MORPHOLOGICAL AND CHEMICAL STUDIES OF COLLAGEN FORMATION

II. Metabolic Activity of Collagen Associated with Subcellular Fractions of Guinea Pig Granulomata

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ABSTRACT

Electron micrographs of thin sections of nuclear, microsomal, and mitochondrial fractions obtained from a carrageenin-induced granuloma showed considerable contamination of the heavier by the lighter fractions. Striated collagen fibrils could be identified in the nuclei + debris fraction. Only a few striated fibrils occurred in the mitochondrial fraction; very fine filaments (diameter 50 A) could be seen in this fraction, but could not be distinguished with certainty from fibrillar material derived from broken nuclei. 35 per cent of the mitochondrial and 80 per cent of the microsomal collagen was extractable by 0.2 M NaCl and could be purified by the standard methods of solution and reprecipitation. The amino acid composition of these collagen fractions determined by ion exchange chromatography was within the range normally found for collagen and gelatin from other mammalian species, allowing for 10 to 20 per cent of some non-collagenous contaminant of the microsomal collagen. Hydroxyproline and proline were isolated by chromatography on paper from hydrolysates of the nuclear, mitochondrial, and microsomal collagen fractions, after incubation of tissue slices with L-^{14}C-proline. The specific activities of the hydroxyproline from these collagens were in the approximate ratio 1:2:6, while that of bound hydroxyproline derived from the supernatant was only 1, indicating primary synthesis of collagen in the microsomes. Attempts to demonstrate incorporation of L-^{14}C-proline into collagen or into free hydroxyproline in cell free systems were unsuccessful, nor was it possible to demonstrate non-specific incorporation of L-^{14}C-valine into TCA-insoluble material by various combinations of subcellular fractions.

INTRODUCTION

Our understanding of the process of fibrogenesis has been greatly increased by the observations that a considerable proportion of the collagen from the skin of young animals could be extracted by cold neutral salt solutions and that fibres could be regenerated from these extracts by, for example, raising the temperature to 37°C (19, 26). The term tropocollagen (18) was introduced to de-

scribe the precursor of the collagen fibre, characterised by electron microscopy. It subsequently became clear that the properties of tropocollagen were the same as those of native collagen molecules, determined by viscosity, sedimentation, osmotic pressure, and light-scattering measurements (2), and that formation of fibres with characteristic 640 A striations was an intrinsic property of this molecule under physiological conditions of pH, temperature, and salt concentration.

The metabolic significance of the collagen fractions extractable by neutral and acid buffers has been studied by several groups of workers (15, 21, 24, 31) who have followed incorporation of labelled glycine and proline into these fractions. The present position has been well reviewed in a recent paper by Jackson and Bentley (25). It is now clear that in the mammalian systems studied, the collagen most recently formed, as measured by its radioactivity, is also the most readily extractable by dilute neutral salt solutions. In this paper this fraction will be referred to as neutral salt-soluble collagen (NSC). As the collagen ages it becomes necessary to use progressively higher salt concentrations and then acid buffers to extract it. Eventually it becomes completely insoluble and can only be extracted after conversion to gelatin. It is noteworthy that collagen fibres produced *in vitro* undergo qualitatively similar changes on ageing at 37°C (17).

The neutral salt and acid buffer extraction procedures which have been used to obtain collagen fractions have been applied only to whole tissues, so that the collagen has been extracted indiscriminately from both intracellular and extracellular structures. In a previous paper (15) we adopted this approach when studying the incorporation of proline and hydroxyproline into slices of a carrageenin-induced granuloma incubated *in vitro*. In the present work we have isolated subcellular fractions from homogenates of the granuloma before extracting the collagen, in order to determine the nature and origin of the intracellular collagen.

The synthesis of the collagen molecule has received less attention than the process of fibrogenesis, and work has been confined mainly to following the incorporation of isotopically labelled amino acids into collagen *in vivo*. This has, however, led to the remarkable observation, first made by Stetten (37), that free hydroxyproline is not incorporated into collagen and that collagen hydroxyproline arises from free proline without passing through the stage of free hydroxyproline. This has been confirmed by a number of workers (11, 15, 38), and a similar relationship has been found between lysine and hydroxylysine (36). This unique behaviour indicates that hydroxylation occurs either at the stage of an "activated" proline (or lysine) or after the proline (or lysine) has been incorporated into a peptide chain.

It was hoped that our subcellular fractions would incorporate amino acids into collagen or at least that they would hydroxylate proline, and that it would be possible to study the hydroxylation step in more detail, but disruption of the cells inactivated both of these systems. C. Mitoma (personal communication) has met with similar difficulties when attempting to observe proline hydroxylation in cell free systems. We have obtained some indication of the metabolic significance of the subcellular fractions by incubating the tissue with ^{14}C-proline before homogenisation and then determining the labelling of the collagen from the subsequently isolated microsomal, mitochondrial, and combined nuclei and debris fractions. Electron microscopy has been used to characterise these fractions.

MATERIALS AND METHODS

Uniformly labeled L-[^{14}C]proline, L-[^{14}C]valine, and L-[^{14}C]glutamic acid were obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England. The proline was diluted with inactive L-proline to give a specific activity of 73,000 CPM/μmole (0.47 μc/μmole). The valine (6.5 μc/μmole) and glutamic acid (10.0 μc/μmole) were not diluted. Chromatography of valine and glutamic acid in butan-1-ol–acetic acid–water (63:10:27) gave a single spot on developing with ninhydrin, and radioautography of the chromatogram showed negligible contamination with other radioactive materials. The proline had been checked previously (15).

Adenosine triphosphate (ATP), diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN), phosphoenol pyruvate (PEP), and PEP-kinase were supplied by C. F. Boehringer, Mannheim, Germany.

Granuloma Production and the Incubation of Slices: These have already been described (15).

Homogenisation of the Tissue: The 7-day-old granuloma could not be homogenised without some preliminary disintegration of the tissue. Two methods were used:

a) 5 gm portions of the tissue were forced through two discs of stainless steel gauze (16 mesh or 40 mesh) mounted in a stainless steel tube, using a close fitting nylon plunger. The apparatus, cooled to 0°C, was mounted in a laboratory sodium press and the tissue forced into a centrifuge tube containing cold 0.25 M sucrose. The fibrous material retained on the gauze was removed and the process repeated for each 5 gm sample.

b) In later experiments a milder procedure was adopted. The tissue was chopped as finely as possible using two 6 inch razor blades.

In both methods the disintegrated tissue was suspended in 2.5 volumes of 0.25 M sucrose and centrifuged for about half a minute at 1000 *g* in order to remove most of the extracellular fluid. The residue was suspended in 2.5 volumes of ice cold 0.25 M sucrose and homogenised for 1.5 minutes in a glass tube with a bore of 3 cm, using a Teflon pestle (diameter 2.95 cm) mounted on a stainless steel shaft. The pestle was driven by a flexible shaft from a ¼ H.P. motor, giving a constant speed of 1,400 RPM, thus allowing frequent up and down motions of the pestle whilst the tube was clamped in an ice bath.

