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Inhibition of Utilization of Glutamic Acid by *Lactobacillus arabinosus*.* (19417)

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Patterns of free amino acids determined by 2-dimensional paper chromatography were studied in a variety of mouse tumors, comparing the neoplastic with normal homologous tissues(1,2). It was found that each normal tissue examined had a distribution of free amino acids characteristic for that tissue, while all of the tumors, regardless of origin or source, showed very similar patterns. One of the most interesting findings was that the glutamine levels in the tumors were uniformly lower than in most of the normal tissues. It appeared feasible to attempt to disturb the protein synthetic mechanisms in tumors by interference with glutamine metabolism, since glutamine may play a strategic role in protein synthesis by virtue of its participation in amide exchange reactions(3-5). Most normal tissues, which have relatively large amounts of free glutamine, would presumably be more resistant to such an interference.

Glutamine metabolism could be disturbed by inhibiting the synthesis of glutamine or by blocking its subsequent utilization in further metabolic processes. *Lactobacillus arabinosus* 17-5 can serve as a good test organism for screening agents which can act in either manner, since this organism can be used both for glutamic acid and for glutamine assay. The present communication describes the results of the tests of several compounds in these assay systems. Methionine sulfoxide and β-hydroxyglutamic acid, previously reported antimetabolites for glutamic acid in *L. arabinosus*(6,7), and γ-glutamylhydrazide, a glutamine antagonist for some strains of hemolytic streptococci(8), have also been studied.

Experimental, assay methods. The essential microbiological procedures have all been described previously(9) and references to the original literature given. All glutamic acid assays were performed at pH 6.0 in a low aspartic acid medium(10) (Medium 1), conditions which minimize lag. In most instances the glutamine assays were performed at pH 7.0 in a high aspartic acid medium(11) (Medium 2), under which circumstances there is a sharp differentiation in response to glutamine and glutamic acid in the low concentration ranges. In other experiments the same conditions were used for glutamine as for glutamic acid. Solutions of all of the substances tested and glutamine were sterilized by Seitz filtration. When determination of growth was made at only one time interval, the turbidity readings were made at 24 hours. Several experiments were performed in which readings were made at numerous time intervals. All

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TABLE I. Inhibition of Growth by β-Hydroxyglutamic Acid† "A" and Its Reversal with L-Glutamic Acid (Medium 1).

Amt of test substance/tube,* γ	Optical density BHG "A"	Optical density BHG "B"	Amt of glutamic acid/tube, γ	Optical density No addition	Optical density 500 γ of BHG "A"
0	.179	.213	0	.0	.0
100	.223	.237	10	.030	.0
200	.223	.226	20	.061	.0
300	.223	.257	30	.095	.0
400	.162	.255	40	.144	.008
500	.084	.251	50	.196	.019
600	.014	.240	60	.244	.123
900	.0	.280	90	.359	.310
1200	.0	.293	120	.445	.352
1500	.0	.255	150	.468	.396

* All tubes contained 50 γ of L-glutamic acid. † BHG.

readings were made in a room maintained at 37°, so that the tubes were at the same temperature during the entire experiment. *Compounds tested.* Nine of the compounds tested were kindly furnished by Dr. Karl Pfister of Merck and Co., Inc. Of these, L-methionine sulfoxide(12,6), L-γ-glutamyl hydrazide(8), DL-β-aspartyl hydrazide(8), L-γ-methylglutamate(13), and DL-pyrrolidone carboxylic acid(14) were secured by the indicated literature procedures. New synthetic methods, to be described in a later publication from the laboratories of Merck and Co., Inc.,‡§ were employed in the preparation of DL-α-methylglutamic acid (m.p. 168-70° decomp.); DL-β-hydroxyglutamic acid ("A" form, m.p. 198° decomp.; "B" form, as hydrate, m.p. 180-5° decomp.; anhydrous "B" form, m.p. 157-8° decomp.). (It is possible that a β-hydroxyglutamic acid reported to melt at 75° as a trihydrate and then to solidify and remelt at 185°(16) is identical with the "B" form of hydroxyglutamic acid mentioned above). The previously described γ-methylamide of L-glutamic acid(15) was kindly furnished by Dr. F. C. Meyer of the Research Department of the Monsanto Chemical Co.

