Biochimica et Biophysica Acta, 522 (1978) 354—362

BBA 68353

MULTIPLE FORMS OF γ-GLUTAMYLTRANSFERASE IN NORMAL HUMAN LIVER, BILE AND SERUM

NILS-ERIK HUSEBY

Institute of Medical Biology, University of Tromsø, 9001 Tromsø (Norway)

(Received July 18th, 1977)

Summary

A study of the multiple forms of γ-glutamyltransferase ((γ-glutamyl)-peptide:amino acid γ-glutamyltransferase, EC 2.3.2.2) in normal human liver, bile and serum are reported. An amphiphilic form of the enzyme was demonstrated in all three samples. When solubilized with detergent, estimated values for Stoke's radius of 48 Å and sedimentation coefficient of 5 S were obtained for this form. A hydrophilic form was also present in serum and bile, which showed identical properties to the enzyme form obtained after papain-treatment of the three samples. The Stoke's radius was found to be 37 Å, and the sedimentation coefficient 5 S.

It was concluded that the heterogeneity of enzyme activity found both on gel filtration and on electrophoresis was due to aggregates of the amphiphilic form with lipids and other proteins, and could not be ascribed to the presence of isoenzymes.

Introduction

γ-Glutamyltransferase ((γ-glutamyl)-peptide:amino acid γ-glutamyltransferase, EC 2.3.2.2) has been purified from normal human liver [1], rat hepatoma [2] and human, sheep and rat kidney [3—6]. As the enzyme is associated with membranes, treatment with detergents is necessary to solubilize the activity. By removing the detergents, the activity is recovered in large aggregates of membrane-like material. Thus, most of the activity does not enter polyacrylamide gels on electrophoresis, and the activity elutes in the void volume on gel filtration [1,3,5]. Treatment with proteolytic enzymes such as papain, trypsin and bromelain of membrane preparations and aggregates, produces a soluble form of γ-glutamyltransferase that does not aggregate [1,4—6]. The enzyme activity is not significantly altered by this treatment [1,5]. Similar behaviour has been reported for several other membrane bound enzymes and proteins and ascribed

to the presence of hydrophobic domains, which bind these proteins to the hydrophobic interior of membranes [7—9]. The hydrophobic domains seem to be removed by proteolytic action [6,8,9].

Frequent and large increases of γ-glutamyltransferase activity in serum are seen in patients with hepatobiliary diseases [10], and heterogeneity of the activity in both normal and pathological serum has been demonstrated by gel filtration and electrophoresis [10]. The diagnostic potential of serum γ-glutamyltransferase measurements might improve by analysis of specific enzyme forms, and several studies on the activity patterns after electrophoresis have been reported [11—13]. However, no clearcut relation of these patterns to specific organs or different diseases has been shown. As multiple forms of the enzyme could arise from an ability of γ-glutamyltransferase to participate in the formation of aggregates, depending on the lipids, lipoproteins or amphiphilic proteins present, a comparison of the molecular γ-glutamyltransferase in serum, bile and liver was undertaken. The influence of detergent and papain was studied, and a test for a hydrophobic domain was performed on the different molecular forms.

Materials and Methods

L-γ-glutamyl-*p*-nitroanilide, glycylglycine, bovine serum albumin (once crystallized), catalase (twice crystallized, from beef liver), papain (twice crystallized, from Papaya Latex), alcohol dehydrogenase (once crystallized, from yeast) were obtained from Sigma Chemical Co., USA, ferritin (twice crystallized) from Nutritional Biochemical Corp., USA, sodium deoxycholate and cetyltrimethylammonium bromide from Merck, W.Germany, Triton X-100 from BDH Chemicals, England, while Lubrol W was a gift from ICI, Oslo, Norway. Blue Dextran was bought from Pharmacia Fine Chemicals, Sweden, Ultrogel Aca 34 from LKB Produkter, Sweden and Agarose from L'Industrie Biologique Francaise, France.