Differential Centrifugation: The method was that of Schneider and Hogeboom (35). The nuclei + debris fraction was sedimented for 10 minutes at 1,000 *g*. The sediment was resuspended in half the original volume of sucrose and again centrifuged. The combined supernatants were centrifuged in the model L Spinco ultracentrifuge at 10,000 *g* for 10 minutes and the mitochondrial sediment was resuspended in 5 volumes of 0.25 M sucrose and centrifuged as before. The combined mitochondrial supernatants were then centrifuged at 105,000 *g* for 35 minutes. The translucent microsome pellet obtained was usually washed by resuspending in 10 ml of sucrose and then resedimented at 105,000 *g*. The microsome pellet did not resuspend evenly owing to aggregation, and could be centrifuged down in 20 minutes. In some experiments the pellet was also surface washed by layering on 2 ml of sucrose and quickly pouring off and draining the tubes.

Density Gradient Centrifugation: Linear concentration gradients of sucrose were prepared by allowing 1 M and 2 M sucrose solutions to flow from 2.2 ml wedge-shaped Perspex vessels into a small (0.3 ml) mixing chamber with magnetic stirrer, through a short length of glass capillary cooled in ice, into the bottom of a cooled 5 ml plastic centrifuge tube. The flow rate was adjusted by inserting short lengths of capillary tube between the reservoirs and the mixing chamber so that 4 ml of mixed sucrose solution flowed in 15 minutes. This slow flow rate ensured that the levels of solution in the two reservoirs fell at the same rate. The effect of density difference was minimised by holding the mixing chamber at the same height as the reservoirs. Samples of solution from different levels in the gradient tube were taken, using a screw-operated tuberculin syringe with a special 18 gauge needle (Fig. 1).

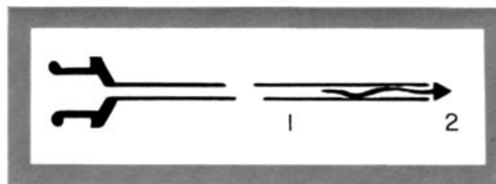


FIGURE 1

Syringe needle for taking samples from density gradient tube. *1*, 18 gauge needle shaft; *2*, cone of solder retained by wire spring to form an annular opening.

The gradient tube was lowered and raised with respect to the needle by a rack and pinion mechanism. In this way the density gradient could be checked by measurement of the refractive index of samples taken from different levels. Microsomes or mitochondria were prepared in 0.88 M sucrose and resuspended. 1 ml samples containing material from about 3 gm tissue were layered carefully on to the sucrose gradient and the tubes spun in the S.W. 39 rotor of the Spinco model L ultracentrifuge at 120,000 *g* for 1 to 2 hours. Most of the particles were concentrated in fairly discrete layers which could be sampled with the syringe with 90 per cent recovery. The refractive index of each sample was determined in an Abbé refractometer; the sucrose was removed by dialysis and the hydroxyproline extractable by hot 5 per cent trichloroacetic acid (TCA) determined. The dry weight of the TCA-insoluble precipitate was also measured.

Chemical Analysis: Total nitrogen was determined by the Kjeldahl method. Total nucleic acid was measured by the extinction at 268.5 mμ of a hot 5 per cent TCA extract as described by Logan *et al.* (29). DNA was measured in the same extract by the colorimetric method of Schneider (34) and RNA was estimated by the difference of the two values. RNA could not be measured by the Schneider procedure owing to interfering substances, probably carrageenin (32). Hydroxyproline was determined by the method of Neuman and Logan (30) as modified by Leach (28). The hydroxyproline content was taken as a direct measure of the collagen present in a sample. Amino acid analysis of collagen was carried out by Dr. J. E. Eastoe, using a semimicro modification of the method of Moore and Stein (Eastoe, 7, 41).

Isolation and Purification of Collagen from Subcellular Fractions: Neutral salt-extractable collagen was obtained by suspending the particulate fraction in

5 to 10 volumes of 0.2 M NaCl, 0.0067 M versene, pH 7.4, at 4°C overnight. The suspension was centrifuged at 105,000 *g* for ½ hour and the collagen was precipitated from the supernatant by raising the NaCl concentration to 20 per cent (*w/v*). The precipitate after centrifugation was redissolved in 10 ml of 0.2 M NaCl and reprecipitated by the addition of solid NaCl to give a 16 per cent solution. After centrifugation the precipitate was dissolved in 5 ml of 0.05 M acetic acid and dialysed overnight at 0°C against 0.05 M acetic acid.

Acid-soluble collagen was extracted by suspending the NaCl-extracted particles in approximately 2 times their volume of 0.5 M sodium citrate buffer, pH 3.5, overnight. The supernatant containing the soluble collagen was dialysed overnight against 0.05 M acetic acid, and then treated with hot 5 per cent TCA according to Fitch *et al.* (9). The collagen was thus gelatinised and non-collagen protein was precipitated. Radioactive insoluble collagen was isolated as described by Jackson (24), but for analytical purposes the method of Fitch *et al.* (9) was employed. When collagen was isolated from tissue slices previously incubated with radioactive amino acids, 1 mg each of inactive L-proline and L-hydroxyproline were added as carrier amino acids at each solution of the collagen. All hydrolyses were performed with 6 N HCl at 110°C for 18 hours in sealed tubes.

Isolation of Proline and Hydroxyproline: Proline and hydroxyproline were isolated by paper chromatography (15).

Purification of TCA-Insoluble Protein for Counting: Radioactive subcellular particles were washed with cold 10 per cent TCA and then heated for 30 minutes at 100°C with 5 per cent TCA containing 1 mg/10 ml of unlabelled amino acid. The TCA-insoluble protein was washed with cold 5 per cent TCA, then dissolved in 0.4 N NaOH and reprecipitated with HCl/5 per cent TCA. Enough HCl was added to neutralise the NaOH. After a further wash with cold TCA the lipids were extracted with hot ethanol and ethanol-ether. Finally the protein was dried, homogenised in ether, and plated by filtering the suspension through discs of Whatman no. 1 paper on a 1.8 cm^2 perforated polythene counting disc.

Unless the solution in alkali and reprecipitation step was included, the unincubated control samples often showed high counts due to adsorbed radioactivity.

Counting of Samples: Hydroxyproline and proline isolated from collagen were counted at infinite thinness (15). The protein samples were counted near infinite thickness and were corrected, after weighing, to infinite thickness using the correction curve given by Calvin *et al.* (3).

Preparation and Incubation of Homogenates of Granuloma and Liver in Vitro: The method of preparation of subcellular fractions was modified for the study of amino acid incorporation into homogenate systems. The homogenisation was carried out in the medium X described by Zamecnik and Keller (40), using a ratio of 1 volume of tissue to 1 volume of medium. This was to avoid excessive dilution of the supernatant fraction containing the activating enzymes. Liver was treated according to Zamecnik and Keller (40). The time of homogenisation was reduced to 45 seconds to minimise damage to the particulate fractions, and in all cases the nuclei + debris fraction was removed before incubation. The microsomes and mitochondria were isolated as described above, but were not washed before incubation.

