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THE MOLECULAR FORMS OF γ-GLUTAMYL TRANSFERASE IN BILE AND SERUM OF ICTERIC RATS

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Summary

Alterations in the molecular form of γ-glutamyl transferase ((5-glutamyl)-peptide:amino-acid 5-glutamyltransferase, EC 2.3.2.2.) were studied in the bile and serum of rats under surgical ligation of the bile duct. Polyacrylamide gel electrophoresis of the bile, followed by the enzyme stain, revealed a major, slowly migrating broad band and a minor, faster migrating band. The former was converted to the latter upon limited proteolysis of the bile with a very small amount of papain. This conversion was accompanied by a decrease in molecular size of the enzyme. Both enzyme forms were specifically adsorbed to a concanavalin A-Sepharose column. Most of the papain-treated enzyme preparation could be eluted from the column by α-methyl-D-glucoside, a haptenic sugar of this lectin. On the other hand, the predominant form of the enyzme in the untreated bile was eluted only in the presence both of the sugar and Triton X-100. Based on the chromatographic behavior of the two enzyme forms (detergent-solubilized and protease-solubilized form) purified from rat renal brush border membrane on concanavalin A-Sepharose column, it was concluded that the predominant form of the enyzme in the bile was the detergent-solubilized form and that the minor component represents the protease-solubilized enzyme. The serum from icteric rats was also found to contain both types of the enzyme. However, the relative amount of the protease-solubilized form to the detergent-solubilized form in the serum was much greater than that in the bile. These findings suggested that γ-glutamyl transferase in the hepatobiliary membrane systems was solubilized into the bile mainly as the detergent-solubilized form, and that, during the process of translocation into the blood circulation, the enzyme was partly converted to the protease-solubilized form by some protease-like action.

Introduction

γ-Glutamyl transferase ((5-glutamyl)-peptide:amino-acid 5-glutamyltransferase, EC 2.3.2.2) catalyzes transfer of the γ-glutamyl moiety of glutathione or its S-substituted derivatives to various amino acids or peptides. This enzyme has received considerable attention as a diagnostic tool for hepatic dysfunction [1—4]. It is believed that the serum enzyme is derived from the hepatobiliary systems [5] although a higher activity is also detected in a number of other transmural tissues. This enzyme is an integral component of surface membranes and hence believed to comprise some hydrophobic domain structure for its anchorage into the membrane/lipid bilayer [6,7]. The enzyme molecule detected in the serum and bile must have been released in some way from surface membrane of hepatobiliary systems, since most of integral proteins of biological membrane could only be solubilized either by the presence of detergents or by limited proteolysis leaving their anchoring domain within the membrane/lipid bilayer [6—10]. For instance, renal γ-glutamyl transferase is known to be solubilized from brush border membranes with detergents (Lubrol-PX, sodium deoxycholate, Triton X-100, etc.) or with proteases (papain, bromelain, trypsin, etc.) [6,7,10]. The highly purified γ-glutamyl transferase of rat kidney is of a glycoprotein nature and composed of two non-identical subunits [6,7], the catalytic site of the enzyme being localized in the small subunit [11,12]. A considerable difference in molecular weight was observed between the renal enzyme preparation solubilized by detergent and that solubilized by proteases [6,7]. The molecular size of the small subunit remained unchanged during the limited proteolysis of the detergent-solubilized enzyme, while that of the large subunit decreased significantly [6,7]. This finding suggested that the large subunit of the enzyme is involved in anchoring the enzyme molecule into the renal brush border membrane, and hence, polypeptide chain(s) cleaved proteolytically from the large subunit may represent the hydrophobic domain of the enzyme molecule.

Based on the knowledge of these different molecular forms of the renal enzyme, one can assume that the molecular form of the enzyme released into the bile and serum under some hepatobiliary dysfunction might be either the detergent-solubilized form or the protease-solubilized form or both. The determination of the enzyme form in the bile and serum seems to be important for understanding pathologic state of various types of hepatobiliary dysfunction.

This paper deals with the determination of the enzyme forms in the bile and serum of rats under surgical ligation of the bile duct.

Materials and Methods

Chemicals. L-γ-Glutamyl-*p*-nitroanilide, papain and bovine serum albumin were purchased from Sigma Chemical Co. Triton X-100 was obtained from Nakarai Chemical Co. (Kyoto). Glycylglycine was purchased from the Protein Research Foundation (Osaka) and Sepharose 4B from Pharmacia Chemical Co. Concanavalin A was purified from Jack bean meals by the method of Agrawal and Goldstein [13]. Concanavalin A-Sepharose was prepared as described previ-

ously [14]. About 5 mg of the lectin was found to be coupled to 1 ml of Sepharose beads.

