STUDIES ON IMPROVEMENTS IN THE MEDIUM FOR *LACTOBACILLUS ACIDOPHILUS* IN THE ASSAY FOR DEOXYRIBONUCLEIC ACID[1]

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Lactobacillus (*Thermobacterium*) *acidophilus* strain R-26 has been proposed as an assay organism for the determination of deoxyribonucleic acids (Hoff-Jørgensen, 1952). In attempting to use this organism for deoxyribonucleic acid determinations in our laboratory, it was noted that growth of the culture was very poor unless crude supplements, such as yeast extract or liver extracts, were added to the medium. The highly significant response observed with these supplements warranted further investigation.

The unique requirement of *L. acidophilus* strain R-26 for a deoxyriboside which is not replaceable by vitamin B_{12} also suggested that the use of this organism for further studies on nucleic acid metabolism would be extremely fruitful.

EXPERIMENTAL METHODS

The culture of *L. acidophilus* strain R-26 was obtained from Dr. Hoff-Jørgensen. Stock cultures were maintained on 10 per cent litmus milk medium (Difco) to which 0.1 per cent cysteine and 0.5 per cent yeast extract (Difco) were added. The inoculum medium and the original assay medium used in these studies were the same as those reported by Hoff-Jørgensen.

The assay medium consisted of the following constituents per 100 ml of double strength medium; casein (acid hydrolyzed), 2.5 g; casein (enzyme hydrolyzed), 0.5 g; sodium acetate, 3.0 g; glucose, 3.0 g; "tween" 80, 0.1 g; DL-cysteine, 0.05 g; DL-tryptophan, 0.02 g; KH_2PO_4, 0.2 g; K_2HPO_4, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.02 g; $MnSO_4 \cdot 4H_2O$, 0.004 g; $FeSO_4 \cdot 7H_2O$, 0.002 g; adenine, 2.0 mg; guanine hydrochloride, 2.0 mg; thymine, 2.0 mg; cytidylic acid, 5.0 mg; riboflavin, 0.1 mg; nicotinic acid, 0.1 mg; calcium pantothenate, 0.1 mg; *p*-aminobenzoic acid, 0.1 mg; pteroylglutamic acid, 0.005 mg; thymidine, 0.8 mg and adjusted to pH 6.8 with 20 per cent NaOH. The casein hydrolyzates used in these studies were obtained from Nutritional Biochemicals Corporation.

The assay was carried out in 18- by 150-mm pyrex test tubes which had been previously calibrated at 660 mμ. Each tube contained a total vol of 10 ml of medium. Cells from a 24-hr culture were washed twice with sterile saline by centrifugation and diluted 1:100 with sterile saline. One drop of suspension was used as the inoculum for each tube in the assays. All the tubes in each assay were inoculated with the same pipette in order to minimize variation in the size of the inoculum. Growth of the cells was determined by turbidity measurements (optical density at 660 mμ) after 20 hr incubation at 37 C. Acid production was determined by titration of the tube content with 0.1 N NaOH.

Determinations of the increase in turbidity due to the addition of crude supplements showed that the greatest differences in growth were observed after 16 to 24 hr of incubation. Titration of the lactic acid production after a 20-hr incubation also showed a response attributable to crude supplementation. After an extended incubation period, these differences in response (turbidity or acid production) to crude supplementation were essentially eliminated.

Yeast extract (Difco) was used as the standard material for the active substance(s). The response curve to graded levels of yeast extract supplementation was obtained by determining the difference

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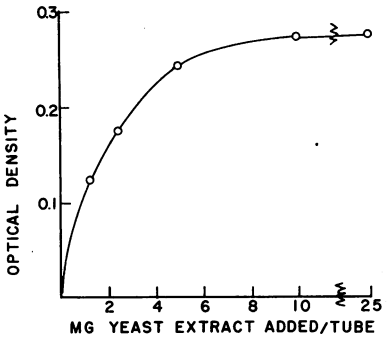

Figure 1. 20-hr turbidimetric growth response (as measured by optical density at 660 mμ) of *Lactobacillus acidophilus* strain R-26 to the addition of graded levels of yeast extract to the basal medium.

in turbidity between the nonsupplemented and supplemented tubes after a 20-hr incubation at 37 C.

RESULTS AND DISCUSSION

The response curve obtained with the addition of graded levels of yeast extract is shown in figure 1.

A number of known bacterial growth stimulatory substances were tested for activity and found to be inactive. Investigations of the stability of the stimulatory material in yeast extract showed it to be stable to autoclaving in 3 N HCl or 3 N NaOH for 16 hr at 20 lb pressure. Ashed samples of yeast extract were inactive.

