to the amount of phosphorus in the phospholipid fractions. Previous investigations[16] have shown that the sensitivity and range of the method permit the analysis of phospholipids in tissue extracts.

Acknowledgements

This investigation has been made possible through a 'Charles Nicolle' Scholarship from the Pasteur Institute of Tunis. I wish to thank Dr. M. KCHOUK for helpful advice and consultation.

Department of Chemistry, Pasteur Institute of Tunis (Tunesia) N. M. NESKOVIC*

1 D. ABRAMSON AND M. BLECHER, *J. Lipid Res.*, 5 (1964) 628.
2 V. P. SKIPSKI, R. F. PETERSON AND M. BARCLAY, *Biochem. J.*, 90 (1964) 374.
3 L. SEMINARIO DE BOHNER, E. F. SOTO AND T. DE COHAN, *J. Chromatog.*, 17 (1965) 513.
4 H. WAGNER, *Fette, Seifen, Anstrichmittel*, 63 (1961) 1119.
5 E. HABERMANN, G. BANDTLOW AND B. KRUSCHE, *Klin. Wochschr.*, 39 (1961) 816.
6 W. M. DOIZAKI AND L. ZIEVE, *Proc. Soc. Exptl. Biol. Med.*, 113 (1963) 91.
7 H. JATZKEWITZ, *Z. Physiol. Chem.*, 336 (1964) 25.
8 LJ. RAKIC, D. KOSTIC AND N. NESKOVIC, unpublished.
9 O. S. PRIVETT AND M. L. BLANK, *J. Lipid Res.*, 2 (1961) 37.
10 O. S. PRIVETT, M. L. BLANK AND W. O. LUNDBERG, *J. Am. Oil Chemists' Soc.*, 38 (1961) 312.
11 S. N. PAYNE, *J. Chromatog.*, 15 (1964) 173.
12 Y. POMERANZ AND O. CHUNG, *J. Chromatog.*, 19 (1965) 540.
13 M. L. BLANK, J. A. SHMIT AND O. S. PRIVETT, *J. Am. Oil Chemists' Soc.*, 41 (1964) 371.
14 H. JATZKEWITZ, *Z. Physiol. Chem.*, 326 (1961) 61.
15 K. RANDERATH, *Thin-Layer Chromatography*, Verlag Chemie, Weinheim and Academic Press, New York, London, 1963, p. 63.
16 N. NESKOVIC, *Arch. Inst. Pasteur Tunis*, 43 (1966) 513.

Received October 11th, 1966

* Present address: Institute of Biochemistry, Faculty of Medicine, Visegradska 26, Beograd, Yugoslavia.

The separation of collagen molecular subunits by gradient elution chromatography

PIEZ and co-workers[1–3] have developed a simple linear gradient technique using acetate buffers for the elution of the α-, β- and γ-subunits of heat-denatured collagen or parent gelatin from CM-cellulose. Experiments have shown that their method does not give a complete picture of the subunit structure, particularly with acid-soluble collagen. In many cases incomplete resolution has also been reported (see for example TRISTRAM, WORRALL AND STEER[4]). Further, the column effluent from the chromatographic separation is monitored either by chemical analysis or by optical absorption in the region of 230 mμ. In the latter case the use of acetate buffers can cause difficulties, as these have a high absorption at this wavelength, and any small changes in acetate concentration can effect the stability of the baseline. The separation and properties of these subunits are most important in determining collagen structure, therefore, the gradient elution system reported here could have important applications.

Experimental

The preparation and purification of the neutral salt-soluble and acid-soluble collagen from bovine calf-skin has been described previously[5].

