Connective Tissue VIII. Factors Effecting Collagen Synthesis by Sponge Biopsy Connective Tissue.* (28484)

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The *in vitro* synthesis of collagen has been demonstrated in sponge biopsy connective tissue(1), carrageenin granulomata(2), rat uterus(3), rat cartilage(4) and rat bone(5) by either conversion of C^{14}-lysine to C^{14}-hydroxylysine or C^{14}-proline to C^{14}-hydroxyproline. Recently, homogenates from chick embryo have been reported capable of converting C^{14}-proline to C^{14}-hydroxyproline(6). Ascorbic acid was reported to be essential in the hydroxylation of proline to hydroxyproline in carrageenin granulomata from guinea pig(7,8). Designation of the hydrogen atoms of proline involved in the hydroxylation has been attempted by two groups of workers(8,9), each using both C^{14}- and tritiated-proline; their results are not in agreement.

Prockop *et al.*(10), Scharpenseel *et al.* (11), and Fujimoto *et al.*(12), using O_{18} as a tracer, have reported that atmospheric oxygen rather than the oxygen of water is required for synthesis of collagen. The metabolism of collagen is still obscure. This study deals with a systematic survey of the optimal conditions for collagen synthesis in sponge biopsy connective tissue.

Methods. Sponge biopsy connective tissue, 2 weeks old, was obtained by the method of Boucek and Noble(13). Tissue slices (1.00 g) were incubated in 5 ml Krebs-Henseleit medium(14) with 1 ml saline containing 1 μc uniformly labelled L-proline-C^{14} (Schwarz BioResearch, Inc., specific activity 1.09 μc/mg or 800 μc/mg) at 37°C, pH 7.35-7.45 under an atmosphere of 95% O_2-5% CO_2, unless otherwise stated. After 6 hours of incubation, the slices were removed, blotted and homogenized in 0.85% sodium chloride containing 0.25% L-proline. The mixture was centrifuged at 15,000 $\times$ *g* for one-half hour and decanted. The residue was extracted with 0.1 N NaOH overnight and centrifuged at 105,000 $\times$ *g* for 1 hour. This extraction was repeated once for 2 hours. To avoid the contamination of noncollagenous protein and C^{14}-proline in the incubation medium, the NaCl solution and the base were discarded. Collagen was extracted from base-soluble residue by Fitch's method(15). The purity of the collagen was indicated by demonstrating an hydroxyproline content of 13%. The collagen samples were hydrolyzed in 6N HCl at 110° for 16 hours in sealed tubes. Aliquots were taken for N_2 determination by Kjeldahl digestion and Conway diffusion techniques (16). Radioactivity was counted in a low background (2 counts per minute) gas flow counter with micromil window. Our counting efficiency was 40%. Data were not corrected for counting efficiency. Specific activity was expressed as cpm/mg protein. Hydroxyproline and proline were separated on Whatman #4 paper in 2 solvent systems, 77% ethanol or water saturated phenol in a vapor phase of NH_3 (0.3% NH_4OH) and HCN (5% NaCN). Both solvents were proven to be satisfactory for separation of hydroxyproline from proline. Glutamic acid synthesis was negligible during 6 hours of incubation. The amino acids were eluted from the paper with water. Aliquots were taken for counting and colorimetric determinations. For hydroxyproline, Neuman and Logan's method(17) was used; for proline, that of Troll and Lindsley(18). Specific activities were expressed as cpm/mg of amino acid, and that of hydroxyproline used as the measure of collagen synthesis. All specimens were incubated in triplicate. Data represent the mean of each 3 samples. In most instances, variations from the mean were within 20%.