A partially hydrolyzed solution of ribonucleic acid (Merck and Co.) was found to elicit a partial response for yeast extract activity. Subsequent determinations of the purine and pyrimidine requirements for *L. acidophilus* strain R-26 showed that the pyrimidine, uracil, was required by this organism. The uracil requirement for *L. acidophilus* strain R-26 had been reported previously by Jeener and Jeener (1952). However, the ability of *L. acidophilus* strain R-26 to grow on the basal medium of Hoff-Jørgensen indicated that the uracil requirement was, at least, partially satisfied in this medium, and other studies have suggested that the uracil activity of the original medium was associated with the cytidylic acid component, probably as a contaminant.

A standard response curve was obtained turbidimetrically when graded levels of uracil were added to a medium containing a new source of cytidylic acid (free of uracil). A number of possible compounds that could possess uracil activity were then assayed for uracil activity. Uridylic acid, orotic acid, and uracil deoxyriboside were more active than uracil (molar basis), however, the maximum growth response was the same for all the uracil active compounds tested. Uridine was unable to replace the uracil requirement of *L. acidophilus* strain R-26. Ureidosuccinate was also tested for uracil activity, since a number of investigators have reported evidence for the conversion of ureidosuccinate to uracil via orotic acid (Cooper and Wilson, 1954; Anderson, *et al.*, 1955; and Lieberman and Kornberg, 1953). Ureidosuccinate was, however, completely inactive.

Subsequent studies showed that the uracil requirement of *L. acidophilus* strain R-26 was also satisfied by growing the cells under an atmosphere containing 10 per cent CO_2. Newton *et al.* (1954), Deibel (1952) and Pappenheimer and Hottle (1940) had previously implicated CO_2 in the nucleic acid metabolism of a number of bacteria.

Adenine, guanine, uracil, and cytosine were required in the medium for a maximum response to purine and pyrimidine supplements. Hypoxanthine was only partially active as a purine source and 5-amino, 4-imidazolecarboxamide was inactive as a purine source.

Reevaluation of the growth response of *L. acidophilus* strain R-26 to graded levels of yeast extract in the presence of optimum uracil levels showed that a significant early growth response (20-hr incubation) to yeast extract supplementation remained. This response was of sufficient magnitude to warrant further study for other stimulatory agents contained in yeast extract.

The bacterial growth stimulatory substances tested previously were reinvestigated in the presence of adequate uracil in the medium, and pyridoxal phosphate was found to be active. Pyridoxal, pyridoxine, and pyridoxamine were also active. A maximum response to vitamin B_6 supplementation was obtained at a level of 1μg per tube with either pyridoxal phosphate, pyridoxal, pyridoxine or pyridoxamine.

When the casein component of the medium was replaced with a purified amino acid mixture (Schweigert *et al.*, 1949) pyridoxal phosphate did not exert a stimulatory response. The vitamin B_6 activity of alanine for a number of bacteria had been reported previously (Møller, 1950; Holden and Snell, 1949; Holden *et al.*, 1949; and Snell, 1945); therefore the absence of a response to

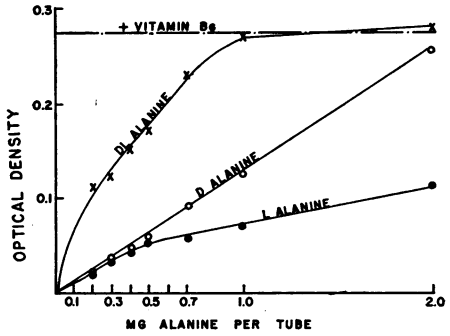

Figure 2. Vitamin B_6 activity of alanine for *Lactobacillus acidophilus* strain R-26 grown on the original medium plus uracil (20-hr incubation).

vitamin B_6 when the amino acid mixture (containing DL-alanine) was used indicated alanine may have vitamin B_6 activity for *L. acidophilus* strain R-26. The vitamin B_6 activity of D-alanine, L-alanine, and DL-alanine was determined, using the original casein medium plus uracil. The vitamin B_6 activity of alanine after 20-hr incubation is shown in figure 2.

D-Alanine and DL-alanine would replace the vitamin B_6 requirement for *L. acidophilus* strain R-26. However, the racemate was twice as active on a molar basis as the D-isomer. L-Alanine would replace approximately 50 per cent of the vitamin B_6 activity. These results indicate a synergistic response with the D and L forms of alanine, possibly a balance effect. It should be noted here that the casein component of the medium used in these studies contains a considerable amount of L-alanine (approximately 8 mg per tube, Block and Bolling, 1950). Supplementation of this medium with vitamin B_6 plus DL-alanine did not surpass the response due to vitamin B_6 alone. The maximum response of *L. acidophilus* strain R-26 to vitamin B_6 plus uracil was, however, still not equal to the maximum response observed with yeast extract.

