ENZYMATIC HYDROLYSIS OF ANALOGOUS SATURATED AND UNSATURATED PEPTIDES

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Two types of peptidases are present in tissues, one of which catalyzes the hydrolysis of the saturated, RCHCONHCHR'COOH, the other that of the unsaturated, RCHCONH(=CHR')COOH = RCHCON-C(CH₂R')-COOH, peptide bonds (cf. (1)). These are designated, respectively, peptidases and dehydropeptidases. We have reported the rates of hydrolysis of variously constituted dehydropeptides in extracts of rat tissues (24). The present study consists in a comparison of such rates with those of analogous saturated peptides under nearly identical experimental conditions. Comparison has been made between glycyl-DL-alanine and glycyldehydroalanine, glycyl-DL-phenylalanine and glycyldehydrophenylalanine, and chloroacetyl-DL-alanine and chloroacetyldehydroalanine, studied in extracts of rat kidney, liver, and hepatoma. Acetyl-DL-alanine, chloroacetyl-DL-phenylalanine, acetylglycine, and glycylglycine were also studied in kidney digests, and the former two compounds compared, respectively, with acetyldehydroalanine and chloroacetyldehydrophenylalanine.

EXPERIMENTAL

The digests were composed of 1 cc. of fresh, aqueous rat tissue extract, or 2 cc. of 0.15 M borate buffer at pH 8.0 and 1 cc. of either water or 0.025 M neutralized dehydropeptide or 0.050 M neutralized racemic peptide solution. No metallic or other activator was added. The hydrolysis at 37° of the dehydropeptides was followed by the rate of evolution of ammonia (2) and that of the peptides by measuring the CO₂ evolved after treatment with ninhydrin (5), both rates being corrected for the respective

1 Substrates containing an α tertiary carbon atom, such as glycylaminoisobutyric acid and favorably constituted di(acylamino)propionic acids, are also hydrolyzed in rat tissues (14). The relation, if any, between the enzymes acting upon such substrates and those catalyzing the hydrolysis of peptides with a hydrogen atom on the α-carbon is not yet clear.

2 The extracts were prepared by grinding the freshly removed tissue with clean sand in a glass mortar and suspending in water, followed by light centrifugation to remove the sand and tissue débris. The extracts were used within a half hour of preparation.
controls. In agreement with the opinion expressed by Neurath et al. (6), we have found the specific and accurate ninhydrin method the most satisfactory of contemporary procedures for measuring the rate of peptide hydrolysis.

The saturated peptides employed were racemic mixtures, and the hydrolysis was followed in each case almost to completion. As others have noted (7-9), both optical forms of the glycyl peptides were hydrolyzed. With extracts of liver and hepatoma, the rates of hydrolysis of the two antipodes of glycyl-DL-alanine and glycyl-DL-phenylalanine were so different that it could be assumed that the more susceptible isomer was completely hydrolyzed before there was appreciable hydrolysis of the less susceptible isomer (Fig. 1). In such cases, it was necessary to employ higher extract concentrations to follow the hydrolysis of the less susceptible than for the more susceptible isomer. With kidney extracts, the rate of hydrolysis of the more susceptible isomer of glycyl-DL-phenylalanine is also considerably greater than that of the less susceptible isomer. How-

Under our conditions, the ninhydrin procedure results in a negligible hydrolysis of all the peptides studied except glycylglycine, which is split to a few per cent (cf. (5)).

By the "more susceptible isomer" we refer to that member of the pair of antipodal peptides which is more rapidly hydrolyzed, without commitment as to whether the two isomers are hydrolyzed by the same or different enzymes.
Fig. 2. Hydrolysis curves of 0.05 M glycyl-DL-alanine (×) and chloroacetyl-DL-alanine (●) with rat kidney extracts containing 0.08 and 0.6 mg. of N per cc., respectively.

