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# Occurrence of γ-Glutamyl Transpeptidase Activity in Several Mycobacteria Including *Mycobacterium leprae*[1]

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The presence of the enzyme, γ-glutamyl transpeptidase (EC. 2,3, 2.2) (γ-GT) in several animal and plant tissues is well known ([17, 19]). The function of this ubiquitous enzyme in relation to the utilization and breakdown of glutathione for the functional and metabolic needs of the cell is a subject of intense study ([16, 17]). The location of this enzyme in high concentrations in cell membranes of transmural tissues of vertebrates has led to the hypothesis that it is involved in absorptive processes ([1, 9, 20, 28]). Meister ([14, 15, 16]) has proposed that that γ-GT has a key function in amino acid transport and translocation, providing convincing evidence for the presence of what is now well established as the "γ-glutamyl cycle." At the present time, very little is known about the presence of γ-GT or its functional significance in microorganisms. Available information on the chemical composition of the cell wall of mycobacteria indicates the presence of γ-glutamyl residues in the D-isoglutaminyl portions of the murein units in the formation or metabolism of which enzymatic processes concerned may involve γ-GT. We report in this communication the detection of γ-GT activity in some mycobacteria, including *Mycobacterium leprae* obtained from biopsy material from lepromatous patients. Preliminary findings on the acceptor specificity of the enzyme activity from these sources is also reported.

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## MATERIALS AND METHODS

**Substrates and acceptor peptides.** L-γ-glutamyl-p-nitroanilide, glycyl-L-alanine, glycyl-D-alanine, glycyl-L-aspartic acid, glycyl-D-aspartic acid, DL-α, ε-diaminopimelic acid, and glycylglycine were obtained from Sigma Chemical Company (St. Louis, Missouri, U.S.A.). Glycyl-L-asparagine and glycyl-D-asparagine were products of ICN Pharmaceuticals, Inc. (Cleveland, Ohio, U.S.A.). All the other peptides and amino acids used in the study were from British Drug House or Nutritional Biochemicals Corporation (Cleveland, Ohio, U.S.A.). Glycyl-D-amino acids were analyzed by thin layer chromatography for possible contamination with glycylglycine, and the latter could not be detected.

**Bacterial cell suspensions.** Cultivable mycobacteria were grown and harvested as follows: *Mycobacterium phlei* and *Mycobacterium smegmatis* were obtained from the collection of the Haffkine Institute, Bombay. *Mycobacterium phlei* (NCTC 8151) were grown in Sauton's medium; *Mycobacterium smegmatis* (NCTC 10265) were cultivated in Middlebrook's medium containing 6% glycerol. At the end of 10 days' incubation, cells were harvested by centrifugation. After washing four times with ice cold saline, the cells were suspended in saline and used for experiments at appropriate dilutions to give the desired cell count. C44-ICRC bacilli and FMR36/A, mycobacterial strains isolated from human leprous biopsies, were grown in modified Dubos medium as described by Bapat, *et al.* ([3, 4]) for 20 days, harvested by centrifugation, thoroughly washed, and used as suspensions in saline at suitable cell counts.

***Mycobacterium leprae.*** Suspensions were prepared by processing freshly obtained human biopsy material by the method

adopted from that of Talwar, *et al.* ([25]) as described in previous work from this laboratory ([10]). Briefly, the ear lobe specimens, after being cleared of blood clots and superficial layers of skin, were rinsed with ice cold saline containing penicillin (100 u/ml). After chopping the tissues into small pieces, they were homogenized by hand in a glass homogenizer to obtain a uniform suspension, which was centrifuged at 75 × *g* for 10 min. The supernatant was re-centrifuged for 30 min at 4000 × *g*, the sediment suspended in 5 ml of 0.25% trypsin, placed in a shaking waterbath at 37°C for 15 min, and re-centrifuged at 4000 × *g* for 30 min. The pellet was then suspended in 5 ml ice cold distilled water and centrifuged at 1000 × *g* for 10 min. The supernatant was re-centrifuged at 4000 × *g* for 30 min. The pellet was then subjected to another cycle of low speed centrifugation to remove traces of tissue contamination. Supernatants containing bacilli were checked by acid-fast staining of smears using methylene blue counterstain. Bacilli were counted according to the method of Shepard and McRae ([21]) and diluted to give the required counts.