Enzyme. γ-Glutamyl transferase was purified from rat kidney using Triton X-100 (detergent-solubilized form of the enzyme) or papain (protease-solubilized form of the enzyme) as described previously [7].

Assays. The enzyme activity was measured with L-γ-glutamyl-*p*-nitroanilide as substrate at 37°C according to the method of Orlowski and Meister [15]. The reaction mixture contained, in a total volume of 1 ml, 2.5 mM L-γ-glutamyl-*p*-nitroanilide, 50 mM glycylglycine, 10 mM $MgCl_2$ and 0.1 M Tris-HCl buffer (pH 9.0). The reaction was started by adding the enzyme sample and the increase in the absorbance at 410 nm was recorded in a high sensitivity spectrophotometer (Model SM-401, Union Giken). 1 unit of the enzyme was defined as the amount of the enzyme required for the formation of 1 μmol of *p*-nitroaniline per min at 37°C. Specific activity of the enzyme was expressed as units per mg of protein. Protein concentration was determined by the method of Lowry et al. [16] with bovine serum albumin as the standard.

Rats, operation and enzyme preparation. Male white Wister rats weighing between 150 and 200 g were used in all experiments. Prior to operation they were given laboratory chow and water ad libitum. Surgical operation was performed in the morning between 8.00 a.m. and 10.00 a.m. under ether anesthesia without a prior fasting period. Ligation of the bile duct was performed at 1 cm distal to the common hepatic duct. At day 7 after operation, blood samples of icteric rats were collected from the carotid artery. To the blood samples were added 2 mM EDTA, followed by centrifugation at 3000 rev./min for 10 min at 4°C. The plasma thus obtained was stored immediately at −20°C. After bleeding, the icteric rats were subjected to laparotomy, and the bile was obtained by puncture of swollen bile duct. The bile samples thus obtained were centrifuged at 5000 rev./min for 10 min at 4°C, and the supernatant fraction was stored immediately at −20°C and kept at this temperature until use.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis of the enzyme samples was carried out in 7.5% polyacrylamide gel according to the method as described previously [11]. Before electrophoresis the bile samples (0.2 ml) were dialyzed against 100 ml of 2.5 mM Tris-HCl buffer (pH 8.6) at 4°C for 12 h. Staining of the enzyme activity in the gel was performed by the method of Miller et al. [17]. The gels were incubated at 37°C for 20 min in a solution containing 2.5 mM L-γ-glutamyl-*p*-nitroanilide/10 mM $MgCl_2$/20 mM glycylglycine/0.1 M Tris-HCl buffer (pH 9.0). Densitometric scanning of the stained gels was carried out immediately after staining in a Simazu Dual-Wavelength Scanner CS-900 at 410 nm for the enzyme activity.

Affinity chromatography. The enzyme samples were thawed and dialyzed against 19 mM Tris-HCl buffer (pH 7.4) containing 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 0.1 M NaCl at 4°C for 12 h. They were centrifuged at 15 000 × *g* for 20 min at 4°C, and the supernatant fractions thus obtained were used for affinity chromatography. Columns containing about 2 ml of concanavalin A-Sepharose were equilibrated with the same buffer solution as used for dialysis of the enzyme samples. The enzyme samples (1—5 ml) were applied and the columns were washed with the same buffer solution. Elution of the adsorbed enzyme

from the column was carried out with the above buffer containing 0.2 M α-methyl-D-glucoside, and then with the buffer containing 0.2 M of the sugar and 1% Triton X-100. The flow rate was 10 ml per h and fractions of 1 ml were collected and assayed for enzyme activity.

Results

Molecular forms of bile γ-glutamyl transferase

Fig. 1 shows the zymogram pattern of γ-glutamyl transferase of the bile preparation obtained from rats under surgical ligation of their bile duct. At least two electrophoretically different enzyme forms appeared to be present, a major broad band (band a) migrating slower than a minor band (band b) (Fig. 1A). After mild treatment of the bile with a very small amount of papain, the band a completely disappeared, and a sharp single band appeared at a position corresponding to that of the band b (Fig. 1B). Thus the enzyme in band b seems to arise from the species in band a through limited proteolysis of the bile by the action of some protease(s). If such a change in molecular form as observed above were caused by limited proteolysis, the molecular size of the

