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ISOLATION OF A NON-COLLAGENOUS RETICULIN COMPONENT AND ITS PRIMARY CHARACTERIZATION

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Summary.—A reticulin component has been extracted from the saline insoluble residue of pig renal cortex and liver by a distilled water dispersion method. The water dispersed component, that could be precipitated by addition of NaCl up to 0·15 mol/l, was proved to be a protein distinct from collagen in that it contained no hydroxyproline and 9% glycine. The carbohydrate content was 4%, consisting mainly of galactose and mannose. The protein showed an affinity for the silver impregnation method, as used for reticulin. Antibodies to it, raised in rabbits, showed typical anti-reticulin staining by immunofluorescent techniques. It is suggested therefore that this protein, having peculiar solubility characteristics, may be a significant, non-collagenous component of reticulin.

IT is well known that histological techniques readily differentiate between collagen and reticulin. Collagen is identified as coarse fibres which are birefringent between crossed polars, are stained red by the van Gieson method and yellow, brown or pink by silver impregnation techniques. On the other hand, reticulin is identified as fine branching fibres which are isotropic between crossed polars, are either unstained or only faintly pink after treatment by the van Gieson method but stain black after silver impregnation. There is some controversy over the relationship between these two types of fibrous proteins. Some consider that collagen and reticulin are fundamentally identical (*e.g.*, Mallory and Parker, 1927); others believe them to differ only in their physical properties (*e.g.*, Foot, 1927; Dublin, 1946; Windrum, Kent and Eastone, 1955), whereas others believe that reticulin and collagen are different entities, both chemically and physically (Glegg, Eidinger and Leblond, 1954; Puchtler, 1964). The reason for this controversy is that very little is known about the chemical identity of reticulin because of a lack of suitable methods for isolating it in a pure form (Glegg *et al.*, 1953; Windrum *et al.*, 1955; Milazzo, 1957; Sulitzeanu *et al.*, 1967).

This communication deals with the isolation of a new protein component of reticulin.

MATERIALS AND METHODS

Material.—Pig kidney and liver were obtained 1 hour after the animals were killed. The kidney cortex was separated by dissection from the rest of the kidney and stored at − 20° as was the liver.

Separation and purification.—All procedures were carried out at + 4°. A modification of the water dispersion method for the isolation of amyloid was used (Pras *et al.*, 1968): 50 g of

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pig kidney cortex or 100 g of pig liver were homogenized in 400 ml of 0·15 mol/l NaCl in a Sorval high speed homogenizer for 5 min and the homogenate was centrifuged at 10,000 rev/min (12,000 *g*) for 30 min. An MSE high speed 18 centrifuge with 8 × 50 rotor was useds for all centrifugations. The supernatant solution was discarded and the sediment wa. homogenized again with 400 ml of 0·15 mol/l NaCl and the mixture centrifuged as before These operations were repeated 8 times in order to wash out most of the soluble proteins and other soluble components of the kidney and liver. Salt was now removed from the residue by homogenziation with 400 ml of distilled water and centrifugation of the suspension at 18,000 rev/min (38,000 *g*) for 1 hour. With this centrifugal force, the protein to be isolated is spun down and the supernatant (Sup. I) contained only small quantities of protein that did not precipitate with 0·15 mol/l NaCl; the supernatant was discarded. The residue was then homogenized with 200 ml of distilled water and was centrifuged at 10,000 rev/min for 1 hour. This yielded a supernatant (Sup. II) which contained 1–1·5 mg/ml of protein that precipitated when sodium chloride was added to give a final concentration of 0·15 mol/l. The residue was homogenized again with 200 ml distilled water and after centrifugation at 10,000 rev/min the supernatant (Sup. III) contained 0·5–0·8 mg/ml protein that precipitated with 0·15 mol/l NaCl. This procedure could be repeated again to collect some more of the protein.

Protein precipitated by 0·15 mol/l NaCl from Sup. II and III showed similar properties; the precipitates from these supernatant fractions were combined and stored at 4° or were frozen at − 20°.

The total yield of the water dispersed protein from 50 g (wet weight) of kidney cortex was 300–320 mg of dry protein; 100 g (wet weight) of liver yielded 125 mg of dry protein.

Histological investigations on the protein.—The protein was studied in (a) air-dried smear preparations; (b) cryostat sections of the frozen pellet of the protein after centrifugation. The preparations were tested by the Gordon and Sweet silver impregnation method, the periodic and acid–Schiff reaction, the van Gieson staining technique for collagen and by Congo red.

