Biochimica et Biophysica Acta, 649 (1981) 155–161
Elsevier/North-Holland Biomedical Press

BBA 79440

ALTERATIONS IN LABELING OF CELL-SURFACE GLYCOPROTEINS FROM NORMAL AND DIABETIC RAT INTESTINAL MICROVILLOUS MEMBRANES

LUCIEN R. JACOBS

Department of Internal Medicine, University of California, School of Medicine, Davis, CA 95616 (U S A)

(Received April 6th, 1981)

Key words Glycoprotein, Glycosylation, Surface labeling, Streptozotocin diabetes, Microvillous membrane, (Rat intestine)

The effect of chronic streptozotocin-induced diabetes was studied on intestinal microvillous membrane surface carbohydrate groups. After 7 weeks of diabetes, purified microvillous membranes were prepared from rat small intestine and surface galactoproteins identified by labeling with galactose oxidase/sodium boro[^{3}H]hydride. Membrane surface sialic acid residues were labeled using the sodium metaperiodate/sodium boro[^{3}H]hydride technique. Membranes were solubilized in SDS and protein labeling analyzed by acrylamide electrophoresis. Membranes from diabetic rats showed an 81% increase in galactoprotein labeling ($P < 0.02$) while labeling of sialic acid residues was unchanged. The greatest increase in galactoprotein labeling occurred in protein monomers of M_r 116 000–200 000, where there was a 155% increase in labeling ($P < 0.005$). These results indicate that intestinal microvillous membrane protein glycosylation is altered in chronic diabetes. This increase in surface membrane carbohydrates could explain the decreased rates of proteolytic degradation previously described for at least one microvillous protein. An increase in membrane galactose groups has also been noted in hepatocyte and kidney glomerular basement membranes, which suggests the presence of a systematic change in membrane protein glycosylation occurring as a result of the diabetic state.

Introduction

Prolonged elevation of blood glucose in diabetes may result in structural and functional alterations of both circulating and membrane-bound proteins. Glucose appears to react nonenzymatically with lens-α-crystallins [1], red cell membrane protein [2], albumin [3], collagen [4], and the basic myelin protein of nerve [5]. There is, in addition, evidence that the structural changes in diabetic microangiopathy can result from hyperglycemia [6].

Other studies have also demonstrated significant changes in the glycoconjugate composition of different cell membranes from diabetic subjects Both rat liver plasma cell and red blood cell membranes show evidence of a decrease in sialic acid [7,8], while rat liver membranes also show an increase in cell surface galactose groups [9]. Analyses of human kidney glomerular basement membranes have also demonstrated a decrease in sialic acid but an increase in glucose and galactose [10,11]. This suggests that there may be systematic alterations in cell membrane glycoprotein biosynthesis in the diabetic animal.

Alterations in membrane protein glycosylation may also be important in membrane protein turnover. The intestinal microvillous membrane protein sucrase-isomaltase is increased in amount in the diabetic rat as a result of a decrease in degradation [12]. Since microvillous proteins are regulated in part by luminal proteases [13], changes in luminal surface protein carbohydrate groups could influence proteolytic degradation. The purpose of this study was to determine whether the intestinal microvillous

Abbreviation SDS, sodium dodecyl sulfate.

membrane and its luminally exposed proteins undergo an alteration in glycosylation following the induction of diabetes.

Materials and Methods

20 fasting male Sprague-Dawley rats (Simonsens, Gilroy, CA) weighing 150–200 g received a single intravenous injection of streptozotocin (Sigma, St. Louis, MO) (70 mg/kg body wt.) in order to induce experimental diabetes. Streptozotocin was dissolved in 0.05 M citrate buffer (pH 4.5) to give a final concentration of 30 mg/ml. Rats were kept in metabolic cages to facilitate the daily collection of urine which was tested for evidence of glycosuria. Only those animals with blood sugars greater than 300 mg/ml, 2 days after injection were considered as diabetic. Both diabetic and control rats were fed a laboratory stock diet and water ad libitum. Body weights were recorded weekly and food intake twice weekly for the ensuing 7 weeks, after which time the rats were killed following an overnight fast and the small intestines removed in order to prepare microvillous membranes.

