ENZYMATIC TRANSFER AND HYDROLYSIS INVOLVING GLUTAMINE AND ASPARAGINE*

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(Received for publication, February 11, 1957)

The occurrence in bacteria of transferases, enzymes which catalyze the
exchange of the β-aspartyl group of asparagine and the γ-glutamyl group
of glutamine with hydroxylamine or labeled ammonia, has been described
(1–4). Transferase activities have also been found in extracts of mammal-
ian tissues (5), in the mold Neurospora crassa,1 and in extracts of green
plants (6–8). Whereas transferase activity in plant (7), mammalian (5),
and Neurospora preparations1 is dependent upon the presence of manganese
together with phosphate or arsenate, these ions do not appear to be neces-
sary for transferase activity in bacterial preparations (2). Recently,
however, purified preparations of Proteus vulgaris were also found to possess
a second type of glutamotransferase which is activated by manganese (9).
Transfer reactions leading to the synthesis of glutamyl peptides from
 glutamine were catalyzed by preparations of Bacillus subtilis (10). As
in the case of the bacterial transferases, the synthesis of glutamyl peptides
by B. subtilis does not require the presence of any cofactors.

Enzymes called synthetases, which catalyze the synthesis of glutamine
or glutamohydroxamic acid from glutamic acid and either ammonia or
hydroxylamine, were found in animal (11–13), plant (14, 15), and microbial
(16, 17) systems. A similar system in lupine seedlings, which synthesizes
asparagine from aspartic acid and ammonia, has recently been described
(18). Synthetase activities require the presence of ATP2 and magnesium
ions. Enzymes were also found which catalyze the hydrolysis of glutamo-
and aspartohydroxamic acids (2, 19).

The relationship among the various enzymatic reactions mentioned
above is not clear. It would be of great interest to ascertain whether all
the four reactions, (1) the synthesis of the amide, (2) the hydrolysis of the
amide, (3) the transfer reactions leading to the formation of the corre-
sponding hydroxamic acids, and (4) the hydrolysis of the hydroxamates,
are due to the activity of one enzymatic system or whether several enzyme

* Aided in part by a grant from the Hadassah Medical Organization, Jerusalem.
1 Grossowicz, N., unpublished data (1951).
2 The following abbreviations are used: ATP = adenosine triphosphate; ADP =
adenosine diphosphate; GHA = γ-glutamyl hydroxamic acid; AHA = β-aspartohy-
droxamic acid.
GLUTAMINE AND ASPARAGINE systems are involved. Some workers consider the transferase activities as being dissociated from the synthetase systems, at least in the bacterial preparations studied (2, 9). Others, like Elliott (14), do not commit themselves. On the other hand, Meister and his associates (19-21) believe that the four reactions are all catalyzed by the same enzyme.

Fig. 1. The effect of manganese, phosphate, and arsenate on the glutamotransferase activity of M. phlei. The reaction mixture for the determination of the effect of manganese contained L-glutamine, 0.02 M; NH₄OH-HCl, 0.02 M; MnSO₄, in various concentrations; phosphate, 0.002 M; and bacterial protein, 9.4 mg., pH 5.8. Total volume 2 ml. Incubation for 1 hour at 37°. For determination of the optimal concentrations of phosphate and arsenate the system contained L-glutamine, 0.02 M; NH₄OH·HCl, 0.02 M; MnSO₄, 0.005 M; phosphate or arsenate as indicated; and bacterial protein, 3.5 mg., pH 5.8. Total volume 1 ml. Incubation for 1 hour at 37°.

The present study was conducted on the transfer reactions involving the amide group of asparagine and glutamine, and the synthesis and hydrolysis of their corresponding hydroxamic acids in cell-free extracts of Mycobacterium phlei.

EXPERIMENTAL

Cultures of M. phlei were prepared and cell-free extracts obtained according to the procedures which have already been described (22).
Hydroxamic acids were determined by the method of Lipmann and Tuttle (23) and hydroxylamine by that of Csaky (24). Ammonia was estimated by nesslerization (22). Amino acids were identified by paper chromatography and estimated quantitatively by the method of Giri et al. (25).

