TRANSAMINATION OF D-AMINO ACIDS BY BACILLUS SUBTILIS

CURTIS B. THORNE, CARMEN G. GÓMEZ, AND RILEY D. HOUSEWRIGHT

Camp Detrick, Frederick, Maryland

Received for publication September 27, 1954

A wide variety of transaminases active on L-amino acids has been demonstrated in microorganisms (Feldman and Gunsalus, 1950; Fincham, 1951), mammalian tissues (Cammarata and Cohen, 1950; Rowsell, 1951), and plants (Wilson *et al.*, 1954). Cohen (1939, 1940) did not find transaminase activity with any of several D-amino acids when he tested minced pigeon breast muscle or purified enzyme preparations although other workers (Euler *et al.*, 1939; Braunstein and Azarkh, 1939) reported that certain D-amino acids were slightly active when tested with mammalian tissue preparations. It has been suggested (Herbst, 1944) that the reports which ascribed activity to D-amino acids may have been in error because of inadequate analytical methods. Stumpf (1951) reported that D-alanine behaved as an active amino donor in reaction mixtures containing α-ketoglutaric acid and enzyme preparations from plants. It was not clear, however, whether D-alanine participated directly in transamination or whether it contributed the amino group in some indirect manner.

In studies on the mechanisms of synthesis of D-glutamic acid and glutamyl polypeptide by *Bacillus subtilis*, we observed that sonic extracts of this organism catalyzed a series of transamination reactions involving D-amino acids. The results of some of our studies on these D-amino acid transaminations are presented here.

EXPERIMENTAL METHODS

Culture and medium. Bacillus subtilis, ATCC 9945, was grown on a shaker at 37 C in 500 ml Erlenmeyer flasks containing 100 ml of medium composed of Difco nutrient broth, 0.8 per cent; Difco yeast extract, 0.3 per cent; glucose, 0.1 per cent; and L-glutamic acid, 0.5 per cent, at pH 7.5. The inoculum was one ml of a spore suspension containing 10^7 spores per ml or one ml of an 18 hour culture grown on the same medium. After 16 to 18 hours the cells were harvested by centrifugation and washed once with 0.05 M phosphate buffer at pH 8.0.

Cell-free extracts. Extracts were prepared by treating a thick suspension of cells in a Raytheon sonic oscillator for 20 minutes. The clear supernatant solution obtained by centrifugation of the sonic-treated material at 12,000 $\times$ G for 20 minutes was used as the source of enzyme. Such extracts contained 6 to 8 mg of nitrogen per ml after dialysis, and they maintained their D-amino acid transaminase activity for several weeks when stored at -20 C.

Test for transaminase activity. For testing transaminase activity, reaction mixtures usually contained 0.1 ml of enzyme, 20 μg of pyridoxal phosphate, and 100 μM of the appropriate substrates in a final volume of 1 ml of 0.05 M phosphate buffer at pH 8.0. The mixtures were incubated in stoppered tubes at 37 C for the desired period of time, and the reactions were stopped by holding the tubes in boiling water for 5 minutes. The precipitated protein was removed by centrifugation, and the supernatant solutions were used for analysis. Variations in this procedure are given as they occur.

Analytical methods. Total glutamic acid, aspartic acid, and alanine were determined quantitatively by paper chromatography (Housewright and Thorne, 1950). L-Glutamic acid was determined manometrically with glutamic acid decarboxylase by the method of Umbreit and Gunsalus (1945) except that sonic extracts of *Escherichia coli* were used instead of dried cells. D-Glutamic acid was estimated by subtracting the amount of the L-isomer from the total. D-Alanine was determined manometrically with D-amino acid oxidase from hog kidneys (Krebs, 1935). Pyruvic and α-ketoglutaric acids were determined by a modification of the method of Cavallini *et al.* (1949) in which the 2,4-dinitrophenylhydrazones are separated by paper chromatography. Phenol-water (3:1) was used as the solvent. Nitrogen was determined by the method of Johnson (1941).