Intestinal microvillous membrane vesicles were purified according to the method of Hopfer et al. [14] using the mucosal scrapings of the entire small intestine. The final brush border membrane fraction (2.5–5.0 mg protein/ml) purified a minimum of 9-fold over the original whole homogenate as estimated from the increase in sucrase specific activity Only membranes which purified more than 12-fold over the homogenate were used for radioactive labeling with specific probes to identify galactose residues and sialic acid moieties. Additional confirmation of microvillous membrane purification was obtained by electron microscopy studies. Membranes were fixed and stained in 3.6% glutaraldehyde/0.2 M cacodylate buffer (pH 7.3) for 1 h at room temperature, followed by 5% OsO_4 in 0.2 M cacodylate buffer (pH 7 3) for a further 3 h. The membranes were imbedded in Spurrs low-viscosity resin. The thin sections were stained with uranyl acetate and lead citrate and examined with an AEI EM-801 electron microscope at 80 kV.

Labeling of membrane galactose residues

Terminal galactose groups were labeled according to the method of Gahmberg and Hakomori [15]. 1 ml of purified membranes were resuspended with 13 units of galactose oxidase (U.S Biochemicals, Cleveland, OH) in 0 02 M phosphate buffer (pH 7.5) for 30 min at 37°C. The membranes were reisolated by centrifugation at 24 000 × *g* at 6°C and the pellet was washed twice in 1 ml of the phosphate buffer. The membranes were then resuspended, in 1 ml of the phosphate buffer and incubated with 400 μCi sodium boro[^{3}H]hydride (170 mCi/mmol; New England Nuclear, Boston, MA) in 80 μl 0.01 M sodium hydroxide for 15 min at 6°C. The labeled membranes were separated from the reaction medium by centrifugation at 24 000 × *g* and washed twice in the phosphate buffer, prior to resuspension in 0.3 ml 0.02 M phosphate buffer (pH 7.5).

Labeling of membrane sialic acid residues

N-Acetyl neuraminic acid residues were labeled by the method of Lenten and Ashwell [16] using the modification of Gahmberg and Andersson [17]. Purified membranes suspended in 0 1 ml 0.02 M phosphate buffer (pH 7.5) were incubated with 1 mM sodium metaperiodate (Sigma, St. Louis, MO) at 0°C for 15 min after which they were separated and washed twice in the phosphate buffer. The membranes were resuspended in 1 ml of the phosphate buffer prior to reduction with sodium boro[^{3}H]-hydride using the same technique and conditions as described above for the labeling of galactose residues.

Membrane solubilization and polyacrylamide gel electrophoresis

7.5% polyacrylamide gels (3 0% stacking and 7 5% separation gel) were prepared according to the method of Laemmli [18]. Membranes (100 μg protein) were first solubilized by heating in 2% SDS (B.D.H. Laboratories, Poole, U.K.) with 5% 2-mercaptoethanol for 3 min at 100°C and then applied to the gel. Samples were run in duplicate. Following electrophoresis at 3 mA/gel, one gel was sliced into 2-mm slices and dissolved in 30% hydrogen peroxide/ 4.8% ammonium hydroxide by incubating at 50°C for 6–8 h. Radioactivity was determined in a Beckman liquid scintillation counter, LS 8000 using an automatic quench correction. The efficiency of recovery of the counts applied to the gel was 70%. The efficiency of tritium counting was 25%. A

duplicate gel from the same experiment was stained with Coomassie brilliant blue G-250 [19] and densitometry was performed by scanning the protein bands at 600 nm in a Gilford gel scanning spectrophotometer

Molecular weight measurements

Weights of protein monomers separated by SDS-acrylamide electrophoresis were estimated using standard protein subunits: myosin (200 000), phosphorylase B (94 000), bovine serum albumin (68 000), ovalbumin (43 000) and carbonic anhydrase (30 000) (Bio-Rad, Richmond, CA).