Results

Glutamotransferase—It has been shown that extracts of M. phlei are high in asparaginase and low in glutaminase activity (22). These extracts were tested for transferase activities by experiments carried out under a wide range of hydrogen ion concentrations (pH 5.0 to 9.5) in the presence of manganese, phosphate, and ATP. No exchange was found to occur between asparagine and hydroxylamine. On the other hand, a vigorous exchange between glutamine and hydroxylamine was observed.3 The glutamotransferase of M. phlei was found to require the presence of manganese and phosphate ions. The optimal concentration of manganese was 3.7 × 10⁻³ M (Fig. 1) and magnesium could not be substituted for manganese. By treating extracts with an anion exchange resin (Amberlite IRA-400) it could be shown that phosphate was essential for the transfer reaction. Arsenate could be substituted for phosphate, and accelerated the reaction to almost 4 times the rate. Whereas the optimal concentration of phosphate was 1.5 × 10⁻³ M (Fig. 1) and arsenate was optimal at a concentration of 5.0 × 10⁻³ M (Fig. 1). ATP was not necessary but at fairly high concentrations (5.0 × 10⁻⁴ M) could replace inorganic phosphate. When phosphate was present, ATP was ineffective (Table I). The affinity of the enzyme for hydroxylamine was high and a maximal reaction rate was obtained at a concentration of 1 × 10⁻² M NH₂OH

2 Lichtenstein, Y., unpublished experiments (1953).

### Table I

**Influence of Adenosine Triphosphate on Glutamotransferase Activity of M. phlei Extracts**

<table>
<thead>
<tr>
<th>Additions</th>
<th>μmoles GHA formed per mg. protein per hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP present, × 10⁻⁴ M</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>0.03</td>
</tr>
<tr>
<td>Phosphate (0.6 × 10⁻³ M)</td>
<td>0.31</td>
</tr>
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The reaction mixture contained L-glutamine 0.02 M, NH₄OH·HCl 0.02 M, MnSO₄ 0.005 M, phosphate and ATP as indicated, and bacterial extract (10 mg. of protein). Total volume 2 ml. Incubation at 37° for 1 hour.
Fig. 2. Hydroxylamine concentration and the activity of the glutamotransferase of *M. phlei*. The system contained L-glutamine, 0.033 M; NH$_2$OH·HCl, as indicated; MnSO$_4$, 0.005 M; Na$_2$AsO$_4$, 0.002 M; and bacterial protein, 2.5 mg., pH 5.8. Total volume 1 ml. Incubation for 1 hour at 37°.

Fig. 3. Glutamine concentration and the activity of the glutamotransferase of *M. phlei*. The system contained L-glutamine, as indicated; NH$_2$OH·HCl, 0.02 M; MnSO$_4$, 0.005 M; phosphate, 0.0017 M; and bacterial protein, 4.8 mg., pH 5.8. Total volume 2 ml. Incubation for 30 minutes at 37°.
Higher concentrations of hydroxylamine (5 to 20 $\times$ 10$^{-2}$ M) inhibited the formation of hydroxaminate considerably (16 to 45 per cent inhibition). On the other hand, saturation of the enzyme with glutamine was not attained even at a concentration of glutamine as high as 2.0 $\times$ 10$^{-1}$ M (Fig. 3). The rate of formation of GHA was proportional to the

![Graph showing pH dependence of glutamotransferase activity](image)

Fig. 4. The influence of hydrogen ion concentration on the glutamotransferase activity of *M. phlei*. The reaction mixture contained L-glutamine, 0.02 M; NH$_2$OH$\cdot$HCl, 0.02 M; MnSO$_4$, 0.005 M; ATP$^\circ$ (when this experiment was performed, it was not yet clear that ATP was not required in this reaction), 0.0012 M; phosphate, 0.01 M; pH, as indicated; bacterial protein, 9.4 mg. Total volume 2 ml. Incubation for 50 minutes at 37°.

concentration of enzyme as long as the quantity of protein in the reaction mixture was less than 7 mg. per ml. Fig. 4 shows the dependence of the glutamotransferase activity on the hydrogen ion concentration; the highest activity was obtained at pH 5.6 to 5.8 and declined rapidly on both sides of the pH curve.