Incubation System: The complete system contained 10 μmoles of PEP, 2 μmoles of ATP, 0.01 ml of PEP-kinase, and 1 μc of the radioactive amino acid. The particulate suspension was equivalent to 20 gm wet weight tissue per milliliter suspension in the case of granuloma particles, and 10 gm wet weight liver per milliliter in the case of liver particles. In each case 0.4 ml of particles suspended in medium X and 0.3 ml of 105,000 *g* supernatant were added. The final volume of 1.0 ml was incubated for 50 minutes under nitrogen at 37°C.

Amino Acid Activation: Amino acid-dependent hy-

FIGURE 2

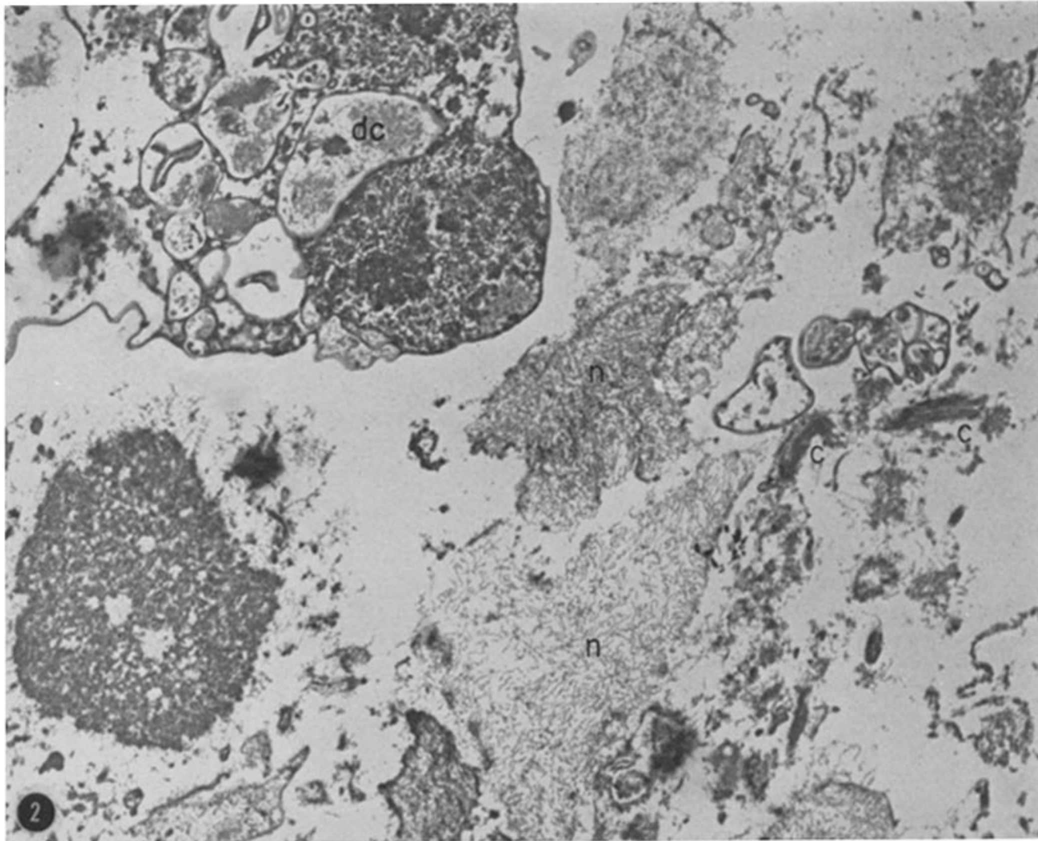
Low power electron micrograph of a section of the nuclear fraction pellet from carrageenin-induced granuloma, showing nuclei at various stages of disruption (*n*) and partially disintegrated cells (*dc*). A group of collagen fibrils occurs at *c*. × 9,000.

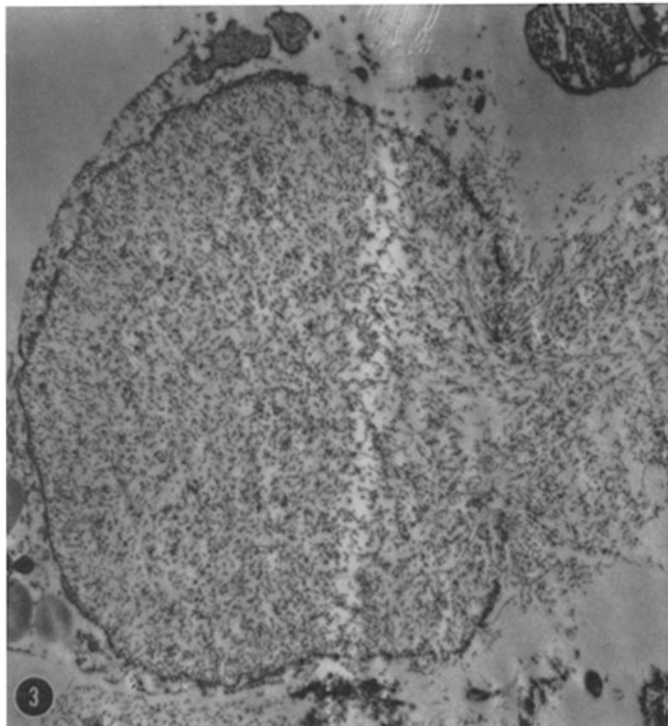
FIGURE 3

Filamentous material emerging from a disrupted nucleus in the nuclear fraction. × 14,000.

FIGURE 4

A bundle of collagen fibrils in the nuclear fraction. × 50,000.


dc
n
n
c
c
2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droxamide formation was measured by the method of Hoagland *et al.* (22), usually in the presence of the following amino acids, each at a concentration of 1 mM: tryptophan, tyrosine, leucine, valine, alanine, glycine, methionine, lysine, proline. The reactions were stopped by the addition of 1 ml ethanol before adding the $FeCl_3$ reagent, since this was found to give clearer supernatants and more reproducible blanks.

The 105,000 *g* supernatant was prepared by the method of Hoagland *et al.* (22).

Electron Microscopy: The pellets of the mitochondrial and microsomal fractions obtained by differential centrifugation were removed *in toto* from the centrifuge tubes and placed immediately in 1 per cent OsO_4 in 0.25 M sucrose buffer at pH 7.3 and at a temperature between 0° and 4°C. (0.88 M sucrose was used in the case of the fractions obtained by density gradient centrifugation.) In order to avoid including the larger masses of debris in the nuclear fraction, the surface layer of the pellet was resuspended and spun down again to give a fresh pellet, which was then treated as above. The pellet in the fixative was cut into small fragments and these were allowed to fix for 1 to 2 hours. Dehydration and embedding were carried out by standard procedures, using Araldite embedding as described by Glauert and Glauert (13). Some of the specimens were treated with phosphotungstic acid (PTA) in absolute alcohol for 2 to 4 hours prior to embedding, to enhance the contrast of the sectioned material (Huxley, 23). Sections were cut on a Haanstra or a Huxley ultramicrotome and examined in a Siemens Elmiskop I operating at 80 kv, using double condenser illumination and at instrumental magnifications ranging from 2,500 to 40,000.

