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Electron Microscopy and Physical Characterization of the Carcinoembryonic Antigen†

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ABSTRACT: Carcinoembryonic antigen (CEA), a glycoprotein material purified from human tumors, has been visualized by electron microscopy. At neutral pH, it consists largely of relatively homogeneous, morphologically distinctive twisted rod or cruller shaped particles, with dimensions 9 × 40 nm. The particle length is considerably diminished at pH 4.0, which correlates with a known diminution of charge. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis indicated a molecular weight of 180,000 in the peak region of the CEA band for both 10 and 15% acrylamide. When native CEA was treated with neuraminidase, reduced, and alkylated, a relatively compact random coil was produced, whereas reduction and alkylation without neuraminidase treatment produced a less compact random configuration, as determined by sedimentation studies and by electron microscopy. Electrophoretic migration, however, was apparently unaffected by reduction and alkylation. Thus the characteristic CEA particle appears by several lines of evidence to be substantially folded into a recognizable tertiary structural arrangement.

Carcinoembryonic antigen (CEA)[1] is a macromolecular glycoprotein antigen isolated from adenocarcinomas of the digestive system (Gold and Freedman, 1965a,b). The CEA produced by tumor cells passes into the circulation where it can be detected by radioimmunoassay (Thomson et al., 1969; Egan et al., 1972). These findings suggested that CEA might be of value in the diagnosis of cancer. The literature in this area has been reviewed by Terry et al. (1974).

Although the carbohydrate composition of CEA varies for different preparations (Terry et al., 1974), the amino acid composition is quite constant for the approximately one-third of the molecule which is protein (Terry et al., 1972, 1974). CEA has a sedimentation coefficient of 6.8 S (Coligan et al., 1972).

Since a molecule of this size is amenable to molecular electron microscopic techniques, we attempted to characterize preparations of this material morphologically, using techniques recently applied to other glycoproteins (Slayter and Codington, 1973; Slayter et al., 1974). At the same time, in order to obtain precise information about size and physical dispersity, sedimentation and electrophoretic studies were undertaken on the same material utilized for electron microscopy. Native, neuraminidase treated, and reduced and alkylated materials were analyzed in the study reported here.

Materials and Methods

Preparation of CEA. CEA used in these studies was purified as described by Coligan et al. (1972).

Radioimmune Assay for CEA. CEA was measured by double antibody radioimmune assay (Egan et al., 1972; Egan, 1974).

Neuraminidase Digestion. Vibrio cholerae neuraminidase (500 units/ml; 1.6 units/μg; General Biochemicals, Chagrin Falls, Ohio) was added to lyophilized CEA in a ratio of 1 unit/35 μg of CEA. For each milliliter of neuraminidase, 100 μl of sodium citrate buffer (0.2 *M* sodium citrate–0.01 *M* $CaCl_2$, pH 5.5) was added. After approximately 48 hr at 37°, the reaction mixture was dialyzed against deionized H_2O at 4°, lyophilized, and stored at 4°.

Amino acid analysis was performed on CEA samples that had been hydrolyzed with *p*-toluenesulfonic acid by the method of Liu and Chang (1971). A Beckman Model 121 H amino acid analyzer was used.

Carbohydrate Analysis. Values for neutral sugars were obtained by the gas chromatographic procedure of Clamp et al. (1972). Amino sugars were determined on the amino acid analyzer during the amino acid analysis. Sialic acid was measured by the method of Warren (1959).

Reduction and Alkylation. Samples to be reduced and alkylated were dissolved at a concentration of 6–7 mg/ml in 0.1 *M* Tris-HCl buffer (pH 8.3) containing urea (9 *M*) and EDTA (2 m*M*). After deaeration with a stream of nitrogen (30 min), sufficient 1.0 *M* dithiothreitol was added to make the concentration 10 m*M*. After 4 hr at room temperature under a stream of nitrogen, the reaction mixture was cooled

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[1] Abbreviations used are: CEA, carcinoembryonic antigen; the subscripts mi and na after CEA designate the individuals from whose tumors the CEA was isolated. Both preparations were isolated from liver metastases of primary adenocarcinomas of the colon. These preparations are chemically very similar, but not identical, although they are antigenically indistinguishable (Terry et al., 1974).

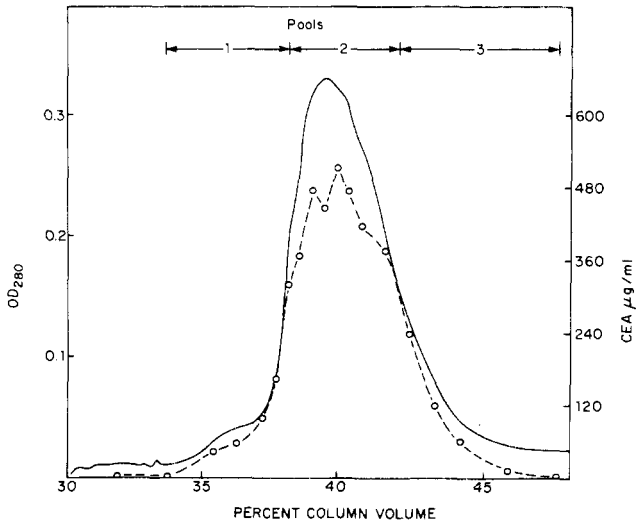

FIGURE 1: Plot of optical density and CEA activity as a function of effluent volume from a Sephadex G-200 column. (—) OD_{280}; (O- - -O) CEA μg/ml.

to 0° in crushed ice, and recrystallized 1 *M* iodoacetamide was added to a final concentration of 20 m*M*. After 1 hr in the dark at room temperature, the reaction mixture was dialyzed against deionized H_2O at 4° and lyophilized.

