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MULTIPLE SMITH-DEGRADATIONS OF CARCINOEMBRYONIC ANTIGEN (CEA) AND OF ASIALO CEA*

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ABSTRACT

Carcinoembryonic antigen (CEA) and asialo CEA were subjected to multiple Smith-degradation (*i.e.*, for each degradation, application in sequence of periodate oxidation, borohydride reduction, and mild hydrolysis with acid; borohydride-*t* was substituted for unlabelled borohydride). High yields of modified glycoproteins were obtained at each stage. After three complete degradations and a further periodate–borohydride-*t* treatment, the carbohydrate content of CEA and of asialo CEA had decreased from 45–50% to 11–12% (*i.e.*, 90% removal of carbohydrate). Glycerol was always one of the products obtained after each degradation, but threitol and erythritol were not detected. The second degradation caused a substantial loss of 2-acetamido-2-deoxyglucose, which is consistent with the location of some of this monosaccharide towards the terminal (non-reducing) end of the oligosaccharides. The "core" region of the oligosaccharides is composed of galactose, mannose, and 2-acetamido-2-deoxyglucose. After the fourth oxidation, 2-acetamido-2-deoxyglucose was 50–60% of the total content of residual carbohydrate. After the first degradation, there was a progressive loss in antigenic activity, but this was associated with a small amount of hydrolysis of the protein moiety of CEA.

INTRODUCTION

In a previous paper[1], it was shown that periodate oxidation followed by reduction with sodium borohydride and mild hydrolysis with acid (Smith degradation) removed about half of the carbohydrate content of CEA without significantly changing its antigenic activity. The multiple Smith-degradations now reported were carried out to ascertain whether more monosaccharides could be removed without loss of antigenic activity, and also to obtain information about the sequence of monosaccharides in the oligosaccharides attached to the polypeptide chain. Multiple Smith-degradations have been applied successfully to several glycoproteins [*e.g.*, α_1-acid glycoprotein[2], fetuin[3], thyroglobulin[4], ovalbumin (chicken-egg)[5], and

*Dedicated to the memory of Dr. Hewitt G. Fletcher, Jr.

transferrin[6]]. In most of these investigations, the oxidations were performed on glycopeptides but, for fetuin and α_1-acid glycoprotein, the native and asialo glycoproteins were used. Four Smith-degradations did not completely remove the carbohydrate from fetuin or α_1-acid glycoprotein, although very little remained in asialo α_1-acid glycoprotein. We have found that only 90% of the carbohydrate was removed after subjection of CEA to three Smith-degradations and an additional periodate–borohydride-*t* treatment, and there was also some proteolysis.

EXPERIMENTAL AND RESULTS

CEA. — CEA was purified, as described previously[7], from large, liver metastases of carcinoma of the colon. Two preparations were used in this work, and these are designated 3/7E and 3/9F. Each was from one tumour.

Asialo CEA. — The sialic acid was removed completely from CEA by using neuraminidase from *Vibrio cholerae.* The neuraminidase was purchased, as a solution in 50mM sodium acetate buffer (pH 5.5), containing sodium chloride (0.16M) and calcium chloride (9mM), from Behringwerke A.G., Marburg-Lahn, W. Germany. The concentration of the enzyme was 500 units per ml. CEA (3/9F) (23 mg) was dissolved in the neuraminidase solution (2 ml, 1000 units) and was kept at 37° for 5 h. The solution was then applied to a column of Biogel P-10, and asialo CEA was eluted with water in the void volume. The fractions containing asialo CEA were combined and the solution was freeze-dried. Asialo CEA had the same antigenic activity as native CEA (see Table I).

