FUNCTION OF THE VITAMIN B_6 GROUP: PYRIDOXAL PHOSPHATE (CODECARBOXYLASE) IN TRANSAMINATION

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Vitamin B_6 has for some time been implicated in protein metabolism. More recently a specific function of pyridoxal in the coenzome of tyrosine decarboxylase has been found (1), and the discovery of this function extended to include amino acid decarboxylases in general (2, 3). All members of the vitamin B_6 group (pyridoxine, pyridoxal,[1] pyridoxamine) are converted into this coenzyme (termed *codecarboxylase* (4) in recognition of its function in amino acid decarboxylases) by those organisms using them as a source of vitamin B_6 (5). The amino acid decarboxylases so far shown to require the coenzyme include tyrosine (2, 5, 6), lysine (7), arginine (2, 3), ornithine (2), glutamic acid (3), and dopa (3, 4-dihydroxyphenylalanine[2]).

The exact structure of the coenzyme is not certain but the properties are sufficiently well known to indicate that it is a phosphorylated derivative of pyridoxal and to suggest the possible position of linkage. The naturally occurring and the synthetic preparations of coenzyme, which possess similar properties (2, 8), are referred to here as pyridoxal phosphate, or codecarboxylase, without further specification of structure. The function of codecarboxylase in amino acid decarboxylation assures it a place in any consideration of protein metabolism but does not necessarily mean that this is the only function of the vitamin B_6 group.

Schlenk and Snell (9) have recently reported that tissues of rats on a vitamin B_6-deficient diet possess low transaminase activity as compared with those of animals supplied adequate amounts of vitamin B_6. The transamination rate of deficient tissues could at times be stimulated by the addition of pyridoxal and adenosine triphosphate, a procedure known to activate the tyrosine decarboxylase system of dried cell preparations of *Streptococcus faecalis* (6).

Snell (10) has also demonstrated interconversion of pyridoxal and pyridoxamine by heating with amino and keto acids respectively, and has

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[1] We are indebted to the Research Laboratories of Merck and Company, Inc., for the pyridoxal used in these experiments.

[2] Green, D. E., personal communication.

suggested that biological reactions to which these systems are analogous may exist. The biological reaction of the conversion of pyridoxamine into pyridoxal (in the form of pyridoxal phosphate) in the presence of pyruvate has been demonstrated by resting cells of *Streptococcus faecalis* (5).

Studies, to date, of transaminase enzymes have left some uncertainty as to the presence of a coenzyme; Kritzmann (11) reported that a coenzyme is required for the aspartic transaminase, whereas Cohen (12) was unable to find evidence that a coenzyme is involved in transamination. Leloir and Green (13) have isolated two transaminase enzymes in highly purified condition and reported that no evidence was found of dissociable prosthetic groups or of significant amounts of B complex vitamins.[3]

The recent demonstration of transamination in lactic acid bacteria (14) offers a new approach to this problem. Cohen and Lichstein (15), using *Streptococcus faecalis* R grown in the pyridoxine-deficient medium (16), were able to demonstrate a decreased rate of tyrosine decarboxylation but not of transamination. They therefore concluded that vitamin B_6 is not involved in transamination. It now appears that their results may be questioned on the basis of the presence of small amounts of pyridoxine in the medium, which, although not enough to saturate the tyrosine enzyme, may have been sufficient for activity of the transamination system.

In this study, vitamin B_6 in the form of pyridoxal phosphate (codecarboxylase) has been shown to function in transamination. Improved methods for the production of an active transaminase system have been found, and dried cell preparations and cell-free transaminase enzymes have been prepared. Further knowledge of the properties of the bacterial transaminase enzymes has been obtained.

