STUDIES ON THE METABOLISM OF MYCOBACTERIUM TUBERCULOSIS

V. The Effect of Amino Acids on the Growth of M. tuberculosis var. hominis[1]

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Received for publication December 11, 1953

Proskauer and Beck (1894) studied the nitrogen requirements of the tubercle bacillus by employing amino acids in various concentrations and combinations, and reported that asparagin, leucine, alanine, and glycocoll supported growth. Armand-Delille *et al.* (1913) obtained good growth of tubercle bacilli in the presence of arginine and of glycine but not in the presence of histidine. They recommended a synthetic medium containing both arginine and glycine since better growth was obtained in this medium than in a peptone-bouillon medium. Long (1922) found that the human strain H37 grew moderately well in a synthetic medium containing DL-alanine and L-alanine, but grew only slightly in the presence of leucine and histidine, and not at all in the presence of tryptophan, phenylalanine, or tyrosine. He felt that the benzene nucleus, present in the last three amino acids, was toxic for the microorganisms in small amounts. Crimm and Martos (1944) reported that no single amino acid when added to a peptone-glycerol broth or to an "amigen"-glycerol broth significantly increased the amount of surface growth of the H37 strain, as determined by weighing the pellicle. In addition seven individual groups of three amino acids each were added separately to a basal semisynthetic medium containing DL-leucine and 0.01 per cent "amigen" (a nonantigenic, enzymatic hydrolyzate of casein), and although two of the groups produced more growth than the other five, the growth was approximately 50 per cent less than was obtained in the 1.0 per cent amigen medium alone. They concluded from these findings that the amino acids, DL-phenylalanine, DL-serine, and L-asparagin, seemed more favorable for the growth of the strain H37 than any of the other amino acids examined. Marshak (1951), employing a semisynthetic medium which contained ammonium chloride, mineral salts, "tween 80", and bovine serum albumin, investigated the effect of a number of amino acids on the growth of the strains H37Rv and H37Ra. Growth was measured turbidimetrically, and the generation time of the tubercle bacilli in each medium was calculated. Marshak found that L-glutamic acid, L-asparagin, L-glutamine, L-histidine, and DL-aspartic acid increased the rate of growth of both strains, while DL-alanine increased the rate of growth of only the H37Ra strain.

The conflicting nature of the above reports is obvious, and while it is impossible to define all of the reasons for the discrepancies, these previous investigations do appear inadequate for one or more of the following reasons: (a) only a few amino acids were examined for their effect on the growth of *M. tuberculosis* var. *hominis*, (b) a basal synthetic medium was not employed, (c) the amino acid being examined was not the single source of nitrogen, (d) several concentrations of amino acids were not employed so the optimum concentration for growth may not have been used, (e) quantitative estimations of the amount or rate of growth were not made.

The present study, therefore, is concerned with the individual effect of a large number of amino acids in various concentrations on the growth of the virulent human variety of *M. tuberculosis* (H37Rv) under conditions designed to eliminate the above enumerated objections.

METHODS

With a few exceptions, which will be described in detail, the methods employed were similar to those reported in the first paper of this series (Youmans and Youmans, 1953*a*).

Three basal media were employed. The first was the modified Proskauer and Beck (P & B) synthetic medium which has been employed in previous studies (Youmans and Karlson, 1947). This medium contains 0.5 per cent asparagin, 0.5 per cent monopotassium phosphate, 0.15 per cent magnesium citrate, 0.05 per cent potassium

[1] This investigation was aided, in part, by a research grant from Parke, Davis & Company, Detroit 32, Michigan.

sulfate, and 2.0 per cent glycerol dissolved in water redistilled from glass. The second medium was a modification in which both the asparagin and magnesium citrate were omitted from this P & B medium; and the third basal medium lacked only the nitrogen source, asparagin.

Each amino acid, in the highest concentration employed, was dissolved in each of the three basal media, and three tenfold dilutions were made in the respective medium. After the hydrogen ion concentration had been adjusted to a pH of 7.0 with 40 per cent NaOH, each medium was tubed in 5.0 ml amounts into ten 18 by 150 mm pyrex test tubes previously rinsed with water redistilled from glass, capped with aluminum caps, and sterilized by autoclaving for 20 minutes at 15 lb. Two tubes of each medium were inoculated with each small inoculum (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} mg) of the virulent H37Rv strain of *M. tuberculosis* var. *hominis*, incubated at 37 C, and examined daily for evidence of growth as described earlier (Youmans and Youmans, 1953*a*). By appropriate treatment of the data obtained by recording the first day when growth appeared with each inoculum, the generation time for the tubercle bacilli in each medium could be estimated (Youmans and Youmans, 1949). At least two determinations were made with the different concentrations of each amino acid.