Enzyme assays

Sucrase activity was assayed by the Tris-glucose oxidase method [20] with sucrose at 28 mM in the reaction mixture. Leucyl-β-naphthylamide, a specific substrate for the microvillous amino-oligopeptidase was used to identify peptidase activity [21]. Enzyme activity was expressed in international units (i.u.) – mmol substrate hydrolyzed per min. Plasma glucose levels were measured by the Tris-glucose oxidase technique [20] and protein was determined by the method of Lowry et al. [22].

The data were analyzed using Student's *t*-test for unpaired observations

Results

All animals demonstrated glycosuria of at least 0.5% 1 week following the streptozotocin. Just prior to killing, the fasting mean blood glucose level of the diabetic rats was 571 ± (S.E.) 32 mg/100 ml compared to 113 ± 10 mg/100 ml in the age-matched controls ($P < 0.001$). 4 days after streptozotocin, food intake increased in the diabetic rats and remained elevated thereafter. During the seventh week of the study, the diabetics consumed 35.4 ± 3.2 g/day compared to the 23.4 ± 1.1 g/day eaten by the controls ($P < 0.001$). Despite their greater food intake, the final body weight of the diabetic rats was only 241 ± 2 g compared to 422 ± 11 g in the controls ($P < 0.001$). Although the diabetic rats weighed less than the controls, their small intestinal mucosal weights were significantly heavier, 5.4 ± 0.2 mg compared to the control weight of 3.9 ± 0.2 g ($P < 0.001$).

Analysis of the intestinal mucosal homogenates (Table I) revealed that protein content and amino-oligopeptidase activity did not change significantly in the diabetic group, whereas sucrase specific activity increased by 3.2-fold compared to controls ($P < 0.005$). Microvillous membranes were purified from the intestinal mucosal homogenates and digestive hydrolase activity was measured (Table II). Sucrase specific activity in the diabetic group was still significantly elevated when compared to the controls ($P < 0.001$), while amino-oligopeptidase activity remained unchanged.

Purified microvillous membranes from both diabetic and normal rats were then used to examine the labeling of surface membrane carbohydrate residues of membrane proteins (Fig. 1). Microvillous membranes from normal rat intestine purified 15.5 ± 1.7-fold over the original whole homogenate, compared to the 15.7 ± 2.1-fold purification obtained for the diabetic group ($P > 0.05$). Electron micrographs of the microvillous membrane fractions revealed a uniform field of vesicles, mostly oriented right-side-out. Micrographs from the different groups appeared comparable with regard to the type of membrane vesicles obtained and their morphology (results not shown).

As shown in Fig. 1 and Table III, there was an 81%

TABLE I

PROTEIN CONTENT, SUCRASE AND AMINO-OLIGOPEPTIDASE SPECIFIC ACTIVITY IN MUCOSAL HOMOGENATES FROM CONTROL AND DIABETIC RAT INTESTINE

Results are mean ± S.E. n s., not significant.

	Control (n = 23)	Diabetic (n = 15)	Significance of difference
Total intestinal mucosal protein (mg)	463 ± 29	540 ± 50	n.s
Sucrase (mU/g protein)	45.5 ± 5.4	147.5 ± 14.6	$P < 0.001$
Amino-oligopeptidase (mU/g protein)	6.5 ± 1.0	6.0 ± 0.6	n.s.

TABLE II

SUCRASE AND AMINO-OLIGOPEPTIDASE SPECIFIC ACTIVITY IN PURIFIED MICROVILLOUS MEMBRANES FROM CONTROL AND DIABETIC RAT INTESTINE

Results are means ± S E. n s , not significant

Enzyme activity	Control (n = 16)	Diabetic (n = 14)	Significance of difference
Sucrase	535 ± 59	1423 ± 207	$P < 0.001$
Amino-oligopeptidase	25.9 ± 10 1	30.5 ± 13.1	n s

increase in galactoprotein labeling of diabetic membranes as compared to the control group ($P < 0.02$). Solubilization of membranes and analyses of their constituent protein monomers by SDS-gel electrophoresis (Fig. 1 and Table III) revealed that the greatest increase in labeling occurred in glycopeptides of M_r 116 000–200 000, where there was a 155% increase in radioactive labeling of galactose residues