Methods

Growth of Culture and Preparation of Enzymes—Streptococcus faecalis R (American Type Culture Collection, No. 8043) was used. Cells of this organism which possess very active transaminase enzymes for the systems

(1) Glutamic acid + oxalacetic acid $\rightleftharpoons$ aspartic acid + α-ketoglutaric acid
(2) Glutamic acid + pyruvic acid $\rightleftharpoons$ alanine + α-ketoglutaric acid

can be obtained by growing the culture in a medium composed of 1 per cent each of tryptone, yeast extract, and K_2HPO_4 and 0.2 per cent glucose. In this medium the final pH is 6.8 to 7.0. The neutral medium yields cells with distinctly more active transamination systems than the medium

[3] Dr. Green has now been able to recover pyridoxal phosphate from the purified transaminase and to assay the coenzyme with dopa decarboxylase apoenzyme (personal communication).

with a final pH of 4.5 to 5, as used for the maximum production of tyrosine decarboxylase (16). The cells were harvested from the neutral medium after 12 to 15 hours incubation at 37° by centrifugation with a Sharples supercentrifuge and washed once with 0.9 per cent saline. Dried cell preparations were obtained by suspending the washed cells in distilled water and drying in shallow layers over Drierite in vacuum desiccators.

The transaminase enzymes were liberated from the cells by the autolysis procedure which Gale and Epps (7) previously used to obtain cell-free amino acid decarboxylases (3, 7, 8). The autolysates could be freed of cell débris by centrifugation with a Beams ultracentrifuge to yield water-clear cell-free enzyme preparations. Resolution of the enzymes was accomplished by aging; this will be discussed with the data.

Transaminase apoenzymes were produced by growing the culture in a synthetic medium modified from that used for the production of tyrosine apoenzyme (17). The changes in the medium were a decrease in the level of glucose to 0.3 per cent and an increase in the buffer content to 1 per cent. The culture was neutralized with sterile N NaOH after growth was well under way so that the pH was maintained between 7.5 and 6.8. The apoenzyme was obtained by growing the culture in the medium with alanine instead of vitamin B_6 and the holoenzyme (intact enzyme) was obtained from cells grown in the same medium with 3 γ of pyridoxal per 10 ml.

Transamination Experiments and Analysis for Amino Acids—Incubations for transamination were carried out in Y-shaped tubes, the cell suspensions (or cell preparations), buffer, and other additions being placed in one arm and the substrates, as the neutral salts, in the other. For the experiments reported in this paper all incubations were under anaerobic conditions at 37°. With this culture the reactions could as well be run without anaerobic precautions. Several Y-tubes were connected to a manifold, placed in a water bath, evacuated, and filled with nitrogen. After 10 minutes equilibration, the contents of the two arms were mixed, and the transamination reaction was allowed to proceed for the desired time and then stopped by the addition of 0.5 ml. of 10 per cent sulfuric acid.

Aspartic acid was determined by the chloramine-T method of Cohen (12). Glutamic acid was determined by the use of a specific glutamic acid decarboxylase preparation from *Escherichia coli* (3). The oxalacetic acid was removed by heating in acid solution, after which the samples were adjusted to pH 5.0 for analysis.

EXPERIMENTAL

Inasmuch as highly active transaminase enzymes have only recently been shown to exist in bacteria (14), and because the systems previously studied were accompanied by an aspartic acid decarboxylase which ren-

dered both the analysis and interpretation of the results difficult, it seemed desirable to extend the studies of bacterial transamination to systems not so complicated by side reactions. Therefore, the transaminase systems of *Streptococcus faecalis* R were studied in living cells, and then extended to dried cell preparations, and to cell-free water-clear preparations in which permeability and related phenomena were eliminated.

A second series of experiments dealt with the function of pyridoxal phosphate (codecarboxylase) as the coenzyme of transamination.

Transaminase Systems of Streptococcus faecalis—A study of the formation of aspartic acid, by the method of Cohen (12), with a dried cell preparation of *Streptococcus faecalis*, has shown that quantities of the preparation beyond 20 mg. do not give appreciably greater amounts of aspartic acid after 30 and 60 minutes incubation (Fig. 1). When transamination was

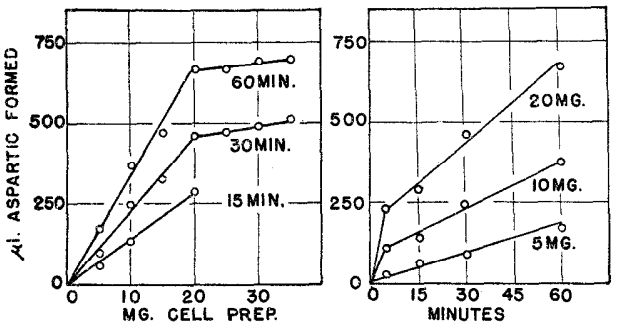

FIG. 1. Transamination by dried cell preparation of *Streptococcus faecalis* R. Dried cell preparation as indicated; 2 ml. of 0.1 M phosphate buffer, pH 8.3, 0.5 ml. of 0.12 M glutamate (1340 microliters), 0.3 ml. of 0.2 M oxalacetate; incubated under N_2 at 37° for the times indicated.