RESULTS

Transaminase activities of sonic extracts. Paper chromatograms showed that glutamic acid was

TABLE 1

*Synthesis of glutamic acid by transamination of aspartic acid and alanine with α-ketoglutaric acid**

Substrates	Glutamic Acid			
	Total	L	D	
	μM per ml	μM per ml	μM per ml	per cent
None	2.6			
α-Ketoglutaric acid	4.5	3.8	0.7	15.6
α-Ketoglutaric acid + L-aspartic acid	42.4	41.6	0.8	1.9
α-Ketoglutaric acid + D-aspartic acid	34.0	3.5	30.5	89.7
α-Ketoglutaric acid + L-alanine	40.6	4.9	35.7	87.9
α-Ketoglutaric acid + D-alanine	39.4	4.3	35.1	89.1

* 0.1 ml of undialyzed extract, 20 μg of pyridoxal phosphate, and 100 μM of each of the appropriate substrates in a final volume of 1.0 ml. 0.01 M phosphate buffer, pH 7.4. Incubated 4 hours.

synthesized when sonic extracts were incubated with α-ketoglutaric acid and either isomer of aspartic acid or alanine. Both isomers of glutamic acid were active in transaminating with pyruvic acid to form alanine, but only the D-isomer of aspartic acid was active. No amino acids were formed when enzyme was omitted from reaction mixtures or when heated enzyme was used.

Table 1 shows quantitative data on the synthesis of glutamic acid from α-ketoglutaric acid and both isomers of aspartic acid. The glutamic acid formed in the reaction mixture with D-aspartic acid contained 89.7 per cent of the D-isomer, while that formed in the reaction mixture with L-aspartic acid contained only 1.9 per cent of the D-isomer. An undialyzed extract was used, and it contained a small amount of free glutamic acid as shown in the analysis of the reaction mixture containing no added substrates. With crude extracts some glutamic acid was always formed in reaction mixtures containing α-ketoglutaric acid and no added amino donor, and it was a mixture of the D- and L-isomers. This could be reduced, but not eliminated, by dialysis. In reaction mixtures containing α-ketoglutaric acid and D-aspartic acid, alanine was formed in addition to glutamic acid. This did not occur in detectable quantities when L-aspartic acid was used. The alanine is believed to result from transamination between D-aspartic acid or D-glutamic acid and pyruvic acid which originated from the oxalacetic acid produced from aspartic acid. When oxalacetic acid was incubated with D-aspartic acid and enzyme, alanine was formed, and when D-glutamic acid and oxalacetic acid were incubated with enzyme, both aspartic acid and alanine were formed.

Table 1 also shows quantitative data on the synthesis of glutamic acid from α-ketoglutaric acid and the two isomers of alanine. The glutamic acid produced when α-ketoglutaric acid and either L- or D-alanine were substrates contained approximately 90 per cent of the D-isomer. As shown in figure 1, extracts contained an active alanine racemase (Wood and Gunsalus, 1951; Stewart and Halvorson, 1953) which converted both isomers of alanine to a DL-mixture. Apparently L-alanine did not transaminate with α-ketoglutaric acid at an appreciable rate since most of the glutamic acid formed when either isomer of alanine was added was the D-isomer. The racemase was present, and the added L- and D-isomers

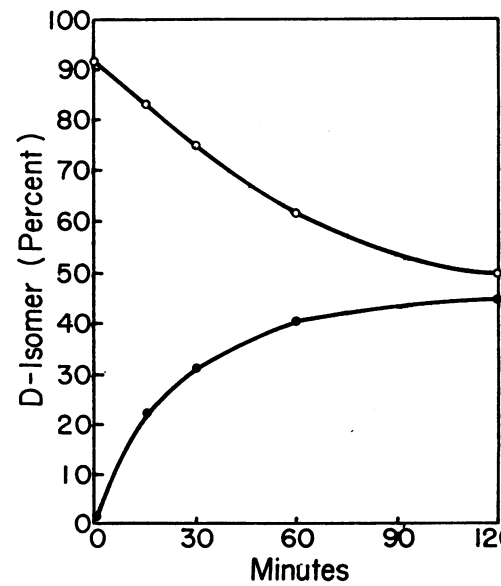

Figure 1. Alanine racemase activity of a sonic extract of *Bacillus subtilis*. Reaction mixtures contained 0.5 ml of enzyme, 500 μM of alanine, and 50 μg of pyridoxal phosphate in a final volume of 5 ml; 0.01 M phosphate buffer at pH 7.4. ○ = values obtained in reaction mixture with D-alanine added and ● = values obtained in reaction mixture with L-alanine added.