RESULTS

Characterisation of the Subcellular Fractions

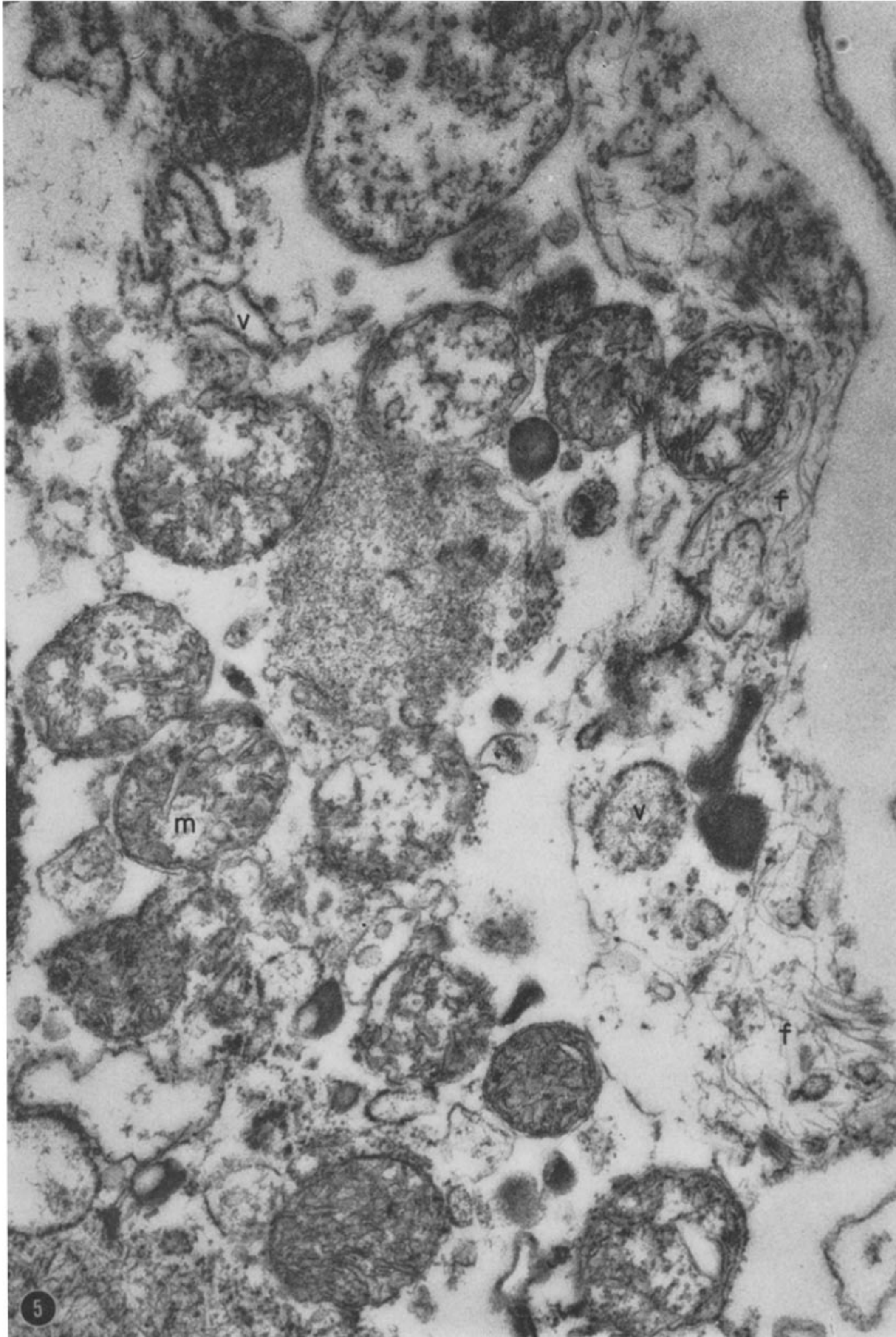
The abundance of collagen fibres in the 7-day carrageenin granuloma made homogenisation a difficult process, and much of the damage to nuclei and mitochondria could probably be accounted for by the procedures necessary to secure adequate disintegration of the tissue. The tendency of mitochondrial and microsomal particles to aggregate, especially after centrifugation into a pellet, also made clear-cut centrifugal fractionation difficult. After resuspension a fraction could be spun down again at a much lower speed; almost all the resuspended microsomal material, for example, sedimented in 5 minutes at 20,000 *g*. This rendered washing inefficient and led to considerable contamination of the heavier by the lighter fractions. The extent of this contamination is evident from the electron micrographs of the sectioned pellets. The fractions were also characterised by analysis for total N_2, DNA, RNA, and hydroxyproline. (The nature of the hydroxyproline will be considered in a separate section.) The results of these analyses are expressed as the per cent distribution of each substance between the different fractions.

Nuclear Fraction: Most of the particulate material sedimented with this fraction (62 per cent of N, 90 per cent of hydroxyproline, 90 per cent of DNA, 56 per cent of RNA). The unusually high proportion of RNA appearing in the nuclear fraction was due partly to unbroken cells and partly to sedimentation of clumped cytoplasmic material at low speed. Electron microscopy confirmed the heterogeneous nature of the nuclear fraction (Fig. 2) and showed the presence of both nuclear and cytoplasmic material at various stages of disruption. The nuclear component, which was most abundant, ranged from intact nuclei to filamentous debris, apparently derived from nucleoplasm. Fig. 3 shows this filamentous material streaming out from a disrupted nucleus. A few almost complete cells were observed, but much of the cytoplasmic material present occurred as isolated pieces of disrupted cells together with some isolated mitochondria and vesicles from the endoplasmic reticulum. Striated collagen fibrils were relatively abundant, more so in some preparations than in others. Collagen fibrils usually occurred in groups, as in Fig. 4; sometimes these fibrils were accompanied by fine filamentous material.

Mitochondrial Fraction (Fig. 5): This fraction (12 per cent N, 3.5 per cent hydroxyproline, 10 per cent DNA, 10 per cent RNA) was con-

FIGURE 5

The mitochondrial fraction (extracted in 0.25 M sucrose). Some damage is apparent in the internal structure of the mitochondria (*m*). A few contaminating microsomal vesicles (*v*) are present. The filaments (*f*) at the right-hand side of the micrograph are probably cytoplasmic in origin. × 40,000.


v
f
m
v
f
5

taminated by many partially disintegrated nuclei and by a high proportion of vesicles from the endoplasmic reticulum. It was difficult to judge the proportion of vesicles in the mitochondrial fraction without taking a very large number of micrographs, but it was estimated roughly to be between 15 and 40 per cent. Clumps of filamentous material were also present; some of these filaments were similar in appearance to those shown in Fig. 3 and were clearly derived from disintegrated nucleoplasm. Other filaments (*e.g.*, *f* in Fig. 5) may have been cytoplasmic in origin (see Discussion). Other contaminating cellular material included fragments of nuclear and cytoplasmic membrane.