Sedimentation coefficients were calculated from plots of log distance vs. time, and the usual corrections made for solvent viscosity and density (Gibbons, 1972; Schachman, 1957). CEA concentrations were about 2 mg/ml.

Electron microscopy was carried out on a Siemens 1A electron microscope at 80 kV with a 70 μm objective aperture and a magnification of 27,000×. Calibration was based upon photographs of indanthrene olive crystals (Labaw, 1964). Micrographs were recorded sufficiently close to focus to make metal grain at the 2.0-nm level clearly resolved on the original plates. Plates were enlarged four times photographically, using a contact intermediate to reverse contrast so that the shadowing metal would appear light. Solutions of glycoproteins, dialyzed exhaustively against 0.15 *M* ammonium acetate, were sprayed at a concentration of about 0.1 mg/ml through a high-pressure spray gun at freshly cleaved mica. Ten different randomly selected preparations of CEA were shadow cast with platinum by rotary shadowing as described previously (Slayter and Lowey, 1967).

In making the measurements from the micrographs, a number of entire fields were measured in order to reduce selectivity, and sufficient total numbers were obtained to give smooth distribution curves. From similarly prepared samples of other molecules of known size and shape, we have determined (H. S. Slayter, unpublished results) that a correction of 2.5 nm must be applied in order to compensate for the distortion in thickness of the original particles by the cap of replicating metal. A discussion of limits of error in this type of measurement appears elsewhere (Slayter and Codington, 1973).

Acrylamide Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out essentially as described by Weber and Osborn (1967), except that pretreatment with mercaptoethanol was omitted in certain cases. The gels consisted of either 2.6% acrylamide and 0.13% bisacrylamide, 5.0% acrylamide and 0.14% bisacrylamide, 7.5% acrylamide and 0.17% bisacrylamide, 10% acrylamide and 0.23% bisacrylamide, or 15% acrylamide and 0.35% bisacrylamide. Segrest and Jackson (1972) noted that the molecular weights of glycoproteins were overestimated relative to proteins at low acrylamide concentrations. They showed that for glycoproteins up to 50,000, a better empirical calibration of the molecular weights of glycoproteins using protein standards was possible when the acrylamide concentration was above 10%.

Sodium dodecyl sulfate (0.1%) was included in the gels and in the tray buffer (0.05 *M* sodium phosphate buffer, pH 6.0 or 8.5). Electrophoresis was carried out for 3–30 hr. In the longer runs, the pyronin y marker migrated off the end of the gel, and, therefore, bovine serum albumin was used as a correlating tracker. Gels were stained either with Coomassie Brilliant Blue or by the periodic acid Schiff reaction (Glossmann and Neville, 1971). The relative distance traveled compared to the tracker position was calculated for the position of maximum density as determined with a Joyce-Loebl densitometer. Control gels were run with various samples including bovine serum albumin, IgG, myosin, tectin, actin, trypsin, and myoglobin as calibration.

Results

Electron Microscopy of CEA. Figure 1 shows the Sephadex G-200 elution profile of CEA_{mi} which is characteristic for most CEA samples at this final step of purification. The specific activities (mg of CEA activity as determined by radioimmunoassay/mg of dry weight) of CEA_{mi} pools 1, 2, and 3 were 72.6, 100, and 95%, respectively. The chemical compositions of these pools and of pools from a second preparation of CEA used in these studies are shown in Table I. Electron micrographs of platinum shadowed replicas of CEA isolated from each pool were prepared and scanned for qualitative appearance. Average lengths and widths were measured.

Several striking features were immediately seen to differentiate these preparations, particularly in the region of peak antigenic activity (pool 2), from other preparations of glycoproteins such as those from tumor cell surfaces (Slayter and Codington, 1973), or blood group substances, A, B, H, amniotic i, and plasma i (Slayter et al., 1974) (see Plate 1a). CEA particles were found to be generally rod-like but shorter and thicker, relative to molecular weight, by a factor of 4 than the other glycoproteins of this approximate composition studied by electron microscopy. Typically, 85% of individual particles in fields of CEA taken from the region of peak antigenic activity on elution from G-200 were characterized as quite homogeneous short rods, with a number average length and width of about 41 and 9 nm, respectively, after correction for shadowing metal (Table II). CEA_{na} pools 2 and 3 gave similar results to pool 2 of CEA_{mi}. Material in the pools peripheral to this peak averaged only about 75% characteristic particles, with the remaining material consisting mainly of particles substantially longer and thinner with peak length of about 80 nm and peak width about 4 nm.

Histograms of combined length measurements at neutral pH in the region of peak antigenic activity (CEA_{mi} pool 2, CEA_{na} pools 2 and 3) show a peak length of about 50 nm (Figure 2a). Calculated number and weight average lengths for the same material were 43 and 49 nm, respectively. Selected particles from this type of preparation are shown in Plate 2 at higher magnification. Groups a, b, and c are characteristic particles found in these CEA preparations. A frequent feature is the crooked backbone sometimes presented in projection as a squashed W form (Plate 2b and c)

Table I: Chemical Composition of Sephadex G-200 CEA Pools.[a]

