コラーゲンの生合成について

緋野邦夫*, 手塚経夫**

(*昭和大学医学部歯学教室; **千葉大学医学部医学化学教室)

コラーゲンの生成に関しては、多くの問題がある。プロリンのオキシプロリンへの転化は、Stetten 以来有名であるが、Gly・Pro・X のくり返しが何故できるか。コラーゲン線維の X 線回折上現れる高次体と非高次体との関係、糖質とコラーゲン生成との関連なども重要な問題である。コラーゲン生成に関与するアスコルビン酸の作用、コータンゾンの作用なども、その機能については不明のままだ残されている。

我々は、特にコラーゲンの生合成の問題を中心に、1959年以来生化学的検索を行なって来た。現在までの知識で、以上の問題について不完全ながらシエマを描くことが出来ると思われる。

1. 引用腫の生成 : 結合組織細胞は、酸性モロ糖類に閉鎖されている。肉芽腫を誘起する物質の多くはポリオキシポリアミノである。寒天はこのような状態に合致し肉芽腫を作る事が示された。これより抽出したコラーゲンのアミノ酸組成はこれ迄の報告と略一致するが、Hypro/Pro は時と共と増えるようであった。

2. 肉芽腫の核酸代謝 : 肉芽腫の核分画には P* がかりどおり入る。また、上清の酸溶性分画の 2', 3'-ヌクレオチドにも入る。後者の塩基組成は、A：U：G：C=10：10：16：16 であった。これらのタンノーパーラーは、コータンゾン注射により著しく塩分けられるようにある。

3. 引用腫の蛋白合成 : 引用腫スライスのコラーゲン合成には、酸素、飼、エネルギー源が必要である。プロリンはペプチドに入ることが、オキシプロリンは入らない。Cell-free の系では ATP 添加はそれ程必要でない。RNase にも割合よく耐える。ヒアロミダーゼは C14 アミノ酸のとりこみを減じ、このとりこみは 8,500 x g。30 分でおける大顆粒分画にもっともよく起る。この分画のとりこみも、コータンゾン注射で著しく変化する。ここにとりこまれた C14 アミノ酸は、コラーゲナーゼ処理で、80%が 60％エタノール可溶性となる。なお中間体として、酸溶性ペプチドが出来、これが 8,500 x g 上清とインキュベートすると、TCA でおけるようになる。

4. プロリンの水酸化 : これが酸素酸反応であることは確かである。我々のデータもこれを示唆する。大顆粒分画で出来たペプチド中のプロリンは、ほとんど水酸化をうけていない。これと上清とインキュベートすると、反応程度反応がすむ。

5. アスコルビン酸と融合組織 : アスコルビン酸欠乏で肉芽腫産生は著しく悪くなる。肝内酵素では phosphoglucomutase が著しくおちる (Ganguli et al.). 吾々および亀山もこれを追試し、アスコルビン酸欠乏の比較的早期にこの酵素活性が低下しているのを認めた。同時に移植スポンジ中のグルコースミン、オキシプロリンを定量し、これらが平行して下っているのを見た。

以上の所見から考えられるコラーゲン合成のシエマとして:
① 核内にて GC 含量の多いメトセンジャー RNA が作られる (コーチゾンで↑).
② 核膜につながる endoplasmic reticulum 中へこれが出で行く、E.R. はアミロサイソフ
リドを多く含み、核から細胞膜の方へのびて行きつつ、中でコラーゲン合成を行なう。ここで (Gly-Pro-X)n が出来る。

③ (Gly-Pro-X)n は、細胞質中で転移反応をうけコラーゲン分子となる。コバリサツカリドはアスコルビン酸欠乏、コーチゾン処理で↓。

議

石橋 貞彦氏
1) Cortisone なる化合物は一般にコラーゲン合成に inhibitory に似るというデータが多い様ですが、それと矛盾しないでしょうか。
2) 上と関連して、GC content の高い RNA がコラーゲンの messenger であるという可能性についても、如何ですか。

手塚 統夫氏
1) Collagen 生成の変される疾患で、尿中に hypro-peptide が多くなると云う報告がありますが、cortisone 注射の時に8,500×g 粗粒の incorporation が増しているもの、この位の peptide ではないかと思います。私の研究では、結合組織でも肝でも cortisone の作用が同じで、核酸およびこれにdepend した蛋白合成を促進するが、結合組織では更に二次的機序で最終的にコラーゲン分子を作るので、ここが犯されると考えます。今的研究データから考えて、cortisone を長期投与すれば、endoplasmic reticulum に含まれる HA が増減し、このため8,500×g 粗粒は取れなくなると考えております。
2) GC-rich の RNA が messenger であることは前から考えられていますが、P'の割合でとらえられるのみで、まだ単離しておりません。現在やつてお

