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Purification and Amino Acid Composition of Monomeric and Polymeric Collagens

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The preparations and amino acid compositions of highly purified tropocollagen and insoluble polymerized collagen are described. These collagens appear to be very suitable for comparative studies in an investigation of the cross-linkages that are introduced into tropocollagen during the formation of polymerized collagen.

The first requirement for chemical structural studies on a protein is that it should be pure. In studies on tropocollagen, Piez, Lewis, Martin & Gross (1961) and Bornstein & Piez (1965) have satisfied the criteria of purity by denaturing the tropocollagen and isolating the purified α- and β-chains before subjecting these to chemical degradation techniques. Gallop (1955) purified ichthyocol, the tropocollagen of carp swim bladder, in a form suitable for structural analysis (Blumenfeld & Gallop, 1966).

The isolation of pure insoluble collagen in the native state has proved to be difficult. Eastoe (1955) considered that the purest form of insoluble collagen for amino acid analysis could only be obtained after gelatinization and further purification of the derived gelatin. Gelatinization was usually carried out on insoluble collagens that had previously been subjected to a pretreatment with acid, alkali or denaturing agent (Eastoe, 1955; Veis & Cohen, 1956, 1958; Veis, Anesey & Cohen, 1961; Veis & Anesey, 1965; Fleischmajer & Fishman, 1965). Other authors (Bowes, Elliott & Moss, 1953, 1957; Drake, Davison, Bump & Schmitt, 1966) have attempted to prepare pure native insoluble collagen by exhaustive extraction of non-collagenous materials. A comparison of the amino acid analyses of these insoluble collagens and the derived gelatins indicates that the insoluble collagens still contain some non-collagenous matter. In particular, a tyrosyl- and hexosamine-rich protein (Bowes, Elliott & Moss, 1958) cannot easily be removed completely from insoluble collagen. The report by Jackson, Leach & Jacobs (1958) that their purest form of tropocollagen contained no hexosamine is of special relevance.

The literature quoted above suggests that the complete removal of non-collagenous proteins is only possible by chemical methods that cause some destruction of the collagen structure. It would be preferable for chemical structural studies to be made on native insoluble collagen in a form as pure as the best derived gelatins.

Previous studies (Steven, 1964) suggested that the use of crude α-amylase from *Bacillus subtilis* in a pretreatment of connective tissue might provide a satisfactory way of overcoming these purification problems.

The present paper reports the preparation and amino acid analysis of tropocollagen isolated by conventional techniques and insoluble polymerized collagen fibrils isolated by this enzymic technique.

MATERIALS

Skin was obtained from a 5-day-old calf for the preparation of acetic acid-soluble tropocollagen. Polymerized collagen was obtained from mature human and bovine Achilles tendon by employing a pretreatment with crude bacterial α-amylase, supplied by Cambrian Chemical Co., London, S.E. 16.

METHODS

Preparation of acetic acid-soluble tropocollagen. Crude acetic acid-soluble calf-skin collagen was prepared (Steven & Tristram, 1962) followed by an ethanol purification procedure based on the scheme presented by Jackson & Cleary (1967). The tropocollagen was dissolved in 0·1 N-acetic acid and precipitated by the addition of precooled ethanol (−20°) to give a final ethanol concentration of 30% (v/v). The precipitate of tropocollagen was redissolved in 0·1 N-acetic acid, brought to pH 7 with N-NaOH and precooled ethanol added to give a final ethanol concentration of 14% (v/v). The collagen was precipitated by centrifugation and a sample removed for amino acid analysis. A study of the amino acid composition of collagen fractions obtained during the above procedure indicated that the precipitation technique removed physically associated non-collagenous materials from the tropocollagen, yielding a cleaner preparation of tropocollagen at each successive stage of this preparation.