Carbamoylated CEA (3/7E). — To a solution of CEA (10 mg) in 8M urea (1.0 ml) which contained *N*-ethylmorpholine acetate buffer (pH 8.0), potassium cyanate (50 mg) was added and the mixture was kept at 37° for 16 h. Glacial acetic acid (1 ml) was then added, and the solution was dialysed with water for 24 h and freeze-dried. Amino-acid analysis of the product showed that only lysine residues had been

TABLE I

COMPARISON OF THE AFFINITY OF THE MODIFIED CEA SAMPLES FOR ANTI-CEA, MEASURED USING THE RADIOIMMUNOASSAY

	Asialo CEA (3/9F) I_{50} (*ng/ml*)[a]	*CEA (3/7E)* I_{50} (*ng/ml*)[a]
Before oxidation	55	55
1st periodate–borohydride-*t*	49	45
1st Smith-degradation	88	225
2nd Smith-degradation	210	415
3rd Smith-degradation	315	1000
4th Smith-degradation	1000	—
Carbamoylated CEA (3/7E)		97

[a] I_{50} is the concentration required to produce 50% inhibition of binding between CEA-^{125}I and anti-CEA.

modified. The lysine content of the native CEA (3/7E) was 2.7% of the total amino acids (mole %), whereas the lysine content of the carbamoylated CEA was 0.7%, At least 80% of the *N*-terminal lysine was carbamoylated[8], and carbamoylated CEA had half of the antigenic activity of native CEA (Table I).

Multiple Smith-degradations. — The procedure was the same for both CEA and asialo CEA. The procedure for the multiple Smith-degradation of CEA (3/7E) was as follows:

(*a*) *Periodate oxidation.* A solution of CEA (23 mg) in 0.2M sodium acetate buffer (pH 3.8, 20 ml) containing sodium periodate (5mM) was kept at room temperature (20°) in the dark for 42 h. Ethylene glycol (5 μl) was then added to reduce the remaining periodate.

(*b*) *Dialysis.* Visking tubing (Scientific Instrument Centre Ltd., London) was used for dialysis after immersing it in boiling water for 15 min and then washing it extensively with water. The CEA solutions were dialysed with water (10 litres) for not more than 7 h at room temperature. Lower yields were obtained when the solutions were dialysed for longer periods of time.

(*c*) *Reductions*[1,8] *with sodium borohydride*-t. Sodium carbonate–sodium hydrogen carbonate buffer (pH 8.9, 20 ml) was added to the dialysed solution of oxidised CEA. Sodium borohydride-*t* (40 mCi/mmole, 40 mg) was added and the mixture was kept at room temperature for 16 h. The solution was then dialysed and freeze-dried. In order to remove any non-covalently bound tritium label and also to remove any glycerol arising from the Visking tubing[2], a solution of the freeze-dried material in water (1 ml) was applied to a column (1.6 × 35 cm; volume, 70.5 ml) of Biogel P-10. The column was eluted with water, and 2.3-ml fractions were collected. The modified CEA (17 mg) was obtained from the void fractions, which were combined and freeze-dried; the residue was analysed for monosaccharides (Table II).

Measurement of radioactivity. — The amount of radioactivity (tritium) was measured by using a Packard Tri-Carb liquid scintillation counter, and calculated as disintegrations per min (d.p.m.). Aqueous samples (0.02 ml) were added to the scintillation fluid (10 ml) which was a mixture of toluene (3.8 ml), *p*-dioxane (3.8 ml), methanol (2.3 ml), butyl PBD (0.07 g), and naphthalene (0.8 g). In order to determine the number of aldehyde groups which were reduced by the sodium borohydride-*t*, it was necessary to know whether or not any isotope-effect was associated with the reduction of an aldehyde group by sodium borohydride-*t*. The specific activity of sodium borohydride-*t* was determined by The Radiochemical Centre, Amersham, U.K., using an excess of a ketone when an isotope effect does not affect the determination. In our experiments, sodium borohydride-*t* was used in excess and an isotope effect could give a product with a lower specific activity than that expected. D-Galactose was reduced with an excess of sodium borohydride-*t* (40 mCi/mmole) to galactitol-*t*. A known excess of unlabelled galactitol was added and the galactitol was recrystallized to constant activity. The specific activity of the undiluted galactitol was calculated to be 10 mCi/mmole, proving that there was no significant isotope-effect associated with the reduction of the aldehyde group.