The mitochondria, forming the bulk of the remainder of this fraction, were fairly typical in appearance; they possessed clearly defined limiting outer membranes and showed only slight evidence of swelling. Some damage was apparent in the internal structure, however, and the cristae were usually disorganised, as compared with the corresponding structures observed in sections of whole tissue (Fig. 7). This damage may have resulted from the use of 0.25 M sucrose in the fractionation procedures.

Only a few striated collagen fibrils could be identified in sections of mitochondrial pellets. In over 60 electron micrographs (at a magnification of 8,000), only four recognisable collagen fibrils could be found, and these occurred in short lengths. The micrographs were taken from five separate pellets and usually more than one section from each pellet was examined. The proportion of striated fibrils present is probably higher than that suggested by these observations, as fibrils at an angle to the plane of the section would be missed, but, even so, it seems unlikely that the collagen in this fraction as measured by its content of hydroxyproline can be explained as being due solely to the presence of fibrils.

Microsomal Fraction (Fig. 6): Electron microscopical examination showed that the microsomal fraction (7 per cent N, 0.3 per cent hydroxyproline, 5 per cent RNA, no DNA) was by far the most homogeneous of the three fractions and consisted very largely of rough and smooth surfaced vesicles. The rough surfaced vesicles resembled closely the particle studded membranes of the endoplasmic reticulum in fibroblasts in intact tissue (Fig. 7).

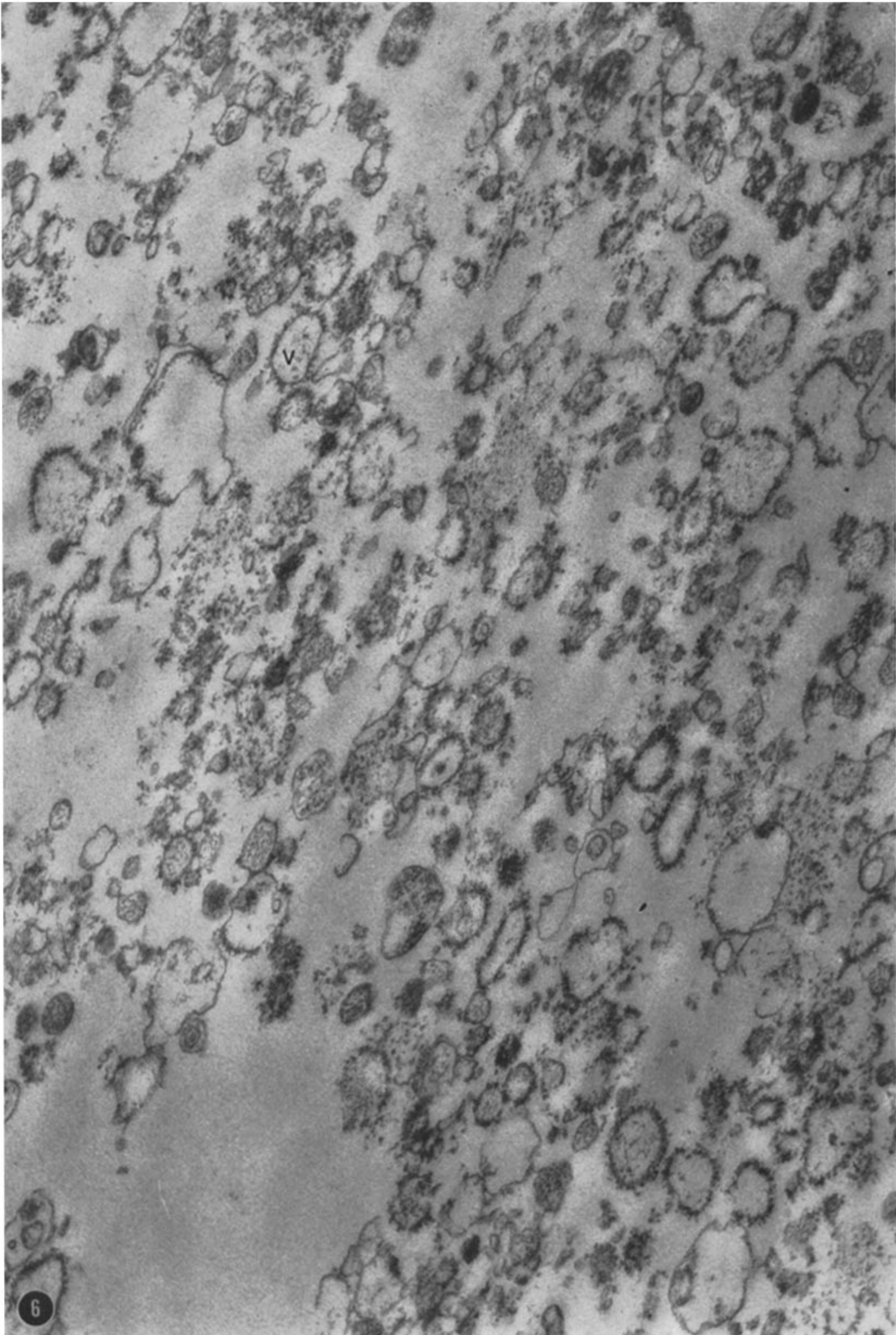
No striated fibrils could be seen in sections of this fraction, indicating that the hydroxyproline present was probably non-fibrous in origin. The microsomal fraction was also characterised by an apparent absence of the fine filaments observed in the mitochondrial fraction. Neither the filaments derived from disintegrating nucleoplasm nor those occurring in the cytoplasm of intact fibroblasts (Chapman, 5) could be identified in pellets of this fraction.

Characterisation of Microsomal and Mitochondrial Collagen

Since 90 per cent of the total hydroxyproline of the granuloma sedimented with the nuclear fraction, the proportions of the extractable collagens were essentially those found in the whole tissue. Mitochondria and microsomes contained much higher proportions (35 per cent and 80 per cent, respectively) of NSC (Table I). The small proportion of microsomal collagen which was not extracted by neutral solvents was not further examined, but some attempts were made to bring the residual mitochondrial collagen into solution, on the assumption that it might be present in non-fibrous form associated with particulate material. Various agents (digitonin, 1 per cent; deoxycholate, 2 per cent; sonic disruption, 15 kc, 15 minutes) known to break up mitochondria were used, but of these only sonic disruption brought more than 10 per cent of the residual 65 per cent into solution. It is also noteworthy that there was little or no citrate-extractable collagen (< 5 per cent) left in the mitochondria after 0.2 M NaCl extraction, although 0.45 M NaCl extracted an additional 15 to 20 per cent of the collagen. The residual mitochondrial collagen was therefore indistinguishable in its solubility behaviour from insoluble collagen. There

FIGURE 6

The microsomal fraction (extracted in 0.25 M sucrose). A high proportion of particle studded vesicles (*v*) is present. × 35,000.


