TYROSINE DECARBOXYLASE

II. Pyridoxine-deficient Medium for Apoenzyme Production

W. D. BELLAMY and I. C. GUNSALUS
Laboratory of Bacteriology, Cornell University, Ithaca, New York

Received for publication March 8, 1945

Previous communications from this laboratory have established the function of pyridoxine derivatives[1] in the tyrosine decarboxylase system of *Streptococcus faecalis*, and a synthetic medium has been described for the production of cells active in this function (Bellamy and Gunsalus, 1943, 1944). Cells grown in media deficient in pyridoxine derivatives have been shown to contain the tyrosine decarboxylating enzyme but not the coenzyme (Gunsalus and Bellamy, 1944a). Pyridoxal (Snell, 1944) will activate this enzyme in the living cells (Gunsalus and Bellamy, 1944b) but dried cell preparations from them are active only if adenosine triphosphate (ATP) is supplied in addition to pyridoxal (Gunsalus, Bellamy, and Umbreit, 1944).

The growth of cells deficient in coenzyme appeared to offer a simpler method for the preparation of the decarboxylase apoenzyme than would the more conventional method of isolation and resolution of the enzyme. Therefore, the present work was undertaken. The main objective has been the production of the greatest possible yield of tyrosine decarboxylase apoenzyme with minimum coenzyme content per unit of medium. This differs from the previous study in which the primary purpose was to find which vitamin or vitamins function in the tyrosine system and to produce as active a tyrosine decarboxylase as possible (Bellamy and Gunsalus, 1944).

The method of apoenzyme production utilized the discovery of Snell and Guirard (1943) that an excess of alanine will support growth of *Streptococcus faecalis*, strain R, in the absence of pyridoxine derivatives. The lack of coenzyme in cells grown with alanine indicates that vitamin B_6 analogues convertible into coenzyme are not synthesized from alanine during growth. This constitutes an exception to the working hypothesis used by Snell and Guirard (1943), who suggest that alanine might be used as an essential nucleus for the synthesis of pyridoxine. However, evidence of synthesis of pyridoxine derivatives by other lactic acid bacteria has been reported by Bohonos, Hutchings, and Peterson (1942).

In the present paper, a medium for the production of a very active tyrosine decarboxylase apoenzyme in *Streptococcus faecalis*, strain R, is reported, and some of the factors affecting its production are discussed. These cells respond quantitatively to pyridoxal, and dried cell preparations from them respond to pyridoxal in the presence of ATP.

[1] The term "vitamin B_6 group" has been applied to the compounds pyridoxine, pyridoxal, and pyridoxamine (Snell, 1944, J. Am. Chem. Soc., **66**, 2082; Harris, Heyl, and Folkers, 1944, J. Am. Chem. Soc., **66**, 2088), and should be used in place of "pyridoxine derivatives."

EXPERIMENTAL

Method. The method of approach was that of Mueller as used previously in establishing the function of pyridoxine derivatives in the tyrosine decarboxylation system (Bellamy and Gunsalus, 1944). The quantity of growth and the tyrosine decarboxylase content of the cells, both as harvested and in the presence of pyridoxal, were determined with each change in the medium. In addition to the activity per unit of cells, $Q_{CO_2}(N)$, the activity per unit of growth medium was recorded. The latter, called Q(M), is defined as the μl of CO_2 evolved from tyrosine per hour by the cells harvested from 10 ml of medium. These values serve a useful purpose in this type of study because they show when a factor is limiting the total enzyme production without respect to growth. For the exploratory studies, cultures were grown in 10-ml amounts in 16-mm test tubes, the cells centrifuged down, suspended in 1.5 ml of water or saline, and 0.5 ml used per Warburg cup. By this procedure several changes in medium could be studied at one time with the expenditure of only slightly more time and material than is required for nutritional studies, or for the bioassay of vitamins.

As one of the purposes of this study was the production of apoenzyme for use in studies of the coenzyme, it was desirable that the medium be prepared as cheaply as possible; thus, the emphasis on the highest possible Q(M).

