

# OCCURRENCE OF D-AMINO ACIDS IN SOME NATURAL MATERIALS\*

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Although the naturally occurring  $\alpha$ -amino acids are usually of the L configuration, examples continue to accumulate of the occurrence in natural products of D-amino acids (1). Certainly the most surprising reports of this type have been those of Kögl and coworkers on the isolation of D-glutamic acid from cancerous tissue. Since his original publication in 1939 (2), and despite the fact that his findings have not been confirmed in other laboratories (3), Kögl has repeatedly reaffirmed his original contention and reinforced it with additional experimental data (4).

The recent studies of antibiotics have provided notable and indisputable instances of the occurrence of D-amino acids in natural products. Even the penicillins yield on acid hydrolysis an amino acid (penicillamine) of D configuration.

In the course of a study of the amino acid metabolism of penicillin-producing molds, it seemed of interest to examine the mold materials for the presence of D-amino acids and to compare the results with those from some other antibiotic-producing organisms. Since this study was begun, Konikova and Dobbert (5) have reported the presence of large quantities of D-amino acids in hydrolysates of *Bacillus brevis*, Dunn *et al.* (6) have found evidence for D-glutamic acid in hydrolysates of *Lactobacillus arabinosus*, and Holden and Snell (7) have clearly demonstrated the presence of D-alanine in the latter organism and in some other lactobacilli.

## EXPERIMENTAL

The analytical procedure applied to all of the natural substances was as follows: The finely ground, dried material was hydrolyzed by being heated under a reflux for 24 hours with 12 volumes of 6 N hydrochloric acid. The excess acid was removed by repeated evaporation *in vacuo*, and the residue was redissolved in water. Total N was determined by the Kjeldahl meth-

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od before hydrolysis of the materials. Total  $\alpha$ -amino N was determined after hydrolysis by the method of Pope and Stevens (8). The content of D-amino acids was estimated by incubation of the hydrolysate with D-amino acid oxidase and colorimetric determination of the keto acids produced (9, 10). In all cases, oxygen consumption was measured and, in general, showed a close correlation to keto acid production, although it was invariably somewhat higher.

The enzyme preparation was essentially that of Behrens (11), an extract in pyrophosphate buffer being used (12). Incubations were carried out by the usual Warburg technique for a period of 5 hours at 38°, a small crystal of thymol being added to suppress microbial growth. The longer period of incubation was employed to insure more nearly complete oxidation of the amino acids. In all cases, control samples were run in which a known quantity of D-valine was added to the hydrolysate of the natural material. The added amino acid was shown to be 97 to 100 per cent oxidized. Under the conditions of these experiments, D-glutamic acid reacted only to the extent of about 30 per cent, D-threonine of about 20 per cent, and D-lysine of about 5 per cent. D-Alanine, D-valine, D-leucine, and D-aspartic acid were completely oxidized. These results correspond reasonably well with other published data (13, 14).

Dried cells of *B. brevis*, *L. arabinosus*, *Torulopsis utilis*, and *Bacillus subtilis* were obtained through the kindness of Dr. J. C. Lewis, Albany, California. *B. brevis* (ATCC 10,068) was grown in shallow layer culture on a tryptone-glucose-salts medium (15), *L. arabinosus* (ATCC 8014) in stagnant culture in 5 gallon jars on trypsin-digested casein (2 per cent), yeast extract (0.25 per cent), glucose (2 per cent), and salts, *B. subtilis* (ATCC 6633) (butanol-extracted cells obtained from the isolation of subtilin as described by Fevold *et al.* (16) from aerated vat-grown cells) on a sucrose-yeast extract-citrate-salts medium (17), and *T. utilis* (ATCC 9950) was grown in aerated vat culture on pear juice-ammonia-salts (18). Samples of *Penicillium chrysogenum* mycelia, collected at 20, 40, and 60 hours after the start of the fermentation in the commercial production of penicillin, were obtained through the kindness of Dr. K. S. Pilcher, Berkeley, California.

In Table I are summarized the results of the determinations. For comparison, a hydrolysate of casein similarly analyzed is included. It will be noted that the hydrolysates of *P. chrysogenum* mycelia contained insignificant amounts of D-amino acids as estimated by this technique. On the other hand, hydrolysates of *L. arabinosus* and *B. brevis* contain relatively large amounts, in the latter case over 10 per cent of the total amino acids. Hydrolysates of *B. subtilis* and *T. utilis* appear to contain small but still significant amounts of D-amino acids.

Because of the possibility that any D-amino acids occurring in acid hydrolysates of natural materials might be artifacts resulting from racemization during hydrolysis, another technique was sought which would avoid this possibility. There is considerable evidence in the literature (19, 20) that a number of D-amino acids are largely excreted in the urine when fed to experimental animals. This fact has indeed been utilized by Kögl (4) in attempting to establish the occurrence of D-glutamic acid in malignant tissue.