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**Table I**

Rates of Hydrolysis of Saturated and Unsaturated Peptides with Rat Tissue Extracts

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Micromoles × 10 substrate hydrolyzed per hr. per mg. N in extract of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kidney*</td>
</tr>
<tr>
<td>Glycyldidehydroalanine</td>
<td>1,620</td>
</tr>
<tr>
<td>Glycyl-DL-alanine (one optical form)</td>
<td>6,000</td>
</tr>
<tr>
<td>&quot; (2nd &quot; &quot; &quot; )</td>
<td>1,020</td>
</tr>
<tr>
<td>Glycyldidehydrophenylalanine</td>
<td>520</td>
</tr>
<tr>
<td>Glycyl-DL-phenylalanine (one optical form)</td>
<td>10,300</td>
</tr>
<tr>
<td>&quot; (2nd &quot; &quot; &quot; )</td>
<td>360</td>
</tr>
<tr>
<td>Chloroacetyldidehydroalanine</td>
<td>100</td>
</tr>
<tr>
<td>Chloroacetyldl-alanine (one optical form)</td>
<td>600§</td>
</tr>
</tbody>
</table>

* Glycylglycine is hydrolyzed with a rate value of about 400; chloroacetylglycine is not hydrolyzed.
† Primary tumors induced in Osborne-Mendel rats by feeding p-dimethylaminoazobenzene; donated by Dr. J. White. Necrotic areas removed.
‡ The corresponding value when 0.025 M glycyl-L-phenylalanine was used was 2300.
§ Acetyl-DL-alanine (only one optical form) is hydrolyzed at a rate value of 310, compared with that of 18 for acetyldidehydroalanine. Chloroacetyl-DL-phenylalanine (only one optical form) is hydrolyzed at a rate value of 1200, compared with that of 0 for chloroacetyldidehydrophenylalanine.
ever, in the case of glycyl-\textit{dL}-alanine, the hydrolysis of both optical isomers more nearly approaches that of the other, and the rate of hydrolysis of each isomer can only be roughly approximated (Fig. 2). Only one optical form of chloroacetyl-\textit{dL}-alanine (Figs. 1 and 2), of acetyl-\textit{dL}-alanine, and of chloroacetyl-\textit{dL}-phenylalanine was hydrolyzed.

The initial reaction rates for the hydrolysis of the peptides were approximated from the slope of the respective curves (micromoles of substrate hydrolyzed plotted against time); the data are given in Table 1.

In the absence of definitive information as to which optical isomer of the racemic peptides was being hydrolyzed, no designation can be made at this time, but presumably the susceptible isomer of acetyl-\textit{dL}-alanine, chloroacetyl-\textit{dL}-alanine, and chloroacetyl-\textit{dL}-phenylalanine and the more susceptible isomer of the glycyl peptides is the \textit{L}, or "natural" form. The nearly equal rates of hydrolysis of glycyl-\textit{L}-phenylalanine and of the more susceptible isomer of glycyl-\textit{dL}-phenylalanine support this possibility (Table I). The rates noted for the saturated peptides may be minimum for the most part, (a) since they were obtained on compounds hydrolyzing in the presence of their optical enantiomorphs, which might exert an inhibitory influence, and (b) since no possible activator was added (8, 10).

Nevertheless, the rates of hydrolysis of the more susceptible forms of the saturated peptides are in every case considerably greater than those of the analogous dehydropeptides (Table I).

**DISCUSSION**

Bergmann and Schleidt (1) showed that purified preparations of dipeptidase, aminopeptidase, and carboxypeptidase, which were highly active toward saturated peptides, had no effect upon unsaturated peptides. The converse has not yet been shown to be true, for no attempt has been made to prepare purified dehydropeptidases free of activity toward the saturated peptides. Nevertheless, some further evidence that the peptidases and dehydropeptidases are distinct enzyme systems may be derived from a comparative study of liver and hepatoma. The malignant transformation of a tissue may serve in effect, in the case of a number of closely related enzymes, as a fractionation procedure. Thus, in the consideration of the possible multiple nature of dehydropeptidase I (4), considerable weight was laid on the fact that, whereas the hydrolysis rate of glycyldehydroalanine and alanyldehydroalanine increased very considerably when liver was transformed into a hepatoma, the hydrolysis rate of glycyldehydrophenylalanine and N-methylglycyldehydroalanine decreased. Assuming that the cancerous transformation of a tissue does not result in qualitative changes in the enzymes concerned, the enrichment in activity toward glycyldehydroalanine and the concomitant decrease in activity toward glycyldehydrophenylalanine (4) (Table I) strongly suggest that the hydrolysis of these substrates is catalyzed by different enzymes. The fact that the malig-
nant transformation of liver is accompanied by a decrease in the rate whereby both optical forms of glycyl-DL-alanine are hydrolyzed (Table I) also suggests that glycyldehydroalanine and glycyl-DL-alanine are hydrolyzed by different enzyme systems. It is of some interest to note that, per mg. of tissue N, the hydrolysis rate of those saturated peptides studied, namely glycyl-DL-alanine, glycyl-DL-phenylalanine, and chloroacetyl-DL-alanine, decreases when liver becomes neoplastic (Table I), whereas it has been reported that the rate of hydrolysis of the more susceptible isomer of DL-leucylglycine and of DL-alanylglycine increases (11, 12). This difference suggests that alanylglycine and glycylalanine may be attacked by different peptidases, a possibility which had been advanced on other grounds for the corresponding D peptides by Maschmann (8). In any event, the use of carefully defined cancer tissue in comparative studies with the normal tissue of origin is sometimes capable of contributing to the solution of certain biochemical problems of fundamental interest (cf. (13)).