Serum was pooled from normal persons and had a γ-glutamyltransferase activity of 32 U/l. Bile was collected from a biliary drain on the third day after a ventricular ulcer operation. The enzyme activity in the bile sample was 550 U/l. Liver was obtained 15 h post mortem from a victim of heart infarction. Liver homogenate was prepared in 4 vols 0.1 M Tris buffer (pH 8.5) using a Waring Blendor for 2 min at full speed. The supernatant, after centrifugation for 15 min at 12 000 × *g*, was used for further analysis, the activity of γ-glutamyltransferase being 1250 U/l. Detergent treatment of serum, bile and total liver homogenate (prior to centrifugation), was performed with 0.5% deoxycholate/0.5% Lubrol W unless otherwise stated. Each sample was incubated for 2 h at room temperature before centrifugation at 12 000 × *g* for 15 min. Treatment of serum, bile and total liver homogenate with papain (1 mg papain/10 mg protein) was performed at pH 7.4, as described [1]. The recovery of γ-glutamyltransferase activity in the supernatant after centrifugation for 15 min at 12 000 × *g* was 85—95%.

Gel filtration was performed on a column (2 × 80 cm) of Ultrogel Aca 34 with 0.1 M Tris (pH 8.5) as eluant at room temperature. In some experiments, 0.5% deoxycholate was added. The column was calibrated using ferritin, cata-

lase, alcohol dehydrogenase and albumin as standard proteins. Blue Dextran was used for determination of the void volume. The Stoke's radii and the molecular weights of γ-glutamyltransferase were estimated as described [14], using the inverse error function of $(1 - K_d)$, K_d being the partition coefficient. Polyacrylamide gel electrophoresis was carried out using the standardized procedure and gel system 1a, described by Maurer [15]. Bromophenol Blue was used as marker. Agarose electrophoresis was performed as described [16] with 0.5% Triton X-100, 0.5% Triton X-100 plus 0.25% deoxycholate, or 0.5% Triton X-100 plus 0.05% cetyltrimethylammonium bromide in the agarose gels. Sucrose gradient centrifugations were performed in a Beckman Spinco L 2 65B ultracentrifuge. Linear gradients from 5 to 20% sucrose were made in 50 mM Tris (pH 8.5). In some experiments deoxycholate was added to final concentrations of 0.5%, 0.25% and 0.1%. Samples were layered on top of the gradients and centrifuged for 18 h at 40 000 rev./min in a SW 50.1 rotor at 4°C. The sedimentation coefficient was estimated as described [17], using catalase, alcohol dehydrogenase and albumin as standard proteins. The linearity of gradients was checked by refractometry.

γ-Glutamyltransferase activity was determined at 37°C in an assay mixture of 4 mM γ-glutamyl-p-nitroanilide/50 mM glycylglycine in 0.1 M Tris buffer (pH 7.6, 37°C), as described [18]. One unit of activity is the amount of enzyme that converts 1 μmol substrate per min. For localization of activity in gels after polyacrylamide or agarose electrophoresis, the gels were cut in 2-mm slices and incubated in 1 ml assay mixture at 37°C from 30 min to 6 h, depending on the activity present. The incubation was stopped by adding 1 ml 3 M acetic acid and the absorbance measured at 405 nm.

Results

Gel filtration

After gel filtration on Ultrogel Aca 34 of both liver homogenate (Fig. 1A) and bile (Fig. 1B), most of the γ-glutamyltransferase activity eluted in the void volume. While activity in the liver homogenate also was present as a shoulder next to the void peak, bile contained a small molecular form of γ-glutamyltransferase eluting near albumin. The activity of serum was separated into 3 fractions of comparable sizes (Fig. 1C): the first was eluted in the void volume and the last fraction was eluted at the same elution volume as the small molecular form found in bile. When the samples to be chromatographed were treated with detergents, the distribution of γ-glutamyltransferase activity in the gel filtration eluates changed. For both liver and bile, a middle peak of activity emerged, while correspondingly less activity was found in the void volume. Detergent-treated serum, likewise, revealed lesser activity in the void volume, while the middle peak was increased (Fig. 1, A—C, dashed lines). The assigned Stoke's radius for this middle peak ranged from 55 to 65 Å, depending on the incubation procedure and detergent used. The last activity peaks in bile and serum were unchanged by this detergent treatment.

