# NUCLEIC ACIDS AND THE BIOSYNTHESIS OF COLLAGEN

By P. H. Springell*

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*Summary*

The study of skin protein fractions obtained by salt fractionation indicates that collagen is associated to a greater extent with the ribonucleoprotein fraction than with the deoxyribonucleoprotein fraction. The gelatin derived from the ribonucleoprotein fraction following *in vitro* incubation of foetal lambskin in the presence of glycine-2-$^{14}$C was appreciably radioactive. Pre-treatment of the skin with crystalline ribonuclease resulted in a marked inhibition of glycine incorporation into total gelatin, whereas crystalline deoxyribonuclease had little effect. It is therefore concluded that ribonucleic acid rather than deoxyribonucleic acid is associated with the biosynthesis of collagen.

Methods of extracting collagen from tissues were compared and discussed in relation to possible nucleic acid contamination.

## I. Introduction

The importance of nucleic acids in the biosynthesis of proteins has become increasingly evident in recent years (Brachet 1955). The extension of these studies to collagen has been hampered by its relative metabolic inertness (Orekhovich 1952; Harkness *et al.* 1954), particularly in adult mammals. Although previously *in vitro* studies were confined to long-term experiments (Gerarde and Jones 1953), in the present work a short-term *in vitro* system with foetal sheepskin pieces was made possible by use of the isotopic tracer technique.

## II. Experimental

### (*a*) *Materials*

(i) *Foetal Sheepskin.*—Foetuses (15–23 cm from base of neck to base of tail) from freshly killed ewes were kept warm until ready for incubation within an hour of slaughter. Portions of skin were dissected from the mid-dorsal region.

(ii) *Adult Sheepskin.*—Snippets of skin were taken from the edge of fresh pelts and freed from the bulk of the wool.

(iii) *Kangaroo Tail Tendon.*—This was air-dried material which had not been subjected to any chemical treatment.

(iv) *Commercial Gelatins.*—Davis and Difco gelatins were used.

(v) *Crystalline Enzymes.*—Crystalline ribonuclease (Armour, lot 3014X) and crystalline deoxyribonuclease (Worthington, lot 289) were used.

(vi) *Glycine-2-$^{14}$C.*—A 1 mc (19·4 mg) sample of methylene-labelled glycine (Radiochemical Centre, Amersham) was dissolved in 100 ml distilled water, and stored in the frozen state until required.

*Biochemistry Unit, Wool Textile Research Laboratories, C.S.I.R.O., Parkville, Vic.

### (*b*) *Methods*

(i) *Incubation.*—About 2 g (wet weight) foetal skin was placed in an incubating medium consisting of 40 ml of solution with a final concentration of 0·13M potassium phosphate buffer, pH 7·4, 0·13M sodium chloride, 1: 20,000 "Merthiolate", and 2 ml radioglycine solution. In experiments where pre-incubation with nucleases was undertaken, the medium consisted in the first instance of 20 ml 0·05M potassium phosphate buffer, pH 7·4, containing 0·003M magnesium sulphate and 2 ml radioglycine solution with or without 5 mg of the appropriate nuclease. At the termination of the pre-incubation period of 2 hr, a further 20 ml of solution containing 0·25M potassium phosphate buffer, pH 7·4, 0·26M sodium chloride, and 1 : 10,000 "Merthiolate" was added. The incubation and pre-incubation were carried out in 150 ml bubblers (5 by 15 cm) for a total of between 28 and 32 hr at 37°C in a slow stream of moist oxygen containing 5 per cent. carbon dioxide. Four incubations were carried out simultaneously. Controls were treated similarly, but were kept at 0°C without gassing.

(ii) *Isolation of Skin Proteins.*—The skin pieces were removed from the medium, blotted on filter paper, and stored in 50-ml centrifuge tubes at −15°C before further treatment. Fresh foetal or adult sheepskin in non-radioactive experiments were also frozen before use.