**Sonicates.** Sonicates of the ICRC bacillus were also used in several experiments. These were prepared by subjecting freshly harvested and washed cell suspensions in a volume of 4 ml in 0.1 M phosphate buffer (pH 7.2) to ultrasonic disintegration in a sonicator at 2.5 A with a 4 mm probe. During sonication, at all times the temperature was kept below 6–8°C. The cell free preparations used were obtained by removing cell debris and intact cells by centrifugation (5000 × *g* for 30 min).

**Enzyme assay.** γ-GT activity was assayed in a final volume of 1 ml containing 0.1 M Tris HCl buffer, pH 8.0, 4 mM L-γ-glutamyl-p-nitroanilide, and 50 mM glycylglycine, or di- or tripeptide or amino acid acceptor. The reaction was started by adding the enzyme (cell suspensions or sonicate) in an appropriate volume to a preincubated (at 37°C) buffer substrate-acceptor mixture. The reaction was followed by measuring the increase in absorbance at 410 nm in the supernatant clarified by centrifugation of the reaction mixture. Heated samples (100°C, 10 min) were also used as controls in all experiments, and the increases in the absorbance (0.005 to 0.025/hr at 10 min) were always no greater than those in samples with no enzyme added. Additional controls lacking acceptor were also included and usually showed around four fold higher increases in absorbance than the controls lacking enzyme or with heated enzyme. Enzyme activity was expressed as μmoles or nanomoles of p-nitroaniline liberated per minute or hour by a known number of bacilli (or mg of bacterial protein nitrogen) at 37°C based on the conditions of individual experiments as described in Results. The protein content of the sonicate was estimated by Lowry's method ([13]) and the nitrogen content of bacterial suspensions by a micro-Kjeldahl procedure ([2]).

**Experiments with *M. leprae* suspensions.** These were carried out essentially under the same conditions as those for the other mycobacteria except that glycyl D-amino acid dipeptides were tested as acceptors in the γ-GT reaction in place of glycylglycine. This was a necessary procedure since any γ-GT activity observed with glycylglycine as the acceptor in *M. leprae* suspensions may arise from contamination of the preparation with the host tissue enzyme, which would result in an acceptor specificity corresponding to the human enzyme. Glycyl-D-amino acid dipeptides are known to have no acceptor activity in the mammalian γ-GT reaction. In view of the small quantity of bacilli available, reactions were incubated for 16–18 hr at 37°C under sterile conditions. After recording increases in absorbance, the reaction mixtures were checked for contamination by staining and plating. After suitable deductions for blanks and controls, enzyme activity was expressed as nanomoles of p-nitroaniline liberated per hr at 37°C per 1 × $10^7$ bacilli.

## RESULTS

Experiments with cultivable mycobacterial cell suspensions clearly indicated the presence of γ-GT activity (Table 1). In addition to the three mycobacterial species shown in Table 1, several other organisms were tested and showed measurable activities. *Mycobacterium phlei* was tested and showed little, if any, activity, however.

With glycylglycine as the acceptor, C44-ICRC bacilli had an approximate specific activity of 33, followed by 30 for a similar

TABLE 1. *γ-glutamyl transpeptidase (γ-GT) activity of mycobacterial cell suspensions.*

| Acceptor dipeptides | γ-GT activity[a] | | |
|---|---|---|---|
| | *M. smegmatis* | C44-ICRC bacilli | FMR/36A bacilli |
| Glycylglycine | 25.8 (100) | 33.3 (100) | 30.1 (100) |
| Glycyl-D-asparagine | 11.3 (44) | 8.2 (25) | 12.3 (41) |
| Glycyl-D-aspartic acid | 9.2 (35) | 8.9 (27) | 9.0 (30) |
| Glycyl-D-alanine | 17.8 (69) | 16.5 (50) | 17.4 (58) |

[a] Enzyme activity is expressed as μmoles of p-nitoaniline liberated per hr per mg N of mycobacterial cells. Controls were run without acceptor. Values in parenthesis are enzymatic activities relative to glycylglycine as the acceptor as 100%.

mycobacterial isolate, FMR/36A, and 26 for *M. smegmatis*. It was significant that these mycobacteria had significant γ-GT activity with glycyl-D-amino acid dipeptide acceptors (Table 1). In parallel experiments with rat kidney homogenates none of these compounds showed any acceptor activity (Fig. 1). Among these compounds, glycyl-D-alanine was found consistently to have the highest acceptor activity.

