THE OCCURRENCE OF d-AMINO ACIDS IN GRAMICIDIN AND TYROCIDINE

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Amino acids having a dextro configuration have been found in natural material from two sources.1 Jacobs and Craig obtained d-proline from ergotinine (4) and later other ergot alkaloids, and Ivánovics and Bruckner found d(-)-glutamic acid as the principal constituent of the P-antigen of Bacillus anthracis and other microorganisms of the related Bacillus mesentericus-subtilis group (5). Inasmuch as gramicidin and tyrocidine were obtained from a related aerobic spore-forming organism, Bacillus brevis (6-8), it was thought to be of interest to investigate the configuration of the amino acids present in these two polypeptides.

The enzymatic method (9) which was used made it possible to discover the presence of d-amino acids at a time when the quantities of pure material available were too small to permit the isolation and polarimetric identification of individual amino acids. This method depends upon the measurement of oxygen uptake and ammonia production when the specific d-amino acid oxidase of Krebs (10) acts upon a hydrolysate. Mention was made earlier of the finding of d-amino acids in gramicidin and tyrocidine (6);

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1 Kögl and Erxleben (1) have described the preparation from tumor proteins of amino acids which, judged on a basis of optical rotation, had small proportions of the d forms present. Later investigations by a number of workers have shown that the findings were probably explained by racemization and fractionation during isolation of the amino acids rather than by the occurrence of the d forms in the original material (see e.g. Dunn (2) and Schoenheimer and Ratner (3)).
the data are presented here, and an accompanying communication reports the actual isolation of \(d\)-leucine from gramicidin (8).

The possibility existed that the observed oxidation might have been brought about by some enzyme, other than \(d\)-amino acid oxidase, also present in the more or less crude enzyme preparation employed. However, there are a number of indications that this cannot be the case. Another communication shows that about 92 per cent of the total nitrogen of hydrolysates of both gramicidin and tyrocidine is present in typical \(\alpha\)-amino acids and ammonia (8). Since the ammonia production by the oxidase was practically equivalent to the oxygen uptake, and corresponded to a considerable proportion of the total nitrogen, it was necessary to conclude that it was actually the result of the oxidation of \(d\)-amino acids. This conclusion has been made still more convincing through the removal, according to the procedure of Negelein and Brömel (11) of the major part of the flavin-adenine prosthetic group from the enzyme protein. When the pure flavin-adenine dinucleotide of Warburg and Christian (12) was added back to the protein component, there was a 3-fold increase in the rate of oxidation of gramicidin hydrolysate. This finding is additional proof that the oxidizing enzyme actually was \(d\)-amino acid oxidase.

**EXPERIMENTAL**

*Preparation of Hydrolysates*—Gramicidin and tyrocidine hydrochloride were hydrolyzed in hydrochloric acid containing acetic acid. A carbon dioxide atmosphere was provided during the hydrolysis and until the strong acid had been removed by evaporation. These conditions were such that only a negligible amount of tryptophane was destroyed or racemized (cf. Experiment 4 in Table I). Further details concerning the hydrolysates will be found in another place (8).

*Enzyme Preparation*—A detailed description of the enzymatic method will be given in a separate publication by one of us (L.). In most of the experiments described here a dry preparation was used, obtained by acetone precipitation of an extract of acetone-dried lamb kidney. Of this dry powder a 10 per cent solution was prepared in 0.2 M pyrophosphate of pH 8.3 (12), containing one-fourth its volume of gum ghatti solution (2 gm. of gum ghatti extracted with 100 cc. of hot water). With \(d\)-alanine, 0.25 cc. of
the concentrated enzyme solution absorbed 40 to 70 c.mm. of O₂ in 10 minutes.

Oxidase Experiments—Ordinary respiration vessels of 8 cc. total volume were used, with 0.5 to 1.0 cc. of hydrolysate (neutralized to phenolphthalein) in the main compartment. 0.25 cc. of enzyme solution was placed in the side arm. A small crystal of thymol was added to each vessel to prevent bacterial growth. The ex-

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Hydrolysate</th>
<th>Total α-NH₂-N</th>
<th>Time of oxidation</th>
<th>d-Amino N found</th>
<th>Remarks</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg.</td>
<td>hrs.</td>
<td>From O₂ uptake</td>
<td>From NH₃ produced</td>
<td>From NH₃ per cent of α-NH₂-N</td>
</tr>
<tr>
<td>1</td>
<td>HA²</td>
<td>0.978</td>
<td>17</td>
<td>0.52</td>
<td>0.43</td>
<td>44</td>
</tr>
<tr>
<td>2</td>
<td>HA₄. Hydrolysis</td>
<td>0.85</td>
<td>23</td>
<td>0.35</td>
<td>0.30</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>80% complete</td>
<td></td>
<td></td>
<td>0.36</td>
<td>0.29</td>
<td>34</td>
</tr>
<tr>
<td>2a</td>
<td>&quot;</td>
<td>0.85</td>
<td>4</td>
<td>0.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2b</td>
<td>HA₄. Tryptophane-free fraction</td>
<td>0.626</td>
<td>4</td>
<td>0.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2c</td>
<td>HA₄. Tryptophane fraction</td>
<td>0.419</td>
<td>4</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>HA₅</td>
<td>0.480</td>
<td>19</td>
<td>0.32</td>
<td>0.22</td>
<td>46</td>
</tr>
<tr>
<td>3a</td>
<td>&quot; Tryptophane-free fraction</td>
<td>0.300</td>
<td>19</td>
<td>0.27</td>
<td>0.21</td>
<td>68</td>
</tr>
<tr>
<td>4</td>
<td>&quot;Hydrolyzed&quot; tryptophane</td>
<td>0.20</td>
<td>23</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Experiments were carried out at 37° with air as the gas phase and with sodium hydroxide in the center cup. After equilibration the enzyme was added from the side arm and shaking was continued for 17 to 20 hours to obtain as complete a reaction as possible. The largest part of the oxygen uptake, however, occurred in the first 3 hours (cf. Experiments 2 and 2a in Table I) indicating the presence of fairly rapidly reacting d-amino acids.
At the end of the experiment the sodium hydroxide was removed and 2.5 or 3.0 cc. of 5 per cent trichloroacetic acid were added to the main compartment of the vessel. The contents were then transferred to a small centrifuge tube. After centrifugation, ammonia was determined in an aliquot sample. The ammonia was driven over either in vacuo after the addition of borate (Parnas (13)) or by steam distillation from calcium hydroxide, and finally determined by titration. Each experiment was accompanied by a blank determination of the oxygen and ammonia for the enzyme alone. These are deducted in the figures given in Table I. The oxygen taken up by the enzyme blank was not greater than 10 to 20 c.mm.