(1) *Extraction of Skin by Trichloroacetic Acid.*—The procedure used was a modification of the method of Fitch, Harkness, and Harkness (1955). Skin pieces were extracted with 5 ml of 10 per cent. trichloroacetic acid for 15 min at 90°C. The supernatant was collected by centrifugation, the precipitate disintegrated with 10 ml of 10 per cent. trichloroacetic acid in a glass homogenizer, and the heat treatment and centrifuging were then repeated. The residue was then washed twice with 5 ml of 10 per cent. trichloroacetic acid and the pooled extracts were clarified by centrifugation and filtration. The supernatants were dialysed against several changes of distilled water for at least 72 hr. In experiments with labelled glycine, about 0·5 g of non-radioactive glycine was added prior to dialysis. The dialysed protein was again filtered, and an aliquot withdrawn for examination in ultraviolet light and dry weight determination if necessary. In radioglycine experiments the bulk was precipitated by mixing seven volumes of gelatin solution with one volume of 1M sodium chloride and pouring this into 36 volumes of acetone. The gelatin was allowed to precipitate out overnight at 0°C, and was then collected by centrifugation and finally dried with acetone and ether to yield a fine powder suitable for plating.

The residues were suspended in 5 ml of 10 per cent. non-radioactive glycine overnight (in non-radioactive experiments this was omitted), washed in water, and dried with acetone and ether.

(2) *Extraction of Skin by Autoclaving.*—Skin pieces were autoclaved in 5 ml of distilled water for 3 hr at 15 lb/in.$^2$. The extract was collected by centrifugation, and the residue washed twice more with hot water. The combined extracts and washings were filtered and, in radioglycine experiments, 0·5 g of non-radioactive glycine was added before dialysis. The dialysed protein and the insoluble material remaining after autoclaving were worked up as in the trichloroacetic acid procedure.

(3) *Fractionation of Skin Proteins.*—The procedure used was based on that of Mirsky and Pollister (1946) and Wiest and Heidelberger (1953). The thawed skin pieces were disintegrated with 6 ml of 0·14M sodium chloride – 0·01M potassium phosphate buffer, pH 6·8, in a glass homogenizer. The residue was collected by centrifugation and was then re-extracted twice more with sodium chloride–potassium phosphate. The pooled supernatants were clarified by further centrifugation, approximately 0·5 g of non-radioactive glycine was added, followed by 2 ml of 5M acetic acid to precipitate the ribonucleoprotein which was then allowed to settle overnight. The ribonucleoprotein was washed with 0·14M sodium chloride, distilled water, and then dried with acetone and ether before plating.

The residue was extracted with 6 ml of 1M sodium chloride and left overnight in the cold before removal of the supernatant by centrifugation. The precipitate was re-extracted with two further lots of 1M sodium chloride and the combined supernatants were clarified by further centrifugation and filtration. After the addition of 0·5 g of non-radioactive glycine, the deoxyribonucleoprotein fraction was dialysed against 1M sodium chloride for 72 hr, then precipitated with 5 per cent. trichloroacetic acid, washed with more trichloroacetic acid, and then dried with acetone and ether before plating.

TABLE 1

RECOVERY OF EXTRACTS AND RESIDUES FROM SHEEPSKIN

| | Foetal | Adult |
|---|---|---|
| Wet weight of whole skin (mg) | 2000 | 2000 |
| Dry weight of whole skin (mg) | 224 | 510 |
| Extraction with trichloroacetic acid | | |
| Dry weight of dialysed extract (mg) | 22 | 127 |
| Dry weight of residue (mg) | 127 | 252 |
| Extraction by autoclaving | | |
| Dry weight of dialysed extract (mg) | 73 | 187 |
| Dry weight of residue (mg) | 83 | 183 |

Subsequent to counting, both nucleoprotein fractions were autoclaved and gelatins isolated by the procedure described above.

(iii) *Treatment of Commercial Gelatins and Kangaroo Tail Tendon.*—The gelatins were either dissolved and examined as such, or dialysed as well; in other instances they were subjected to the autoclaving or trichloroacetic acid treatments. Kangaroo tail tendon collagen was converted to gelatin by means of trichloroacetic acid or by autoclaving.

(iv) *Ultraviolet Spectrophotometry.*—A Beckman model DU spectrophotometer was used. The samples examined were diluted to yield solutions of 0·01–0·02 per cent. The $E^{1\%}_{1\,\mathrm{cm}}$ values were calculated following dry weight determinations on suitable aliquots.

