OPTICAL PURITY OF AMINO ACID ENANTIOMORPHS

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(Received for publication, May 4, 1951)

A variety of procedures has been devised for the preparation of the enantiomorphic forms of amino acids by resolution. The choice of a given procedure may depend upon considerations of economy, ease of manipulation, yield, and purity of the final products. Although the first three of these factors have received attention, the question of optical purity of amino acid enantiomorphs has usually been neglected. This is surprising in view of the fact that highly accurate appraisal of the purity of these compounds has long been possible through the use of certain well known, optically specific enzyme systems. For example, Zeller (27) has suggested the use of $L$-amino acid oxidase preparations for the detection of $L$ isomer contamination in $D$-amino acids. The present report is concerned with application of such enzyme systems to an evaluation of the purity of the enantiomorphic forms of several of the amino acids prepared by the resolution methods reported from this Laboratory (9-17, 20).

The effectiveness of these methods has hitherto been based on evidence from two sources. Firstly, manometric measurements of ninhydrin-developed carbon dioxide during the digestion period indicate 50 per cent hydrolysis of the racemic $N$-acylated amino acid or amino acid amide, implying that the action of the respective acylase or amidase is optically specific. Secondly, determinations of the specific optical rotation of the $L$- and $D$-amino acids finally obtained yield values for the enantiomorphs nearly equal in magnitude and opposite in sign, supporting the concept of the optical specificity of the enzyme concerned.

We are, however, reluctant to ascribe a high order of effectiveness to any resolution procedure based on this type of evidence alone. The mano-

1 The term "resolution" denotes a procedure whereby both enantiomorphs are prepared in pure form from a racemate. For amino acids, two general procedures have been employed: namely, (a) the formation of diastereoisomeric mixtures of $N$-acylated racemic amino acids with optically active bases, which was introduced by Fischer (1) (for the literature cf. (2)), and more recently (b) the use of the enzymatic synthesis of $N$-acylated amino acids with anilides (3-8) and of enzymatic hydrolysis of $N$-acylated amino acids (9-19), amino acid amides (20), and amino acid esters (21-24). The preparation of one isomer from a racemate by selective enzymatic destruction of its enantiomorph has been described (25, 26). All of these procedures derive of course from the pioneer work of Pasteur.
metric measurement of ninhydrin-developed carbon dioxide in solutions of
pure amino acids is good to 0.2 to 0.3 per cent, as pointed out by Van
Slyke, Dillon, MacFadyen, and Hamilton (28) (cf. also (2)). In enzymatic
digests of acylated amino acids or amino acid amides, this error may be
increased to about ±1 per cent. Thus, if complete hydrolysis of the L
form did not occur, or if the enzymes were not exclusively asymmetric in
their action and slowly attacked the D forms, the divergence from the
theoretical 50 per cent of hydrolysis might not be noted. In either event,
one of the isomers examined could be contaminated by as much as 1 per
cent of the other without detection.

The customary criterion of optical purity, namely that of optical rota-
tion, is not sufficiently sensitive to reveal this amount of contamination.
A simple example will suffice to show this. If the specific optical rotation
of L-methionine for a 2.00 per cent solution in 2 N HCl is accepted as
+23.0°, the angular rotation in a 2 dm. tube will be +0.92°. If the L-
methionine contained 1 per cent of the D form, the angular rotation would
be expected to be +0.90° and the specific optical rotation +22.5°. Even
with a good polarimeter and careful observations, it would be difficult to
accept the differences between these readings as significant. There is no
"theoretical value" for an optical rotation, but only one on which most
observers agree. The area of agreement, however, may be several per cent
wide. It is therefore difficult to determine whether the spread in values
in the literature for the rotation of the amino acid isomers is due to individ-
ual experimental variations or to contamination of the isomers by their
respective enantiomorphs. A more sensitive procedure than that of mea-
surement of the extent of the hydrolysis of the derivative, or of the optical
rotation of the products, is needed to define more closely the criteria of
optical purity to be expected by this, or for that matter, any resolution
method.

By employing preparations of L- and D-amino acid oxidases and of bac-
terial decarboxylases, we have been enabled to determine the level of
possible contamination of one optical isomer by the other to less than 1

2 This may be caused by a possible reversal of hydrolysis, inhibition by products
of the reaction, or loss of activity of the enzyme. In some cases (cf. (20)), the hy-
drolysis apparently proceeds to completion only in very low initial concentrations of
the substrate. Our current procedure is to employ initial concentrations of the
racemates no higher than 0.1 M.