	CEA_{mi}			CEA_{na}[b]	
	Pool 1	Pool 2	Pool 3	Pool 2	Pool 3
	Moles of Carbohydrate/10^5 g of Carbohydrate				
Fucose[c]	92.9	112.9	95.3	42.2	43.2
Mannose	42.3	87.1	69.8	69.6	72.3
Galactose	149.1	138.6	126.2	155.3	148.1
N-Acetylglucosamine[d]	171.7	172.3	174.5	197.5	199.5
N-Acetylgalactosamine	52.2	19.8	36.1	24.4	22.1
Sialic acid[e]	29.4	27.7	35.0	37.4	39.6
	Moles of Amino Acid/10^5 g of Protein				
Lys[d]	30.1	21.1	35.3	25.3	26.8
His	19.9	12.1	14.9	13.8	14.4
Arg	34.3	24.1	28.3	29.8	28.6
Asp	105.1	163.4	126.0	140.9	143.6
Thr	111.7	92.4	85.2	89.5	83.3
Ser	111.4	118.5	101.3	106.4	94.3
Glu	83.2	94.8	106.9	96.8	85.5
Pro	78.5	79.9	78.6	79.6	82.4
Gly	59.0	57.8	61.6	68.1	76.9
Ala	60.2	61.8	66.9	66.6	63.8
Cys	15.9	12.0	14.0	12.6	17.6
Val	59.5	49.3	51.3	38.6	59.0
Met	10.2	2.9	0	2.3	2.3
Ile	29.5	29.1	20.8	35.4	37.8
Leu	73.3	67.6	56.1	73.1	73.1
Tyr	30.7	40.9	36.4	39.4	34.8
Phe	23.3	10.8	35.4	18.3	19.3
% by wt carbohydrate	52.3	50.5	44.3	62.8	64.6
% by wt protein	33.0	35.0	51.3	33.3	36.7

[a] All samples were dried over P_2O_5 prior to weighing for analysis. The failure to achieve 100% total weight recovery in several samples is attributed to rapid H_2O regain. [b] Pools 2 and 3 of CEA_{na} had a G-200 elution volume equivalent to the ascending and descending halves, respectively, of CEA_{mi} pool 2, and were similar to this pool in antigenic activity. [c] Neutral sugars were determined by gas chromatography according to the method of Clamp et al. (1972). [d] Amino sugars and amino acids were determined on a Beckman 121 H amino acid analyzer according to the method of Liu and Chang (1972). [e] Determined by the thiobarbituric acid assay (Warren, 1959).

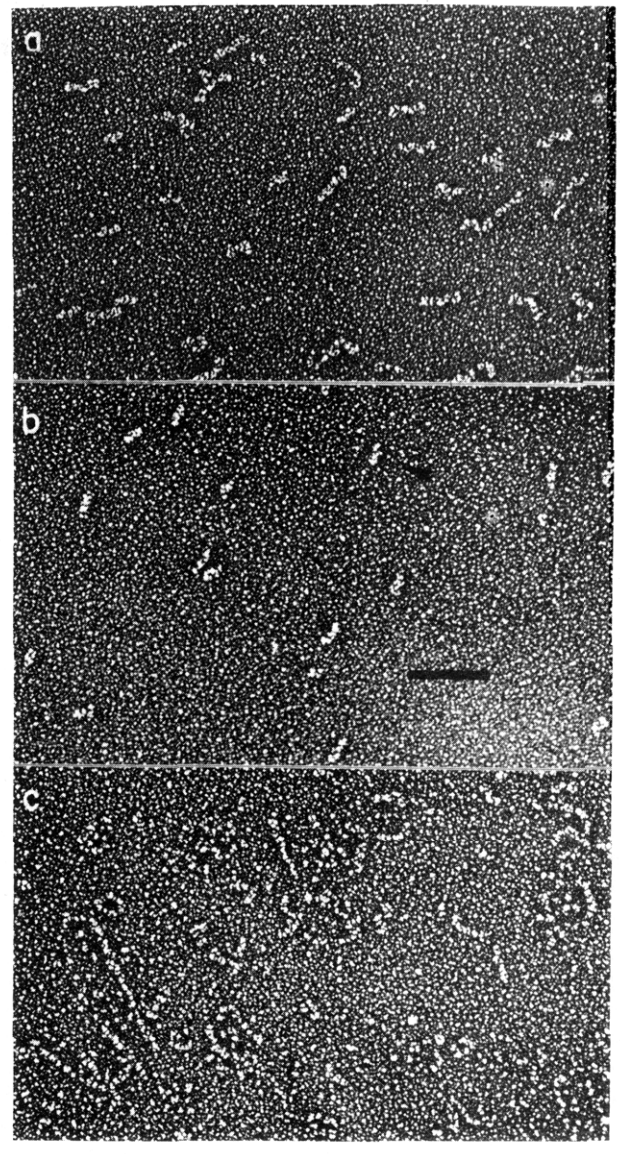

PLATE 1: CEA at pH 7.0 (a); pH 4.0 (b); and pH 9.5 (c). Replicas are rotary shadow cast with platinum. Magnification 100,000×. Bar indicates 0.1 μm.

