Chemical and Physical Properties of the Human Urinary Glycoprotein with Gastric Antisecretory Activity

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The chemical and physical properties of the high-molecular-weight glycoprotein ($s_{20,w}^0 = 8S$; $V_e = V_0$ on Sephadex G-200) with gastric antisecretory activity extracted from the urine of pregnant women were studied. Gel filtration in the presence of sodium dodecyl sulphate and sodium dodecyl sulphate/polyacrylamide-disc-gel electrophoresis indicated subunit mol.wts. of 16000 ± 1500 and 13000 ± 1000 respectively. Reaggregation of the subunits and partial recovery of the biological activity were observed on removal of the detergent. The partial C-terminal sequence was found to be Phe-Tyr-Leu-Val-OH, whereas glycine appears to be the N-terminal amino acid. The carbohydrate composition was examined; all galactosamine was found to be O-glycosidically linked to the polypeptide chain.

Inhibitors of gastric secretion have been found in human urine; these have been grouped under the general name of 'urinary gastric secretory depressant' or simply 'urogastrone' (Gray *et al.*, 1940), where the term 'gastrone' generically indicates the factor inhibiting all forms of gastric acid stimulation (Thomson, 1966). Several authors (Friedman, 1951; Gregory, 1955; Mongar & Rosenoer, 1962; Morimoto & Yamamoto, 1969; Lawrence *et al.*, 1971) described extraction procedures and partial chemical and physiological characterizations of purified antisecretory fractions. However, these studies did not lead to a clear elucidation of the chemical nature and physiological properties of the various isolated inhibitors.

Sandweiss *et al.* (1938, 1939) observed that peptic ulcers rarely occur in women during pregnancy. This seemed to suggest that women might be protected under these circumstances by a hyperproduction of gastrone inhibiting the gastric secretion.

Indeed we succeeded in isolating from the urine of women at the third or fourth month of pregnancy (which corresponds to the period of maximal gastric hypokinesia and hypochylia) a high-molecular-weight glycoprotein showing a marked gastric antisecretory activity (Carrea *et al.*, 1973).

In the present paper we report the chemical and structural characterization of this inhibitor. The subunit structure and molecular weight in the presence of sodium dodecyl sulphate and the C- and N-terminal amino acids have been determined, and the carbohydrate moiety has been more thoroughly investigated. This work is a necessary preliminary to

a study of the biosynthesis and physiological mechanism of the glycoprotein, as well as to the development of an immunological assay.

Materials and Methods

Preparation of the human urinary gastric inhibitor

Human urinary gastric inhibitor (hereinafter called the 'inhibitor') was obtained from the urine of women at the third or fourth month of pregnancy as previously reported (Carrea *et al.*, 1973). The purification procedure included chromatography through a column of De-Acidite H, benzoic acid extraction, fractionated precipitations with organic solvents and gel filtration on Sephadex G-200. The yield was 1.7 mg of glycoprotein/litre of urine.

Molecular-weight determination

Sodium dodecyl sulphate-gel filtration. The procedure was the following modification of the method of Fletcher *et al.* (1970). Sephadex G-200, equilibrated with 0.5% sodium dodecyl sulphate in 0.05M-Tris/acetic acid buffer, pH 8, was packed into a column (1 cm $\times$ 100 cm). The flow rate was 2.5 ml/h and fractions (2 ml) were collected. The E_{278} of the effluent was monitored. The chromatographic experiments were carried out at room temperature (20°C). The following standards were used for column calibration: Blue Dextran 2000, bovine serum albumin (mol.wt. 69000), aldolase (40000), pepsin (35000), trypsin (23300), lysozyme (14300) and cytochrome *c* (12400).

Samples of the inhibitor and standards were incu-

bated at 2mg/ml in 2% (w/v) sodium dodecyl sulphate, pH8, at 37°C for 24h. Portions (0.5ml) of each were separately applied to the column.

Sodium dodecyl sulphate/polyacrylamide-disc-gel electrophoresis. The procedure of Weber & Osborn (1969) was basically followed. The acrylamide concentration was 15% (w/v). The standard proteins were: bovine serum albumin, ovalbumin (mol.wt. 43000), trypsin, haemoglobin (mol.wt. 15500), lysozyme and cytochrome c. Test samples and standards were incubated at 37°C for 24h in 0.05M-sodium phosphate buffer, pH7.2, containing 1% sodium dodecyl sulphate. Portions (10–20 µg) of each were loaded on to the gel.