TABLE 2

*Configuration of glutamic acid and alanine isolated from transamination reaction mixtures**

Substrates	Glutamic Acid			Alanine		
	Formed	Isolated		Formed	Isolated	
	μM	μM	per cent D	μM	μM	per cent D
α-Ketoglutaric acid	17.3	16.8	26.1			
α-Ketoglutaric acid + D-aspartic acid	1,428.6	1,349.6	88.3	1,567.4	1,544.9	50.4
α-Ketoglutaric acid + L-aspartic acid	36.0	27.1	15.0	trace		
Pyruvic acid	trace			trace		
Pyruvic acid + D-aspartic acid	trace			2,495.5	2,324.7	46.5

* See text for experimental details.

of alanine were rapidly converted to mixtures of the two.

Configuration of glutamic acid and alanine isolated from reaction mixtures. Alanine and glutamic acid were isolated from reaction mixtures to permit analysis and determination of their configuration in the absence of any substances that might possibly interfere. Initially, 3.5 ml of enzyme and 200 μg of pyridoxal phosphate in a total volume of 21.5 ml with 0.2 M substrates and 0.01 M phosphate at pH 7.4 were incubated aseptically for 16 hours. The enzyme and substrates had been sterilized by filtration. After 16 hours an additional 3.8 ml of enzyme were added to each flask, and enough pyruvic acid or α-ketoglutaric acid was added to the appropriate flasks to make the final concentration of total added keto acid 0.2 M. The final volume in each flask was 30 ml. The flasks were allowed to incubate 17 hours more, and the glutamic acid and alanine were isolated by ion exchange chromatography on columns of "dowex 50" resin (Stein and Moore, 1949). Results of analyses of the isolated compounds are shown in table 2. The glutamic acid isolated from the reaction mixture containing α-ketoglutaric acid and D-aspartic acid was 88.3 per cent D, while that isolated from the mixture containing α-ketoglutaric acid and L-aspartic acid was only 15.0 per cent D. The glutamic acid isolated from the mixture containing α-ketoglutaric acid as the only added substrate was 26.1 per cent D. An undialyzed sonic extract was used, and it contained small amounts of glutamic acid and alanine. When D-aspartic acid and α-ketoglutaric acid or pyruvic acid were the substrates, the isolated alanine was approximately 50 per cent D. This was to be expected since alanine racemase was present.

Relative stabilities of L- and D-amino acid transaminases. Fresh extracts were active with both D- and L-aspartic acids in the synthesis of glutamic acid from α-ketoglutaric acid, but the activity with D-aspartic acid was usually much greater than that with the L-isomer. The data in table 3 illustrate the relative stabilities of the L- and D-amino acid transaminases. The fresh extract had approximately equal activities with L- and D-aspartic acids. When the extract was dialyzed for 70 hours at 5 C, the activity with both isomers was decreased, but the D-isomer was more active than the L-isomer. When the extract was allowed to stand at 5 C for 72 hours, it retained full activity with D-aspartic acid, but the activity with L-aspartic acid was reduced to

TABLE 3

*Relative stabilities of L- and D-amino acid transaminases**

Prep. No.	Treatment	Glutamic Acid Produced from		
		α-Kg	α-Kg + L-Asp	α-Kg + D-Asp
		μM per ml	μM per ml	μM per ml
I	None (fresh extract)	2.2	24.8	26.5
II	Prep. I dialyzed 70 hours at 5 C (0.01 M phosphate buffer, pH 7.4)	0.8	2.1	11.0
III	Prep. I stored 72 hours at 5 C	3.1	5.4	27.5

* 0.1 ml of enzyme, 20 μg of pyridoxal phosphate, and 100 μM of appropriate substrates in a final volume of 1.0 ml. 0.01 M phosphate buffer, pH 7.4. Incubated 2 hours. Abbreviations: α-Kg = α-ketoglutaric acid and Asp = aspartic acid.