For the chromatography, a water-jacketed column with adjustable pistons at each end (LKB Produkter) was filled with CM-cellulose (Schleicher and Schull, Batch No. 16064) to give a bed 3.3 × 33 cm. The gradient was produced in a nine-chamber Varigrad (Buchler Instruments). The buffer was pumped on to the column by means of a constant flow pump (Beckman Instruments) at 100–150 ml/h. The column effluent was passed through the flow cell (1 cm) of a recording spectrophotometer (Beckman Model DB) set at 230 mμ. The effluent was then collected in 10 ml aliquots in a refrigerated fraction collector (Beckman Model 132). The collagen was dissolved in the starting buffer, to give solutions containing about 100 mg/40 ml, heat-denatured at 40° or 45°, and pumped on to the column kept at the temperature of denaturation. It was found that the collagen was more soluble in the initial phosphate buffer of the gradient than in the acetate buffer of PIEZ *et al.*[1].

Both upward and downward buffer flow in the column were tried, the latter being found to give the best results.

Results

Early experiments using the system of PIEZ *et al.*[1] showed that their gradient did not elute all the protein from the column[5]. A step-wise gradient with acetate buffers of higher ionic strength made it possible to resolve thermally denatured collagen into the α- and β-subunits, plus four larger components, designated A,B,C and D[6]. Optical rotation and sedimentation data[6] showed these to be of relatively high molecular weight and having the high helical contents characteristic of collagen.

For the reasons given above regarding the use of acetate buffers, and because step-wise gradients require manual changing of the buffers at specific points, a gradient elution system using phosphate buffers was developed. This uses 250 ml of buffer per chamber of the Varigrad as follows:

Chamber 1 and 2: 0.05 *M* NaH_2PO_4 + 0.05 *M* NaCl

Chamber 3: 0.05 *M* NaH_2PO_4 + 0.2 *M* NaCl

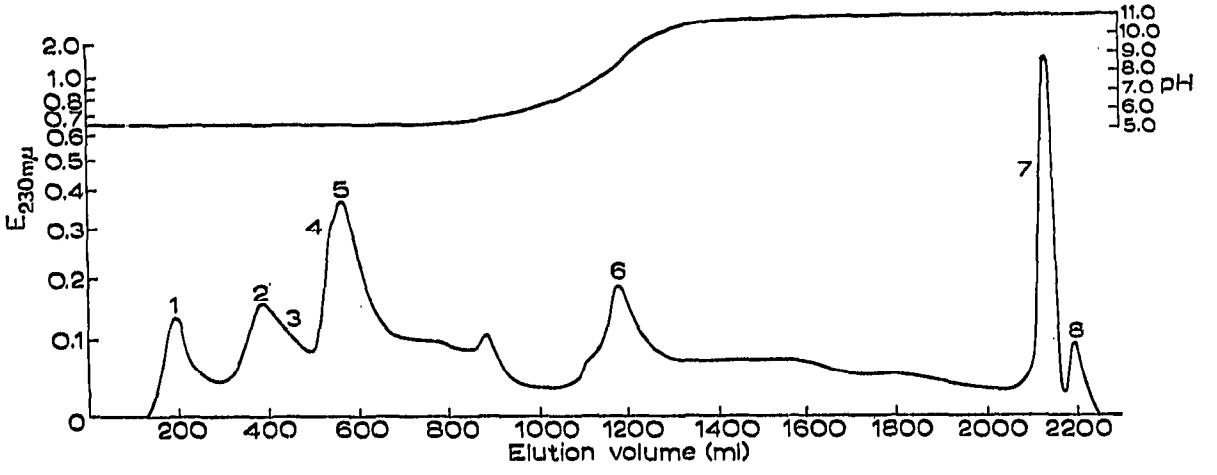

Fig. 1. Chromatography of 98 mg of acid-soluble collagen on CM-cellulose at 40° using a phosphate buffer gradient (see text). The upper curve shows the pH gradient produced.