ります。

石橋 貞彦氏
1) 8,500g の顕粒の性質についてどうお考えか、もう一度御説明下さい。
2) コラーゲンが細胞外へ出て行く時は、その顕粒が出て行くとお考えですか。

手塚 統夫氏
1) 大顕粒は、内皮に近いコラーゲン分子をもつ endoplasmic reticulum の破片と考えられます。従って、比重は均一ですが、大きさは不均一でもよく、ヒアルロン酸様物質をある程度含んでおります。核酸含量は極めて低くなっています。
2) この顕粒は、実験室細胞をこわした為に生じたものですから、そのまま細胞外へ出すとは考えられません。核近い endoplasmic reticulum が接続膜近くに動いて行くにつれ、内部のペプチドが伸ばると考えますが、これが細胞膜につながっているかどうか、私はデータを持っておりません。電顕上そのような報告はありませんが、プロリンの酸化化反応を考えると、一旦細胞質中へ出たコラーゲン分子が分岐されて後線維を作る考えた方がよいように思います。
Studies on the Connective Tissue

II. Nucleic Acids and the Related Substances in the Granuloma Induced by the Agar-Implantation

Tsunao Tetsuka* and Kunio Konno**

(*Department of Physiological Chemistry, Chiba University Medical School;
**Department of Biochemistry, Showa University School of Medicine)

Collagen is a main proteinous component and characterized by its several peculiar properties. Among these properties, the amino acid composition is quite different from other proteins, namely, collagen contains little aromatic and sulfur-containing amino acids and considerably large amount of glycine, proline, hydroxyproline, and also acidic and basic amino acids1.

In the last decade, our knowledge about the protein biosynthesis has been increasing rapidly. The major part of this problem was clarified by Hoagland et al. That involves the amino acid activation, formation of amino acyl-s-RNA and its transfer to the peptidic chain on ribosomal special RNA2. The latter was called as "messenger RNA" because it seemed to be a replica of DNA and to play a role of passing the word of heredity from gene to newly synthesized protein3. It has been supposed that the amino acid residues in protein are corresponding to the triplet codes of nucleotides in RNA. These triplet codes were partially determined recently by Ochoa et al.4 and Nierenberg et al5. If collagen is synthesized by this way, the connective tissue cells may contain some specific RNA, which has a rather deviated base composition corresponding to the characteristic amino acid composition of collagen.

As another system of synthesizing protein, the authors would like to refer to the mechanism concerning the bacterial cell wall synthesis6. Now it is not yet clarified whether such a mechanism is active in mammalian tissue or not. However, it is possible to suppose that the synthesis of collagen is brought about in a way similar to the bacterial cell wall synthesis, and there may be precursors of uridine and cytidine derivatives. In order to clarify the mechanism of collagen synthesis, the authors attempted to see a pattern of nucleic acid and its derivatives in the connective tissue.

EXPERIMENTAL S

Preparations: Granuloma was produced by the agar implantation in the back of albino rats as reported previously7.

Reagents: Four nucleoside 5'-monophosphates and 3',5'-cyclic AMP were the
gift of the Takeda Chemical Industries, Ltd. Nucleoside 2'- and 3'- monophosphates were the alkaline digests of RNA, which was prepared by the phenol extraction from rat liver, followed by the fractionation into four nucleoside 2'- and 3'-monophosphates with the use of Dowex 1×8 (formate) column chromatography. As the nucleotide-hydrolysing enzymes, 3'-nucleotidase [EC 3.1.3.6] was prepared from rye grass by the method of Shuster and Kaplan, and 5'-nucleotidase [EC 3.1.3.5] was partially purified from bull semen after Heppel and Hilmoe. Intestinal phosphatase [EC 3.1.3.1] was a product of Worthington Biochemical Corporation and 5'-AMP deaminase [EC 3.5.4.6] was kindly supplied by Dr. S. Minakami, Dr. T. Hashimoto and Dr. T. Ishii of the Department of Nutrition and Physiological Chemistry of this University, who had prepared this enzyme from rabbit muscle by the method of Nikiforuk and Colowick. All other reagents were the commercial products.