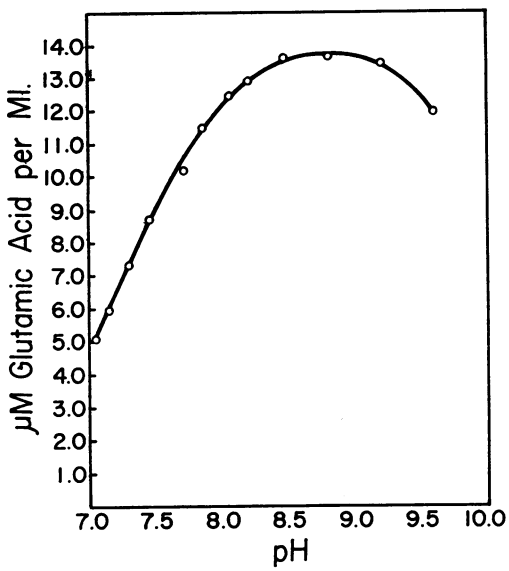

Figure 2. The effect of pH on the transamination reaction between α-ketoglutaric acid and D-aspartic acid. Reaction mixtures contained 0.1 ml of enzyme, 100 μM of α-ketoglutaric and D-aspartic acids, and 20 μg of pyridoxal phosphate per ml; 0.05 M tris-hydroxymethyl aminomethane buffer. Incubation time, 30 minutes.

about 22 per cent of the original. All these tests were done with added pyridoxal phosphate, and thus the loss in activity could not be restored completely by the addition of coenzyme.

Effect of pH. The effect of pH on transamination between α-ketoglutaric and D-aspartic acids is shown in figure 2. The optimum pH was in the range of 8.5 to 9.0. The same reaction with L-aspartic acid proceeded at an optimum rate over a broader range of pH 7 to 8.

Transaminase activity of a partially purified preparation. By fractionation with ammonium sulfate a preparation was obtained which was active with D-amino acids but not with the L-isomers. An equal volume of saturated ammonium sulfate was added to an aliquot of a fresh extract which was active in transaminating both D- and L-amino acids. The precipitate which formed was discarded, and solid ammonium sulfate was added to the supernatant solution until it was saturated. The resulting precipitate was dissolved in 0.05 M phosphate buffer at pH 8 and dialyzed against the same buffer for 17 hours. The enzyme solution was then brought to 60 per cent saturation with ammonium sulfate by the addition of an appropriate volume of a saturated solution. The resulting precipitate was discarded and the supernatant solution was brought to 70 per cent saturation with ammonium sulfate by the addition of more saturated solution. The precipitate which formed was dissolved in 0.05 M phosphate buffer at pH 8 and dialyzed against the same buffer for 20 hours. All these operations were performed at 6 C.

TABLE 4

*D-Amino acid transaminase activity of a partially purified preparation**

Substrates	Glutamic Acid	Alanine
	μM per ml	μM per ml
α-Ketoglutaric acid	0	
α-Ketoglutaric acid + D-aspartic acid	10.2	
α-Ketoglutaric acid + L-aspartic acid	0	
α-Ketoglutaric acid + D-alanine	15.1	
α-Ketoglutaric acid + L-alanine	10.4	
Pyruvic acid		0
Pyruvic acid + D-aspartic acid		13.9
Pyruvic acid + L-aspartic acid		0
Pyruvic acid + D-glutamic acid		8.5
Pyruvic acid + L-glutamic acid		0

* The reaction mixtures contained 100 μM of substrates, 80 μg of pyridoxal phosphate, and 0.15 mg of enzyme nitrogen in 1.0 ml of 0.05 M phosphate buffer at pH 8.0. Incubated 30 minutes.

TABLE 5

*Transamination balance**

Compound	Concentration of Compound					
	I. Substrates D-Alanine + α-ketoglutaric acid			II. Substrates D-Glutamic acid + pyruvic acid		
	0 hr	1 hr	change	0 hr	1 hr	change
	μM/ml	μM/ml	μM/ml	μM/ml	μM/ml	μM/ml
Glutamic acid...	0	15.1	+15.1	44.3	25.1	−19.2
Pyruvic acid....	0	15.0	+15.0	25.7	6.9	−18.8
α-Ketoglutaric acid.....	39.8	24.2	−15.6	0	20.6	+20.6
Alanine.....	42.6	27.6	−15.0	0	20.1	+20.1

* Reaction mixtures consisted of 1.5 ml of a partially purified enzyme preparation, 400 μg of pyridoxal phosphate, and substrates as given in the table in a final volume of 10.5 ml. 0.05 M phosphate buffer at pH 8.0.

