METABOLIC STUDIES OF RICKETTSIAE

V. Metabolism of Glutamine and Asparagine in Rickettsia mooseri

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The presence of glutamic-aspartic transaminase activity in suspensions of purified Rickettsia mooseri (Bovarnick and Miller, 1950; Hopps et al., 1956) suggested that this organism might possess other types of enzymes which catalyze amino or amido nitrogen transfer reactions. Since deamidases of glutamine and asparagine are widely distributed enzymes (Zittle, 1951; Waelsch, 1952), it was thought possible that they might also occur in rickettsiae. This report presents evidence that suspensions of purified R. mooseri deamidate glutamine and asparagine to the corresponding dicarboxyl amino acids. These rickettsiae also transfer the glutamyl and aspartyl residues of the amides to hydroxylamine with the formation of the respective hydroxamic acids.

**MATERIALS AND METHODS**

*Rickettsia suspensions.* Purified suspensions of R. mooseri were prepared by previously described methods (Wisseman et al., 1951; Hopps et al., 1956). Pertinent biological and chemical data on the preparations studied are given in table 1. Particulate suspensions of normal yolk sacs were prepared by carrying crude 20 per cent suspensions through two centrifugation steps as described previously (Hopps et al., 1956).

**Deamidase assay methods.** A qualitative (1) and a quantitative (2) method were employed for the demonstration and determination of deamidase activities.

(1) For qualitative demonstration of deamidase action, the rickettsial suspensions were incubated for 2 hr in Warburg vessels under our previously described standard conditions for manometric experiments (Wisseman et al., 1951). Glutamine or asparagine in concentrations of 50 μmoles per ml served as substrates. After the incubation the contents of the vessels were centrifuged for 30 min at 15,000 × g to sediment the rickettsiae.

(2) For quantitative determination of the amino acids formed by deamidation, mixtures consisting of 0.2 ml of rickettsial suspensions, 0.3 ml of 0.013 M phosphate buffers of the desired pH, and 0.1 ml of 0.25 M solutions of either glutamine or asparagine were placed in Hopkins type centrifuge tubes and incubated for 1 hr in a water bath at 36 C. Addition of 1 drop of 37 per cent formaldehyde to these mixtures or to identical control mixtures at zero time was employed to interrupt enzymatic deamidation. To prevent nonenzymatic deamination of glutamine (Vickery, Pucher, and Clark, 1935) heating or excessive pH values were not used for this purpose. The rickettsiae were then sedimented, and samples of 25 μl of the supernatant fluids were applied to Whatman no. 1 paper sheets for chromatography. After two successive descending developments with the butanol-acetic acid-water mixture of Partridge (1948), the elution and quantitative determination of the separated amino acids were carried out as described previously (Hopps et al., 1956).

**Amino acyl transferase assay methods.** A qualitative (1) and a quantitative (2) method were employed for the demonstration and estimation of amino acyl transferase activities.

(1) For qualitative demonstration of transferase activities, the rickettsiae were incubated for 2 hr in Warburg vessels as previously described (Wisseman et al., 1951); hydroxylamine hydrochloride, neutralized with KOH, and either glutamine or asparagine in concentrations of 50
Figure 1. Paper chromatogram showing the deamidation of glutamine and asparagine by Rickettsia mooseri. 1, Glutamine guide; 2, glutamic acid guide; 3, supernatant fluid from rickettsial incubation with glutamine; 4, supernatant fluid from rickettsial incubation with asparagine; 5, aspartic acid guide; 6, asparagine guide. Glutamine and glutamic acid gave purple spots, aspartic acid a dark blue spot, and asparagine an orange yellow spot with the ninhydrin reagent of Levy and Chung (1953).