The medium used previously in establishing the function of pyridoxine derivatives in the tyrosine decarboxylase system was used as a starting point for this study. Several changes were required in order to improve the activity of the R strain. These included the substitution of hydrolyzed casein for gelatin, an increase in the level of carbohydrate, the addition of alanine, and an adjustment in the source and level of folic acid. The casein was substituted for gelatin in order to avoid the high level of glycine which Snell and Guirard (1943) had found to inhibit growth.

Culture. Streptococcus faecalis, strain R, was used in preference to the 10C1 strain employed in the previous study of tyrosine decarboxylase because improved growth in the synthetic media and more reproducible activity were obtained with the R culture. The use of the R strain also enabled us to take advantage of the considerable amount of information available, in the papers of Snell and others, regarding the nutritive requirements of this organism. The main drawback in the use of the culture was the maximum $Q_{CO_2}(N)$ (tyrosine) of 400 so far obtained, as compared to 2,000 to 3,000 with the 10C1 strain (Gunsalus and Bellamy, 1944b). However, in spite of the low maximum activity, the ratio between the activity of the deficient cells and those stimulated with pyridoxal was greater with the R culture. The higher coenzyme content of the 10C1 strain could not be avoided because alanine would not substitute for pyridoxine derivatives with this culture. Therefore, a low level of pyridoxine was added to support growth so that a small amount of coenzyme was formed.

The stock culture was maintained at refrigerator temperature in an agar composed of 1 per cent tryptone, 0.3 per cent K_2HPO_4, 0.3 per cent $CaCO_3$, 0.1 per cent glucose, 1.5 per cent agar, and 10 per cent by volume of liver extract (prepared by heating 1 pound of ground liver in two liters of water in flowing steam

for one hour and straining off the solids). Before use the culture was transferred from the stock agar to a buffered tryptone yeast extract broth (Wood and Gunsalus, 1942) and incubated at 30 to 35 C. The synthetic medium was inoculated with one drop (0.5 per cent) of a 12- to 24-hour broth culture which had been diluted with 20 volumes of sterile distilled water. By maintaining the culture in active condition and improving the base medium, the decarboxylase activity of this culture was increased from $Q_{CO_2}(N)$ of 400 to about 3,000. Also, the growth was nearly doubled, thus increasing the Q(M) from around 100 to nearly 2,000.

Medium for Apoenzyme Production

A satisfactory medium for the production of tyrosine decarboxylase apoenzyme is given in table 1. This medium is autoclaved 15 minutes at 15 pounds' pressure, cooled, inoculated with 0.02 per cent (0.5 per cent of a culture diluted 20 times) of a 12- to 24-hour broth culture, and incubated 15 to 20 hours at 37 C. The cells are harvested by centrifuge, suspended in saline, and used in Warburg experiments at a level of 1 to 3 mg of cells per flask. To test the tyrosine decarboxylase activity pyridoxal, or coenzyme, must be added, as the enzyme is almost entirely devoid of coenzyme and thus does not decarboxylate tyrosine. The pyridoxal is converted into coenzyme by the living cells.

As indicated in table 2, the cell suspension shows a very low rate of tyrosine decarboxylation ($Q_{CO_2}(N)$ 75), which is increased about twentyfold by the addition of pyridoxal, i.e., to $Q_{CO_2}(N)$ 2,700. The rate is not increased further by the addition of ATP. These cells are as active in the decarboxylation of tyrosine as are cells grown in a medium containing pyridoxal, and differ from such cells mainly in their lack of coenzyme.