Gel filtration was also performed with 0.5% deoxycholate in the eluant. The enzyme activity of detergent-treated samples eluted under these conditions in one main peak at the same elution volume for all samples (Fig. 2A). Assuming

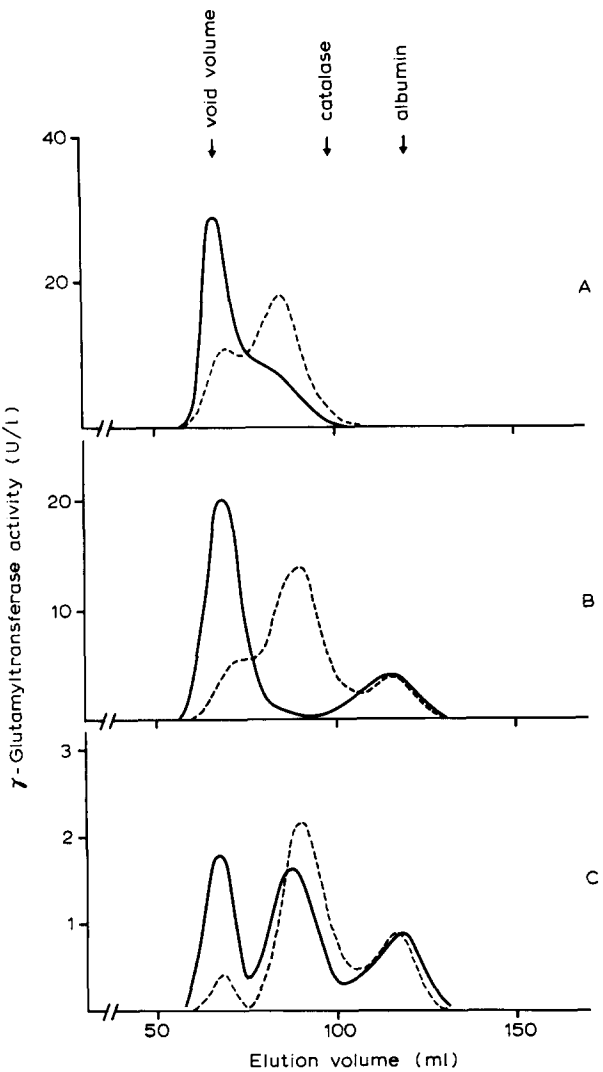

Fig. 1. Gel filtration on Ultrogel Aca 34 with 0.1 M Tris (pH 8.5) as eluant. The solid lines show the distribution of γ-glutamyltransferase activity after chromatography of liver homogenate (A), bile (B) and serum (C). The dashed lines show the distribution of γ-glutamyltransferase activity when the samples were preincubated with 0.5% deoxycholate/0.5% Lubrol W at room temperature for 2 h. Volumes of 0.3 ml liver homogenate, 0.5 ml bile and 0.2 ml serum were applied. The elution positions of Blue Dextran, catalase and albumin are indicated.

the enzyme · detergent complex to be globular, a Stoke's radius of 48 Å and a molecular weight of 175 000 could be estimated. For serum and bile, a shoulder was seen just after the main peak, with an elution volume equal to that of the small molecular form of serum and bile found when gel filtration was performed without deoxycholate present in the eluant (Fig. 1, B, C). Treatment with papain of liver homogenate, bile or serum, yielded an enzyme preparation that eluted with one activity peak of identical elution volume for all three samples (Fig. 2B). This elution volume was also identical to that of the

