Formation of Collagen Hydroxyproline *in vitro*

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Stetten & Schoenheimer (1943) fed [^{15}N]proline to young rats and showed it to be a good source of the hydroxyproline of the carcass protein. Later, Stetten (1949) found that the efficiency of dietary [^{15}N]hydroxyproline was less than one-twentieth of that of proline as a precursor of collagen hydroxyproline. These findings are of considerable interest since they are contrary to the general observation that proteins are built up from their constituent free amino acids (Loftfield, 1957). The only close parallel, discovered subsequently, is the formation of collagen hydroxylysine from lysine (Sinex & Van Slyke, 1955; Piez & Likins, 1957), but not from free hydroxylysine (Sinex & Van Slyke, 1957). Stetten (1949) suggested that free hydroxyproline was not an intermediate in collagen biosynthesis and that hydroxylation of the proline residues occurred at the protein or peptide stage. The hydroxylation of an unreactive aliphatic carbon atom is a reaction which would be expected to require some form of preliminary activation of the substrate and although it is not impossible that this might take place in a protein it appears much more likely that a low-molecular-weight substrate would be involved. However, a number of workers (Robertson & Schwartz, 1953; Gould & Woessner, 1957) have endeavoured to obtain evidence for a collagen-like protein intermediate lacking the hydroxyl groups of hydroxyproline in the tissues of scorbutic guinea pigs. They assumed that the impaired collagen synthesis in these animals resulted from inability to hydroxylate such an intermediate protein. It was shown that administration of vitamin C to scorbutic animals led to increases in hydroxyproline and parallel decreases in proline in collagen-forming tissues, but since no isolation of the hypothetical precursor was attempted the results are not conclusive.

This work was undertaken with the object both of studying the hydroxylation of proline in relation to collagen formation and of analysing this process in terms of current theories of protein synthesis. In order to obtain information about intermediates it was decided to use the granuloma induced in guinea pigs by subcutaneous injections of carrageenin (Robertson & Schwartz, 1953), since this tissue synthesizes collagen rapidly *in vivo* and appeared to be a promising tissue for experiments *in vitro*. Moreover, Jackson (1957) had already studied the incorporation *in vivo* of labelled glycine into the neutral-salt-soluble, citrate-soluble and insoluble collagen fractions of this tissue and had confirmed the results of Harkness, Marko, Muir & Neuberger (1954) for the corresponding fractions from rabbit skin. We have based our approach on the work of these authors, using labelled proline and hydroxyproline in place of glycine and using tissue slices instead of whole animals.

MATERIAL AND METHODS

Carrageenin. This was a commercial dried extract of Irish Moss obtained from the Seaplant Chemical Corporation, New Bedford, Mass., U.S.A. and was used without further purification.

Amino acids. Commercial L-proline was found to contain 3–4% of hydroxyproline, which could not readily be removed by recrystallization. A pure sample was prepared from the mixed reineckates of proline and hydroxyproline, which had been obtained from a gelatin hydrolysate (Neuberger, 1945). The reineckates were dissolved in 30% (v/v) methanol and passed through a column of Zeo-Karb 225 resin in the hydrogen form. The reinecke acid emerged in the effluent and after washing the column the proline and hydroxyproline were eluted with aq. N-NH_3 soln.

After removal of the NH_3 by evaporation the two imino acids were separated from each other and from small amounts of other amino acids by displacement chromatography on Zeo-Karb 225 resin (200-mesh) with aq. 0·1 N-NH_3 soln. as the displacing agent (Partridge & Westall, 1949). The pooled proline fractions contained less than 0·5% of hydroxyproline.

Unlabelled L-hydroxyproline was obtained from British Drug Houses Ltd. It contained less than 0·2% of proline and was chromatographically pure.

Uniformly labelled L-[^{14}C]proline was obtained from The Radiochemical Centre, Amersham, Bucks. Inactive L-proline was added to give a specific activity of 73 000 counts/min./μmole (0·47 μc/μmole). Chromatography in butan-1-ol–acetic acid–water (63:10:27) gave a single spot on developing with ninhydrin, and radioautography of the chromatogram showed that apart from the proline spot there was a faint spot in the valine position and a very faint streak extending from the proline spot towards the origin. This contamination was regarded as negligible.

Uniformly labelled L-[^{14}C]hydroxyproline was prepared from combined neutral-salt-soluble collagen and acid-soluble collagen extracted from granuloma slices incubated with labelled proline and isolated as described below. Tissue slices (20 g.) were incubated with 40 μc of uniformly

labelled proline ($5{\cdot}4 \times 10^6$ counts/min.) and from the hydrolysed soluble collagens 1·4 mg. of hydroxyproline was isolated, having a specific activity of 5600 counts/min./μmole.

Chemical estimations. Hydroxyproline was determined by the method of Neuman & Logan (1950) and proline by the ninhydrin method of Troll & Lindsley (1955). Raffinose was estimated by the orcinol method of Roe, Epstein & Goldstein (1949) and urea by the determination of NH_3 released during incubation with urease as described by Conway (1950). Total N was estimated by the Kjeldahl method. Approximate estimations of amino N were made by the colorimetric ninhydrin method of Meyer (1957).

Granuloma production. Male albino guinea pigs weighing 400–500 g. were shaved on the abdomen and injected subcutaneously with 5 ml. of a 1% solution of carrageenin in 0·9% NaCl soln. during ether anaesthesia. At the required interval after the injection the animals were killed by a blow on the neck and the granulomata were peeled away from the abdominal muscle and dermis and chilled in cold saline. The wet weight varied between 10 and 20 g.

The 7-day-old granuloma has been used for most of the metabolic experiments, since Jackson (1957) showed that the rate of collagen synthesis reached its maximum between the sixth and seventh day. At this stage the tumour weighed from 10 to 25 g. and consisted of a central gelatinous region containing little collagen, but large numbers of blood cells suspended in fluid dispersed throughout the gel. The paper-electrophoretic pattern of the fluid expressed from the gel was similar to that given by guinea-pig serum. This central region, occupying perhaps half of the bulk of the granuloma, was surrounded and penetrated by bands of highly cellular fibrous tissue which on the dorsal surface contained variable amounts of muscle derived from the panniculus carnosus.

Incubation of granuloma slices. Blocks of tissue were cut from the granuloma with scissors and 0·5 mm. slices were cut on a Stadie–Riggs microtome (Stadie & Riggs, 1944). Slices cut from the dorsal surface were often contaminated by muscle fibre and were rejected; the rest were kept at 0° in a beaker packed in ice chips.

