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Note

Oxidation products of ovarian cyst mucins. Characterisation by density-gradient analysis*

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Density-gradient methods in the ultracentrifuge are well-known to be very useful in application to macromolecules where density-variation can occur (*e.g.*, in the nucleic acids). The methods are useful in the glycoprotein field because the buoyant densities of the two constituents are well-separated; proteins band[1] at about 1.3 g/mL in cesium chloride, whereas carbohydrates band[2] at about 1.6 g/mL. Most of the mucus-type glycoproteins band[3–5] in the range 1.45–1.55 g/mL, the values being closely correlated with the particular ratio of carbohydrate to protein; the zone-shape is also informative[3]. The purpose of this note is to show how density-gradient methods were of material help in deciding the mechanism of the limited reaction between mucus glycoproteins and hydrogen peroxide[6,7].

EXPERIMENTAL

Methods. — Density-gradient experiments were carried out with an MSE Centriscan analytical ultracentrifuge fitted with u.v. absorption optics. Solutions were of ~1.5 mg/mL of glycoprotein in sodium acetate buffer (pH 5.6) containing ~33% (w/w) of Cs_2SO_4, used in preference to CsCl because it gives steeper gradients. Recording of the distribution at equilibrium (~20 h) were made at 260 nm. Procedures established previously[3,8] were followed for the determinations of buoyant density. Values of the standard deviation of the mass-distribution (σ_T), which is a measure of the total spread of the pattern, were determined from the

$$\sigma_T^2 = \frac{\int_a^b (r - r^0)^2 C dr^2}{\int_a^b C dr^2} \quad ; a < r < b \qquad (1)$$

equation (*1*), where C is the total concentration at the radial displacement r; and r^0, a, and b are the values of r characterising the peak concentration, and the upper

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and lower solution limits, respectively. All data processing was done with the aid of a Wang 720C computer, fitted with 4K extended memory.

Number-average molecular weights for the glycoproteins were determined from intermediate-speed sedimentation equilibrium experiments in a Beckman Model E ultracentrifuge, according to procedures developed earlier[10,11].

Theory of the density-gradient analysis. — The mass-distributions are broad, extending over more than half the ultracentrifuge cell. In such cases, the original and extended theories of Meselson *et al.*[12], and Ifft and Vinograd[13] do not adequately represent the concentrations near the solution limits. Accordingly, the equations specifically designed for broad distributions[8] were used. These are complicated in form, but are easily programmable for small computers. For example, the equation describing the distribution of the i^{th} component banded near the centre of the cell may be written as in Eq. (*2*) where h is the distance from the band-centre

$$c_i(r) = K_i \cdot c_i^0 \cdot \exp(p_i h_i^2 - q_i h_i^3) \qquad (2)$$

r_i^0 ($h_i = r - r_i^0$); c_i^0 the initial concentration of species i; and K_i, p_i, and q_i are functions of the macromolecular properties (M_i, the molecular weight; ρ_i^0, the buoyant density) and the experimental variables (speed of rotation, absolute temperature, and salt distribution parameter). In a given experiment, K, p, and q are constant, depending solely on the species i. Where the value of ρ_i^0 falls outside the limits of the cell, expansions based on displacements from the solution limits were derived: they have forms somewhat similar to Eq. (*2*), but differ substantially in detail. The full defining equations are given in the reference cited[8].

Two assumptions were made. First, the molecular weights of all species in a particular glycoprotein fraction are assumed to be the same, and equal to M_n, the number-average molecular weight multiplied by the appropriate solvation factor[13]. Second, the density-variation in the glycoprotein is assumed to be adequately represented by a finite number, N, (usually 30) of species whose buoyant densities ρ_i^0 are separated by a constant difference $\Delta\rho$, and whose initial concentrations c_i^0 are given by the expression (*3*), where, C^0 is the total concentration (thus $\Sigma c_i^0 = C^0$)

$$c_i^0 = \frac{C^0 \Delta\rho}{\sigma\sqrt{2\pi}} [(\rho_i^0 - \bar{\rho}^0)^2/2\sigma^2]; \quad i = 1,2 \ldots N \qquad (3)$$

and σ ($=\sigma(\rho^0)$) the standard deviation of the density distribution. Eq. (*3*) is the Gaussian approximation for a finite number of components.

In use, a value of σ has first to be assumed; $\Delta\rho$ then follows from the arbitrary limitation $\Delta\rho = 3\sigma/N$, when each c_i^0 value may be calculated for the experimentally-known value of C^0. The concentration of this species at each point in the cell is calculated from Eq. (*2*), and then the calculations extended to each species i. Summation at each point over all species produces a distribution curve for comparison with the original; recycling for a different value of σ is continued until the fit is

judged adequate. It is emphasised that $\sigma(\rho^0)$ computed in this way not only makes full allowance for the spreading due to density-variation in the specimen, but also makes a good approximation to the spreading due to diffusion (see below).

