PURINE AND PYRIMIDINE ANTAGONISM IN A PYRIMIDINE-DEFICIENT MUTANT OF NEUROSPORA

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Inhibitions of the growth of organisms by closely related structural analogues of several water-soluble vitamins are well known. Similar types of antagonism in the case of purine and pyrimidine metabolites are those between the purine bases, adenine and guanine, and benzimidazole (1) and between barbituric acid and uracil in the growth of *Staphylococcus aureus* (2). In such cases the antagonism is usually considered to be due to competition between the analogue and the metabolite in enzyme systems where the normal metabolite is involved.

Antagonisms between two structurally related, naturally occurring substances are less common but have been demonstrated to exist between amino acids of similar structure (3, 4) and between thiamine and pyridoxine (5). A most striking relationship between normal metabolites is that reported by Raska (6) in which pellagra was produced experimentally in dogs when adenine alone or in conjunction with phosphate was fed in daily doses of 400 to 500 mg. An explanation similar to that mentioned above for the antagonism between metabolites and structural analogues not occurring naturally has also been offered for the closely related natural substances.

The present study is concerned with the inhibition of growth of the pyrimidine-deficient *Neurospora* mutant, No. 1298, by the naturally occurring purine ribonucleotides and ribonucleosides. This experimentally produced strain, unlike the wild type, is unable to synthesize the pyrimidine ribonucleosides on a medium containing inorganic salts, carbohydrate, and biotin. Normal growth takes place, however, when the medium is supplemented with either cytidine or uridine or the corresponding nucleotides (7). It has been found that adenosine and adenosine-3-phosphate (yeast adenylic acid) inhibit the utilization of the pyrimidine compounds to a varying degree. An amount of adenine nucleoside which is sufficient to inhibit growth completely on the quantity of cytidine used has no inhibitory effect on an equivalent amount of uridine. The addition of an equimolar amount of uridine to a mixture of cytidine and adenosine in

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which no growth takes place results in the elimination of the antagonism. In contrast to the effect of adenosine and adenylic acid on this mutant strain of *Neurospora*, adenine shows no inhibitory properties at comparable concentrations. A similar inhibitory effect on the utilization of the pyrimidine nucleosides was found for guanosine and guanylic acid, but larger amounts of these compounds were required to produce inhibition under the same conditions. Guanine like adenine failed to cause inhibition at moderate concentrations.

EXPERIMENTAL

The growth response of the mold to various concentrations of supplements and inhibitors as compared to that in the absence of inhibitor was determined from the dry weight of mycelium produced in liquid culture after incubation for 3 days at 25°. The composition of the basal medium, the method of inoculation, and the determination of the weight of mycelium were the same as previously described (7, 8). The concentrations of the pyrimidine derivatives used in the determination of the inhibitory effects of the purine compounds were those which produced an approximately half maximum growth of the mold. In this range an amount of mold which can be readily weighed is obtained, and growth response is most sensitive to small changes in the concentration of added supplement. The growth of the mold was found from the average value of determinations made in triplicate.

Uridylic acid, in the form of the diammonium salt, cytidylic acid, uridine,[1] and guanine[2] were prepared by methods devised in this laboratory (9). Guanosine was isolated from yeast nucleic acid as described by Levene (10). The cytidine, guanylic acid, adenosine, and adenosine-3-phosphate were commercial samples.[3]

Antagonism by Adenosine and Adenosine-3-phosphate—The effect of adenine, adenosine, and adenosine-3-phosphate on the growth activity of cytidine, uridine, cytidylic acid, and uridylic acid was determined by adding increasing amounts of each purine compound to the basal medium supplemented with a constant amount of growth factor. The amount of cytidine or uridine used was 0.5 mg. per 25 ml. of basal medium. The growth of the mold in the presence of varying amounts of adenine, adenosine, and adenosine-3-phosphate was determined and plotted as the percentage of growth obtained in the absence of inhibition. The data for

[1] Loring, H. S., and Ploeser, J. McT., unpublished work.