studied for various lengths of time with 10 and 20 mg. of the preparation, measurable amounts of aspartic acid were formed in less than 10 minutes. A decreased rate of reaction occurred with longer periods of incubation, presumably because the reaction approached equilibrium. The rate of the two transamination reactions, (1) glutamate-aspartate and (2) glutamate-alanine, were studied in both the forward and backward directions with the dried cell preparations. The data in Table I show that the preparations catalyzed both reactions with reasonable rates. As had been previously reported for animal tissue (12), the rate of the forward reactions, *i.e.* glutamate utilization, is greater than the reverse reactions. The rates obtained in the 5 minute incubation period may well be near the maximum values obtainable with these cells, for the reactions had not proceeded far enough to approach equilibrium and thus decrease the reaction rate.

The equilibrium of the glutamic-aspartic reaction is shown in Fig. 2; the forward reaction was measured by the determination of aspartic acid

TABLE I

Transamination Rates of Dried Cell Preparations of Streptococcus faecalis R

10 mg. of dried cells, 2 ml. of 0.1 M phosphate buffer, pH 8.3, 0.5 ml. of 0.12 M amino acid, 0.3 ml. of 0.2 M keto acid; incubation at 37° under nitrogen.

Reaction	Incubation time	Glutamic acid formed or used	Transamination	Q_T (N)*
	min.	*microliters*	*per cent*	
Glutamate + oxalacetate	5	127	10.2	760
	15	192	15.3	385
	30	310	24.8	310
Aspartate + α-ketoglutarate	30	132	10.6	130
	60	190	14.8	95
	120	266	20.8	65
Glutamate + pyruvate	5	98	7.8	590
	15	150	12.0	300
	30	190	15.3	190
Alanine + α-ketoglutarate	5	11	0.9	65
	15	14	1.0	30
	30	14	1.0	15

* Q_T (N) = microliters of substrate transaminated per hour per mg. of cell nitrogen.

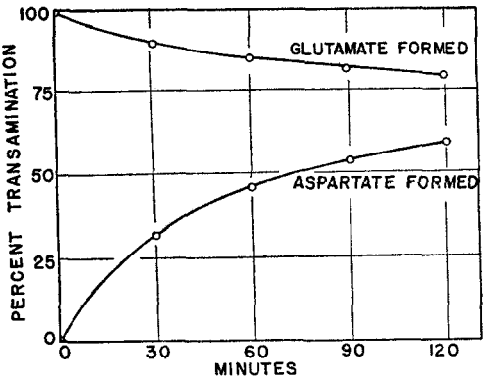

FIG. 2. Reversibility of glutamate-aspartate transaminase of *Streptococcus faecalis* R. 20 mg. of dried cell preparation; 2 ml. of 0.1 M phosphate buffer, pH 8.3. For the lower curve, 0.5 ml. of 0.12 M glutamate, 0.3 ml. of 0.2 M oxalacetate; analyzed by the chloramine-T method. For the upper curve, 0.5 ml. of 0.25 M *dl*-aspartate, 0.3 ml. of 0.2 M α-ketoglutarate; analyzed by the glutamic decarboxylase method. Incubation at 37° under nitrogen.

formation, with glutamic and oxalacetic acids as substrates, and the reverse reaction was measured by the appearance of glutamic acid, with aspartic

and α-ketoglutaric acids as substrates. In these studies *dl*-aspartate was used in double the desired concentration of the natural isomer and the assumption made that the unnatural form does not interfere with the reaction. The curves are virtually identical with those reported by Albaum and Cohen (18) for oat seedlings. The fact that the two curves do not approach one another more closely may indicate that one or more of the substrates is involved in other processes, the products from which