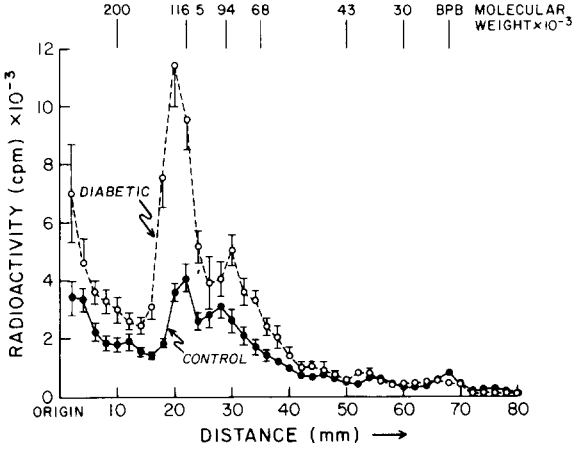

Fig. 1 SDS-acrylamide electrophoresis of intestinal microvillous membrane surface glycoproteins labeled at the galactose residue. Membranes from control and diabetic rat intestine were purified and labeled by the galactose oxidase/sodium boro[^{3}H]hydride technique (see Materials and Methods). 100 μg membrane protein was solubilized by heating for 2 min at 100°C in 2% SDS, and applied to a 7 5% gel Following electrophoresis, the gel was cut into 2-mm slices, dissolved in 30% H_2O_2 and radioactivity was determined in a scintillator counter The comparison gel was stained with Coomassie blue G-250 and scanned (Fig. 3). Positions of protein standards and the bromphenol blue (BPB) dye front are indicated at the top. Points on the curve represent the mean ±S E. of results obtained from four animals in each group

in the diabetic group ($P < 0.005$). Labeling of membrane sialoglycoproteins (Fig. 2) like galactoprotein labeling also revealed a major labeled peak in the 116 000–200 000 dalton range However, in contrast to the membrane galactoproteins, the sialoglycoproteins failed to show a significant difference in terminal sialic acid labeling between the normal and diabetic groups Representative examples of densitometric scans of solubilized microvillous membrane proteins separated by SDS-acrylamide electrophoresis and stained for protein with Coomassie brilliant blue are shown in Fig 3. Apart from a tendency to show three clear protein bands in the diabetic membranes (broken line) in the M_r 94 000–68 000 range, there was little qualitative difference between membranes from normals and diabetics In general, labeling of

TABLE III

RADIOACTIVE LABELING OF INTESTINAL MICROVILLOUS SURFACE MEMBRANE GALACTOPROTEINS FROM NORMAL AND DIABETIC RAT INTESTINE

Results are means ± S.E n s , not significant

Molecular weight (×10⁻³)	Radioactivity (×10⁻³ cpm)		Significance of difference
	Control (n = 4)	Diabetic (n = 4)	
>200	12 7 ± 1 5	21 6 ± 3 6	n s
200–116 5	14 4 ± 1 7	36 7 ± 4.4	$P < 0.005$
116 5–94	11 1 ± 1.5	18 2 ± 2 2	$P < 0.05$
94–68	6 5 ± 0 8	11 4 ± 1 3	$P < 0.02$
68–43	4 2 ± 0 5	5 6 ± 1 0	n s
43–30	2 4 ± 0 6	3 0 ± 0 2	n.s
<30	3 5 ± 1 2	2 9 ± 0 5	n s
Total cpm/ 100 μg protein	54 8 ± 5 6	99 4 ± 11 4	$P < 0.02$