[2] Loring, H. S., and Ali, S. A., unpublished work.

[3] Cytidine and guanylic acid were kindly provided by the National Biochemical Corporation, New York. Adenosine and adenosine-3-phosphate were obtained from Schwarz Laboratories, Inc., New York.

cytidine and the three purine compounds and for uridine and adenosine are shown in Fig. 1. It can be seen that adenosine was twice as inhibitory of cytidine activity as was adenosine-3-phosphate. When uridine was used as the growth factor, approximately five times as much adenosine was required to produce the same degree of inhibition. Free adenine failed to inhibit the growth of the mold on cytidine at a concentration equivalent to 0.6 mg. of adenosine and indeed was slightly stimulatory at some concentrations. Similarly, no significant inhibition of uridine in the presence of adenine was observed at a concentration equivalent to 4.0 mg. of adenosine.

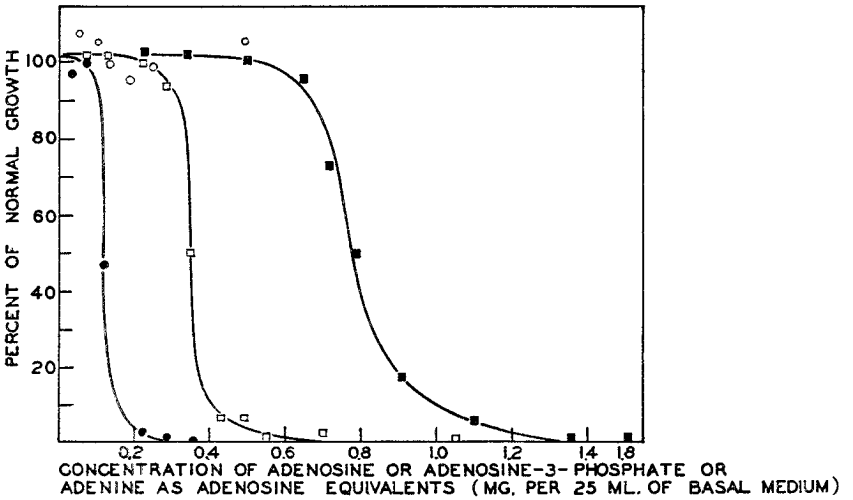

FIG. 1. The effect of adenine, adenosine, and adenosine-3-phosphate expressed as adenosine or adenosine equivalents on the growth of *Neurospora* mutant No. 1298; ○ adenine, ● adenosine, and □ adenosine-3-phosphate in the presence of 0.5 mg. of cytidine; ■ adenosine in the presence of 0.5 mg. of uridine.

In the case of cytidylic acid and uridylic acid, the amounts employed to give about half maximum growth were 1 mg. of cytidylic acid and 1 mg. of diammonium uridylate per 25 ml. of basal medium. Addition of the three adenine compounds in similar amounts to those used for the pyrimidine nucleosides gave inhibition curves of the same type as those shown in Fig. 1. Free adenine as with cytidine and uridine did not affect the utilization of the pyrimidine ribonucleotides. The molar ratios of antagonist to metabolite to give 50 per cent inhibition in the case of the four pyrimidine compounds and adenosine and adenosine-3-phosphate were calculated from the respective inhibition curves and are given in Table I. Of the four pyrimidine compounds it may be seen that cytidylic acid was the most readily inhibited, the molar ratio of adenosine to cytidylic acid for 50 per

cent inhibition being 0.13. In contrast to cytidylic acid, the inhibition ratio for adenosine and uridylic acid was 0.41. It is evident that adenosine-3-phosphate was less inhibitory in all cases than the corresponding nucleoside. Cytidine, like cytidylic acid, was more strongly inhibited than uridine, but each pyrimidine nucleoside was affected to a lesser degree by adenosine than was the corresponding nucleotide.