3 Definite hydrolysis of the amides of D-proline, D-alanine, and D-leucine has been
observed with preparations of mushrooms (29).

4 This is of course true only up to a certain limit. It is possible that an amino
cid isomer may contain as much as 2 per cent of its enantiomorph, and its rotation
value may still fall within the area of general acceptance for such values. When,
however, the rotation value for any one isomer falls more than 5 per cent below the
median of the range of values for this isomer, it must be viewed with suspicion.
part in 1000. All amino acids capable of being examined in this fashion, and which were obtained by the resolution procedures described (9–17, 20), were found to contain less than 0.1 per cent of the respective enantiomorph. In the cases of L-alanine and L-serine, which could be readily investigated in larger amounts, less than 0.01 per cent of the enantiomorph was noted. It is evident that the enzymatic resolution procedure described yields amino acid isomers of high optical purity. It is proposed (a) that the readily available oxidase and decarboxylase preparations be routinely employed when possible to supplement optical rotation data in the characterization of amino acid isomers, and (b) that a standard of optical purity greater than 99.9 per cent for each isomer be adopted to define an adequate resolution of both isomers from a racemic amino acid.

EXPERIMENTAL

Enzyme Preparations—Cobra venom, obtained from Hynson, Westcott and Dunning, Inc., and rattlesnake venom from Ross Allen's Reptile Institute were used as sources of L-amino acid oxidase (27, 31). The latter material was used to test the D-aminophenylacetic acid, D-valine, D-isoleucine, and D-alloisoleucine preparations, the former to test all other D isomers. D-Amino acid oxidase solutions were prepared by extraction of an acetone powder of fresh hog kidney (31). Lyophilized Clostridium welchii (strain SR 12) cells were employed as a source of glutamic acid (30, 32, 33) and aspartic acid (32, 33) decarboxylases.

General Procedure—The conventional Warburg manometric technique was employed. The gas phase was air, and in certain experiments 2,2,4-trimethylpentane was used as the manometric fluid. The usual procedure was as follows: 1000 μM of the amino acid isomer to be tested were weighed

4 The oxidases react with L and D isomers other than those enantiomorphic with the amino acid studied. However, if the starting racemic amino acids are pure, no trouble is encountered from this source. The assumption that these enzymes are optically specific is supported by the data.

5 Not all of the resolved isomers could be tested in this fashion. Neither cobra venom (31) nor rattlesnake venom L-amino acid oxidase, for example, was found to oxidize L-alanine, L-histidine, L-lysine, L-ornithine, L-serine, L-threonine, or L-allothreonine at suitable rates, and consequently the D forms of these amino acids were not studied. Hog kidney D-amino acid oxidase reacts poorly, if at all, with D-lysine or D-threonine. Enough of the resolved isomers could be assayed, however, by these means (see Table II) adequately to test the effectiveness of the resolution method. It is possible that the other amino acids could be oxidized by enzymes from other sources.

6 By suitably enlarging and redesigning the Warburg vessels, there is no reason why this procedure could not be extended to larger quantities of the more insoluble amino acids, and their possible contamination studied at levels of 0.01 per cent or less.
into the main compartment of the vessel and 1.5 to 2.5 cc. of buffer were added. The enzyme preparation (0.3 to 0.4 cc.) was placed in the side bulb. For the studies with \(\text{d-amino acid oxidase}\), 0.1 \text{M} sodium pyrophosphate buffer of pH 8.1 was employed. For \(\text{l-amino acid oxidase}\), a 0.1 \text{M} potassium phosphate buffer of pH 7.4 was used, and 25 units of crystalline catalase were added to the main compartment. With the preparation of cobra venom employed, 5 mg. provided a sufficient excess to insure rapid oxidation; with the rattlesnake venom preparation, 15 mg. were used with

**Table I**

*Determination of Optical Purity of Amino Acids; Recovery Experiments with Added Enantiomorphs*