TABLE II

CARBOHYDRATE ANALYSIS OF MODIFIED CEA MATERIAL

Stage of degradation of CEA	*Carbohydrate (mole %)*	*Fucose*[a]	*Galactose*[a]	*GlcNAc*[a]	*Mannose*[a]	*Sialic acid*[a]
CEA (3/7E)						
Before oxidation	45.4	39	58	104	21	5
1st IO_4^-/B^3H_4	30.6	—	30	102	21	—
1st acidic hydrolysis	34.2	—	35	112	24	—
2nd IO_4^-/B^3H_4	26	—	27	83	20	—
2nd acidic hydrolysis	27.2	—	28	85	23	—
3rd IO_4^-/B^3H_4	21	—	23	65	17	—
3rd acidic hydrolysis	18.4	—	21	56	15	—
4th IO_4^-/B^3H_4	12.2	—	15	37	9	—
Asialo CEA (3/9F)						
Before oxidation	51	42	65	110	38	—
1st IO_4^-/B^3H_4	40.4	—	28	147	27	—
1st acidic hydrolysis	44.4	—	47	154	40	—
2nd IO_4^-/B^3H_4	22.2	—	32	54	25	—
2nd acidic hydrolysis	19.4	—	31	43	15	—
3rd IO_4^-/B^3H_4	14	21	21	34	15	—
3rd acidic hydrolysis	15.8	—	21	41	17	—
4th IO_4^-/B^3H_4	10.8	—	17	25	12	—
Asialo CEA (3/7E)						
Before oxidation	44.4	39	58	104	21	—
1st IO_4^-/B^3H_4	26.6	—	24	88	21	—
1st acidic hydrolysis	25.6	—	24	83	21	—

[a]The carbohydrate analyses were performed as described previously[7]; expressed in moles/10^5 g. Glycerol was detected in each of the oxidized–reduced samples before acidic hydrolysis, but neither threitol nor erythritol was detected (g.l.c. of the trimethylsilyl ethers).

Acidic hydrolysis. — A solution of the freeze-dried material (16.5 mg), obtained from the sodium borohydride-*t* reduction, in 0.05M H_2SO_4 (1 ml) was kept at 37° for 22 h. The solution was neutralized with M NaOH (0.2 ml) and then applied to a column (1.6 × 35 cm) of Biogel P-10. The column was eluted with water, and 2.3-ml fractions were collected. The resulting elution profile showed two peaks of radioactivity. The first peak (fractions 8–16) was due to the modified CEA, and the second peak (fractions 22–40) was due to the products released by acidic hydrolysis. The fractions containing the modified CEA were combined and freeze-dried, and the residue was analysed for monosaccharides (Table II).

In another experiment, the neutralized solution was applied to a column (2.5 × 47 cm) of Biogel P-2 which had been calibrated with 2-acetamido-^{14}C-2-deoxy-D-glucose and di-*N*-acetyl-^{14}C-chitobiose (Fig. 1). All of the radioactively labelled products released were eluted after di-*N*-acetyl-^{14}C-chitobiose. Some carbohydrate-containing material was contained in the fractions 40–52 (see Fig. 1) which contain only a small amount of radioactivity. The similarity of the ratio of the monosac-

charides to those in the modified CEA is consistent with the possibility that trailing of the modified CEA occurred on the column and that these monosaccharides were not released. The amount of intact monosaccharide released (fractions 53–71) was negligibly small in each of the acidic hydrolyses. Most of the radioactivity in these fractions was due to glycerol-*t* which was detected by g.l.c. as its trimethylsilyl ether.