Antagonism by Guanosine and Guanosine-3-phosphate—An entirely analogous situation was found in the case of the guanine compounds. Guanosine and guanylic acid inhibited the utilization of each of the four pyrimidine nucleosides or nucleotides while guanine at comparable concentrations had no effect. The effect of guanosine and guanosine-3-

TABLE I

Molar Ratios of Antagonist to Metabolite for 50 Per Cent Inhibition

Metabolite	Weight metabolite used per 25 ml. medium	Antagonist	Moles antagonist / Moles metabolite
	mg.		
Cytidylic acid	1	Adenylic acid	0.27
" "	1	Adenosine	0.13
Cytidine	0.5	Adenylic acid	0.60
"	0.5	Adenosine	0.24
Uridylic acid	0.86	Adenylic acid	0.6
" "	0.86	Adenosine	0.41
Uridine	0.5	Adenylic acid	3.2
"	0.5	Adenosine	1.4
Cytidine	0.5	Guanosine	0.68
"	0.5	Guanylic acid	1.29
Uridine	0.5	Guanosine	3.0
"	0.5	Guanylic acid	7.8

phosphate on mold growth in the presence of 0.5 mg. of cytidine or uridine is shown in Fig. 2. It may be seen that approximately twice as much guanosine or guanylic acid was required to produce the same amount of inhibition as for the adenine compounds. Guanine in an amount equivalent to 10 mg. of guanosine per 25 ml. of basal medium did not affect the growth of the mold in the presence of 0.5 mg. of cytidine or uridine. The molar ratios of guanosine or guanylic acid to cytidine and uridine to produce 50 per cent inhibition are also shown in Table I.

Antagonism in Mixtures of Pyrimidine Nucleosides and Nucleotides—The surprising difference in the ability of adenosine to inhibit growth on uridine as compared to cytidine suggested that the antagonism was involved to a different degree in the reactions concerned in the utilization of the two compounds. If the reaction inhibited was the deamination of cytidine to

uridine rather than the utilization of cytidine *per se*, then it should be possible to eliminate the inhibition of cytidine by the addition of sufficient uridine to avoid the necessity of deamination. It was desirable, therefore, to determine the amount of uridine that would cause the reversal of the antagonism in an inhibitory mixture of cytidine and adenosine. A series of flasks containing 0.25 mg. of cytidine and 0.27 mg. of adenosine in 25 ml. of basal medium was supplemented with increasing amounts of uridine from 0.05 to 0.5 mg. The molar ratio of adenosine to cytidine was 1.0, which in the absence of uridine produces complete inhibition. The effect of the

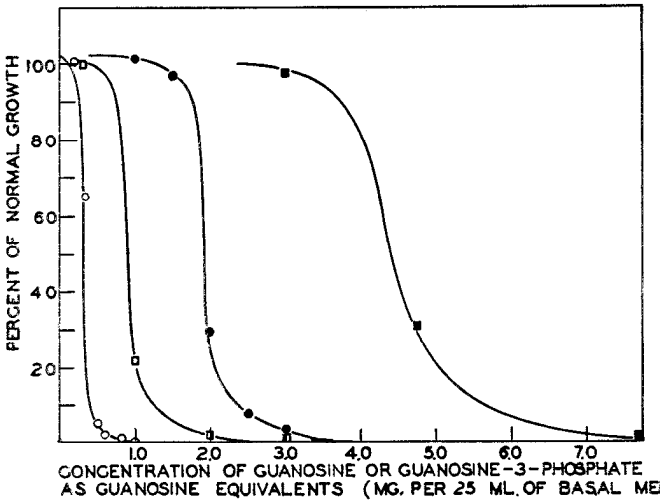

FIG. 2. The effect of guanosine or guanylic acid expressed as guanosine or guanosine equivalents on the growth of *Neurospora* mutant No. 1298; ○ guanosine, and □ guanylic acid in the presence of 0.5 mg. of cytidine; ● guanosine, and ■ guanylic acid in the presence of 0.5 mg. of uridine.