In attempting to compare the response to vitamin B_6 using the original casein medium plus uracil and the medium in which the casein was replaced by the purified amino acid mixture, it was noted that the addition of enzymatic digested casein to the amino acid medium resulted in a pronounced stimulation of growth. The activity in the enzymatic digest of casein was stable to 16-hr autoclaving at 20-lb pressure in 3 N HCl or 3 N NaOH. Glutamine, cystine, carnosine, and anserine were also tested for activity and were found to be inactive in replacing the casein response. These results suggested that the stimulatory agent in enzymatic digest of casein is not one of the strepogenin compounds described by Sprince and Wooley (1944) or the histidine peptides described by Peters *et al.* (1953) and Snell (1945). Enzymatic digest of casein added at a level of 25 mg per tube (10 ml medium) would support optimum growth in the amino acid medium.

The response to enzymatic casein in the amino acid medium was compared with a medium in which the casein was replaced by amino acids added in the same ratio as those described by Block and Bolling (1950) for the amino acid content of casein. This mixture of amino acids (to stimulate casein) would not replace the activity of the enzymatic casein, indicating that the response is not due to amino acid imbalance effects or a limiting amino acid supplied by casein. The growth response to added L-alanine in the original casein medium which contained a high level of L-alanine may also be indicative of an amino acid imbalance in the original casein medium. The growth response to an amino acid-enzymatic casein medium plus uracil and vitamin B_6 was equivalent to the growth response observed when optimum amounts of yeast extract were supplemented to the original medium.

A modified medium was formulated on the basis of the results described above and the composition of this medium is shown in table 1. No stimulatory responses were observed when graded levels of yeast extract were added to this medium.

Since *L. acidophilus* strain R-26 is used as an assay organism for deoxyribonucleic acid (Hoff-Jørgensen, 1952), the deoxyribonucleoside (and deoxyribonucleotide) activities of a number of compounds were determined, using the original medium plus uracil and the modified amino acid medium of table 1. The response to the deoxyribonucleoside compounds was determined by turbidity readings after 20-hr incubation. Thymidine was used as the reference standard and the molar activities of the compounds were expressed as percentage of thymidine activity per mole. The deoxyribonucleic acid derivatives used in these studies were obtained from the California Foundation for Biochemical Research, Los Angeles, California.

Figure 3 shows the 20-hr incubation response

TABLE 1

Composition of double strength modified amino acid-casein medium for Lactobacillus acidophilus strain R-26

Constituent	Amount/100 ml	
	g	*mg*
Casein (enzyme hydrolyzed)	0.5	
L-Glutamic acid		80
L-Asparagine		80
DL-Lysine		40
DL-Serine		40
DL-Threonine		40
DL-Alanine		40
L-Cysteine		40
L-Proline		20
DL-Methionine		40
L-Leucine		40
DL-Isoleucine		40
DL-Valine		40
L-Histidine		20
DL-Phenylalanine		20
DL-Tryptophan		20
L-Arginine		20
L-Tryosine		20
Glycine		20
Glucose (anhydrous)	3.0	
Sodium acetate (anhydrous)	3.0	
"Tween" 80	0.1	
KH_2PO_4	0.2	
K_2HPO_4	0.2	
$MgSO_4 \cdot 4H_2O$	0.02	
$MnSO_4 \cdot 4H_2O$	0.004	
$FeSO_4 \cdot 7H_2O$	0.002	
Thymine		2.0
Adenine		2.0
Guanine		2.0
Uracil		2.0
Cytidylic acid		5.0
Guanylic acid		3.0
Adenylic acid		3.0
Riboflavin		0.1
Nicotinic acid		0.1
Calcium pantothenate		0.1
p-Aminobenzoic acid		0.1
Pteroylglutamic acid		0.005
Pyridoxal phosphate		0.1
Thymidine		0.8

Adjusted to pH 6.8 with 20% NaOH.

curve to graded levels of thymidine, using the original medium plus uracil and the modified medium of table 1. The lower levels of thymidine (up to 2 μg per tube) gave identical response in both media; however, higher levels of thymidine gave no additional response in the original medium plus uracil. The use of the modified medium (table 1), however, showed additional increments in turbidity due to the addition of thymidine to a level of 24 μg per tube. This increase in range of the response curve to deoxyribonucleosides and the increase in growth due to the use of the modified medium indicate that the original medium may give erroneous assay results due to impurities of a stimulatory nature in the test supplements.