Results. Results of initial screening tests. In the concentrations used (40 to 3000 γ per tube) only 2 of the substances were markedly inhibitory for growth on glutamic acid and none of them inhibited the utilization of glutamine. Methionine sulfoxide was not inhibitory under the present conditions of testing, even when the concentration present was 60 times that of glutamic acid. Although L-γ-glutamylhydrazide inhibits the utilization of glutamine in some streptococci(8), it did not inhibit the utilization of glutamine in the present test system. A more detailed description of the inhibitory properties of α-methylglutamic acid and β-hydroxyglutamic acid "A" will follow.

Inhibition of growth on glutamic acid by β-hydroxyglutamic acid "A". Table I shows the results of an experiment in which were tested the inhibitory effects of the racemates of the two diastereoisomers of β-hydroxyglutamic acid (BHG). The "A" acid was markedly inhibitory while the other isomer showed no inhibition and even possibly a slight stimulation of growth. It is seen that the inhibition is competitive with glutamic acid, since increasing amounts of glutamic acid in the presence of a constant amount of inhibitor decreased the extent of inhibition.

TABLE II. Inhibition of Growth by α-Methylglutamic Acid (All Tubes Contained 50 γ L-glutamic Acid; Medium 1).

Amt of α-methylglutamic acid per tube, γ	% of normal growth Exp. No. 1	Exp. No. 2	Exp. No. 3
100	56.2	92.7	55.5
200	37.5	71.1	54.5
300	16.3	18.7	48
400	6.9	3.9	—
500	5.9	2.3	34.9

‡ Rogers, E. F., Conbere, J. P., Leanza, W. J., and Pfister, K.

§ The authors are grateful to Dr. W. W. Umbreit of Merck and Co., for his interest which made possible the cooperation of the St. Louis and Rahway laboratories in this work.

TABLE III. Reversal of Inhibition by α-Methylglutamic Acid with L-Glutamic Acid (Medium 1).

Amt of glutamic acid/tube, γ	Optical density				
	Exp. 1		Exp. 2		
	Control	500 γ inhibitor/tube	Control	500 γ inhibitor/tube	1500 γ inhibitor/tube
10	.029	.0	.022	.0	—
20	.059	.0	.046	.0	—
30	.095	.011	.071	.006	.014
40	.143	.030	.120	.027	—
50	.192	.063	.168	.060	—
60	.244	.108	.219	—	.116
90	.357	.254	.343	—	.264
120	.445	.399	.432	—	.371
150	.468	.466	.458	—	.421

Inhibition of growth on glutamic acid by α-methylglutamic acid. In Table II are the results of 3 different experiments showing the inhibitory effects of different concentrations of α-methylglutamic acid (AMG) on growth in the presence of 50 γ of glutamic acid. Although inhibition was found in each experiment, the extent of inhibition differed from one experiment to the other. This was also found to be the case in a number of other similar experiments not reported in detail. A possible explanation for this will be suggested in a subsequent section. Increasing concentrations of glutamic acid produced a reversal of the inhibition exerted by a given amount of AMG (Table III), showing the competitive nature of the inhibition.