For isotope experiments 10 g. wet wt. of slices was transferred into 50 ml. of ice-cold phosphate saline medium 4A of Krebs (1950) in a 250 ml. conical flask. The organic constituents were replaced by 22·4 mM-glucose and the following concentrations of amino acids: L-leucine (0·76 mM), L-isoleucine (0·38 mM), L-valine (0·85 mM), L-threonine (0·84 mM), L-methionine (0·13 mM), L-arginine (0·95 mM), L-lysine (1·1 mM), L-tyrosine (0·22 mM). After the addition of labelled proline the flasks were gassed with O_2 and stoppered and incubated at 37°.

In experiments designed to estimate the distribution of proline or hydroxyproline between the slices and the medium the amino acid supplements were omitted and the glucose concentration was reduced to 11·2 mM in order to reduce interference by glucose in the subsequent estimation of raffinose.

Intracellular and extracellular distribution of proline and hydroxyproline when incubated with slices of granuloma. Tissue slices were incubated in the presence of urea, raffinose, L-proline and L-hydroxyproline and after time for equilibration they were separated from the medium. The concentration (c) of each substance in the medium and the total amount (m) in the slices were then determined. The quantity m/c then gives the fluid space which the substance would occupy if it were present in the slices at its extracellular concentration. Since urea distributes itself uniformly between extracellular and intracellular water, the urea space is equal to the total slice water. Raffinose on the contrary does not enter the cells (Helmreich & Cori, 1957) and the raffinose space is therefore equal to the extracellular water. The intracellular water is thus obtainable by difference. The extracellular imino acid in the slice is given by the product of its concentration in the medium and the raffinose space. The amount of intracellular imino acid is then obtained by subtracting this from the total slice imino acid. The intracellular concentration can then be calculated since the amount of intracellular water is known.

About 1 g. wet wt. of granuloma slices was added to 5 ml. of ice-cold medium supplemented with L-proline (3·47 mM) or L-hydroxyproline (3·05 mM), glucose (11·2 mM), raffinose (6·7 mM) and urea (10 mM). The flasks, set up in triplicate or quadruplicate, were stoppered and incubated in air at 37° for 35 min. with shaking and cooled in ice–water for a few minutes. The slices were removed from the supernatant with a glass hook and allowed to drain on the sides of the flask.

The drained slices were weighed in tared specimen tubes, transferred to a small mortar and ground with 1 g. of sand and 2 ml. of saturated picric acid solution. After adding a further 8 ml. of saturated picric acid solution the protein precipitate was centrifuged and a sample (8 ml.) of the supernatant passed down a 1·6 cm. × 1·0 cm. column of Dowex-2 (200-mesh) in the chloride form to remove picric acid. The effluent and washings were made up to 15 ml.

The supernatant from the incubation flask was centrifuged for 5 min. to remove debris and a 5 ml. sample deproteinized with 5 ml. of saturated picric acid solution. A sample (8 ml.) of the supernatant was passed through a similar column of Dowex-2 and the effluent and washings were made up to 15 ml. The total volume of the supernatant was obtained from the following expression: vol. of medium added plus loss in weight of slices during incubation minus dry weight of protein in the supernatant. Proline, hydroxyproline, urea and raffinose were estimated in suitable fractions of the 15 ml. effluent. In each experiment tissue blanks were obtained by incubating slices in suspending medium without supplements. Recoveries were measured by the addition of known amounts of L-proline, L-hydroxyproline urea and ruffinose to the separated slices and supernatant immediately before deproteinization. With this technique, quantitative recoveries (96–99%) were obtained. When trichloroacetic acid was used to precipitate protein the imino acid recoveries were low.

Determination of free proline and hydroxyproline in granuloma slices. The method was essentially the same as that described above for the tissue blanks except that (i) double quantities of tissue and medium were used; (ii) the slices were separated and washed by centrifuging as in the L-[^{14}C]proline-incorporation experiments described in the next section; (iii) the picric acid was removed by taking up the amino acids on Zeo-Karb 225 (200-mesh), and washing away the picric acid and eluting with aq. N-NH_3 soln. This last procedure had the effect of concentrating the solution, since the volume of NH_3 soln. eluate was quite small.

Proline and hydroxyproline were determined after evaporating the NH_3 and taking up in a small volume of

water. On account of the small amounts of hydroxyproline to be determined the Neuman & Logan method was scaled down by a factor of two.

Estimation and purification of collagen from granuloma slices. After incubation the flasks were chilled in ice–water and the contents centrifuged at 0°. The supernatant was removed and the packed slices were rapidly washed by suspending in cold medium and again centrifuging. The packed slices and the combined supernatant and washings were stored at −20°.

To obtain a finely divided tissue suitable for extraction of soluble collagen the frozen packed slices were crudely sliced and dropped into acetone–solid CO_2. The brittle slices were blotted free from acetone and powdered in a mortar previously cooled with the acetone–solid CO_2 mixture. The powder was transferred to a chilled 50 ml. centrifuge tube and allowed to stand at 0° for a few minutes to enable any CO_2 to evaporate before extracting soluble collagen. The extraction procedure was based on that used by Jackson (1957) and is shown in Fig. 1. All operations were carried out at 4°.

The powder was washed twice with 15 ml. of water to extract free tissue amino acids. Trichloroacetic acid was added to the extract to a concentration of approx. 20% and proline and hydroxyproline were isolated as described below. Since this water extract might also have contained a metabolically active water-soluble collagen, the trichloroacetic acid precipitate was heated for 30 min. with 5 ml. of hot 5% (approx.) trichloroacetic acid to extract gelatin (Fitch, Harkness & Harkness, 1955). The trichloroacetic acid was extracted with ether and the solution concentrated to give water-soluble fraction *A*.

The washed powder was extracted overnight with four successive portions of 0·2 M-NaCl soln. buffered with 7 mM-ethylenediaminetetra-acetic acid (EDTA), pH 7·4 (15 ml./10 g. wet wt. of tissue). The combined extracts contained the neutral-salt-soluble collagen. This was precipitated by adding solid NaCl to a final concentration of 16% and the precipitate centrifuged at 26 000 *g* in the Spinco Model L Ultracentrifuge for 30 min. This precipitate was redissolved in 0·2 M-NaCl soln., pH 7·4, to which a little inactive proline had been added, and dialysed overnight against two changes of 2 l. of 0·2 M-NaCl soln. buffered with 0·01 M-phosphate, pH 7·4. The collagen was again precipitated by adding an equal volume of cold 30% (w/v) NaCl soln. and the precipitate centrifuged at 26 000 *g*. This precipitate was gelatinized by heating to 80° in a small volume of water.

The supernatant from the first precipitation of neutral-salt-soluble collagen contained the water-soluble collagen fraction described by Jackson (1957). The NaCl was removed by dialysis against large volumes of water in the cold, and the precipitated protein was spun down. The supernatant was concentrated and the gelatin extracted by heating to 90° with 5% trichloroacetic acid. The trichloroacetic acid was removed by ether extraction and the solution concentrated to give water-soluble fraction *B*.

