16 hours at 38–39°.

Hydroxyaspartic acid	Galvanometer readings for each reversing agent				
	None	Pantothenic acid, 1 γ per 10 cc.	l(+)-Aspartic acid, 10 γ per 10 cc.	β-Alanine, 10 γ per 10 cc.	Asparagine, 10 γ per 10 cc.
γ per 10 cc.					
0	43.5	44.0	45.5	45.0	45.0
10	42.0	45.5	44.8	41.8	44.8
30	10.0	43.0	44.0	41.0	43.5
50	7.0	45.5	45.0	41.8	44.5
100	3.5	34.0	17.5	30.0	11.5
200	1.5	8.5	6.0	3.5	6.5
300	2.0	4.0	5.0	3.8	4.0
		100 γ per 10 cc.	100 γ per 10 cc.	100 γ per 10 cc.	100 γ per 10 cc.
0	46.0*	47.0	44.8	43.0	44.0
100	43.0*	44.0	42.0	39.5	22.0
300	7.5*	7.5	26.0	4.0	4.5
1,000	4.0*	5.0	15.8	4.5	5.0
2,000			4.5		
		3000 γ per 10 cc.	3000 γ per 10 cc.	3000 γ per 10 cc.	3000 γ per 10 cc.
0		45.8	48.5	46.0	48.0
10,000		5.0	47.5	5.2	4.0

* Supplemented with 10 γ of pantothenic acid per tube.

aspartic acid below levels of 200 to 300 γ per 10 cc. of analogue but was completely ineffective at higher concentrations of the inhibitor. Asparagine gave similar results but did not appear to be as active as β-alanine.

The effect of added pantothenic acid on the molar ratio of hydroxyaspartic acid to aspartic acid for maximum inhibition is shown in Table V. Addition of 5 γ of pantothenic acid per 10 cc. resulted in a change of the antibacterial index from 3 to 20 in this and similar experiments. The anti-

bacterial index determined both with and without added pantothenic acid varied with the temperature of incubation. For example, in tests with incubation for 16 hours at 37–38° a change of antibacterial index from 10 to 30 was obtained on the addition of 5 γ of pantothenic acid per 10 cc. of medium.

Addition of 50 γ of pyridoxamine, 0.1 γ of biotin, 5 γ of riboflavin, 5 γ of nicotinic acid, 0.2 γ of folic acid, 5 γ of thiamine, or 1 γ of *p*-aminobenzoic

TABLE V

Effect of Pantothenic Acid on Hydroxyaspartic Acid Toxicity

Test organism, *Escherichia coli;* incubated 18 hours at 38–39°.

Hydroxyaspartic acid	*l*(+)-Aspartic acid	Galvanometer reading	
		Without pantothenic acid	With pantothenic acid, 5 γ per 10 cc.
γ per 10 cc.	*γ per 10 cc.*		
0	0	57.5	58.5
10	0	30.0	54.5
30	0	4.0	28.0
50	0		10.0
100	0		4.5
0	30	50.0	52.0
30	30	13.0	53.0
100	30	6.0	51.0
300	30	4.0	13.5
500	30		9.0
1000	30		4.0
0	100	53.5	52.0
100	100	19.0	53.0
300	100	4.0	39.0
1000	100		15.0
2000	100		6.0

Antibacterial index = 3 without pantothenic acid, 20 with added pantothenic acid.

acid per 10 cc. of medium in place of pantothenic acid did not affect the toxicity of hydroxyaspartic acid for *Escherichia coli.*

Even though the rate of growth of the organism in a medium supplemented with all the vitamins (except pantothenic acid) in the quantities listed above was found to be greater than in a medium supplemented with pantothenic acid alone, the antibacterial index determined in the medium containing 5 γ of pantothenic acid per 10 cc. was found to be 3 to 9 times that determined in the medium supplemented with the nutrilite mixture. The latter antibacterial index was almost identical with that obtained in a medium containing none of these vitamins.

The relative ability of aspartic acid and glutamic acid to reverse the toxicity of hydroxyaspartic acid is shown in Table VI. Glutamic acid and aspartic acid were equally effective except at high concentrations, at which glutamic acid began to show decreasing activity. Oxalacetic acid, malic acid, succinic acid, and fumaric acid were found in similar tests to be completely ineffective in reversing the inhibition.

TABLE VI

Reversal of Hydroxyaspartic Acid Toxicity by l(+)-Glutamic Acid

Test organism, *Escherichia coli;* incubated 16 hours at 37°.