Time-course of growth on glutamic acid in presence and absence of AMG. In Fig. 1 is shown the 3-dimensional plot of the time-course of growth with glutamic acid alone under the standard assay conditions, employing time, optical density, and glutamic acid concentration as the variables. It is seen that little additional growth occurred after 24 hours at the lower concentrations and that suitable growth curves for assay purposes were obtained between 22 and 30 hours. In Fig. 2 are plotted the results of an experiment similar in all respects to that shown in Fig. 1 with the exception that all tubes contained 1500 γ of AMG. A comparison of the results in Fig. 1 and 2 shows clearly the marked inhibitory effect of the AMG. However, the inhibition was not absolute, since there was some growth in the presence of inhibitor at 41 hours in the tubes containing the 4 highest concentrations of glutamic acid. Although the growth with 150 γ of glutamic acid in the presence of AMG was only 28% of the control at 24 hours, it was 75% of the normal value at 30 hours. Similarly, in the presence of 120 γ of glutamic acid the inhibited growth was 9% of the control at 24 hours, but 59% at 30 hours. It is evident from this that although readings taken at only one time interval may give good qualitative evidence of the inhibitory potentialities of a compound in the present test series, little quantitative significance can be attached to such results. Inhibition ratios calculated from such data would be of little value. The variability observed in the degree of inhibition found for a given amount of AMG when growth was measured at 24 hours (Table II) could possibly have been caused by different rates at which the inhibition was overcome in the individual experiments.

Mechanism of action of α-methylglutamic acid and β-hydroxyglutamic acid "A". Results with methionine sulfoxide and an unresolved mixture of BHG indicated that these substances were active by virtue of their ability to prevent the amidation of glutamic acid(6,7). Small amounts of glutamine were able to overcome the inhibition, and the utilization of glutamine itself was not appreciably affected. The same mechanism is probably operative in the present experiments with AMG and BHG. Neither compound inhibited the utilization of glutamine. Actually there may have been a slight stimulation by the AMG and BHG "A", both of which were strongly inhibitory in the utilization of glu-

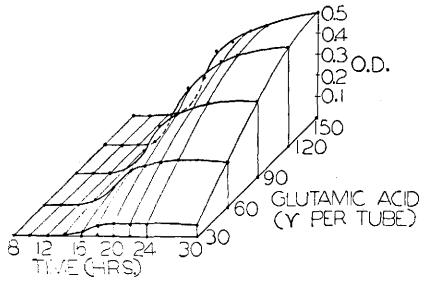

FIG. 1. Time-course of growth on different amounts of L-glutamic acid.

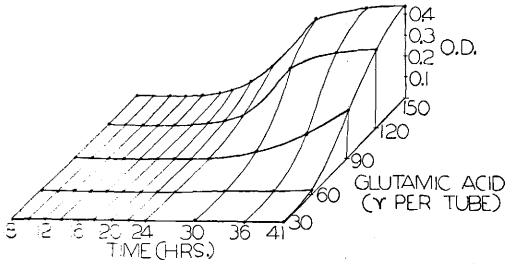

FIG. 2. Time-course of growth on different amounts of L-glutamic acid in the presence of 1500 γ of AMG per tube.

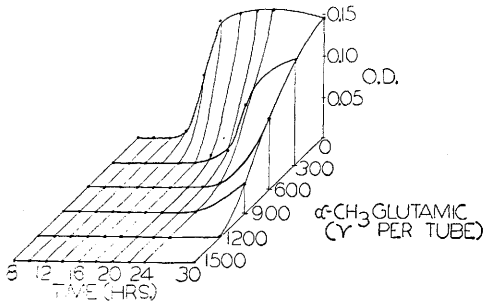

FIG. 3. Time-course of growth on 50 γ of L-glutamic acid in the presence of varying amounts of AMG.

tamic acid. In an experiment in which the time-course of growth was followed it was found that there was no growth for 18 hours when amounts of AMG from 300 to 1500 γ per tube were used in the presence of 50 γ of glutamic acid (Fig. 3). At this time the control tubes had attained almost maximal growth. However, at 30 hours the tubes containing 300 γ of AMG had 86% of the growth found in the controls, and the tubes containing 600 and 900 γ of AMG had begun to show a rapid growth. This emphasizes the importance of the consideration of the time factor in the reversal as well as the inhibition studies. It is doubtful in the present instance that the inhibition is overcome because of the destruction of the inhibitor. It would rather appear that there is a very slow rate of synthesis of glutamine going on. When a small critical concentration of glutamine has been attained the utilization of glutamic acid may proceed independently of the ability of the organism to synthesize glutamine.