Table 4 shows data obtained with this preparation. No amino acids were produced when the enzyme was incubated with pyruvic acid or α-ketoglutaric acid without added amino donor. Both D-aspartic and D-glutamic acids were active in transaminating with pyruvic acid, but the L-isomers were inactive. Similarly, D-aspartic acid transaminated with α-ketoglutaric acid to form glutamic acid, but L-aspartic acid was inactive. Although not shown in the table, the glutamic acid produced from α-ketoglutaric acid and D-aspartic acid was composed entirely of the D-isomer. Glutamic acid was produced when α-ketoglutaric acid and either D- or L-alanine were incubated together with the enzyme, but the D-isomer was the more active of the two. The apparent activity with L-alanine is probably the result of the presence of alanine racemase in this partially purified transaminase preparation. This was not tested.

Transamination balance. Table 5 shows the results of studies on transamination balances. The enzyme used was a partially purified preparation similar to the one described above. In the reaction between D-alanine and α-ketoglutaric acid, 1 μM of each of the substrates disappeared while 1 μM of each of the products, glutamic acid and pyruvic acid, was formed. A similar balance was obtained when the reverse reaction was studied. These data provide evidence that these reactions with D-amino acids are true transamination reactions.

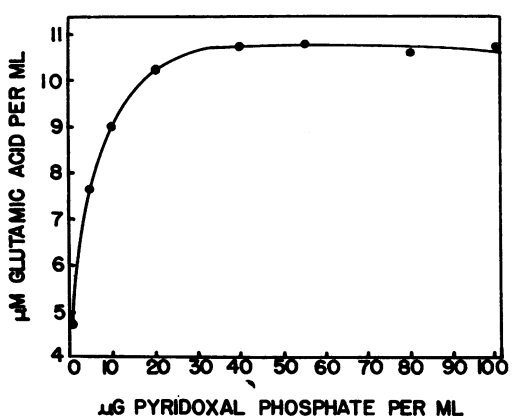

Figure 3. The effect of pyridoxal phosphate on the transamination reaction between α-ketoglutaric acid and D-aspartic acid. Reaction mixtures contained 0.1 ml of a partially purified enzyme preparation (0.15 mg of nitrogen), 100 μM of α-ketoglutaric and D-aspartic acids, and pyridoxal phosphate as given in the figure, in a final volume of 1 ml; 0.05 M phosphate buffer at pH 8.0. Incubation time, 30 minutes.

Effect of pyridoxal phosphate. Figure 3 shows the effect of pyridoxal phosphate on the reaction between α-ketoglutaric acid and D-aspartic acid when a partially purified preparation was used. This preparation was the same as that described above, i.e., the fraction precipitated by 70 per cent saturation with ammonium sulfate. Although the preparation was active in the absence of any added pyridoxal phosphate, it did not exhibit maximum activity until 20 to 40 μg of pyridoxal phosphate per ml were added.

DISCUSSION

The optimum medium for production of glutamyl polypeptide by growing cultures of *B. subtilis* contains a large amount of L-glutamic acid, but the glutamic acid of the polypeptide contains from 20 to 80 per cent of the D-isomer (Thorne *et al.*, 1954). A glutamic acid racemase has not been demonstrated in this organism, but the finding of these D-amino acid transamination reactions suggests the following series of reactions as a mechanism for indirect conversion of L-glutamic acid to the D-isomer: (1) transamination between pyruvic acid and L-glutamic acid produces L-alanine and α-ketoglutaric acid; (2) the alanine is racemized; and (3) transamination between α-ketoglutaric acid and D-alanine results in the formation of D-glutamic acid.

The discovery of D-amino acid transamination suggests an important role for alanine racemase. It appears that in *B. subtilis* alanine may be the key compound involved in D-amino acid metabolism. It will be interesting to test other organisms and tissues for D-amino acid transamination and to learn whether the reactions occur in a number of species or whether they are peculiar to *B. subtilis* and closely related organisms.

ACKNOWLEDGMENT

The authors wish to acknowledge the technical assistance of Aaron Freeman and Raymond Gregoire.