The residue after extraction of neutral-salt-soluble collagen was then extracted overnight with three successive 10 ml. portions of sodium citrate–HCl buffer (0·5 M; pH 3·8; Britton, 1955), and finally with 10 ml. of sodium acetate buffer (0·2 M; pH 3·8; Britton, 1955). Citrate-soluble collagen was precipitated from the combined extracts by adding solid NaCl to a final concentration of 5%. The precipitate was centrifuged in the Spinco Ultracentrifuge as before and the supernatant was rejected. The acid collagen was then redissolved in 3–4 ml. of 0·01 N-acetic acid containing about 10 mg. of proline and dialysed for 24 hr. against 2 × 2 l. of 0·01 M-sodium and potassium phosphate buffer, pH 7·4. After dialysis the sac content was spun as before and the precipitate was gelatinized in water at 80°.

Insoluble collagen was isolated from the alkali-washed residues as described by Jackson (1957). All the collagen fractions were hydrolysed at 110° with 6 N-HCl for 18 hr. in sealed tubes.

Extraction of free proline and hydroxyproline. The free hydroxyproline and proline present in the supernatant from the incubation and in the water washings of the powdered frozen slices were isolated after deproteinization with cold 20% trichloroacetic acid. The trichloroacetic acid supernatants were extracted three times with an equal volume of ether to remove trichloroacetic acid and then the amino acids were desalted and chromatographed as described below.

Desalting and chromatography. All amino acid solutions were desalted before chromatography and before counting on short columns of Zeo-Karb 225 (200 mesh) in the hydrogen form. The amino acid solution was run on to a column containing about 1 g. of resin (or more if a large amount of salt was present) and the column was washed with three or four volumes of water. Amino acids were eluted with aq. N-NH_3 soln. and the eluates taken to dryness *in vacuo*.

One-dimensional chromatograms were run in the following solvent systems: (1) water-saturated phenol in an atmosphere of ammonia; (2) butan-1-ol–acetic acid–water (63:27:10); (3) pyridine–amyl alcohol–water (35:35:30). Two-dimensional chromatograms were run in the first two of these in succession.

Isolation of proline and hydroxyproline. The hydrolysates were dried *in vacuo*, dissolved in water and when necessary the solutions were desalted. The dried eluates were redissolved in 0·2 ml. of water and applied as a streak to sheets of Whatman no. 3 MM paper. Marker spots (containing 10 μg. of proline, hydroxyproline, glycine and leucine) were applied 3 cm. from either end of the streak and a descending chromatogram was developed for 27 hr. in phenol. The chromatogram was then dried at 60–70° in a current of air and the positions of the proline and hydroxyproline were detected in strips cut in the direction of the run, so as to include the marked spots and approx. 1 cm. of the edges of the streak. These strips were dipped in a 0·1% solution of ninhydrin in acetone and air-dried, and the proline and hydroxyproline revealed as pink and red spots respectively by steaming over a boiling-water bath (Clarkson, 1955). Other amino acids were detected by subsequent heating in the oven. Strips corresponding to the proline and hydroxyproline were then cut from the rest of the chromatogram and the amino acids eluted with water as in a descending chromatogram. The concentrated eluates were rechromatographed with the same technique in butanol–acetic acid–water for 36 hr., and the proline and hydroxyproline were detected and eluated as before. Imino acids isolated from collagen hydrolysates were usually found to be free from other ninhydrin-reacting material at this stage. However, chromatographed extracts of tissue amino acids or supernatant amino acids often showed contamination with small amounts of other ninhydrin-reacting material and therefore a further chromatogram