C- and N-Terminal analysis

Carboxypeptidase A digestion. Inhibitor (30mg) was dissolved in 7.5ml of 0.2M-N-ethylmorpholine/acetate buffer, pH8, containing 1% sodium dodecyl sulphate and 1.25 µmol of norleucine as the internal standard, and incubated at 37°C for 24h.

2.5mg of carboxypeptidase A-DFP (from bovine pancreas, Sigma Chemical Co., St. Louis, MO, U.S.A.), solubilized according to Needleman (1970), was added (E/S ratio 1:12) and the digestion performed at 37°C. Portions (1.5ml) were removed at selected time-intervals, acidified to pH2 and freeze-dried. The residue was analysed with a Technicon autoanalyser.

Leucine aminopeptidase digestion. Inhibitor (16mg) was dissolved in 8ml of 0.01M-Tris/5mM-MgSO₄ buffer, pH8.5, containing 1.25 µmol of norleucine.

Leucine aminopeptidase (from pig kidney; type III, Sigma) was added (900 µg in 4ml of the same buffer; E/S ratio of 1:18), after activation at 37°C for 30min. The digestion was performed at 37°C. Portions (2ml) were removed, acidified, freeze-dried, and analysed with the autoanalyser.

Control experiments were performed by incubating the enzymes under the same conditions in the absence of substrate.

Amino acid analysis

Analyses were performed as previously reported (Carrea *et al.*, 1973).

Carbohydrate analysis

Total hexoses, methylpentoses, total hexosamines and sialic acid were determined as previously described (Carrea *et al.*, 1973). Glucosamine and galactosamine were analysed by ion-exchange chromatography on the autoanalyser after sample hydrolysis with 3M-HCl in sealed tubes under N₂ at 105°C for 8h.

Individual neutral sugars were determined as trimethylsilyl derivatives by g.l.c. (Sweeley *et al.*, 1963), after sample hydrolysis with 1M-HCl at 105°C for 90min in sealed tubes under N₂, and chromatog-

raphy on a mixed-bed column consisting of equal amounts of Dowex 50X8 and Dowex 1X2. Mannitol was added as the internal standard. Operating conditions were as follows: liquid phase 3.8% SE30; 140°–190°C; ΔT , 1.25°C/min; nitrogen flow, 25ml/min.

Alkali treatment

Procedure I. Inhibitor (5mg) was incubated in 2.5ml of 0.2M-NaOH/0.5M-NaBH₄ at 37°C for 60h in the dark (Nichols & Bezkorovainy, 1973). Excess of borohydride was destroyed by acidification with acetic acid, and boric acid was removed by repeated evaporation under vacuum with a HCl/methanol mixture (1:1000, v/v). Glucosamine and galactosamine were determined, after acid hydrolysis, with the autoanalyser.

Procedure II. This procedure was essentially as described by Bhargava & Gottschalk (1967). Inhibitor (15mg) was treated with 4ml of 0.2M-NaOH at 100°C. Portions (0.4ml) were collected at various times, cooled at 20°C and treated with 1.5ml of 0.1M-borate/0.65M-NaBH₄ buffer, pH9, for 6–7h to reduce the released hexosamine.

Excess of borohydride and boric acid was eliminated as described above. Bound hexosamine was determined after acid hydrolysis by the method of Elson & Morgan (1933).

Gastric antisecretory activity in pylorus-ligated rats

This assay was performed as previously described (Lugaro *et al.*, 1965).

Results and Discussion

Subunit structure of the glycoprotein inhibitor

Native inhibitor is characterized by a high value of the sedimentation coefficient ($s_{20,w}^0 = 8S$) and is not retained by Sephadex G-200. Also, electrophoretic migration in highly porous polyacrylamide gels is very slow (Carrea *et al.*, 1973). A quantitative estimate of the molecular weight of native material has not been made, but the above results clearly show the high-molecular-weight nature of the molecule.

Carrea *et al.* (1973) showed that this urinary glycoprotein splits down to smaller fragments under mild alkaline conditions. This suggested that the inhibitor was composed of several subunits. This hypothesis is now confirmed by the present investigation of the behaviour of the glycoprotein in the presence of dissociating agents.