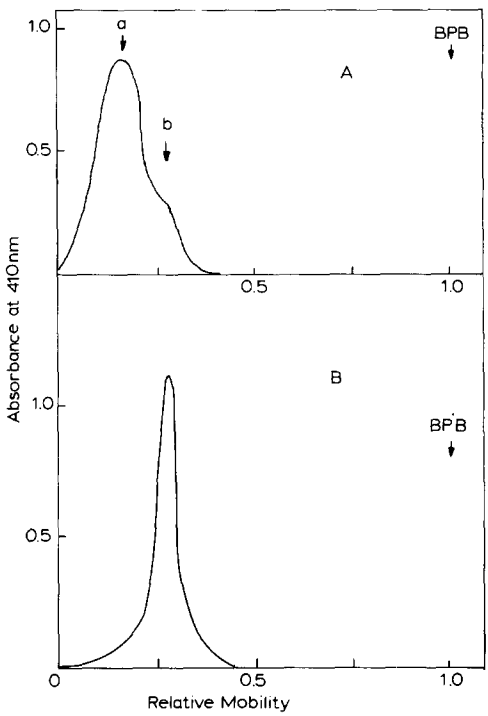

Fig. 1. Polyacrylamide disc gel electrophoresis. The bile preparation (0.2 ml) containing 0.03 units of γ-glutamyl transpeptidase was dialyzed against 100 ml of 2.5 mM Tris-glycine buffer (pH 8.6) at 4°C for 12 h. The bile samples thus obtained were incubated with 10 mM 2-mercaptoethanol in the presence or absence of papain (5 μg/ml) at 37°C for 20 min. They were subjected to polyacrylamide gel (7.5%) electrophoresis. After electrophoresis, the gels were stained for the enzyme activity and the densitometric scanning of the stained gels was performed as described under Materials and Methods. Bromophenol blue (BPB) was used as a tracking dye. A, incubated in the absence of papain; B, incubated in the presence of papain.

enzyme in band b should be smaller than that in band a. To test this, the bile samples were subjected to chromatography on Sephadex G-200 column before and after mild treatment with papain. As expected, most of the enzyme activity in the native untreated bile was eluted in the void-volume fractions and a very small amount of the enzyme was eluted as the second peak in the retarded fractions (Fig. 2A). On the contrary, the enzyme in the papain-treated bile appeared as a single peak in an inner-volume position, which was exactly identical with that for the second peak on the chromatogram of the untreated bile (Fig. 2B). The chromatographic pattern of the enzymes in the native and papain-treated bile samples was similar to that of the purified preparation of renal γ-glutamyltransferase detergent-solubilized and protease-solubilized, respectively (Fig. 2C). Upon ultrogel chromatography, the molecular size of the native bile enzymes (a major and a minor component) was found to be the same as those of purified renal γ-glutamyltransferase detergent-solubilized and protease-solubilized [7], respectively (data not shown). These results strongly suggest that the predominant form of the bile enzyme is similar to the renal detergent-solubilized form and that proteolytic treatment converts the bile detergent-solubilized form into protease-solubilized form with concomitant decrease in molecular size. The change in the electrophoretic mobility of the bile enzyme upon proteolysis as described earlier (Fig. 1) can also be interpreted as showing that the enzyme in band a and band b represent the detergent-solubilized form and the protease-solubilized form of the enzyme, respectively.

Chromatographic behavior of the bile and serum γ-glutamyl transferase on concanavalin A-Sepharose column

The renal γ-glutamyl transferase is a glycoprotein [10,11]. The bile enzyme can also be a glycoprotein. The liver contains various types of glycosidase that might cause some modification of the oligosaccharide moiety of glycoproteins [18]. The conversion of the bile detergent-solubilized form to protease-solubilized form might be accompanied by the loss of the oligosaccharide moiety of the enzyme. To test this possibility, the bile preparations were subjected to chromatography on a concanavalin A-Sepharose column, which is expected to bind specifically the mannose-like oligosaccharide moiety of glycoproteins. Both of the bile detergent-solubilized and protease-solubilized forms of the enzyme were found to be adsorbed to the lectin-conjugated column (Fig. 3). This suggested that the mannose-like oligosaccharide moiety of the enzyme responsible for the binding to the lectin remained intact. However, the elution profiles of the two enzyme forms were quite different from each other. The bile protease-solubilized form was readily eluted from the column with α-methyl-D-glucoside (Fig. 3B), while the bile detergent-solubilized form was eluted only with a solution containing both the sugar and Triton X-100 (Fig. 3A). That this difference in the chromatographic behavior between two forms of the bile enzyme results from the proteolytic modification of the parent enzyme molecule in the bile was confirmed using the well-characterized renal detergent-solubilized and protease-solubilied forms of enzyme preparation. Although the data were not shown, combined use of the sugar and the detergent was required for the elution of the Triton X-100-solubilized renal enzyme,

