Spectrophotometric estimation of protein in presence of ultraviolet-absorbing impurities

Several workers have recommended the spectrophotometric estimation of serum proteins at wavelengths in the region of 200–220 mμ (TOMBS *et al.*[1], MURPHY AND KIES[2]). This communication emphasizes the specificity of this procedure for protein in presence of certain ultraviolet-absorbing impurities. The following experiment is presented as an example of the use of this procedure in the chromatographic fractionation of proteins from a crude tissue extract.

An extract of wheat leaves was prepared as described by WRIGLEY AND WEBSTER[3] and freed of low molecular weight compounds by passage through Sephadex G-50. When this preparation was fractionated by column chromatography on DEAE-cellulose, a series of peaks (at fractions 14, 20 and 26) appeared in the profiles of

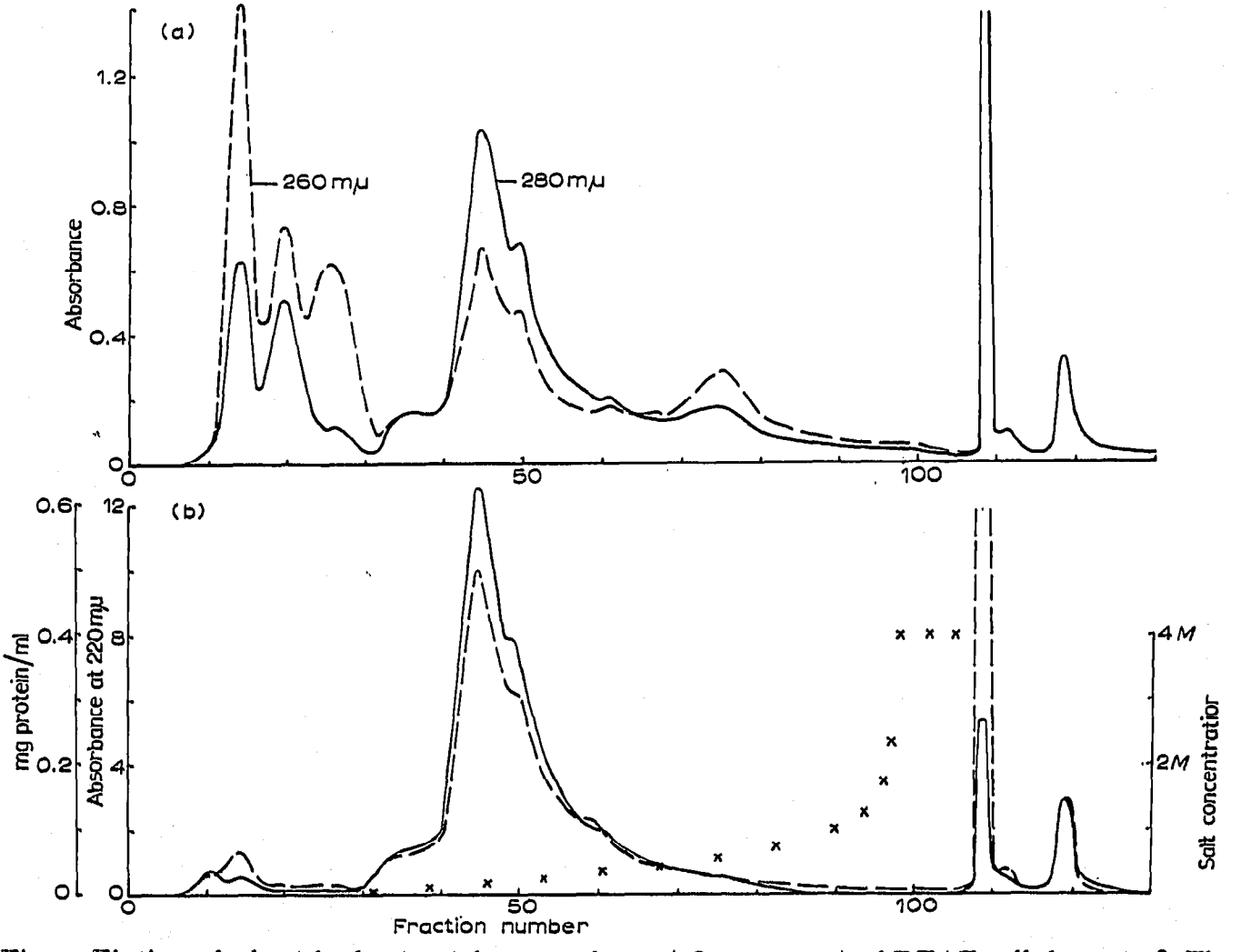

Fig. 1. Elution of wheat leaf extract from a column (38 × 0.9 cm) of DEAE-cellulose at 4°. The sample (36 mg protein) was applied in 9 ml 0.01 *M* Tris adjusted to pH 7.5 with hydrochloric acid. The column was eluted with a gradient of sodium chloride (effluent concentration indicated by crosses) in 0.01 *M* Tris pH 7.5. Fraction 108 corresponds to the start of elution by 0.1 *N* sodium hydroxide. The flow rate was 0.65 ml/min. Fractions were collected at 5 min intervals. (a) Broken line, absorbance at 260 mμ; solid line, absorbance at 280 mμ. (b) Broken line, absorbance at 220 mμ; solid line, protein concentration estimated by the method of LOWRY *et al.*[4]