**γ-GT activity in cell free extract of C44-ICRC—acceptor profile.** Among the various dipeptides, tripeptides, and amino acids, glycylglycine was the best acceptor in the γ-GT activity catalyzed by sonicates of C44-ICRC (Table 2). Both stereoisomers of glycylalanine were found to have acceptor activity which was 60% of the glycylglycine activity. The three tripeptides tested also exhibited acceptor activity without undergoing hydrolysis to the dipeptides. Among the amino acids, only L-asparagine, DL-methionine, and DL-α, ε-diaminopimelic acid demonstrated accep-

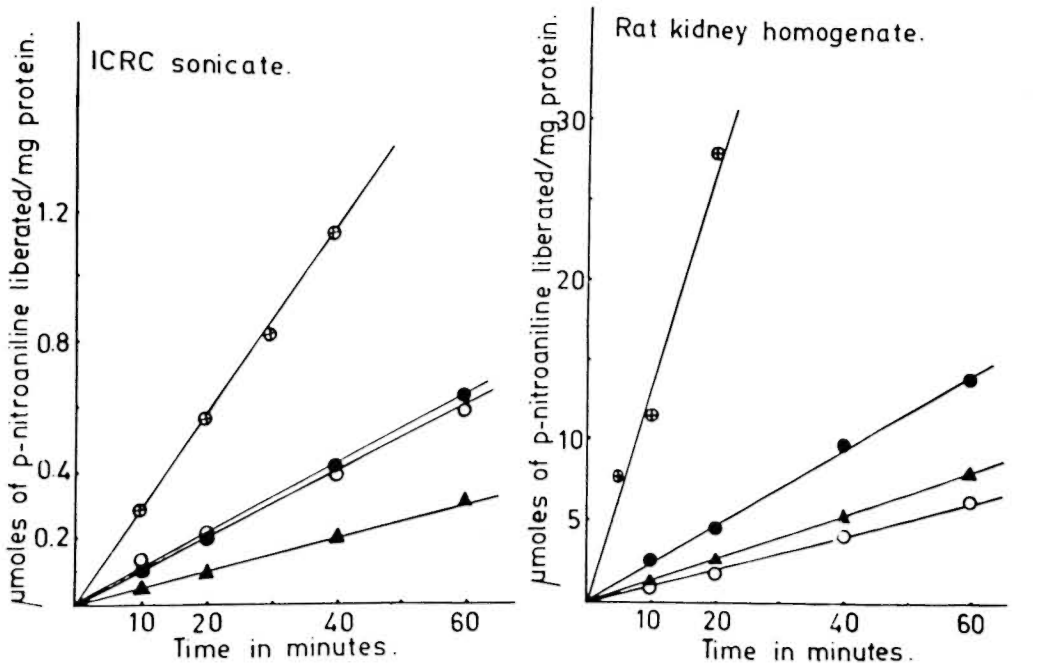


FIG. 1. Acceptor activity of glycyl dipeptides in γ-glutamyl transpeptidase in C44-ICRC bacillus sonicate and rat kidney homogenate.
▲—▲—▲ without acceptor (control);
⊕—⊕—⊕ with glycylglycine as acceptor;
●—●—● glycyl-L-asparagine;
○—○—○ glycyl-D-asparagine.

TABLE 2. *γ-GT activity in sonicates of the C44-ICRC bacillus. Acceptor profile.*

