Non-competitive Sulphanilamide Antagonists for Yeasts

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SUMMARY: Sulphanilamide inhibition of the growth of certain yeasts and yeast-like organisms was overcome in a non-competitive manner by methionine, adenine and histidine, which are therefore regarded as end-products of enzyme systems for which *p*-aminobenzoic acid is essential. The methionine system was the most sensitive to sulphanilamide, followed in order by those of adenine and histidine.

In the presence of methionine, adenine and histidine together, the test organisms were still sensitive to sulphanilamide, implying that *p*-aminobenzoic acid is concerned in still another system, or systems. The nature of these systems is obscure, but the inhibition is not overcome by purines, pyrimidines or pteroylglutamic acid, nor by single amino-acids, except in one case when DL-cysteine showed some effect. In another case, a low concentration of choline chloride was effective.

Evidence of the vital functions of *p*-aminobenzoic acid (*p*-AB) may be obtained by the method of inhibition analysis using sulphanilamide (S-amide) (Shive & Roberts, 1946). The biosynthesis of methionine and of the purine skeleton seems to be dependent on enzyme systems in which *p*-AB plays an essential role, since methionine and purines overcome S-amide inhibition in a non-competitive manner (Harris & Kohn, 1941; Snell & Mitchell, 1942; Lampen & Jones, 1947). This conclusion is supported by other studies which showed that *p*-AB can be replaced by purines and amino-acids in the growth of mutant strains of *Escherichia coli* (Lampen, Jones & Roepke, 1949) and by adenine, methionine and histidine for the growth of a yeast (Cutts & Rainbow, 1950).

In other cases, S-amide inhibition is overcome by relatively high concentrations of thymine or by relatively low concentrations of pteroylglutamic acid (PGA). It is therefore concluded that PGA is concerned in the biosynthesis of thymine (Lampen & Jones, 1947; Winkler & de Haan, 1948). As a result of extensive inhibition analysis of *Esch. coli*, the latter workers found that serine is an S-amide antagonist, and Lampen *et al.* (1949) showed that other amino-acids have some effect. In the present study, purines, pyrimidines, amino-acids, PGA and choline were tested as antagonists of S-amide bacteriostasis of some yeast and yeast-like organisms, and the results compared with those already obtained by a different technique (Cutts & Rainbow, 1950).

METHODS

The basal medium used was that described by Cutts & Rainbow (1950).

Test organisms and inocula. Four test organisms were used; cultures of each were maintained on malt agar slants and transferred 2-3 times weekly as required.

(a) The strain of *Saccharomyces cerevisiae*, designated 'Yeast 47' by Rainbow (1948). This organism requires exogenous sources of biotin, pantothenate and

p-AB. Inocula were prepared by transferring a little surface growth from a malt agar slant to 6 ml. of basal medium containing 0.1 μ g. *p*-AB/ml. and incubating at 25° for 18–24 hr. The cells were then washed twice (centrifuge) in 6 ml. portions of sterile 0.85 % saline and finally suspended in a further 6 ml. portion.

(b) A strain of top fermentation brewer's yeast (*S. cerevisiae*) isolated by us from a brewery pitching yeast and designated 'Yeast 70'. This strain required biotin and inositol, synthesized pantothenate slowly, but was independent of exogenous *p*-AB. Inocula were suspensions in sterile saline of a little of the surface growth from a malt agar slant.

(c) *Kloeckera brevis*, B768 (Emery, McLeod & Robinson, 1946). This strain was independent of exogenous *p*-AB. Inocula were prepared as for (b) above.

(d) *Schizosaccharomyces octosporus*, an organism requiring for growth exogenous adenine, methionine and histidine, but not *p*-AB (Northam & Norris, 1951). Inocula were grown in basal medium supplemented with (μ g./ml.): DL-methionine (100), adenine HCl (50) and L-histidine HCl (25) and otherwise treated as in (a) above.

Auxanographic tests were carried out as previously described (Cutts & Rainbow, 1950). For each series, the following substances were tested for ability to overcome sulphanilamide inhibition: *purines and pyrimidines*—adenine, guanine, xanthine, cytosine, thymine and uracil; *amino-acids*—DL-alanine, L-arginine, L-asparagine, L-aspartic acid, DL-cysteine, L-cystine, glycine, L-glutamic acid, L-histidine, L-hydroxyproline, DL-isoleucine, L-leucine, L-lysine, DL-methionine, DL-norleucine, DL-norvaline, L-proline, DL-phenylalanine, DL-serine, DL-threonine, DL-tryptophan, L-tyrosine and DL-valine; other substances—*p*-AB, PGA and choline chloride.