In preliminary experiments of this type, adult male rats weighing 300 to 400 gm. were fed *ad libitum* a diet consisting of vitamin-test casein 20, dextrin 44, Crisco 25, salt mixture (21) 4, Alphacel 2, and cod liver oil 5

TABLE I  
*Analyses for D-Amino Acids in Acid Hydrolysates*

Substance	Total N	Total amino N	D-Amino N
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Casein.....	12.4	9.90	0.02
<i>P. chrysogenum</i> (20 hrs.).....	4.1	2.20	0.01
“ “ (40 “ ).....	3.1	2.20	0.01
“ “ (60 “ ).....	3.4	2.20	0.01
<i>B. subtilis</i> .....	9.1	5.10	0.10
	9.1	5.96	0.11
<i>T. utilis</i> .....	7.3	4.63	0.08
	7.3	4.78	0.08
<i>L. arabinosus</i> .....	7.2	4.92	0.33
<i>B. brevis</i> .....	10.9	7.04	0.81
	10.9	6.97	0.84

parts, respectively. Water-soluble vitamins were supplied in the form of a daily supplement of 5 ml. of a solution containing 10 mg. of thiamine, 10 mg. of riboflavin, 10 mg. of niacin, 12 mg. of pyridoxine, 100 mg. of calcium pantothenate, 5 gm. of choline, and 5 gm. of Wilson's liver extract (1:20) dissolved in 500 ml. To 100 gm. of this basal diet was added 0.027 mole of a DL-amino acid. The rats were kept in metabolism cages so arranged that the urine dropped into collecting tubes kept at dry ice temperature. Analyses of the urines by the previous technique indicated a very small apparent content of D-amino acids from the control diet, but distinct amounts from the feeding of DL-amino acids. The amounts of different D-amino acids excreted under the conditions of the experiments varied widely, aspartic acid being excreted to the extent of 17 per cent, and alanine 1 per cent.

Despite the apparent variability in recovery to be expected from different D-amino acids, it seemed worth while to carry out feeding experiments

of a similar sort with the natural materials. In Table II are given the results obtained in experiments with diets containing *L. arabinosus* and *B. brevis*. When these products were included in the rat diets, half an equal weight of casein and half of dextrin were removed. Animals were first placed on the control diet for 3 days, then fasted for 12 hours and fed the experimental diet for 3 days, during which time urine was collected. At the end of another 12 hour fast, urine collection was stopped. The animals did not eat the diets containing *B. brevis* well, but appreciable amounts were consumed.

The results are expressed in terms of mg. of amino acid N per gm. of food consumed, and are given for the untreated urines and for the same

TABLE II

*Analyses for D-Amino Acids in Urine Samples*

The results are given in mg. per gm. of food consumed.

Diet	Food consumed	Total amino N in urine		D-Amino N in urine	
		Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis
	<i>gm.</i>				
Basal .....	70.0	0.28	0.45	0.004	0.009
“ .....	71.0	0.28	0.42	0.004	0.008
“ + 30% <i>L. arabinosus</i> .....	52.5	0.15	0.37	0.007	0.04
“ + 30% “ “ .....	42.0	0.15	0.37	0.007	0.03
“ + 30% <i>B. brevis</i> .....	33.0	2.42	5.95	0.24	0.37
“ + 30% “ “ .....	14.0	2.36	5.81	0.16	0.23
“ + 15% “ “ .....	34.0	2.93	4.53	0.11	0.16

urines subjected to a mild acid hydrolysis (1 N sulfuric acid for 3 hours at 90°) designed to convert pyrrolidone-2-carboxylic acid, if present, to glutamic acid. It will be seen that there was a very significant excretion of D-amino acids after the feeding of *B. brevis*, though less certain results from the feeding of *L. arabinosus*.

In preliminary experiments to determine the nature of the D-amino acids excreted after the feeding of *B. brevis* cells to rats, the urines have been examined for amino acids by two-dimensional paper chromatography. Also, aliquots of the urine samples have been treated with D-amino acid oxidase and examined for keto acids by the method of Cavallini *et al.* (22). The most prominent amino acid appeared unmistakably to be aspartic acid (blue color with ninhydrin;  $R_f$  0.17 (phenol);  $R_f$  0.27 (collidine-lutidine)), and in the enzyme-treated urines a keto acid corresponding in chromatographic behavior to that derived from aspartic acid was observed.

## DISCUSSION

The results of the experiments with *L. arabinosus* are consistent with the findings of Holden and Snell (7). Qualitative evidence was obtained that D-alanine was the major reactive component of our hydrolysates of *L. arabinosus*, and the small excretion of D-amino acids after feeding of *L. arabinosus* correlates with the small excretion of D-alanine itself fed under similar conditions.

In the case of *B. brevis*, the total quantity of D-amino acids found in acid hydrolysates, while high, was not nearly so large as that reported by Konikova and Dobbert (5). Whether differences in cultural conditions or in the strain employed can account for these differences in composition is problematical. It should, of course, be pointed out that the enzymatic method employed in this study will not detect certain D-amino acids and will estimate others only partially. However, essentially the same methods were employed in both studies. The feeding experiments with *B. brevis* confirmed the existence of D-amino acids in the original material. The indications that D-aspartic acid is an important component of acid hydrolysates of *B. brevis* cells and that it is excreted in the urine after feeding of unhydrolyzed material are of particular interest because it is apparently the first instance of the finding of D-aspartic acid in this organism.

Further studies are being directed toward identification and quantitative determination of the different D-amino acids in several natural materials.

## SUMMARY

A study has been made of the occurrence of D-amino acids in acid hydrolysates of cells of *Lactobacillus arabinosus*, *Bacillus brevis*, *Bacillus subtilis*, *Torulopsis utilis*, and mycelia of *Penicillium chrysogenum*. The finding of significant quantities of D-amino acids in the first two materials provides qualitative confirmation of earlier reports.

Additional evidence of the occurrence of D-amino acids in these natural materials was obtained in feeding experiments with rats. Inclusion in a purified diet of unhydrolyzed cells of *B. brevis* led to the excretion of significant amounts of D-amino acids in the urine. Chromatographic data indicated that a principal component of the urine was D-aspartic acid.

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