Preparation and analysis of the acid soluble fraction: With specified intervals after the intraperitoneal injection of radioactive phosphate (2–5mc), a rat was anesthetized by Nembutal and ether. The granulomas produced on both sides of the back were removed as fast as possible, and separated from the agar kept inside of them, followed by the freezing with dry ice-acetone. The granuloma tissue thus obtained was minced, homogenized with 10% perchloric acid, and centrifuged (at 4,000 r.p.m.) for 10 minutes. The supernatant obtained was neutralized with potassium hydroxide, and fractionated with Dowex 1×8 (formate) column chromatography after the method of Horie.

Preparation and analysis of RNA: The granuloma tissue obtained as mentioned above was treated by freezing and thawing, and its RNA was extracted by the phenol method.

RNA of the microsomal and the pH 5 fractions was obtained as follows. The tissue was minced, sonicated (9kc, 20 minutes) and fractionated in accordance with Hogeboom and Schneider in 0.25 M sucrose solution. Among the fractions obtained, sediment of 105,000×g for 60 minutes and the precipitable fraction at pH 5.2 from the supernatant were collected. RNA of each fraction was prepared by the use of phenol extraction. The contaminating phenol in these RNA fractions was removed by washing 3 times with ether.

Analytical methods: Inorganic phosphate was determined by the methods of Fiske and SubbaRow and, in some cases, of Berenblum and Chain. Ribose was estimated by orcinol reaction. The quantity of base components of each nucleotide was calculated from the ultraviolet absorption at 260mù. The determination of radioactivites of the P³²-labeled compounds was carried out by the use of an end-window Q-gas flow counter and sometimes also by a windowless Q-gas flow counter.

Paper chromatography of nucleotides, nucleosides and bases: The solvent systems used were as follows: a) 69% n-butyric acid-0.85% NaOH, b) iso-butyric acid-M NH₄OH-0.1 M EDTA (100:60:1.6), c) saturated ammonium sulfate-0.1 M
acetate buffer (pH 6)-isopropanol (79:19:2), d) 95% ethanol-5% ammonium citrate buffer (pH 4.4) (73:22), e) iso-propanol-conc. HCl (170:41, added by water to 250), f) water-saturated-n-butanol-15 N NH₄OH (100:1).

RESULTS

Acid soluble fraction of the granuloma: Two weeks after the implantation of agar, 2—5mc of radioactive phosphate were injected to rats intraperitoneally. The granuloma was removed after specified intervals, and the acid soluble fraction was obtained by adding 10% PCA. The fraction neutralized with potassium hydroxide was put on a column (Dowex 1 x 8, formate, 0.9 x 35cm) and fractionated by the gradient elution method. As shown in Figs. 1—3, there are four big peaks (C, A, G, U,) which seem to correspond to nucleoside monophosphates because of their elution patterns, while ATP shows rather a small peak. The ultraviolet absorption spectra of these fractions showed that they probably contained cytosine (C), adenine (A), guanine (G) and uracil (U), respectively, as mentioned later. All these facts seemed to show that the pattern of acid soluble nucleotides was different from those of other tissues."
**Fig. 2** Elution pattern of the acid soluble fraction 60 minutes after P³²-injection

**Fig. 3** Elution pattern of the acid soluble fraction 6 hours after P³²-injection
The peaks for the radioactivity soon after the P$^{32}$ injection were in accord with the peaks of ATP and other nucleoside triphosphates and also with the peak of sugar phosphate. The small peaks of radioactivity also appeared at the locus of nucleoside monophosphates, and were proved later as corresponding to the nucleoside 5'-monophosphates. Two hours or more after the P$^{32}$-injection, nucleoside 2'- and 3'-monophosphates became radioactive, and the total radioactivities of these 2', 3'-nucleotides surpassed those of 5'-nucleotides 5—6 hours after the injection.

**Effect of some conditions on nucleoside 2', 3'-monophosphates:** The amount of nucleoside 2', 3'-monophosphates diminished markedly when a granuloma-bearing rat had been kept at the cold temperature. The acid soluble nucleotide pattern, as shown in Figs. 1—3, was reproducibly obtained, when the rat was kept at the room-temperature from May to September.