(v) *Radioactivity Measurements.*—Aluminium discs (25 mm diameter, 4 mm thick, 1 $cm^2$ well) were used for mounting radioactive samples. As far as possible sufficient material was used to give samples of infinite thickness, but where this was not possible, counts were corrected to infinite thickness. A $\pm 5$ per cent. standard error of counting was not exceeded. Radioactivities were measured using a thin-end window Geiger-Müller tube (EHM $2^S$) with a conventional scaler unit.

## III. Results

While investigating the most suitable method for extraction of total collagen from sheepskin, it was observed that when both adult and foetal skins were extracted

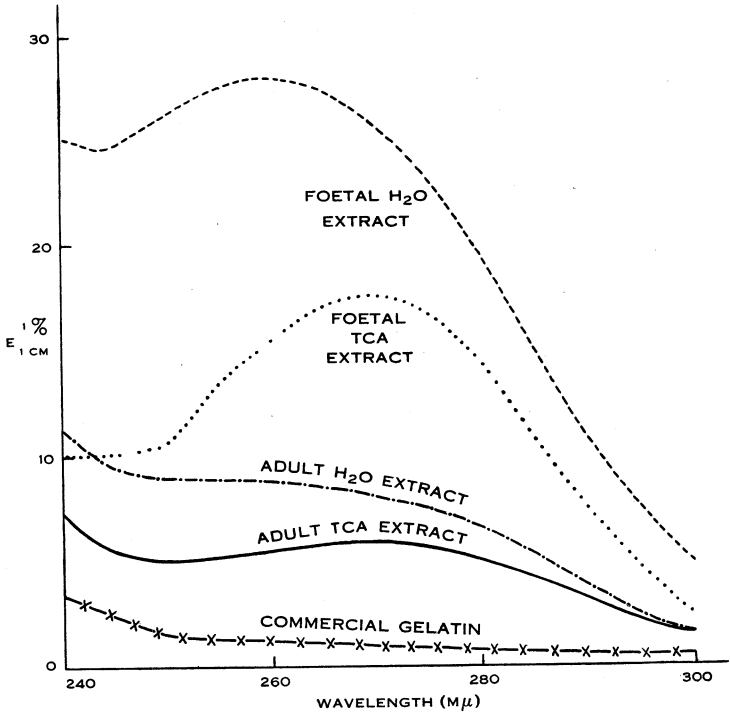


Fig. 1.—Ultraviolet absorption spectra of extracted adult and foetal sheepskin (from foetus No. 21) and of commercial gelatin (undialysed Davis gelatin). TCA, trichloroacetic acid.

with hot trichloroacetic aicd, less material was removed than by autoclaving (Table 1). Furthermore, the dry weights of the dialysed trichloroacetic acid extracts were smaller than those obtained by the alternative procedure.

The extracts obtained by the two methods also differed in their ultraviolet absorption characteristics (Fig. 1). The absorption peaks of the trichloroacetic acid extracts were located at 270 mμ, while those of the autoclaved extract were at 260 mμ. The extinction coefficients of the latter were also higher. For adult sheepskin extracts the absorption curves were lower and much flatter in the 250–280 mμ region.

Commercial gelatins showed a much lower absorption than was found with skin extracts. Moreover, neither autoclaving nor trichloroacetic acid treatment had any influence on its absorption characteristics. As with skin, kangaroo tail tendon gave a larger yield of gelatin when extracted by autoclaving than by the alternative method, but the ultraviolet absorption characteristics of the two extracts were similar and closely resembled those of commercial gelatin.

TABLE 2

RADIOACTIVITY OF GELATIN EXTRACTED FROM FOETAL SHEEPSKIN FOLLOWING IN VITRO INCORPORATION OF RADIOGLYCINE

| Extraction Procedure | Foetus 19 (counts/min) | Foetus 20 (counts/min) | Foetus 23 (counts/min) |
|---|---|---|---|
| Trichloroacetic acid | 20 | 68 | 2544 |
| Autoclaving | 131 | 168 | 8180 |

When foetal sheepskin was incubated with radioglycine and the radioactivity of the total gelatins isolated by the two procedures compared, it was evident (Table 2) that more radioactivity had been incorporated into gelatin obtained by autoclaving.