Fig. 1 shows the elution profile for the glycoprotein when subjected to gel filtration through Sephadex G-200 equilibrated with 0.5% sodium dodecyl sulphate. The three peaks obtained (1, 2 and 3) contain material with identical amino acid and carbohydrate compositions corresponding (within the

Fig. 1. Gel-filtration profile of human urinary gastric inhibitor on Sephadex G-200 in the presence of sodium dodecyl sulphate

Inhibitor (10mg) was subjected to gel filtration on a column (1.8 cm $\times$ 100 cm) of Sephadex G-200, equilibrated with 0.5% sodium dodecyl sulphate in 0.05 M-Tris/acetic acid buffer, pH 8. The flow rate was 8 ml/h. Before being applied to the column, the inhibitor was incubated in 3 ml of 2% (w/v) sodium dodecyl sulphate, pH 8, at 37°C for 24 h.

Table 1. Amino acid and carbohydrate compositions of human urinary gastric inhibitor peaks 1, 2 and 3

Before analysis peaks 1, 2 and 3 were freed of sodium dodecyl sulphate by means of exhaustive dialysis and gel filtration on Sephadex G-25 (equilibrated with 0.01 M-phosphate, pH 7). For details of the analyses, see the Materials and Methods section.

Component	Composition (μ mol/mg)			
	Human urinary gastric inhibitor	Peak 1	Peak 2	Peak 3
Carbohydrate				
Glucose	0.31	0.30	0.30	0.29
Galactose	0.44	0.46	0.44	0.45
Mannose	0.30	0.31	0.31	0.30
Fucose	0.21	0.21	0.20	0.20
Sialic acid	0.28	0.27	0.29	0.29
Glucosamine	0.27	0.26	0.26	0.28
Galactosamine	0.07	0.07	0.07	0.06
Amino acid				
Aspartic acid	0.56	0.57	0.57	0.55
Threonine	0.63	0.64	0.65	0.62
Serine	0.43	0.45	0.44	0.42
Glutamic acid	0.61	0.62	0.64	0.62
Proline	0.35	0.37	0.34	0.36
Glycine	0.48	0.48	0.49	0.47
Alanine	0.36	0.36	0.38	0.37
Valine	0.16	0.17	0.15	0.17
Cysteine*	0.45	0.42	0.44	0.41
Methionine	0.03	0.05	0.03	0.05
Isoleucine	0.16	0.16	0.17	0.15
Leucine	0.49	0.48	0.49	0.47
Tyrosine	0.14	0.13	0.13	0.15
Phenylalanine	0.24	0.23	0.22	0.22
Lysine	0.24	0.24	0.23	0.25
Hystidine	0.17	0.17	0.18	0.19
Arginine	0.22	0.22	0.20	0.21

* Values represent the sum of cysteine and cysteic acid.

experimental error) to that of native inhibitor, as shown in Table 1.

After removal of sodium dodecyl sulphate by dialysis, and freeze-drying, peaks 1 and 2 were separately re-incubated in sodium dodecyl sulphate and subjected to gel-filtration through Sephadex G-200 as described in the legend to Fig. 1. The material of peak 1 gave an elution profile similar to that reported in Fig. 1 (gel filtration of inhibitor). The material of peak 2 gave a pattern of two peaks. The elution volumes in this case coincided with those of peaks 2 and 3 in Fig. 1. Thus peak 1, which is eluted in the void volume (see Fig. 1), is composed of undissociated (or slightly dissociated) inhibitor. Peak 2 is a product of partial dissociation of the starting material and peak 3 represents the fully dissociated form.

When gel filtration was performed in the presence of 0.25% β -mercaptoethanol according to Kobayashi *et al.* (1972) after reduction of the glycoprotein [2% (v/v) β -mercaptoethanol, 2% (w/v) sodium dodecyl sulphate, pH 8.2, 24 h at 40°C in the dark] no component of lower molecular weight was observed.

The relative amounts of eluted glycoprotein corresponding to the three peaks depended on the incubation conditions. 8 M-urea and 6 M-guanidinium chloride were less effective dissociating agents of the glycoprotein than was sodium dodecyl sulphate.

Removal of sodium dodecyl sulphate from the material eluted in peaks 2 and 3 by means of exhaustive dialysis, and gel filtration through Sephadex G-25 (equilibrated with 0.01 M-phosphate, pH 7) caused re-association of the glycoprotein. In fact the material was eluted in the void volume of a column of Sephadex G-200 similarly equilibrated. This shows that the subunits of the inhibitor, formed in the presence of sodium dodecyl sulphate, can re-associate to higher-molecular-weight species. Whether or not they revert to the same aggregated form of native inhibitor is not known. The product of re-association retained about 30% of the biological activity of the native glycoprotein when assayed for antisecretory activity in the rat.