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Source from literature</th>
<th>Concentration</th>
<th>Enzyme system</th>
<th>(\text{O}_2) consumed (microatoms) or CO(_2) produced ((\mu)l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\mu)M</td>
<td>(\mu)M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>(10)</td>
<td>0</td>
<td>10,000</td>
<td>(\text{d-amino acid oxidase})</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>10,000</td>
<td>(\text{l-amino acid oxidase})</td>
</tr>
<tr>
<td></td>
<td>(10)</td>
<td>0</td>
<td>1,000</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>(11)</td>
<td>1,000</td>
<td>0</td>
<td>(\text{l-amino acid oxidase})</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,000</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>(17)</td>
<td>1,000</td>
<td>2.0</td>
<td>(\text{Glutamic acid decarboxylase})</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>(10)</td>
<td>1,000</td>
<td>1.0</td>
<td>(\text{Aspartic acid decarboxylase})</td>
</tr>
</tbody>
</table>

* The experimental details are given in the text.

The data indicate that, when 1000 \(\mu\)M of the isomer were used, no \(\text{d-valine and d-isoleucine, and 30 mg. with d-aminophenylacetic acid and d-alloisoleucine. The procedures for glutamic acid and aspartic acid decarboxylase were carried out as described (30, 32, 33)}. Each test included control vessels without substrate, and vessels to which 1, 2, and 10 \(\mu\)M of the susceptible enantiomorph were added. All experiments were carried out in duplicate. The flasks were tipped after a 10 to 15 minute equilibration period and read at intervals until gas evolution or consumption was complete (15 to 120 minutes).

**Results**

The results of several representative experiments are summarized in Table I. The data indicate that, when 1000 \(\mu\)M of the isomer were used, no
evidence of contamination with its enantiomorph was obtained. Experiments in which as little as 1 \(\mu M\) of the susceptible isomer was added showed

### Table II

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Source*</th>
<th>Concentration of enantiomorph</th>
<th>Amino acid</th>
<th>Source*</th>
<th>Concentration of enantiomorph</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Alanine</td>
<td>(10, 20)†</td>
<td>&lt;0.01</td>
<td>D-Phenylalanine</td>
<td>(11)†</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>&quot;</td>
<td>A</td>
<td>1.1</td>
<td>&quot;</td>
<td>A</td>
<td>2.1</td>
</tr>
<tr>
<td>&quot;</td>
<td>B</td>
<td>1.3</td>
<td>L-Tyrosine</td>
<td>(11)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>(10)†</td>
<td>&lt;0.1</td>
<td>D-Tyrosine</td>
<td>(11)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>&quot;</td>
<td>A</td>
<td>&lt;0.1</td>
<td>L-Tryptophan</td>
<td>(11)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>&quot;</td>
<td>B</td>
<td>5.8</td>
<td>D-Tryptophan</td>
<td>(11)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>d-Methionine</td>
<td>(10)†</td>
<td>&lt;0.1</td>
<td>L-Serine</td>
<td>(10)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>&quot;</td>
<td>A</td>
<td>2.8</td>
<td>D-Glutamic acid</td>
<td>(17)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>&quot;</td>
<td>B</td>
<td>3.5</td>
<td>D-Aspartic &quot; &quot;</td>
<td>(10)‡</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>L-Norleucine</td>
<td>(12)†</td>
<td>&lt;0.1</td>
<td>L-Isoleucine</td>
<td>(15)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>&quot;</td>
<td>B</td>
<td>16.3</td>
<td>&quot;</td>
<td>A§</td>
<td>1.0</td>
</tr>
<tr>
<td>D-Norleucine</td>
<td>(12)†</td>
<td>&lt;0.1</td>
<td>D-Isoleucine</td>
<td>(15)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>&quot;</td>
<td>A</td>
<td>12.4</td>
<td>&quot;</td>
<td>A§</td>
<td>0.6</td>
</tr>
<tr>
<td>L-Valine</td>
<td>(10)†</td>
<td>&lt;0.1</td>
<td>D-Alloisoleucine</td>
<td>(15)†</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>&quot;</td>
<td>A</td>
<td>2.8</td>
<td>D-Alloisoleucine</td>
<td>(15)†</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>d-Valine</td>
<td>(10)†</td>
<td>&lt;0.1</td>
<td>S-Benzyl-L-cysteine</td>
<td>(20)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>L-Norvaline</td>
<td>(12)†</td>
<td>&lt;0.1</td>
<td>S-Benzyl-L-cysteine</td>
<td>(20)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>d-Norvaline</td>
<td>(12)†</td>
<td>&lt;0.1</td>
<td>L-Ethionine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Leucine</td>
<td>(10)†</td>
<td>&lt;0.1</td>
<td>D-Ethionine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td>A</td>
<td>1.4</td>
<td>&quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>(11)†</td>
<td>&lt;0.1</td>
<td>L-Aminophenylacetic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td>A</td>
<td>&lt;0.1</td>
<td>D-Aminophenylacetic acid</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* A and B are available commercial sources. The figures in parentheses represent bibliographic references.
† Obtained also by a new and improved acylase preparation from hog kidney prepared by Dr. S. Birnbaum.
‡ Obtained by the action on chloroacetyl-L-aspartic acid of a specific aspartic acid acylase from hog kidney prepared by Dr. S. Birnbaum.
§ The stereochemical purity of these commercial specimens is unknown; i.e., it is possible that they may be mixtures of isoleucine and alloisoleucine (cf. (15)).
|| Preparation to be published.