For the calculation of the percentage of d-amino acid, given in next to the last column of Table I, the more reliable figures from the ammonia production were used. Compared with the ammonia values, the oxygen consumption was usually from 15 to 30 per cent above the values to be expected from the equation,

\[ R-\text{CHNH}_2-\text{COOH} + \frac{3}{2}\text{O}_2 = R-\text{CO}-\text{COOH} + \text{NH}_3 \]

In a recent paper on d-amino acid oxidase, Klein and Handler (14) reported similar discrepancies with a number of amino acids.

Similar experiments were performed with the reconstituted enzyme. The removal of the prosthetic group was carried out by the procedure described by Negelein and Brömel (11). Without the use of a cooled centrifuge it was not feasible to remove completely the flavin-adenine. On addition of a pure preparation of flavin-adenine dinucleotide\(^2\) the rate of oxidation of d-alanine by the protein component rose 3-fold. Experiments with gramicidin hydrolysate and the tryptophane-free fraction of it, in which the protein was employed with and without the added flavin component, gave a similar result. Fig. 1 shows the initial rates of oxidation in one of the experiments.

The initial rates found with both crude and purified enzyme preparations are always higher than would be expected if d-leucine alone were present. There is thus an indication of the presence of another and faster reacting d-amino acid. The tryptophane fraction contained no detectable d-amino acid (Table I) and, in

\(^2\) A sample of pure barium flavin-adenine dinucleotidate has been kindly supplied to the senior author by Professor Otto Warburg.
confirmation of this finding, crystalline L-tryptophane was isolated (8).

Experiments with Tyrocidine—Oxidase experiments showed appreciable quantities of d-amino acids in tyrocidine hydrolysates. Ammonia production indicated that somewhat more than 20 per cent of the α-amino acids were of the d configuration. This might be considered as a minimum value, since with tyrocidine the ammonia determinations indicated only about 50 per cent of what would have corresponded to the oxygen consumed. In addition, the analysis of ammonia produced required the correction for a rather large amount of preformed ammonia in the tyrocidine hydrolysate. A dicarboxylicamino acid fraction prepared from tyrocidine showed very little, if any, d-amino acid.

**DISCUSSION**

The d-leucine and L-tryptophane recovered from gramicidin and the 68 per cent of d-amino acids found in the tryptophane-free fraction are sufficient evidence that the amino acids of gramicidin are not present as racemates. The d-leucine β-naphthalenesulfonate isolated corresponds to about one-half the total of 45 per cent determined as d-amino acids by the oxidase method. Since a large part of the remaining amino acid is alanine (8), there is a

![Fig. 1. Initial oxygen uptake by acid hydrolysate of gramicidin and d-amino acid oxidase protein. ( without added flavin-adenine dinucleotide; O with added flavin-adenine dinucleotide.](http://www.jbc.org/)

[http://www.jbc.org/]
considerable possibility that all or part of it could be present as d-alanine. The rather high rate of oxidation points in this direction.

Gramicidin, then, presents a picture of a polypeptide composed of amino acids, some of which occur in the d form and others of which occur in the l form. This interesting type of structure, which was also indicated in the polypeptide component of ergotinine (4), may well be responsible for the resistance of gramicidin (and tyrocidine) to ordinary proteolytic enzymes. Possession of a structure of this sort may conceivably be important in connection with the known antibacterial and toxic properties of these polypeptides, either by contributing a toxicity in itself, or by making difficult the destruction and removal of a molecule toxic for other reasons.

The findings of Ivánovics and Bruckner (5) and the above results furnish two cases of the occurrence of d-amino acids in the aerobic sporulating bacteria. Berger, Johnson, and Baumann have described d-peptidase activity in a number of microorganisms (15), one of which, Bacillus megatherium, belongs to the same general group. Inasmuch as the organisms of this group are wide-spread in nature, and are found for example in certain cheeses, it seems possible that the animal organism is, on occasion, confronted with the task of disposing of d-amino acids liberated in the intestinal tract. If this should be the case, it would provide one use for the oxidase, occurring in animal tissues, specifically capable of breaking down d-amino acids.

**SUMMARY**

Enzymatic assay with d-amino acid oxidase indicates that 45 per cent of the α-amino acids of gramicidin hydrolysates have the d configuration. Tyrocidine appears to contain d-amino acids amounting to 20 per cent of its α-amino acids.

**BIBLIOGRAPHY**

2. Dunn, M. S., in Luck, J. M., Annual review of biochemistry, Stanford University, **10**, 104 (1941).
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