RESULTS

In table 1 is listed the generation time in hours of the tubercle bacilli grown in the medium from which both asparagin and magnesium citrate had been omitted. In those cases where such a calcu-

TABLE 1

The effect of amino acids on the growth of H37Rv when added to modified P & B medium from which asparagin and magnesium citrate had been omitted

AMINO ACID	GENERATION TIME IN HOURS			
	Amino acid concentration in per cent			
	1.0	0.1	0.01	0.001
β-Alanine	0	10^{-2}*	10^{-3}*	10^{-3}*
DL-Alanine	10^{-4}*	10^{-4}*	10^{-3}*	10^{-3}*
D-Arginine HCl	0	10^{-3}*	10^{-3}*	10^{-3}*
L-Asparagin	21.7	21.7	22.7	27.6
DL-Aspartic acid	10^{-3}*	24.0	27.6	10^{-3}*
DL-α-Amino-n-butyric acid	0	0	10^{-3}*	10^{-3}*
α-Amino-isobutyric acid	10^{-3}*	10^{-4}*	10^{-4}*	30.0
Cysteine HCl	I	I	10^{-3}*	10^{-4}*
L-Cystine	I	I	0	10^{-4}*
L-Glutamic acid	30.0–10^{-4}*	22.7–24.0	24.0–26.4	10^{-4}*
Glycine	10^{-3}*	10^{-4}*	10^{-3}*	10^{-3}*
L(−)-Histidine	10^{-3}*	26.4–27.6	26.4–27.6	26.4
DL-Isoleucine	0	10^{-4}*	10^{-4}*	10^{-3}*
L-Leucine	0	10^{-4}*	10^{-4}*	10^{-3}*
DL-Norleucine	0	10^{-3}*	10^{-4}*	10^{-4}*
DL-Lysine HCl	0	10^{-3}*	10^{-4}*	10^{-4}*
DL-Methionine	0	10^{-3}*	10^{-4}*	10^{-4}*
DL-Phenylalanine	0	10^{-2}*	10^{-4}*	10^{-4}*
L-Proline	31.2–10^{-4}*	27.6	10^{-4}*	10^{-3}*
DL-Serine	0	10^{-3}*	10^{-3}*	10^{-4}*
DL-Threonine	10^{-3}*	10^{-4}*	10^{-4}*	10^{-4}*
L(−)-Tryptophan	0	10^{-3}*	10^{-4}*	10^{-3}*
3,5-L-Diiodotyrosine	I	I	10^{-3}*	10^{-3}*
L(−)-Tyrosine	I	I	10^{-4}*	10^{-4}*
DL-Valine	10^{-2}*	10^{-4}*	10^{-4}*	10^{-4}*
Controls 10^{-4}*				

* Smallest inoculum (in milligrams) which grew; 0 = no growth; I = insoluble.

lation could not be made since growth was limited to only some of the more heavily inoculated tubes, the smallest inoculum which did grow during 5 weeks of incubation is recorded. In the few cases when the results obtained from two determinations were not the same, both values are given. This basal medium did not permit growth of as small inocula of the H37Rv strain as did the other two basal media, thereby allowing better definition of the effect of each amino acid on growth.

The addition of L-asparagin to this medium produced the most rapid and the heaviest growth. The presence of L-glutamic acid or DL-aspartic produced a slightly slower rate of multiplication. Of the other amino acids only L(−)-histidine, and L-proline in certain concentrations, supported growth of all inocula. Although DL-alanine did not support growth of the smaller inocula, the amount of growth of the larger inocula was much heavier than in the control. Growth was inhibited somewhat by DL-α-amino-n-butyric acid and L-cystine. The remaining amino acids, except in the usually inhibitory 1.0 per cent concentration, did not affect growth significantly.

In the basal medium from which only the asparagin had been omitted, the finding of greatest interest was the rapid rate of growth of the tubercle bacilli in the control medium. All inocula, even when prepared from carefully washed suspensions, grew in this medium, and the generation time (22.7 hours) did not differ significantly from that obtained in the presence of asparagin. Other experiments, however, have shown that the total mass of culture which develops following prolonged incubation is much less in the nitrogen-free medium than in the same medium containing asparagin.

In view of the rapid rate of growth of the organisms in the basal medium alone it was impossible to define the effect, if any, of most of the amino acids on the H37Rv strain. However, the inhibitory action of DL-α-amino-n-butyric acid and L-cystine was again evident. Also, in this medium cysteine HCl was inhibitory for the smaller inocula.