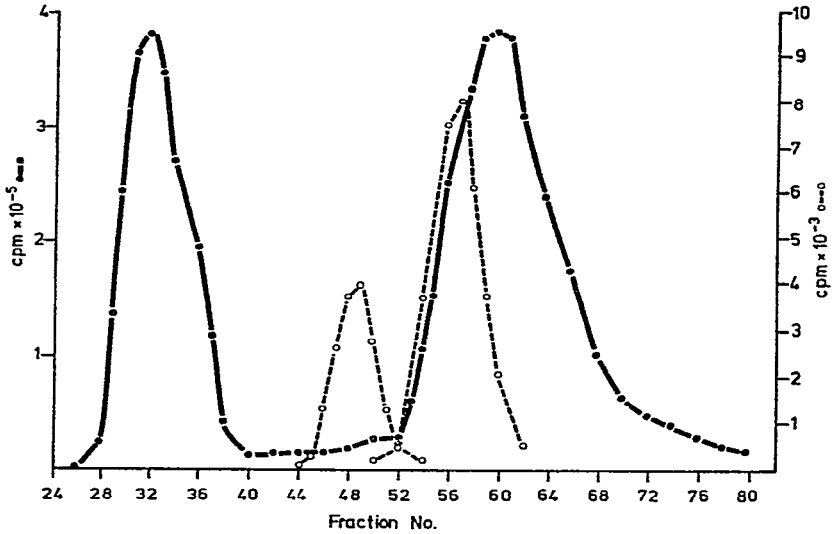

Fig. 1. The elution profiles obtained from a column (2.5 × 47 cm) of Biogel P-2; ●——●, products from the first Smith-degradation of asialo CEA; ○----○, di-*N*-acetyl-^{14}*C*-chitobiose (fractions 44–54) and 2-acetamido-^{14}*C*-2-deoxy-D-glucose (fractions 50–62).

G.l.c. analysis of the products from the first Smith-degradation of asialo CEA (3/9F):

Fractions (2.2 ml)	*Product (mg)*	D-*Galactose*[a]	*GlcNAc*[a]	D-*Mannose*[a]
28–39	12.5	47	154	40
40–52	1.7	13	34	8
53–71	13.2[b]	1	1.5	0.5

[a]Expressed in moles/10^5 g. [b]Most of this weight is due to sodium sulphate which is obtained from the neutralization of the 0.05M H_2SO_4. Glycerol (determined as its trimethylsilyl ether) was only detected in the material from fractions 53–71.

A constant amount of radioactivity per mg was associated with the modified CEA after the acidic hydrolysis (Table III), and was probably associated with the protein moiety. Oxidation of some amino acids can occur when they are linked in peptides, although more oxidation occurs with free amino acids[9]. The amino acids occurring in CEA which might be oxidized are cystine, tryptophan, tyrosine, and histidine. Westwood *et al.*[1] have shown that cystine is only oxidised at higher concentrations of periodate (0.5M), although 61% of the tryptophan and 57% of the tyrosine are destroyed at the periodate concentration used in these experiments[8].

Small amounts of histidine and arginine are also destroyed. The radioactive labelling of the protein could be caused by reduction by sodium borohydride-*t* of the oxidised products of these amino acids. However, it has been shown[8] that CEA is still labelled with tritium, albeit to a lesser extent, after sequential treatment with sodium borohydride-*t* and 0.05M H_2SO_4.