Preparation of polymerized collagen. Achilles tendons taken from aged humans at post mortem and 3-year-old cows were treated with crude bacterial α-amylase (Steven,

1964) and the fibrils of polymerized collagen dispersed in dilute acetic acid. Fibrils were collected by adjusting the pH to 6–7 with N-NaOH and were then purified by redispersing in acetic acid and precipitating six times with addition of saturated NaCl to give a final concentration of approx. 5% (w/v) NaCl. This procedure was designed to remove physically associated non-collagenous materials.

Electron-microscopic examination of the polymerized collagen fibrils. Polymerized collagen was dispersed in 0·1 N-acetic acid and a small sample placed on carbon-coated grids. The excess of liquid was drained off and the grids were subjected to shadowing with gold–palladium at an angle of 15°. Other grids were stained positively and negatively with 1% phosphotungstic acid or 1% $Pb(NO_3)_2$.

The purity of the polymerized collagen was further checked by determining the quantity of impurity remaining as an insoluble residue (Jackson & Cleary, 1967) after heating polymerized collagen in 5% (w/v) trichloroacetic acid for 30 min. at 100°. On cooling a small quantity of flocculent precipitate was removed by centrifugation and washed clean of the supernatant gelatin. The gelatin and insoluble residue were then submitted to Kjeldahl digestion and distillation, and samples were employed for nitrogen analysis by nesslerization. The impurity represented 0·4% of the total nitrogen of the original polymerized collagen.

Hydrolysis of tropocollagen and polymerized collagen samples. Samples of collagen were hydrolysed under N_2 in sealed glass tubes at 100° for 24 hr. according to the method described by Eastoe (1955). The hydrolysates were filtered and the excess of HCl was removed by rotary evaporation at 50°. Four samples, from each collagen hydrolysate, containing approx. 15 μmoles of mixed amino acids were analysed. The three best analyses were averaged to provide the results expressed in Table 1. Analyses were carried out on a Technicon autoanalyser, with a resin column (125 cm. high × 0·6 cm. internal diam.) with 5 ml. of added methanol to each of the first two Autograd chambers and 'chromobeads type A'. The Technicon standard amino acid mixture with the inclusion of hydroxyproline, norleucine, ornithine and hydroxylysine was employed to identify and estimate the peaks of each individual amino acid in the collagen hydrolysates. The losses of threonine, serine and glutamic acid have been calculated on the basis of 97, 95 and 97% recoveries respectively (Eastoe, 1955). Values for NH_3 tended to rise for each successive analysis of a particular hydrolysate, indicating atmospheric contamination with NH_3. For this reason these values have been excluded from Table 1.

Methionine and cystine chromatographed as a single peak, but, since the collagen samples only yielded a trace amount of this peak, it was not considered worth while to estimate these residues individually. Oxidation products of methionine have not been estimated in the present work. Synthetic hydroxylysine (obtained from L. Light and Co. Ltd., Colnbrook, Bucks., and Sigma Chemical Co., St Louis, Mo., U.S.A.) chromatographed as a double peak, presumably the partially resolved *n*- and allo-isomers; in this study the two peaks are presented as a combined value and referred to as 'hydroxylysine'. These two peaks are mentioned further in the Discussion section.

RESULTS

Electron-microscope pictures of polymerized collagen are presented in Plates 1–3. The amino acid composition of acid-soluble tropocollagen and