The evidence reported in the present paper strongly indicates that the native glycoprotein is composed of a single species of subunit whose molecular weight equals that of the material eluted as peak 3 in Fig. 1.

Sodium dodecyl sulphate/polyacrylamide-disc-gel electrophoresis showed two components, in agreement with results obtained from sodium dodecyl sulphate-gel filtration. The mobility of the components was not changed by the addition of β -mercaptoethanol. Therefore S-S bonds, if present, are intra- and not inter-chain.

Molecular weight determination for the subunits

The subunit molecular weight was measured by sodium dodecyl sulphate-gel filtration and sodium dodecyl sulphate/polyacrylamide-disc-gel electro-

Fig. 2. Plot of V_e/V_0 against molecular weight of standard proteins subjected to gel filtration on a column of Sephadex G-200 in the presence of sodium dodecyl sulphate

For further details, see the Materials and Methods section.

Fig. 3. Plot of electrophoretic mobility against molecular weight of standard proteins electrophoresed in polyacrylamide gels in the presence of sodium dodecyl sulphate

The concentration of acrylamide was 15% (w/v), and that of sodium dodecyl sulphate 0.1%. The electrophoretic mobilities were relative to Bromophenol Blue. For details, see the Materials and Methods section.

phoresis. As shown in Fig. 2, the former method gave a subunit mol.wt. of 16000 ± 1500 . The product of partial disaggregation, eluted with peak 2 of Fig. 1, had a mol.wt. of about 49000, corresponding to three times the subunit molecular weight. It should be stressed that these results represent only very approximate values, since it is known that several glycoproteins exhibit anomalous behaviour on gel filtration (Whitaker, 1963). The subunit mol.wt. calculated from the mobility of the faster component in sodium dodecyl sulphate/polyacrylamide-disc-gel electrophoresis was 13000 ± 1000 (Fig. 3).

C- and N-Terminal analyses

Carboxypeptidase digestion of the inhibitor, previously incubated in the presence of sodium dodecyl sulphate, showed valine to be the C-terminal amino acid (Fig. 4). The kinetics of the enzymic hydrolysis is consistent with the partial sequence Phe-Tyr-Leu-Val-OH. Approximately 1 mol of valine/mol of subunit was released on the basis of a subunit mol.wt. of 14500. This result confirms the molecular-weight determinations reported above. It also provides evidence of the homogeneity of the subunits, at least regarding their polypeptide backbone.

The results of the leucine aminopeptidase digestion

Fig. 4. Carboxypeptidase A digestion of human urinary gastric inhibitor

Amino acids released (mol/mol of inhibitor subunit) are plotted against digestion time. The subunit mol.wt. is assumed to be 14500 (average of the values obtained by disc-gel electrophoresis and gel filtration in sodium dodecyl sulphate). For further details, see the Materials and Methods section. $\square$, Valine; $\blacksquare$, leucine; $\circ$, tyrosine; $\bullet$, phenylalanine.

Table 2. Leucine aminopeptidase digestion of human urinary gastric inhibitor

For further details, see the Materials and Methods section. Results are expressed as mol of amino acid released/mol of inhibitor subunit (assuming a mol.wt. for the subunit of 14500). tr, Trace.

Digestion time (min)...	Amino acid released (mol/mol of subunit)			
	20	90	180	420
Amino acid				
Glycine	tr	0.10	0.22	0.49
Serine	tr	0.10	0.18	0.42
Threonine	tr	0.11	0.20	0.46
Valine	tr	0.12	0.21	0.48

of the inhibitor are summarized in Table 2. It is noteworthy that glycine is kinetically released in the same proportion as serine, threonine and valine during the enzymic digestion, despite the fact that the enzyme is markedly less specific for glycine than for all the other amino acids (Smith & Hill, 1960). Therefore glycine might be the *N*-terminal amino acid. The stability of carboxypeptidase in sodium dodecyl sulphate media made it possible to carry out the enzymic digestion on the sodium dodecyl sulphate-denatured glycoprotein. Under these conditions the substrate should be more easily degraded. Leucine aminopeptidase digestion of the native undissociated glycoprotein was mandatory because the presence of sodium dodecyl sulphate interferes with the enzymic activity. Thus the result showing glycine as the *N*-terminal residue should be accepted with caution. Control experiments have shown that spurious amino acids are not formed in significant amounts in either of the enzymic digestions.