Table II: Lengths and Widths across CEA_{mi} G-200 Elution Pattern.[a]

Pool	% Short Fat	% Long Skinny	Lpk[b]	$\bar{L}_n$[c]	$\bar{L}_w$[d]	$[\bar{L}_w/\bar{L}_n]$	Wpk[e]	$\bar{W}_n$[f]
1	71	29	42.5	37.5	45.5	1.2	9.0	10.5
2	85	15	42.5	42.5	49.5	1.2	9.0	11.5
3	77	23	30.0	46.5	55.5	1.2	9.0	9.5

[a] Dimensions are given in nm and all values are corrected for 2.5 nm of metal replica. The molecules are classified by inspection as "short and fat" or "long and skinny". The dimensional values are for the "short and fat" species. [b] Lpk is most frequent length. [c] $\bar{L}_n$ is number average length. [d] $\bar{L}_w$ is weight average length. [e] Wpk is most frequent width. [f] $\bar{W}_n$ is number average width.

or as a bent or straight three-lobed structure (Plate 2a). The variable distribution of metal replica density reflected principally in crystallite size is interpreted to mean that a significant variation in height in the third dimension occurs. Plate 2 (d–f) illustrates various configurations observed with a very low frequency. These would be consistent with various stages of partial unfolding from the characteristic fat configuration to a thinner, longer rod. Highly diagrammatic photographs of wire models are shown adjacent to the various parts of Plate 2, indicating approximately to scale the extent to which folding must occur in a single polypeptide backbone to account for the observed configuration.

Number and weight averages are normally calculated in order to provide a more meaningful comparison between the electron microscopic parameters and sedimentation equilibrium data, since the length distributions are slightly skewed toward longer lengths. This skewness could result in a somewhat higher molecular weight value obtained in a sedimentation experiment which would not be comparable to the peak value of electron microscopic length found in a strict frequency distribution. The ratio of $\bar{L}_w/\bar{L}_n$, which is considered a good index of heterogeneity, is 1.2, indicating substantial homogeneity (Slayter and Codington, 1973).

When the pH was lowered to 4.0 or raised to 9.5, gross conformational changes were observed (see Figure 2b and c and Plate 1b and c). At pH 4.0, a shorter, fatter, more homogeneous species was observed, with a number average

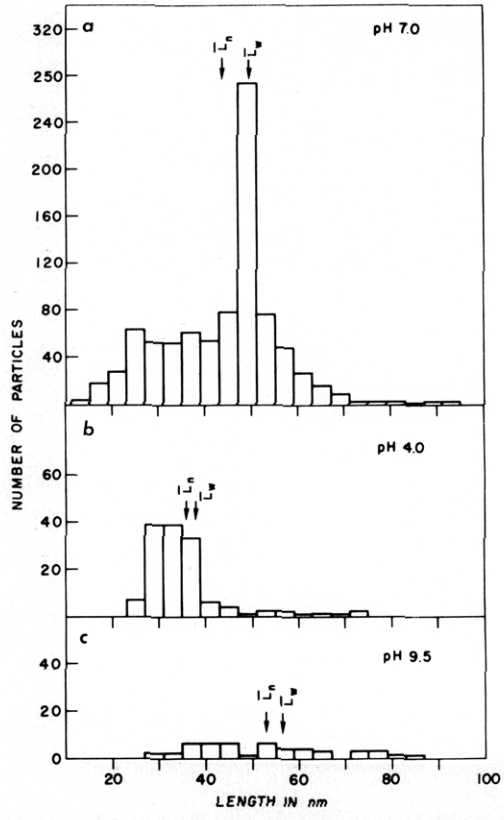

FIGURE 2: Histograms showing length distributions of CEA molecules at: (a) pH 7.0; (b) pH 4.0; (c) pH 9.5. $\bar{L}_n$ and $\bar{L}_w$ are number average and weight average length, respectively.

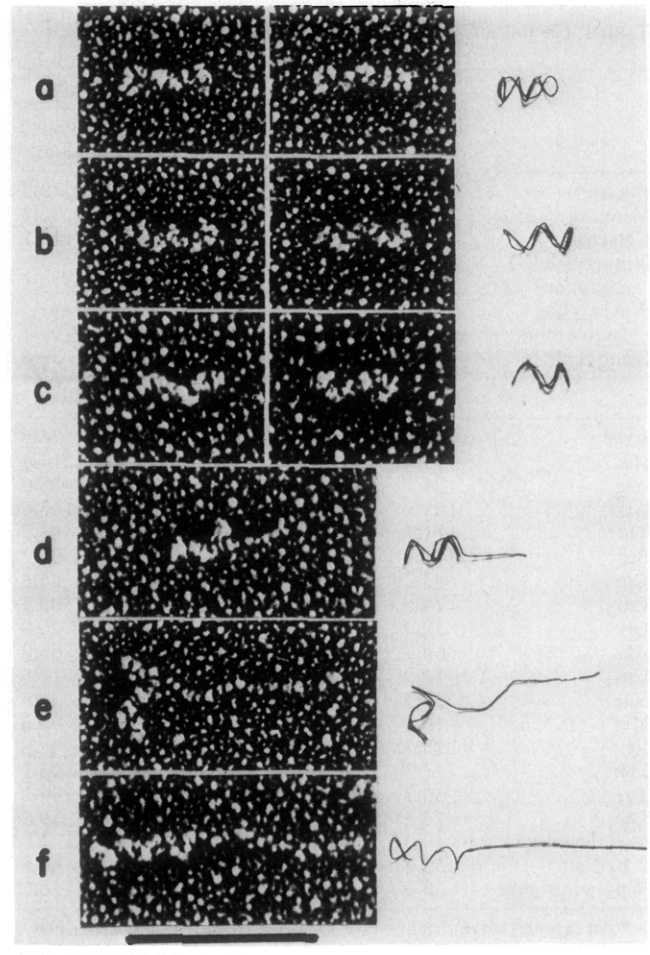

PLATE 2: Selected particles from a CEA preparation at pH 7.0 showing the various detailed configurations observed (a–c). Particles shown in (d–f) occur occasionally and may represent stages in unfolding of the particle as indicated in the adjacent photographs of wire models. These are approximately to scale, and are intended to indicate in principle and diagrammatically, the extent to which the single polypeptide backbone must be folded to account for the observed size and shape of the particle. Magnification 245,000×. Bar indicates 0.1 μm.