Synthetases—All attempts to find glutamo- or aspartosynthetase activities in extracts of *M. phlei* failed. Both systems were tested over a wide range of pH values (4.0 to 13.0) and in the presence of ATP with MgSO$_4$,
and either cysteine or KCN (13,14). At high pH values (9.0 to 13.0) some non-enzymatic formation of hydroxamic acid took place in the presence of either active or boiled enzyme.

Hydrolysis of Aspartohydroxamic Acid—Dialyzed extracts of M. phlei were found to catalyze the hydrolysis of AHA into aspartic acid and hydroxylamine. The pH curve of the reaction shows a sharp peak at pH 7.6 to 7.7 and differs considerably from the pH curve of the asparaginase, which shows optimal activity over a wider pH range (pH 8.0 to 9.0) (Fig. 5). The splitting of AHA was strongly inhibited by L- and D-asparagine and to a lesser extent by L-aspartic acid. At a ratio of L-asparagine to L-aspartohydroxamic acid as low as 1:20, the hydrolysis of AHA was diminished by approximately 40 per cent (Fig. 6). On the other hand, the addition of L-AHA in concentrations 15 times as high as that of L-asparagine did not appreciably (20 per cent) inhibit the hydrolysis of the amide. Both the affinity of L-AHA for the enzyme and the rate of
Fig. 6. The effect of analogues of AHA on the hydrolysis of AHA. The system contained \( L \)-asparagine; \( L \)-aspartic acid; \( L \)-AHA and NH\(_4\)Cl, as indicated; Tris buffer, pH 7.7, 0.017 M; and bacterial protein, 2.7 mg. Total volume 1 ml. Incubation for 1 hour at 37°C.

Fig. 7. The activity of the AHA-hydrolyzing enzyme as a function of the concentration of AHA. The system contained \( L \)-AHA, as indicated; Tris buffer, pH 7.7, 0.033 M; and bacterial protein, 3.2 mg. Total volume 1 ml. Incubation for 1 hour at 37°C.
decomposition of the hydroxamate did not differ greatly from the corresponding values for L-asparagine (22) and were fairly high (Fig. 7).

DISCUSSION

In our experiments, extracts of M. phlei have been found to possess a manganese-activated glutamotransferase. However, no measurable glutaminase activity was detected. There was no aspartotransferase activity in spite of the presence of a strong asparaginase (22). Thus it would seem that in M. phlei the reactions of hydrolysis and transfer are catalyzed by different enzymes. It is of considerable interest that neither asparto- nor glutamosynthetase activities were observed in M. phlei under conditions optimal for these reactions in animal, plant, and other bacterial systems (11 to 13, 15, 16, 18). It seems therefore that the asparaginase and glutamotransferase of M. phlei are in no way connected with the corresponding synthesizing enzyme systems.

We also found that extracts of M. phlei have the ability of catalyzing the hydrolysis of AHA to aspartic acid and hydroxylamine. It is worth noting that, whereas the hydrolysis of AHA is strongly inhibited by the presence of asparagine and to a lesser extent by aspartic acid, the hydrolysis of L-asparagine is not affected by the presence of AHA. AHA and asparagine appear to have similar affinities for the enzymes which catalyze their hydrolysis and similar rates of enzymatic decomposition. Hence the one-sided inhibition of AHA splitting by L-asparagine could scarcely be explained unless it be assumed that the hydrolysis of AHA and of asparagine by extracts of M. phlei is due to different enzymes.

The glutamotransferase of M. phlei, unlike that of Proteus vulgaris (2, 5), has been shown to require the presence of manganese together with phosphate or arsenate. These requirements are identical with those of the animal enzyme (5) and that of Neurospora crassa. ATP has no influence on the enzymatic activity when tested in the presence of an excess of phosphate. However, in the absence of added phosphate (or arsenate) high concentrations of ATP activate the system to almost the same extent as would the addition of phosphate. This effect can be explained by the liberation of inorganic phosphate from ATP by ATPase and myokinase found in extracts of this organism (26).