TABLE III

MULTIPLE SMITH-DEGRADATION OF CEA (3/7E)

Stage	*Yield (mg)*[a]	*D.p.m.* ($\times 10^{-6}$)	*D.p.m./mg* ($\times 10^{-6}$)
Before oxidation	23.1	—	—
1st IO_4^-/B^3H_4	17.1	890	52.0
1st acidic hydrolysis	14.7	314	21.4
Products released	—	688	—
2nd IO_4^-/B^3H_4	9.2	356	38.7
2nd acid hydrolysis	7.5	166	22.1
Products released	—	220	—
3rd IO_4^-/B^3H_4	6.0	265	44.1
3rd acidic hydrolysis	5.0	106	20.1
Products released	—	157	—
4th IO_4^-/B^3H_4	2.6	398	15.3

MULTIPLE SMITH-DEGRADATION OF ASIALO CEA (3/9F)

Stage	*Yield (mg)*[a]	*D.p.m.* ($\times 10^{-6}$)	*D.p.m./mg* ($\times 10^{-6}$)
Before oxidation	21	—	—
1st IO_4^-/B^3H_4	15.9	1150	72.3
1st acidic hydrolysis	12.5	268	21.4
Products released	—	457	—
2nd IO_4^-/B^3H_4	8.4	472	56.2
2nd acidic hydrolysis	6.5	146	23.4
Products released	—	284	—
3rd IO_4^-/B^3H_4	3.5	342	97.7
3rd acidic hydrolysis	2.3	80	35
Products released	—	224	—
4th IO_4^-/B^3H_4	0.4	148	370

[a]The yields of material were higher than shown, because 0.5–1.0 mg was used at each stage for carbohydrate analysis and for inhibition studies using the radioimmunoassay for CEA.

From the amount of radioactivity released during the acidic hydrolysis, an estimate was obtained of the extent of periodate oxidation and of the number of aldehyde groups produced. This value was similar to the one obtained using the results from monosaccharide analysis of the oxidised–reduced products in the first Smith-degradation, when larger amounts of CEA were used. The values calculated from the measurements of radioactivity were much less accurate in the second and third Smith-degradations.

Determination of the antigenic activity of the modified CEA molecules. — These measurements were performed by using the radioimmunoassay for CEA described by Laurence *et al.*[10]. The results are given in Table I. I_{50} is the concentration of CEA or modified CEA required to produce 50% inhibition of the binding between CEA-^{125}I and goat anti-CEA.

Estimation of the mol. wt. of the modified CEA molecules by using gel-filtration. — A column (1.6 × 100 cm) of Biogel A-1.5 m was prepared and calibrated by using a range of standards (see Fig. 2). The standard proteins and modified CEA samples

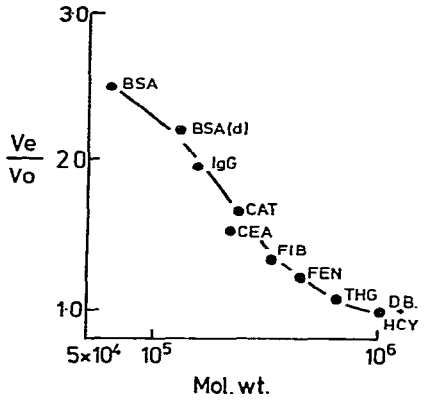

Fig. 2. Calibration of the column of Biogel A-1.5 m. Ve = Volume at which standard was eluted. Vo = Void volume of column (determined using blue dextran 2000). BSA, bovine serum albumin; BSA(d), bovine serum albumin dimer; IgG, γ-globulin G; CAT, catalase; CEA, carcinoembryonic antigen; FIB, human fibrinogen; FEN, ferritin; THG, thyroglobulin; DB, blue dextran 2000; HCY, haemocyanin. The inclusion volume of the column was determined by using pyridoxal phosphate.

