Quantitative Studies on Mutants of *Bacterium aerogenes* Requiring Nicotinic Acid and Histidine

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In the microbiological assay of amino-acids and vitamins various species of lactobacilli are the micro-organisms most frequently employed. The medium used for the cultivation of the lactobacilli and of most other micro-organisms at present used in assay procedure is complex, requires expensive ingredients and much time in preparation. The mutant strains of fungi such as *Neurospora* do indeed grow in simple media, but the harvesting of the mycelium is more laborious than either the turbidimetric or acidimetric measurements of bacterial growth. These disadvantages might be overcome by the use of a bacterium, in wild or mutant form, which would grow in simple medium and give a quantitative response to the required growth factor.

The assay of nicotinic acid with a strain of *Proteus vulgaris*, HX 19, was described by Grossowicz & Sherstinsky (1947). The medium contained casein hydrolysate, carbohydrate and mineral salts. The quantitative response of a mutant of *Escherichia coli* requiring arginine to the presence of arginine in simple medium and in the presence of protein hydrolysates has also recently been reported by Lampen & Jones (1947). As a preliminary study of the suitability of such mutants for use in microbiological assay the quantitative response of certain spontaneous and X-ray induced mutants of *Bacterium aerogenes* (*Aerobacter aerogenes*) to nicotinic acid and to histidine has been investigated. The mutants used in the present experiments were prepared and isolated by Devi, Pontecorvo & Higginbottom (1949).

EXPERIMENTAL

Micro-organisms. The mutant strains were all derived from *Bacterium aerogenes*, A10C, which grew well in a medium containing only glucose and inorganic salts. Of the two strains requiring nicotinic acid, 893 was a spontaneous mutant and 3A3 was obtained by X-irradiation. Both failed to grow in simple medium in the absence of nicotinic acid. The growth requirements for nicotinic acid could be met by the corresponding amide or by relatively large amounts of quinolinic acid.

The three histidine-requiring mutants were obtained by X-irradiation. One, 9B2, would grow, after a lag period, in the simple medium, and has been described as an 'adaptable' mutant (Devi *et al.* 1949). The other two mutants, 297 and 506, were probably identical and failed to grow in the absence of histidine. So far as could be ascertained the growth

requirements of these mutants were specific for nicotinic acid or amide or for histidine. Stock cultures of these mutant strains were carried on agar slopes of 'complete' medium.

Media. The 'complete' medium (pH 7.0) contained glucose 10 g.; peptone, 10 g.; yeastrel, 5.0 g. and agar 15 g./l. of tap water.

The 'simple' medium contained glucose 20 g.; $(\text{NH}_4)_2\text{SO}_4$, 1.0 g.; KH_2PO_4 , 3.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.04 g./l. of glass-distilled water. This was later modified to contain glucose, 20 g.; $(\text{NH}_4)_2\text{SO}_4$, 3.0 g.; KH_2PO_4 , 10.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.04 g.; sodium citrate, 18.5 g. in 1 l. glass-distilled water. The pH was maintained at 6.9–7.1 except where stated otherwise.

The basal (simple) medium was prepared in double the above concentration to allow for dilution by the addition of the standard solutions.

Standard solutions. Stock solutions containing 200 $\mu\text{g.}/\text{ml.}$ were prepared by dissolving 50 mg. L-histidine monohydrochloride, nicotinic acid or nicotinamide in 250 ml. glass-distilled water. The stock solution of quinolinic acid contained 68 mg. of the acid in 250 ml. glass-distilled water and thus contained the mol. equivalent of 200 $\mu\text{g.}$ nicotinic acid/ml. These solutions could be stored in the refrigerator for 2 weeks without deterioration, although the solution of nicotinamide was less stable than that of the corresponding acid. From the stock solutions fresh dilutions were prepared for each experiment.