Table 1. *Composition of monomeric and polymeric collagens*

Amino acid	Composition (residues/1000 residues)			
	Tropocollagen (calf skin)	Polymerized collagen (bovine Achilles tendon)	Polymerized collagen (human Achilles tendon)	Acid-processed gelatin* (human Achilles tendon)
Hydroxyproline	93·7	84·6	89·6	92·1
Aspartic acid	50·2	46·1	46·5	48·4
Threonine	19·0	16·9	17·1	18·5
Serine	39·4	33·5	36·8	36·9
Glutamic acid	79·5	76·8	81·6	72·3
Proline	120·8	120·2	121·9	126·4
Glycine	331·9	336·7	337·0	323·7
Alanine	108·9	113·2	109·0	110·7
Valine	14·6	21·1	19·0	25·4
Methionine + Cystine	Trace	Trace	Trace	5·7
Isoleucine	9·1	12·3	9·7	11·1
Leucine	27·1	25·0	26·1	26·1
Tyrosine	4·5	2·8	3·4	3·6
Phenylalanine	12·8	13·8	12·8	14·2
Hydroxylysine	5·6	9·9	9·2	8·9
Ornithine	2·5	4·7	1·9	—
Lysine	26·9	25·0	22·4	21·5
Histidine	4·7	5·7	6·1	5·0
Arginine	48·7	51·3	49·4	49·0
	999·9	999·1	999·5	999·9

* Results taken from Eastoe (1955) for acid-processed gelatin.

two samples of polymerized collagen are presented in Table 1 together with Eastoe's (1955) results for gelatin derived from human tendon after acid pretreatment.

DISCUSSION

The appearance of the polymerized collagen fibrils in the electron microscope clearly indicate that it is a highly purified form of collagen, containing only little (if any) adhering ground substance and possessing all the staining characteristics of native insoluble collagen.

The total amino acid composition of polymerized collagen samples and Eastoe's (1955) results for the derived gelatins agree remarkably well. Since the latter were derived from the purest form of gelatin, it is suggested that polymerized collagen must also be a highly pure form of collagen.

Some minor differences are worth discussing at this stage. The present results indicate slightly more hydroxyproline and glycine in polymerized collagen than in the acid gelatin. The hydroxyproline values are always open to criticism (Piez & Morris, 1960). Similarly, glycine values must be considered to be a close approximation rather than absolute values, owing to the excessive quantity of the amino acid that must be estimated in collagen hydrolysates. With these qualifications in mind, the present results indicate that the polymerized collagen is at least as pure, if not purer than, Eastoe's (1955) gelatin.

In the purest samples of tropocollagen examined in this work, only one peak appeared in the position of hydroxylysine, whereas all preparations of polymerized collagen yielded two peaks chromatographing in the positions of *n*- and allo-hydroxylysine. The appearance of the second peak in polymerized collagen corresponding to allo-hydroxylysine does not account for the increase in the total hydroxylysine residues found in polymerized collagen relative to tropocollagen. This increase in hydroxylysine with polymerization cannot be explained at present, although Fleischmajer & Fishman (1965) also found a similar increase in total hydroxylysine in human skin gelatins compared with tropocollagen.

Ornithine was detected in all collagen hydrolysates examined, confirming the earlier work by Jackson *et al.* (1958). Ornithine is usually considered to be derived from alkaline degradation of arginine, but this explanation is not acceptable in the present work since alkaline pretreatment has been avoided.

It may be concluded that the pretreatment of tendon with crude α-amylase appears to provide a highly purified preparation of native polymerized collagen fibrils as judged by electron-microscope and amino-acid analysis. This polymerized collagen fulfils all requirements for chemical studies on the modifications that must take place in tropocollagen to polymerize into polymerized collagen. Work has already been presented on the selective rupture of the polypeptide chains at or near the site of cross-linking (Steven, 1966).