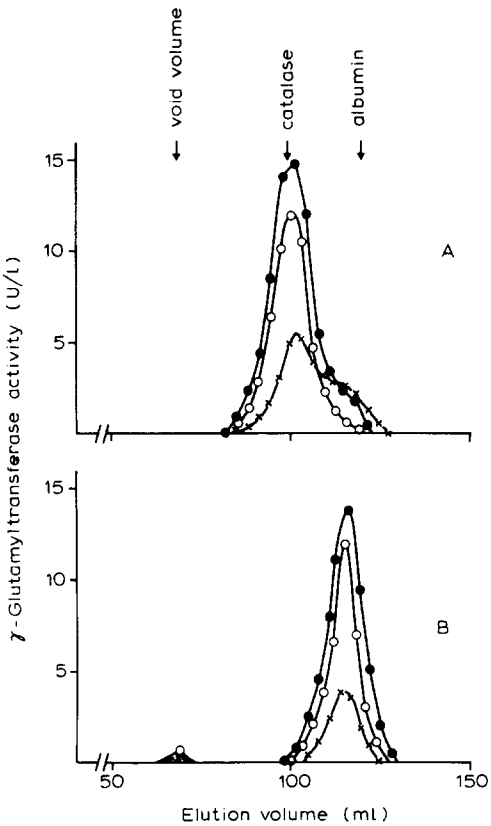

Fig. 2. Gel filtration on Ultrogel Aca 34. A: Liver homogenate, bile and serum were incubated with 1% deoxycholate for 2 h at room temperature before chromatography with 0.1 M Tris (pH 8.5)/0.5% deoxycholate as eluant. B: Liver homogenate, bile and serum were incubated with papain as described in Materials and Methods, before chromatography with 0.1 M Tris (pH 8.5) as eluant. The curves show the distribution of γ-glutamyltransferase activity after gel filtration of liver homogenate —○—, bile —●—, and serum —X—. The applied volumes were 0.15 ml, 0.30 ml and 2.0 ml, respectively. The elution position of Blue Dextran, catalase and albumin are indicated.

small enzyme component found for untreated serum and bile (Fig. 1, B, C). The Stoke's radius was estimated to be 37 Å, and the molecular weight to be 80 000.

Polyacrylamide gel electrophoresis

Electrophoresis of papain-treated liver homogenate, bile and serum, revealed γ-glutamyltransferase activity in one zone of the gels. The same relative mobility for the activity zone was detected for all 3 samples (Fig. 3, A—C). With samples from the last peak after gel filtration of bile and serum, an identical band of activity was obtained. After electrophoresis of untreated liver homogenate, bile and serum (Fig. 3, D—F), activity was found both in the concentrating gel and on top of the separating gel. Liver homogenate showed no bands of activity, but activity was spread halfway through the separating gel. For both bile and serum, an activity band with mobility equal to that found in the papain-treated samples was recovered. Serum also showed two more bands with

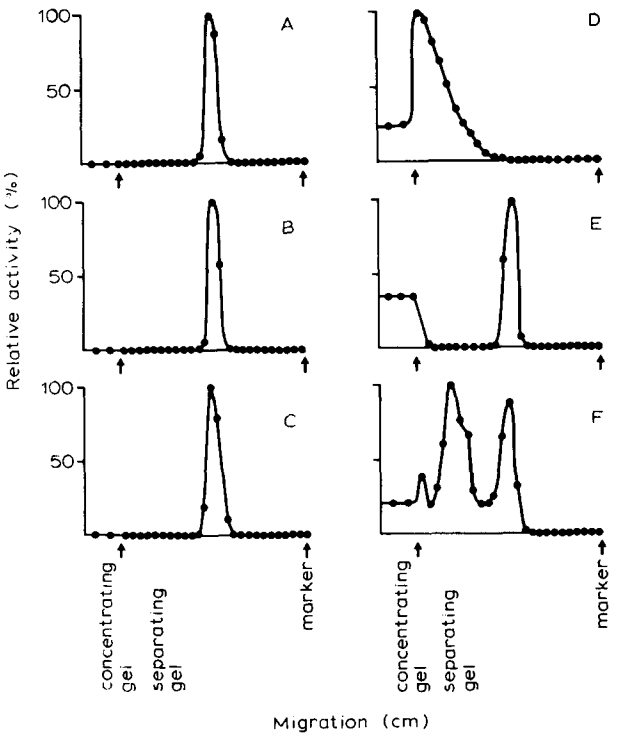

Fig. 3. Relative distribution of γ-glutamyltransferase activity after polyacrylamide gel electrophoresis. The enzyme activity in 2 mm slices of the gels was measured, and the highest activity set to 100%. The figures give the relative distribution of activity in papain treated samples. (A: liver homogenate, B: bile and C: serum) and in untreated samples (D: liver homogenate, E: bile and F: serum). The migration is from left to right, the position of the concentrating and the separating gels are indicated.

shorter relative mobilities. These bands also appeared when electrophoresis was performed on the second peak from gel filtration of serum. Similar bands could be detected in samples from the second peaks after gel filtration of detergent pretreated liver homogenate and bile. However, activity was still found on top of the separating gel when electrophoresis was performed in the presence of 0.1—0.2% deoxycholate (not shown).