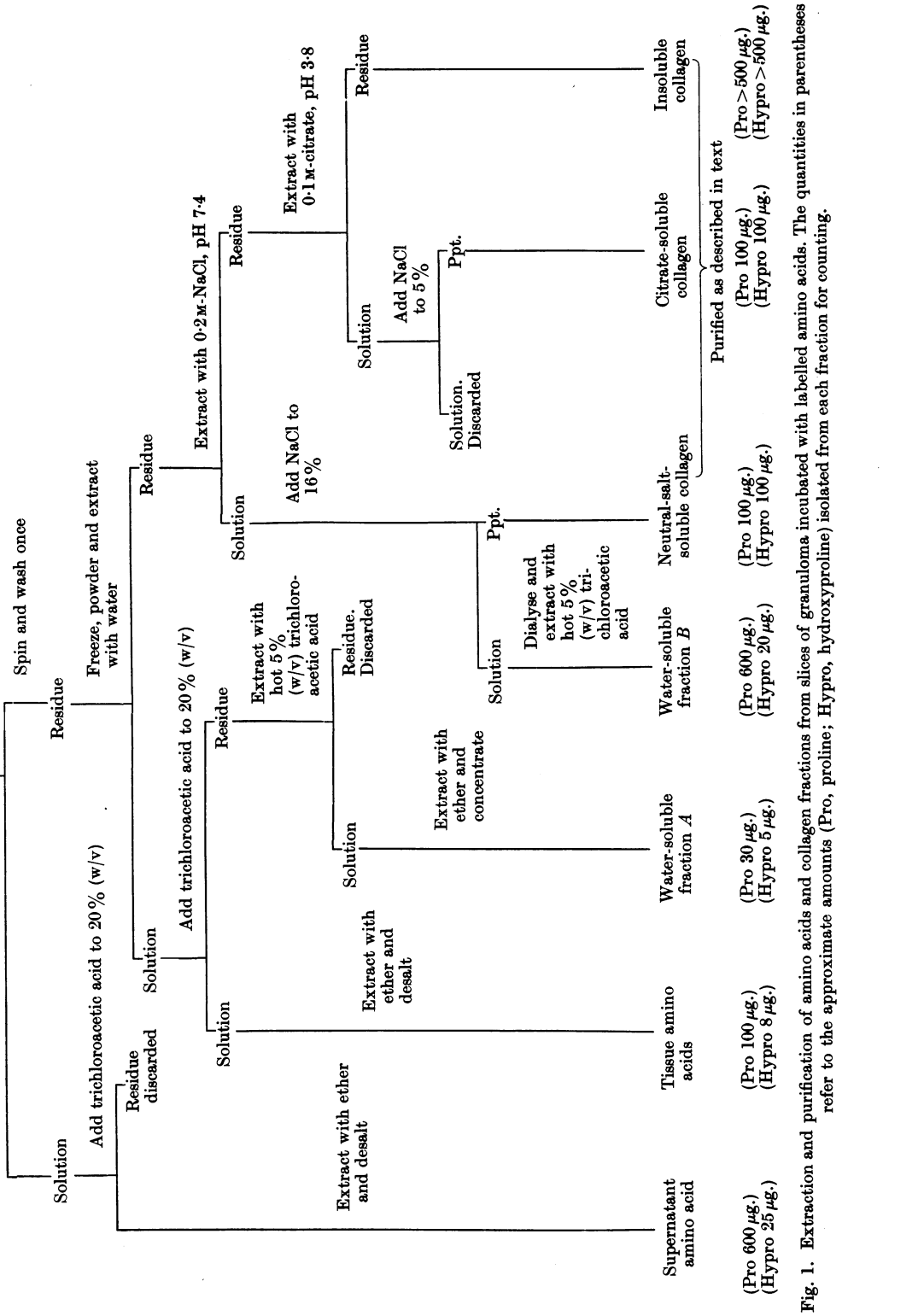

Fig. 1. Extraction and purification of amino acids and collagen fractions from slices of granuloma incubated with labelled amino acids. The quantities in parentheses refer to the approximate amounts (Pro, proline; Hypro, hydroxyproline) isolated from each fraction for counting.

was run in the pyridine–amyl alcohol–water system, or the nitrous acid procedure described below was used.

In later experiments the phenol chromatogram was omitted and the hydrolysate was first treated with nitrous acid which deaminated all the α-amino acids to the corresponding α-hydroxy acids. After this a single run in the butanol–acetic acid–water usually sufficed to separate proline and hydroxyproline in a chromatographically pure state.

Isolation of glutamic acid. In one experiment glutamic acid was isolated from the phenol chromatogram of the free amino acid fractions, and desalted and counted as described below.

Nitrous acid treatment of the mixed amino acids. The method adopted was based on that of Schweet (1954). Ice-cold sodium nitrite solution (3 ml. of 40 %, w/v) was pipetted slowly beneath the surface of 10 ml. of ice-cold conc. HCl and the precipitated NaCl allowed to settle. Samples of the brown supernatant were transferred by Pasteur pipette to the amino acid solution in a test tube, in the ratio of 1·3 ml. to approx. 20 mg. of amino acid mixture, and the reaction mixture was immediately shaken in an oil bath at 120° for 2·5 min. The solution was cooled and evaporated to dryness in a desiccator. Proline and hydroxyproline were desalted and chromatographed in butanol–acetic acid–water as previously described. This procedure gave clean bands of proline and hydroxyproline both from collagen hydrolysates and from tissue and supernatant amino acid fractions. However, a second butanol–acetic acid–water run was necessary for the hydroxyproline from the amino acid fractions since very slight trailing of the strongly radioactive proline band gave rise to large errors in the specific activity of the hydroxyproline, which contained only about 0·1 % of the total proline counts.

This method gave 80–90 % recovery of proline and hydroxyproline from collagen hydrolysates. The recovery of small amounts of hydroxyproline from large amounts of other amino acids was much lower (about 50 %).

Counting of isolated proline and hydroxyproline. All amino acid samples after elution from the chromatogram were desalted before plating for counting. This was necessary since between 2 and 4 mg. dry wt. of material could be extracted from strips of paper of the size normally used and this was sufficient to introduce considerable self-absorption errors when counting at infinite thinness. The use of paper washed with acetic acid or water for chromatography was not satisfactory since the washing greatly reduced the sensitivity of the ninhydrin colour test for proline and hydroxyproline.

From here onwards the technique was essentially that of Campbell (1955). The samples were dissolved in water and 0·1–0·2 ml. samples pipetted on to 2 cm.^{2} polythene planchets to which 10 μl. of 0·2 % cetyltrimethylammonium bromide had been added. The samples were dried in a desiccator or at 60° and counted either for 5 min. or for 1000 counts, whichever gave the greater number of counts, in a Geiger end-window counter. After counting, the planchet contents were dissolved in water and the proline or hydroxyproline was estimated by the methods given above. The relation between counts/min. and μg. of imino acid present was linear up to at least 200 μg. The specific activities of proline and hydroxyproline samples measured before and after the complete isolation procedure agreed within 10 %.

RESULTS

Free proline and hydroxyproline in the granuloma

A number of experiments were made to determine the nature of the free proline and hydroxyproline pools in the granuloma, in order to clarify the interpretation of the results of isotope-incorporation experiments. The free amino acids of the granuloma (about 18 mg. of trichloroacetic acid-soluble amino nitrogen/100 g. wet wt. of 8-days granuloma) were studied qualitatively by two-dimensional paper chromatography. The only unusual feature was the free hydroxyproline (1–2 mg./100 g. wet wt.), which was also present in the blood of guinea pigs carrying the granuloma (< 0·5 mg./100 ml.). The free proline content was much larger (4–6 mg./100 g.) and of the same order as that of other amino acids present. The increase in proline and hydroxyproline in the slices and supernatant during a typical incubation is shown in Fig. 2. It

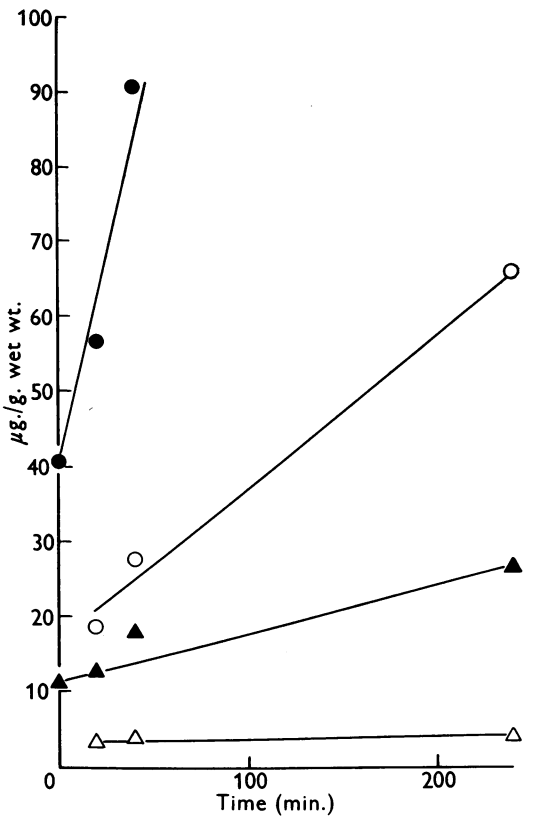

Fig. 2. Increase in free proline and hydroxyproline during incubation of tissue slices. ●, Total proline; ○, slice proline; ▲, total hydroxyproline; △, slice hydroxyproline. The 4 hr. point for total proline (300 μg./g. wet wt.) lay on the same line as the early points.

was much greater for proline than for hydroxyproline and in both cases it was directly proportional to incubation time. The increase in total amino nitrogen paralleled the increase in free proline, and paper chromatograms showed large increases in all the free amino acids present, suggesting that the proline arose from protein breakdown. The low hydroxyproline production shows that only a small proportion of this proline comes from collagen. Steinberg & Vaughan (1956) have observed similar increases in free amino acids during incubation of liver slices.