TABLE 1

Biochemical and biological data on purified suspensions of Rickettsia mooseri

<table>
<thead>
<tr>
<th>Lot No.</th>
<th>Total Nitrogen, μg per ml</th>
<th>Complement-fixing Titer with Egg antiserum</th>
<th>Mouse Toxicity Titer</th>
<th>Oxidation of Substrates, μl O₂ per 10 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Rickettsial antiserum</td>
<td></td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>M41-1</td>
<td>546</td>
<td>1:320</td>
<td>1:160</td>
<td>1:240</td>
</tr>
<tr>
<td>M42-1</td>
<td>563</td>
<td>1:40</td>
<td>1:320</td>
<td>1:480</td>
</tr>
<tr>
<td>M40-4</td>
<td>453</td>
<td>1:80</td>
<td>1:160</td>
<td>1:240</td>
</tr>
<tr>
<td>M43-1</td>
<td>1667</td>
<td>1:160</td>
<td>1:640</td>
<td>1:1280</td>
</tr>
</tbody>
</table>

μmoles per ml were employed as substrates. At the end of the incubation period, the procedure of Lipmann and Tuttle (1945) was used to deproteinize the contents of the vessels and to develop the color reaction of hydroxamic acids with ferric chloride.

(2) For quantitative determination of the amounts of hydroxamic acid formed, mixtures consisting of 0.5 ml of rickettsial suspensions, 0.8 ml of 0.013 M phosphate buffers of the desired pH, 0.2 ml of 0.25 M glutamine or asparagine, and 0.2 ml of neutralized 0.25 M hydroxylamine hydrochloride were incubated for 1 hr at 36 C. Deproteinization and colorimetric determination of hydroxamic acids were carried out after Lipmann and Tuttle (1945).

Keto acid determination. Total keto acids in metabolic mixtures were determined after the procedure of Friedemann and Haugen (1943) and expressed as α-ketoglutaric acid equivalents. The absorption spectra of the dinitrophenylhydrazones of keto acids were determined in samples which had been treated in the same manner as in the quantitative colorimetric procedure for keto acids.
TABLE 2
Keto acid accumulation resulting from the oxidation of glutamic acid and glutamine by Rickettsia mooseri

<table>
<thead>
<tr>
<th>Glutamic Acid</th>
<th>Glutamine</th>
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<tbody>
<tr>
<td>Oxygen utilized</td>
<td>&quot;Total keto acids&quot; formed</td>
</tr>
<tr>
<td>µmoles</td>
<td>µmoles</td>
</tr>
<tr>
<td>4.5</td>
<td>1.1</td>
</tr>
</tbody>
</table>

0.0017 M Arsenite added

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th>1.0</th>
<th>1.15</th>
<th>1.15</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>4.7</td>
<td>1.5</td>
<td>1.0</td>
<td>1.15</td>
<td>1.15</td>
</tr>
</tbody>
</table>

RESULTS
Qualitative paper chromatography of the contents of Warburg vessels in which rickettsiae had been incubated for 2 hr either with glutamine or with asparagine revealed the presence of considerable quantities of glutamic acid or aspartic acid, respectively, in the metabolic mixtures. A typical chromatogram has been traced and reproduced in figure 1.

Glutamine was oxidized by the rickettsial suspensions at high rates similar to those for glutamic acid. This makes glutamine second only to glutamic acid as a metabolite of purified rickettsiae in vitro, since all other known substrates of the metabolism of *R. mooseri* are oxidized at rates which are small by comparison with those for glutamic acid (Wisseman et al., 1952). No

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Figure 2. Absorption spectra of dinitrophenylhydrazones of the keto acids derived from the rickettsial oxidation of glutamic acid and of glutamine. (The spectrum of the hydrazone of α-ketoglutaric acid is shown for comparison.)
oxygen uptake was observed in rickettsial suspensions that were incubated with asparagine. The results of these manometric experiments are listed in table 1.

The deamidation of asparagine leads to aspartic acid which is not oxidized by R. mooseri to a significant extent (Bovarnick and Miller, 1950), whereas the deamidation of glutamine results in glutamic acid which is oxidized by these rickettsiae along the previously established pathway.