RESULTS AND DISCUSSION

All relevant analyses, both physical and analytical, are summarised in Table I. No reference is made here to the carbohydrate analyses, published earlier[6], as they show no consistent changes on oxidation. Similarly, analyses for only 6 of the 17 amino acids are included, the remaining 11 showing no consistent variation[6].

The total standard deviations of the mass-distributions [found from Eq. (*1*)] always increased on oxidation. For 603AmS, for example (the patterns for which were published previously[6]), the original gave $\sigma_T = 0.136$ cm, increasing to 0.152 cm on oxidation. For 610 (Fig. 1), corresponding values were 0.083 and 0.100 cm. The standard deviations of the buoyant densities, obtained by the simulation procedure described, are given for each parent and its oxidation product in Table I.

In general, the results presented in Table I are fully consistent with the conclusions on the nature of the reaction given earlier[6,7]; accordingly, what follows relates only to those aspects of the suggested mechanism which are clarified by the new analysis.

The most significant observation is that, whereas the total standard deviations of the mass-distributions increased, the standard deviations of the buoyant densities were essentially unchanged by oxidation, within the precision of the method. An increase in the total width of a distribution could arise either from a decrease in molecular weight or an increase in the width of the density distribution. The observed near-constancy of the density distributions shows that the change in the patterns can be ascribed solely to the observed decrease in molecular weight. This finding implies that the reaction is not accompanied by disproportionation between the peptide and carbohydrate components of the molecule.

The latter conclusion is particularly relevant to the glycoprotein-oxidation system, where the products so far characterised are those occurring after some 20 h under the stated conditions, where near-plateau characteristics exist, *i.e.*, the viscosity is nearly time-independent. However, very prolonged reaction (7 days) did cause further degradation, and the possibility would exist that the 20-h products are mixtures of parent and degraded substance.

The assumption of constancy of molecular weight in a sample is necessary for the repeated application of Eq. (*2*). The assumption is less restrictive than might be thought, for it is well-recognised[3] that the spreading of glycoproteins in density-gradients is largely determined by their density dispersion. Thus, apparent molecular weights calculated on single-species theory from density-gradient distributions are frequently an order of magnitude less than the true molecular weight[3]. Accordingly, a relatively severe error may be made in selecting a value of M for Eq. (*2*) without seriously affecting the $\sigma(\rho^0)$ value finally obtained. The values of $\sigma(\rho^0)$ quoted are therefore good approximations.

TABLE I

PHYSICAL AND ANALYTICAL CHANGES IN MUCUS GLYCOPROTEINS[a] OCCURRING ON REACTION WITH H_2O_2

Properties	*Glycoprotein*									
	603AmS	*603AmS/ox*	*485*	*485/ox*	*603/43-50/Pr*	*603/43-50/Pr/ox*	*531/Pr*	*531/Pr/ox*	*610/Pr*	*610/Pr/ox*
$10^{-3} \times M_n^0$	469	149	502	154	413	111	421	287	378	205
ρ^0 (g/mL)	1.356	1.362	1.306	1.310	1.346	1.365	1.341	1.348	1.321	1.319
$\sigma(\rho^0)$ (g/mL)	0.021	0.022	0.011	0.011	0.019	0.017	0.013	0.015	0.012	0.012
Proportion of peptide[b]	7.6	7.6	12.0	10.7	8.0	6.3	14.0	13.7	17.2	14.1
Asp[c]	4.9	6.2	2.8	3.7	3.0	3.9	1.7	2.5	1.6	2.9
Thr	19.0	20.7	27.1	27.7	27.4	29.2	33.0	31.9	33.4	30.1
Ser	16.3	17.3	18.1	20.0	18.0	20.0	23.2	22.8	22.9	23.0
Glu	4.7	5.0	4.1	4.3	1.0	1.2	2.4	3.8	2.2	4.5
Pro	15.1	14.2	16.0	14.7	18.3	15.8	13.7	12.0	14.2	11.9
His	3.2	0.4	2.8	0.8	2.2	0.1	1.1	0.3	1.2	0.2

[a]The parent glyucoproteins were either native low-mol.wt., ammonium sulphate-soluble, fractions, or Pronase-treated derivatives of higher-mol.wt. fractions ("Pr"). They are fully described by Donald[14]. The products of their metal-catalysed reaction with H_2O ("ox") have been fully described earlier[6,7]. [b]Values are the proportion of peptide in the glycoprotein as percentage (w/w). [c]Values are in μmol of amino acid/100 μmol of total amino acids.