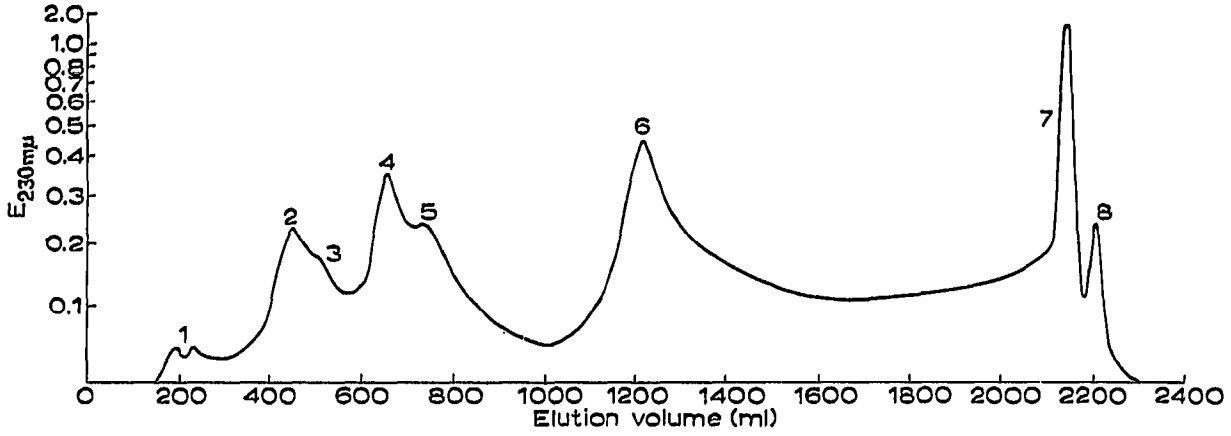

Fig. 2. Chromatography of 101 mg of acid-soluble collagen on CM-cellulose at 45° using a phosphate buffer gradient (see text).

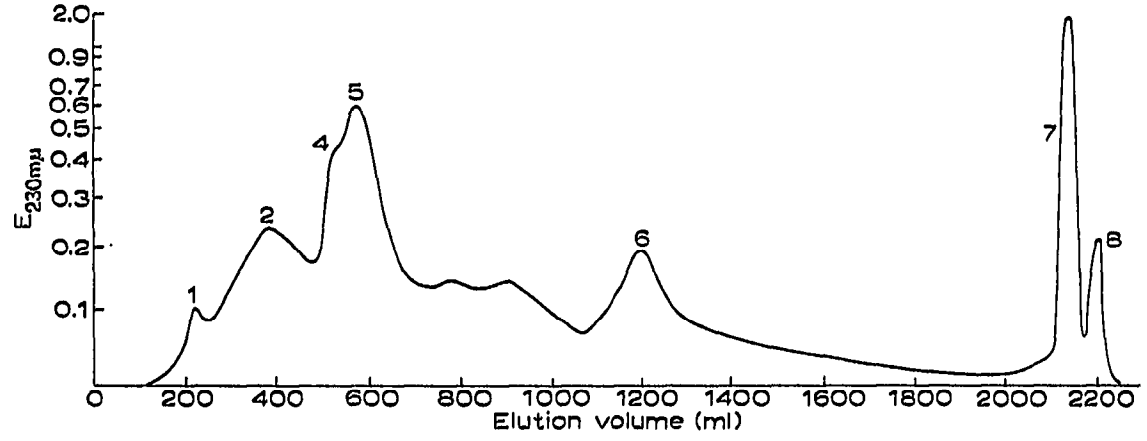

Fig. 3. Chromatography of 104 mg of neutral salt-soluble collagen on CM-cellulose at 45° using a phosphate buffer gradient (see text).

Chamber 4 and 5: 0.05 *M* Na_2HPO_4 + 0.2 *M* NaCl + 0.02 *M* NaOH
Chamber 6 and 7: 0.05 *M* Na_2HPO_4 + 0.2 *M* NaCl + 0.04 *M* NaOH
Chamber 8 and 9: 0.05 *M* Na_2HPO_4 + 0.5 *M* NaCl + 0.06 *M* NaOH

Elution was continued until two litres of the buffer was used. The column was then eluted with a further 500 ml of the last buffer only. This system includes both a salt and a pH gradient. The gradient was arranged so that the pH remained constant at 5.1 until a higher salt content than that recommended by PIEZ *et al.*[1] for eluting the α- and β-subunits had passed through the column. The pH was then gradually increased (Fig. 1) to 11.0, at which value the remaining protein was removed from the column. Using this gradient without any protein on the column gave a very steady baseline for the first litre of eluent. The baseline then gradually rose a total of 0.07 absorption units for the remainder of the run. The chromatograms shown have been corrected for this slight shift in the baseline.