Inoculum. Nicotinic acid solution (5 ml.) or histidine solution (5 ml.) was added to 5 or 10 ml. basal medium (depending on the micro-organism being used) and the mixture sterilized at a pressure of 10 lb./sq.in. for 10 min. When cool, the solution was inoculated from a stock culture on 'complete' agar medium and incubated for 21–24 hr. at 37°. The culture was then centrifuged and the bacterial sediment washed twice with 10 ml. quantities of sterile quarter-strength Ringer solution. The washed bacteria were suspended in 10 ml. quarter-strength Ringer solution, spun at a low speed to remove clumps, and the uniform suspension suitably diluted so that the turbidity of the inoculum (read on the Spekker absorptiometer) was the same in each experiment. The inoculum was freshly prepared for each assay.

Assay procedure. The assays were carried out in ordinary tubes 6 in. long and $\frac{1}{2}$ in. internal diameter. Graduated doses of the standard solutions in 5 ml. were added to the tubes, 5 ml. of the basal medium (double strength) added to each tube and the contents well mixed. Triplicate tubes were set up for each dose. The tubes were then capped with inverted specimen tubes and sterilized at a pressure of 10 lb./sq.in. for 10 min. When cool 0.05 ml. of the inoculum was added to each tube except the three used as controls.

After shaking well, the tubes were incubated in a water bath at 37°. Turbidimetric measurements of growth using the Spekker photoelectric absorptiometer were made after

incubation for 17 hr. and the results expressed as extinctions (i.e. as the Spekker drum readings). Acidimetric measurements were made after 1, 2 or 3 days incubation, using 0.1, 0.05 or 0.02N-NaOH with bromothymol blue as indicator. To each tube 1 ml. of 0.04% aqueous solution of bromothymol blue was added and the contents titrated with NaOH containing 100 ml. bromothymol blue/l., using a comparator.

RESULTS

Nicotinic acid-requiring mutants 893 and 3A3. The response of the strain 893 to doses of 0.02, 0.04, 0.1, 0.2, 0.4 and 1.0 μ g. nicotinic acid in 10 ml. basal medium was determined by both acidimetric and

Fig. 1. Dose-response curves, strain 893, to nicotinic acid in the original basal medium; x—x, turbidimetric measurements after 17 hr. at 37°; O—O, acidimetric measurements after 43 hr. at 37°.

turbidimetric methods. The response varied with the method of measurement (Fig. 1). The growth response to the lower doses of nicotinic acid was very poor. This 'lag' varied slightly in different determinations, but usually ceased with doses over 0.2 or 0.3 μ g. nicotinic acid. No 'lag' was shown in acidimetric titrations.

No delay in growth or acid production was obtained with nicotinamide (Fig. 2).

The simplicity of the medium or its low buffering action may have influenced the growth response to nicotinic acid. Therefore, before further study of the mutants, various modifications of the medium, which might increase the extent of growth and acid production while still maintaining a relatively simple composition, were investigated using the parent strain.

Modifications of the basal medium. The extent of growth or acid production was not appreciably affected by increasing the concentration of glucose, by the addition of 0.1% xylose or by increasing the amount of ammonium sulphate present up to 0.6%. A mixture of the purines, adenine, guanine, uracil

and xanthine, each in a concentration of 0.001%, inhibited both growth and acid production. Alanine, glycine and glutamic acid (nitrogen equivalent to that in 0.1% ammonium sulphate) did not satisfactorily replace ammonium sulphate as the source of nitrogen, although the addition of asparagine or glutamic acid to the basal medium gave increased growth and acid production.

By the addition of 0.75% vitamin-free casein hydrolysate (Ashe Laboratories Ltd.) to the basal medium, the growth as measured turbidimetrically was doubled, although acid production was only increased by one-third. The responses were similar,

Fig. 2. Dose-response curve, strain 893, to nicotinic acid and amide in original basal medium; turbidimetric measurements (Exps. I and II) after 17 hr. at 37°.

but much less marked, with a mixture of lysine and glutamic acid in the amounts presumed to be present (Schmidt, 1945) in 0.75% casein hydrolysate. Proline inhibited both growth and acid production, but this inhibition was removed by the addition of lysine.