Tests in liquid media were carried out as described by Northam & Norris (1951). Observations were made on duplicate cultures.

RESULTS

Inhibition of Yeast 47

Auxanographic tests were carried out to identify substances which could overcome the inhibition of the growth of Yeast 47 by sulphanilamide (S-amide). The substances listed above were first tested in the basal medium containing 1000 μ g. S-amide and 0.5 μ g. *p*-AB/ml. In this medium, little growth took place after 48 hr. incubation, during which period observations were made. After 96 hr. growth was heavy and general on all plates, except around the point of application of norleucine. The results are recorded in Table 1. Apart from *p*-AB, DL-methionine, adenine, guanine and DL-cysteine each overcame S-amide inhibition, whilst norleucine accentuated the inhibition.

For the second series of auxanographs, DL-methionine, adenine and guanine were added singly to portions of basal medium containing a concentration of *p*-AB decreased to permit inhibition of growth by 1000 μ g. S-amide/ml. In these tests, S-amide inhibition was overcome by adenine or by guanine in the medium containing methionine, or by DL-methionine in the media containing

adenine or guanine. Norleucine was again inhibitory in the two latter media.

In basal medium containing S-amide, *p*-AB, DL-methionine and adenine, inhibition of Yeast 47 was overcome only by L-histidine and by *p*-AB itself. By including L-histidine HCl in the basal medium, as well as adenine and methionine, and diminishing the *p*-AB concentration still further to permit of inhibition, Yeast 47 was found too tolerant of S-amide for further results to be obtained, unless the inoculum used for the tests was one of deficient cells obtained by growth in the defined *p*-AB-free medium described in (*d*) above. In this way, DL-cysteine was shown to overcome S-amide inhibition in the presence of methionine, adenine and histidine.

For the final auxanographs, adenine, methionine, histidine and DL-cysteine were included in the basal medium containing S-amide and *p*-AB. Under these conditions and using an inoculum from the *p*-AB free defined medium,

Table 1. *Antagonists of sulphanilamide inhibition of various organisms*
(Results were obtained by auxanograph technique; observations up to 48 hr. of incubation at 25°.)

Basal medium containing			
Supplements ($\mu\text{g./ml.}$)	S-amide ($\mu\text{g./ml.}$)	<i>p</i> -AB ($\mu\text{g./ml.}$)	S-amide inhibition overcome by
Yeast 47			
None	1000	0.5	Adenine, guanine, DL-methionine, DL-cysteine, <i>p</i> AB
DL-Methionine (100)	1000	0.1	Adenine, guanine, <i>p</i> -AB
Adenine HCl (50)	1000	0.1	DL-Methionine, <i>p</i> -AB
Guanine (50)	1000	0.1	DL-Methionine, <i>p</i> -AB
DL-Methionine (100) adenine HCl (50)	1000	0.005	L-Histidine HCl, <i>p</i> -AB
DL-Methionine (100)* adenine HCl (50)* L-histidine HCl (25)*	1000	0.001	DL-Cysteine HCl, <i>p</i> -AB
DL-Methionine (100)* adenine HCl (50)* L-histidine HCl (25)* DL-cysteine HCl (250)*	1000	0.0003	<i>p</i> -AB
Yeast 70			
None	1000	0	Adenine, guanine, DL-methionine, <i>p</i> -AB
DL-Methionine (100) adenine HCl (50)	3000	0	L-Histidine, L-leucine, L-tyrosine, <i>p</i> -AB
DL-Methionine (100) adenine HCl (50) L-histidine HCl (25)	3000	0	No demonstrable inhibition
<i>Kloeckera brevis</i>			
None	1000	0	DL-Methionine, PGA, <i>p</i> -AB
DL-Methionine (100)	1000	0	Adenine, <i>p</i> -AB
Adenine HCl (50)	3000	0	No demonstrable inhibition
<i>Schizosaccharomyces octosporus</i>			
DL-Methionine (100)* adenine HCl (50)* L-histidine HCl (25)*	1000	0	Choline chloride, <i>p</i> -AB

* Tests carried out using an inoculum grown on a *p*-AB-free medium.

Yeast 47 was still sensitive to S-amide, the inhibition being overcome only by *p*-AB of all the substances tested.

Non-competitive nature of the anti-S-amide action of purines and amino-acids. For Yeast 47, a competitive relation was found to exist between *p*-AB and concentrations of S-amide between 300 and 3000 $\mu\text{g./ml.}$ For lower concentrations of S-amide, the ratio of the concentrations (S-amide)/(*p*-AB) required just to restore full growth (i.e. the antibacterial index) diminished, as might be expected when the enzyme systems involved are no longer saturated with respect to *p*-AB and S-amide. At the other extreme, the inhibition due to 10,000 $\mu\text{g.}$ S-amide/ml. was not completely overcome by *p*-AB.