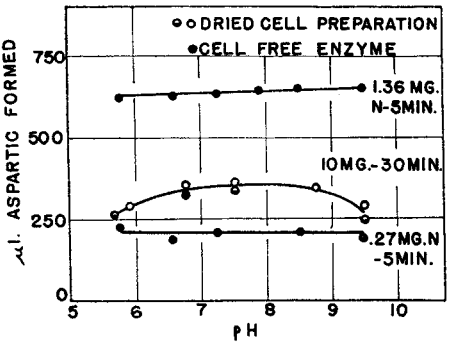

FIG. 3. Influence of pH on glutamate-aspartate transaminase activity. 10 mg. of dried cell preparation, 0.27 or 1.36 mg. of N of cell-free transaminase, 2 ml. of 0.1 M phosphate buffer, pH 8.3, 0.5 ml. of 0.12 M glutamate, 0.3 ml. of 0.2 M oxalacetate; incubated 5 or 30 minutes under nitrogen at 37°.

TABLE II

Transaminase Activity of Cells and Cell-Free Enzyme

Conditions as in Table III; incubation 5 minutes.

Preparation	Bacterial nitrogen	Aspartate formed	Transamination	Q_T (N)
	mg.	*microliters*	*per cent*	
20 mg. dried cells	2.68	234	17.8	1,047
Cell-free enzyme from 20 mg.				
1.0 ml.	1.36	650	49	5,700
0.5 "	0.68	555	44	9,800
0.2 "	0.27	235	19	10,500

do not appear in the reaction as written. By using shorter incubation periods, 5 to 10 minutes, the side reactions appear to be largely eliminated.

The data in Fig. 3 indicate the effect of pH on the glutamic-aspartic transaminase enzyme of *Streptococcus faecalis* R. Both dried cells and cell-free transaminase preparations were used, and the rate of reaction found to be only slightly dependent on the pH within the range of 5.7 to 9.5. The relatively sharp optimum in the alkaline range as reported for animal tissue (11), plant cells (18), and *Escherichia coli* (14) is not apparent

for the *Streptococcus faecalis* preparations. The data for the latter resemble those reported by Cohen (12) and by Kritzmann (11) for purified transamination preparations.

TABLE III

Function of Pyridoxal Phosphate in Transamination with Dried Preparations of Streptococcus faecalis R

10 mg. of cell preparation, 2 ml. of 0.1 M phosphate buffer, pH 8.3, 0.5 ml. of 0.12 M glutamate, 0.3 ml. of 0.2 M oxalacetate; synthetic pyridoxal phosphate or pyridoxal as indicated; incubation at 37° under nitrogen.

Preparation	Glutamate used	Aspartate formed	Transamination*	Q_T *
5 min. incubation				
	microliters	*microliters*	*per cent*	
Grown with pyridoxal	95	84	7	110
" without pyridoxal	25	9	1	20
+ 10 γ pyridoxal phosphate†	128	94	8	135
+ 50 " "	107	86	7	115
Grown with pyridoxal	117	123	10	145
" without pyridoxal	0	0	0	0
+ 12 γ pyridoxal phosphate	101	137	10	140
+ 10 " "	57	109	7	100
30 min. incubation				
Grown with pyridoxal	322		25	64
" without pyridoxal	136		11	27
+ 4 γ pyridoxal phosphate	186		15	38
+ 20 " " + 1 mg. adenosine triphosphate	223		17	45
Grown without pyridoxal	0		0	0
+ 15 γ pyridoxal phosphate	131		10	26
+ 1500 γ pyridoxal phosphate‡	287	358	24	32

* Average of glutamate and aspartate values.

† Pyridoxal phosphate is expressed in pyridoxal equivalents as estimated by tyrosine decarboxylase assay (8).

‡ 20 mg. of cells.

The cell-free transaminase, for which data are shown in Table II, was prepared by autolysis, as indicated under "Methods." For the particular preparation used in this case, 100 mg. of the dried cell preparation were

incubated in 5 ml. of 0.1 M phosphate buffer, pH 8.3, for 24 hours at 37° in the presence of toluene. After autolysis the suspension was centrifuged, to remove the bulk of the débris, and the supernatant cleared in a Beams ultracentrifuge. The resulting solution, which was almost clear, contained considerable transaminase activity. Aging the solution for 24 hours in the refrigerator caused more protein to precipitate. This was removed with the Beams ultracentrifuge to yield the water-clear supernatant, for which the data are given in Table II. The activity of this preparation on a protein basis (Q_T (N) 10,500) compares favorably with the purified transaminase which Cohen (12) isolated from animal tissue. The bacterial transaminase, however, is far from pure.