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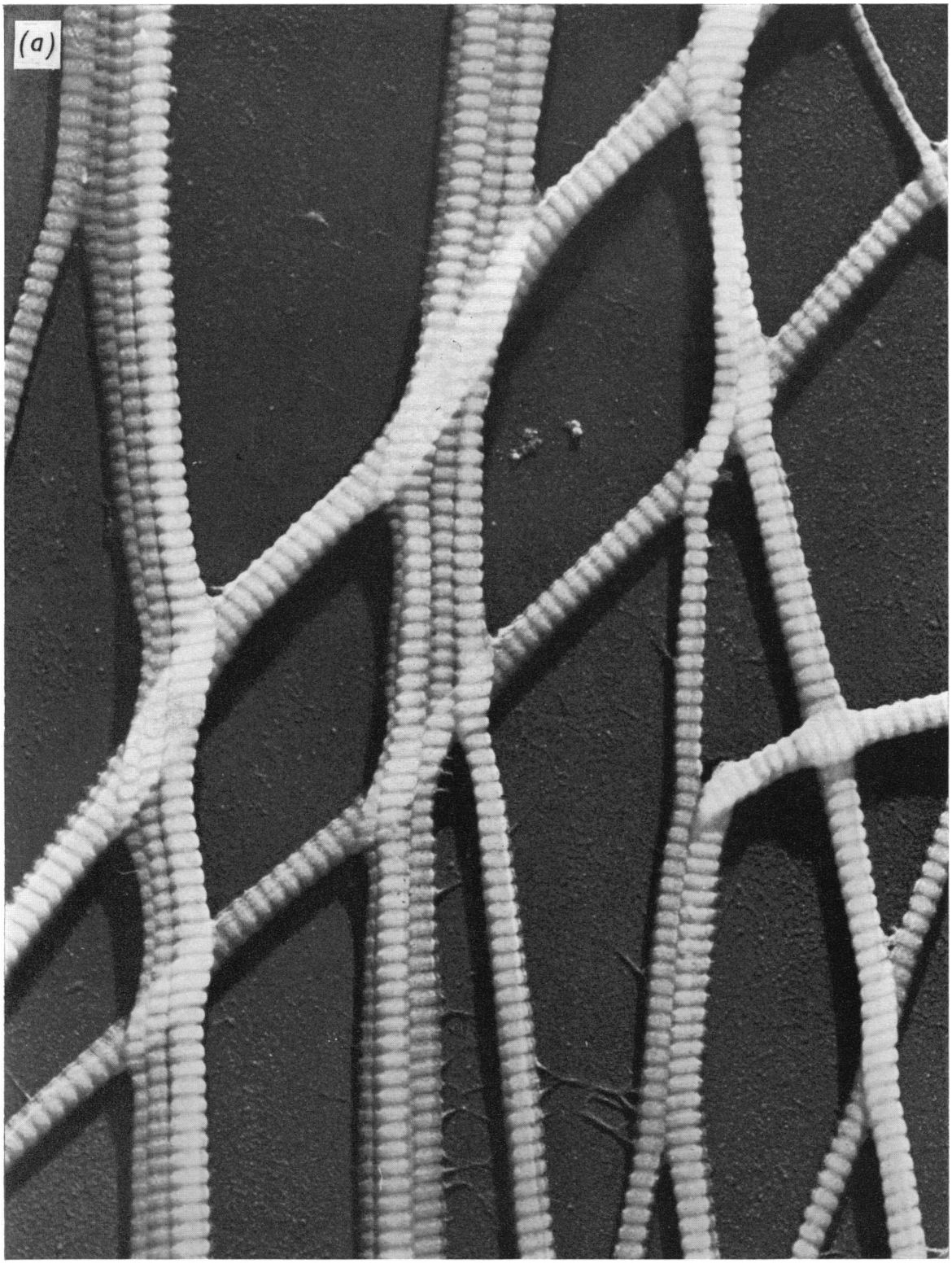
EXPLANATION OF PLATES 1–3