Hydroxyaspartic acid	Galvanometer readings for reversing agent	
	l(+)-Aspartic acid, 0 γ per 10 cc.	*l*(+)-Glutamic acid
γ per 10 cc.		
0	48.0	
30	48.5	
100	8.0	
300	1.0	
	30 γ per 10 cc.	30 γ per 10 cc.
0	47.5	47.3
100	40.0	43.0
300	6.0	8.0
1000	1.0	5.0
	100 γ per 10 cc.	100 γ per 10 cc.
0	48.3	47.5
100	44.5	44.6
300	21.0	27.3
1000	1.0	1.0
	300 γ per 10 cc.	300 γ per 10 cc.
0	47.0	53.1
100	46.0	48.4
300	44.0	37.0
1000	20.5	6.0
3000	1.0	1.0

Isoserine, which bears the same structural relationship to hydroxyaspartic acid as β-alanine to aspartic acid, did not inhibit growth of *Escherichia coli* at a concentration of 10 mg. per 10 cc.

DISCUSSION

In a number of instances the amount of analogue required to cause growth inhibition is in a constant molar ratio to the amount of metabolite

present over a wide range of concentration. This is true only when the other components of the medium are the same and when the size of inoculum and time and temperature of incubation are not allowed to vary. This may be interpreted mathematically by assuming that inhibition of growth of a microorganism results from competition of the analogue with a metabolite for a specific enzyme and that the analogue-enzyme complex is not capable of carrying out the normal function of the metabolite.

Competition of an inhibitor (analogue, in our case hydroxyaspartic acid), I, with a substrate (metabolite, in our case aspartic acid), S, for an enzyme has often been represented by the following equations where P represents the product and ES and EI represent the enzyme-substrate complex and enzyme-inhibitor complex, respectively.

$$E + S \rightleftharpoons ES \rightarrow E + P$$

$$E + I \rightleftharpoons EI$$

By mass law,

$$\frac{[E][S]}{[ES]} = K_S \tag{1}$$

where K_S is the dissociation constant of the enzyme-substrate complex, and

$$\frac{[E][I]}{[EI]} = K_I \tag{2}$$

where K_I is the dissociation constant of the enzyme-inhibitor complex. By dividing Equation 2 by Equation 1, one obtains

$$\frac{[I]}{[S]} = \frac{K_I[EI]}{K_S[ES]} \tag{3}$$

If $[E_t]$ represents the total enzyme concentration, both free and combined, by definition

$$[E_t] = [E] + [EI] + [ES] \tag{4}$$

In application of these equations to inhibition of growth of microorganisms, the following limitations simplify the problem. (1) Under the limiting conditions of defined basal medium, size of inoculum, and time and temperature of incubation, the total amount of growth of a microorganism is a function of the amount of product (P) formed by the inhibited enzyme reaction when this reaction becomes the limiting factor of growth, specifically when growth of the organism is being inhibited by the analogue. Since the total amount of growth is constant for a defined inhibition, the

amount of product (P) formed during the constant time of incubation is constant. Since the rate of formation of the product is directly proportional to $[ES]$ and the time of incubation is constant, then $[ES] = C_{ES}$, a constant concentration of enzyme-substrate complex. (2) For concentrations of substrate and inhibitor approaching that of enzyme saturation, $[E] \rightarrow 0$ and is negligible in comparison with $[EI]$. Since $[ES]$ is constant and $[E]$ is negligible in comparison with $[EI]$, by assuming $[E_t]$ to be constant in the bacterial cell during growth, it is then apparent from Equation 4 that $[EI] = C_I$, a constant concentration of enzyme-inhibitor complex. The assumption that $[E_t]$ is constant in the cell during bacterial growth has been made by Wyss (15) in applying similar equations to the inhibition of growth of *Escherichia coli* by sulfanilamide to show that the inhibition was competitive. Effects of substrates on synthesis and stability of enzymes have been noted (16), but, by growing the organism in a medium containing the substrate before testing with an inhibitor, these effects presumably become negligible.

Hence, by substitution of C_{EI} and C_{ES} respectively in Equation 3, we obtain Equation 5 which may be applied to the instances under discussion

$$\frac{[I]}{[S]} = \frac{K_I C_{EI}}{K_S C_{ES}} = K \tag{5}$$

where K is the molar ratio of analogue to metabolite within the bacterial cell for a given inhibition. If it is assumed that this concentration ratio within the cell is a function of the concentration ratio in the medium, then the latter ratio is also constant for a given inhibition.

If the metabolite, S, is utilized by an organism in several enzyme systems to synthesize several products, P_1, P_2, P_3, etc., then a specific analogue, I, may be capable of preventing the conversion of S to one or more of these products. If one of these conversions, for example $S \rightarrow P_1$, is inhibited to the largest extent and becomes the limiting factor for growth, Equation 5 applies to that particular enzyme system, and the K value (becoming K_1 for this particular case) is the molar ratio for a defined inhibition. For maximum inhibition, K_1, a property of the enzyme effecting the conversion, $S \rightarrow P_1$, would indeed be the antibacterial index.

