vantage can be overcome by substituting a solute which does not absorb (see Table I) or by using such dilute solutions that the buffer solution alone can be used as a blank. Because of the sensitivity of the method, extreme care must be taken in preparing the dilutions of the unknown solution.

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Received August 25th, 1960

Biochim. Biophys. Acta, 45 (1960) 382–384

The heterogeneity of ovomucoid

This report concerns the behaviour of some ovomucoid preparations on gradient elution from columns of triethylaminoethylammonium-cellulose. In all the four preparations examined, which were obtained by using trichloroacetate to precipitate other egg-white proteins[1,2], it was possible to show the presence of substantial impurities of unrelated proteins, in agreement with the results of an investigation by zone electrophoresis[3]. Indications were also obtained of some degree of heterogeneity of the ovomucoid itself.

The results of one run are shown in Fig. 1. Of the three main peaks emerging in succession as the NaCl concentration is increased, only the second showed antitryptic

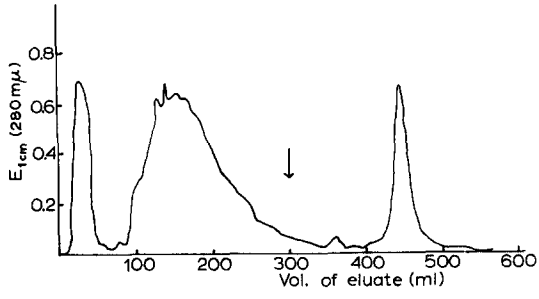

Fig. 1. Chromatography of ovomucoid (preparation B of Table I) on triethylaminoethylammonium-cellulose (Serva Entwicklungslabor, Heidelberg, Germany). The sample (20 ml, E_{1cm} 6.5 at 280 mμ, previously dialysed free of salts) was applied to a column 4.5 cm high and 2 cm diameter, equilibrated before use with 0.004 *M* sodium phosphate buffer (pH 6.8). Elution was effected first by gradient to 0.2 *M* NaCl, 0.004 *M* phosphate (pH 6.8); beginning at the point indicated by the arrow, a second gradient to 1.0 *M* NaCl, 0.004 *M* sodium phosphate (pH 6.8) was applied. The eluate was collected in fractions of 3.4 ml at a rate of 0.3 ml/min. Temperature, 2°.

activity[4] and appreciable amounts of neutral sugar[5]. The first and third peaks therefore contained contaminating materials different from ovomucoid, presumably protein in nature, since they showed absorption maxima near 280 mμ. "Deproteinising" by the SEVAG procedure[6] selectively removed the material of the first peak. The third peak varied most in quantity from one preparation to another (Table I).

TABLE I

CHROMATOGRAPHIC COMPONENTS OF OVOMUCOID PREPARATIONS*

*Preparation***	*A*	*B*	*B****	*C*	*D*
First peak	9	11	1	16	14
Second peak	71	49	62	52	57
Third peak	5	12	9	10	3

* As % of total material applied to column, measured by absorption at 280 mμ. Chromatographic conditions as in Fig. 1.

** Preparations A, B and C were made from fresh hen's egg white by addition of sodium trichloroacetate to a concentration of 5 % at pH 3.5, followed by precipitation of the ovomucoid from the supernatant by 2 vol. 95 % ethanol at 0° and pH 6.0[2]. Preparation D was bought from L. Light and Co, Colnbrook, England, and stated to have been obtained by the trichloroacetic acid–acetone method[1].

*** "Deproteinized" before chromatography by twice shaking overnight at room temperature with 0.25 vol. *n*-pentanol–chloroform (3:1, v/v)[6].

Some degree of heterogeneity of the ovomucoid itself was suggested not only by the broad and asymmetric shape of the second peak, but also by a partial resolution into components in the case of preparation C (Table I), the E_{1cm} at 280 mμ falling to 0.15 at an eluate volume of 143 ml between peaks of 0.28 at 122 ml and 0.48 at 170 ml. After each run, therefore, the fractions composing the early, middle and late parts of the ovomucoid peak were pooled separately to give three approximately equal portions. These pooled fractions were then compared with respect to the following properties:

(*a*) *Antitryptic activity*. The residual trypsin activity, measured[4] with *p*-toluenesulphonyl-L-arginine methyl ester as substrate, was determined as a function of the amount of inhibitor added, expressed in terms of its absorption at 280 mμ; the inhibitory potencies thus found differed by less than the experimental error of approx. 5 % for the different fractions.

(*b*) *Content of neutral sugars*, determined by the absorption at 500 mμ developed with an orcinol–H_2SO_4 reagent[5]. Using solutions adjusted to the same E_{1cm} at 280 mμ, the results for the different fractions differed by less than 5 %.

(*c*) *Nature of the neutral sugars present*. After hydrolysis in 1.0 *N* HCl at 95° for 16 h, the HCl and the bulk of the amino acid and peptide hydrolysis products were absorbed on a column of the anion-exchange resin Amberlite IRA 400 in the bicarbonate form. The sugars remaining in solution were subjected to paper chromatography, using butanol–pyridine–water (6:4:3, v/v/v) as moving phase and aniline hydrogen phthalate to reveal the spots[7]. As judged visually, the 3:1 molar ratio of mannose to galactose found in earlier analyses of ovomucoid[8,9] was unchanged in each fraction.

(*d*) *Sialic acid content*. Using the diphenylamine method[10] with N-acetylneura-

minic acid as standard, and assuming that an E_{1cm} of 0.45 at 280 mμ corresponds to a concentration of 1 mg/ml for all fractions, the values for the sialic acid content ranged from 0.9–1.3 % for the early fractions from various runs, while the late fractions gave higher values of 1.8–2.3 %. These results suggested that the basis of the heterogeneity might lie in variations in sialic acid content, those molecules containing more of the acid being held more firmly by the anion exchanger. However, with the "direct Ehrlich" reaction[11], no fraction gave a value greater than 0.5 % sialic acid, which corresponds to appreciably less than one residue per molecular weight of approx. 28,000[1,2]. This matter cannot therefore be regarded as settled.

Heterogeneity of ovomucoid was first indicated by the observation that it exhibits reversible boundary spreading in moving-boundary electrophoresis[12], and at the unusually low ionic strength of 0.01, resolution into components has been observed[2,13]. By zone electrophoresis at an ionic strength of 0.1, however, no signs of such heterogeneity were found.[3] Recently, fractions of ovomucoid have been obtained by elution from carboxymethylcellulose between pH 3.5 and 4.3 (ref. 14).

The work reported here suggests a simple batch procedure for the purification of ovomucoid, which has been successfully applied to 2 g preparation B on a column of 20 g triethylaminoethylammonium-cellulose. Conditions were as described for Fig. 1 except that, instead of elution by gradient, contaminants were washed out with 0.004 *M* sodium phosphate buffer (pH 6.8) containing 0.01 *M* NaCl, and the ovomucoid was then eluted by raising the NaCl concentration to 0.2 *M*. Analysis of this material by gradient elution showed no trace of the first and third peaks given by crude preparations. The similarity in the properties of the early and late fractions described above is ground for believing that the ovomucoid so prepared is substantially free of contamination by unrelated protein.

I wish to acknowledge the willing technical assistance of Mr. A. D. LLOYD and, in part of the work, of Mrs. M. FORD DE SANTOS.

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Received August 8th, 1960