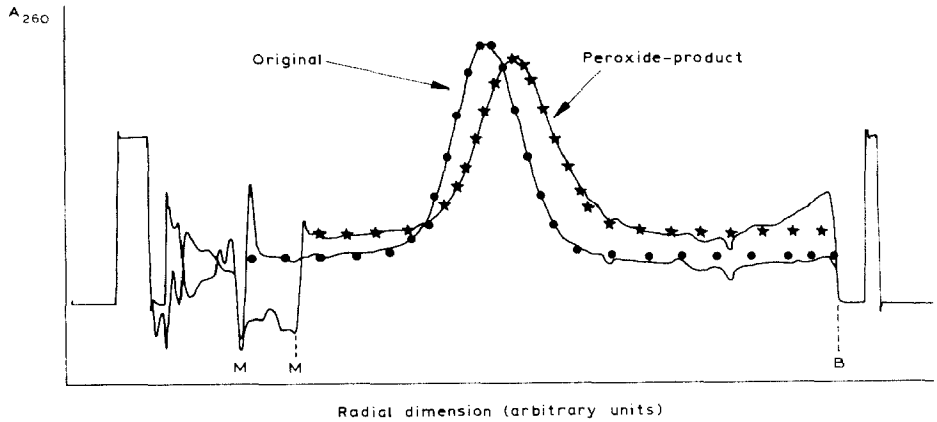

Fig. 1. Density-gradient patterns for glycoprotein 610 and its hydrogen peroxide-treated product, and the corresponding simulations. Ultracentrifuge-recorder traces of u.v.-absorption scans for the glycoprotein and its oxidised derivative (solid lines), with superimposed points (● for original, ★ for product), calculated by the Gaussian-distribution method described in the text. Note the small difference in meniscus positions (denoted *M*) between the two cells; the positions of the cell bottoms (B) were identical.

To sum up, the analysis described above gives further weight to the initial interpretation of the metal-catalysed oxidative degradation of mucus glycoproteins, *i.e.*, that the first step in the reaction is the destruction of backbone histidyl residues with corresponding lysis of the histidyl peptide bond.

Comparison of results on the two native and three Pronase-treated glycoproteins suggested that most of the histidyl residues occur in the glycosylated region[14] of the molecule, rather than in the naked segment. Furthermore, since the molecular-weight changes indicated rather low degrees of fragmentation (only 3 backbone peptide bonds need to be broken to account for the changes in 603AmS, for example, where a total of 15 His residues occur), it follows either that most of the histidine residues exist in groups, or that some are largely protected from attack by extensive local glycosylation. Evidence favouring the latter possibility came from a study of the oxidation in much higher concentrations of Cu^{2+}, where degradation was extensive but no greater loss of histidine occurred.

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REFERENCES

1 D. J. COX AND V. N. SCHUMAKER, *J. Am. Chem. Soc.*, 83 (1961) 2439–2445.
2 R. J. ERIKSON AND W. SZYBALSKI, *Virology*, 22 (1964) 111–122.
3 J. M. CREETH AND M. A. DENBOROUGH, *Biochem. J.*, 117 (1970) 879–891.
4 K. R. BHASKAR AND J. M. CREETH, *Biochem. J.*, 143 (1974) 669–679.
5 J. M. CREETH, K. R. BHASKAR, AND J. R. HORTON, *Biochem. J.*, 167 (1977) 557–569.
6 J. M. CREETH, B. COOPER, A. S. R. DONALD, AND J. R. CLAMP, *Biochem. J.*, 211 (1983) 323–332.
7 B. COOPER, J. M. CREETH, AND A. S. R. DONALD, *Biochem. J.*, 228 (1985) 615–626.
8 J. M. CREETH AND J. R. HORTON, *Biochem. J.*, 161 (1977) 449–463.
9 N. SUEOKA, *Proc. Natl. Acad. Sci. U.S.A.*, 45 (1959) 1480–1490.
10 J. M. CREETH, K. R. BHASKAR, A. S. R. DONALD, AND W. T. J. MORGAN, *Biochem. J.*, 143 (1974) 159–170.
11 J. M. CREETH AND S. E. HARDING, *J. Biochem. Biophys. Methods*, 7 (1982) 25–34.
12 M. MESELSON, F. W. STAHL, AND J. VINOGRAD, *Proc. Natl. Acad. Sci. U.S.A.*, 43 (1957) 581–588.
13 J. B. IFFT AND J. VINOGRAD, *J. Phys. Chem.*, 66 (1962) 1990–1998.
14 A. S. R. DONALD, *Biochim. Biophys. Acta*, 317 (1973) 420–436.