Preparation of antisera.—An emulsion was prepared consisting of 10 ml of the dispersed protein in water (2 mg protein/ml) and 10 ml of complete Freund's adjuvant. Each rabbit received 1 ml of this emulsion subcutaneously, divided between 5 injection sites (*i.e.*, 0·2 ml of emulsion, 0·2 mg of protein for each injection site). The animals were bled from the ear vein 4 weeks after immunization. Anti-rabbit gamma globulin was obtained from the serum of a goat which had been immunized with the globulin fraction of normal rabbits and it was conjugated with fluorescein.

Immunofluorescence.—The indirect fluorescent staining technique was used (Weller and Coons, 1954). Quick-frozen unfixed blocks of tissue from various pig and rat organs were cut (6 μm thick) in a cryostat. The sections were mounted on alcohol-cleaned glass slides and pre-treated for 20 min with the antisera to the isolated protein diluted 1 : 20–1 : 100. They were then rinsed in buffered saline for 30 min and treated with the fluorescein-conjugated goat anti-rabbit globulin fraction diluted 1 : 30 for 20 min; rinsed in buffered saline for 60 min; mounted in glycerol containing 10% buffered saline; covered with glass coverslip and examined.

Inhibition studies were made by incubating the isolated protein with antisera raised against it for 1 hour at 37°. Centrifugation removed the immune complexes. Similar studies were made on the isolated protein after it had been treated with alkali (see below).

Analytical methods.—Neutral sugars were determined by the anthrone method (Yemm and Willis, 1954) and by the orcinol–sulphuric acid technique of Francois, Marshal and Neuberger, 1962). Thin layer chromatography (Merck DC-fertigplatten cellulose F) was performed after hydrolysis of the protein in 2 mol/l H_2SO_4, the solvent being acetone : butan-1-ol : water (5 : 4 : 1 by vol).

Amino acid content was determined by means of a Locarte, London, automatic amino acid analyser. Hydroxyproline was determined according to Woessner (1961). Electrophoretic studies were made by the techniques of Weber and Osborn (1969) with 0·2% sodium dodecyl sulphate concentration and 7·5–10·0% polyacrylamide gel.

RESULTS

General appearance

The water-dispersed protein derived from pig kidney cortex or liver had a

straw colour and a clear serous appearance. The supernatants contained 0·4–1·6 mg of protein per ml checked by the Folin and Biuret methods. The protein could be sedimented completely by centrifugation at 18,000 rev/min (38,000 *g*) for 2 hours. When various salts were added (NaCl, buffered saline, $CaCl_2$, phosphate buffer), the clear solution became opalescent within seconds and the protein precipitate had settled within an hour.

Solubility in sodium chloride

To test the solubility of the water-dispersed protein in various concentrations of sodium chloride, 2 ml of the water-dispersed protein, containing 0·5 mg protein, were mixed with an equal volume of solutions of sodium chloride of concentrations ranging from 0·2 to 1·0 mol/l NaCl. After allowing to stand for 1 hour, the mixtures were sedimented at 500 *g* for 5 min and the concentration of protein in the supernatant fluids was measured by the absorbance at 280 nm. It was found that the protein precipitated in the presence of NaCl at concentrations between 0·1 and 0·24 mol/l (Fig. 1). At lower salt concentrations or at concentrations greater than 0·3 mol/l it remained dispersed and would not sediment even when centrifuged at 10,000 *g*.

Effect of heat

Prolonged heating of the protein in 0·15 mol/l NaCl at 100° for 3 and 6 hours failed to solubilize or gelatinize the suspension. The whole of the material remained unaffected; the optical density of the supernatant fluid was not increased by heating.

Solubility in urea and guanidine and the effect of alkali

The water-dispersed protein could be solubilized both in 8 mol/l urea and in 6 mol/l guanidine hydrochloride solutions. These solvents did not appreciably reduce its particle size, since most of it could be sedimented by centrifugation at 18,000 (38,000 *g*).

Treatment of the protein with 0·05 N NaOH immediately cleared the opalescent solution even when the protein was suspended in 0·15 mol/l saline. After treatment for 1 hour the protein was not sedimentable by centrifugation at 18,000 *g* for 2 hours. Neutralization, by the addition of acid, or dialysis against 0·15 mol/l NaCl precipitated the protein again.