TABLE 3

DISTRIBUTION OF RADIOACTIVITY IN TOTAL AND FRACTIONATED FOETAL SHEEPSKIN

| Fraction | Foetus 14 (counts/min) | | Foetus 15 (counts/min) | |
|---|---|---|---|---|
| | Incubated | Control | Incubated | Control |
| Total skin gelatin | 1490 | 58 | 1660 | 63 |
| Residual skin | 658 | 32 | 1000 | 40 |
| Total ribonucleoprotein | 1902 | 70 | 372 | 72 |
| Ribonucleogelatin | 2424 | 23 | 263 | 31 |
| Ribonucleoprotein residue | 1220 | 14 | 165 | 14 |
| Total deoxyribonucleoprotein | 282 | 10 | 94 | 10 |
| Deoxyribonucleoprotein residue | 1165 | 19 | 530 | 25 |

In subsequent work, the autoclaving rather than the trichloroacetic acid technique was adopted, because of the better yield of gelatin and the higher specific activity of the gelatin obtained in this way.

The distribution of radioactivities in two fractionation experiments is given in Table 3. It was found that in each case more glycine was incorporated into the incubated samples than into the controls, and that the soluble protein* derived from

*Although no attempt at quantitative recovery was made, this material amounted to about 10 mg.

the autoclaved ribonucleoprotein fraction had incorporated appreciable radioactivity. Although direct radioactivity measurements of the gelatin derived from the deoxyribonucleoprotein fraction could not be carried out because only traces of this gelatin were recovered, the indications were that since the deoxyribonucleoprotein residue after autoclaving showed a sharp rise in radioactivity, only relatively inert or weakly radioactive material had been removed by the autoclaving.

TABLE 4

INFLUENCE OF CRYSTALLINE RIBONUCLEASE AND DEOXYRIBONUCLEASE ON GLYCINE INCORPORATION INTO TOTAL SKIN GELATIN

| Nature of Pretreatment | Foetus 16 (counts/min) | Foetus 17 (counts/min) | Foetus 22 (counts/min) | Foetus 23 (counts/min) |
|---|---|---|---|---|
| None (cold control) | — | — | 51 | 67 |
| None (incubated) | 1534 | 1870 | 1036 | 8180 |
| Ribonuclease | 645 | — | 536 | 3919 |
| Deoxyribonuclease | — | 1751 | 1318 | 8470 |

Pre-incubation of sheepskin with both ribonuclease and deoxyribonuclease was undertaken and the effect on glycine incorporation noted. Table 4 shows that ribonuclease inhibits glycine uptake into the total skin gelatin fraction by a factor of two, whereas deoxyribonuclease has little effect.

Examination of these gelatins in the ultraviolet indicated that both the shape of the absorption curves and the extinction coefficients at the absorption maximum of 260 m$\mu$ (Table 5) remained virtually unaltered as a result of the nuclease action.

TABLE 5

INFLUENCE OF CRYSTALLINE RIBONUCLEASE AND DEOXYRIBONUCLEASE ON $E_{1\,cm}^{1\%}$ VALUES OF EXTRACTED GELATINS AT 260 M$\mu$

| Nature of Pretreatment | Foetus 16 | Foetus 17 | Foetus 22 | Foetus 23 |
|---|---|---|---|---|
| None (cold control) | — | — | 24·3 | 29·3 |
| None (incubated) | 18·7 | 14·0 | 23·7 | 30·5 |
| Ribonuclease | 18·3 | — | 24·0 | 34·0 |
| Deoxyribonuclease | — | 15·0 | 23·1 | 30·5 |

## IV. DISCUSSION

The results in Tables 3 and 4 indicate that no glycine incorporation occurs in the absence of oxygen and at low temperature. The fact that collagen was associated with the ribonucleoprotein fraction, that the derived gelatin was labelled to an appreciable extent, and that ribonuclease inhibited glycine incorporation into the total skin gelatin, may mean that ribonucleic acid is associated with collagen synthesis. Conversely, the failure to obtain measurable quantities of gelatin from the deoxyribonucleoprotein fraction, together with the likelihood of the virtual absence of labelling in such gelatin and the absence of an effect by deoxyribonuclease on glycine uptake by total skin gelatin, may be taken as an indication that deoxyribonucleic acid is not concerned with collagen synthesis to an appreciable extent.