**TABLE 3**

<table>
<thead>
<tr>
<th>Hydroxamic acid formation by Rickettsia mooseri</th>
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<tbody>
<tr>
<td>Additions</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>Glutamine</td>
</tr>
<tr>
<td>Asparagine</td>
</tr>
<tr>
<td>Hydroxylamine-HCl</td>
</tr>
<tr>
<td>Glutamine + hydroxylamine-HCl</td>
</tr>
<tr>
<td>Asparagine + hydroxylamine-HCl</td>
</tr>
</tbody>
</table>

Of the above substances 50 \( \mu \)moles per ml were added to the standard mixture for manometric experiments with rickettsiae (Wisseman et al., 1951); the total experimental volume was 3.0 ml and contained 1 ml of rickettsial suspension M46-1. Optical density at 500 \( \mathrm{m}_{\mu} \) was considered a relative measure of the quantities of hydroxamic acids formed. Asterisks indicate samples in which turbidity contributed to the optical density.

(Wisseman et al., 1952). The following experiments were designed to illustrate this in detail.

The oxidation of glutamic acid in *R. mooseri* leads to the accumulation of small quantities of the keto acid intermediates of the Krebs cycle, i.e., \( \alpha \)-ketoglutaric acid, oxalacetic acid, and pyruvic acid; the addition of arsenite to rickettsial suspensions that oxidize glutamate introduces a metabolic block that prevents the oxidation of \( \alpha \)-ketoglutarate and leads to a decrease in oxygen consumption and an accumulation of nearly stoichiometric quantities of \( \alpha \)-ketoglutaric acid (Wisseman et al., 1952).

Table 2 shows the amounts of oxygen consumed and of total keto acids accumulating during rickettsial oxidation of glutamic acid and of glutamine, in the absence or presence of arsenite.

It is readily apparent that while the actual quantities of oxygen consumed and of keto acids accumulated differed from one experiment to another, the ratios of the two quantities were very similar for glutamic acid and for glutamine in the experiments without arsenite as well as in those where arsenite had been added.

When the absorption spectra of the keto acid dinitrophenylhydrazones in dilute sodium hydroxide were plotted and compared for the keto acids, resulting from the rickettsial oxidations of glutamine and of glutamic acid, it became apparent that they were identical except for a parallel displacement owing to concentration differences (figure 2). The absorption spectrum of the dinitrophenylhydrazone of pure \( \alpha \)-ketoglutaric acid is shown for comparison and differs from that of

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*Figure 3. pH Dependence of glutamine and asparagine metabolism in *Rickettsia mooseri*
the mixture of the three keto acids which arise from the metabolism of *R. mosseri*.

These various findings illustrate identical features of the rickettsial degradation of glutamic acid regardless of whether this compound is added directly to rickettsial suspensions or is derived from the rickettsial deamidation of glutamine. They suggest that deamidation of glutamine, followed by glutamic acid oxidation, is the principal if not sole pathway by which *R. mosseri* metabolizes this amide.

The rickettsiae transferred the amino acyl residues from glutamine or asparagine to hydroxylamine. This type of transferase reaction has been studied in detail by Waelsh (1952) and his collaborators. To demonstrate the transfer reaction, rickettsial suspensions were incubated with mixtures of hydroxylamine and either glutamine or asparagine, and the experimental mixtures were analyzed for hydroxamic acids, which are the reaction products of amino acyl residues with hydroxylamine. Table 3 lists the results of such an experiment. The relative amounts of hydroxamic acids present indicated that significant quantities of these compounds were formed only when rickettsiae, hydroxylamine, and one of the amino acid amides were incubated together.

In the presence of hydroxylamine, glutamine was oxidized to a much lesser extent than in a control experiment without added hydroxylamine (table 3). This finding is not surprising since hydroxylamine by virtue of being a carbonyl reagent is an inhibitor of a variety of enzymatic reactions.

The quantitative methods described above were employed to determine the pH dependence of the rickettsial deamidases and transferases. The results are presented graphically in figure 3.

Particulate suspensions of normal yolk sac which contained several hundred times more egg material than was carried over into the rickettsial suspensions during purification, did not exhibit significant deamidase and amino acyl transferase activities.