The water dispersed product as a protein

Protein determination by the Folin method established that up to 90% of the dry weight consisted of protein. The ultra-violet absorption spectrum of the water dispersed product showed a nonspecific absorption spectrum. The presence of only a slight hump at 280 nm (Fig. 2) was suggestive of only few tryptophan and tyrosine residues.

Amino acid analysis, by Autoanalyzer, of the native preparations of the water-dispersed protein and of the alkali-treated preparation showed a preponderance of acidic amino acids. Of the other amino acids leucine was particularly abundant, as was alanine. Only a low content of cysteine and unmeasurable amounts of hydroxyproline were found. Separate determination of hydroxyproline by the method of Woessner (1961) showed that there was not more than 0·2% (w/w)

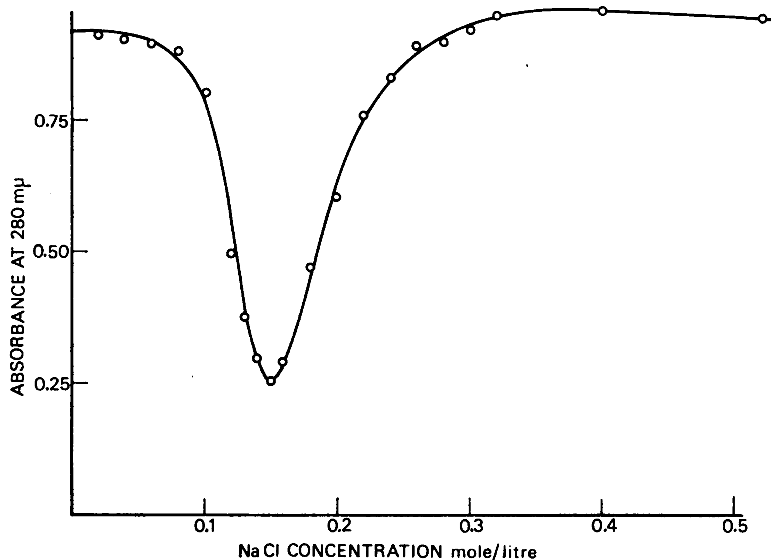


FIG. 1.—Solubility curve of the water dispersed reticulin component on sodium chloride. Absorbance readings at 280 nm of a number of supernatant fluids, after centrifugation at 500 *g*.

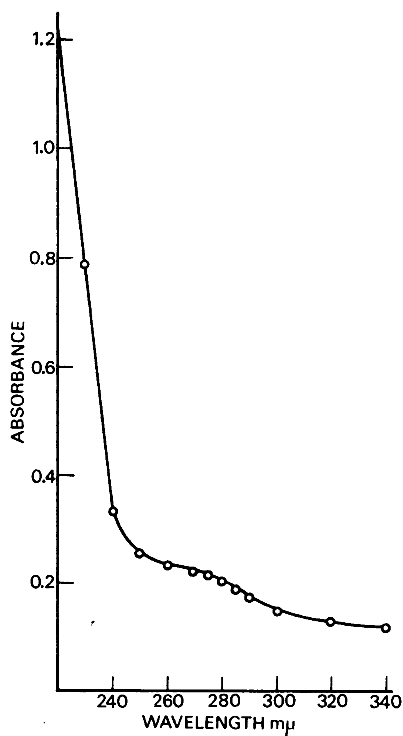


FIG. 2.—Absorbance of the water dispersed reticulin component at 280 nm. Protein concentration 0·2 mg/ml.

of hydroxyproline. Very similar amino acid analyses were found on the protein, whether it was derived from the kidney or the liver and the results were not influenced by treatment with alkali (Table I).

TABLE I.—*Amino Acid Composition of the Water Dispersed Reticulin Component: Results expressed as Residues/100 Residues*

	Native kidney	Alkali-treated kidney	Native liver	Alkali-treated liver
Hydroxyproline*	traces	0	0·2	traces
Aspartic acid	9·08	8·63	8·20	8·65
Threonine	5·50	5·73	5·91	5·85
Serine	7·14	6·00	6·63	6·70
Glutamic acid	11·44	10·66	10·97	10·79
Proline	4·46	4·73	5·75	5·49
Glycine	8·67	9·05	9·00	9·04
Alanine	8·92	8·93	9·54	9·58
Half cystine	1·22	0·55	0·73	0·54
Valine	5·70	6·16	5·54	5·19
Methionine	2·14	1·55	2·14	1·99
Isoleucine	4·07	4·88	3·85	3·78
Leucine	10·76	10·83	9·76	9·75
Tyrosine	2·85	3·12	3·07	3·14
Phenylalanine	3·94	4·62	3·75	4·10
Histidine	1·85	1·75	1·98	1·99
Lysine	6·62	6·28	7·00	7·30
Arginine	5·63	6·54	6·18	6·11
	99·99	100·01	100·00	99·99

* Determined also separately (Woessner, 1961).