TABLE IV

GEL-FILTRATION VALUES USING A COLUMN OF BIOGEL A-1.5 m FOR ASIALO CEA (3/9F) AND THE PRODUCTS OF SMITH-DEGRADATION

	Ve/Vo ±*s.d.*[a]	*Mol. wt., whole molecule*	*Polypeptide*[b] *chain*
Asialo CEA	1.64 ±0.17	225,000	110,000
1st IO_4^-/B^3H_4	1.56 ±0.19	260,000	
1st acidic hydrolysis	2.06 ±0.26	145,000	81,000
2nd IO_4^-/B^3H_4	1.83 ±0.29	190,000	
2nd acidic hydrolysis	2.29 ±0.31	109,000	87,000
3rd IO_4^-/B^3H_4	2.14 ±0.27	130,000	
3rd acidic hydrolysis	2.56 ±0.31	58,000	49,000
Carbamoylated asialo CEA (3/9F)		225,000	

[a]The gel-filtration elution profiles were not normal but skew curves. The mean elution volume (Ve) was not therefore the peak height. The standard deviations for these skew curves were calculated using the method described by Udny Yule and Kendall[15] [b]Calculated using the values for the percentage of carbohydrate obtained by monosaccharide analysis (see Table II).

were eluted from the column using 0.1M Tris-HCl (pH 7.5) containing 0.2% w/v of sodium azide. The results are shown in Table IV.

Qualitative, N-terminal, amino-acid analysis of the modified CEA samples. — The modified CEA molecules were subjected to *N*-terminal, amino-acid analysis by using the dansyl chloride method of Hartley[11]. The product obtained from the first oxidation–reduction of asialo CEA (3/9F), and the product from the first Smith-degradation of asialo CEA (3/9F) had only lysine as the *N*-terminal amino acid. No evidence of proteolysis has been observed in any product from the first oxidation–reduction, but after the first Smith-degradation, native CEA (3/7E) had only about 20% of its *N*-terminal amino acid as lysine. Since proteolytically degraded CEA is almost inactive in the radioimmunoassay[7], this 20% of material, presumably with an intact polypeptide chain and full activity, is probably responsible for virtually all of the activity obtained (20% of 225 ng/ml, see Table I). The product obtained after the second Smith-degradation of asialo CEA (3/9F), however, had only ~25% of its *N*-terminal amino acid as lysine, and after the third degradation, there was very little *N*-terminal lysine. The amino acids then detected as *N*-terminal, in addition to lysine, were serine, threonine, glycine, valine, proline, leucine, isoleucine, and phenylalanine.

DISCUSSION

When conclusions are drawn from experiments involving modification of CEA, consideration must be given to the microheterogeneity of CEA, the extent of which is as yet unknown. There is good evidence that the protein moiety of CEA does not vary from preparation to preparation. The amino-acid analyses of various CEA preparations are similar; also, the first 24 amino acids from the *N*-terminal end have been sequenced in five different preparations of CEA from liver metastases of carcinoma of the colon and the same sequence was found in each case[12]. The same five monosaccharides have been found in every preparation of CEA studied, *viz.*, 2-acetamido-2-deoxyglucose, galactose, mannose, fucose, and sialic acid; 2-acetamido-2-deoxygalactose has been found in some preparations[7]. Although the amounts of 2-acetamido-2-deoxyglucose and galactose are reasonably constant in the preparations of CEA studied, the amounts of mannose, fucose, and sialic acid do vary[7]. The comparison between our analytical results and those of other groups has been reported and discussed in a previous paper[7]. Two preparations of CEA (3/7E and 3/9F) were used in this study. The amounts of the monosaccharides present were similar in the two preparations, with the exception of the amount of mannose.

Multiple Smith-degradations of CEA and asialo CEA, using oxidizing conditions (pH 3.8, 20°) chosen to give a reasonably rapid oxidation without overoxidation[13], proceeded in a similar manner; after three degradations and a further periodate–borohydride-*t* treatment, the product from CEA and that from asialo CEA had similar carbohydrate contents. Sialic acid therefore does not have the same effect on the sequential oxidation of CEA that it has on the sequential oxidation of α_1-acid glycoprotein[2]. Several pieces of structural evidence emerge from the results. The first