V
6

seemed to be too few striated collagen fibrils to account for all of this fraction, but on the other hand it is difficult to suggest an alternative source. The neutral salt-extractable collagen from microsomes and mitochondria was precipitated with 20 per cent sodium chloride in the usual way. A small amount (15 to 20 per cent) of the hydroxyproline was not precipitated, presumably corresponding to the water-soluble fraction B obtained from the whole granuloma (15). Purification by solution and reprecipitation proceeded in exactly the same way as for collagen extracted from the whole tissue. Experiments described below using microsomes obtained from tissue slices incubated with ^{14}C-proline showed that all the bound ^{14}C-hydroxyproline from this fraction coprecipitated with carrier neutral salt-extractable collagen obtained from the nuclear fraction, providing further evidence for their identity.

A more detailed characterisation was provided by a complete amino acid analysis on 0.7 mg of microsomal collagen extracted from 200 gm of granuloma. Samples of mitochondrial and nuclear collagen were analysed in parallel. The detailed results will be published elsewhere, and only those relevant to the present discussion are shown in Table II (Eastoe, 41).

It appears that the collagens extracted from the nuclear and mitochondrial fractions have amino acid compositions within the normal range for collagens from other species. The hydroxyproline and proline and glycine contents of the microsomal collagen were 10 to 20 per cent lower than normal, while the amounts of tyrosine, lysine, and several other amino acids not shown in Table II were higher than normal. Although it is possible that this difference represents a genuine difference in the amino acid composition of the first-formed collagen, it is more likely due to contamination of the sample by 10 to 20 per cent of another protein. The content of hydroxyproline and hydroxylysine relative to their non-hydroxylated precursors was not significantly different from that found for collagen from other sources.

The Metabolic Activity of the Collagens Associated with Subcellular Fractions

The microsomal, mitochondrial, and nuclear + debris fractions were isolated after 45 minutes incubation of slices with L-[^{14}C]proline. The specific activities of the hydroxyproline and proline isolated from purified NSC are shown in Table III. The microsomal NSC hydroxyproline has the highest specific activity in each experiment, suggesting that it probably represents the earliest collagen formed. There was some protein-bound hydroxyproline in the homogenate supernatant, but the low specific activity of this fraction (Table III) showed that the microsomal collagen had not been adsorbed from the supernatant.

The hydroxyproline isolated from the mitochondrial NSC had a lower specific activity than that from the microsomes. However, it was still somewhat higher than that from the nuclei + debris NSC, and one must consider whether the radioactivity could be due to contamination of the mitochondria by microsomes. Electron micrographs indicated that the mitochondrial fraction contained between 15 and 40 per cent of microsomal material, and calculation on the basis of this rough estimate showed that much of the radioactivity in the mitochondrial NSC could have been due to microsomal material. However, the radioactivity in the insoluble collagen from the mitochondria could not have originated in this way, since the microsomes contain only small amounts of insoluble collagen. This suggested that there might be two morphologically distinct collagen-containing fractions in the mitochondria, and, on the assumption that they might be microsomal vesicles and collagen fibrils respectively, attempts were made to achieve a separation by centrifuging to equilibrium in a sucrose density gradient. Since collagen fibres have a density of 1.4, they should be readily separable from most other subcellular fractions with densities in the neighbourhood of 1.2. The gradient extended from 1.0 M sucrose (ρ = 1.13) to 2.0 M sucrose (ρ = 1.26). Five distinct fractions could be seen

FIGURE 7

Part of a fibroblast in intact 7-day granuloma for comparison with the homogenised and centrifuged fractions. The appearance of the nucleus (*n*), endoplasmic reticulum (*er*), and mitochondria (*m*) are typical of fibroblasts in this tissue. Collagen fibrils (*c*) occur just outside the cytoplasmic membrane (*cm*). × 45,000.