A stable powder with tyrosine decarboxylase activity may be prepared by drying these cells *in vacuo* over drierite. The cells are harvested as for living cell experiments but are washed once with saline and suspended in distilled water before drying. For best results the cell suspensions are placed in petri dish halves—not over 10 ml each—and each placed in a separate pyrex desiccator containing 2 to 3 pounds of drierite. Upon evacuation with an oil pump to 1 mm or less of mercury pressure, the contents of the petri dish soon freeze and drying occurs from the frozen state. With sufficient desiccant and a high vacuum, the material dries completely within 10 to 15 hours to yield a fluffy powder. This dry cell powder retains virtually all of the decarboxylase activity present in the fresh cells and may be kept indefinitely over drierite without appreciable decrease in activity. The data in figure 1 show the decarboxylase response of the dried preparation to pyridoxal and to pyridoxal plus ATP. The preparation differs from the living cells in the requirement for ATP in addition to pyridoxal for activation, whereas with living cells pyridoxal is sufficient. The preparation alone, or with ATP added, shows practically no tyrosine decarboxylation.[2]

[2] These cell preparations have been adapted to the quantitative determination of pyridoxal and of (codecarboxylase) pyridoxal phosphate (Umbreit, Bellamy, and Gunsalus, 1945, Arch. Biochem., 7, 185–199).

TABLE 1

Medium for tyrosine decarboxylase apoenzyme production

	QUANTITY PER 10 ML
Acid-hydrolyzed casein	100 mg.
Glucose	100 mg
K_2HPO_4	50 mg
Sodium acetate	20 mg
Salts B*	0.05 ml
Alanine	2 mg
Cystine	2 mg
Tryptophane	1 mg
Sodium thioglycollate	1 mg
Adenine sulfate	50 μg
Guanine hydrochloride	50 μg
Uracil	50 μg
Nicotinic acid	50 μg
Riboflavin	10 μg
Calcium pantothenate	10 μg
SLR factor†	0.025 μg
Biotin	0.01 μg
pH	7.2–7.3

* Salts B = $MgSO_4 \cdot 7H_2O$ 10 g; NaCl 0.5 g; $FeSO_4 \cdot 7H_2O$ 0.5 g; $MnSO_4 \cdot 4H_2O$ 0.5 g; Water 250 ml.

† Kindly furnished by the Research Laboratories of Merck and Co., Inc.

TABLE 2

Tyrosine decarboxylase activity of Streptococcus faecalis R cells

Growth: Medium as table 1.
Nephelometer reading 110 ≅ 0.66 mg bacterial nitrogen per 10 ml
Evelyn reading 660λ = 47

Warburg cups: 1 ml 0.075 M phthalate buffer pH 5.0; 0.5 ml cell suspension ≅ 1 to 3 mg cells; pyridoxal, ATP, or water to 2.5 ml; 0.5 ml M/30 tyrosine, from side arm.

ADDITIONS TO WARBURG CUPS	TYROSINE DECARBOXYLATION	
	$Q_{CO_2}(N)$*	$Q_{CO_2}(M)$†
None	75	55
10 μg pyridoxal	2700	2200
10 μg pyridoxal + 1 mg ATP	1800	1450

* = μl CO_2 per hour per mg bacterial nitrogen.

† = growth x $Q_{CO_2}(N)$; = μl CO_2 per hour per cells from 10 ml medium.

Factors Which Influence the Production of Tyrosine Decarboxylase

Nicotinic acid. The level of nicotinic acid in the growth medium has been reported to influence the tyrosine decarboxylase activity of the cells aside from its effect on growth (Bellamy and Gunsalus, 1943). With lower levels of nico-

tinic acid, however, the glycollytic and the tyrosine decarboxylase activity of the cells decreased simultaneously, thus indicating the possibility that the reduction in tyrosine decarboxylation was the result of an effect on the energy metabolism of the cells rather than directly on the decarboxylase. This contrasted with pyridoxine deficiency which affected the tyrosine system without altering the rate of glycolysis (Bellamy and Gunsalus, 1944). After the requirement of ATP for the conversion of pyridoxal into coenzyme was found, the importance of the energy level of the cell to the tyrosine decarboxylase was more apparent. As might be expected, the effect of nicotinic acid on apoenzyme production was less than on the production of the complete enzyme. Although reasonably good activity was obtained with 0.25 and 0.5 μg nicotinic acid per 10 ml, the Q(M) could be increased a third if 10 to 50 μg were added (table 3). As nicotinic acid

