Enzyme induction in Escherichia coli is stimulated by certain 5-amino-2,4-bis(substituted-amino)pyrimidines (Kunkee, 1960a). Other 2,4-diaminopyrimidines have been reported to be antagonists of folic acid (Hitchings et al., 1952; Doctor, 1956). Since there are indications that the stimulation of enzyme induction involves nucleic acid metabolism (Kunkee, 1960b) and since folic acid is involved in nucleic acid precursor synthesis (Rogers and Shive, 1948), we wished to determine what effect the 5-amino-2,4-bis(substituted-amino)pyrimidines might have on folic acid metabolism. E. coli has no growth requirement for folic acid and enzyme induction in E. coli is not affected by folic acid either in the presence or in the absence of the compounds which stimulate induction (Kunkee, 1960a). Therefore, to discover a relationship between folic acid and the substituted pyrimidines, we studied the folic acid requirement for growth of streptococci.

MATERIALS AND METHODS

In these experiments, an adaptation of the assay for folic acid given in the Difco Manual (1953) was used.

Streptococcus lactis strain R (ATCC 8043, Streptococcus faecalis) was maintained on Microassay culture agar (Difco), and Micro inoculum broth (Difco Manual, 1953). The assay for folic acid was carried out in tubes containing 2.5 ml of folic acid assay medium (Difco) to which 0 to 1.5 μg folic acid (Nutritional Biochemicals Corp.) and various amounts of test compound were added. The final solution was brought to 5 ml and autoclaved. After inoculation the cultures were incubated for 24 hr in stationary tubes at 37 C.

Growth was determined by both turbidity and protein measurements. For turbidity readings the cultures were centrifuged in the cold for 30 min at 1000 × G, the pellets resuspended with 2.5 ml of 0.9 per cent NaCl, and the turbidity read at 700 μm. It was noticed that after incubation a slight brown precipitate was present in the culture tubes to which the 5-amino-2,4-bis (substituted-amino)pyrimidines had been added. To prevent the brown color from interfering with the turbidity readings, the readings were made at 700 μm.

The protein determinations were made on cells from 3-ml samples which had been centrifuged and washed once with 3 ml of cold 0.9 per cent NaCl. The pellets were resuspended with 0.6 ml of 0.5 N NaOH and heated momentarily at 100 C. The protein was then determined, using the method of Lowry et al. (1951).

All turbidity and optical density measurements were made in 10 by 75 mm cuvettes in the Coleman Junior spectrophotometer.

RESULTS

Using the folic acid assay (Difco Manual, 1953), we obtained a typical growth response curve, as measured by turbidity, with Streptococcus lactis and concentrations of folic acid of 0 to 1.5 μg per ml. Increased growth was found when the cells were incubated in the presence of folic acid and any of the 5-amino-2,4-bis (substituted-amino)pyrimidines which stimulate enzyme induction (Kunkee, 1960a). There was little or no growth with the substituted pyrimidine alone; thus the compounds did not have folic acid activity, but acted to make a given concentration of folic acid more effective. In the first experiments, growth was measured as turbidity of cultures after incubation for 24 hr. The assumption that the turbidity measurements were a reflection of growth was verified by protein determinations on the 24-hr cultures.

In figure 1 is seen the increased growth response, as measured by protein formation, of
S. lactis in the presence of 5-amino-2,4-bis(2-thenylamino)pyrimidine. The protein formation also increased with increasing concentration of the substituted pyrimidine (figure 2). It can be seen that there was little protein formation except when folic acid was present.

Colonies counts were also made on the cultures to determine if the increase in growth, as measured by turbidity and protein formation, was also apparent as an increase in viable cells. There was an increase in colony formers in the presence of the substituted pyrimidines (figure 1), and determinations with Petroff-Hausser bacteria counter showed about the same degree of increase in cells. Microscopic examination revealed no change in morphology of the cells grown in the presence of the substituted pyrimidines.

It was found that under the conditions of the assay the bacteria responded not only to folic acid but also to thymine, the formation of which has been shown to be folic acid-dependent (Rogers and Shive, 1948). However, in experiments with thymine without folic acid, it was found that there was no increased growth when 5-amino-2,4-bis(furfurylamino)pyrimidine was present. Thus, the effect of the substituted pyrimidine was a sparing of folic acid, not of thymine. This is consistent with the results in a previous paper (Kunkee, 1960b), where it was shown that the action of the substituted pyrimidines involved uracil metabolism but not thymine metabolism.

**DISCUSSION**

The substituted pyrimidines can be considered to be structural analogues of the pteridine portion of folic acid (schema 1). Thus, one explanation for the sparing effect is that the substituted

![Figure 1](image1)

**Figure 1.** Growth of *Streptococcus lactis* on various levels of folic acid in the presence and absence of substituted pyrimidines. (○) Control; (●) 5-amino-2,4-bis(2-thenylamino)pyrimidine-acetic acid, 36 μg per ml.

![Figure 2](image2)

**Figure 2.** Growth of *Streptococcus lactis* on various levels of 5-amino-2,4-bis(2-thenylamino)pyrimidine in the presence and in the absence of folic acid. (●) Folic acid, 0.4 μg per ml; (○) no folic acid.

![Schema 1](image3)

**Schema 1**
pyrimidines inhibit nonuseful metabolism of folic acid.

Other 2,4-diaminopyrimidines have been reported to be antagonists of folic acid in S. lactis (Hitchings et al., 1952). None of the 5-amino-2,4-bis(substituted-amino)pyrimidines tested showed any toxicity of S. lactis at the concentrations used (up to 100 µg per ml). However, the compounds which inhibited enzyme induction (Kunkee, 1960a), such as 2,4,5- and 2,4,6-triaminopyrimidines, also inhibited growth of S. lactis. Furthermore, 2,4-bis-furfurylamino-5-nitropyrimidine, which had no effect on enzyme induction, had no effect in the folic acid assay. The correlation of activity of the test compounds in stimulating enzyme induction and in sparing folic acid is presumptive evidence that these activities are directly related.

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SUMMARY

5-Amino-2,4-bis (substituted-amino) pyrimidines, which stimulate enzyme induction, also showed a sparing effect on the folic acid requirement of Streptococcus lactis. That is, there was increased growth in the presence of the compounds plus folic acid, and there was no growth in the presence of the substituted pyrimidines unless folic acid was present. It was suggested that the 5-amino-2, 4-bis(substituted-amino)-pyrimidines inhibited the catabolism of the added folic acid.

REFERENCES