Neutral sugars

The values of neutral sugars were 3·8% of the dry weight in the native proteins and 4·1% after alkali treatment. Using the orcinol sulphuric acid method of Francois *et al.* (1962) almost all the colour developed within 15 min, suggesting that the sugars were galactose and mannose. This suggestion was confirmed by thin layer chromatography in which the spots corresponded to the galactose and mannose markers. Glucuronic acid content was 0·15% of the dry weight.

The physical nature of the material

The water-dispersed material was tested by sodium dodecyl sulphate–acrylamide gel electrophoreses. With a 10% acrylamide gel the material did not penetrate the gel but remained as a band at the origin. With 0·2% sodium dodecyl sulphate in a 7·5% acrylamide gel there was still a large band at the origin but 2 strong and 2 weak fast moving bands were also distinguished. When this material was subjected to treatment with alkali before electrophoresis the band at the origin was less strong and the fast moving bands were enhanced.

Lipids

Treatment of the water-dispersed protein with chloroform–methanol 3 : 1 (v/v) solubilized 4% of its dry weight. The same delipidation could be achieved by adding one volume of methanol to the 0·5 N NaOH protein solution. The lipid material stayed in solution in the 50% methanol even after protein had been precipitated after neutralization. No attempts were made in this study to establish the significance or to characterize the lipid component.

Histological staining

The water-dispersed material was either spread on to microscope slides or it was sedimented at 18,000 rev/min, frozen rapidly and cut at 10 μm in a cryostat, the sections being mounted on to slides. Both the smear preparations and the reactions of this material were uncoloured by the periodic acid–Schiff, the van Gieson and the Congo red staining methods. On the other hand, both types of preparation stained with the Gordon and Sweet silver impregnation method, producing jet black fibres which were reminiscent of reticulin fibres in histological sections treated in this way for disclosing reticulin (Fig. 3).

Immunofluorescence studies, which will be reported in detail in a separate publication, showed that the antibodies to the water-dispersed material localized precisely on basement membranes and reticulin fibres in many tissues (Fig. 4, 5).

DISCUSSION

Reticulin is the name given to a particular histological form of extracellular connective tissue fibres. These can be recognized by the fact that they are fine, branched fibres which are isotropic between crossed polars, are relatively unstained by the van Gieson method for collagen but which are blackened by certain silver impregnation techniques. Such reticulin fibres are widely distributed in animal tissues, being particularly conspicuous in the basement membranes of the glomeruli and tubules of the kidney and in the supportive architecture of the vasculature and lung alveoli.

A simple water dispersion technique has now been used to isolate a unique protein component from pig kidney and liver. This protein is insoluble in physiological saline and buffers; it was dispersed by distilled water and it was this property which has made it possible to separate it from other connective tissue fibrous proteins such as collagen and elastin, and from proteins derived from cell membranes. When this protein material was studied in smear preparations, or in sections of the re-precipitated material, it did not stain with the van Gieson technique, it was isotropic and the silver impregnation method showed it to consist of blackened fibres. Moreover immunofluorescence studies showed that the antibody to this material became localized precisely on reticulin fibres in frozen sections of a number of different tissues (Fig 4, 5) (also in preparation).

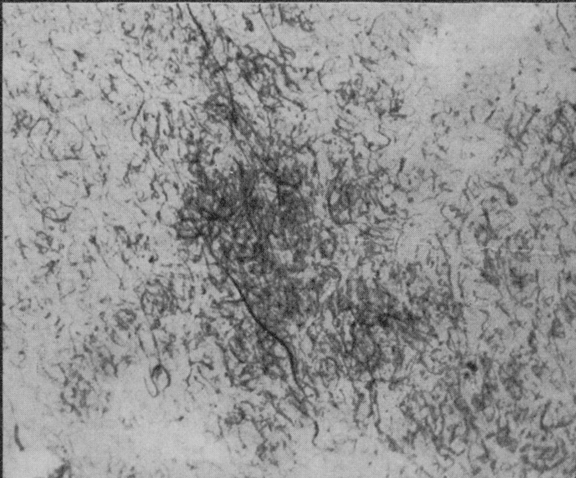
Thus there is evidence that this isolated protein material has the histological and the antigenic properties associated with reticulin. Although at present it is not possible to decide whether it forms a major, or only a minor, component of reticulin, the following evidence indicates that it may be a major component. First, it was obtained in high yield (310 mg dry weight from 50 g wet weight kidney); secondly it was strongly immunogenic (1 mg of this protein was sufficient to raise a strong antibody), and the antibody produced an intense immunofluorescent stain when used at low concentration (as low as 1 : 100 dilution).