---

**Fig. 4**  Elution pattern of the acid soluble fraction of the granuloma after incubation with P$^{32}$ *in vitro*

(The granuloma was minced in the ice-cold, and an aliquot of the minced tissue (about 1 g wet weight) was incubated in the medium which contained 0.016 M Tris-HCl buffer, pH 7.4, 0.001 M MgSO$_4$, and 0.1 mc of P$^{32}$ in the physiological saline (final 3 ml), at 37°C for 60 minutes with shaking. The minced tissue was washed with the ice-cold phosphate buffer after the incubation, and frozen in dry ice-acetone. Extraction and analysis of the acid soluble fraction was the same as mentioned above.)
A granuloma one week after the agar implantation showed essentially the same pattern as that two weeks after the implantation. Fig. 4 shows a pattern of acid soluble nucleotides obtained from the minced granuloma which has been incubated with $^{32}$P, glucose and magnesium ions in Tris-HCl buffer at 37°C for 60 minutes. Marked decrease of the ultraviolet absorption is observed in the peaks of nucleotides, especially in the nucleoside monophosphates. It is noted that the unadsorbable fraction shows a large peak which seems to be due to nucleosides and/or base components. A peak designated as Ur in the figure which has not been observed in the tissue before the incubation does not contain phosphates and is likely due to uric acid because of its characteristic ultraviolet absorption spectrum (Fig. 5) and the positive murexide reaction. The acid soluble fraction of granuloma obtained from a scorbutic guinea pig was analyzed in the same way (Fig. 6). The granuloma was ill-developed and revealed a haemorrhagic change in it. The peaks of nucleoside monophosphates are low, while the peaks of ATP and ADP are rather high; this pattern resembles those of other tissues such as liver, erythrocyte, etc.

It is well known that an adrenocortical hormone affects the connective tissue metabolism. The present authors attempted to know the hormonal effect on the nucleotide metabolism of the granuloma. Dexamethasone phosphate (Decadron, Nippon Merck-Banyu Co., Ltd.) had been injected intraperitoneally into a granuloma-bearing rat 24, 12 and 6 hours before the extirpation (each dose was about 0.4mg/100g). No change occurred in the pattern of acid soluble nucleotides except the relative specific radioactivities of nucleoside $2',3'$-monophosphates. As the amount of $^{32}$P incorporated into the granuloma might be influenced by various factors, the present authors adopted the specific radioactivities of nucleoside $5'$-monophosphates as a reference and designated the relative specific radioactivity of $2',3'$-nucleotide as a ratio to the value of corresponding $5'$ nucleotide.

In fact, the relative specific radioactivity was calculated only for the adenosine
Fig. 6 Elution pattern of the acid soluble fraction of the granuloma in a scorbic guinea pig
(A guinea pig of 200-250 g body weight was fed with the ascorbic acid-deficient diet during this experiment. Two weeks after the arrest of the body weight gain, agar implantation was done, and the granuloma aged 1 week was used as the sample. The granuloma was ill-developed and the haemorrhagic change was shown in the granuloma. P32 was injected 60 minutes before extirpation.)

monophosphate to avoid contamination of other phosphorus compounds. This value was increased with time after P32-injection. One hour after the injection, the value was around 0.03 in a normal granuloma, while 0.3 in a decadron-treated granuloma. This effect was seen also 6 hours after the injection, in which 0.34 for the normal but 2.6 for the decadron-treated granuloma.

**Paper chromatography of the nucleoside monophosphates:** As can be seen in Figs. 1—3, the presence of nucleoside 2'- and 3'-monophosphates was suggested. The peaks A, C, G and U showed the characteristic ultraviolet absorption spectra corresponding to those of adenosine, cytidine, guanosine and uridine, respectively (Fig. 7).

In the case of cytidylic acid, 5'- and 3'-isomers have values of 2.10 and 2.00 for the ratio of E280/E260 at acidic pH, while 2'-isomer has a value of 1.80 and the sample C shows a similar ratio to the latter. For adenylic acid, cyclic AMP may be eluted also following to 5'-AMP in this system. To identify which isomers the samples were, paper chromatography was carried out (Table I). The results indicated that the
Ultraviolet absorption spectra of each nucleotide fraction (Each fraction was purified by charcoal absorption followed by lyophilization. The nucleotide was dissolved in 0.01 M Tris-HCl buffer (pH 7.4), 0.5 ml. An aliquot of it was added by 2 ml of water and its spectrum was taken, ———; 0.1 ml of 6 N HCl was added, ·······; further 0.1 ml of 15 N NH₃OH was added, ---·--.)

Fig. 7 Ultraviolet absorption spectra of each nucleotide fraction

Components of each nucleotide: Table II shows the molar ratio of phosphorus and pentose to each base. Phosphate was released by wet ashing or by the action of intestinal phosphatase. Pentose was determined after periodate oxidation, when 5'-nucleotides showed scarcely any orcinol reaction. For the sample C and U, color developed very slightly even after long period of heating in the orcinol reaction, so that it was suggested that the samples were pyrimidine nucleotides.