addition of the uridine is shown in Fig. 3, *A*, where the weight of mycelium found for each concentration of uridine was plotted against the total weight in mg. of cytidine and uridine used as the growth supplement. The curve showing the growth of the mold on either pyrimidine nucleoside in the absence of inhibitor is also presented. It may be seen that the growth-promoting properties of the mixtures were almost completely inhibited until the molar ratio of the cytidine to uridine approached 1. When the ratio reached 1, the inhibition was strikingly eliminated, and as more uridine was added, the amount of growth was approximately that found with either cytidine or uridine when no inhibitor was present. As shown in the cytidine-adenosine curve in Fig. 1, 0.27 mg. of adenosine in the

presence of 0.5 mg. of cytidine, a molar ratio of 0.5, gave 98 per cent inhibition. Thus it is evident that the inhibition was a specific one and that the addition of an equivalent quantity of cytidine instead of uridine would not have overcome the effect of the adenosine.

An experiment performed with 0.4 mg. of cytidylic acid and 0.18 mg. of adenosine, an amount giving complete inhibition, gave a similar elimination of antagonism when increasing amounts of uridylic acid were added. Inhibition was nearly 100 per cent when the ratio of uridylic acid to cytidylic acid was less than 1, but, when the ratio became 1, inhibition no longer was observed. In this case when an additional equivalent of cytidylic acid

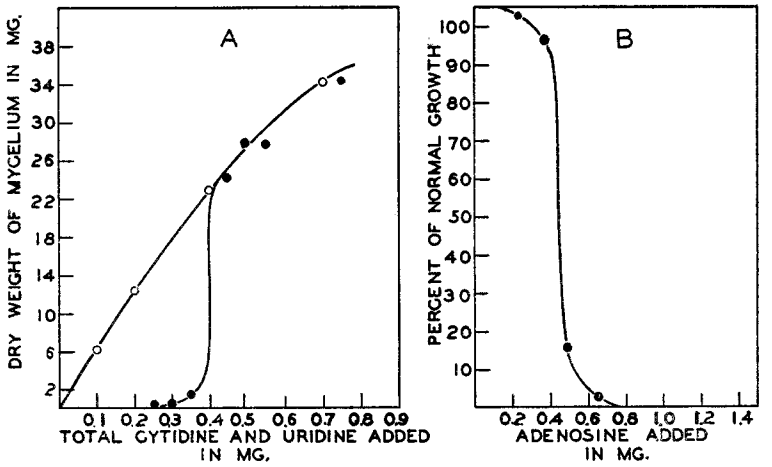

FIG. 3, *A*. The effect of uridine on an inhibitory mixture of 0.25 mg. of cytidine and 0.27 mg. of adenosine in comparison with the normal growth curve on cytidine or uridine; ○ normal growth curve on cytidine or uridine, ● growth curve on cytidine-adenosine mixture with varying amounts of uridine.

FIG. 3, *B*. The effect of adenosine on an equimolar mixture of 0.25 mg. of cytidine and 0.25 mg. of uridine.

was added to the 0.4 mg. of cytidylic acid-0.18 mg. of adenosine mixture, the amount of growth corresponded to about 95 per cent inhibition.

In an attempt to reverse the guanosine inhibition of cytidine, increasing amounts of uridine were added to a mixture of cytidine and guanosine which is completely inhibitory (0.25 mg. of cytidine and 0.5 mg. of guanosine; *cf.* Fig. 2). The antagonism was also eliminated in this case by the addition of an equimolar amount of uridine, 0.25 mg. of the latter permitting growth equal to 82 per cent of that expected from either 0.5 mg. of cytidine or the same quantity of uridine with no inhibitor added.

The striking elimination of the inhibition of cytidine by an equimolar amount of uridine provided evidence that the antagonism of adenosine was

concerned in a highly specific way with the deamination of cytidine to uridine, possibly by inhibition of a cytidine deaminase present in the mold. A possible explanation of the antagonism for uridine alone could be the blocking of the reverse reaction, the amination of uridine to cytidine. A larger amount of adenosine would be required for the blocking of this reaction than for the deamination, as shown by the larger amounts required to inhibit growth on uridine. If this were the case, one would expect the amount of adenosine which inhibits the utilization of uridine alone to have no effect on an equimolar mixture of cytidine and uridine, both being available for growth.