It was necessary to consider at some point the possibility that free hydroxyproline could not penetrate the fibroblast wall, since absence of its incorporation into collagen could be interpreted in terms either of a permeability barrier or of a pathway not involving free hydroxyproline. Although it seemed likely that hydroxyproline could penetrate into fibroblasts, since it was known to be taken up by ascites cells (Christensen, Riggs, Fischer & Palatine, 1952) and by intestinal mucosa (Wiseman, 1956), it was decided to test the point directly. Urea, raffinose, proline and hydroxyproline spaces were therefore measured on slices of 12-day-old granuloma, since this appeared to be more fibrous, with less extracellular space than 7-day tissue. The results, given in Table 1, show that proline and hydroxyproline were taken up by the cells of the granuloma to similar extents and were in fact concentrated within them. Unfortunately it is still possible that any lack of incorporation of free hydroxyproline could be due to a permeability barrier. The cell population of the granuloma is heterogeneous, so that although histological examination showed a predominance of fibroblasts it is possible, though unlikely, that all intracellular hydroxyproline is contained within other cells (muscle cells, macrophages and other white corpuscles) and that fibroblasts are impermeable to hydroxyproline. Furthermore, these experiments do not exclude an intracellular-permeability barrier which could prevent access of exogenous hydroxyproline to the site of collagen synthesis, but which would not prevent utilization of endogenous hydroxyproline. These two possibilities are difficult to test experimentally and, since there is no independent support for them, they will be provisionally disregarded.

These experiments also showed negligible oxidative metabolism of proline or hydroxyproline during the 35 min. incubation period. This was confirmed in a number of other experiments on 6-day and 8-day tissue, which had been designed to detect direct oxidation of proline to hydroxyproline. No such conversion was observed with ordinary analytical techniques, and neither proline nor hydroxyproline was appreciably oxidized. Experiments with L-[^{14}C]proline showed that less than 0·1% of the radioactivity appeared in the free glutamic acid after incubation for 4 hr., showing that this pathway of proline breakdown is not of great importance in this tissue.

Specific activity of the proline pool

The change in specific activity of the proline of the supernatant amino acid and tissue amino acid fractions with time is shown in Table 2. The most remarkable feature of these results is the practically constant specific activity of the tissue amino acid proline. It changed very little between 10 min. and 4 hr., although the specific activity of the supernatant amino acid proline was two to four times as great throughout this period. Since the tissue amino acid fraction contains both extracellular and intracellular amino acids, the average specific activity of the intracellular proline pool may not have been constant, nevertheless it must have been much lower than either that of the extracellular proline or that of the total tissue amino acid proline. No reliable estimate of the specific activity of the intracellular proline could be made on

Table 1. *Distribution of* L-*proline and* L-*hydroxyproline between slices and medium*

Calculation of the results is described under Methods. The errors are standard deviations calculated from the results of four to six replicate incubations.

	Urea	Raffinose	Proline	Hydroxyproline
Space (expressed as % wet wt. of slices after incubation)	85·5±2	72±2	107±4	103±1
Intracellular concentration / Extracellular concentration	1	0	2·6±0·6	2·3±0·5

Table 2. *Specific activity of the proline pool*

Time of incubation (min.)	10	20	30	45	240
Supernatant amino acid (counts/min./μmole)	14 700	13 500	12 200	10 600	4 000
Tissue amino acid (counts/min./μmole)	2 700	2 100	2 600	2 900	2 000

account of the large proportion of extracellular space in the tissue, for even the washed slices retained over 50% by weight of medium, as measured by raffinose space.

Rate of uptake of [^{14}C]*proline into collagen*

After a lag period of 5 min. the specific activity of the neutral collagen proline increased linearly with time for at least 45 min. (Fig. 3). After 4 hr. the specific activity was about double that at 45 min. Calculation of the rate of collagen synthesis from Fig. 3 requires a knowledge of the specific activity of the intracellular proline pool and it will be considered later.

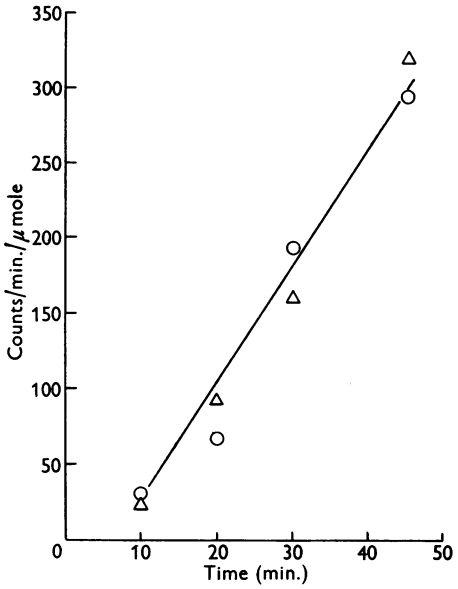

Fig. 3. Increase in specific activity of proline (○) and hydroxyproline (△) from neutral-salt-soluble collagen during incubation of tissue slices.

The specific activities of proline and hydroxyproline isolated from various collagen fractions after incubation for 4 hr. are shown in Tables 3 and 4. The relative activities of the neutral-salt-soluble, citrate-soluble and insoluble collagens are in accord with earlier experiments performed *in vivo* showing the uptake of [^{14}C]glycine into corresponding fractions from rabbit skin (Harkness *et al.* 1954) and from carrageenin-induced granuloma (Jackson, 1957). The total counts incorporated into neutral-salt-soluble collagen after 4 hr. were 20 times those in either citrate-soluble collagen or insoluble collagen. Since Jackson (1957) has shown that it is only the insoluble collagen which undergoes a large increase in total mass, the neutral collagen must be its main precursor.

The two water-soluble fractions were studied since it appeared possible that they might represent precursors of neutral-salt-soluble collagen. The fraction *A* probably contained any intracellular collagen in solution. It will be seen from Fig. 1 that the amount of hydroxyproline isolated was very small (15 μg. from 30 g. of slices). Fraction *B*, which corresponded to the 'water-soluble fraction' of Jackson (1957), contained considerably more hydroxyproline. Both fractions were quite highly labelled, although of lower specific activity than the neutral-salt-soluble collagen (Table 4). Unfortunately both were crude mixtures of proteins and contained only a small proportion of collagen, as can be seen from their relative contents of proline and hydroxyproline (Fig. 1). This difficulty was

Table 3. *Specific activities (counts/min./μmole) of proline and hydroxyproline from collagen fractions, after incubation for 4 hr.*

Specific activities in parentheses in the last column are based on low counts (<5/min.). The other insoluble-collagen specific activities are one-seventeenth of those obtained from a separate experiment in which 17 times as much [^{14}C]proline was added.