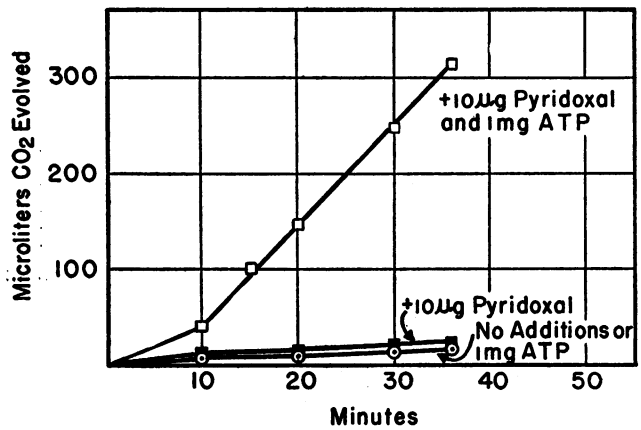

FIG. 1. TYROSINE DECARBOXYLATION BY DRIED CELL PREPARATIONS

Twenty-hour cells centrifuged, washed in 1/5 volume saline, suspended in a small volume of water, vacuum dried over drierite. In Warburg cups: 0.44 mg bacterial nitrogen; 1.0 ml 0.075 M phthalate buffer pH 5.0; 0.5 ml M/30 tyrosine suspension, in side arm; water or other additions to 3.0 ml.

is relatively cheap, the 50 μg level was used in the base medium. With the increased nicotinic acid content of the medium the pH falls more rapidly during growth and reaches a lower final value (table 3). This may account for the better activity with the excess of nicotinic acid.

Alanine. Snell and Guirard (1943) have shown that alanine must be added at a higher level than is present in hydrolyzed casein if the R strain of *Streptococcus faecalis* is to be grown in the absence of pyridoxine derivatives. The influence of the addition of alanine to the medium is shown in table 4. One mg of additional alanine per 10 ml was sufficient for maximum growth but did not support maximum enzyme production. The 2-mg level, however, was satisfactory for this purpose.

Purines and pyrimidines. It first appeared that the level of purines in the medium used previously was too low, and, indeed, some improvement in the yield of enzyme was obtained if the level of adenine was increased. However,

with adenine, as with nicotinic acid, the effect upon the production of the active enzyme was greater than upon the production of apoenzyme. For growth and apoenzyme production 50 μg each of guanine, uracil, and adenine were used per 10 ml of medium. Usually guanine plus uracil yielded reasonably active tyrosine decarboxylase. Occasionally, however, the activity was very poor, but reached the maximum value when 50 μg adenine were added per tube in addition to the guanine and uracil. In the process of developing a satisfactory

TABLE 3

Influence of nicotinic acid on tyrosine decarboxylase

Warburg cups as in table 2

NICOTINIC ACID IN MEDIUM	GROWTH	FINAL pH	TYROSINE DECARBOXYLATION	
			$Q_{CO_2}(N)$	$Q_{CO_2}(M)$
μ/10 ml	*turbidity**			
0	20	6.3	1100	132
0.12	75	5.1	1080	480
0.25	95	4.8	2100	1200
0.5	110	4.6	1900	1200
10	105	4.2	2400	1500
50	105	4.2	2570	1620
100	105	4.2	2570	1620

* Nephelometer reading, each scale unit $\cong$ 6 μg of bacterial N per 10 ml.

TABLE 4

Influence of alanine on tyrosine decarboxylase

Warburg cup as in table 2

ALANINE ADDED TO MEDIUM	GROWTH	FINAL pH	TYROSINE DECARBOXYLATION	
			$Q_{CO_2}(N)$*	$Q_{CO_2}(M)$*
mg/10 ml	*turbidity**			
0	32	4.7		
1	110	4.3	2300	1420
2	110	4.3	2880	1900
5	110	4.2	2360	1560
10	95	4.2	3260	1780

* See table 3.

medium as much as 250 μg of adenine were required in some batches to obtain optimum enzyme production. This variation in the amount of adenine required was finally eliminated when a satisfactory level of the SLR factor was established.

