Demonstration of the Biosynthesis of Collagen in Rat Skin

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The increase in the tensile strength of collagen formed in wound tissue has been extensively used as a measure of the synthesis of collagen. Another method is to study changes in the amount of collagen. Certain collagen fractions can be isolated, or the total amount of collagen can be determined by estimating the amount of hydroxyproline. The use of tracer-techniques has also become a widely used method. The use of labelled proline methods increased when the separation of labelled proline and hydroxyproline was achieved by oxidation and toluene extraction.[1] The incorporation of labelled proline into collagen is traced, and the radioactivity of proline or hydroxyproline is determined.

In this communication we wish to present a method for sensitive and convenient demonstration of the biosynthesis of collagen in rat skin. It involves the isolation and acid hydrolysis of collagen, followed by separation of labelled glycine, proline, and hydroxyproline by thin layer chromatography, and then measurement of the radioactivity of the separated amino acids with a thin layer scanner and a methane gas flow counter, described first by Wenzel.

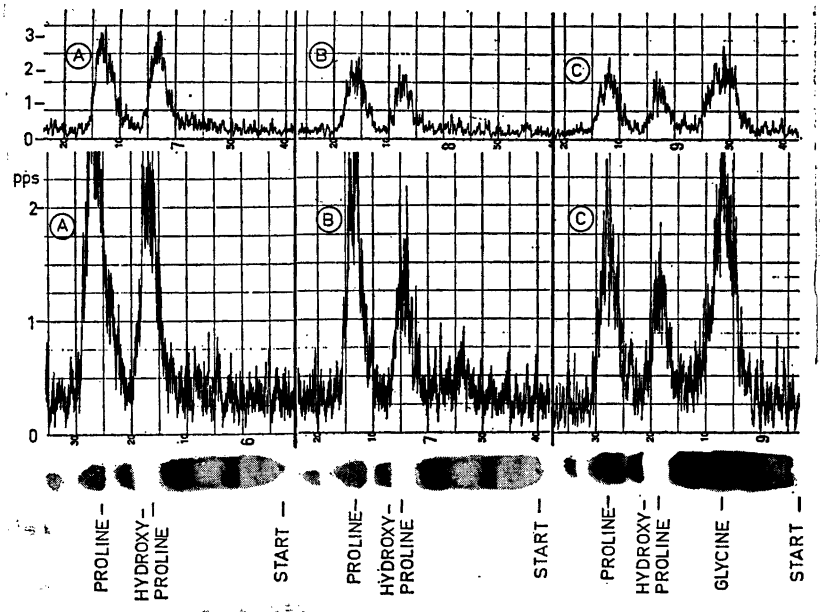

Fig. 1. Radioscans of the separation of proline, hydroxyproline, and glycine by thin layer chromatography from hydrolysate of neutral salt soluble rat skin collagen. Development distance, 17 cm. R_F values: proline, 0.45; hydroxyproline, 0.31; glycine, 0.18. A) 6 h experiment (only labelled proline was injected); the applied material contained 5 μg hydroxyproline; B) 12 h experiment (only labelled proline was injected); the applied material contained 10 μg hydroxyproline; C) 24 h experiment (both labelled glycine and proline were injected); the applied material contained 10 μg hydroxyproline. The instrument settings used were: operation voltage, 3040 V; time constant, 10 sec; sensitivity, 800 mV; scanning speed, 120 mm/h; slit width, 2 mm, without window; measuring range, upper curves: 10 pps, lower curves: 3 pps. The use of the lowest measuring range of 3 pps was possible by replacing the Berthold ratemeter by an Ekco Ratemeter M5190 (Ekco Electronics Ltd., Essex, England). Other details in text.

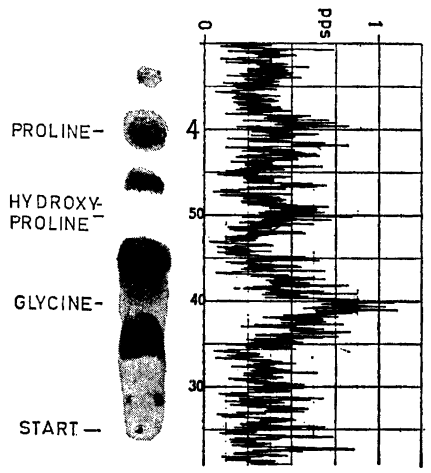

Fig. 2. Radioscan of the separation of proline, hydroxyproline, and glycine by thin layer chromatography from hydrolysate of acid soluble rat skin collagen. Test animals were killed 24 h after the injection of labelled proline and glycine. The applied material contained 20 μg hydroxyproline. Measuring range was 3 pps. Other instrument settings and details were as for Fig. 1.

et al.[2] This method as a whole has not previously been used in investigating collagen, although the separation of collagen amino acids by chromatographical means has been, of course, known for a long time.