	Tissue amino acid	Neutral-salt-soluble collagen	Citrate-soluble collagen	Insoluble collagen
Hydroxyproline	—	1480	70	(4·6) 3·8
Proline	3900	910	50	(5·3) 3·9
$\frac{\text{Hydroxyproline}}{\text{Proline}}$	—	1·6±0·1 (average of six 4 hr. experiments)	1·3	(0·9) 1·0

Table 4. *Specific activities of hydroxyproline from the water-soluble fractions*

	Time of incubation (hr.)	Hydroxyproline (counts/min./μmole)		
		From neutral-salt-soluble collagen	From water-soluble fractions	
			A	*B*
Expt. C. 28	0·5	144	79	—
Expt. C. 30	2	470	—	315

partly overcome by isolating hydroxyproline, which arises only from the collagen. However, it is likely that this hydroxyproline was metabolically heterogeneous and that it was derived both from denatured neutral-salt-soluble collagen (or possibly from a neutral-salt-soluble collagen precursor) and from degraded unlabelled collagen (gelatin) arising during incubation.

Conversion of proline into hydroxyproline

The specific activity of the neutral-salt-soluble collagen hydroxyproline was of the same order as that of the proline, showing that most of it must have been derived directly from proline. At early times (less than 40 min.) it was usually lower (the 20 min. point of Fig. 3 was the only exception), whereas later it was equal to or higher than the specific activity of the proline (Fig. 3 and Tables 3 and 5). If all the collagen hydroxyproline had been derived directly from proline (free or bound) without accumulation of intermediates, a constant specific-activity ratio of unity would have been expected, so that this variable ratio indicates some more complex process. It is especially difficult to understand how the collagen hydroxyproline could under any circumstances attain a specific activity higher than that of the proline. The earliest explanation considered was that it was an experimental artifact due to a protein impurity containing unlabelled proline. The neutral collagen was therefore purified further by three different methods, applied to different samples: (i) it was dissolved at pH 7·4 and precipitated with 15% NaCl; (ii) it was redissolved at pH 3·8 and precipitated with 5% NaCl; (iii) it was redissolved at pH 7·4 and the fraction taken which underwent reversible gelling when warmed to 37° (Fessler, 1956). None of these procedures affected the hydroxyproline/proline specific-activity ratio or the hydroxyproline/nitrogen ratio. Further discussion of this problem is deferred until later.

The ratios of the specific activity of hydroxyproline to that of proline from the citrate-soluble and insoluble collagen fractions was lower than the corresponding ratio for the neutral-salt-soluble fraction (Table 3). This is possibly because much of the radioactivity in these fractions passed through the neutral-salt-soluble collagen stage at relatively early times during the incubation when the ratio was lower than it was after 4 hr.

In order to test for free hydroxyproline as an intermediate in the formation of collagen hydroxyproline, unlabelled L-hydroxyproline was added to the incubation mixture (Table 5). At mM concentration it had little effect, but at 10 mM the specific activities of both proline and hydroxyproline were considerably reduced. There was apparently some inhibition of collagen synthesis without any dilution of hydroxyproline radioactivity relative to proline, showing that free hydroxyproline could not be an intermediate. Inhibition by hydroxyproline of incorporation of proline into proteins has also been observed by Steward, Pollard, Patchett & Witkop (1958).

A study of the radioactivity of the free hydroxyproline from the tissue and supernatant amino acid fractions leads to similar conclusions about its role in collagen synthesis (Table 5). There was no great difference between specific activities of the two fractions, which were both less than half that of the neutral-salt-soluble collagen hydroxyproline. In itself this specific activity was not so low as to exclude free hydroxyproline from the role of intermediate, since the pool might have been metabolically heterogeneous. However, addition of unlabelled hydroxyproline did not trap any additional counts in this fraction, whose specific activity therefore fell sharply. It seems likely that this labelled free hydroxyproline arises from the irreversible breakdown either of labelled collagen or of some hydroxyproline-containing precursor. After these experiments were completed it was

Table 5. *Effect of adding unlabelled hydroxyproline* (1) *on the incorporation of* L-[^{14}C]*proline into collagen and* (2) *on the specific activity of free hydroxyproline*

Specific activities have been multiplied by the factor 4000/specific activity of tissue amino acid proline, in order to correct for differences in the specific activity of the amino acid pool. Figures in parentheses are total counts isolated.

		Specific activities of proline and hydroxyproline (counts/min./μmole)				
		Neutral-salt-soluble collagen				
Incubation time (hr.)	Concn. of hydroxyproline added (mM)	Hydroxyproline	Proline	Hydroxyproline / Proline	Tissue amino acid Hydroxyproline	Supernatant amino acid Hydroxyproline
2	0	780	680	1·15	—	88 (22)
2	10	440	400	1·10	—	0·6 (36)*
4	0	1510	930	1·64	370 (27)	380 (73)
4	1	1450	860	1·69	—	—
4	10	830	520	1·60	3·4 (20)*	1·6 (88)*

* Values are based on low counts (<5/min.) and therefore approximate.

Table 6. *Comparison of* [^{14}C]*proline and* [^{14}C]*hydroxyproline as sources of neutral-salt-soluble collagen hydroxyproline*

Labelled amino acid added	Incubation time (hr.)	Activity added (counts/min.)	Counts/min./μmole: Supernatant amino acid: Proline	Supernatant amino acid: Hydroxyproline	Tissue amino acid: Proline	Tissue amino acid: Hydroxyproline	Neutral-salt-soluble collagen hydroxyproline
Proline	0	150 000	—	—	—	—	2·1
Proline	2	150 000	5200	—	2400	—	465
Hydroxyproline	2	27 000	—	2900	—	3500	2·3

found that the labelled proline used contained traces (0·03 %) of labelled hydroxyproline, which could be isolated from it by adding carrier, and treating with nitrous acid and chromatographing several times in butanol–acetic acid–water. This hydroxyproline accounted for a considerable proportion of the counts in the free hydroxyproline fractions. However, the 2 hr. experiment (Table 5), for which L-[^{14}C]proline purified by butanol–acetic acid–water chromatography was used, shows that some of the radioactivity was genuinely derived from proline. The presence of this labelled hydroxyproline does not therefore affect the conclusions drawn from these experiments.

To obtain final confirmation that free hydroxyproline could not be incorporated into collagen, labelled hydroxyproline was incubated with tissue slices in the usual system and the specific activity of the amino acid and neutral-salt-soluble collagen fractions were determined (Table 6). The incorporation of labelled hydroxyproline was negligible. The 2 counts/min. which did appear were quite possibly due to slight contamination of the neutral collagen by labelled free hydroxyproline, since in a control experiment similar activity appeared in proline when neutral-salt-soluble collagen was isolated in the presence of labelled proline added after incubation.