length of 36 nm and a width of about 10 nm. At pH 9.5, a significant number of longer and distinctly thinner particles appeared, ranging up to about 80 nm, with a number average length of 53 nm, and a width of about 4 nm. It is noteworthy that unfolded CEA particles at high pH appeared in micrographs to be randomly coiled rather than extended. Thus, it is likely that the figures for length obtained from these preparations are not representative of the maximum extended length, possibly due to internal disulfide cross-linking, since the contour length could not be followed through maximally unfolded particles. Particles of intermediate length are presumed to be only partially unfolded. These are similar to particles found at low frequency in CEA_{mi}, pool 2, and at higher frequency in the adjacent pools, which show a lower specific antigenic activity. It is tempting to speculate that these may be denatured particles, possibly arising in the native preparation by some step in the preparative procedure, for example, in the perchloric acid treatment. However, when the pH 9.5 treated material was returned to pH 4.0 and then pH 7.0, the characteristic morphology returned and CEA activity remained.

Molecular Weight by Sodium Dodecyl Sulfate–Polyacrylamide Electrophoresis. Apparent molecular weights of CEA were determined at increasing acrylamide concentration. A plot of apparent molecular weight as a function of percent acrylamide approaches a value of about 180,000 asymptotically, based upon an empirical calibration against protein standards (myosin, spectrin, bovine serum albumin and its dimer, actin, IgG, and trypsin.) Although these standards do not contain large amounts of carbohydrate, the discrepancy between migration rates of proteins and glycoproteins has been shown to be minimized at acrylamide concentrations above 10% (Segrest and Jackson, 1972). CEA is very soluble compared with the aggregating membrane glycoprotein investigated by Segrest and Jackson (Grefrath and Reynolds, 1974). Since our value of 180,000 is consistent with a value obtained by short column sedimentation equilibrium (Dr. James Morris, personal communication), we used this figure in calculations realizing that it was derived from the peak of density within a migrating band which was broader than bands obtained with purified proteins of similar molecular weight.

Modification of CEA. In the following reactions, the removal of sialic acid was determined by the thiobarbituric acid assay. The success of the reduction and alkylation was determined by the complete disappearance of cysteine and the appearance of *S*-carboxymethylcysteine during amino acid analysis. CEA_{na} pool 3 (composition in Table I) was used for these experiments. The sedimentation rates reported for comparison were determined by a single run with each sample at the same concentration in a multicell rotor.

Neuraminidase treatment of CEA, so that virtually all sialic acid was removed, resulted in a morphological type by electron microscopy which was essentially indistinguishable from untreated CEA (Plate 3a). The sedimentation coefficient (6.8 S) was unchanged compared with untreated CEA (Table III).

Table III: Sedimentation Coefficients and Calculated Diameters[a] of Particles of Various CEA Preparations.

	S	D_{max}[c,d] Number Average Measured EM	D_{max}[e] Calcd as a Rod	D_{max}[e] Calcd as a Flexible Coil	D_{max}[e] Calcd as a Sphere
Untreated	6.8[b]	42	40	28	21
Neuraminidase treated only	6.8	42	40	28	21
Neuraminidase treated; reduced and alkylated	5.3	27	52	36	27
Reduced and alkylated only	5.1	110 ex.[f] 64 col.	54	38	28

[a] Dimensions are in nm. [b] Determined by Coligan et al. (1972). This value was obtained by extrapolation to infinite dilution. [c] D_{max} is the maximum diameter of a particle in solution or in an electron microscope image. [d] The correspondence between the D_{max} measured and D_{max} calculated for various models indicates that in the untreated and neuraminidase treated material a rod model fits well, whereas a sphere model fits better for the neuraminidase treated and reduced and alkylated CEA, and an extended flexible coil fits better for the reduced and alkylated only CEA. [e] D_{max} is calculated as indicated in the results section assuming a rod, sphere, or disc model, whichever seems most appropriate in a given instance. [f] Both extended (ex.) and collapsed (col.) forms were found after reduction and alkylation, and were averaged separately.

After neuraminidase treatment followed by reduction and alkylation, the sedimentation coefficient was reduced to 5.3 S (Table III), and the shape by electron microscopy was drastically transformed (Plate 3b) to a globular conformation. This material exhibited a marked tendency to aggregate in electron microscope preparations, although there was no evidence for such aggregation in the sedimentation pattern. The smallest unit in the micrograph is probably representative of the gross conformational state of CEA in this modified condition (see arrows), although variation in compactness probably results in flattening in the third dimension with a concomitant increase in the cross section.

After reduction and alkylation only, the sedimentation coefficient was reduced only slightly further to 5.1 S (Table III). However, the morphological appearance was distinct from that found after neuraminidase treatment or neuraminidase treatment followed by reduction and alkylation (Plate 3c). While a substantial fraction of the particles was found extended (see arrows) to a peak length of about 60 nm (Table III), other material appeared globular or aggregated. Unlike the pH effect, this change appeared irreversible, since the characteristic conformation was lost as measured by sedimentation and electron microscopy.

In order to examine these conformational changes further for clues as to structure, we carried out a theoretical correlation between S and R_{max}, the maximum radius of the particles in solution or in the electron microscope image.