To study this question an equimolar mixture of 0.25 mg. of cytidine and 0.25 mg. of uridine was supplemented with different amounts of adenosine from 0.23 to 1.4 mg., and the amount of mold growth determined in each case. The mycelium weights expressed as per cent of growth in the absence of inhibitor and the amounts of adenosine added are shown graphically in Fig. 3, *B*. When 0.27 mg. of adenosine was used, an equimolar mixture of all three components was present, and the growth of the mold was not inhibited, as was expected from the curve shown in Fig. 3, *A*. However, as the ratio of adenosine to cytidine and uridine was increased, mold growth was inhibited in a fashion similar to that found for uridine alone. Thus for 50 per cent inhibition, the same adenosine-uridine ratio of about 1.4 was found in this experiment where cytidine was present as with uridine and adenosine alone. The effect of adenosine on uridine is probably concerned, therefore, with the utilization of uridine for growth directly rather than with its conversion to cytidine.

To ascertain whether the inhibitory effects of adenosine and guanosine on a mixture of cytidine and uridine were additive, increasing amounts of guanosine from 0.2 mg. to 1.0 mg. were added to flasks containing 0.25 mg. of cytidine, 0.25 mg. of uridine, and 0.45 mg. of adenosine in 25 ml. of basal medium. This mixture allows about 30 per cent of the normal growth of the mold to take place; *cf.* Fig. 3, *B*. The addition of the guanosine resulted in further inhibition.

To demonstrate that the antagonism observed between adenosine and cytidine is a competitive one, *i.e.*, that a constant ratio of antagonist to metabolite will give the same degree of growth regardless of the actual concentration of metabolite present, flasks were set up containing 0.25, 0.50, 0.75, and 1.0 mg. of cytidine per 25 ml. of basal medium. To these were added 0.07, 0.14, 0.21, and 0.28 mg. of adenosine, respectively. The percentage of normal growth obtained (as calculated from the standard growth curve (8)) was approximately the same at each concentration level of antagonist and metabolite, *i.e.*, 8, 6, 9, and 7 per cent, respectively.

Although the antagonism between cytidine and adenosine was also

demonstrated to exist in the pyrimidine-deficient mutant of *Neurospora* No. 263-1895-3a[4] (7), it could not be shown in the wild type which is able to synthesize its pyrimidine requirements. In determining this fact 5 mg. of adenosine were added to 25 ml. of basal medium, and the growth of the wild type measured in the usual way. The amount of growth did not differ significantly in the presence of adenosine from that found with the unsupplemented medium alone.

DISCUSSION

The *Neurospora* mutant No. 1298, unlike its wild type counterpart, fails to grow on the basal medium alone but grows when the medium is supplemented with either of the two pyrimidine ribonucleosides or ribonucleotides. As these compounds are essential constituents of ribonucleic acid, it is evident that the failure of growth in their absence is due to a deficiency in ribonucleic acid synthesis. Because normal growth is obtained with either cytidine or uridine or the corresponding nucleotides, it is also apparent that the mold can accomplish the amination of uridine with the formation of cytidine or the deamination of cytidine to uridine as well as the phosphorylations necessary for the formation of nucleic acid from the nucleosides.

The difference in the levels at which cytidine and uridine are inhibited suggests that the inhibition of at least two different reactions is involved. Evidence that the deamination of cytidine to uridine is inhibited is provided by the fact that cytidine was more readily inhibited than uridine and because the cytidine inhibition could be readily eliminated by the presence of an equimolar quantity of uridine. In the latter instance the conversion of cytidine to uridine was no longer required for nucleic acid synthesis and the inhibition of this reaction would not be expected to have the same inhibitory effect on the utilization of the two compounds for growth. At concentrations of adenosine which inhibited growth on uridine, however, it appears that it is the utilization of uridine which is affected rather than its conversion to cytidine, as this inhibition was not removed by the presence of cytidine. The nature of the reaction concerned in this case is not apparent.