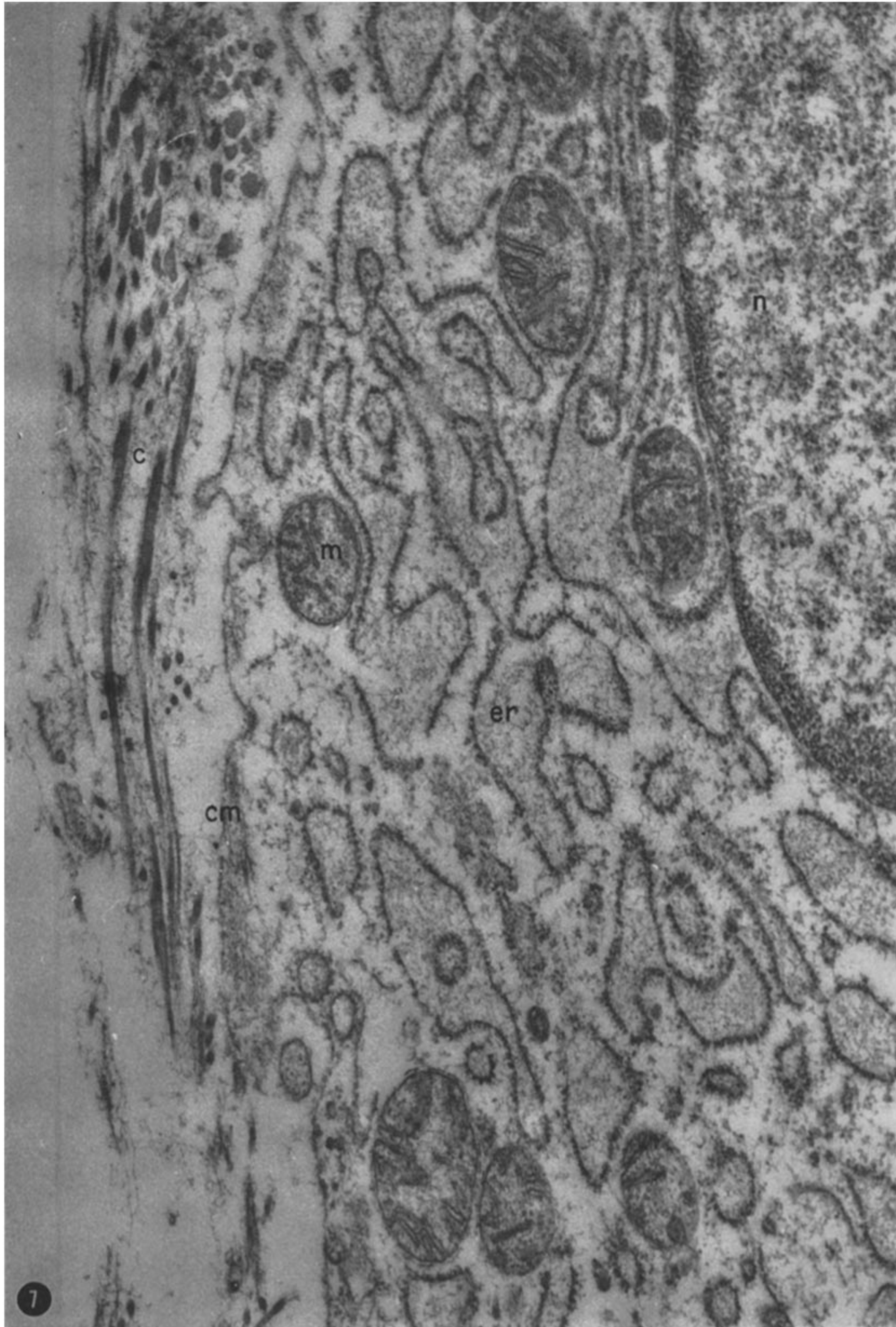

c
m
n
er
cm
7

TABLE I

The Extraction of Collagen from Subcellular Fractions

Approximately 80 gm of tissue were used in each experiment (hypro = hydroxyproline).

Fraction	Extracted successively with				
	0.2 M NaCl (NSC fraction)		0.5 M Citrate	Hot 5 per cent TCA	
Nuclei + debris					
Mg hypro/100 gm wet wt.	2.3		3.7	30	
Per cent hypro in fraction	6.5		10.0	83	
Mitochondria	Exp. 1	Exp. 2	Exp. 1	Exp. 1	Exp. 2
Mg hypro/100 gm wet wt.	0.13	0.54	0.04	1.25	0.22
Per cent total hypro in fraction	31	35	2.4	66	63
Microsomes					
Mg hypro/100 gm wet wt.	0.056	0.054*	—	0.013	0.011
Per cent total hypro in fraction	80	80	—	20	20

* Extracted with 0.45 M NaCl.

Further Extraction of Mitochondria Extracted with 0.2 M NaCl‡

	Hydoxyproline extracted by				
	Deoxycholate, 2 per cent	Digitonin, 1 per cent	Sonic disruption	Citrate, 0.5 M	NaCl, 0.45 M
Mg hypro/100 gm tissue	0.11	0.141	0.32	0.04	0.078
Per cent total mitochondrial hypro	6.3	8.05	18	2.4	21.6

‡ These figures are taken from different experiments.

after centrifuging for 1 hour at 120,000 *g* and included one which had reached the bottom of the tube. Hydroxyproline was present in all fractions, and there was no clear-cut association with any particular one, although the concentration in the two heavier fractions was about twice that in the lighter ones. The significance of this is doubtful, since extraction with 0.45 M sodium chloride showed no differences in the proportion of extractable collagen in the different fractions, and no collagen fibrils could be seen in electron micrographs of sections of the heaviest fraction.

Microsomal material was also fractionated in this way to give three fractions and some intermediate opalescent material. Again all fractions contained hydroxyproline, the heaviest with twice the concentration of the lighter fractions. In both microsomes and mitochondria the heavy fractions were aggregated into floccules, while the lighter ones were evenly dispersed and opalescent. Electron micrographs of the microsomal material showed only smooth surfaced vesicles in the two lighter fractions and mainly particle studded vesicles in the heaviest one, but no identifiable collagen could be seen, confirming the results on the unfractionated microsomes (Fig. 6).

Metabolic Activity of Homogenates

Attempts to obtain incorporation of proline into collagen in homogenates were unsuccessful. Neither was there any evidence for the hydroxylation of proline in such systems, since both the hydroxyproline in the tissue free amino acid fraction and that isolated from the collagen were not radioactive. Further experiments were made to see if any general incorporation of amino acids into TCA-precipitable protein would take place in this system.

A preliminary incubation of slices of granuloma with L-[^{14}C]proline and L-[^{14}C]glutamic acid showed that relative incorporation of these amino

TABLE II

Amino Acid Composition of Collagen from Different Sources (Residues per 1,000 Residues)

	Neutral salt-extractable collagen				Gelatin	
	Nuclear	Mitochondrial	Microsomal	Rabbit skin*	Ox skin‡	Ox bone‡
Glycine	324	300	263	276	333	314
Proline	116	110	102	104	129	119
Hydroxyproline	104	100	80	91	98	101
Lysine	25.8	28.3	36.1	29.7	27.8	26.2
Hydroxylysine	8.8	—	7.6	4.5	5.5	6.4
Tyrosine	3.2	4.8	11.3	1.6	1.5	2.9
Cystine	0	0	0	0	0	0
Proline/hydroproline ratio	1.11	1.10	1.27	1.14	1.32	1.18

* Jackson *et al.* (27).
‡ Eastoe (6).

TABLE III

The Specific Activities (cpm/μmole) of Hydroxyproline and Proline from Neutral Salt-Soluble Collagen Extracted from Subcellular Particles

Experiment No.	Microsomes		Mitochondria		Nucleic + debris		Supernatant	
	Hypro	Pro	Hypro	Pro	Hypro	Pro	Hypro	Pro
E 3	1,050	—	—	—	153.0	130.0	—	—
E 5	215	184	—	—	64.0	95.0	106	61.5
E 6	567	—	256	184	83	74.0	91	97.5
E 7	1,910	—	722	769	319	407	472	322
E 6 (mitochondrial insoluble collagen)	—	—	70	—	—	—	—	—

10 gm slices were incubated with 2 μc L-^{14}C-proline (300,000 CPM) for 45 minutes before homogenisation. The specific activity of the free proline isolated from the slices was 3,800 CPM/μmole.

acids into the TCA-insoluble protein of the microsomes and mitochondria and debris fractions was similar to that reported in liver. However, isolated granuloma microsomes and mitochondria had little or no incorporation activity in comparison with liver microsomes and mitochondria (Table IV). The exchange of liver supernatant for granuloma supernatant reduced the incorporation into the liver microsomes by 90 per cent. This would suggest that either the amino acid–activating enzymes or the supernatant RNA or both were inactive. It is also possible that small amounts of carrageenin present in the granuloma supernatant may have inhibited the liver system, but this was not checked directly.

When the granuloma microsomes or mitochondria were incubated with liver supernatant, there was no marked incorporation of a radioactive amino acid, which suggested that the particles also were inactive. Various possible cofactors were added in attempts to obtain an active system, but without success. Addition of all the common amino acids, 0.001 M DPN and TPN, 0.01 mg of a liver concentrate containing a mixture of nucleotide cofactors, and 0.01 M Coenzyme A produced no stimulation of incorporation.