However, if P_1 is supplied in excess of growth requirements to the organism, the effect of I on the conversion, $S \rightarrow P_1$, is of no consequence in inhibiting the growth of the bacteria. If the analogue cannot combine with other enzymes which convert S to P_2, P_3, etc., the analogue becomes ineffective as a growth inhibitor. But in many cases, the analogue may also prevent other reactions, say $S \rightarrow P_3$, in which case addition of P_1 in excess of growth requirements would not render the analogue ineffective as a growth inhibitor, but on the contrary, this second enzyme system,

which becomes the limiting process for growth, is affected. Equation 5 applied to this particular enzyme system would give a higher K value, K_3, which would then be the molar ratio for a given inhibition (or in case of maximum inhibition, the antibacterial index) of the organism when grown in the presence of P_1. Thus, addition of the product (or its equivalent) of an inhibited enzyme system merely necessitates a change in ratio of analogue to metabolite sufficient to inhibit the enzyme system with the next higher K value in order again to prevent growth of the organism.

The change in molar ratio for maximum inhibition, the antibacterial index, resulting from addition of pantothenic acid (or β-alanine) to the medium is an example of the effect of addition of the product (or its equivalent) of the inhibited enzyme system to the medium. The inhibited enzyme system is no longer indispensable, since the product is already present; however, another enzyme system is affected, as is revealed by a higher antibacterial index corresponding to this other enzyme system.

Thus, aspartic acid, aside from its share in the synthesis of proteins, has been found to be an intermediate in the biological synthesis by *Escherichia coli* of essential metabolites. The functioning of the enzyme system which synthesizes the β-alanine portion of pantothenic acid is blocked by hydroxyaspartic acid reacting in competition with aspartic acid. Since isoserine, which might have been formed by decarboxylation of hydroxyaspartic acid, was ineffective in inhibiting growth, competition between isoserine and β-alanine is eliminated as a possibility; so, the decarboxylation of aspartic acid to β-alanine is the probable step in the synthesis of pantothenic acid which is blocked. These results do not preclude the possibility that the aspartic acid combines with other groupings such as the pantoyl group before decarboxylation. Asparagine is as effective as aspartic acid in preventing the toxicity of hydroxyaspartic acid at low concentrations but is not converted to any appreciable extent into aspartic acid above a definite level of the inhibitor.

Of growth factors and amino acids studied other than aspartic acid, only glutamic acid effectively prevented the toxicity of hydroxyaspartic acid over a range of concentrations. Glutamic acid was as effective as aspartic acid up to 100 γ per 10 cc. of medium. Beyond that the effectiveness of glutamic acid decreased and was not enhanced by oxalacetic acid. The most logical explanation of this effect is that the transamination reaction converting glutamic acid and oxalacetic acid to aspartic acid and ketoglutaric acid is dependent upon the amount of glutamic acid available and not upon oxalacetic acid which *Escherichia coli* presumably produces in sufficient quantities. The enzyme system may not be capable of converting large amounts of glutamic acid to aspartic acid, or large amounts of hydroxyaspartic acid may inhibit the functioning of the enzyme.

Lichstein and Cohen (17) have recently shown that *Escherichia coli*, among other organisms, readily converts glutamic acid to aspartic acid by the transamination reaction.

Although resting *Escherichia coli* cells decompose aspartic acid to fumaric acid and ammonia by a reversible process (18), fumaric acid showed no ability to reverse hydroxyaspartic acid toxicity on rapidly growing *Escherichia coli*. Succinic acid, malic acid, and oxalacetic acid were also ineffective.

This method of investigation has shown promising results in connection with the study of other transformations, details of which will be presented in forthcoming papers.

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SUMMARY

dl-para-Hydroxyaspartic acid and *meso*-diaminosuccinic acid have been found to inhibit competitively some of the functions of aspartic acid in *Escherichia coli*. Hydroxyaspartic acid also inhibited growth of *Leuconostoc mesenteroides* which requires aspartic acid for growth.

The inhibition of growth of *Escherichia coli* by hydroxyaspartic acid was also prevented over wide ranges of concentration by glutamic acid which must therefore serve as a precursor of aspartic acid. Pantothenic acid, β-alanine, and asparagine were effective in preventing the toxicity of hydroxyaspartic acid for *E. coli* only at low levels of hydroxyaspartic acid concentration. The effect of supplying pantothenic acid in the medium raised the antibacterial index to a higher value. A mathematical interpretation of the antibacterial index is given, and the following conclusions are drawn. (1) Aspartic acid is a precursor in the biosynthesis by *E. coli* of the β-alanine portion of pantothenic acid. (2) β-Alanine is also used by the organism for pantothenic acid synthesis. (3) Under the conditions of testing, asparagine is not converted to aspartic acid to any appreciable extent when the concentration of hydroxyaspartic acid is above a given level. (4) At least one additional biosynthetic transformation involving aspartic acid is prevented by sufficient amounts of hydroxyaspartic acid.

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