The non-competitive nature of the action of methionine, adenine and histidine in overcoming the S-amide inhibition of Yeast 47 was demonstrated in liquid medium containing 1000 $\mu\text{g.}$ S-amide/ml. Fig. 1 shows that methionine behaved as a non-competitive antagonist. The concentration of *p*-AB required to restore full growth was decreased from 1.0 to 0.3 $\mu\text{g.}$ *p*-AB/ml. (approximately a threefold increase in the antibacterial index) by 100 $\mu\text{g.}$ of DL-methionine/ml. This concentration of methionine partially overcame the inhibition in the presence of 0.1 $\mu\text{g.}$ *p*-AB/ml., but higher methionine concentrations, which would be expected to be more effective if the relation were competitive, were no more effective.

The non-competitive anti-S-amide activities of adenine and histidine are shown in Figs. 2 and 3 respectively. The effect of adenine was evident in a medium containing 100 $\mu\text{g.}$ DL-methionine/ml., 30 $\mu\text{g.}$ adenine HCl/ml. producing a further threefold increase in the antibacterial index. In a separate experiment, guanine was found to be equally effective. To demonstrate the non-competitive anti-S-amide activity of histidine, the presence in the medium of both DL-methionine (100 $\mu\text{g./ml.}$) and adenine HCl (100 $\mu\text{g./ml.}$) was necessary. In this case, the inhibition in *p*-AB-free medium was partially overcome by 100 $\mu\text{g.}$ L-histidine HCl/ml.; 1000 $\mu\text{g.}$ L-histidine/ml. was no more effective. Thus for Yeast 47 methionine, adenine and histidine are non-competitive S-amide antagonists, which suggests that these are end-products of enzymic systems for which *p*-AB is essential.

The order in which methionine, adenine and histidine exert antisulphonamide activity. The effects on the antibacterial index of additions of methionine, adenine and histidine, singly and in all possible combinations, to the basal medium was determined by tube tests (Table 2). The antibacterial index was almost unaltered by additions of adenine or histidine, or by adenine + histidine, but was increased some 2.5 times by methionine. The effect of histidine and methionine together was no greater than that of methionine alone, but the combination adenine + methionine resulted in a further 2.5-fold increase of the index. The effect of histidine was evident only when adenine and methionine are both present.

It therefore appears that the anti-S-amide actions of these substances are exerted in a definite order, the methionine system being the most sensitive to S-amide, that of adenine intermediate and that of histidine the least sensitive. The successive effects of the three substances is shown in Fig. 4.

Inhibitions of other yeasts

To examine whether the results obtained with Yeast 47 were of more general significance, other yeasts and yeast-like organisms were tested. Only qualitative tests were made, by means of the auxanographic technique, with the minor

Fig. 1

Fig. 1. S-amide inhibition (1000 µg. S-amide/ml.) of Yeast 47 overcome by DL-methionine. The numbers above the curves show the respective concentrations of *p*-AB (mµg./ml.).

Fig. 2

Fig. 2. Inhibition of Yeast 47 in presence of 1000 µg. S-amide + 100 µg. DL-methionine overcome by adenine at various concentrations of *p*-AB (*p*-AB in mµg./ml. as shown by the numbers above the respective curves).

Table 2. *Effects of methionine, adenine and histidine on the antibacterial index with Yeast 47*

(Antibacterial index = 1000/µg. *p*-AB/ml. required just to restore full growth in 48 hr. at 25°. Basal medium contained 1000 µg. S-amide/ml.)

Supplements (µg./ml.)	Antibacterial index (10 ³)
Nil	1.0
Adenine HCl (50)	1.0
L-Histidine HCl (25)	1.0
Adenine HCl (5) + L-histidine HCl (25)	1.0
DL-Methionine (100)	2.5
DL-Methionine (100) + L-histidine HCl (25)	2.5
DL-Methionine (100) + adenine HCl (50)	6.0
DL-Methionine (100) + adenine HCl (50) + L-histidine HCl (25)	60.0

modification that, since none of the organisms tested required a source of exogenous *p*-AB, this substance was omitted from the basal medium, in order that the sensitivity to S-amide should not be diminished. The results are recorded in Table 1.

Fig. 3

Fig. 3. Inhibition of Yeast 47 in presence of S-amide (1000 µg./ml.) + DL-methionine (100 µg./ml.) + adenine HCl (100 µg./ml.) overcome by L-histidine at various concentrations of *p*-AB. *p*-AB concentrations are indicated by the numbers (mµg./ml.) above the respective curves.