SUMMARY

Sonic extracts of *Bacillus subtilis* catalyzed a series of transamination reactions involving D-amino acids. D-Glutamic acid was synthesized

from α-ketoglutaric acid and D-aspartic acid or D-alanine. Alanine was produced from pyruvic acid and D-aspartic acid or D-glutamic acid. Extracts contained an active alanine racemase which converted either isomer of alanine to a mixture of the two. When fresh extracts were used, L-amino acids also were active in transamination; but, upon aging of extracts, the activity with L-amino acids disappeared or was reduced, while the activity with D-amino acids was maintained. By fractionation with ammonium sulfate a preparation was obtained which was specific for D-amino acids.

Pyridoxal phosphate appears to be the coenzyme for D-amino acid transamination.

The pH optimum for the reaction between α-ketoglutaric acid and D-aspartic acid was in the range of 8.5 to 9.0.

REFERENCES

BRAUNSTEIN, A. E., AND AZARKH, R. M. 1939 Transamination of l- and d-amino acids in normal muscle and in malignant tumors. Nature, **144**, 669–670.

CAMMARATA, P. S., AND COHEN, P. P. 1950 The scope of the transamination reaction in animal tissues. J. Biol. Chem., **187**, 439–452.

CAVALLINI, D., FRONTALI, N., AND TOSCHI, G. 1949 Keto-acid content of human blood and urine. Nature, **164**, 792–793.

COHEN, P. P. 1939 Transamination in pigeon breat muscle. Biochem. J. (London), **33**, 1478–1487.

COHEN, P. P. 1940 Transamination with purified enzyme preparations (transaminase). J. Biol. Chem., **136**, 565–584.

EULER, H. VON, HELLSTROM, H., GUNTHER, G., ELLIOTT, L., AND ELLIOTT, S. 1939 Enzymatische Versuche an Sarkomen und chlorophylldefekten Gersten-Mutanten. Hoppe-Seyler's Z. physiol. Chem., **259**, 201–203.

FELDMAN, L. I., AND GUNSALUS, I. C. 1950 The occurrence of a wide variety of transaminases in bacteria. J. Biol. Chem., **187**, 821–830.

FINCHAM, J. R. S. 1951 Transaminases in *Neurospora crassa*. Nature, **168**, 957–958.

HERBST, R. M. 1944 The transamination reaction. Advances in Enzymol., **4**, 75–97.

HOUSEWRIGHT, R. D., AND THORNE, C. B. 1950 Synthesis of glutamic acid and glutamyl polypeptide by *Bacillus anthracis*. I. Formation of glutamic acid by transamination. J. Bacteriol., **60**, 89–100.

JOHNSON, M. J. 1941 Isolation and properties of a pure yeast polypeptidase. J. Biol. Chem., **137**, 575–586.

KREBS, H. A. 1935 Metabolism of amino acids. III. Deamination of amino acids. Biochem. J. (London), **29**, 1620–1644.

ROWSELL, E. V. 1951 Transamination to pyruvate and some other α-keto-acids. Nature, **168**, 104–106.

STEIN, W. H., AND MOORE, S. 1949 Chromatographic determinations of the amino acid composition of proteins. Cold Spring Harbor Symposia Quant. Biol., **14**, 171–190.

STEWART, B. T., AND HALVORSON, H. O. 1953 Studies on the spores of aerobic bacteria. I. The occurrence of alanine racemase. J. Bacteriol., **65**, 160–166.

STUMPF, P. K. 1951 Transaminases in higher plants. Federation Proc., **10**, 256.

THORNE, C. B., GÓMEZ, C. G., NOYES, H. E., AND HOUSEWRIGHT, R. D. 1954 Production of glutamyl polypeptide by *Bacillus subtilis*. J. Bacteriol., **68**, 307–315.

UMBREIT, W. W., AND GUNSALUS, I. C. 1945 The function of pyridoxine derivatives. Arginine and glutamic acid decarboxylases. J. Biol. Chem., **159**, 333–341.

WILSON, D. G., KING, K. W., AND BURRIS, R. H. 1954 Transamination reactions in plants. J. Biol. Chem., **208**, 863–874.

WOOD, W. A., AND GUNSALUS, I. C. 1951 D-Alanine formation: A racemase in *Streptococcus faecalis*. J. Biol. Chem., **190**, 403–416.