Still another suggestion that the peptidases and dehydropeptidases may be distinct systems arises from the fact that, whereas the dehydropeptides of alanine are hydrolyzed in tissue extracts much faster than the dehydropeptides of phenylalanine, the reverse holds true for the more susceptible (but not for the less susceptible) antipodes of the corresponding saturated peptides (4) (Table I).

The hydrolysis rates of the susceptible optical isomer of acetyl-DL-alanine, chloroacetyl-DL-alanine, and chloroacetyl-DL-phenylalanine, and of the more susceptible isomer of glycyl-DL-alanine and glycyl-DL-phenylalanine, are all greater than those of the corresponding analogous dehydropeptides in the same tissue extract (Table I). Little can be said in this connection of the less susceptible antipodes of the saturated peptides. Those of acetyl-DL-alanine, chloroacetyl-DL-alanine, and chloroacetyl-DL-phenylalanine are not hydrolyzed at all, while those of glycyl-DL-alanine and glycyl-DL-phenylalanine in kidney and liver extracts are apparently hydrolyzed at rates approximating those of the corresponding dehydropeptides (Table I). Further investigations will lay emphasis on the use of optically pure L and D peptides, which will also permit the use of more accurate kinetic treatment.

The only saturated peptide studied which was not hydrolyzed by aqueous extracts of kidney was chloroacetylglycine (Table I). This is in contrast to the ease of hydrolysis of the susceptible isomer of chloroacetyl-DL-alanine. Glycylglycine was hydrolyzed at a rate approximating that of glycylamino-
isobutyric acid (14), which was considerably less than that of glycyl-DL-alanine. It may be suggested that the presence of a center of optical asymmetry in a favorable position within the peptide is frequently conducive to increased susceptibility to peptidase action.

SUMMARY

The rates of hydrolysis in fresh, aqueous rat tissue extracts were determined for glycyl-DL-alanine, glycyl-DL-phenylalanine, chloroacetyl-DL-alanine, acetyl-DL-alanine, and chloroacetyl-DL-phenylalanine, and the rates compared both among themselves and with those of the corresponding analogous dehydropeptides. Both optical isomers of the glycyl peptides of alanine and phenylalanine were hydrolyzed, one at a faster rate than the other, but only one isomer of the chloroacetyl and acetyl peptides was hydrolyzed. Chloroacetylglycine was not hydrolyzed, and glycylglycine was hydrolyzed in kidney at a rate approximately one-fifteenth that of the more susceptible isomer of glycyl-DL-alanine.

The more susceptible isomers of the racemic saturated peptides were hydrolyzed in extracts of kidney, liver, and hepatoma at considerably faster rates than those of the corresponding dehydropeptides. The neoplastic transformation of liver results in a decrease in the hydrolysis rate of glycylalanine, which is in contrast with the increase in hydrolysis rate of glycyldehydroalanine. The more susceptible antipodes of the saturated peptides of phenylalanine were hydrolyzed in kidney, liver, and hepatoma at a faster rate than those of alanine, whereas the converse held true of the corresponding dehydropeptides. The question of the separate identity of the peptidases and dehydropeptidases was discussed.

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