Function of Pyridoxal Phosphate in Transamination—Two methods of approach have been used to determine whether pyridoxal phosphate functions as the coenzyme of transamination. First, cells grown in a pyridoxal-deficient medium containing an excess of alanine were tested for transaminase activity alone and with added pyridoxal phosphate. The transaminase activity of cells grown in the same medium with added pyridoxal was used for comparison. Second, the cell-free transaminase enzyme was partially resolved to yield apoenzyme, and the ability of pyridoxal phosphate to restore activity determined.

Cells harvested from a medium deficient in pyridoxal and dried preparations from them were almost devoid of transaminase activity for the glutamate-aspartate system (Table III). The addition of synthetic pyridoxal phosphate to these cell preparations stimulates the transamination rate to a level comparable with that obtained for cells harvested from the same medium with excess pyridoxal present during growth. The stimulations were consistent and reproducible. In addition, there was usually good agreement between the glutamic acid disappearance and the aspartic acid formation. The stimulation of the transamination reaction with pyridoxal in the absence of adenosine triphosphate is in contrast to the results obtained with the tyrosine decarboxylase preparations (6). This is very probably due to the presence of appreciable quantities of adenosine triphosphate in those cells harvested from the neutral medium (see, for example, O'Kane and Umbreit (19)).

Attempts to resolve the cell-free transaminase by the methods used to resolve the amino acid decarboxylases (7, 8) were partially successful. Simple dialysis was not effective in removing the coenzyme, but aging coupled with dialysis did accomplish some resolution, apparently through the destruction of the coenzyme rather than by dissociation. The data in Table IV show that a variety of treatments resulted in varying degrees of resolution, and that the activity could be restored with pyridoxal phosphate. Pyridoxal was also effective in some cases, especially in the presence of

adenosine triphosphate, as was found to be the case with the tyrosine decarboxylase apoenzyme obtained by resolution of the enzyme from cells grown with pyridoxal (2, 8).

TABLE IV

Function of Pyridoxal Phosphate in Transamination with Cell-Free Transaminase

Conditions as for Table III.

Preparation	Additions	Glutamate used	Aspartate formed	Stimulation
	5 min. incubation			
		microliters	*microliters*	*per cent*
Autolysate held 21 days and centrifuged in Beams ultracentrifuge	None	40	45	
	10 γ pyridoxal phosphate	179	189	320
	21 " "	151	145	220
Autolysate dialyzed and held at 5° 11 days	None	97	73	
	10 γ pyridoxal phosphate	121	136	50
Same, held 13 days	None	94		
	10 γ pyridoxal phosphate	166		77
	25 " " + 1 mg. adenosine triphosphate	175		85
Autolysate held 9 days, 5°	None	200		
	12 γ pyridoxal phosphate	240		20
Same, dialyzed 20 hrs.	None	48		
	12 γ pyridoxal phosphate	104		110
	30 min. incubation			
Autolysate held 6 days, then dialyzed and held 4 days at 5°	None	289		
	3 γ pyridoxal phosphate	358		24
Autolysate held 6 days	None	238		
	15 γ pyridoxal phosphate	393		63
	50 " " + 1 mg. adenosine triphosphate	289		25

SUMMARY

Conditions for growing cells of *Streptococcus faecalis* R with highly active transaminase enzymes have been devised and the enzymes have been obtained in dried cell preparations and in a water-clear, cell-free condition.

Pyridoxal phosphate, previously shown to function as the coenzyme of amino acid decarboxylases, has been shown to function as the coenzyme of the glutamate-aspartate transaminase. This has been accomplished by two methods: (1) by growing cells in a medium deficient in pyridoxal to yield transaminase apoenzyme and activating the enzyme with synthetic pyridoxal phosphate; (2) by resolving the cell-free enzyme from organisms grown with pyridoxal and restoring the activity of this apoenzyme with pyridoxal phosphate.

The function of the vitamin B_6 group in protein metabolism is therefore at least partially explained by its action in amino acid decarboxylation and in transamination.

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