When the individual amino acids were incorporated in the basal medium which also contained asparagin, there was no evidence of any growth stimulating effect. Moreover, the presence of asparagin decreased the previously noted inhibitory effect of certain amino acids.

DISCUSSION

The finding of greatest interest in this study was the observation that the H37Rv strain grew in the basal synthetic medium which contained no added organic nitrogen source. All inocula grew, and the rate of growth was similar to that obtained when asparagin was present. The mass of growth which developed during prolonged incubation, however, was not as great. The phenomenon of growth of mycobacteria without a nitrogen source has been noted by others (Dingle and Weinzirl, 1932; Marshak and Schaefer, 1952; Schaefer *et al.*, 1949). Dingle and Weinzirl (1932) referred to the unpublished work of Weinzirl and Ott who had noted that virulent tubercle bacilli (strain H37) would continue to grow in Long's medium from which the asparagin had been eliminated, but that growth would stop in the absence of both asparagin and glycerol. Schaefer *et al.* (1949) observed that in their ammonium chloride-free "tween-albumin"-mineral salt medium the optical density of tubercle bacilli (strain H37Rv) "increased to 3 times the initial amount, provided glucose or glycerol was present in a concentration suitable for growth", after which lysis of the organisms occurred. They suggested as an explanation that bacteria grown in an excess of nitrogen stored sufficient nitrogen so that under conditions of a nitrogen deficiency the organisms still were able to synthesize new bacterial substance. However, in the present study, in order for an inoculum of 10^{-6} mg (200 to 500 bacteria) to become visible (approximately 150,000,000 bacteria), the tubercle bacilli would have to undergo at least 19 or 20 cell divisions in the nitrogen-free medium. It seems unlikely that the cells in the inoculum could have stored sufficient nitrogen for this number of cell divisions. Furthermore, the H37Rv strain has been maintained by serial transfer on this nitrogen-free medium for over three years (Youmans and Youmans, 1953*b*). Marshak and Schaefer (1952) by chemical methods found that the cells of the second generation, grown in a nitrogen deficient medium, contained approximately 40 per cent of the nitrogen measured in the original inoculum. Lysis would occur as the nitrogen content of the cells became depleted. Schaefer *et al.* (1949) suggested that the lysis might be due to the "tween 80" employed as a dispersing agent in their medium since this surface active agent might change the cell membrane in some fashion so that the normal resistance to the autodigestive

processes was lost. In the present study there was no gross evidence of lysis.

The nitrogen metabolism of the organisms appeared to be influenced somewhat by the magnesium citrate since when it was omitted from the nitrogen-free basal medium, the smallest inocula did not grow. No growth of any of the inocula occurred in the absence of glycerol which confirms the observations of Weinzirl and Ott (Dingle and Weinzirl, 1932) and Schaefer *et al.* (1949).

Because of the small inocula employed, the growth of this strain of *M. tuberculosis* in a medium containing no added source of organic nitrogen might be due to nitrogenous impurities or to the utilization of atmospheric ammonia. The possibility, however, that these organisms have some capacity to fix atmospheric nitrogen also must be considered. To test this possibility studies are currently being conducted in collaboration with Dr. R. H. Burris and Dr. P. W. Wilson of the University of Wisconsin employing isotopic nitrogen. The results will be reported in a separate communication.

The growth of this strain of *M. tuberculosis* var. *hominis* in a medium without an added source of nitrogen at a rate comparable to that obtained in media containing a source of organic nitrogen complicates the interpretation of the experimental findings. The results obtained using the medium from which the magnesium citrate also had been omitted are more definitive since growth in this medium was poor.

SUMMARY

Twenty-five amino acids were tested for their suitability as nitrogen sources for the growth of the H37Rv strain of *M. tuberculosis* var. *hominis* by incorporating them in various concentrations into three different basal media: (1) modified Proskauer and Beck (P & B) medium, (2) modified P & B medium from which asparagin had been omitted, (3) P & B medium free of both asparagin and magnesium citrate. In the last medium only L-asparagin, L-glutamic acid, and DL-aspartic acid served as good stimulants to growth, whereas L(−)-histidine, L-proline, and possibly DL-alanine stimulated growth to a lesser degree.

Growth of the tubercle bacilli in the basal medium deficient only in asparagin was just as rapid as growth in the same medium to which had been added an adequate nitrogen source.

The addition of the single amino acids to the complete P & B medium did not in any case result in a stimulation of growth.

Regardless of the medium employed the majority of the amino acids were inhibitory in a concentration of 1.0 per cent, and DL-α-amino-n-butyric acid, L-cystine, and cysteine were inhibitory in lower concentrations.

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