The effect of the purines and pyrimidines on tyrosine decarboxylase production varies with the strain of *S. faecalis* employed. For example, with the 10C1 strain the tyrosine decarboxylase formation is decreased rather than increased by doubling the concentration of purines. Also, increased levels of uracil or guanine were harmful. In this connection, see Snell and Mitchell (1941) and Stokes (1944).

The effect of thymine, which is also important in the nutrition of these cultures, will be discussed along with the folic acid complex.

Folic acid and related compounds. The folic acid requirement may be met by a number of somewhat related substances including thymine, folic acid (concentrate), and SLR factor. For a discussion of the relationship of these compounds, see Stokes (1944) and Stokes *et al.* (1944). When poor growth and variable tyrosine decarboxylase apoenzyme production were traced to the folic acid in use, Dr. Keresztesy of Merck and Company kindly supplied us with

TABLE 5

Influence of various members of the folic acid complex on growth and tyrosine decarboxylase production

SOURCE OF "FOLIC ACID"	GROWTH	FINAL pH	$Q_{CO_2}(N)$	$Q_{CO_2}(M)$
μg/10 ml	*turbidity*			
SLR factor				
0	0			
0.0025	75	4.5	2300	1660
0.025	120	4.2	2840	1880
0.25	115	4.2	2550	1600
Thymine*				
5	5	6.6		
50	89	4.3	2700	1460
100	98	4.4	3100	1800
400	84	4.3	2600	1300
Folic acid* concentrate				
0.0025	5			
0.01	16			
0.1	120	4.2	1560	1040
1.0	125	4.2	1310	940

Base as listed in table 1, except SLR factor omitted.

* We wish to thank Dr. E. E. Snell, Department of Chemistry, University of Texas, for this material.

sufficient SLR factor for the continuation of the work. This material, at a level of 0.025 μg per 10 ml, will support maximum growth and activity of the cells. The dried cell preparations so far used in the study of the coenzyme have been prepared with this factor in the medium.

More recently, however, it has been found that in the base medium (table 1) thymine at 100 μg per tube and folic acid at 0.1 μg per tube will replace the SLR factor. The folic acid was in the form of a concentrate of 5,000 potency. The effect of these three substances at several levels is given in table 5. In each case, levels which were below the amount required for maximum growth resulted in a decreased activity. This seemed to be accompanied by a higher final pH than was found in the media which yielded optimum activity.

Thiamine. It was found that thiamine does not influence growth or apoenzyme production by this culture. Therefore, it may be omitted from the base medium.

Age of culture. As an acid reaction favors decarboxylase production, sufficient carbohydrate and a long enough incubation period for the medium to become strongly acid are desirable. To insure an excess of carbohydrate, 1 per cent glucose was added to the medium in place of the 0.5 per cent used previously.

A study of the influence of the age of the culture on the tyrosine decarboxylase showed that the activity reached a maximum within 1 to 2 hours after the pH fell below 5.0. With an adequate medium, and the size of inoculum used in this work, the culture reaches maximum growth and a low pH within 8 to 10 hours at 37 C. Therefore, if this were the only consideration, cells could safely be harvested after 10 to 12 hours' incubation. However, the rate of decarboxylation without added pyridoxal is greater in young cultures than in older ones. For this reason, it is beneficial to harvest cells as old as compatible with the stability of the enzyme. The ATP level of the cells will also be lower in aged cultures so that less decarboxylation occurs upon addition of pyridoxal. In the present study, the best balance of these factors was realized by harvesting 16- to 20-hour cultures.

Cell preparations obtained under the above conditions usually contain very little ATP, as indicated by low Q_{CO_2} with pyridoxal in the absence of ATP. However, if a preparation does contain larger amounts of ATP, this may be removed by incubating the dried cells in a phthalate buffer at pH 5.0 for several hours at 0 C.