To counter the poorer acid production compared with growth in the presence of casein hydrolysate modification of the buffering agents was investigated. Sodium acetate (0.16–4.0%) or the corresponding amounts of potassium acetate inhibited growth and acid production. The concentration of potassium dihydrogen phosphate was increased by two, three, four and six times that in the original basal medium. The greater the phosphate content the greater the amount of growth and particularly the acid production (titratable acidity). This effect was slightly enhanced by increasing the amount of glucose and ammonium sulphate to 3 and 0.3% respectively (Table 1).

Table 1. *The effect of addition of increased amounts of phosphate and other substances on growth and acid production by Bact. aerogenes A10C*

(Media A, B, C and D contained respectively two, three, four and six times the amount of KH_2PO_4 in the basal medium.)

Medium	Additions (%)	Turbidimetry after 17 hr. at 37° (Extinction E)	Acidimetry after 43 hr. at 37° (ml. 0.05N-NaOH)
A	Nil	0.486	9.5
	$(\text{NH}_4)_2\text{SO}_4$, 0.2	0.453	9.7
	Glucose, 1	0.463	9.8
	$(\text{NH}_4)_2\text{SO}_4$, 0.2 + glucose, 1	0.518	10.1
B	Nil	0.570	13.0
	$(\text{NH}_4)_2\text{SO}_4$, 0.2	0.663	13.7
	Glucose, 1	0.555	14.0
	$(\text{NH}_4)_2\text{SO}_4$, 0.2 + glucose, 1	0.645	14.3
C	Nil	0.691	15.6
	$(\text{NH}_4)_2\text{SO}_4$, 0.2	0.720	16.8
	Glucose, 1	0.650	16.5
	$(\text{NH}_4)_2\text{SO}_4$, 0.2 + glucose, 1	0.661	17.6
D	Nil	0.733	23.3

Although the highest growth response was obtained with a four- to six-fold increase in phosphate, discoloration of the medium occurred during sterilization. To avoid the necessity of sterilizing the

reaction in the various modifications of the medium was always adjusted to between pH 6.9 and 7.1.

The final modified basal medium, the composition of which was given on p. 391, contained increased amounts of ammonium sulphate and potassium dihydrogen phosphate and in addition sodium citrate. This basal medium was used for all subsequent

Fig. 3. Dose-response curve, strains 893 and 3A3, with nicotinic acid in original and modified basal medium. Acidimetric measurements after 48 hr. at 37°; x—x, strain 893 in original medium; o—o, strain 893 in modified medium; Δ—Δ, strain 3A3 in original medium; ●—●, strain 3A3 in modified medium.

glucose and phosphate portions of the medium separately, a threefold increase in phosphate was adopted. Additional buffering associated with a further increase in both growth and acid production was obtained when potassium (2%) or sodium (1.85%) citrate was included in the medium with three times the original amount of phosphate. The

Fig. 4. Dose-response curve, strains 893 and 3A3, to nicotinic acid in original and modified basal medium. Turbidimetric measurements after 17 hr. at 37°; x—x, strain 893 in original medium; o—o, strain 893 in modified medium; Δ—Δ, strain 3A3 in original medium; ●—●, strain 3A3 in modified medium.

quantitative studies of the mutant strains of *Bact. aerogenes*.

Quantitative response to nicotinic acid and amide. The dose-response curves of strains 893 and 3A3 to nicotinic acid in the original and the modified basal medium are given in Fig. 3 (titratable acidity) and Fig. 4 (turbidity measurement). The maximum

response, whether measured by acidimetric or turbidimetric methods, was greatly improved by the use of the modified medium, but the 'lag' in growth response to small doses of nicotinic acid in the turbidity method remained.

The addition of 0.3% vitamin-free casein hydrolysate to the modified basal medium did not affect the 'lag' in response to nicotinic acid (Fig. 5). Indeed the response was rather less in the presence than in the absence of casein hydrolysate except at the higher dose levels. The growth response to nicotinamide was greater at all dose levels in the presence of casein hydrolysate.