The sedimentation equation

$$S = \frac{M(1 - \bar{\nu}\rho)}{A6\pi\eta} \frac{1}{R_e}$$

can be used to relate sedimentation coefficient to the radius of the equivalent sphere where M = molecular weight, $\bar{\nu}$ = partial specific volume, ρ = solvent density, A = avogadro's number, η = solvent viscosity (Tanford, 1961). R_e is related to R_G, the radius of gyration, by a constant of proportionality which varies empirically. Examination of data concerning the relationship between R_e and R_G (H. S. Slayter, to be published; Slayter, 1965; Tanford, 1961) for various molecular species, including serum albumin, myosin, fibrinogen, collagen, and several glycoproteins, leads us to set $R_e = 0.55R_G$, for purposes of internal comparison here. For a rod model R_G equals $0.578R_{max}$; for a spherical model R_G equals $0.775R_{max}$; and for a flexible coil R_G is approximately $0.41R_{max}$ (Tanford, 1961). Calculation of $2R_{max}$ or D_{max} for various preparations of CEA vs. measured S resulted in values which were remarkably similar to the electron microscopic results (Table III), particularly for a rod model.

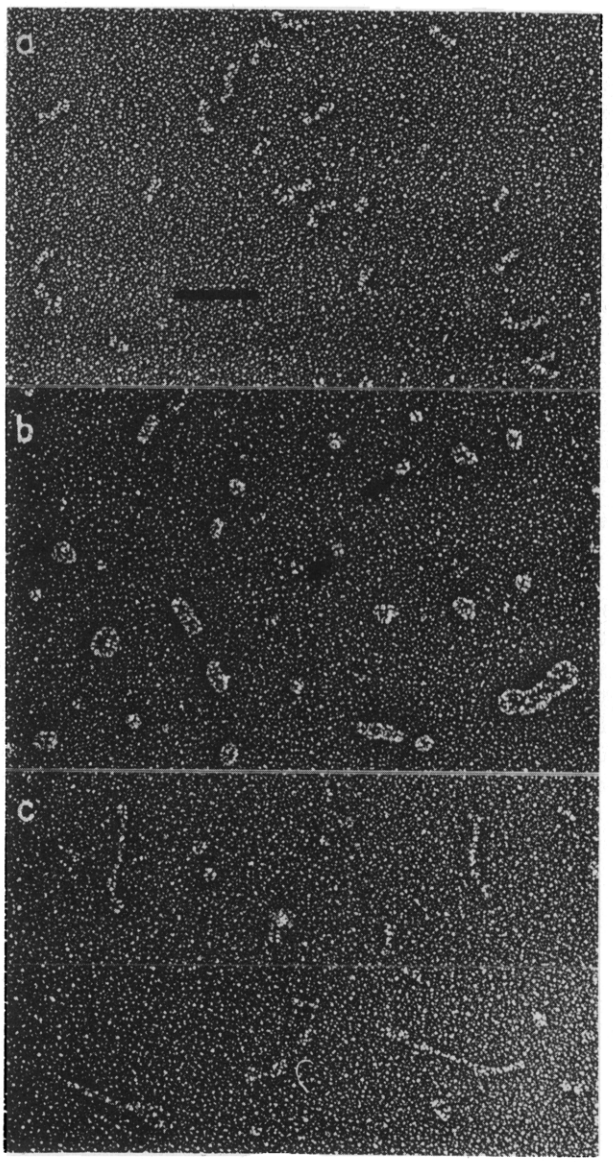

PLATE 3: Micrographs of CEA at pH 7.0, after treatment with (a) neuraminidase; (b) neuraminidase plus reduction and alkylation; (c) reduction and alkylation. Magnification 100,000×. Bar indicates 0.1 μm.

Sodium dodecyl sulfate–polyacrylamide gels were run to determine whether the molecular weight of the CEA was decreased appreciably by treatment with neuraminidase and/or reduction and alkylation. The treated CEA samples were examined on sodium dodecyl sulfate–5% polyacrylamide gels at pH 6.0 and pH 8.5 in the presence and absence of β-mercaptoethanol. The apparent molecular weight cal-

culated from measurements on standard proteins run at the same time were essentially identical with the untreated CEA (approximately 180,000). The apparent molecular weights were calculated from the CEA band peak as determined by a densitometer. Staining with Coomassie Blue or periodic acid Schiff gave identical results. The one exception was the CEA which had only been treated with neuraminidase. It had an apparent molecular weight of 200,000 at pH 8.5 in the absence of β-mercaptoethanol.

CEA bands on stained gels are quite broad relative to those normally obtained with proteins. This migration heterogeneity is apparently not due to gel overloading, since gels loaded with 10, 30, 100, and 200 μg of CEA all gave the same results. This electrophoretic heterogeneity is probably due to the known microheterogeneity of the polysaccharide portion of most, if not all, glycoproteins (Spiro, 1970). This variability in the amount of carbohydrate per CEA molecule would probably affect the amount of sodium dodecyl sulfate bound per gram of glycoprotein (Segrest and Jackson, 1972). Different fractions of CEA obtained from isoelectric focusing are known to vary in their carbohydrate composition and percent protein by weight (Coligan et al., 1973, Terry et al., 1974). This variable composition would lead to different amounts of sodium dodecyl sulfate binding per unit weight of CEA and thus band broadening upon polyacrylamide electrophoresis. Since the treated CEA samples did migrate identically with intact CEA, it appears that removal of the sialic acid and/or reduction-alkylation does not appreciably alter the apparent molecular weight.

