FAILURE OF FOLIC ACID TO ANTAGONIZE SULFANILAMIDE NON-COMPETITIVELY IN THE GROWTH OF LACTOBACILLUS ARABINOSUS 17-5*

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It has been postulated by Lampen and Jones (1) that, though less potent than p-aminobenzoic acid (PABA), folic acid (FA) is a growth factor for Lactobacillus arabinosus 17-5, and that PABA and p-aminobenzoyl-glutamic acid (PABG) indirectly are growth stimulants for this organism because they are utilized for the synthesis of FA. It was shown by us in the preceding paper (2) that (a) the aged FA solution which is 80 per cent decomposed is almost 100-fold more active than the solution of intact FA; (b) PABA and PABG are likewise at least 100-fold more active than the intact FA; and (c) when the pH of the medium is maintained at 6.2 to 6.6 during growth, the aged FA, PABA, and PABG complete their growth stimulation during a period of less than 40 hours; in contrast, the stimulation of growth by the intact FA is conditioned by a 60 hour or longer period of incubation which, apparently, is required for the splitting of the inactive FA into an active form. These facts permitted the conclusion that FA per se is inactive and that its decomposition products or products derived therefrom support the growth of L. arabinosus.

It was also postulated (1) that sulfonamides block the synthesis of FA via PABA, and that growth in the presence of FA is not subject to inhibition by these drugs for the reason that, there being no need for a synthesis of FA, there does not exist a susceptible reaction site for sulfonamides to block. On this basis, FA was considered a non-competitive antagonist to sulfonamides. If this were so, the growth in the presence of an optimal amount of FA and a sulfonamide should be expected to run simultaneously parallel to, or coincide with, the growth in the absence of the drug. The data presented here show that sulfanilamide (SA) inhibits the growth of L. arabinosus in the presence of FA. However, whenever a reversal occurs, it occurs at a period very much later than the conclusion of the log

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phase of the growth in the absence of sulfanilamide. It therefore follows that FA per se is not an antagonist.

EXPERIMENTAL

The experimental conditions are the same as those described previously. As in the previous paper (2), in the determination of the degree and rate of growth, one failed to observe the informative intermediary changes if the measurement was made merely at the end of a 24 or 48 hour period. Preliminary experiments convinced us of the necessity of observing the entire growth curve to obtain precise information about the effects of various factors or combinations of factors.

Results

In our experiments, 0.05 to 0.1 γ of FA per ml. represents the optimal concentration to support the growth of L. arabinosus. A 10- to 100-fold increase in FA concentration (1.0 to 5 γ per ml.) does not appreciably increase either the rate or degree of growth. The data presented in Fig. 1 (Curves 2 and 2a) and Fig. 2 (Curves 3 and 3a) show that the growth supported by 0.01 γ of FA per ml. (85 per cent of the maximal) is completely inhibited by 1.0 γ of SA per ml. It will also be seen (Fig.

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**Fig. 1.** Effect of aging on the antisulfanilamide activity of FA. Curve 1, 0.01 γ of FA aged for 48 hours before inoculation; Curve 1a, same as Curve 1 but containing 1 γ of SA; Curve 2, 0.01 γ of fresh FA per ml. of medium inoculated without aging; and Curve 2a, same as Curve 2 but containing 1 γ of SA.
2, Curves 2 and 2a) that the growth supported by 0.1 γ of FA per ml. is inhibited by 1 γ of SA per ml. In this case, however, at a time when the drug-free system has reached the end of the log phase, the drug-containing system begins to show reversal of inhibition, which is complete after a period of 12 hours; a lag from the control of at least 12 hours persists at all time intervals. A disparity of at least 6 hours between the drug-free and drug-containing systems (Curves 1 and 1a) persists even in the presence of 5 γ of FA per ml. which is 100-fold in excess of the optimal growth requirement. In no case, therefore, do rates of the growth of *L. arabinosus*

17-5 in SA-containing and SA-free systems run simultaneously parallel or coincide. The most critical argument against the concept that FA is a non-competitive antagonist to sulfanilamide is provided by the systems containing 0.01 γ of FA with and without 1 γ of SA per ml. Here, despite the fact that the growth in the SA-free system is 85 per cent of the optimal growth, FA is incapable of antagonizing the complete inhibition by sulfanilamide.

That the solutions of FA in sterile medium yield products which antagonize sulfanilamide is illustrated by the data presented in Fig. 1. Solutions of 0.01 γ of FA per ml. of medium which had been aged in the incu-
bator for 48 hours before inoculation manifested antisulfanilamide action at a very much earlier period than did the systems in which FA was not allowed to age before inoculation. Indeed, it is to be noted that 0.01 γ of FA per ml. of medium after aging for 48 hours showed antisulfanilamide action, while the corresponding system not subjected to aging failed to antagonize sulfanilamide (Fig. 1, Curve 2a, and Fig. 2, Curve 3a).