Influence of α-methylglutamic acid on the time-course of growth on glutamine alone and on a mixture of glutamine and glutamic acid. The standard growth curves for glutamine were very similar to those found for glutamic acid (Fig. 1) when assays were performed in Medium 1. When varying amounts of AMG were added to 50 γ of glutamine virtually identical growth curves were obtained (Fig. 4). No increased time-lag in growth was noted at any concentration of inhibitor tested. There was a slight depression in the extent of growth below that in the control tubes in all of the samples containing AMG, but this was the same regardless of the concentration of AMG. A comparison of Fig. 3 and 4 shows clearly the remarkable difference between the effect of AMG on the utilization of glutamic acid and glutamine. It was previously suggested that AMG acts by preventing the synthesis of glutamine from glutamic acid, but that once small amounts of glutamine become available the bacteria can utilize the remainder of the glutamic acid. From this it would be expected that small amounts of glutamine should enable growth to take place on small quantities of glutamic acid in the presence of

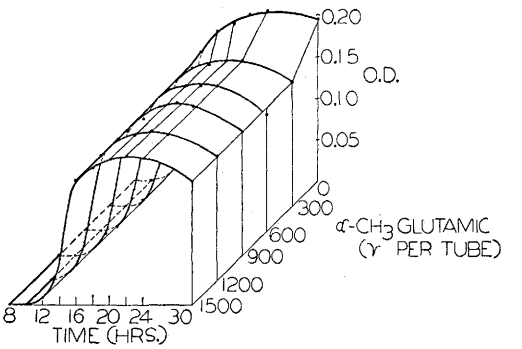

FIG. 4. Time-course of growth on 50 γ of L-glutamine in the presence of varying amounts of AMG.

a large amount of inhibitor. An experiment was performed in which each tube contained a mixture of 35 γ of glutamic acid and 15 γ of glutamine in the presence of varying amounts of AMG up to 1500 γ per tube. When 50 γ of glutamic acid alone was used in the presence of 1200 γ and 1500 γ of AMG per tube, there was no growth at all in 30 hours (Fig. 3). In the present experiment there was visible growth in all tubes by 16 hours. Even in the tubes containing 1200 γ and 1500 γ of AMG growth amounting to 89% and 78% of normal was found at 30 hours, showing clearly the marked protection afforded by small amounts of glutamine. However, some inhibition of growth by AMG was noted in the earlier time periods. It may well be that AMG also inhibits some steps in glutamic acid utilization other than amidation.

Inhibition of the α-ketoglutarate-glutamine system. It was shown previously that α-ketoglutarate does not replace glutamine or glutamic acid for growth in Medium 2 (9). Good growth occurred with α-ketoglutarate in the presence of small amounts of glutamine. The results suggested that the role of glutamine is catalytic. It was of interest to see whether this system could be inhibited by some of the substances tested for glutamic acid antagonism. From the results in Table IV it is seen that methionine sulfoxide and AMG are weakly inhibitory and that BHG "A" appears to be a somewhat stronger inhibitor. These findings are being extended in further studies.