The relatively high absorption in the 260 m$\mu$ region of the skin-derived gelatins (Fig. 1) indicates possible contamination with nucleic acids or their degradation products, particularly in the extracts of autoclaved foetal skins. Furthermore, the above findings suggest that the richer the tissue in nucleoprotein, the greater the difference between extracts obtained by trichloroacetic acid treatment and by autoclaving.

Rough estimates of the possible nucleic acid content may be obtained from phosphorus analyses and extinction coefficients by assuming that nucleic acid

TABLE 6

NUCLEIC ACID CONTENT OF GELATINS AS CALCULATED FROM PHOSPHORUS ANALYSES AND EXTINCTION DATA

| Sample | Phosphorus Content (g/100 g gelatin) | $E^{1\%}_{1\,cm}$ at 260 m$\mu$ | Calculated Nucleic Acid Content (g/100 g gelatin) | |
|---|---|---|---|---|
| | | | From Phosphorus Content | From $E^{1\%}_{1\,cm}$ |
| Difco gelatin | 0·04 | 1·57 | 0·4 | 0·5 |
| Davis gelatin | 0·03 | 1·26 | 0·3 | 0·4 |
| Foetus 19 | | | | |
| Autoclaved extract | 0·20 | 20·9 | 2·2 | 7·2 |
| Trichloroacetic acid extract | 0·15 | 10·8 | 1·7 | 3·7 |
| Foetus 23 | | | | |
| Autoclaved extract | 0·10 | 30·5 | 1·1 | 10·5 |
| Trichloroacetic acid extract | 0·10 | 24·0 | 1·1 | 8·3 |

contains 9 per cent. phosphorus and that the extinction per mole of phosphorus is 10,000 (Table 6). It is evident that for commercial gelatins all the nucleic acid could be accounted for in terms of phosphorus, while the extracted gelatins contain less phosphorus than might be expected from the extinction data. One reason for this could be a partial hydrolysis of the phosphate groups during the extraction procedure and their removal by dialysis. Whatever the true explanation, it would appear that the extracted foetal skin gelatins could contain at least 1 or 2 per cent. nucleic acid and possibly even as much as 10 per cent.

The presence of such nucleic acid material could influence the interpretation of results obtained in studies where labelled glycine is used for following collagen metabolism. Because of the known rapid turnover rates of nucleic acids in biological systems (Smellie 1955), incorporation of glycine into nucleic acids would be expected. In fact, Table 2 shows that, following *in vitro* incubation of foetal sheepskin with radioglycine, greater incorporation is associated with the gelatin from the autoclaved extracts, i.e. with the gelatin containing a larger amount of material absorbing at 260 m$\mu$, than with gelatin from the trichloroacetic acid extracts. The practice of isolating the glycine from an acid hydrolysate and taking its radioactivity as a

measure of collagen metabolism may not entirely eliminate the effect due to nucleic acid contamination, because of the possibility of conversion of some of the labelled purines into glycine under conditions of the hydrolysis (Bendich 1955).

If no hydrolysis of the labelled protein is undertaken, as was the case in the present study, and if incubation with nucleases (Table 4) had some influence on the nucleic acid content of the isolated gelatins, then the differences in radioactivity might be a reflection of such changes in nucleic acid content. It would appear from Table 5, however, that the action of nucleases has had no effect on the amount of extractable material absorbing at 260 m$\mu$.

The choice of a suitable extraction method was clearly not an easy one. Similar contamination could be expected in several of the other methods currently used for collagen extraction, particularly from tissues rich in nucleoprotein. Several of these same methods, in fact, have also been employed for extraction of nucleic acids or their derivatives. Thus trichloroacetic acid itself has been used for this purpose (Schneider 1945), while dilute citrate has been used for both procollagen extraction (Orekhovich *et al.* 1948) and for removal of ribonucleic acid from deoxyribonucleic acid (Dounce 1943), and sodium chloride was used for the isolation of salt-soluble collagen (Gross, Highberger, and Schmitt 1955) and of nucleoproteins (Mirsky and Pollister 1946). Although sodium phosphate does not appear to have been used for nucleic acid extraction, Schmitt, Gross, and Highberger (1953) have reported that collagen extracts prepared in this way have strong absorption at 260 m$\mu$.

## V. Acknowledgments

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