Smith-degradation of CEA caused destruction of all of the fucose and sialic acid, and about half of the galactose. Mannose and 2-acetamido-2-deoxyglucose were not degraded to any significant extent. Since the amount of intact monosaccharide released on acidic hydrolysis was negligibly small, these oxidised monosaccharides must be either terminal or preceding oxidisable monosaccharides. Glycerol, but not threitol or erythritol, was detected by g.l.c. analysis of the products obtained after each degradation. There cannot therefore be any 4-substituted galactose or mannose residues in CEA. It is likely, therefore, as fucose and sialic acid have only been found in terminal positions in glycoproteins, that CEA oligosaccharides terminate in sialic acid-(2→6 or 2)-galactose or fucose-(1→6 or 2)-galactose. Evidence for the terminating sequence sialic acid–galactose has previously been obtained from experiments using D-galactose oxidase and sodium borohydride-*t* on asialo CEA[7]. Since the sum of the fucose and sialic acid content of CEA exceeds the amounts of oxidised galactose, these terminating sequences cannot be the only ones. There must be others where the penultimate monosaccharide is resistant to oxidation.

The second Smith-degradation caused destruction of 2-acetamido-2-deoxyglucose; this was more extensive in the case of asialo CEA (3/9F). There was also some oxidation of galactose, but mannose was only oxidised in the case of asialo CEA (3/9F). The considerable loss of 2-acetamido-2-deoxyglucose (as compared with the loss of the other monosaccharides) is consistent with a terminating sequence of fucose (or sialic acid)–galactose–2-acetamido-2-deoxyglucose.

The third and fourth oxidations decreased the content of all three remaining monosaccharides (galactose, mannose, and 2-acetamido-2-deoxyglucose). After four oxidations, the total carbohydrate remaining was only 11–12% (*i.e.*, 90% removal of carbohydrate). This amount could presumably be decreased by a further Smith-degradation. After four oxidations, the content of 2-acetamido-2-deoxyglucose (expressed as a percentage of the total carbohydrate) was 60% for degraded CEA (3/7E) and 46% for degraded asialo-CEA (3/9F). The fact that carbohydrate was still left after the four degradations is consistent with the view that there is either a large number of (1→3) linkages in CEA or extensive branching, or both. The "core" region of the oligosaccharide chains, *i.e.*, the region near the polypeptide chain, contains galactose, mannose, and 2-acetamido-2-deoxyglucose, with mannose probably only occurring in this region.

It is possible, assuming that the oligosaccharide chains are similar in composition and size (they may even be homologous), to obtain an approximate value for the numbers of chains per polypeptide unit. The monosaccharide-analysis results[7] and the results of the degradation of CEA to glycopeptides are consistent with these assumptions. The ratios of the monosaccharides in the native CEA (3/7E) (see Table II) are mannose–galactose–2-acetamido-2-deoxyglucose–fucose, 1:3:5:2. The ratios for asialo CEA (3/9F) are 1:2:3:1. The mol. wt. of the total carbohydrate in the native CEA is ~90,000, and in the asialo CEA ~100,000 (mol. wt. of CEA is 200,000). In the native CEA, the chains will have a mol. wt. of ~2,000*n*, where *n* is the number of mannose residues per chain, and for the asialo CEA the value will be 1300*n*

(calculated by summing the mol. wts. of the individual monosaccharides). The number of oligosaccharide chains in the native CEA is therefore $\sim 90{,}000/2000n$ $(= 45/n)$, and in the asialo CEA $\sim 100{,}000/1300n$ $(= 77/n)$.