Discussion. The purpose of the present investigation was to find inhibitors of glutamic acid and glutamine utilization in a microbiological test system which would also be effective in mammalian tissues. α-Methylglutamic acid (AMG) and one of the diastereoisomeric racemates of β-hydroxyglutamic acid were more potent inhibitors of glutamic acid utilization for *L. arabinosus* than any compounds previously described. All of the experiments performed were consistent with the interpretation that these compounds acted by preventing the synthesis of glutamine from glutamic acid. Tests are in progress to determine whether the amidation of glutamic acid in animal tissues is also inhibited by these substances. Preliminary tests with mice of the $C57_6$ strain weighing between 14 and 18 g have shown that these animals can tolerate for at least 10 days 30 mg of AMG administered daily by the intraperitoneal route without showing any toxic symptoms or loss in weight. An indication of the *in vivo* antagonism of AMG to glutamic acid also was obtained when it was found that this compound significantly enhanced the incidence of audiogenic seizures over the controls in a susceptible strain of mice (dba) tested under standard conditions (17). Marked reductions in seizure incidence and in fatalities below the control levels were obtained when glutamic acid was given. Tests are now being conducted with AMG in tumor-bearing animals. AMG has also been found to be a potent inhibitor of a bacterial glutamic acid decarboxylase and a somewhat weaker inhibitor of brain glutamic decarboxylase. These results will be reported subsequently.

TABLE IV. Inhibition of the α-Ketoglutarate–Glutamine System (All Tubes Contained 2.5 mg α-Ketoglutaric Acid and 30 γ of Glutamine; Medium 2).

Amt of inhibitor /tube, γ	Optical density		
	Methionine sulfoxide	α-Methylglutamic acid	β-Hydroxyglutamic acid A
0	.309	.301	.333
100	—	.330	.231
200	—	.290	.215
300	.288	.297	.210
400	—	.286	.194
500	—	.261	.171
600	.286	—	—
900	.268	—	—
1200	.254	—	—
1500	.223	—	—
1800	.244	—	—
2400	.222	—	—
3000	.210	—	—

Summary. 1. Ten compounds were tested for inhibition of utilization of glutamic acid and glutamine by *Lactobacillus arabinosus*. 2. DL-α-methylglutamic acid and one of the diastereoisomeric racemates of β-hydroxyglutamic acid were potent competitive inhibitors of glutamic acid utilization. None of the compounds tested was a glutamine inhibitor. 3. The results indicated that the two active compounds prevented the synthesis of glutamine from glutamic acid by the bacteria. 4. Experiments in which the time-course of growth was followed showed that under cer-

tain conditions the inhibition by α-methylglutamic acid could be overcome. A possible explanation was suggested for this finding and the importance of taking readings at several time intervals was emphasized.

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Virulence Enhancing Activities of Aureomycin on *Candida albicans.** (19418)

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Personal observations and a number of reports in the literature(1) indicate that proliferation of fungus-like organisms, notably *Candida albicans* may occur in patients treated with penicillin, aureomycin, chloramphenicol. and terramycin. Not only is there a numerical increase in the oral cavity, intestinal or urogenital tract, etc., but localized or disseminated disease with serious, and at times fatal consequences may develop.

The mechanism of stimulation of growth and increase in pathogenicity of fungi in patients treated with antibiotics is not fully understood. The frequency of these complications has become of sufficient impact to warrant the American Council on Pharmacy and Chemistry of the A.M.A. to issue "a warning statement to be included in aureomycin hydrochloride, chloramphenicol and terramycin hydrochloride labeling"(2). The Council suggests that susceptible bacteria are suppressed by the bacteriostatic action of the drugs, and monilia or other yeast-like organisms may then replace the normal or abnormal bacterial flora, thus paving the way for secondary mycotic infection. The following experiments may shed some light on these problems.

Material and methods. Candida albicans. A strain isolated from the oral cavity of a patient was used for all the tests. The strain grew in fluid media with sediment and no pellicle formation, fermented dextrose, maltose and sucrose, did not attack lactose. On Sabouraud's agar it produced white, creamy colonies, on blood agar plates dull grey colony forms and on corn meal agar streaks, it developed the characteristic mycelium and yeast cell clusters. It was therefore considered a typical *Candida albicans*. For animal tests, the growth from Sabouraud's agar slants was washed off with normal saline solution and

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