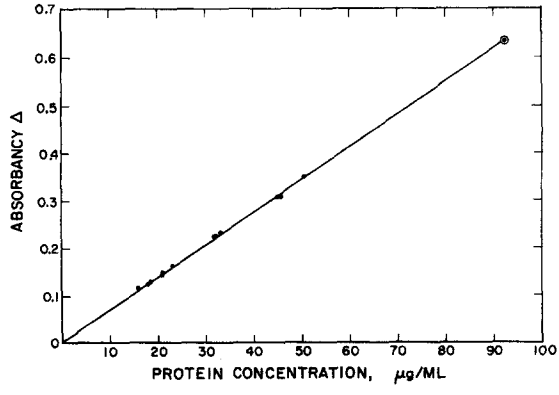
Note on spectrophotometric determination of proteins in dilute solutions

One of the most common methods of measuring protein concentrations involves determination of the absorption of the protein solution at 260–280 mμ[1]. While useful, this procedure has several major limitations: (1) proteins vary with respect to the concentrations of aromatic amino acids which absorb at these two wavelengths and therefore vary with regard to their molar absorbancies; (2) the method is insensitive to protein concentrations of less than 100 μg/ml. The colorimetric method of LOWRY *et al*[2] is more sensitive but it is also more time consuming. WESTLEY *et al*[3] have recently proposed another procedure useful in the range of 0.01–1.0 mg but also requiring preparation of reagents, development of color, etc. WADDELL'S procedure[4] for determining microgram quantities of protein eliminates both objections (lack of sensitivity and need for the color development). The purpose of the present communication is to emphasize the usefulness of the latter method. It is rapid, accurate and can be used with very dilute protein solutions such as are frequently encountered in chromatographic separations. SPIES *et al*[5] have also shown that absorption in this region of the u.v. is proportional to protein concentration. BENDIXIN[6] has reported that WADDELL'S procedure for blood and cerebrospinal fluid proteins compares satisfactorily with either Kjeldahl nitrogen values or LOWRY'S colorimetric method.

WADDELL'S method has been used routinely in this laboratory for some time. WARBURG AND CHRISTIAN'S method[7] for determining protein concentrations from the 280 mμ/260 mμ absorbancy ratio could not be used with solutions of brain proteins, perhaps because of their low aromatic amino acid contents. In addition, the latter method was not sensitive enough for following chromatographic fractionation of small quantities of protein. In order to demonstrate the usefulness of WADDELL'S procedure, protein solutions made from a Versatol** standard have been utilized for much of the data presented. A Beckman DU spectrophotometer was used to measure the absorbancy of these solutions at 215 and 225 mμ.

As seen in Fig. 1, Beer's Law was obeyed over a concentration range of 20–100 μg/ml. We have obtained similar results using either albumin or a guinea-pig-brain



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** Obtained from Warner-Chilcott, Laboratory Supply Division, New York, N.Y.

protein as standards. The ordinate in Fig. 1, *i.e.*, absorbancy Δ, is the difference between the absorbancies at 215 and 225 mμ.

Table I lists the various buffers which have been found to be satisfactory. Because of the high absorption at 215 mμ, acetate, succinate, citrate, phthalate and barbiturate buffers cannot be used in 0.1 *M* concentration. With proper blanks, 0.005 *M* solutions of all these buffers are satisfactory.

TABLE I

SATISFACTORY BUFFERS FOR DETERMINATION OF PROTEIN CONCENTRATION FROM 215–225 mμ ABSORPTION

Protein	*Buffer*	*Protein concentration (mg/ml)*	
		Weight	*u.v. data*
Versatol	0.1 *M* borate (pH 9.8)	10	10.1
Versatol	0.1 *M* phosphate (pH 7.2)	10	9.1
Versatol	0.1 *M* tris* (pH 8.8)	10	9.2
Versatol	0.005 *M* acetate (pH 5.1)	10	9.2
Versatol	0.005 *M* succinate (pH 6.3)	10	9.1
Albumin	0.01 *N* HCl	10	9.4
Brain protein	0.1 *M* phosphate (pH 7.3)	13.5	13.7

* Tris(hydroxymethyl)aminomethane.

STRICKLAND *et al.*[7] have said that this method was not satisfactory when applied to electrophoretically separated fractions of serum proteins. Their protein fractions were dissolved in 0.1 *N* NaOH which cannot be used as a solvent in WADDELL'S procedure because of its high absorption in this region of the spectrum. This objection while valid for solutions which contain 0.1 *N* NaOH would not apply if 0.005 *N* alkali was used (Table II). NaCl and $(NH_4)_2SO_4$ solutions commonly encountered in protein-fractionation procedures do not interfere. This method has been utilized quite successfully in following the chromatographic separation of guinea-pig-brain proteins on CM-cellulose columns. For eluting, a NaCl gradient (0–1.0 *M*) was used in 0.005 *M* sodium acetate buffer (pH 5.1).

This spectrophotometric method has great advantages over other procedures in simplicity, rapidity and sensitivity. Since $(NH_4)_2SO_4$ does not interfere with the determination, it would be particularly useful in following salt fractionations.

The only observed limitation of the method is that many salts and acids commonly present in protein solutions absorb in this region of the spectrum. This disad-

TABLE II

DETERMINATION OF PROTEIN CONCENTRATION BY U.V.-ABSORPTION; COMPARISON OF WADDELL'S METHOD WITH THE TECHNIQUE OF WARBURG AND CHRISTIAN

Protein	*Buffer*	*Protein concentration (mg/ml)*		
		Weight	WARBURG AND CHRISTIAN[1]	WADDELL[4]
Albumin	0.005 *M* acetate	18	14.7	17.5
Albumin	0.005 *N* NaOH	18	10.1	17.8
Albumin	0.1 *M* borate	18	11.7	17.5

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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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

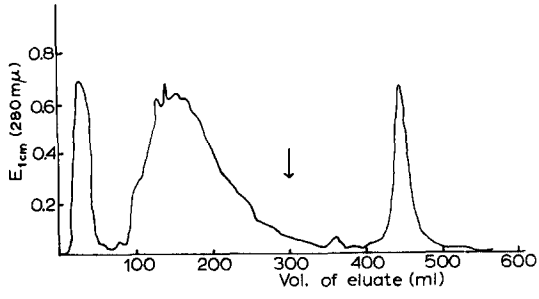

Fig. 1. Chromatography of ovomucoid (preparation B of Table I) on triethylaminoethylammonium-cellulose (Serva Entwicklungslabor, Heidelberg, Germany). The sample (20 ml, E_{1cm} 6.5 at 280 mμ, previously dialysed free of salts) was applied to a column 4.5 cm high and 2 cm diameter, equilibrated before 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°.