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TABLE I. Collagen Synthesis in Sponge Biopsy Connective Tissue Slices; Effect of Alterations in Medium.*

	Specific activity (counts/min/mg)		
		From collagen	
	Collagen	Hydroxy-proline	Pro-line
Exp 1			
Complete medium†	71	335	277
Without KH_2PO_4	62	311	267
" glucose	81	346	329
" CaCl	66	292	268
Exp 2			
Complete medium†	63	264	236
Without NaCl	3	3	17
" $NaHCO_3$	19	67	78
" Na citrate	85	264	263
" $MgSO_4$	94	266	333
Exp 3			
Complete medium† with amino acid mixture‡	25	76	124
Without amino acid	86	287	394
" $CaCl_2$	21	78	117
" NaCl	38	100	246
" $MgSO_4$	29	106	153
" Na citrate	24	113	107
" KH_2PO_4	12	42	43
" $NaHCO_3$	28	82	112
" glucose	18	62	74

* The sponges used in Exp 1 and 2 were soaked in distilled water, and in Exp 3 were soaked in 0.85% NaCl before implantation. C^{14}-proline used in Exp. 1 and 2 had specific activity 800 μc/mg and in Exp 3 had specific activity 1.09 μc/mg.

† Krebs-Henseleit medium(14) contained NaCl 44.5 mg, $NaHCO_3$ 12.5 mg, KH_2PO_4 5.0 mg, $MgSO_4 \cdot 7H_2O$ 3.0 mg, sod. citrate 1.5 mg, $CaCl_2$ 1.5 mg and glucose 1.0 mg per 5 ml.

‡ Containing 0.02 μmole of L-tryptophan, 0.05 μmole each of L-methionine, L-cystine and L-histidine-monohydrochloride, 0.1 μmole each of L-alanine, L-serine, L-phenylalanine, glycine, L-tyrosine and L-arginine monohydrochloride and 0.2 μmole each of L-isoleucine, L-valine, L-threonine, L-leucine per ml of medium.

Results. The effect on collagen synthesis of eliminating, one at a time, each individual ingredient from the incubation medium is shown in Table I. The most striking effect was observed when sodium chloride was not present. The synthesis of collagen was reduced to approximately 1/100 that of the control level. The specific activity of both collagen protein and collagen proline was also reduced. When $NaHCO_3$ was eliminated from the medium, collagen synthesis was reduced to ¼ of the control level. The elimination of other components from the complete medium exerted no appreciable effect on collagen synthesis.

Also in Table I are shown the effects on collagen synthesis of adding an amino acid mixture, containing the 9 amino acids essential for growth, to the incubation medium and then omitting, one at a time, each of the ingredients of the Krebs-Henseleit solution. A definite depression of collagen synthesis (¼-⅓) occurred in all of the samples to which the amino acid mixture was added. A further 50% decrease in collagen synthesis was observed when KH_2PO_4 was omitted from the medium.

The effect of varying pH on collagen synthesis is shown in Table II. The highest value for collagen synthesis as measured by the specific activity of hydroxyproline was obtained at an initial pH of 7.4 and for proline in collagen and total collagen at a pH of 8.4. These differences in maxima may indicate a specific optimal pH for the greatest activity of the particular hydroxylating enzyme or enzymes concerned in conversion of proline to hydroxyproline.

The effect of freezing, crushing and duration of incubation on collagen synthesis is shown in Table III. When incubation time was increased from 3 to 6 hours, the increase in collagen synthesis was approximately 10-fold. This finding is suggestive of a non-linear relationship between time and connective tissue formation. However, it may be dependent upon unrecognized factors present

TABLE II. Influence of pH on *In Vitro* Synthesis of Collagen.*†

	Specific activity (counts/min/mg)		
		From collagen	
pH	Collagen	Hydroxy-proline	Proline
3.4	13	10	268
4.4	35	14	298
5.4	72	29	371
6.4	154	213	778
7.4	162	760	814
8.4	248	288	1,227
9.4	188	217	898
10.4	75	62	629

* Sponges used in this exp were soaked in 0.85% NaCl before implantation. C^{14}-proline had specific activity 1.09 μc/mg.

† Same as Table I.

TABLE III. Influence of Incubation Time on *In Vitro* Synthesis of Collagen.*†

Hr	Specific activity (counts/min/mg)		
		From collagen	
	Collagen	Hydroxy-proline	Proline
3	68	305	329
6	561	2,647	2,550
6 (frozen & crushed)‡	5	7	11

* Same as Table II, except specific activity of proline was 800 μc/mg.
† Same as Table I.
‡ The sponges were frozen on dry ice and crushed to powder before incubation.

in those experiments, partially inhibiting connective tissue formation during the early hours of incubation.