The treatments described above (three Smith degradations and a fourth oxidation–reduction) produce material containing 37 and 25 moles of 2-acetamido-2-deoxyglucose per 10^5 g of product from the native and asialo CEA samples, respectively. Since the material which remains contains 11–12% of carbohydrate, these values correspond to values of 42 and 31 moles of 2-acetamido-2-deoxyglucose per mole of oxidised native and asialo CEA, respectively. If the degraded oligosaccharide units contain, on average, one residue of 2-acetamido-2-deoxyglucose, for the native CEA, n would be 1 and the molecule would contain 45 oligosaccharide chains, each comprising one mannose, three galactose, five 2-acetamido-2-deoxyglucose, and two fucose residues. A non-integral number between two and three is obtained by these calculations for the asialo CEA (3/9F), perhaps indicating less homogeneity in the carbohydrate part of this preparation, but if $n = 2$, the molecule would contain 39 chains, each with two mannose, four galactose, six 2-acetamido-2-deoxyglucose, and two fucose residues. Therefore, assuming that no oligosaccharide chain has been completely degraded in the multiple Smith-degradations, the number of oligosaccharide chains in CEA is approximately 40, with a mol. wt. of 2,000–2,500.

It can be seen from the data in Table IV that, after each Smith-degradation, the mol. wt. of the modified CEA decreased, as was expected. There was, however, an apparent increase in mol. wt. after each oxidation–reduction sequence. This result could be due to intermolecular links which are broken down by acidic hydrolysis. From the estimation of the mol. wt. of the polypeptide moiety, it appears that some proteolysis occurred, especially during the third acidic hydrolysis. This is evident from the *N*-terminal analyses. After the third acidic hydrolysis, lysine could hardly be detected as an *N*-terminal amino acid. Since CEA has 6 disulphide bridges[14], some proteolysis could occur without change in mol. wt.; only after three acidic hydrolyses does the change in mol. wt. become clearly evident. The antigenic activities of the products are compared in Table I. Although there is little loss in activity after the first acidic hydrolysis[1,8], there is a progressive loss in activity with each subsequent stage. Variations in the loss of antigenic activity, however, were observed between different samples of CEA and even using the same preparation of CEA in two separate, but similar, experiments. Variations in the extent of proteolysis were thought to be responsible, although it is difficult to explain why the proteolysis is more severe in subsequent degradations. It is conceivable that, during the latter stages, the oxidised protein moiety is less protected by carbohydrate from acidic hydrolysis. From the data in Table I, it can be seen that, after the third Smith-degradation, modified CEA had an activity of 1000 ng/ml and modified asialo CEA had an activity of 315 ng/ml. Although some loss in activity could be due to removal, from the "core" region, of monosaccharides which are involved in the antigenic determinants of CEA, it is more likely, from the acidic hydrolysis experiments on native CEA[7], that the loss in activity is due to proteolysis. In fact, prolonged dialysis (when modified, inactive

CEA molecules of smaller mol wt. might be lost) or isolation of modified CEA from the first few fractions obtained after gel-filtration on Biogel P-10 often gave material with a higher antigenic activity.

Because concentrations of periodate as low as 5mM degrade some of the amino acids in the polypeptide chain of CEA, and the acidic conditions cause rupture of the peptide bonds, it is unlikely that intact, carbohydrate-free polypeptide will be obtained by this method. However, it may be possible to remove all of the carbohydrate from CEA enzymically without proteolysis, and preliminary work with a crude extract of *Trichomonas foetus* seems to corroborate this view.

During the preparation of this manuscript, an account of the work on one Smith-degradation and a further periodate–borohydride treatment of CEA was published by Coligan and Todd[16]. The acid-hydrolysis conditions (0.5M HCl, 25°, 24 h) used in their Smith degradations cause considerable loss (61%) in antigenic activity when applied to native CEA. We have shown[8] that if one of our preparations of CEA is treated with sodium borohydride-*t* followed by 0.05M H_2SO_4 at 37° for 22 h (the latter acid strength being adequate for removal of the hemi-acetal fragments in a Smith degradation), no loss in antigenic activity occurs. Coligan and Todd also reported complete destruction of cysteine and tryptophan in CEA using 30mM periodate at 4° and pH 4.5. We have shown[14] the absence of free sulphide residues in our CEA preparations; six disulphide bonds are present in the molecule, which are cleaved by 0.533M periodate and a large loss of antigenic activity thereby ensues[1].

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