Agarose electrophoresis

Electrophoresis was performed with papain-treated samples and with samples from the activity peaks obtained from gel filtration of deoxycholate-treated liver homogenate, bile and serum. In all experiments, only one zone of activity emerged, but the migration of the zone depended for some samples on the particular detergents present. Fig. 4 shows the typical distribution of activity after electrophoresis of a sample from the void peak (A) and the last peak (B) after gel filtration of serum. The variation in the mobility of the activity zone of the void peak sample indicates that γ-glutamyltransferase in this sample binds detergent. A correspondingly amphiphilic form of the enzyme was found both in the void peak and in the second peaks after gel filtration of all three samples. The activity zones in Fig. 4B do not show any variation depending on

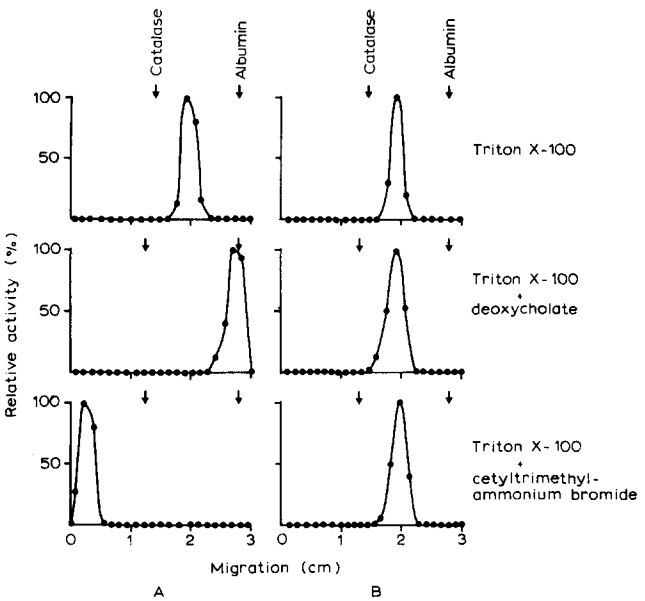

Fig. 4. Relative distribution of γ-glutamyltransferase activity after agarose gel electrophoresis. The electrophoresis was performed with Triton X-100, Triton X-100 and deoxycholate, and Triton X-100 and cetyltrimethylammonium bromide in the gels as indicated, with samples from A: the void volume fraction after gel filtration of serum and B: the third activity peak after gel filtration of serum. The figures give the relative distribution of enzyme activity measured in 2 mm slices of the gels, with the highest activity set to 100%. The positions of catalase and albumin after electrophoresis in the same system are indicated.