Fig. 5. Dose-response curve, strain 893, with nicotinic acid and nicotinamide in modified basal medium with and without casein hydrolysate. Turbidimetric measurements after 17 hr. at 37°; x—x, nicotinic acid without casein hydrolysate; o—o, nicotinic acid with casein hydrolysate; Δ—Δ, nicotinamide without casein hydrolysate; ●—●, nicotinamide with casein hydrolysate.

Factors affecting the 'lag' in growth response. In turbidimetric estimations of growth the incubation time was shorter, and Spekker readings were less time-consuming than the acidimetric estimations. Therefore in an attempt to eliminate the 'lag', and thus perfect the turbidimetric measurements of response, the effect of size of inoculum, reaction of the medium and the length of the incubation period at 37° were studied.

Increase in the density of the inoculum tended to reduce the 'lag' somewhat and also increased the growth response to higher doses of nicotinic acid. The reaction of the medium had a definite effect on the response. At pH 6.0 or below the 'lag' was eliminated, but under such conditions the response

to higher doses of nicotinic acid was much less than at pH 7.0 (Fig. 6). These results stress the importance of careful control of the reaction of the medium. The 'lag' was markedly reduced or eliminated by increasing the incubation period at 37° from the usual

Fig. 6. Effect of hydrogen-ion concentration on the dose-response curve of strain 893 with nicotinic acid; turbidimetric measurements after 17 hr. at 37°.

Fig. 7. Effect of period of incubation at 37° on dose-response curve of strain 893 with nicotinic acid; turbidimetric measurements. (Exps. I and II).

17 hr. to 41 or 48 hr. (Fig. 7). The absence of a 'lag' in the response measured by titration may thus have been due to the use of the longer incubation time of 48 hr. Finally, since there was no 'lag' in response

to the amide, an addition of nicotinamide to give a final concentration of $0.05 \mu\text{g./assay tube}$ was made to the modified basal medium. The growth response of strains 893 and 3A3 to nicotinic acid showed no 'lag' in the presence of suboptimal amounts of nicotinamide (Fig. 8).

Fig. 8. Dose-response curves, strains 893 and 3A3, with nicotinic acid in modified medium with and without suboptimal amounts of nicotinamide. Turbidimetric measurements after 17 hr. at 37° ; x—x, strain 893 without amide; o—o, strain 893 with amide; Δ — Δ , strain 3A3 without amide; $\bullet$ — $\bullet$, strain 3A3 with amide.

Response to quinolinic acid. No growth was obtained with quinolinic acid in doses equivalent to 0.1 – $1.5 \mu\text{g.}$ nicotinic acid. With doses of quinolinic acid about 1000 times that of nicotinic acid a response similar to that with nicotinic acid was obtained. It seems likely that this response was due, not to quinolinic acid itself, but to nicotinic acid present as an impurity or formed during the heat sterilization of the quinolinic acid. Similar results were obtained by Koser, Dorfman & Saunders (1940) with strains of *Proteus* sp. and dysentery bacilli which required nicotinic acid.

The histidine-requiring strains 297, 506 and 9B2. The response of strains 297 and 506 to 0, 10, 20, 30, 40, 50, 75, 100, 125 and $150 \mu\text{g.}$ histidine in the modified medium is given in Fig. 9. A smooth curve was given by strains 297 and 506 indicating a quantitative response of both growth and acid production to graded doses of histidine. The response with strain 9B2, an 'adaptable' mutant, was much greater, $10 \mu\text{g.}$ of histidine giving as much growth as $125 \mu\text{g.}$ histidine with strains 297 and 506.

The dose-response curves of strain 9B2 with histidine in doses from 0 to $30 \mu\text{g.}$ are given in

Fig. 10. The acidimetric titrations in this instance were recorded after incubation for 19 hr. when about three times as much acid was produced as after incubation for 43 hr. with strains 297 and 506.