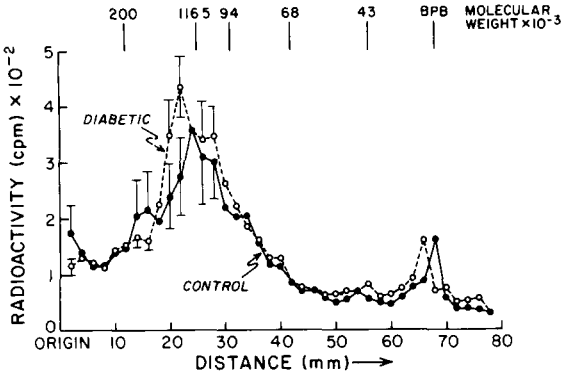

Fig 2. SDS-acrylamide electrophoresis of intestinal microvillous membrane surface glycoproteins labeled at the *N*-acetyl neuraminic acid (sialic acid) residue. Membranes from control and diabetic rat intestine were purified and labeled by the sodium metaperiodate/sodium boro[^{3}H]-hydride technique (see Materials and Methods). 100 μg labeled membrane protein were solubilized and applied to 7.5% gels and electrophoresis was carried out as described for Fig. 1 Points on the curve represent the mean ±S.E of results obtained from five control and seven diabetic rats. BPB, bromphenol blue.

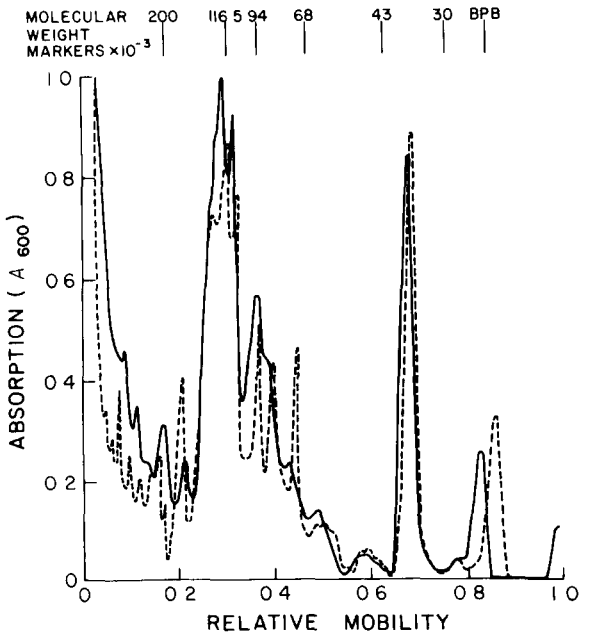

Fig. 3. SDS-acrylamide electrophoresis of intestinal microvillous membrane proteins stained with Coomassie blue G-250. Membranes from control (continues line) and diabetic (broken line) rat intestine were purified, radioactively labeled and solubilized prior to electrophoresis as described for Fig 1 Gels were scanned in a Gilford 250 spectrometer. BPB, bromphenol blue.

membrane glycoproteins appeared to correlate well with the distribution and intensity of protein band staining. The notable exception was the protein band located at 43 000–30 000 daltons (Fig. 3) which failed to label with either the galactose or the sialic acid radioactive ligands.

Discussion

These studies on the labeling of the surface carbohydrates residues clearly demonstrate a specific alteration in the biochemical composition of the intestinal microvillous membrane of chronic diabetic rats. The increased labeling of membrane galactose moieties found in the diabetic group was most marked in the larger molecular weight glycoproteins (Fig. 1, Table III). In contrast, chronic diabetes was not associated with any significant change in the labeling of the terminal carbohydrate residue sialic acid (Fig. 2). Specific changes in microvillous digestive hydrolase activity were also noted; sucrase activity was increased in the diabetic rats while amino-oligopeptidase activity remained unchanged (Tables I and II).

