# IN VIVO INCORPORATION OF LABELED AMINO ACIDS DURING EARLY STAGES OF COLLAGEN BIOSYNTHESIS

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The biosynthesis of collagen has been extensively studied in many tissues by following the rate of uptake of labeled amino acids. These studies have been reviewed recently (Jackson and Bentley, 1960). The various fractions reported by different workers all appear to arise by dispersion of tissue collagen aggregates into the constituent tropocollagen molecules, having specific physico-chemical properties (Gross, Highberger and Schmitt, 1954). The fraction extracted with 0.14M NaCl, however, appears to contain the most recently synthesized collagen (Jackson and Bentley, 1960) and hence is the most important fraction to study in the early stages of collagen biosynthesis. In most studies involving the uptake of isotopically labeled amino acids into collagen a regular pattern was observed showing a rise to a peak of activity followed by a fall to a minimum level. The time of peak activity and the rates of rise and fall depended on the type of extraction procedure and the tissue used, but in general only one peak of radioactivity has been observed. In carrying out experiments in which much earlier, and more frequent time points were studied than had been previously reported, we have noted an unexpected variation in the pattern of amino acid incorporation, both in skin and in open wound granulation tissue. It is the purpose of this report to describe and offer a possible explanation for the double peak uptake curve observed.

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## EXPERIMENTAL:

### Skin

Fifty young female guinea pigs were decapitated in groups of 3 at various time intervals (Figure 1, Table 1) after an intraperitoneal injection of 35 μc of 2-$C^{14}$ glycine. In two other experiments 2-$H^3$ glycine was used (Table 1). Skin was removed from each group, pooled and crushed after being frozen in liquid nitrogen. It was extracted three times with a 0.14M NaCl solution buffered to pH 7.5 with phosphate, and the collagen thus extracted subjected to rigorous purification (Jackson and Bentley, 1960). Radioactivity in the purified collagen was measured in the Tri-carb liquid scintillation spectrometer and corrected to disintegrations per minute by the use of internal standards. Hydroxyproline was determined (Neuman and Logan, 1950) as a measure of collagen concentration.

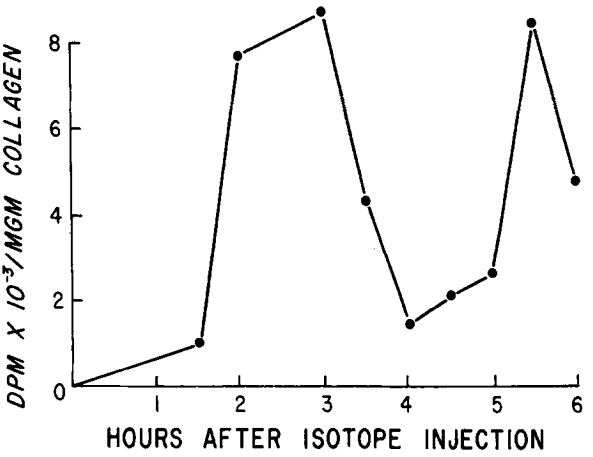


Fig. 1. Specific activity of collagen extracted from skin after incorporation of 2-$C^{14}$ glycine.

### Wound Granulation Tissue

It has long been known that hydroxyproline is unique to collagen and is derived biosynthetically from proline (Stetten, 1949), therefore, because of the difficulty of purifying collagen from the small amounts of wound tissue available and to eliminate possible contamination with other glycine-containing proteins, tritiated proline was used as tracer in the wound study. Circular wounds were

TABLE I

| Hours After Isotope Injection | Purified Collagen Specific Activity DPM per mgm Collagen | | |
|---|---|---|---|
| | Experiment I | Experiment II | Experiment III |
| 0.5 | 6437 | -- | -- |
| 1 | -- | 10402 | -- |
| 1.5 | 9380 | 15753 | 945 |
| 2 | 12822 | 12020 | 7695 |
| 2.5 | -- | 7041 | -- |
| 3 | 6725 | 330 | 8678 |
| 3.5 | -- | 14063 | 4287 |
| 4 | 9707 | 12770 | 1426 |
| 4.5 | -- | -- | 2113 |
| 5 | -- | 8623 | 2614 |
| 5.5 | -- | -- | 8504 |
| 6 | 7743 | -- | 4768 |