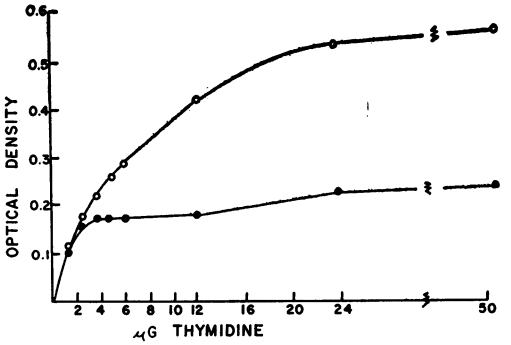

Figure 3. 20-hr turbidimetric response curve of *Lactobacillus acidophilus* strain R-26 to graded levels of thymidine using the original medium plus uracil (closed circles) and the modified medium of table 1 (open circles).

TABLE 2

Activity of deoxyribonucleic acid derivatives for Lactobacillus acidophilus strain R-26

Compound	Per Cent Activity* 20-Hr Turbidimetric Assay	
	Original medium plus uracil	Modified medium
Adenine deoxyriboside	99	73
Cytosine deoxyriboside	101	73
Guanine deoxyriboside	76	66
Hypoxanthine deoxyriboside	78	67
Uracil deoxyriboside	100	86
Deoxyadenylic acid	73	36
Deoxyguanylic acid	11	11
Thymidylic acid	32	22
Deoxycytidylic acid	147	93

* Relative to thymidine activity; thymidine = 100% activity.

The molar activities of the deoxyribonucleoside and deoxyribonucleotide compounds determined by the turbidimetric assay are presented in table 2. The over-all activities of the deoxyribonucleosides tested were somewhat less than those ob-

served for thymidine when either of the two media were used. Adenine deoxyriboside, cytosine deoxyriboside, and uracil deoxyriboside had activities equivalent to thymidine when the original medium plus uracil was used; however, their activities were 27–14 per cent lower than thymidine when modified medium (table 1) was used. The total growth response to deoxyribonucleoside levels, using the modified medium of table 1, was considerably more than that obtained using the original medium plus uracil (figure 3).

The deoxyribonucleotides varied extensively in their deoxyribonucleoside activity. Deoxycytidylic acid was the most active deoxyribonucleotide tested, with an activity equal to that of thymidine. Deoxyguanylic and thymidylic acid were of extremely low activity (11 and 32 per cent, respectively). These variations in the deoxyribonucleotide activities indicate that the assay procedure for deoxyribonucleic acid (Hoff-Jørgensen, 1952) should be modified to include hydrolysis procedures to form the deoxyribonucleosides prior to assay. Further studies on the incorporation of the deoxyribonucleosides and deoxyribonucleotides into the deoxyribonucleic acid of *L. acidophilus* strain R-26 are being initiated in view of these results.

SUMMARY

A significant growth response by *Lactobacillus acidophilus* strain R-26 was observed when crude supplements were added to a medium presumably adequate for this organism. The nutritional requirements of this organism were reevaluated in order to determine the nature of the response and to improve the medium for measuring deoxyribosides with this organism.

The pyrimidines, uracil and cytosine, and the purines, adenine and guanine, were required for maximum growth. The uracil requirement was replaceable by uridylic acid, orotic acid, uracil deoxyriboside, and CO_2; however, ureidosuccinate and uridine were inactive.

Pyridoxal phosphate, pyridoxamine, pyridoxine, or pyridoxal were stimulatory and of approximately equal activity. The response to vitamin B_6 was replaceable by D- or DL-alanine, and partially replaceable by L-alanine. DL-Alanine was twice as active as D-alanine.

Preliminary tests have indicated the presence of a stimulatory factor in enzymatic casein which is probably not an amino acid or known stimulatory peptide. Partial replacement of the casein hydrolyzate with an amino acid mixture also resulted in a significant growth response, indicating a possible amino acid imbalance in the original medium.

A modified assay medium formulated to include the stimulatory substances determined in these studies increased the range of the assay and the total bacterial growth.

The relative activity of a number of deoxyribonucleosides and deoxyribonucleotides for *L. acidophilus* strain R-26 was determined, using the original medium and the modified medium. The activity of deoxyguanylic acid, and deoxyadenylic acid and thymidylic acid, was significantly lower than that of thymidine when either of the two media were used.

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