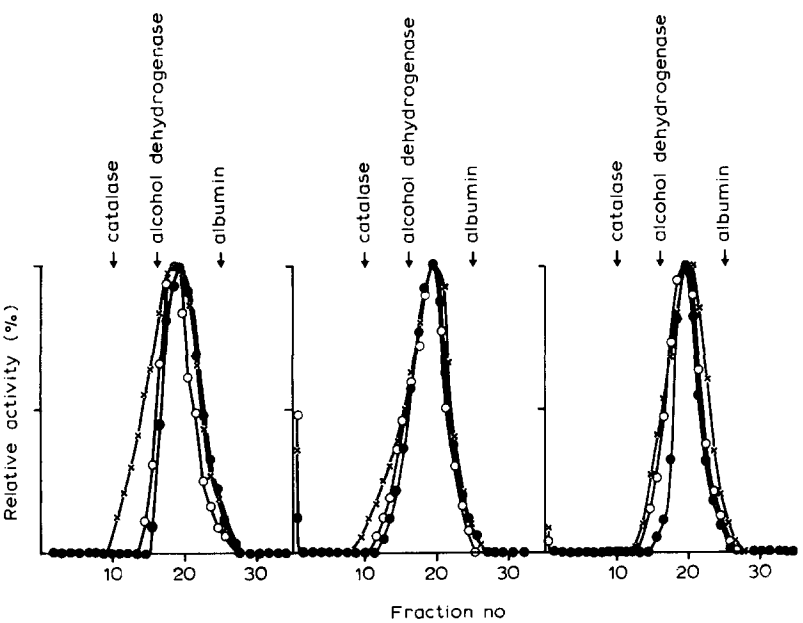

Fig. 5. Sucrose gradient centrifugation of γ-glutamyltransferase. After centrifugation as described in Materials and Methods, 32—34 fractions of the gradients were collected from the bottom. The fraction of highest γ-glutamyltransferase activity is set 100%, and the figures show the relative distribution of γ-glutamyltransferase activity. A: papain treated, B: untreated and C: detergent treated liver homogenate (—○—), bile (—●—) and serum (—X—). The sucrose gradient in C contains 0.5% deoxycholate. The positions of catalase, alcohol dehydrogenase and albumin are indicated.

the particular detergents in the gel. This was also found for samples from the last peak after gel filtration of bile and for all samples treated with papain. Albumin and catalase that were electrophoresed as controls (indicated by arrows in Fig. 4), neither showed any variation in mobility, as expected for proteins unable to bind significant amounts of detergent.

Sucrose gradient ultracentrifugation

After centrifugation of papain-treated liver homogenate, bile and serum, γ-glutamyltransferase activity was confined in one peak (Fig. 5A). A sedimentation coefficient of 5 S was estimated for γ-glutamyltransferase in all samples, using albumin, alcohol dehydrogenase and catalase as standard proteins. Centrifugation of untreated serum, bile and liver homogenate revealed some activity in the pellet, but again most activity was recovered in the gradient at the same density as was found for the papain-treated samples (Fig. 5B). Treatment of the samples with 0.5% deoxycholate, and centrifugation with deoxycholate (0.5%, 0.25% and 0.1%) in the gradients, did not change the position of the activity peak. However, less activity was recovered in the pellet (Fig. 5C).

Discussion

Gel filtration and polyacrylamide gel electrophoresis have demonstrated heterogeneous and different patterns of γ-glutamyltransferase activity in liver homogenate, bile and serum. The changes observed in these patterns after treatment of the sample with detergents are in accord with a solubilization of large aggregates. A completely solubilized form of the enzyme was probably obtained after chromatography with detergent present in the eluant. The estimated Stoke's radius and molecular weight were the same for all three samples analyzed. These values are probably valid for a complex of detergent and enzyme, as the rat kidney enzyme was found to bind a large amount of Triton X-100 [6]. The "charge shift electrophoresis" technique [16] demonstrated that the γ-glutamyltransferase present in the void peak and the middle peaks after gel filtration contains a hydrophobic domain that can bind detergent. Thus, this form found in liver homogenate, bile and serum is capable of complexing with lipids, detergents and amphiphilic proteins to yield the heterogeneous patterns found after chromatography and electrophoresis.

γ-Glutamyltransferase obtained from the last activity peak after gel filtration of bile and serum, was without such a hydrophobic region. The enzyme from these two preparations showed similar properties to the papain-treated enzyme preparations. Thus, identical Stoke's radii and electrophoretic mobilities were obtained. Liver homogenate contained mostly the detergent-binding form, but when homogenate was incubated at 37°C and pH 7.2, a hydrophilic form, similar to the papain-induced preparations, emerged (unpublished data). As this transformation was probably proteolytic, the presence of the hydrophilic enzyme form in serum and bile might be the product of proteolytic reactions in vivo. This form may also be the "soluble" form described by Szewczuk [19].