Experiments I and II show the incorporation of 2-$H^3$ glycine into skin collagen. Experiment III shows the incorporation of 2-$C^{14}$ glycine into skin collagen. (Experiment III is also presented graphically as Figure 1).

made in 50 young female guinea pigs by excising a piece of dorsal skin (area about 12 sq. cm.). The wounds were left uncovered for 10 days, a time at which collagen synthesis is proceeding maximally. At this time 45 μc of 3, 4, $H^3$ proline was injected intraperitoneally into each animal. The animals were decapitated in groups of 4 at regular intervals between 2 and 6 hours after injection. The granulation tissue was excised from the wounds after removal of the scab and extracted in the same manner as was the skin. After removal

of aliquots for assay of total collagen extracted, the extracts were dialysed for 24 hours against distilled water. The dialysates after acidification with acetic acid were heated to 60° C for 1 hour and non-collagenous proteins precipitated by addition of trichloacetic acid (TCA) to a final concentration of 5%. The supernatant was dialysed free of TCA and free amino acids, dried and hydrolysed in sealed tubes with 6N HCl at 138° C. Hydroxyproline was isolated from the hydrolysates using the method of thin layer chromatography (Myhill and Jackson, to be published) and assayed by the micro method of (Woessner, 1961). Radioactivity was measured as above.

## RESULTS AND DISCUSSION:

The results are surprising in that a double peak is seen in the uptake curve. The peaks are present when purified collagen is counted as such after incorporation of $C^{14}$ glycine (Figure 1) or $H^3$ glycine (Table 1) and also when isolated hydroxyproline is counted after use of tritiated proline (Figure 2).

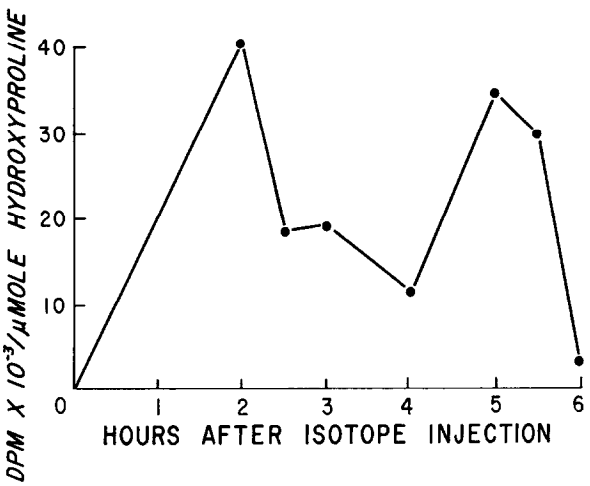


Fig. 2. Specific activity of hydroxyproline isolated from collagen extracted from wound granulation tissue after incorporation of 3,4,$H^3$ proline.

In the skin experiment, where purified collagen was counted as such after incorporation of $C^{14}$ glycine, the existence of two peaks could conceivably be due to the presence of a non-collagenous impurity taking up $C^{14}$ glycine at a different rate. This possibility can be excluded since a similar pattern is seen

upon counting isolated hydroxyproline, this imino acid being practically unique to collagen.

The collagen with the same specific activity extracted at two different times after administration of the label must have been synthesized at the same time, since the specific activity of the free amino acid pool is very greatly different at these two times (Henriques, Henriques and Neuberger, 1955). Thus, one explanation for the phenomenon seems to be that newly formed collagen is extractable with 0.14M NaCl for a short period, i.e., up to two and one half hours after synthesis, at which time it is rendered temporarily unextractable by some mechanism and the specific activity of the extracted collagen falls. Later, this collagen becomes available for extraction once more and the second rise in specific activity is seen. It is extremely unlikely that any increase in the specific activity of the unextractable collagen would be seen at times when extractable collagen activity falls, since the radioactivity of the small amounts of collagen involved would be obscured by the infinitely greater amounts of preformed collagen which would be brought into solution along with it.

A likely site of alteration of the extractability is the cell surface. It has been remarked that during the preparation of tissue for electronmicroscopy, fibrils seen at the cell surface could not be washed off (Porter and Pappas, 1959). A second but less likely explanation is that two forms of collagen are produced, each with a different rate of synthesis. No evidence, however, for the existence of two types of collagen in any particular tissue has been put forward.

Whilst this manuscript was in preparation, the authors were informed by Dr. Jerome Gross (Personal communication) that he had observed a similar double peak while studying the biosynthesis of collagen in chick embryos.

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