Amino acid and carbohydrate composition of the glycoprotein inhibitor

The acid character of the inhibitor is due to the significant amount of bound sialic acid (9%) and also to the prevalence of acidic amino acids as shown in Table 1. The glucosamine/galactosamine ratio is 4:1. Glucose, mannose and galactose are present in the ratio 2:2:3. Good agreement has been found between the quantitative data on hexosamines and neutral sugars obtained by the autoanalyser and g.l.c. respectively and the data obtained by colorimetric analyses. Although glucose is not frequently found in glycoproteins, it can be excluded that its presence in the inhibitor results from contamination of products of degradation of the Sephadex G-200 used for gel filtration. In fact, the glucose content of the inhibitor was unaffected by the use of a different molecular sieve such as Bio-Gel A-1.5m. Further, collagen-like contamination can be excluded because no hydroxylysine was found in the hydrolysates.

Effect of alkali treatment on the inhibitor

All galactosamine appears to be involved in carbohydrate-protein linkages. This conclusion is based on two different lines of evidence.

Analyses of galactosamine after treatment of inhibitor with NaOH and NaBH₄ (procedure I) have shown a 60% fall in content, and the formation of galactosaminitol; glucosamine was unchanged. The threonine, and to a less extent the serine content, suffers a decrease on alkali treatment. This decrease is greater than that observed for galactosamine on a molar basis. Thus it is likely that the alkali treatment also destroys some hydroxy amino acids (McGuire & Roseman, 1967) which are not involved in the *O*-glycosidic linkage.

On the other hand the analyses of the total hexos-

Fig. 5. Decrease in content of bound hexosamines in human urinary gastric inhibitor on treatment with 0.2M-NaOH at 100°C

For further details, see the Materials and Methods section.

amine content after the alkali treatment (procedure II) have shown an approx. 20% decrease (Fig. 5). This value coincides with the percentage of galactosamine relative to the total hexosamine in the inhibitor. It is therefore likely that all galactosamine present in the inhibitor is involved in *O*-glycosidic linkages.

As shown in Fig. 5 extended alkali treatment causes further and slower decrease in hexosamine content. This may be due to the hydrolysis of more stable bonds such as those involving glucosamine (Lee & Scocca, 1972).

Concluding remarks

The effect of sodium dodecyl sulphate, as observed by gel filtration and electrophoresis, proves that the inhibitor undergoes reversible dissociation phenomena in solution. The mol.wt. of the subunits is estimated as 13000 by electrophoresis and 16000 by gel filtration. These values are within the range of uncertainty in molecular weight estimation of glycoproteins provided by the two techniques.

The measured molecular weight is confirmed by the experiments of carboxypeptidase A digestion showing that 1 mol of valine is released per 14000 g of glycoprotein. Removal of the detergent from the dissociated glycoprotein resulted in re-aggregation of the subunits. This observation suggests that in aqueous solution the subunits are kept in some aggregated form by hydrophobic bonds. The strong tendency toward aggregation is evidenced by the observation that the incubation in 2% (w/v) sodium dodecyl sulphate does not promote complete dissociation of the inhibitor. Similar aggregation phenomena, in solutions containing detergents, also have been reported for other glycoproteins (Fletcher *et al.*, 1970; Morawiecki, 1964; Janado *et al.*, 1973).

Several hypotheses can be made as to the site of synthesis of the inhibitor. Its dissociable nature and

the size of the subunits are compatible with a possible origin above the renal glomerule. A duodenal origin of the inhibitor is suggested by studies performed on similar glycoproteins extracted from pig duodenum and urine (G. Carrea & G. Lugaro, unpublished work).

On the other hand the great amount of the inhibitor present in the urine of pregnant women might suggest that the placenta is involved in the synthesis of this glycoprotein, at least during part of pregnancy.

Note added in Proof (Received 15 January 1976)

Since this work was submitted, a paper has been published that describes the isolation from human urine of an inhibitor of the gastric secretion (Gregory, 1975). This inhibitor is not a high-molecular-weight glycoprotein like the inhibitor isolated by us, but a 53-residue polypeptide, and might be identified as the epidermal growth factor. Also, this peptide, though more active than the glycoprotein, is extractable in far smaller amount from urine. Thus at least two different inhibitors of gastric secretion are present in human urine.

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