EXPLANATION OF PLATES

FIG. 3.—An air-dried smear preparation of the water dispersed reticulin component stained by Gordon and Sweet silver impregnation method. Magnification approximately × 640.

FIG. 4.—Indirect immunofluorescent staining of guinea-pig liver. Magnification approximately × 900.

FIG. 5.—Indirect immunofluorescent staining of guinea-pig kidney. Magnification approximately × 900.



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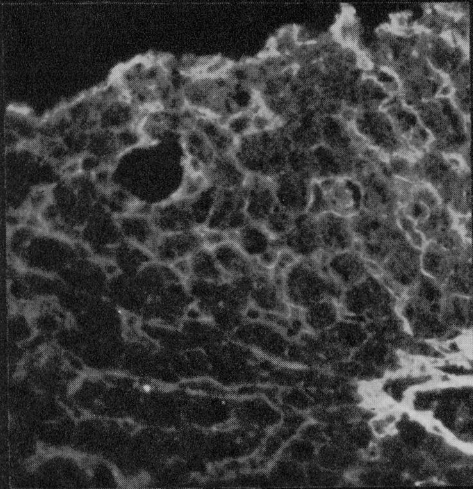

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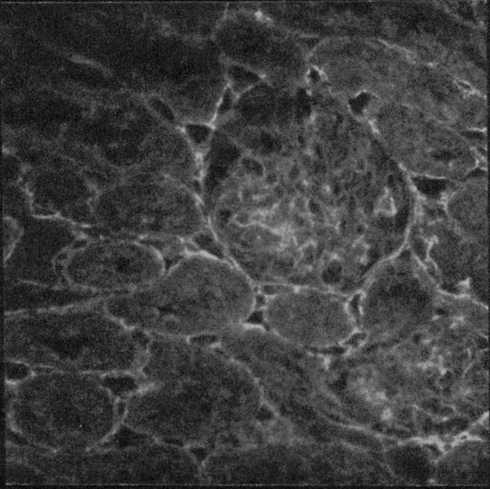

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The amino acid analysis of the purified protein showed that it almost lacked hydroxyproline (less than 0·2%) and that its glycine content was that of normal proteins (9%). Both these properties show that this protein is not a collagen. Moreover, the presence of 4% (by weight) of neutral sugars is incompatible with this protein being a collagen in which the neutral sugars comprise only about 0·5% of the mass.

Thus these results indicate that a component, possibly the major component, of reticulin is a non-collagenous protein having the peculiar property that it can be dispersed in distilled water but it is precipitated by physiological (0·15 mol/l) saline. They are therefore in contradistinction to the claims by Windrum *et al.* (1955) who isolated a protein with an amino acid content which was identical to that of collagen. However, it must be noted that one stage of the purification procedure used by these workers included treatment with 0·05 N NaOH. This treatment would disperse the protein described in this communication and cause it to be lost from the material which they finally analysed. Similarly, Glegg *et al.* (1954), in treating their " reticulin " with tap water or distilled water, would have dispersed this protein into the washing so that it would not have been found in their " reticulin ".

On the other hand, Myers *et al.* (1966) extracted an alkali-soluble product from saline-insoluble material from homogenates of parietal yolk sac carcinoma of white mice and mouse spleen. Antibodies produced to this material were located on reticulin fibres and basement membranes by immunofluorescence techniques, very much as was the protein described in this communication. Unfortunately nothing is known of the chemical composition of the material studied by Myers *et al.* (1966) although, from the method used in its isolation, it is likely to correspond to the protein described here.

Siegfried (1892) first defined reticulin as a substance derived from reticulin fibres after their gelatin content had been removed. The confusion between reticulin and collagen appears to have been a recent one (*e.g.*, Kramer and Little, 1953) and may have arisen predominantly through technical error in which at least one component of the reticulin has been washed away in water washings. The present communication indicates therefore that the original distinction between " reticulin " and collagen should be reinstated.

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