DISCUSSION

Collagen precursors

Some form of soluble collagen is now firmly established as the precursor of the collagen fibre. Orekhovitch & Shpikiter (1957) maintain that citrate-soluble collagen (so-called procollagen) is the precursor, although Harkness *et al.* (1954) and Jackson (1957) have shown quite clearly that the neutral-salt-soluble fraction becomes labelled both more rapidly and more highly than the citrate-soluble fraction. Moreover, Jackson (1957) has pointed out that the method used by Orekhovitch to extract citrate-soluble collagen will also extract neutral-salt-soluble collagen, so that his 'procollagen' is a mixture of the two fractions. Our results confirm that the neutral-salt-soluble collagen fraction represents the earliest stages of collagen synthesis, but before making categorical statements about a particular fraction being a collagen precursor the fractionation procedure should be examined in more detail.

Superficially, neutral-salt-soluble and citrate-soluble collagens appear to be metabolically distinct fractions, but it is becoming increasingly apparent that this is misleading. It seems more accurate to regard them as arbitrary fractions from a continuous series of 'collagens' of decreasing solubility. Once the collagen is in solution its properties are largely independent of the method of extraction, provided that there has been no denaturation. The amino acid compositions of the neutral and citrate-soluble fractions are almost the same (Jackson, Leach & Jacobs, 1958) and there is increasing evidence that the two fractions cannot be distinguished on physicochemical grounds (Peng & Tsao, 1956; Orekhovitch & Shpikiter, 1957; Bensusan & Hoyt, 1958). Both Gross (1956, 1958*b*) and Bensusan & Hoyt (1958) have shown that when acid-soluble collagen is dialysed against neutral buffers it remains in solution, provided that the ionic strength is greater than 0·15. The resulting neutral solutions have the same fibre-forming properties as solutions of neutral-salt-soluble collagen. The arbitrary nature of the distinction is further emphasized by the experiments of Gross (1958*a*), who found that 0·45 M-sodium chloride solution extracted five times as much collagen from skin of young guinea pigs as did 0·2 M-sodium chloride solution.

The continuous gradation of solubilities of the constituents of the collagen fibre has a metabolic parallel in continuously decreasing specific activities of the collagen from consecutive citrate extracts of rabbit skin, corresponding to less and less soluble fractions (Harkness *et al.* 1954; Orekhovitch & Shpikiter, 1957). We have repeated this observation on successive neutral salt extracts of the granuloma. It is therefore incorrect to refer to a collagen fraction isolated by a particular extraction method as the precursor of the collagen fibre since all the solvents used extract more or less fibrous collagen in addition to collagen in solution. The amount of collagen actually present in solution *in vivo* at any one time is very small and presumably appears in the first extract in whatever

solvent is being used, along with varying amounts of collagen derived from the fibre. The 0·2M-neutral-salt-soluble fraction contains only the most recently laid down and hence most highly labelled collagen whereas the citrate extract includes more older material formed before the introduction of the labelled amino acid. The 0·45M-sodium chloride solution extract of Gross (1958*a*) may occupy an intermediate position.

Careful observation of electron micrographs of collagen fibres led Gross, Highberger & Schmitt (1954) to postulate the existence of an elongated collagen particle or molecule (tropocollagen), probably secreted by the fibroblast and subsequently aggregating more or less spontaneously to form collagen fibres (Gross, 1956). The existence of such particles in collagen solutions has been amply confirmed by Boedtker & Doty (1956). On the basis of the evidence from incorporation of isotopic amino acids it has usually been assumed that the neutral-salt-soluble collagen fraction contains the tropocollagen of Gross *et al.* (1954). However, there has been no evidence to exclude a soluble precursor representing an even earlier stage of collagen formation than the neutral-salt extract. Jackson (1957) commented on the bound hydroxyproline content of water-soluble fraction *B* and suggested that it might contain a neutral-salt-soluble collagen precursor. Our measurement of the specific activity of the hydroxyproline of the two water-soluble fractions *A* and *B* (Table 4) show that this can be so only if these fractions contain a high proportion of metabolically inert collagen in addition to the hypothetical precursor. Since no purification was attempted, this is not impossible. However, evidence has recently been obtained which locates more precisely the intracellular source of neutral-salt-soluble collagen. It has been found (N. M. Green & D. A. Lowther, unpublished work) that bound hydroxyproline isolated from the 'microsome fraction' after incubation of granuloma slices with labelled proline has nearly four times the specific activity of the neutral-salt-soluble collagen hydroxyproline. Moreover, this hydroxyproline can be extracted from the microsomes with 0·14M-sodium chloride solution and coprecipitated with carrier neutral-salt-soluble collagen, providing good evidence for the chemical identity of collagen extracted from the fibres by neutral-salt solutions with microsomal collagen, which is probably the first collagen to be formed.

Conversion of proline into hydroxyproline

The results presented in Table 6 show that free hydroxyproline is not incorporated into the neutral-salt-soluble collagen of granuloma slices whereas under the same conditions both proline and glycine (unpublished experiments) are incorporated. Moreover, both the proline and the hydroxyproline of the soluble collagens isolated from slices incubated with L-[^{14}C]proline are approximately equally labelled and the addition of hydroxyproline does not alter their relative specific activities (Table 5). These experiments suggest that collagen hydroxyproline is derived from added proline, as previously suggested by Stetten (1949), and that free hydroxyproline is not an intermediate. Stetten's results have recently been confirmed by Jackson & Smith (1957), who studied the incorporation of [^{14}C]proline into the protein of osteoblasts *in vitro*, by Mitoma & Smith (1957) who used a granulation-tissue mince and by Wolf & Berger (1958) who fed L-[^{14}C]hydroxyproline to rats. The most likely explanation of these results is that proline is hydroxylated only in a bound form (Stetten, 1949) and that the resulting bound hydroxyproline is incorporated directly into collagen. The alternative mentioned above, that there is an intracellular permeability barrier preventing access of exogenous hydroxyproline to the site of collagen synthesis, is difficult to test experimentally and will be provisionally disregarded. Although our results do not allow any direct inferences to be made about the nature of the bound proline which is hydroxylated, the ratio of specific activities of hydroxyproline/proline provokes a number of questions related to this problem. This ratio increases with time from a value of 0·5–0·7 after 10 min. to 1·0 at about 40 min. and to 1·6 after 4 hr. (Fig. 3 and Table 3). Ratios greater than 1 were also obtained *in vivo*, when collagen was isolated from rat skin after feeding L-[^{14}C]proline (A. Neuberger & F. Charconnet, unpublished work). We first considered that the high ratio might be due to a protein impurity containing proline of low specific activity, but further purification of neutral-salt-soluble collagen was without effect. Furthermore, the amounts of such an impurity required would be so large that the amino acid composition of the collagen would be changed. Since neutral-salt-soluble collagen from rabbit skin has almost the same amino acid composition as the other collagen fractions (Jackson *et al.* 1958), and since our neutral-salt-soluble fraction has a normal hydroxyproline content (7·7 g./100 g. of protein nitrogen) this explanation is untenable. Another possibility is that there is a source of unlabelled proline which can enter collagen without mixing completely with the pool of labelled free proline, thus continually diluting collagen proline relative to hydroxyproline. The breakdown of cell proteins could fulfil this role provided that there was some direct coupling of breakdown to collagen synthesis. This could take the form either of an activated proline derived from protein breakdown or of a physical inhomogeneity in the intracellular amino