| Acceptors | Relative enzyme activity[a] | Acceptors | Relative ezyme activity[a] |
|---|---|---|---|
| None | 23 | Tripeptides | |
| Dipeptides | | glycylglycylglycine | 61 |
| glycylglycine | 100 | DL-alanylglycylglycine | 48 |
| glycylglycine ethyl ester | 55 | DL-leucylglycylglycine | 35 |
| glycyl-L-alanine | 64 | Amino acids | |
| glycyl-D-alanine | 60 | glycine | 12 |
| glycyl-L-asp. $NH_2$ | 47 | DL-alanine | 14 |
| glycyl-D-asp. $NH_2$ | 47 | DL-valine | 24 |
| glycyl-D-aspartic acid | 33 | L-lysine | 27 |
| glycyl-D-aspartic acid | 35 | L-glutamine | 22 |
| glycyl-DL-Norvaline | 55 | DL-methionine | 23 |
| glycyl-DL-Valine | 39 | B-alanine | 23 |
| glycyl-DL-Leucine | 28 | DL-norvaline | 27 |
| glycyl-DL-methionine | 20 | L-asparagine | 32 |
| DL-alanyl glycine | 33 | L-arginine | 24 |
| DL-alanyl-DL-norvaline | 25 | DL-ornithine | 27 |
| DL-alanyl-DL-methionine | 22 | DL-α, ϵ-diaminopimelic acid | 33 |
| DL-alanyl-DL-phenyl-alanine | 27 | | |
| benzoyl glycine | 41 | | |
| benzoyl-DL-alanine | 20 | | |
| D-leucyl glycine | 20 | | |

[a] Assay conditions were as described under Materials and Methods. Enzyme activity is expressed in relation to the enzyme activity with glycylglycine as the acceptor (100%).

tor activity. In each instance the activity was distinctly lower than that with glycylglycine. L-glutamine showed almost no acceptor activity.

Interestingly, glycine and DL-alanine brought about a consistent decrease in the "blank" activity (reaction mixtures containing no acceptor), indicating an inhibitory effect of these amino acids on the hydrolytic function of the enzyme ([26, 27]). Such an inhibitory effect of glycine was observed on the γ-GT activity of sonicates of C44-ICRC as well as that of the rat kidney homogenate when glycylglycine was used as an acceptor. At the same level of glycine used (12.5 mM), the magnitude of inhibition in the ICRC system (62%) was greater than in the rat kidney γ-GT (48%). An observation of particular significance is that glycine consistently inhibited the hydrolytic activity associated with the γ-GT in the mycobacterial system. This was in sharp contrast to the rat kidney γ-GT system where glycine served as an acceptor without having an effect on the hydrolytic function (Fig. 2).

**γ-GT activity of *M. leprae* suspensions.** The presence of γ-GT activity in *M. leprae* suspensions was indicated by the data in Table 3. Considerable γ-GT activity was observed in the system with glycylglycine as the acceptor (2–4 times the activities obtained with glycyl-D-amino acid dipeptides). Because contamination of the *M. leprae* suspensions with host tissue enzymes could account for γ-GT activity with glycylglycine as the acceptor, only activities with glycyl-D-amino acid dipeptides as acceptors are presented in Table 3. The data suggest that *M. leprae* possess γ-GT activity similar to the other mycobacteria investigated.

## DISCUSSION

Studies on mammalian γ-GT have led to the conclusion that one of the main functions of this enzyme is the rapid utilization of glutathione with simultaneous transfer of the γ-glutamyl moiety to several physiologically important acceptors. This is followed by a series of enzymatic processes which represent a cyclical breakdown and resynthesis of glutathione, fulfilling several metabolic needs of the cell ([16, 17]). Knowledge regarding the presence of this enzyme in bacteria, and more importantly its physio-