The molecular weight estimated for the hydrophilic form is consistent with the value reported for the papain-treated and purified liver enzyme [1]

obtained by electrophoretic methods, and also with the values reported for the rat kidney enzyme [6]. However, the higher value of 110 000 found by gel filtration for the liver enzyme [1] might be caused by an unsatisfactory gel preparation used at that time.

The sedimentation coefficient of 5 S and Stoke's radius of 37 Å found for the hydrophilic γ-glutamyltransferase correspond well with the 4.9 S and 36 Å reported for the papain-treated enzyme purified from rat kidney [6]. In the presence of deoxycholate the Stoke's radius was estimated to be 48 Å, but a value for the sedimentation coefficient of 5 S was still found. A corresponding discrepancy was revealed for the rat kidney enzyme, where values of 4.5 S and 70 Å were obtained in the presence of Triton X-100. This is probably caused by an abnormally high partial specific volume for the detergent · protein complex. Further studies are needed to determine the amount of detergent bound to the enzyme and to find a reliable molecular weight both for the enzyme and also for the hydrophobic region removed by papain.

Normal serum thus contains two molecular forms of γ-glutamyltransferase. The changes in enzyme heterogeneity after electrophoresis of various pathological sera may result from changes in the serum content of lipids and amphiphilic proteins that may complex with γ-glutamyltransferase. This has also been suggested by Freise et al. [20] who demonstrated that chylomicrons could cause changes in the electrophoretic pattern of the enzyme activity. Thus, such multiple forms should not be termed "isoenzymes". An increased sialic acid content of the enzyme in fetal and different diseased states have been reported [21], and a test for such changes of γ-glutamyltransferase may prove far more usable for diagnostic purposes.

References

1 Huseby, N.E. (1977) Biochim. Biophys. Acta 483, 46—56
2 Taniguchi, N. (1974) J. Biochem. 75, 473—480
3 Miller, S.P., Awasthi, Y.C. and Srivastava, S.K. (1976) J. Biol. Chem. 251, 2271—2278
4 Zelazo, P. and Orlowski, M. (1976) Eur. J. Biochem. 61, 147—155
5 Tate, S.S. and Meister, A. (1975) J. Biol. Chem. 250, 4619—4627
6 Hughey, R.P. and Curthoys, N.P. (1976) J. Biol. Chem. 251, 7863—7870
7 Helenius, A., Fries, E., Garoff, H. and Simons, K. (1976) Biochim. Biophys. Acta 436, 319—334
8 Maroux, S. and Louvard, D. (1976) Biochim. Biophys. Acta 419, 189—195
9 Spatz, L. and Strittmatter, P. (1973) J. Biol. Chem. 248, 793—799
10 Rosalki, S.B. (1975) in Advances in Clinical Chemistry (Bodansky, O. and Latner, A.L., eds.), Vol. 17, pp. 53—107, Academic Press, London
11 Igartua, E.B., Domecq, R. and Findor, J. (1973) Klin. Wochenschr. 51, 272—274
12 Patel, S. and O'Gorman, P. (1973) Clin. Chim. Acta 49, 11—17
13 Hetland, Ö., Andersson, T.R. and Gerner, T. (1975) Clin. Chim. Acta, 62, 425—431
14 Fish, W.W. (1975) in Methods in Membrane Biology (Korn, E.D., ed.), Vol. 4, pp. 189—276, Plenum Press, New York
15 Maurer, H.R. (1971) Disc Electrophoresis and Related Techniques of Polyacrylamide Gel Electrophoresis, Walter de Gruyter and Co., Berlin
16 Helenius, A. and Simons, K. (1977) Proc. Natl. Acad. Sci. U.S. 74, 529—532
17 Martin, R.G. and Ames, B.N. (1961) J. Biol. Chem. 236, 1372—1379
18 Huseby, N.E. and Strømme, J.H. (1974) Scand. J. Clin. Lab. Invest. 34, 357—363
19 Szewczuk, A. (1966) Clin. Chim. Acta 14, 608—614
20 Freise, J., Magerstedt, P. and Schmidt, E. (1976) J. Clin. Chem. Clin. Biochem. 14, 589—594
21 Köttgen, E. and Gerok, W. (1976) Klin. Wochenschr. 54, 439—444