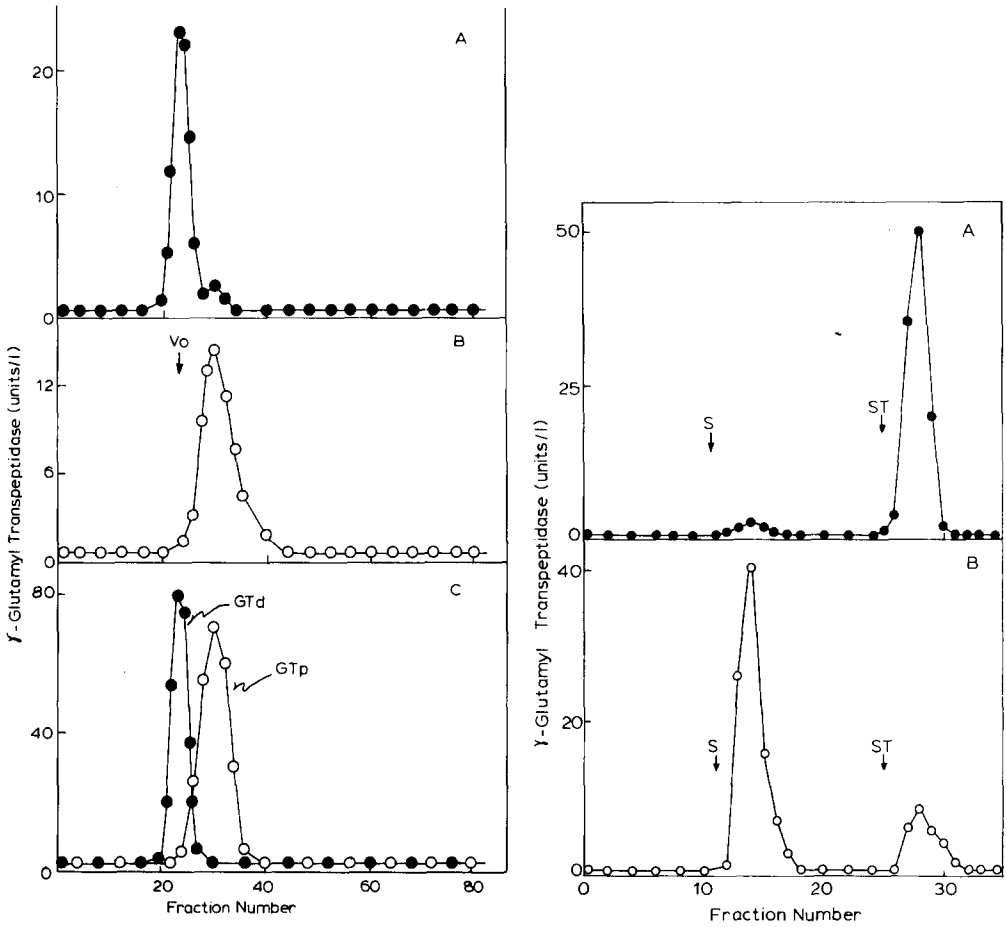

Fig. 2. Gel filtration on Sephadex G-200. The bile samples (3 ml) were dialyzed against 500 ml of 10 mM Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl, 1 mM 2-mercaptoethanol at 4°C for 6 h. After incubation of the bile samples in the presence or absence of papain (5 μg/ml) at 37°C for 20 min, 2 ml of the incubated samples (containing 0.3 units of the enzyme) were subjected to gel chromatography on a Sephadex G-200 column (2.5 X 30 cm) preequilibrated with the same buffer solution as used for dialysis. Fractions of 3.3 ml were collected and assayed for enzyme activity. The elution position of Blue Dextran (V_0) was indicated. A, the bile sample incubated in the absence of papain; B, incubated in the presence of papain; C, two enzyme forms of purified rat renal enzyme (1 unit of the detergent-solubilized form and 2 units of the protease-solubilized form).