absorbance at 280 and 260 mμ (Fig. 1a) prior to salt elution. These fractions probably contained nucleic acid and were essentially free of protein. Colorimetric analysis indicated that protein was virtually absent, no amido black staining was observed after these fractions were submitted to disc electrophoresis (according to WRIGLEY AND WEBSTER[3]) and the spectra of fractions 14, 20 and 26 were similar to the typical nucleic acid spectrum (MUNRO AND FLECK[5]). The absorption spectra of several other chromatographic components (at fractions 37 and 76) also indicated the presence of non-protein ultraviolet-absorbing materials. It is therefore apparent that an elution profile based on absorbance at 280 or 260 mμ gives a misleading indication of protein elution.

On the other hand, the profile of absorbance at 220 mμ (Fig. 1b) was almost identical to that of protein determined colorimetrically, despite the presence of large quantities of non-protein material. Furthermore, protein absorbance at 220 mμ was about ten times higher than at 280 mμ. This meant that by measuring absorbance at 220 mμ protein elution could be followed satisfactorily for a column loaded at a tenth of the usual level without interference from non-protein contaminants. The reduced loading produced better chromatographic resolution. For leaf extracts, prior removal of low molecular weight material was important because of its high absorbance at 220 mμ. (This material also gave high colour yield in the colorimetric estimation of protein.)

The absorbance of relatively pure protein rises rapidly at wavelengths shorter than 240 mμ. In this region, the absorbance of nucleic acid is relatively low. By plot-