The use of this gradient in the separation of the subunits of neutral salt-soluble and acid-soluble collagen at 40° and 45° is illustrated in Figs. 1, 2 and 3. These show that in the region of nine components are present in each case. From a comparison of these results with those from the acetate gradient, it is evident that component 1 in

all three chromatograms corresponds to the fore-peak shown in most elution curves of heat-denatured collagen[1,5,7]. Since components 2, 3, 4 and 5 are eluted under similar conditions of ionic strength and pH to those used with acetate buffers[1,2,7], they must correspond to the α_1-, β_{11}-, β_{12}- and α_2-subunits, respectively. The presence of γ-components can be ignored since these were shown by sedimentation to be present in very small amounts[5].

The chromatograms in Figs. 1 and 2 illustrate the effect of denaturation and column temperature on the separation. It is evident from these that at 45° there is more of component 6 and the later components, than obtained at 40°. These findings are in contrast to those of TRISTRAM *et al.*[4] who found more of the components eluting with the strong buffers (their components 3 and 4) at lower temperatures. Their component 4 would correspond to components 7 and 8 in the present case, since they are all removed from the column with sodium hydroxide solution, and their component 3 to component 6 in the current separation. The components eluting at the higher ionic strength and pH probably correspond to the polymeric forms suggested by several authors from chromatography[4,11], gel-electrophoresis[8,9] and other studies[10]. If components 7 and 8 are polymeric forms, they must be relatively stable in the range 40 to 45°, as they remained on the chromatographic column at these temperatures for approximately 24 h before being eluted. This is in agreement with the findings of HOLLMEN AND KULONEN[8] who showed by gel-electrophoresis that the collagen components did not change essentially after 24 h heating at 40°, but that after one week of heating the amount of the slow-moving fractions, corresponding to the polymeric materials, was reduced. The physical and chemical properties of these chromatographic components are being studied.

This work was financed by grants from the Livestock and Meat Industries Control Board and the Council for Scientific and Industrial Research.

Leather Industries Research Institute,
Rhodes University, Grahamstown
(Republic of South Africa)

D. R. COOPER
R. J. DAVIDSON

1 K. A. PIEZ, E. A. EIGNER AND M. S. LEWIS, *Biochemistry*, 2 (1963) 58.
2 K. A. PIEZ, *J. Biol. Chem.*, 239 (1964) 4315.
3 P. BORNSTEIN AND K. A. PIEZ, *J. Clin. Invest.*, 43 (1964) 1813.
4 G. R. TRISTRAM, J. WORRALL AND D. C. STEER, *Biochem. J.*, 95 (1965) 350.
5 D. R. COOPER AND R. J. DAVIDSON, *Biochem. J.*, 97 (1965) 139.
6 R. J. DAVIDSON AND D. R. COOPER, *J. S. African Chem. Inst.*, in press.
7 H. HEIDRICH AND L. K. WYNSTON, *Z. Physiol. Chem.*, 342 (1965) 166.
8 T. HOLLMÉN AND E. KULONEN, *Acta Chem. Scand.*, 18 (1964) 1027.
9 J. PIKKARAINEN AND E. KULONEN, *Acta Chem. Scand.*, 18 (1964) 2011.
10 P. F. DAVISON AND M. P. DRAKE, *Biochemistry*, 5 (1966) 313.
11 A. VEIS AND J. ANESEY, *J. Biol. Chem.*, 240 (1965) 3899.

Received October 5th, 1966