It has been shown that ADP strongly inhibits the synthesis of glutamine from glutamic acid, ammonia, and ATP (12). Therefore, the failure to demonstrate glutamosynthetase activity in preparations of M. phlei could also be interpreted as being due to the inhibition of the system by ADP liberated by ATPase action. If this assumption were true, enough ADP should accumulate to inhibit glutamosynthetase activity of extracts.

known to possess this enzyme. Thus the following experiment was set up: sonic extracts of *P. vulgaris* were prepared and tested for glutamosynthetase activity. This test was performed in the presence and in the absence of *M. phlei* extracts. It was found that the addition of *M. phlei* extract did not inhibit the glutamosynthetase activity of the *Proteus* preparation (see Table II). The inference that *M. phlei* extracts lack glutamosynthetase activity seems therefore justified.

**Table II**

<table>
<thead>
<tr>
<th>Additions</th>
<th>GHA formed* µmoles</th>
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<tbody>
<tr>
<td>None</td>
<td>3.32</td>
</tr>
<tr>
<td>Extract of <em>M. phlei</em> (4.5 mg. protein)</td>
<td>3.26</td>
</tr>
</tbody>
</table>

The system contained L-glutamic acid, 0.05 M; NH₄OH·HCl (adjusted to pH 7.0), 0.4 M; MgSO₄, 0.01 M; ATP, 0.01 M; Tris buffer, pH 7.0, 0.1 M; *P. vulgaris* extract, 10.8 mg. of protein; extract of *M. phlei* as indicated. Total volume, 2 ml. Incubation at 37° for 45 minutes.

* Corrections were made for the values of the blanks (without glutamic acid) and for the *M. phlei* extract.

**SUMMARY**

1. Extracts of *Mycobacterium phlei* were found to possess glutamotransferase activity and were active in hydrolyzing aspartohydroxamic acid (AHA) into aspartic acid and hydroxylamine. Aspartotransferase and glutamo- and aspartosynthetases were not found.

2. The glutamotransferase was dependent upon manganese (optimal concentration $3.7 \times 10^{-3}$ M) and phosphate (optimal concentration $1.5 \times 10^{-3}$ M) for activity. Magnesium could not be substituted for manganese, whereas phosphate could be replaced by ATP or arsenate; the latter was more active than phosphate.

3. The pH optimum of the glutamotransferase was between 5.6 and 5.8, falling off steeply at more acid or alkaline pH values.

4. The affinities of the transferase system for hydroxylamine and glutamine were investigated. Saturation of the enzyme with hydroxylamine was achieved at a concentration of $1.0 \times 10^{-2}$ M; in 5- to 20-fold higher concentrations, hydroxylamine inhibited the transferase activity (16 to 45 per cent). On the other hand, even as much as $2.0 \times 10^{-1}$ M of glutamine was not enough to saturate the enzyme.

5. The hydrolysis by *M. phlei* extracts of AHA and of asparagine was compared. The pH curve of the AHA-hydrolysis system was very steep.
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(optimal pH 7.6 to 7.7); that of asparaginase showed a much wider range of optimal activity (pH 8.0 to 9.0). Similar enzyme-substrate affinities were found in both systems. However, the hydrolysis of AHA was strongly inhibited by asparagine, whereas AHA did not appreciably affect asparaginase activity.

6. It is suggested that the transfer and hydrolysis reactions discussed are catalyzed by different enzymes.

The participation of Dr. Yehudith Lichtenstein in the early phases of this work is gratefully acknowledged. The authors are indebted to Dr. N. Lichtenstein, from the Department of Biological and Colloidal Chemistry of the Hebrew University, Jerusalem, for his generous gift of L-asparto-hydroxamic acid.

Addendum—After completion of this work, additional experiments were performed with D-AHA in order to elucidate the stereospecificity of the AHA-hydrolyzing enzyme. It was found that the enzyme hydrolyzes L- and D-AHA at a similar rate. We have already reported (22) that the L-asparaginase of M. phlei is inhibited competitively by D-asparagine. Thus, a striking difference in the stereospecificity of the two systems (L-asparaginase and AHA-hydrolyzing enzyme) is found, which presents additional proof for the non-identity of these enzymes.

BIBLIOGRAPHY

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