Fig. 4

Fig. 4. Inhibition of growth of Yeast 47 in basal medium + 1000 µg. S-amide/ml. overcome by *p*-AB under various conditions. Curve I, no additions; curve II, +100 µg. DL-methionine/ml.; curve III, +100 µg. each of DL-methionine and adenine HCl/ml.; curve IV, +100 µg. each of DL-methionine, adenine HCl and L-histidine HCl/ml.

S-amide inhibition of Yeast 70 resembled that of Yeast 47 with respect to methionine, adenine and histidine, except that inhibition in the presence of methionine and adenine was overcome by L-leucine and L-tyrosine, as well as by L-histidine. Cutts & Rainbow (1950) showed that the *p*-AB-sparing effect of suboptimal concentrations of methionine was much enhanced by leucine and tyrosine, and in the present case these amino-acids may act by virtue of their methionine-sparing effect. This conclusion is supported by the fact that, on raising the concentration of DL-methionine to 250 µg./ml. and repeating the test with Yeast 70, no anti-S-amide activity by L-leucine or L-tyrosine could be detected.

With *Kloeckera brevis*, S-amide inhibition was overcome by methionine and adenine; but in the presence of both these substances, the organism was too

tolerant of S-amide to permit of further inhibition analysis. The action of PGA as an anti-S-amide substance was approximately competitive over a limited range, a relatively high concentration ($3\text{ }\mu\text{g./ml.}$) being required to overcome the inhibition by $1000\text{ }\mu\text{g. S-amide/ml.}$ This suggested that the activity of the PGA was due to its conversion by *K. brevis* into free *p*-AB (cf. Koft, Sevag & Steers, 1950).

Fig. 5. Inhibition of growth of *Schizosaccharomyces octosporus* in basal medium + $1000\text{ }\mu\text{g. S-amide/ml.}$ + $100\text{ }\mu\text{g. DL-methionine/ml.}$ + $50\text{ }\mu\text{g. adenine HCl/ml.}$ + $25\text{ }\mu\text{g. L-histidine HCl/ml.}$ Inhibition overcome by *p*-AB in the presence and absence of $1\text{ }\mu\text{g. choline chloride/ml.}$

Schizosaccharomyces octosporus did not require exogenous *p*-AB for growth, but had to be provided with adenine, methionine and histidine, the syntheses of which compounds could not therefore be blocked by S-amide. Nevertheless, the organism showed inhibition by S-amide, which was overcome by $1\text{ }\mu\text{g. choline chloride/ml.}$ (Fig. 5), although the growth rate was then subnormal; concentrations of choline chloride exceeding $1\text{ }\mu\text{g./ml.}$ had no greater effect.

DISCUSSION

As non-competitive antagonists of S-amide inhibition, methionine, adenine and histidine may be regarded as end-products of the activity of certain enzyme systems for which *p*-AB is an essential component. The results confirm those of Cutts & Rainbow (1950), who found that relatively high concentrations of

methionine, adenine and histidine could replace *p*-AB (albeit not completely) for the growth of Yeast 47. With Yeast 47, the difference in sensitivity to S-amide between the systems involved in the syntheses of methionine, adenine and histidine suggests that three different enzyme systems, each requiring *p*-AB, are involved. That *p*-AB is concerned in the synthesis of an intermediate, common to all three substances, seems less likely.

The inhibitory effect of norleucine in the auxanographs of Yeasts 47 and 70 on media containing no methionine is ascribable to the synergistic effect exercised by norleucine and suphonamides and which may be due to interference by norleucine with methionine metabolism, since the latter two substances are structurally analogous.

Guanine is as effective as adenine in overcoming inhibition by S-amide with Yeasts 47 and 70, but it cannot replace adenine in *p*-AB-free medium for Yeast 47 (Cutts & Rainbow, 1950). Thus, Yeast 47 can convert adenine to guanine, but not guanine to adenine. Guanine may therefore exert its action as an antisolphonamide by 'sparing' the product of a reaction inhibited by S-amide, viz. adenine. Comparison may be made with the work of Kerr, Seraidarian & Brown (1951) who found that, whereas isotopically labelled adenine was incorporated into the ribonucleic acid of *Torulopsis utilis* both as adenine and guanine, labelled guanine appeared only as guanine.

There is no evidence that PGA is an essential cell constituent for the yeasts studied. Other tests with Yeast 47, not reported here, confirm that PGA will neither replace *p*-AB nor raise the level of growth obtainable in the presence of methionine, adenine and histidine in *p*-AB-free medium. The explanation of the inactivity of synthetic PGA for yeasts possibly lies in Woods's (1948) suggestion that synthetic PGA may not be identical with (or convertible to) all natural forms of 'folic acid'.

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