Fig. 9. Dose-response curve, strains 506 and 297, with histidine. Turbidimetric measurements after 17 hr. at 37° ; x—x, strain 506; o—o, strain 297. Acidimetric measurements after 43 hr. at 37° ; Δ — Δ , strain 506; $\bullet$ — $\bullet$, strain 297.

Fig. 10. Dose-response curves of strain 9B2 to histidine; x—x, turbidimetric measurements after 17 hr. at 37° ; o—o, acidimetric measurements after 19 hr. at 37° .

DISCUSSION

The behaviour of the nicotinic acid-requiring mutants of *Bact. aerogenes*, designated 893 and 3A3, has conformed to that previously described for similar mutants of other micro-organisms. A lack of growth

response, a 'lag', in the presence of low doses of nicotinic acid has been observed by Bonner & Beadle (1946) with nicotinic-requiring mutants of *Neurospora*. With mutants of *Esch. coli*, Roepke, Libby & Small (1944) found a close correlation between the activity of nicotinic acid at a given pH and the dissociation value of the acid. The dissociation of nicotinic acid decreased with decrease in hydrogen-ion concentration, and it was concluded that only the undissociated acid could enter the cell and be utilized for metabolic purposes.

On the other hand, the relationship between the response to nicotinic acid and the corresponding amide showed a very close parallel with the results obtained in the utilization of glutamic acid and glutamine by *Lactobacillus arabinosus* (*plantarum*) (Lyman, Kuiken, Blotter & Hale, 1945; Hac, Snell & Williams, 1945). As in the present investigation with nicotinic acid and amide, the lag in response to small doses of glutamic acid was not obtained with the corresponding amide. Hac *et al.* (1945) showed that the 'lag' tended to be minimized by increasing the size of the inoculum, by longer periods of incubation and by an acid reaction in the medium. Lyman *et al.* (1945) found that the 'lag' with the acid could be eliminated by the addition of minimal amounts of the amide to the basal medium. They concluded that their results could readily be explained by the assumption that glutamine, rather than glutamic acid, was the substance actually utilized by the micro-organisms. Similarly, it might be concluded that nicotinic acid must be converted to nicotinamide before it can be utilized by the mutants 893 and 3A3. The most important part played by nicotinic acid is in relation to the phosphopyridine nucleotides where it is present as the amide. This would appear to give further support to the suggestion that the first step in the utilization of nicotinic acid is conversion to the amide.

The histidine-requiring strains gave a quantitative growth response to doses of 0–150 μ g. of histidine. The 'adaptable' mutant, 9B2, proved very much more sensitive in its response to the histidine. This may be related to its ability to grow in the basal medium in the absence of added histidine after a delay of at least 24 hr.

The application of these mutants to the actual assay of nicotinic acid or amide and of histidine in

biological material has not yet been attempted. The non-specific effect of the presence of other substances has not been studied with the mutants requiring histidine. The addition of casein hydrolysate to the basal medium did produce a further increase in growth response to nicotinic acid or amide, and it seems likely that further modification of the basal medium would be required should these mutants be used in microbiological assays.

SUMMARY

1. A study has been made of the quantitative response of certain mutants of *Bacterium aerogenes* (*Aerobacter aerogenes*), which require nicotinic acid and histidine, to the required metabolite in a simple basal medium containing glucose and mineral salts. The effect of making various modifications in the mineral salt content of the medium was investigated.

2. The strains requiring nicotinic acid, after incubation for 17 hr., showed a 'lag' in growth response to doses of nicotinic acid under 0.2–0.3 μ g., but not to nicotinamide. The limiting doses at which the 'lag' in growth response ceased varied somewhat in different estimations.

3. The 'lag' was reduced by increasing the size of the inoculum, by lengthening the period of incubation or by increasing the acidity of the medium. It could be eliminated by the addition of minimal amounts of nicotinamide to the basal medium.

4. A quantitative response to histidine in doses from 0 to 150 μ g. was obtained with the non-adaptable histidine-requiring mutants. The 'adaptable' mutant showed a much greater response to histidine.

5. The possibility of the use of these mutants in microbiological assay is briefly discussed.

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