that, within experimental error, stoichiometric amounts of gas were evolved or used. Even in the case of the slightly soluble amino acids, e.g. tyrosine, good recoveries were obtained. Thus the absence of oxygen uptake or carbon dioxide formation in tests with 1000 \(\mu M\) of amino acid indicates that
the isomer tested was contaminated with less than 0.1 per cent of its enantiomorph. Although the conditions employed in the present study permit detection of 0.1 per cent of the enantiomorph, greater sensitivity may be achieved by using higher concentrations of the amino acid to be tested. Thus, in the cases of alanine and serine, it was possible to detect 1 part of the \textit{D} isomer in the presence of 10,000 parts of \textit{L} isomer. This test indicates a maximum concentration of \textit{D}-alanine and of \textit{D}-serine of less than 0.01 per cent for the preparations of \textit{L}-alanine and \textit{L}-serine tested. \textit{L}-Alanine was obtained both by the resolution of acetyl-\textit{DL}-alanine (10) and of \textit{DL}-alanine amide (20).\footnote{To obtain \textit{L} isomers of this purity it is necessary, among other things, to insure the complete absence of free \textit{DL}-amino acid from the starting preparations of acylated \textit{DL}-amino acid or \textit{DL}-amino acid amide. The presence of \textit{D} isomer contamination in the \textit{L}-amino acid isolated (34) can be due only to such an impurity in the starting material; acetyl-\textit{D}-alanine is completely resistant to the action of acylase. In order to obtain \textit{D} isomers of the purity noted, it is our practice to allow digests to stand for several hours beyond the time at which 50 per cent hydrolysis of the starting racemate is observed and to insure the complete solubility of the acyl-\textit{D}-amino acid in acetone.}

All of the amino acid optical isomers prepared by enzymatic resolution in this Laboratory which could be tested by these procedures were found to contain less than 0.1 per cent of the respective enantiomorph (Table II). Several amino acid preparations obtained from two commercial sources were subjected to test.\footnote{These isomers were selected because it is highly probable that the \textit{L} forms were obtained by some method of resolution rather than by isolation from a protein hydrolysate. The estimation of contamination of \textit{L}-amino acids by other \textit{L}-amino acids, when isolation methods from a protein hydrolysate are used, constitutes a separate problem. No test with this degree of sensitivity is yet available to determine the contamination of isoleucine, threonine, or hydroxyproline by their respective allosteroisomers. The ready oxidation by \textit{D}-amino acid oxidase of \textit{D}-isoleucine and \textit{D}-alloisoleucine yields enantiomorphic \textit{\alpha}-keto acids with equal and opposite optical rotations (15, 35). The determination of such values yields a criterion of the stereochemical purity of the amino acids within the limits of error of the polarimetric observations, which may be as much as \pm 1 per cent.}

SUMMARY

Amino acid oxidases and decarboxylases have been employed to determine the level of possible contamination of one optical isomer by the other to less than 1 part in 1000. Application of this procedure to amino acids prepared by the resolution methods reported from this Laboratory showed that the preparations uniformly contained less than 0.1 per cent of the...
enantiomorph. L-Alanine and L-serine, which could be tested at higher concentrations, contained less than 0.01 per cent of the D forms. A survey has been made of certain commercially available optically active amino acids.

BIBLIOGRAPHY

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