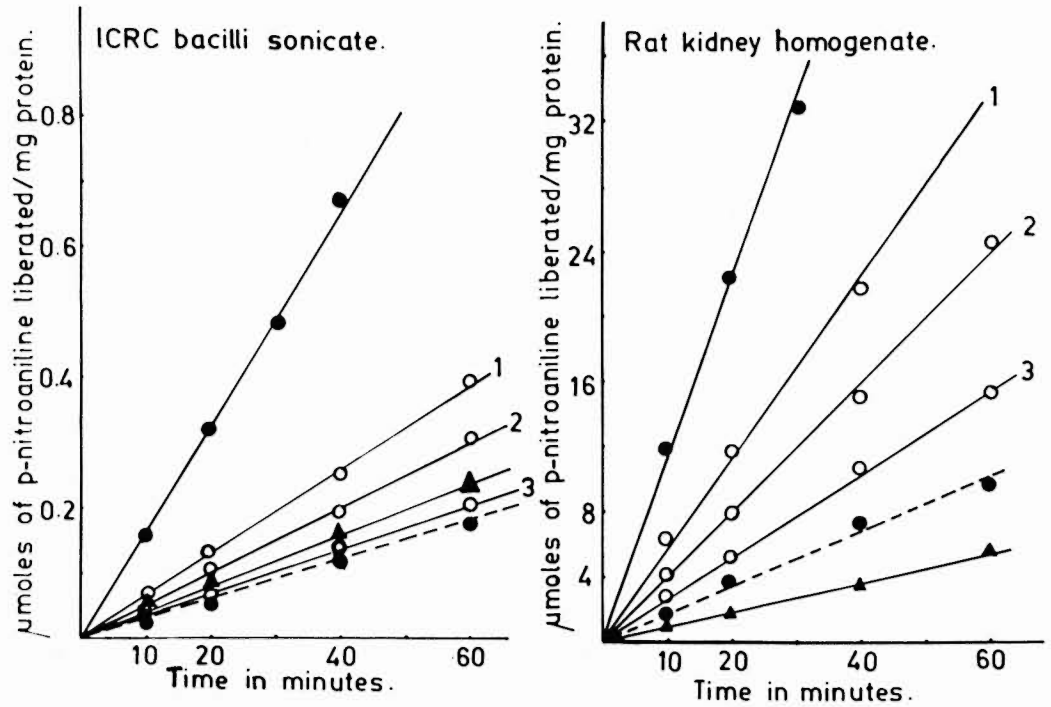


FIG. 2. Effect of glycine in γ-glutamyl transpeptidase activity of C44-ICRC bacillus sonicate and rat kidney homogenate.

▲—▲—▲ without acceptor;
●—●—● with no addition (glycylglycine as acceptor);
○—○—○ 1, 2 and 3 glycine added at 12, 5, 25 and 50 mM concentration respectively;
●---●---● 50 mM glycine (without acceptor).

logical role in microbial systems, is scanty [18, 23, 24].

Our present studies demonstrate the occurrence of γ-GT activity in *M. smegmatis* and in two acid-fast isolates obtained from leprosy patient biopsies and successfully adapted to grow *in vitro*. Of significance is the observation that *M. leprae* suspensions prepared from freshly obtained biopsy material from lepromatous leprosy patients exhibited γ-GT activity. Preliminary screening of a number of other mycobacteria indicate a rather wide distribution of this enzyme in mycobacterial species.

TABLE 3. *γ-GT activity of* Mycobacterium leprae *suspensions.*

| Experiment no. | Morphological index (%)[a] | Enzyme activity[b] with acceptor as: | | |
|---|---|---|---|---|
| | | Glycyl-D-aspartic acid | Glycyl-D-asparagine | Glycyl-D-alanine |
| 1[c] | 2 | 0.61 | 0.86 | 2.30 |
| 2[d] | 2 | 1.47 | 2.33 | 1.85 |
| 3[d] | 3 | 1.17 | 3.12 | 2.27 |

[a] Two to 3% of the bacilli were stained solidly in acid-fast stains.

[b] Enzyme activity is expressed as p-nitroaniline liberated in nanomoles/hr/1.0 × $10^7$ bacilli at 37°C corrected for blanks without acceptor. Activities with glycylglycine as acceptor were in the range of two to four times the values obtained for the acceptors mentioned in the table.

[c] Biopsy material from lepromatous leprosy patient treated with dapsone for 2 weeks.

[d] Untreated lepromatous leprosy patients.

The ability of glycyl-D-amino acid dipeptides to function as acceptors in the mycobacterial γ-GT reaction was strikingly different from that of the mammalian system in which these compounds were inactive. Thus the acceptor specificity of mycobacterial γ-GT for glycyl-D-amino acids may offer a useful tool to distinguish the mycobacterial enzyme from the mammalian (host) counterpart. Another observation of interest is the effect of glycine on mycobacterial γ-GT (inhibition of hydrolytic activity) in contrast to its effect on the mammalian enzyme (serving as an acceptor and not causing inhibition of hydrolytic ac-

tivity). Future studies with purified enzyme preparations may explain these differences from a mechanistic point of view.