The fact that the pyrimidine nucleotides, cytidylic and uridylic acids, are more strongly inhibited by adenosine than are the corresponding nucleosides is in agreement with their less efficient utilization for growth. Similarly adenosine-3-phosphate was less inhibitory than adenosine. These results are in agreement with several others which indicate that the

[4] This mutant was kindly provided by Dr. H. K. Mitchell, Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, California.

nucleosides may play a more central rôle in nucleic acid metabolism than either the free bases or their nucleotides.

The absence of adenosine inhibition in the wild type organism is in keeping with other observations that no inhibition is produced by closely related structural analogues when the substance concerned is not required for growth (11). In the wild type *Neurospora* an efficient mechanism may be present for the conversion of adenosine to adenine which later was found in these experiments not to be inhibitory. An alternative explanation may be that pyrimidine synthesis can be stimulated to balance the increased amount of adenosine present.

Of interest is the highly specific nature of the antagonism of the pyrimidine nucleosides by adenosine and guanosine and the striking reversal of the adenosine-cytidine inhibition by uridine. Unlike most inhibitions by closely related structural analogues in which high antagonist-metabolite ratios are necessary to produce inhibition, growth on cytidine was completely inhibited by an equimolar amount of adenosine. Similarly such a completely inhibitory mixture in the presence of a molecular equivalent of uridine behaved as if no antagonist at all were present. These experiments demonstrate the pronounced effect of the purine and pyrimidine nucleosides on growth in this strain of *Neurospora* and suggest a similar function in the control of growth in other organisms.

SUMMARY

The utilization of the pyrimidine ribonucleosides and ribonucleotides for growth by the pyrimidine-deficient mutant of *Neurospora* No. 1298 can be completely inhibited by the addition of adenosine or adenosine-3-phosphate to the culture medium. Adenosine is the most active antagonist, adenosine-3-phosphate is somewhat less so, and adenine has no antagonistic effect when added in comparable concentrations. The nucleotides are more readily inhibited than the nucleosides, and cytidylic acid and cytidine require less adenosine or adenosine-3-phosphate for inhibition than do uridylic acid or uridine. Guanosine and guanylic acid also inhibit the utilization of the pyrimidine compounds, but somewhat larger amounts are required. Guanine like adenine shows no inhibitory action at moderate concentrations.

The inhibition of cytidine by adenosine is strikingly reversed by the addition of an amount of uridine equal to the cytidine present. Uridine, however, is inhibited by the same concentration of adenosine regardless of whether or not an equimolar quantity of cytidine is present. These results suggest that at least two reactions may be involved in the inhibition, namely, the deamination of cytidine to uridine and the utilization of uridine itself for the synthesis of ribonucleic acid by the mold.

BIBLIOGRAPHY

1. Woolley, D. W., *J. Biol. Chem.*, **152,** 225 (1944).
2. Woods, D. D., *Biochem. J.*, **36,** 4 (1942).
3. Gladstone, G. P., *Brit. J. Exp. Path.*, **20,** 189 (1939).
4. Roblin, R. O., Jr., *Chem. Rev.*, **38,** 255 (1946).
5. Richards, M. B., *Brit. Med. J.*, **1,** 433 (1945).
6. Raska, S. B., *Science,* **105,** 126 (1947).
7. Loring, H. S., and Pierce, J. G., *J. Biol. Chem.*, **153,** 61 (1944).
8. Loring, H. S., Ordway, G. L., and Pierce, J. G., *J. Biol. Chem.*, **176,** 1123 (1948).
9. Loring, H. S., Roll, P. M., and Pierce, J. G., *J. Biol. Chem.*, **174,** 729 (1948).
10. Levene, P. A., and Bass, L. W., Nucleic Acids, American Chemical Society monograph series, New York (1931).
11. Woolley, D. W., and White, A. G. C., *J. Exp. Med.*, **78,** 285 (1943).