These observations are further corroborated by the data presented in Fig. 3 (Curves 1, 1a, 2, and 2a) which show that PABG and aged FA (80 per cent decomposed) antagonize 1 γ of SA per ml. 14 and 10 hours earlier, respectively, than the system containing fresh FA (Curve 3a). The latter system trails its respective control by a period of 20 hours. It will also be seen that fresh FA antagonizes 50 γ of SA per ml. only slightly (Curve 3b), while the complete inhibition by 500 γ of SA per ml. continues at the end of the 48 hour period (Curve 3c).

The gradual reversal by 0.05 to 5.0 γ of FA per ml. of the inhibition by 1 γ of SA per ml. can be related to the liberation of an adequate amount of decomposition products. As reported previously (2, 3), 1.0 to 5.0 γ of FA per ml. could yield 1 to 2 per cent diazotizable amine as PABG after an incubation period of 24 hours. The systems would therefore contain

Fig. 3. A comparison of the antisulfanilamide activity of PABG, aged FA, and fresh FA. Curve 1, 0.01 γ of PABG; Curve 1a, same as Curve 1 but containing 1 γ of SA; Curve 2, 0.01 γ of aged FA; Curve 2a, same as Curve 2 but containing 1 γ of SA; Curve 3, 0.01 γ of fresh FA; Curve 3a, same as Curve 3 but containing 1 γ of SA; Curve 3b, containing 50 γ of SA, and Curve 3c, containing 500 γ of SA.
0.01 to 0.05 \( \gamma \) of PABG per ml. to antagonize 1 \( \gamma \) of SA per ml. and to stimulate growth (see Fig. 3). In the presence of 0.1 \( \gamma \) of FA per ml. the concentration of PABG at the end of the 24 hour period would be about 0.001 to 0.002 \( \gamma \) per ml., which would cause only a partial reversal of the inhibition. This would permit partial growth, which is sufficient, as shown in Table I, to render the medium acidic and thereby render SA ineffective as inhibitor. As a consequence, a complete reversal of inhibition would follow.

**Critical Effect of Changing pH of Medium during Growth on Sulfonamide Inhibition**—The rate of the growth of a lactic acid bacterium is usually measured by titrating the amount of acids produced. Growth media used for this purpose have practically no buffering capacity. The medium used by Lampen and Jones (1), for example, contains only 0.1 gm. per cent of phosphate mixture of pH 6.6 to 6.8. This medium undergoes an increase in \( H^+ \) concentration before a measurable growth occurs. In view of the critical effect of pH on the degree of sulfanilamide inhibition, the relative rates of pH change and growth of *L. arabinosus* 17-5 in the medium (2) which contained a 2.5-fold greater amount of phosphate (0.25 gm. per cent) were investigated. The data are presented in Table I. It will be seen that, on the average, in an SA-containing system a turbidity reading of 2 indicates a change of pH from 6.6 to 6.3 and a reading of 7 indicates a change from pH 6.6 to 6.1. A turbidity of 2 corresponds to a 350-fold increase in the number of cells (an increase from 30,000 (inoculum) to 10,500,000 cells per ml. at the end of an 18 hour period). The very sparing growth which results from the metabolism of glucose in the presence of sulfanilamide is necessarily associated with an increase in the concentration of \( H^+ \). An increase in \( H^+ \) concentration would suppress the in-

### Table I

| Change of pH of Medium during Growth of *L. arabinosus* 17-5 |
|---------------------|---|---|---|---|---|---|---|
| 0.25 per cent phosphate medium + 1 per cent glucose. |
| Growth medium containing pH T. pH T. pH T. pH T. pH T. pH T. pH |
| 0.01 \( \gamma \) of PABA per ml. 6.6 | 2 | 6.5 | 9 | 6.1 | 43 | 5.6 | 81 | 4.9 | 135 | 4.5 |
| 0.01 \( " " \) \( +1 \gamma \) of SA per ml. 6.6 | 0 | 6.5 | 1 | 6.4 | 26 | 2 | 8 | 6.0 | 24 | 5.7 | 51 | 5.0 |
| 0.01 \( " " \) FA per ml. 6.6 | 0 | 6.5 | 1 | 6.5 | 36 | 4 | 11 | 6.0 | 28 | 5.8 | 69 | 5.0 |
| 0.01 \( " " \) \( +1 \gamma \) of SA per ml. 6.6 | 0 | 6.6 | 0 | 6.5 | 06 | 5.5 | 26 | 4 | 56 | 8 | 19 | 5.7 |

* T. turbidity readings with the Klett-Summerson photoelectric colorimeter, No. 54 filter.
hibitory activity of sulfanilamide and represent a condition in which a very much smaller amount of antagonists, PABA or PABG, etc., is required to abolish the inhibition. Schmelkes et al. (4), for example, reported that 20 mg. per cent of sulfanilamide inhibited the growth of Escherichia coli, at pH 7.6, 6.8, and 6.0, 95, 71, and 54 per cent, respectively. The inhibitions with 5 mg. per cent of sulfadiazine at pH 6.7, 5.7, and 4.6 were, respectively, 95, 89, and 17 per cent. Brueckner (5), in experiments with Staphylococcus aureus, observed that at pH 6.5 a 5-fold greater amount of sulfanilamide is required than at pH 7.8, and that at pH 6.5 the SA:PABA ratio is 21,000 and 840 at pH 7.8. A 25-fold smaller amount of PABA is therefore required at pH 6.5 than at 7.8 to antagonize a given amount of SA.