Fig. 3. Affinity chromatography of the bile enzymes on concanavalin A-Sepharose column. The papain-treated and untreated bile samples were prepared as in Fig. 2, and were dialyzed against 100 ml of 10 mM Tris-HCl buffer (pH 7.4) containing 0.1 M NaCl, 1 mM $CaCl_2$ and $MnCl_2$ at 4°C for 12 h. Each of the bile samples (1 ml, 0.14 units of the enzyme) thus prepared was separately subjected to chromatography on a concanavalin A-Sepharose column (1 X 3 cm), which was preequilibrated with the same buffer solution. The column was washed with the same buffer solution and eluted with 0.2 M α-methyl-D-glucoside in the absence (S) or presence (ST) of 0.1% Triton X-200. Each fraction (1 ml) was assayed for the enzyme activity. A, untreated bile sample; B, papain-treated bile preparation.

whereas the sugar alone was sufficient for the elution of the papain-solubilized renal enzyme. Thus in analogy to the situation with the renal enzymes, the chromatographic profiles of the bile enzymes as described above indicate that

the predominant enzyme in the intact bile is the detergent-solubilized form of γ-glutamyl transferase.

γ-Glutamyl transferase in the icteric rat serum was also adsorbed to the concanavalin A-Sepharose column. Also in this case, two enzyme forms were observed, one eluted with the sugar alone and the other with combined use of Triton X-100 (data not shown). However, the relative ratio of the protease-solubilized to the detergent-solubilized form of the enzyme was rather high in the serum (0.4—1.3). This was in marked contrast with the case of the bile in which the detergent-solubilized form of the enzyme is predominant.

Discussion

The present investigation demonstrated that there were two enzyme forms of γ-glutamyl transferase in the icteric rat bile preparations, the major component having slow electrophoretic mobility and a minor component migrating slightly faster. The former was assigned to the detergent-solublized form and the latter to the protease-solubilized form which was assumed to be generated from the detergent-solubilized form by the action of some protease(s). This assignment is based on the fact that the renal enzyme either bound to the brush border membranes or solubilized with a detergent could be converted to the enzyme form of smaller molecular weight by treating with a protease such as papain, bromelain, or trypsin [6,7,10,19,20].

Like renal γ-glutamyl transferase preparations, the bile protease-solubilized form could be eluted from the lectin-conjugated column with the sugar, and the bile detergent-solubilized form could be eluted only with the combined use of the sugar and detergent. Previously, it was postulated that the cleaved peptide moiety of the renal detergent-solubilized form through limited proteolysis might be an anchoring portion which is of hydrophobic nature [6,7,20]. Thus the difference in the chromatographic behavior between the bile detergent-solubilized and protease-solubilized forms may be ascribed to the fact that the detergent-solubilized molecule is more hydrophobic than the protease-solubilized molecule. A similar contribution of hydrophobic interaction has recently been described for other glycoproteins such as interfer on and collagen glycosyltransferase which showed high affinity for concanavalin A-Sepharose column [21,22]. These glycoproteins adsorbed to the column were eluted with the sugar only in the presence of high concentrations of ethylene glycol. Although the data were not shown, Triton X-100 could be replaced by ethylene glycol for the specific elution of the renal detergent-solubilized as well as the bile detergent-solubilized forms of the enzyme from the column. The recent report [23] that concanavalin A possesses the site for hydrophobic compounds per monomeric unit and that this site is independent of the saccharide-binding capacity normally associated with the lectin may be relevant to the present finding.

It is also possible that changes in the terminal oligosaccharide moiety of the bile enzymes may be partly responsible for the difference in the chromatographic behavior. However, this possibility appeared to be ruled out as the chromatographic profiles of the bile enzymes on Sephadex G-200, ultrogel

Aca-34, or on the lectin-conjugated column remained unchanged after sialidase treatment (data not shown).

Affinity chromatography of serum samples on a concanavalin A-Sepharose column also revealed the presence of two enzyme forms. The result of gel chromatography of the serum samples on Sephadex G-200 was consistent with the above result, the one being eluted in the void-volume fractions and the other eluted in an inner-volume fraction as was the case with the bile enzymes (data not shown). The relative amount of protease-solubilized to detergent-solubilized form of the enzyme in the icteric rat serum seemed to vary from one sample to another (0.4—1.3). However, with all serum samples tested, this ratio was relatively high when compared with that for untreated biled preparations (less than 0.05). Comparison of this ratio between the bile and serum suggests that the bile protease-solubilized form may be preferentially transferred to the blood circulation, or alternatively, the bile detergent-solubilized form may be partly converted to the protease-solubilized form by some protease-like activity prior to or after migration into the blood circulation. Since the enzyme level in normal rat serum was quite low (less than 0.6 unit per l), the precise value for the ratio of the two enzyme forms was not obtained, and it could not be determined whether the appearance of the two forms of the enzyme in the serum was specific for obstractive jaundice or not (the enzyme activity of icteric rat serum used in this experiment was 20—60 units per l). During the preparation of this manuscript, we have come across the paper [24] describing the presence of both the hydrophobic and hydrophilic forms of the enzyme in the normal human serum and bile. This is consistent with the present findings.