acid pool whereby amino acids from protein breakdown were preferentially reutilized for synthesis of collagen or other protein. The low specific activity of hydroxyproline relative to proline at early times requires a separate explanation. It is possible that there is a larger accumulation of intermediates between free proline and collagen hydroxyproline than between free proline and collagen proline. Approximate calculations show that an extra pool equivalent to about 1–2 μg. of hydroxyproline/g. of tissue on the hydroxyproline pathway could account for the observed results (Fig. 3). The last two hypotheses are incompatible with hydroxylation of proline after completion of the collagen peptide chain. In general it can be stated that hydroxylation at this stage cannot be reconciled with the hydroxyproline/proline specific-activity ratios without making even more complicated assumptions.

Rate of synthesis of collagen

In order to calculate the rate of turnover of neutral-salt-soluble collagen from the isotope-incorporation data it is necessary to know the specific activity of the amino acid pool from which it was formed. Unfortunately it is impossible to obtain an accurate estimate of this from our data. The tissue amino acid fraction isolated from the washed slices contained a considerable proportion of extracellular amino acid, whose specific activity was presumably close to that of the proline in the incubation medium. This would not matter if there had not been so high a ratio of the specific activity of the proline of the supernatant amino acid fraction to that of the tissue amino acid fraction. This ratio, which fell only slowly during the experiment in spite of the high specific-activity gradient, was probably of the same origin as that observed in liver *in vivo* (Loftfield & Harris, 1956) and in perfused lung (Askonas & Humphrey, 1958), although its magnitude was initially about double that found in these systems. Either there was a pool of free intracellular proline which remained unlabelled and which exchanged only slowly with the pool from which collagen was synthesized, or the continuous turnover of intracellular protein maintained the specific activity of the intracellular proline below that in the incubation medium. The second hypothesis seems the more likely on general grounds, moreover the existence of a high protein turnover receives support from the large amounts of proline liberated from the slices during incubation. It is interesting to contrast the behaviour of hydroxyproline, whose specific activity was almost the same in both supernatant and tissue amino acid fractions (Tables 5 and 6) and which was liberated from the slices at only one-fifteenth of the rate of proline liberation. Unfortunately, this could be explained equally well in terms of either hypothesis.

In view of the uncertainties attached to any estimate of the specific activity of the amino acid pool it is perhaps more informative to assume a maximum rate of synthesis equal to that found by Jackson (1957) *in vivo* and to calculate from this a minimum specific activity for the pool proline. For a 7-day granuloma Jackson found that 6 μg. of collagen hydroxyproline/g./hr. was synthesized, whereas the total neutral-salt-soluble collagen did not change significantly. Since the pool of neutral-salt-soluble collagen hydroxyproline was 40 μg./g. of slices in our experiments, this rate corresponds to 15% replacement/hr. The average specific activity of the free proline pool should therefore be approximately 100/15 times that of the neutral-salt-soluble collagen proline after incubation for 1 hr., since only 5% of the counts in the neutral-salt-soluble collagen were transferred to the acid-soluble and insoluble collagen fractions. From consideration of a 30 min. period during the linear portion of Fig. 3, the average specific activity of the proline pool would be

$$\frac{100 \times 235}{7{\cdot}5} = 3100 \text{ counts/min./}\mu\text{mole.}$$

This is slightly higher than the specific activity of tissue amino acid proline during this period (Table 3). Since this fraction contained considerable amounts of extracellular proline of high specific activity the average specific activity of the intracellular proline was lower than that of the whole tissue amino acid. It is therefore difficult to account for the higher specific activity just calculated, unless the intracellular pool is heterogeneous. Although this is not unlikely, there is insufficient evidence for further discussion.

SUMMARY

1. When slices of granuloma induced by carrageenin were incubated in the presence of L-[^{14}C]proline, both proline and hydroxyproline isolated from the soluble and insoluble collagen fractions were labelled. After a lag period of 5 min. the specific activities of proline and hydroxyproline from neutral-salt-soluble collagen increased linearly with time for at least 45 min. After 4 hr. the total counts in this fraction were 20 times those in the acid-soluble or insoluble collagen fractions, confirming that neutral-salt-soluble collagen represents an early stage in collagen-fibre formation.

2. The specific activity of hydroxyproline from neutral-salt-soluble collagen increased from approximately 0·6 to 1·6 times that of the proline during the incubation for 4 hr. Tentative explanations for this variable ratio were put forward and it

was pointed out that it would be difficult to reconcile with any scheme of collagen synthesis involving the hydroxylation of proline as a final stage.

3. The addition of unlabelled L-hydroxyproline to the incubation medium in the presence of L-[^{14}C]proline had no effect on the ratio of the specific activities of collagen hydroxyproline and proline although the total radioactivity incorporated was reduced.

4. Incubation of the tissue with L-[^{14}C]hydroxyproline did not result in a significant incorporation of radioactivity into collagen.

5. Radioactive free hydroxyproline was isolated from the slices and medium after incubation with L-[^{14}C]proline but its specific activity was only half that of the neutral-salt-soluble collagen hydroxyproline and the total counts present were not increased when unlabelled hydroxyproline was present as a trapping agent.

6. It is concluded that free hydroxyproline is not an intermediate in the formation of the hydroxyproline of collagen.

7. Both proline and hydroxyproline added to the medium were found to be concentrated intracellularly about 2·5 times. The lack of incorporation of free hydroxyproline cannot therefore be due to the impermeability of the cells towards hydroxyproline.

8. The specific activity of the free proline from the slices increased to 20 % of that in the incubation medium within 10 min. but no further increase occurred in spite of the high specific activity of the proline from the medium. Although the latter fell to approximately one-third of its initial value during incubation for 4 hr., owing to dilution by proline liberated from the slices, it was never less than twice the specific activity of the slice proline.

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