Rats were given intraperitoneally ^{14}C-labelled glycine (8.1 mC/mmole, uniformly labelled) and proline (7.0 mC/mmole, uniformly labelled), or only proline (The Radiochemical Centre, Amersham, England). 16 μC of the labelled compounds were injected per 100 g approximately. The test animals were decapitated at 6, 12, or 24 h after the injection, and the skin from the dorsal and abdominal area was flayed, purified from hair and fat tissues and cut into fine pieces with scissors. This material was homogenized for 60 sec with the Ultra-Turrax top drive homogenizer (Janke & Kunkel, KG, Stauffen i.Br., Germany) in small portions in cold (4°) 0.45 M NaCl solution. About 75 ml of a mixture still containing a few whole pieces of skin was obtained from each rat. This 75 ml mixture was then shaken for 24 h at 4° with a Bühler shaker, Type Sm2 (Tübingen, Germany) at 130 rev./min. The resultant mixture was centrifuged for 30 min at 48 000 *g*. From the supernatant fluid, neutral salt soluble collagen was prepared according to the method used by Piez *et al.*[3] The pellet remaining in the centrifuge tube was homogenized in 0.5 M acetic acid and the obtained mixture was shaken for 24 h as above. From the supernatant fluid finally obtained, acid soluble collagen was prepared according to Piez *et al.*[3] The precipitated collagen from both purification procedures was dissolved in 1 ml of 0.1 M acetic acid. These solutions contained from 25 to 120 μg hydroxyproline per ml as estimated according to Stegeman[4] and Woessner.[5] 0.5 ml of these collagen solutions was hydrolyzed for 48 h in 2 ml of 6 N HCl at 103°. The excess of HCl was removed after the hydrolysis by repeated evaporation (four times). The remaining material was dissolved in 0.15 ml of water and 50 μl of this solution was applied to plates coated with 0.35 mm Silica Gel layers. The amino acids were then separated by one-dimensional chromatography using a phenol/water mixture (75:25, w/w) as solvent. The amino acids were then detected with ninhydrin, and the distribution of radioactivity was finally determined with a thin layer scanner and a methane gas flow counter (Laboratorium Prof. Dr. Berthold, 7547 Wilbad, W.-Germany). Experiments were also carried out with a mixture of argon (90 %) and methane (10 %) and its use at any operation voltage did not significantly affect the scanning results.

The results are shown in Figs. 1 and 2. Fig. 1 shows how labelled proline, hydroxyproline and glycine were determined from a hydrolysate of neutral salt soluble collagen on thin layer plates using two different measuring ranges. This figure also shows that the hydroxylation of proline in the rat skin collagen had taken place within 6 h. Fig. 2 shows a result with the hydrolysate of acid soluble collagen (soluble in 0.5 M acetic acid) when the test animals were killed 24 h after the injection of labelled proline and glycine. No radioactivity was detected on those thin layer plates where the three amino acids were separated from collagen in the 12 and 6 h experiments, indicating that even 12 h was not a sufficiently long time for the labelled proline in rat skin collagen to reach the acid soluble form.

This method has been used in our laboratory in the investigation of some aspects of the biosynthesis of collagen. The method could also be used in studying the incorporation of labelled compounds into other proteins, and can be especially valuable in studies on collagen because proline and hydroxyproline are easily separated by thin layer chromatography. Also there is no significant loss of material in the procedure. When comparing the analysis time of this method with others it should be remembered that no time is required here for the preparation of samples. Furthermore, this method has the particular advantage that it enables the incorporation of practically all collagen amino acids to be studied simultaneously and from a single experiment on the same thin layer plates. In addition, it is not necessary to use a separate method for the isolation of the different labelled collagen amino acids, a fact that facilitates the comparison of incorporation of the different amino acids.

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Heteroaromatic Boron Compounds

IV. 3,2-Borazaropyridine. A New Aromatic System

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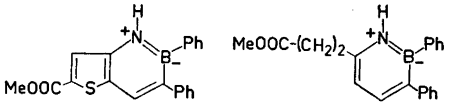
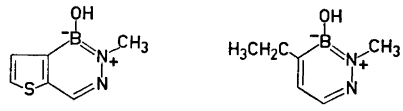
During the last years, there has been a considerable interest in preparing the boraza analogue of benzene, *viz.* borazarene.





The first derivative of this new monocyclic boron-nitrogen containing system was synthesised by Dewar *et al.*[1] in 1962, who reported the preparation of 2,3-diphenyl - 6 - (2 - carbomethoxy-ethyl)-2,1-borazarene (II) by desulphurization of 2-carbomethoxy-5,6-diphenyl-5,4-borazarobenzothiophene (I). Later, White [2] prepared 2-phenylborazarene and Dewar *et al.*[3] recently published the synthesis of some other simple borazarene derivatives. The UV-spectrum of 2-phenylborazarene closely resembles that of 2-phenylpyridine and gives evidence for the aromatic nature of this compound. The above mentioned compounds have only been prepared in small amounts and little is known about their chemical properties.

Gronowitz *et al.*[4-6] have prepared derivatives of the three possible borazarothienopyridines. From these systems, it should be possible to prepare derivatives of the boraza analogue of pyridine, namely 3,2-borazaropyridine, which as far as we know has not been prepared before.

We have succeeded in preparing 4-ethyl-3- hydroxy-2- methyl-3,2- borazaropyridine (IV) in 49 % yield by desulphurization of 4- hydroxy -5- methyl -4,5- borazarothieno

* Deceased.