Discussion

A cardinal question concerning CEA relates to the degree to which it can be considered homogeneous. It may be homogeneous in one sense, such as gross size and shape, or N-terminal sequence, while heterogeneous in other senses, such as amino acid composition, degree of amidation, or acetylation. Purification of CEA is monitored by radioimmunoassay inhibition using antisera to a standard CEA preparation. If this standard material contains impurities, antiserum to it would be capable of perpetuating this impure state. CEA would appear to be homogeneous by the following criteria. Ouchterlony gels run with crude perchloric acid extracts show multiple bands compared with one band with purified CEA when analyzed with antiserum prepared against the crude material (Colligan, unpublished observation). Immunoelectrophoresis shows a single long band migrating in the β region (Coligan et al., 1972). The initial N-terminal sequence is remarkably constant from one preparation to the next (Terry et al., 1972; Chu et al., 1974; Coligan et al., 1975). The chemical composition of CEA, although varying slightly between different preparations especially with regard to sialic acid content, appears to be reasonably characteristic of the molecule (Terry et al., 1974). On the other hand, CEA exhibits significant charge heterogeneity when examined by isoelectric focusing which produces multiple fractions of essentially equivalent antigenicity. This heterogeneity correlates with a variable amount of sialic acid: the more negative the charge on the CEA, the higher the sialic acid content (Coligan et al., 1973). Although removal of the sialic acid with neuraminidase renders the isoelectric focusing patterns more homogeneous and increases the isoelectric point of the bulk of the material, some heterogeneity still remains. Studies with ovomucoid (Beeley, 1971; Melamed, 1967) also lead to the conclusion that sialic acid alone does not account for all the heterogeneity in that glycoprotein.

The electron microscopic results present a picture of substantial homogeneity on a morphological basis, particularly with regard to the $\bar{L}_w/\bar{L}_n$ value of 1.2, which is similar to the value previously obtained for a tumor cell surface glycoprotein (Slayter and Codington, 1973) shown by sedimentation studies to be quite homogeneous.

The shape of the characteristic native particle at neutral pH resembles a twisted rod. Clearly, no single parameter describes width, as there is variation in width as a function of axial position making a mathematical model elusive. The disposition of metal replica, as mentioned in the Results section, leads us to believe that a quasihelical distribution of mass may exist. The images suggest a cruller-like shape. Whether this is due to some form of super-coiling, is the result of a distinctive folding process, or is related to the distribution of carbohydrate, or combinations of the above, is not known.

In any case, CEA is four to five times too short to permit a single extended polypeptide backbone which would have a length of 220 nm based on a calculation of polypeptide length from compositional data (35% protein; 106.5, average residue weight; molecular weight, 180,000; 0.364 nm extension per residue) (Pauling, 1951, 1952). Thus, the roughly 220-nm polypeptide must in some way be folded into a native particle 42 nm long. The bulges in the particle could be due to an irregular mode of folding which is well documented in other proteins such as myosin (Slayter and Lowey, 1967) and fibrinogen (Hall and Slayter, 1959).

The fact that the reduced and alkylated structure appears collapsed rather than extended, as is the case with other glycoproteins which we have examined by electron microscopy, is consistent with fewer glycosidic side chains.[2] In fact, as discussed previously, it is believed that in CEA

[2] The approximately 65% by weight of carbohydrate existing as multiple side chains, believed attached through asparagine linkages to glucosamine residues (Terry et al., 1974), may contribute to this shape. (The values for CEA_{na} in Table I are used as a basis for the calculations made here.) The ratio of *N*-acetylglucosamine to Asx (asparagine plus aspartic acid) residues is about 2.5, so that the number of side chains would apparently be limited by the possible receptor sites (asparagine) on the protein chain to a maximum of about 87. Since there are about 600 monosaccharide residues per 180,000 molecular weight, there would be about seven residues per side chain, if these 600 residues were equally distributed over 87 sites. This figure of seven residues would presumably be the minimal average size of the polysaccharide groups. The precise value to be used for the extension per sugar residue is not known, but the maximal value based on a pyranose structure would be about 0.5 nm. Thus the extension for a chain of seven sugar residues would be approximately 3.5 nm. If the number of carbohydrate chains were smaller, the length would increase. As the side chain length approaches the shadow replica thickness of 2.5 nm, a protuberance would become apparent. In addition, if the side chains were distributed asymmetrically, they could cause a lobulated appearance. Evidence for asymmetric distribution is not yet conclusive, but some indications exist. First, glycopeptides from proteolytic digests of CEA have glucosamine/Asx ratios as high as 10 (Banjo et al., 1974; Coligan and Pritchard, unpublished data). Assuming that the terminal asparagine is not removed from the polysaccharide in the digestion, some chains much larger than the average appear to be present. Secondly, since there seem to be about 575 amino acid residues in the protein chain, about one in seven would appear to be glycosylated if the average polysaccharide grouping comprises seven sugar residues. For such a molecule the maximum diameter on an extended basis, with carbohydrate groups extending on both sides, would be about 7.5 nm. Since in the reduced and alkylated CEA preparations, rods as thin as 3 nm are seen (Plate 3), it is reasonable to expect some lack of symmetry in the distribution of the sugar residues.