The ability for synthesizing collagen was decreased to 1/400 when sponge slices were frozen and crushed before incubation.

The oxygen requirement in collagen synthesis is shown in Table IV. Replacement of oxygen by air decreased the collagen synthesis to ⅓ of the control level. Replacement of oxygen by nitrogen decreased collagen synthesis to approximately 1% of the control value.

Discussion. In previous work on the *in vitro* synthesis of hydroxylysine from lysine in uterine slices, the optimal incubation period proved to be 6 hours(3). In the study above described we have compared the 3 and 6 hour periods of collagen synthesis using the conversion of C^{14}-proline to C^{14}-hydroxyproline as an index of collagen formation. Inasmuch as a 6 hour period of incubation was shown to yield a satisfactory amount of collagen for the present purposes, all other incubations were carried out for this period. Synthesis of collagen was dramatically decreased by individual removal from the medium of the following: sodium chloride and sodium bicarbonate.

In a previous experiment(1), depression of collagen formation to about ¼ to ⅓ the control value occurred when all of the 10 amino acids essential for growth(19) were added to the incubation mixture. This was attributed to competition between the added lysine (0.35 mg to the 5 ml of medium) and the radioactive material. However, in the present study, no lysine was added and a marked depression of collagen formation was still apparent. As can be noted from Table I, the essential amino acids were considered present in sufficient concentrations to maintain growth in the normal animal. However, some of the amino acids of collagen were not present at all or in relatively insufficient quantity. Subsequent trial of an amino acid mixture with a percentage composition similar to that of collagen(20,21) has not depressed the formation of collagen in the *in vitro* system used. The role of the individual amino acids and their optimal concentrations are now being investigated.

The depression of collagen formation produced by the amino acid mixture used in these experiments was not appreciably altered by the omission separately from the incubation medium of each of its individual non-amino acid components, except that when KH_2PO_4 was removed, collagen synthesis was further decreased by 50%. Maximum collagen synthesis occurred between pH 7.4 and 8.4 with striking decreases above or below these levels. An atmosphere of 95% oxygen and 5% carbon dioxide proved to be about two times better than an atmosphere of air; whereas, in an atmosphere of nitrogen, virtually no synthesis took place. In all of the experiments where atmosphere, pH, incubation time were optimally adjusted and the complete Krebs-Henseleit medium was used, the ratio of the specific activities of hydroxyproline and proline found in collagen varied around unity. Variations from this ratio may be in part due to adsorption phenomena, in dealing with which we have found considerable difficulty(3).

Since elimination of sodium chloride from

TABLE IV. Influence of Atmospheric Alterations on *In Vitro* Synthesis of Collagen by Sponge Biopsy Connective Tissue Slices.*†

Atmosphere	Specific activity (counts/min/mg)		
		From collagen	
	Collagen	Hydroxy-proline	Proline
95% O_2–5% CO_2	327	1,474	1,560
Air	113	418	626
N_2	7	17	60

* Same as Table III. † Same as Table I.

the incubation medium produced a most striking depression of collagen synthesis, experiments were done to replace sodium chloride with other chemical compounds at equal molar concentration. In relation to collagen synthesis, none of the compounds could completely replace sodium chloride in the medium. When sodium chloride was replaced by KCl, collagen synthesis was reduced to ⅛ of control level; when replaced by NaI, to 1/30 of control level; when by sucrose or glucose, to 1/10 and ⅓ respectively, of control level. It is believed that besides osmotic pressure, Na^+ and Cl^- ions combined in the form of sodium chloride are essential ingredients in the incubation medium for collagen synthesis and thus aid in incorporation of proline and hydroxyproline by their effect upon transport. It is probable that the importance of other salts in the medium depends at least partially upon the same factor.

The results of our experiment on the oxygen requirement for collagen synthesis in sponge biopsy connective tissue agree with recent work of others(10,11,12). Freezing and crushing almost completely destroyed the ability of the tissue to form collagen. This indicates that intact cells are required for efficient synthesis of collagen.