These preliminary findings indicate the value of pursuing studies to understand the role of γ-GT in mycobacterial metabolism, which may be quite different from that in mammalian systems ([11, 16, 17]). The following considerations specific to mycobacterial cell wall chemistry are noteworthy. The cell walls of mycobacteria contain a basic structure, murein, also designated peptidoglycan or mucopeptide, linked covalently to arabinogalactan mycolate. The basic murein unit consists of repeating units of β-1,4 linked pyranosides of N-acetylglucosamine-N-acetylneuraminic acid tied to L-alanyl-D-isoglutaminylmesodiaminopimelyl-D-alanine. As much as 70% of the cross-linking in the murein in mycobacteria exists as interpeptide bridges between mesodiaminopimelic acid which occurs in an amidated form in the peptidoglycan ([5, 8, 12, 30]). Furthermore, the peptide linkage in which diaminopimelate occurs in the cell wall involves a "γ-glutamyl" of D-isoglutamine and a D-alanyl residue at one of the carboxyl ends. As shown in the present study, the presence of γ-GT in mycobacteria and its acceptor specificity in terms of glycyl-D-amino acid peptides, particularly glycyl-D-alanine, and its acceptor specificity in terms of diaminopimelic acid among the amino acids, suggest significance in regard to cell wall composition and synthesis.

The lack of significant acceptor activity observed in mycobacterial γ-GT with L-glutamine and methionine deserves comment. In mammalian systems, these compounds have the highest acceptor activity among amino acids ([16, 17]). The absence of sulfur-containing amino acids in the peptidoglycan as well as the non-peptidoglycan amino acid pools in mycobacterial cell walls ([6, 7]) may be related to the very low acceptor activity seen with methionine in the present study. Similarly, glutamine may not have the same functional role in mycobacteria as it does in mammalian systems ([1]). Whether γ-GT has a role in the formation and utilization of the polyglutamic acid found in the cell wall of some mycobacteria ([29]) needs to be understood. Delineation of such biochemical phenomena in mycobacteria in general and in *Mycobacterium leprae* in particular, may help in providing clues for the design of new drugs by exploiting the knowledge on cell wall composition and synthesis. This has been the case with a number of well known antibiotics which affect cell wall biosynthesis ([22]). Further studies with purified mycobacterial γ-GT are in progress.

## SUMMARY

γ-Glutamyl transpeptidase (γ-GT) activity, which catalyzes the transfer of the "γ-glutamyl" group of γ-glutamyl compounds to several dipeptide and amino acid acceptors, was found to be present in several mycobacteria, including *M. leprae*, both in cell suspensions and in cell-free sonicates. Glycyl D-amino acids were active as acceptors, particularly glycyl-D-alanine and α, ε-diaminopimelic acid, among the amino acids. Two mycobacterial isolates obtained from biopsy material of lepromatous patients also exhibited similar enzyme activity. The need for further work to delineate the possible role of γ-GT in mycobacterial metabolism is strongly indicated.

## RESUMEN

En varias micobacterias, incluyendo al *M. leprae*, tanto en suspensión como en sonicados libres de células, se encontró presente la actividad de γ-glutamil transpeptidasa (γ-GT) la cual cataliza la transferencia del grupo "γ-glutamil" de los γ-glutamil compuestos a varios dipéptidos y aminoácidos aceptores.

Los glicil-D-aminoácidos fueron aceptores activos, particularmente la glicil-D-alanina y el ácido α-ε-diaminopimélico. Dos preparaciones micobacterianas obtenidas a partir de biopsias de pacientes lepromatosos también mostraron una actividad enzimática similar. Se señala la necesidad del trabajo futuro encaminado a delinear el posible papel de la γ-GT en el metabolismo micobacteriano.

## RÉSUMÉ

On a décelé chez plusieurs mycobactéries, y compris *M. leprae*, et ceci tant dans les suspensions cellulaires que dans les sonicats dépourvus de cellules, l'existence d'une activité γ-glutamyl transeptidase (γ-GT), catalysant le transfert du groupe γ-glutamyl des composés-glutamyl à plusieurs dipeptides et acides aminés servant d'accepteurs. Les acides glycyl D-aminés étaient actifs comme accepteurs et particulièrement la glycyl-D-alanine et l'acide α, ε-diaminopimélique, parmi d'autres acides aminés. Deux isolates mycobactériens recueillis à partir de matériel de biopsies prélevées chez des malades lépromateux, présentaient également une activité enzyma-

tique semblable. On insiste fortement sur la nécessité de poursuivre les travaux en vue de délimiter le rôle possible de la γ-glutamil transeptidase dans le métabolisme mycobactérien.

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