Micrographs of polymerized collagen (by courtesy of Dr J. A. Chapman). (*a*) Shadowed with gold–palladium at 15°. Magnification ×44000. (*b*) Positive staining with 1% phosphotungstic acid, pH 7·0. Magnification ×260000. (*c*) Treated with 1% $Pb(NO_3)_2$, pH 5·5. Magnification ×260000. Lead has been deposited in the holes. (*d*) Negative staining with 1% phosphotungstic acid in N-HCl. Magnification ×260000. The unravelling of the fibril can be seen to expose the protofibrils.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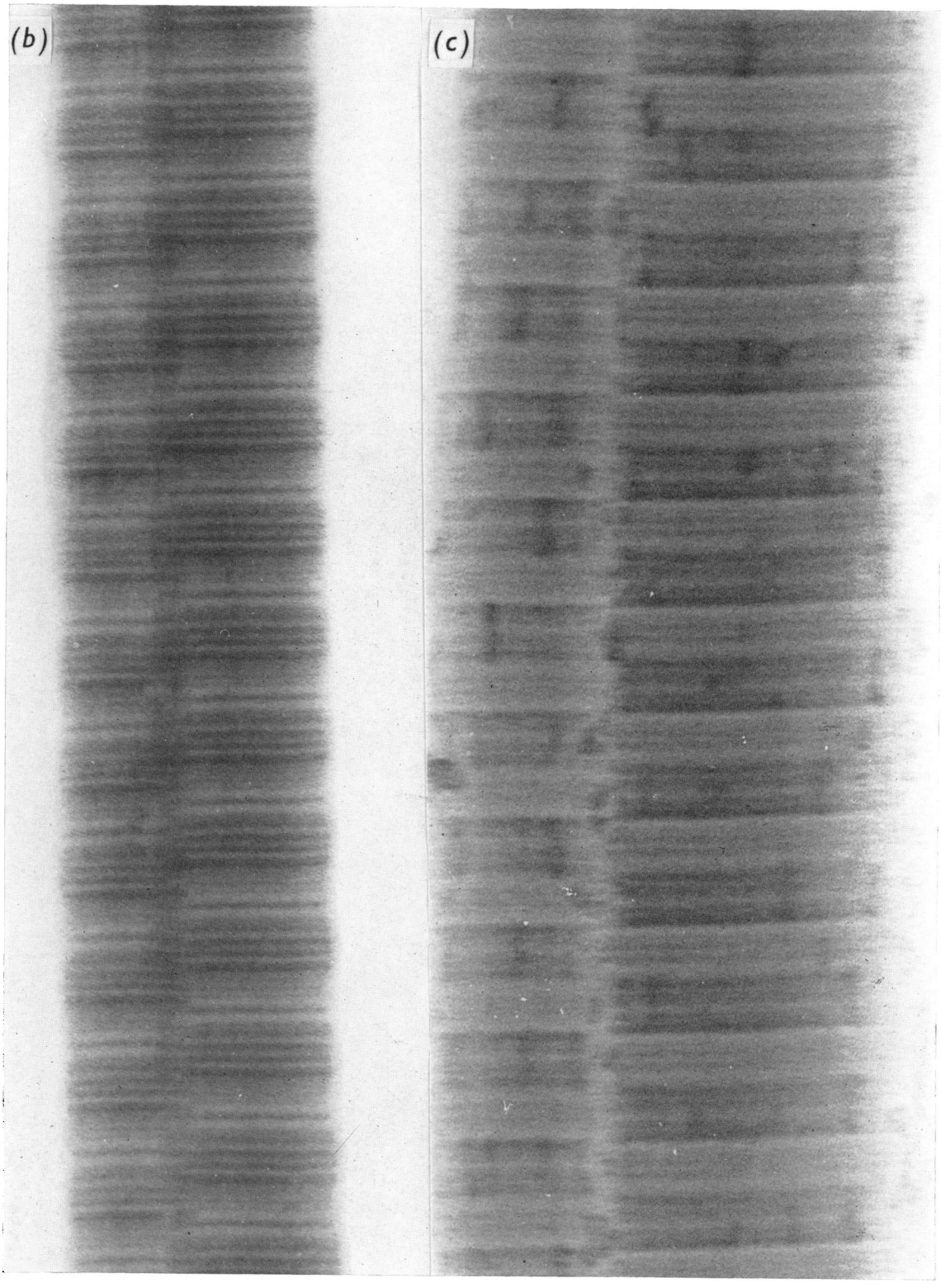
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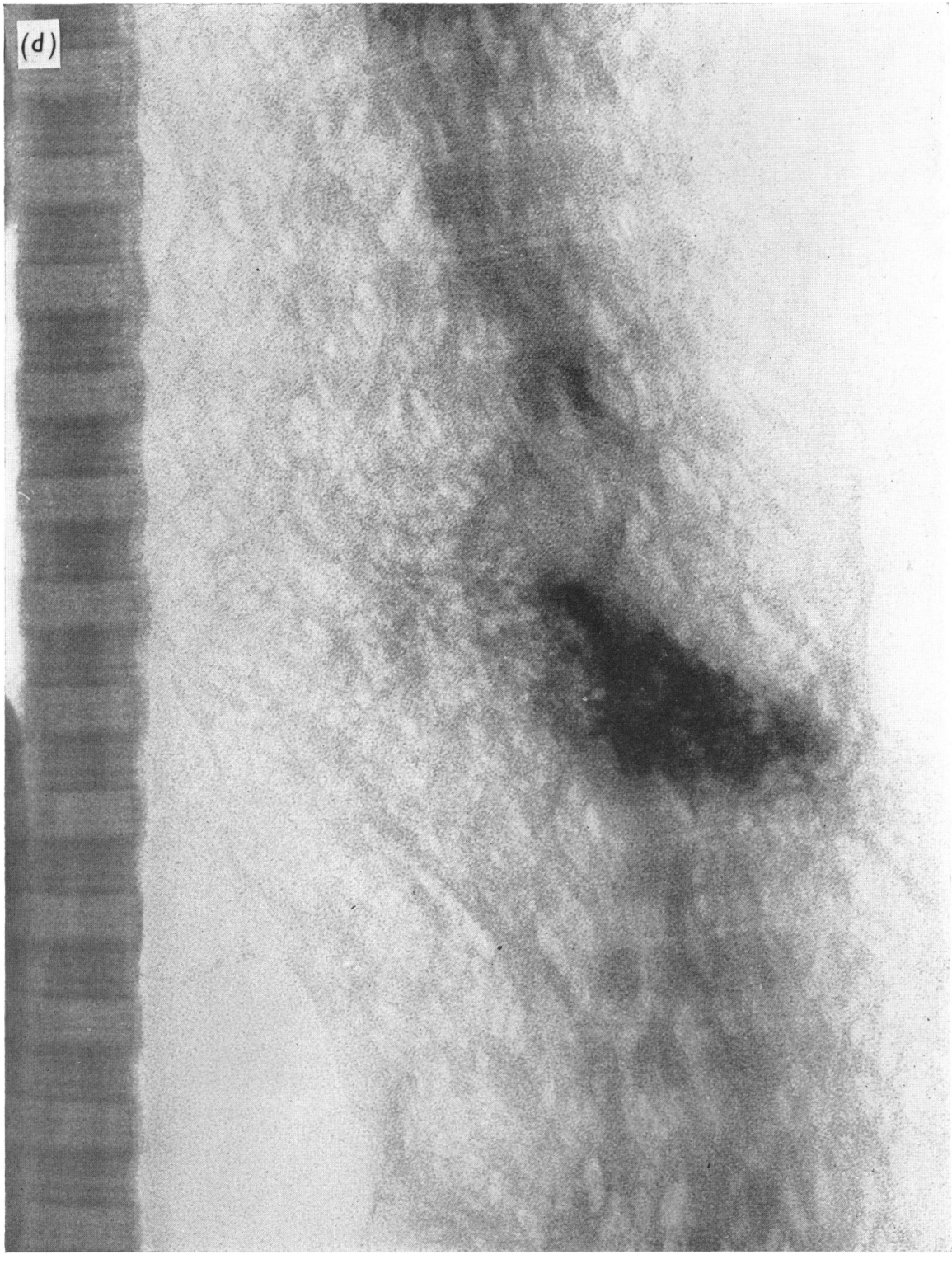
(c)


(d)