It is tempting to speculate about the pathways of collagen synthesis, particularly as to incorporation of proline and hydroxyproline into the collagen molecule. Stone and Meister (8) have called attention to two schemae which have possible application to processes for conversion of proline to collagen imino acid. In pathway 1, proposed by the above workers, the overall reactions involve the linkage of proline in a proline peptide, followed by the formation of hydroxyproline peptide from this material with later incorporation of both substances into collagen. If proline peptide is the only precursor of hydroxyproline in collagen, it is obvious that the ratio of the specific activities of hydroxyproline to proline can never exceed unity. In our experiments, a ratio of 1.21 was the highest found. In most of our studies the ratio was 1.0 or lower.

Summary. The conversion of C^{14}-proline to C^{14}-hydroxyproline has been used as a measure of the synthesis of collagen in sponge biopsy connective tissue *in vitro*. Krebs-Henseleit medium, pH 7.4, under an atmosphere of 95% O_2-5% CO_2 created the most satisfactory conditions we have employed for collagen synthesis *in vitro*. Elimination of NaCl from the incubation medium gave the most striking depression of collagen synthesis. Replacement of NaCl by other chemical compounds did not restore the synthesis of collagen to the control level. An amino acid mixture, containing essential amino acids, had an inhibitory effect on collagen synthesis. Freezing and crushing destroys most of the enzyme system or systems required for collagen synthesis. The data presented are compatible with the concept that all collagen hydroxyproline is derived from proline peptide.

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Studies on Serological Homogeneity of Asian Influenza Strains. (28485)

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Several groups of investigators(1,2,3) have reported evidence of what was interpreted to represent serological heterogeneity of different strains of Asian influenza virus isolated during 1957. Interpretation of these findings has, however, been complicated by the demonstration by Fukumi(4), and others, that Asian strains of influenza virus display marked variations in antibody avidity, perhaps analogous to the PQ variations described by van der Veen and Mulder(5). Fukumi, in a study of 300 strains of Asian virus isolated in Japan during 1957, demonstrated that the strains differed markedly in avidity for antibody and for inhibitors. In an effort to determine whether some of the extreme variations were related to antigenic difference, cross absorption experiments were carried out. These experiments did not support the hypothesis of antigenic diversity as underlying the difference in antibody combining power manifested by these strains.

Preliminary analyses of strains isolated at the Medical General Laboratory (406) in 1960 by hemagglutination inhibition technique suggested possible differences between 1957 and 1960 Asian influenza strains. Since complement fixation techniques employing purified "viral" antigen(6) are thought to offer a means for circumventing the avidity problems associated with the hemagglutination inhibition technique, it appeared of interest to evaluate these differences by these methods. The present report summarizes comparison by hemagglutination inhibition and specific viral complement fixation techniques of 6 Asian influenza strains with 6 such strains isolated during 1960.

Materials and methods. Virus strains. The prototype 1957 strain employed for immunization was A_2/Formosa/313/57, in third allantoic passage. This strain had been isolated in Formosa by Dr. S. P. Wang. The prototype 1960 strain employed for immunization was A_2/Japan/775/60. This strain was isolated at the Medical General Laboratory (406) in April 1960 from a patient at the Tachikawa Air Force Base Hospital near Tokyo. Third allantoic passage material was employed for immunization. In addition to the above strain, the following 1957 strains were examined: A_2/Japan/Adachi-2/1957 and A_2/Japan/Kumamoto/Y-5/1957 strains described by Fukumi(4), which were kindly supplied by Colonel T. Sonoguchi, Japan Self Defense Forces Hospital; A_2/Formosa/T-22/57, which was employed on the initial assumption that it represented an independent isolate but was subsequently found to represent a derivative of the same isolate as A_2/Formosa/313/57: A_2/Japan/115/57, a strain isolated at Medical General Laboratory (406).

The 1960 strains examined included, in addition to the prototype described above: A_2/Japan/538/60, isolated from a patient in Kyushu during March, 1960 at Medical General Laboratory (406); A_2/Japan/691/60, a strain isolated from a patient at Johnson Air

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