These observations establish the fact that during growth a medium with very negligible buffering capacity will undergo a continued shift from neutrality to high acidity. This would both reduce the potency of sulfanilamide and raise the potency of the antagonist and thus readily abolish the inhibition of growth. It is therefore to be expected that if the medium is strongly buffered to prevent it from becoming acid in reaction the in-
hibition by sulfanilamide would present a basically different pattern. Experiments were performed with systems containing an optimal concentration (0.05 \( \gamma \) per ml.) of FA in the presence of 0.25 and 2.5 gm. per cent of phosphate buffer. The initial pH of these systems was 6.8. The results of the experiment are presented in Fig. 4, Curves 1, 1a, 2, 2a, and 2b. Growth turbidities and pH values were determined at various intervals as shown in Fig. 4. It will be seen that growth in 0.25 and 2.5 gm. per cent of phosphate (Curves 1 and 2) is nearly equal in degree and rate. In

![Fig. 5. A comparison of antisulfanilamide activities of PABA, PABG, aged FA, and fresh FA in systems containing 3 gm. per cent of phosphate buffer and 0.5 per cent glucose to maintain the pH at 6.2. Curve 1, 0.01 \( \gamma \) of PABA or PABG; Curve 1a, 0.01 \( \gamma \) of PABA and 1 \( \gamma \) of SA; Curve 1b, 0.01 \( \gamma \) of PABG and 1 \( \gamma \) of SA; Curve 2, 0.01 \( \gamma \) of aged FA; Curve 2a, same as Curve 2 but containing 1 \( \gamma \) of SA; Curve 3, 0.01 \( \gamma \) of fresh FA; and Curve 3a, same as Curve 3 but containing 1 \( \gamma \) of SA.

The weakly buffered medium the pH had changed from 6.8 to 4.0 (Curves 1 and 1a). In the system (Curve 1a) containing 100 \( \gamma \) of SA per ml. when growth had barely started (turbidity 20, 26 hour period) the pH had changed from 6.8 to about 5.0. In this acid region sulfanilamide is ineffective. At this period, in which growth lags behind that of the control by 6 hours, the growth curve, as would be expected, climbs steeply, and reversal is complete at the end of the 40 hour period, when the pH of the medium is 3.9. In contrast, the pH of the reaction in 2.5 gm. per cent of phosphate medium (Curve 2) was 6.0 at the end of the 24 hour period and remained relatively constant throughout the entire growth period,
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Under these conditions growth in the presence of 100 γ of SA per ml. of medium was completely inhibited (Curve 2b), and in 1 γ of SA per ml. at the end of the 40 hour growth period a partial reversal (18 per cent) was evident (Curve 2a).

In another experiment, the phosphate content of the medium was increased to 3.0 gm. per cent and the glucose content was reduced to 0.5 per cent to increase the buffering capacity of the medium and to reduce the production of acid. The pH of this medium at 0 hour was 6.7 and remained at a level of pH 6.2 to 6.3 throughout the growth periods. Since glucose was reduced from 1.0 to 0.5 per cent, the total growth was likewise reduced to one-half of the growth obtained in systems with 1 per cent glucose. The data are presented in Fig. 5. It will be seen that growth in the presence of 0.01 γ per ml. of PABA, PABG, or aged FA follows the usual trend (Curves 1 and 2). The growth in the system with fresh FA (Curve 3) lags behind that of the others by nearly 22 hours. In the presence of 1.0 γ of SA per ml. the inhibitions are antagonized by PABA and PABG (Curves 1a and 1b). Here growth curves lag behind those of the respective controls by about 20 hours. There was a 26 hour interval before antagonism occurred (Curve 2a) in the presence of aged FA (80 per cent decomposed). There was no sign of antagonism to 1.0 γ of SA per ml. by fresh FA at the end of the 58 hour period (Curve 3a). The data show again that when the pH of the medium is maintained at a constant level near neutrality throughout the growth period FA is incapable of acting as a non-competitive antagonist to sulfanilamide.

SUMMARY

Folic acid per se has been shown not to be a growth stimulant for L. arabinosus 17-5. Growth in the presence of FA follows a characteristic induction period corresponding to the time required for the liberation from FA of a sufficient amount of more active products such as PABG, etc. Folic acid per se has likewise been shown to be incapable of functioning as an antagonist to sulfanilamide. A delayed reversal of the inhibition of growth in systems with FA has been shown to be related to an inadequate buffering capacity of the medium. Rendering the medium acid would abolish the inhibitory power of sulfanilamide. In a strongly buffered medium complete inhibition persists without reversal. The postulate that sulfonamides interfere with the synthesis of folic acid via p-aminobenzoic acid is not supported by the experimental facts reported here.

The data derived here with p-aminobenzoic acid or p-aminobenzoylglutamic acid in experiments with L. arabinosus 17-5 do not permit correlation with data with organisms stimulated solely by FA.
BIBLIOGRAPHY

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