Previous studies have also demonstrated an increase in sucrase as well as other disaccharidase activities [22–24]. The present results also confirm the findings of Olsen and Rogers [25] who found that amino-oligopeptidase activity in diabetic rats did not alter. The development of hyperphagia by rats with chemically induced diabetes was also confirmed in this study. An alteration in food intake may in itself effect intestinal hydrolase activity. This question has been specifically examined in diabetic rats where both pair-feeding experiments [24,26] and intestinal bypass studies [27] have provided firm evidence that the diabetic enhancement of microvillous hydrolase activity is independent of intraluminal factors such as food intake or pancreatico-biliary secretions. This would therefore suggest that the alterations in surface membrane glycosylation are probably also independent of any change in intraluminal factors occurring as a result of the diabetic state. The mechanism for these alterations in microvillous membrane glycosylation has at present not been determined. The increased content of terminal galactose/*N*-galactosamine residues might be due to an increase in synthesis or alternatively, a decrease in degrada-

tion. Similarly, although surface sialic acid levels appeared to be normal in the diabetic group, this does not rule out the possibility of changes in rates of synthesis and degradation which might alter membrane protein carbohydrate turnover without actually producing a change in the carbohydrate level.

These results show for the first time that the intestinal cell membrane surface topography is significantly altered in rats with chronic experimental diabetes. It has been suggested that altered glycosylation of brush border proteins might be responsible for the decreased rate of degradation of the enzyme sucrase [12] Although in the present study the glycosylation of purified individual proteins was not studied, the gel electrophoresis results revealed that the increase in galactose residues occurred predominantly in the proteins of M_r 116 000–200 000 which is the weight range in which protein subunits of sucrase-isomaltase [29] and amino-oligopeptidase are found [30]. Both sialic acid and galactose groups have been identified in the carbohydrate portion of amino-oligopeptidase [31], whereas in sucrase-isomaltase, galactose but not sialic acid is present.

There is currently an increasing body of evidence to support the role of luminal proteases and particularly pancreatic proteases in the regulation of intestinal microvillous membrane proteins [13]. Since carbohydrate groups such as sialic acid or *N*-acetylgalactosamine render protection against proteolysis of glycoproteins [33], an increase in superficial carbohydrate moieties might reduce proteolysis and hence membrane protein degradation [34]. Thus, these results strongly support the hypothesis that the decreased rate of microvillous membrane protein degradation seen in diabetes is due to the increase in surface membrane galactose residues.

Investigations of other cell systems have also revealed specific alterations in membrane and membrane-bound protein carbohydrate groups. In the rat, liver plasma cell membrane sialic acid is decreased and surface galactose groups are increased [7–9], while red cell membrane sialic acid is decreased [7]. Similarly, in human kidney glomerular basement membranes, sialic acid is also diminished, whereas galactose and glucose are elevated [10,11]. The present results would therefore appear to support the concept of a systematic and specific alteration in cell membrane glycoprotein biosynthesis occurring in the chronic diabetic rat.

Further studies are needed to determine the role of hormones in the regulation of microvillous membrane proteins in the diabetic and specifically how hormones may regulate the biosynthesis of the digestive hydrolases. The existence of low insulin and elevated glucagon levels has been documented in streptozotocin-diabetic rats [35]. Administration of insulin for several days after alloxan or streptozotocin partly inhibits the increase in digestive hydrolase activity [26,27]. These observations also provide evidence against a possible direct effect of streptozotocin on the intestine, producing the changes in digestive enzyme activity which have been attributed to the diabetic state.

The present data lend further support to the concept that the changes in cell membrane structure and function found in the chronic diabetic occur as a result of an alteration in glycoprotein biosynthesis. It would thus appear that non-enzymatic glucosylation is only one of several factors which may play a role in the pathogenesis of the long-term complications of diabetes mellitus. The relative importance of these different changes in membrane protein glycosylation still remains to be determined.

Acknowledgements

I wish to thank Cathy Norman and Jill Souder for their excellent technical assistance and Bob Munn for carrying out the electron microscopy.