It is well documented that the serum level of membrane-bound enzymes such as alkaline phosphatase increases in biliary obstructive jaundice [25,26]. In hepatobiliary dysfunction with severe obstructive jaundice, some membranous protein of erythrocytes was reported to disappear, or decrease to a considerably low level, and to reappear after surgical removal of the biliary obstruction [27]. A similar releasing mechanism to that for these membrane components might operate in the release of γ-glutamyl transferase from hepatobiliary systems. Cholic acid, a natural detergent secreted into the bile, may be responsible for the solubilization of such membrane components, as the biliary concentration of this compound is known to be high [28].

References

1 Goldberg, J.A., Friedman, O.M., Pineda, E.P., Smith, E.E., Chatteriji, R., Stein, E.H. and Rutenberg, A.M. (1960) Arch. Biochem. Biophys. 91, 61—70
2 Szeczeklik, E., Orlowski, M. and Szewczuk, A. (1961) Gastroenterology 41, 353—359
3 Cohen, M.I. and McNamara, H. (1969) J. Pediatr. 75, 838—842
4 Tamaoki, H., Minato, S., Takei, S. and Fujisawa, K. (1975) Clin. Chim. Acta 65, 21—27
5 Naftalin, L., Child, V.J. and Morley, D.A. (1969) Clin. Chim. Acta 26, 297—300
6 Hurghey, R.P. and Curthoys, N.P. (1976) J. Biol. Chem. 251, 7963—7870
7 Horiuchi, S., Inoue, M. and Morino, Y. (1977) Eur. J. Biochem. 87, 429—437
8 Cook, G.M.W. (1968) Biol. Rev. 43, 363—391
9 Vannier, C., Louvard, D., Maroux, S. and Desnuelle, P. (1976) Biochim. Biophys. Acta 455, 185—199
10 Tate, S.S. and Meister, A. (1975) J. Biol. Chem. 250, 4619—4617
11 Inoue, M., Horiuchi, S. and Morino, Y. (1977) Eur. J. Biochem. 73, 335—342
12 Tate, S.S. and Meister, A. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 931—935
13 Agrawal, B.B.L. and Goldstein, I.J. (1967) Biochim. Biophys. Acta 147, 262—275

14 Inoue, M., Mori, M. and Seno, S. (1973) Symp. Cell Biol. 24, 69—78
15 Orlowski, M. and Meister, A. (1963) Biochim. Biophys. Acta 73, 679—681
16 Lowry, O.H., Rosebrough, N.J., Farr, A. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
17 Miller, S.P., Awasthi, Y.C. and Slivastava, S.K. (1976) J. Biol. Chem. 251, 2271—2278
18 Suzuki, K. (1976) in Biological Roles of Sialic Acid (Rosenberg, A. and Schengrund, C., eds.), pp. 159—182, Plenum Press, New York
19 Zelazo, P. and Orlowski, M. (1976) Eur. J. Biochem. 61, 147—155
20 Hughey, R.P., Coyle, P.J. and Curthoys, N.P. (1979) J. Biol. Chem. 254, 1124—1128
21 Davey, M.W., Sulkowski, E. and Carter, W.A. (1976) Biochemistry 15, 704—713
22 Anttinen, H., Myllylä, R. and Kivirikko, K. (1977) Eur. J. Biochem. 78, 11—17
23 Edelman, G.M. and Wang, J.L. (1978) J. Biol. Chem. 253, 3016—3022
24 Huseby, N.E. (1978) Biochim. Biophys. Acta 522, 354—362
25 Righetti, A.B. and Kaplan, M.M. (1972) Gastroenterology 62, 1034—1039
26 Holdsworth, G. and Coleman, R. (1975) Biochim. Biophys. Acta 389, 47—50
27 Iida, H., Hasegawa, I. and Nozawa, Y. (1976) Biochim. Biophys. Acta 443, 394—401
28 Vyvoda, O.S., Coleman, R. and Holdsworth, G. (1977) Biochim. Biophys. Acta 465, 68—76