there are a maximum of one in seven residues glycosylated, compared with one in three for an extended glycoprotein isolated from mouse mammary adenocarcinoma cells (Slayter and Codington, 1973). It is probable that a more important primary determinant of conformation is the disposition of the polypeptide chain. The maximum calculated extended length of about 220 nm is approximately the maximum length observed in reduced and alkylated CEA preparations, although the probability is high that the radius of gyration of the random coil in solution is on the average substantially less. In all other CEA material examined in the electron microscope, lengths were more typically about 45.0 nm with a slightly lower number average length at pH 4.0 of about 35.0 nm. A calculation of length versus diameter for a rod model, given the observed molecular weight and the $\bar{\nu}$ equal to 0.693, results in the plot shown in Figure 3. (The $\bar{\nu}$ was calculated from published values for carbohydrate (Gibbons, 1972) and amino acid (Schachman, 1957) components.) Clearly, within the limits of uncertainty of width measurement (see Slayter and Codington, 1973), a much larger consistent deviation of measured width from the calculated curve is found for a given length than is noticed for shorter CEA particles. It is estimated that the typical CEA configuration seen at neutral pH is only about one-sixteenth filled. This leads us to conclude that the CEA polypeptide chain becomes more loosely coiled as it collapses filling space in electron microscope replicas out of proportion to its mass. At the same time there is a resultant reduction in its radius of gyration, as indicated by both sedimentation and electron microscopic results. Certainly a significant part of the space-filling structure would be the carbohydrate side chains presumably bristling from the folded polypeptide backbone.

The value of 180,000 for the molecular weight of CEA obtained by polyacrylamide gel electrophoresis, and confirmed by sedimentation equilibrium results, permits a clearer interpretation of the electron microscope findings. Furthermore, the agreement between calculated R_{max} and measured S for native CEA indicates that the CEA conformation which appears most frequently in electron micrographs is consistent with the unit in solution, based upon the data and calculations presented in Table III.

Based upon the amino acid composition, as many as five disulfide bonds could exist and effectively stabilize the structure. Reduction and alkylation of these clearly results in striking changes in conformation, indicated by both the sedimentation and electron microscopic results. This leads to the conclusion that substantial conformational stabilization is due to disulfide linkages, without which the characteristic particle type is replaced by an amorphous population. There tends to be an extended linear form present in the reduced and alkylated preparations (with sialic acid attached), substantially longer than the native particle. Thus, it appears that sialic acid must contribute sufficient negative charge, at neutral pH, to result in a net negative charge on the polymer sufficient to keep it partially extended.

One puzzling result obtained in comparing electrophoretic mobility for the three modified CEA preparations is that the neuraminidase-treated preparation lags behind the untreated, and reduced and alkylated preparations even when the reduced and alkylated preparations are neuraminidase treated. This seems to imply that sodium dodecyl sulfate is not permitted to bind in the same stoichiometry in the neuraminidase-treated CEA compared to the other CEA preparations. This effect is noticed at pH 8.5 in the absence of

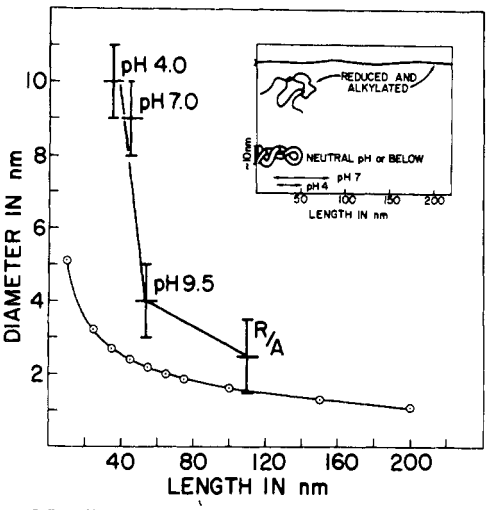

FIGURE 3: CEA diameter vs. length. Circles indicate calculated points based on figures for molecular weight and partial specific volume of 180,000 and 0.693, respectively. Crosses indicate experimental points determined as number averages from electron microscopy, at pH 4.0, 7.0, and 9.5, as well as after reduction and alkylation. The insert diagrams different morphological states of CEA induced by reduction and alkylation and changes in pH. Arrows indicate variations of length within a particular population.

mercaptoethanol but not at pH 6.0, either in the presence or absence of reducing agent. A possible explanation is that an intramolecular reorganization induced by the change in charge distribution occurs during removal of the sialic acid, which denies access by sodium dodecyl sulfate to significant regions of potential hydrophobic bonding sites. In the presence of a reducing agent, the disulfide bonds are probably cleaved, which allows the molecule to unfold and once again expose these hydrophobic regions to sodium dodecyl sulfate. A similar effect has been noted by Segrest and Jackson (1972) with desialized glycoproteins.

Heterogeneity of carbohydrate groups is displayed for a variety of glycoproteins such as transferrin, thyroglobulin, ceruloplasmin, immunoglobulins, and α-1-acid glycoprotein (see Gottschalk, 1969). This has been attributed to the variety of specific, enzymatically catalyzed steps required in the biosynthesis of carbohydrate polymers (O'Brien and Neufeld, 1972).

Many globular proteins are folded in such a way that two or more structural domains are distinguishable. The preservation of the conformation of such domains (generally conserved more faithfully than the exact amino acid sequence) may be related to steric requirements imposed by essential functions (Rossman and Liljas, 1974). Thus, from an evolutionary point of view, it may be contended that tertiary structural parameters determined either directly or indirectly, provide in certain instances, a relevant measure of homogeneity which may be more useful than the precise primary structure in an operational sense. Thus, the electron microscopic appearance of CEA is considered to be a potentially significant determinant of homogeneity.

As a result of the preceding discussion of available data bearing upon the gross physical structure of CEA and the relationship of these data to our electron microscopic results, we wish to suggest an approximate model for CEA incorporating a core folded to about 40.0 nm from which numerous carbohydrate side chains protrude, making an important contribution to both size and shape. Further chemical studies concerned with variations in acetylation and am-

idation, together with partial degradation studies involving electron microscopy, should make possible further clues to the exact conformation of CEA.

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