DISCUSSION

For the production of cells, or cell preparations, of *Streptococcus faecalis* R with a minimum amount of coenzyme and a maximum concentration of tyrosine decarboxylase apoenzyme, a careful balance of the factors which control these activities is necessary. The principle of optimum nutrition for the maximum function of a specific system, without regard to the quantity of growth, holds for the production of apoenzyme devoid of coenzyme, as well as for the production of highly functional cells as shown earlier (Bellamy and Gunsalus, 1944).

The production of an apoenzyme nearly free of coenzyme by growth under controlled conditions offers a promising method for the study of enzyme reactions without the tedious process of resolving an enzyme after its separation from tissue.

The production of an apoenzyme by growth of an organism in the absence of the vitamin required for the formation of the coenzyme constitutes evidence against the universal occurrence of B vitamins in living cells. Thus, the general hypothesis that those B vitamins not required for growth are synthesized by the cell appears not to be valid in this instance. If the hypothesis of universal occurrence were valid it should not be possible to grow cells without a coenzyme as has been done in the present case. Therefore, it is suggested that some cells, of which *Streptococcus faecalis* R appears to be one, can grow and carry on their metabolism without pyridoxine or related compounds possessing vitamin B_6 activity. A somewhat similar case involving folic acid has been reported by Stokes (1944), who indicates that folic acid is not present in *Streptococcus faecalis* R cells grown in thymine.

SUMMARY

By control of the medium, a tyrosine decarboxylase enzyme free of its coenzyme (pyridoxal phosphate) has been produced during the growth of a culture of *Streptococcus faecalis* R.

This appears to constitute an exception to the universal presence of B vitamins in living cells.

Dried cell preparations obtained from deficient cells may be used for the detection and estimation of pyridoxal and pyridoxal phosphate (codecarboxylase).

REFERENCES

BELLAMY, W. D., AND GUNSALUS, I. C. 1943 Growth requirements of *Streptococcus fecalis* for tyrosine decarboxylation. J. Bact., **46**, 573.

BELLAMY, W. D., AND GUNSALUS, I. C. 1944 Tyrosine decarboxylation by streptococci: growth requirements for active cell production. J. Bact., **48**, 191–199.

BOHONOS, N., HUTCHINGS, B. L., AND PETERSON, W. H. 1942 Pyridoxine nutrition of lactic acid bacteria. J. Bact., **44**, 479–485.

GUNSALUS, I. C., AND BELLAMY, W. D. 1944a A function of pyridoxal. J. Biol. Chem., **155**, 357–358.

GUNSALUS, I. C., AND BELLAMY, W. D. 1944b The function of pyridoxine and pyridoxine derivatives in the decarboxylation of tyrosine. J. Biol. Chem., **155**, 557–563.

GUNSALUS, I. C., BELLAMY, W. D., AND UMBREIT, W. W. 1944 A phosphorylated derivative of pyridoxal as the coenzyme of tyrosine decarboxylase. J. Biol. Chem., **155**, 685–686.

SNELL, E. E. 1944 Vitamin activities of "pyridoxal" and "pyridoxamine." J. Biol. Chem., **154**, 313–314.

SNELL, E. E., AND GUIRARD, B. M. 1943 Some interrelationships of pyridoxine, alanine, and glycine in their effect on certain lactic acid bacteria. Proc. Nat. Acad. Sci. U. S., **29**, 66–73.

SNELL, E. E., AND MITCHELL, H. K. 1941 Purine and pyrimidine bases as growth substances for lactic acid bacteria. Proc. Nat. Acad. Sci. U. S., **27**, 1–7.

STOKES, J. L. 1944 Substitution of thymine for "folic acid" in the nutrition of lactic acid bacteria. J. Bact., **48**, 201–209.

STOKES, J. L., KERESZTESY, J. C., AND FOSTER, J. W. 1944 Relation of the *Streptococcus lactis* R factor to "folic acid." Science, **100**, 523–524.

WOOD, A. J., AND GUNSALUS, I. C. 1942 The production of active resting cells of streptococci. J. Bact., **44**. 333–341.