The inactivation of protein-synthesising systems by cell breakage is not uncommon and does not necessarily indicate any fundamental difference between the granuloma and other tissues. For example, microsomes from pigeon, chicken, and turkey livers would not incorporate radioactive

TABLE IV

Incorporation of ^{14}C-Valine into Homogenate Microsomal and Mitochondrial Protein (CPM at Infinite Thickness)

	Experiment		
	E 17	E 19	E 20
Granuloma microsomes, granuloma supernatant	28	—	—
Granuloma microsomes, liver supernatant	13	—	—
Liver microsomes, granuloma supernatant	61	—	—
Liver microsomes, liver supernatant	467	—	—
Zero control	20	3.0	10.0
Granuloma mitochondria, granuloma supernatant		46.0	6.0
Granuloma mitochondria, liver supernatant		15.3	5.0
Liver mitochondria, liver supernatant		407	86.7
Liver mitochondria, granuloma supernatant			21.1

In all cases final volume was 1.0 ml; additions were 0.4 ml of particle suspension, 0.3 ml of 105,000 *g* supernatant, 1 μc of ^{14}C amino acid, 2 μmoles ATP, and 10 μmoles PEP. In experiments E 19 and E 20, PEP-kinase, 0.1 ml, was also added. The tubes were incubated for 50 minutes at 37°C under N_2.

amino acids into TCA-insoluble protein, whereas duck liver microsomes were active (4). Askonas and Humphrey (1) found that homogenisation of spleen, bone marrow, or lymph gland stopped the incorporation of ^{14}C amino acids into γ-globulins, though incorporation into TCA-insoluble material was only partially inhibited.

Some experiments were made which demonstrated the presence of amino acid–activating enzymes in the 105,000 *g* supernatant from the homogenate, using amino acid–dependent hydroxamide formation as a measure of their activity. When a mixture of amino acids was used, the total activity per gram wet weight was 40 per cent of that found with guinea pig liver supernatant, but when proline and glycine were tested separately no significant activity could be found by this method. It appeared that a more sensitive technique such as the amino acid–dependent exchange of pyrophosphate into ATP would be necessary to detect activation of these amino acids.

DISCUSSION

It is likely on general grounds that collagen synthesis follows a pattern similar to that found for other proteins, and the evidence presented in this paper does support this. The high radioactivity of the collagen isolated from the microsomal fraction points to the microsomes and their attached particles either as the site of collagen synthesis or as the site at which the newly synthesised collagen accumulates. It is also significant that amino acid–activating enzymes were present in the 105,000 *g* supernatant. The absolute specific activity of the hydroxyproline from the microsomal NSC varied rather widely, possibly owing to difficulty in obtaining reproducible granulomata, but it was always considerably higher than that derived from other subcellular fractions. In the more active preparations the specific activity was over 25 per cent of that of the amino acid pool after 45 minutes incubation, showing that there can be no large pools of intermediates between the free proline and the completed collagen molecule. In particular, any non-hydroxylated protein precursor of collagen (14) could only be present in very small quantity, unless a second independent pathway of collagen synthesis is postulated.

The high proportion of microsomal collagen extractable by neutral salt solutions (Table II) is in harmony with the data on amino acid incorporation in suggesting that the collagen has been recently synthesised. This correlation may be regarded as an extension of the relationship between age of the collagen and the ease with which it can be extracted by neutral salt solutions (25). It is worth noting in this connection that although overnight extraction was used to ensure maximum yield, 80 per cent of the microsomal NSC dissolved in 0.2 M sodium chloride within half an hour.

The physicochemical properties of the microsomal NSC, in so far as they could be determined with the small quantities available, suggested that it was a typical native collagen. When precipitated with 15 per cent NaCl and centrifuged it formed a transparent gel which on resolution in a small amount of 0.2 M NaCl gave a viscous solution. The amino acid composition differed slightly from that of the NSC derived from nuclear and mitochondrial fractions, but this could well have been due to the presence of 10 to 20 per cent of a contaminating protein, since the purification

was limited by the small amount of material to a single resolution and reprecipitation.

We must now consider how far it is possible to correlate the chemical data with the electron microscopic evidence. The earliest fibrillar material in sections of intact granuloma appeared to take the form of fine intracellular filaments, approximately 50 A in diameter (5). These filaments showed a marked tendency to lie close to and parallel to the outer cell boundary, and frequently occurred in elongated cell processes. Although they exhibited no regular striations, there was some indirect evidence to suggest that these filaments were collagenous in nature and were released into the extracellular phase, where subsequent development took place. Fine striated filaments, 50 to 100 A in diameter, occurred extracellularly, usually in proximity to striated collagen fibrils of characteristic appearance.

The fate of these filaments, both intra- and extracellular, during homogenisation of the tissue is uncertain. A number of unstriated filaments were observed in the mitochondrial fraction, but it was difficult to be sure of their identity because the damaged nuclei present gave rise to filamentous material not easily distinguishable from the cytoplasmic filaments. Filaments were not observed in electron micrographs of the microsomal fraction, but as the total collagen present was only 0.5 to 1 per cent of the dry weight, the significance of such a negative finding is uncertain. If we assume on this evidence that 50 A cytoplasmic filaments were absent from the microsomal fraction, then it is likely that the highly radioactive collagen originated from a prefibrous state or organisation, possibly associated with the microsomal vesicles. On the other hand, some of it may have been derived from 50 A cytoplasmic filaments rendered unrecognisable by homogenisation and centrifugation. Similar considerations will apply to the mitochondrial NSC, much of which probably originates from contamination of the mitochondria by microsomes. This view of the intracellular collagen would be in accord with the observation of Fitton Jackson and Smith (11) that there was considerable accumulation of protein-bound hydroxyproline in tissue culture before any collagen fibrils could be distinguished under the electron microscope.

In principle it is possible to calculate the mean concentration of intracellular collagen from the figures for mitochondrial and microsomal collagen (Table II), but in practice only a very rough estimate can be made because a considerable amount of cytoplasmic material, including unbroken cells, sediments with the nuclei and debris. The figures for the RNA content of this fraction indicate that as much as 50 per cent of the cytoplasmic material may be lost in this way. Taking this into consideration, there are probably between 30 and 100 μg of intracellular NSC per gram of tissue, or between 200 and 700 μg per milliliter of intracellular water (15). Fessler (8) has recently made solubility measurements on newly formed collagen fibres, at 37°C, *in vitro*, and shown that they are in equilibrium with 20 to 50 μg of collagen per milliliter, so that it would not be surprising if fibrogenesis commenced within the cell. The approach to equilibrium would be slow, possibly slower *in vivo* than *in vitro* (20), and equilibrium would probably never be reached within the cell. This can be seen more clearly if we consider the rate of collagen synthesis in the 7-day carrageenin granuloma, which may be calculated from the figures of Jackson (24). His data show that approximately 50 μg collagen was synthesised per gram of tissue per hour, or 350 μg per milliliter of intracellular water per hour, indicating that the intracellular collagen has a replacement time of about 1 to 2 hours. Collagen would, therefore, leave the cell as a mixture of fine fibrils and collagen molecules in various states of aggregation. This leads to a picture of fibrogenesis which is essentially similar to that put forward by other workers who have made detailed electron microscopic studies both *in vivo* (5, 10, 12) and *in vitro* (16, 33, 39).

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