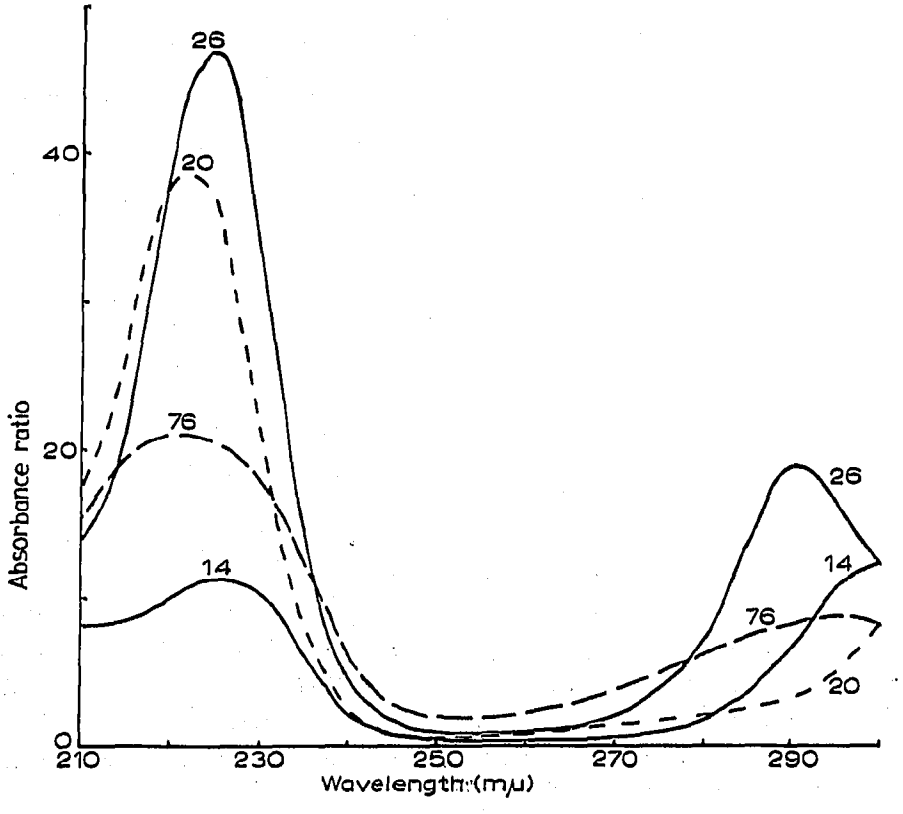

Fig. 2. Plot of wavelength against the ratio of absorbance of fraction 45 to absorbance of each of fractions 14, 20, 26 and 76, from chromatography of leaf extract on DEAE-cellulose (Fig. 1).

ting the ratio of absorbance of fraction 45 (relatively pure protein) to that of various non-protein fractions at a range of wavelengths (Fig. 2) it is evident that, in the region 220 to 225 mμ, absorbance due to protein reaches a maximum relative to absorbance due to contaminants. Absorbance in this region should therefore be reasonably specific for protein and also be very sensitive.

TOMBS *et al.*[1], in recommending spectrophotometric determination of serum protein at 210 mμ as an extremely sensitive procedure, pointed out that absorbance in this region is largely due to the peptide bond. Consequently, proteins from different sources have similar absorption coefficients at this wavelength. Instrumental limitations to analysis at these wavelengths have been discussed by SAIDEL *et al.*[6] and TOMBS *et al.*[1] The use of this procedure places certain restrictions on the choice of buffers. The strong absorbance of succinate, phthalate and barbiturate makes estimations at 220 mμ difficult in their presence. Sodium hydroxide, acetate, glycine and Tris can be used at a concentration of 0.01 *M* and sodium chloride, cacodylate, borate, phosphate and ammonium sulphate are satisfactory up to and above 0.1 *M*. The absorbance of protein at 220 mμ accords with Beer's Law up to 2.0 and is virtually independent of pH between values of 3 and 11.

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Received October 31st, 1967

J. Chromatog., 33 (1968) 534–536

Fractionation of indole compounds on Sephadex G-10*

GELOTTE[1] and PORATH[2] observed that aromatic amino acids and other aromatic compounds are adsorbed to Sephadex and consequently pass through the column slower than non-aromatic compounds of comparable size. Sephadex should adsorb the clavine alkaloids and other indole-containing compounds, since these compounds are aromatic. It was found that the indole-containing fermentation products of *Claviceps purpurea* PRL 1980 are separated from carbohydrates and salts in the fermentation medium and are themselves separated into fractions on a Sephadex G-10 column.

* This research was supported by the Robert A. Welch Foundation Grant No. D-117 and by State-Supported Research Grant, Account No. 191–4762.