**DISCUSSION**

Glutaminase and asparaginase are ubiquitous enzymes which have been reported to occur in a variety of unicellular and multicellular plants and animals (Zittle, 1951; Waelsh, 1952). Two types of deamidases for glutamine and asparagine are known which have been named I and II (Zittle, 1951). Type I requires phosphate ions and splits off the amide groups, yielding ammonia and the free dicarboxylic amino acids. Type II is a special transferase which requires the presence of a keto acid, for example pyruvic acid, to which the ε-amino groups of the amino acid amides are transferred by transamination. The resulting ε-amides of keto acids deamidate spontaneously to yield ammonium ions and the corresponding keto acids. The deamidases of *R. mosseri* are obviously of type I, since they act in the absence of added keto acids and yield glutamic or aspartic acids. Glutaminase in the rickettsiae was most active in the range between pH 7 and 8, which corresponds to the optimal range of glutaminases from various biological sources. The rickettsial asparaginase on the other hand had an optimum of pH 6.1 which is more acid than that frequently found for this enzyme (Zittle, 1951).

It is reasonable to speculate that rickettsial deamidation of glutamine and asparagine might be one of the biochemical reactions by which rickettsiae interfere in vivo with the metabolism of certain of their host cells. Strain L of mouse fibroblasts in which rickettsiae grow well has an absolute requirement for glutamine (Eagle et al., 1956). For HeLa cells which also require glutamine and support the growth of rickettsiae, Levintow (1957a, b) has shown that glutamine from the medium is directly incorporated into the cellular proteins and serves also as a source of the amide group of asparagine. It is worthy of consideration that in heavy rickettsial infection of these tissue culture cells the rickettsial deamidation of glutamine might compete with the synthetic and degradative reactions of the host cell for the limited supply of this compound. As virtually nothing is known about the mechanism by which rickettsiae damage host cells, any lead as to the metabolic lesion caused in such cells by multiplying rickettsiae might result in important insights into the nature of the host-rickettsia relationship.

Data on the pH dependence of enzymatic reactions are useful for comparing analogous enzymes from different biological sources. Since whole intact rickettsiae rather than isolated enzymes were employed in the present studies, observations on the pH dependence of rickettsial enzymes may be overlaid with nonspecific changes of rickettsial physiology; such changes might be of some importance at the pH limits of rickettsial viability.
Glutamyl transfer by rickettsial suspensions was optimal at pH 7 to 8 which is the same pH range as that found in Proteus vulgaris by Grossowicz et al. (1950); the transferases of Proteus are the best known microbial enzymes of this type. Aspartyl transferase in R. mooseri was optimal at the highest pH studied, 8.3. The corresponding enzyme in Proteus has a primary maximum at pH 6.2 and a secondary maximum toward the alkaline range, owing to the fact that in this range the speed of the reverse reaction (cleavage of aspartohydroxamate) becomes minimal. The apparent pH optimum of the rickettsial asparto-transferase at pH 8.3 may be the result of similar conditions, as the asparagine splitting enzyme has a pH optimum at 6.2 and shows a rapid decline toward the alkaline range (figure 3).

The present work, although concerned with rickettsial enzymes whose role in the physiology of the organism is difficult to evaluate, has nevertheless concrete significance. It adds to the increasing array of autonomous metabolic functions which rickettsiae are known to perform. A continued mapping of the metabolic pattern of rickettsiae is desirable for a better understanding of the nature of these intracellular parasites and of their interrelationships with their host cells.

SUMMARY

Suspensions of purified Rickettsia mooseri deamidate glutamine to glutamic acid and asparagine to aspartic acid. They also transfer the glutamyl and aspartyl residues of glutamine and asparagine to hydroxylamine with the formation of the corresponding hydroxamic acids. The possible importance of these metabolic reactions for the physiology of rickettsiae is discussed.

REFERENCES


Levintow, L. 1957b Evidence that glutamine is a precursor of asparagine in a human cell in tissue culture. Science, 126, 611–612.