References

1 Stevens, V.J., Rouzer, C A., Monnier, V.M. and Cerami, A. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 2198–2922
2 Miller, J.A., Gravallese, E. and Bunn, H.F (1980) J. Clin. Invest. 65, 896–901
3 Day, J.F., Thornburg, R.W., Thorpe, S.R. and Baynes, J.W. (1979) J. Biol. Chem. 254, 9394–9400
4 Robins, S.P. and Bailey, A.J (1972) Biochem. Biophys Res. Commun. 48, 76–84
5 Fluckinger, R. and Winterhalter, K H. (1978) in Biochemical and Clinical Aspects of Hemoglobin Abnormalities (Caughey, W S., ed.), p. 205, Academic Press, New York
6 West, K.M. (1978) in Epidemiology of Diabetes and its Vascular Lesions (West, K.M., ed.), pp. 159–189, Elsevier, New York
7 Chandramouli, V and Carter, J R (1975) Diabetes 24, 257–262

8 Durand, G., Dumont, J.P., Appel, M., Durand, D., Davy, J. and Agneray, J. (1980) Horm. Metab. Res. 12, 247–251
9 Chandramouli, V., Lundt, B. and Carter, J.R. (1979) FEBS Lett. 104, 389–392
10 Westberg, N.G. and Michael, A.F. (1973) Acta Med. Scand. 194, 39–47
11 Beisswenger, P.J. and Spiro, R.J. (1972) Diabetes 22, 180–193
12 Olsen, W.A. and Korsmo, H. (1977) J. Clin Invest. 60, 181–188
13 Alpers, D.H. and Tedesco, F.J. (1975) Biochim. Biophys. Acta 401, 28–40
14 Hopfer, U., Nelson, K., Perrotto, J. and Isselbacher, K.J. (1973) J. Biol. Chem. 248, 25–32
15 Gahmberg, C.G. and Hakomori, S.I (1973) J. Biol. Chem. 288, 4311–4317
16 Lenten, L.V. and Ashwell, G. (1971) J. Biol. Chem. 246, 1889–1894
17 Gahmberg, C.G. and Andersson, L.C. (1977) J. Biol. Chem. 252, 5888–5894
18 Laemmli, U.K. (1970) Nature (Lond.) 227, 680–685
19 Blakesley, R.W. and Boezi, J.A. (1977) Anal. Biochem. 82, 580–582
20 Gray, G.M. and Santiago, N.A. (1969) J. Clin. Invest. 48, 716–728.
21 Wojnarowska, F. and Gray, G.M. (1975) Biochim. Biophys. Acta 403, 147–160
22 Lowry, O.H., Rosebrough, N.J , Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
23 Younoszai, M.K. and Schedl, H.P. (1972) J. Lab. Clin. Med. 79, 579–586
24 Caspary, W.F. (1973) Digestion 9, 248–263
25 Olsen, W.A. and Rogers, L. (1971) J. Lab. Clin. Med. 77, 838–842
26 Olsen, W.A , Agresti, H.L. and Lorenzonn, V (1974) in Intestinal Adaptation (Dowling, R.H. and Reicken, E.O., eds.), pp. 179–187, F.K. Schattauer Verlag, Stuttgart
27 Caspary, W.F., Rhein, A.M. and Creutzfeldt, W. (1972) Diabetologia 8, 412–414
28 Olsen, W.A. and Korsmo, H. (1975) J. Lab. Clin. Med. 85, 832–837
29 Conklin, K.A., Yamashiro, K.M. and Gray, G.M. (1975) J. Biol. Chem. 250, 5735–5741
30 Gray, G M. and Santiago, N.A (1977) J. Biol. Chem. 252, 4922–4928
31 Kim, Y.S. and Brophy, E.J. (1976) J. Biol. Chem 251, 3199–3205
32 Cogoli, A., Mosimann, H., Vock, C., Von Balthazar, A-K. and Semenza, G. (1972) Eur. J. Biochem. 30, 7–14
33 Gottschalk, A., Bhargava, A.S. and Murty, V.L.N. (1972) in Glycoproteins· Their Composition, Structure and Function (Gottschalk, A. ed), pp. 810–829, Elsevier, New York
34 Scawen, M and Allen, A. (1977) Biochem. J. 163, 363–368
35 Stearns, S.B. and Benzo, C.A (1978) Horm. Metab. Res. 10, 20–23