Periodate oxidation of each nucleotide: Nucleoside 5'-monophosphate is oxidized easily with periodate, while 2'- and 3'-isomers are resistant to this treatment. Fig. 8 represents the action of periodate on each 2'- and 3'-nucleotide fraction, which scarcely consumes any periodate. Pentose was recovered quantitatively even after 48 hours treatment as mentioned above.

Substrate specificities of nucleotidases: 3'-Nucleotidase partially purified from rye grass contained some unspecific phosphatases. To avoid these effects, 5'-AMP was used as a control for an incubation condition in which 5'-AMP was split slightly. Phosphate released from the samples was extracted with isopropanol as phosphomo-
Table I  Rf values of each mononucleotide fraction in paper chromatography with several kinds of solvents

<table>
<thead>
<tr>
<th>Solvent</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
</tr>
</thead>
<tbody>
<tr>
<td>5′-AMP</td>
<td>0.63</td>
<td>0.39</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>2′-, 3′-AMP</td>
<td>0.72</td>
<td>0.34</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td>3′-, 5′-Cyclic AMP</td>
<td>0.15</td>
<td>0.15</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>Sample 5′A</td>
<td>0.41</td>
<td>0.41</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td>Sample A</td>
<td>0.72</td>
<td>0.25</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>5′-CMP</td>
<td>0.55</td>
<td>0.55</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>2′-, 3′-CMP</td>
<td>0.67a</td>
<td>0.59</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td>Sample C</td>
<td>0.66</td>
<td>0.66</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>5′-GMP</td>
<td>0.26</td>
<td>0.40</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>2′-, 3′-GMP</td>
<td>0.40</td>
<td>0.35</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>Sample G</td>
<td>0.40</td>
<td>0.46</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>5′-UMP</td>
<td>0.36</td>
<td>0.42</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>2′-, 3′-UMP</td>
<td>0.42</td>
<td>0.43</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>Sample U</td>
<td>0.41</td>
<td>0.43</td>
<td>0.43</td>
<td></td>
</tr>
</tbody>
</table>

N.B.  Toyo-Roshi No.53, descending method for solvent a, and ascending for others. For the solvents a, b, c and d, see the text.

*Rf values reported in bibliography are 0.65 for 2′-CMP, and 0.59 for 3′-CMP.

Table II  Molar ratios of each component of nucleoside monophosphate fractions

<table>
<thead>
<tr>
<th>Components</th>
<th>A</th>
<th>C</th>
<th>G</th>
<th>U</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Pentose</td>
<td>0.96</td>
<td>0.90</td>
<td>0.90</td>
<td>0.90</td>
</tr>
<tr>
<td>Phosphate</td>
<td>1.26</td>
<td>1.00</td>
<td>0.98</td>
<td>0.98</td>
</tr>
</tbody>
</table>
Fig. 8 Periodate oxidation of each nucleotide
(The reaction mixture was as follows:
0.02 M sodium acetate buffer (pH 4.6)
3.1 ml, 1.5×10⁻³ M periodic acid 0.1 ml,
containing 5'-AMP (0.104 μmole),
5'-GMP (0.212 μmole), sample A (0.079 μmole),
C (0.133 μmole), G (0.085 μmole), or U (0.086 μmole), respectively.)

lybdate, and its amount and radioactivity were determined. Phosphate released by the
action of bull seminal 5'-nucleotidase was also determined (Table III). In the fraction

Table III Effects of the 3'-nucleotidase and 5'-nucleotidase on each nucleoside monophosphate

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>C</th>
<th>G</th>
<th>U</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pi released by 3'-nuc</td>
<td>17.7%</td>
<td>4.7%</td>
<td>7.2%</td>
<td>10.9%</td>
</tr>
<tr>
<td>leotidase¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radioactivity released</td>
<td>18.1</td>
<td>6.1</td>
<td>8.1</td>
<td>11.1</td>
</tr>
<tr>
<td>by 3'-nucleotidase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pi released by 5'-nuc</td>
<td>5.2%</td>
<td>5.8%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>leotidase¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radioactivity released</td>
<td>9.6</td>
<td>8.5</td>
<td>0</td>
<td>3.5</td>
</tr>
<tr>
<td>by 5'-nucleotidase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N.B. 1) Partially purified from rye grass extract (ca. 4 times). Assay condition: 0.02 M
Tris-HCl buffer (pH 7.4), enzyme preparation containing 1 mg protein, final
volume 2.0 ml, 37°C for 30 minutes.
2) Partially purified from bull seminal fluid (ca. 8 times). Assay condition: 0.13 M
glycine-NaOH buffer (pH 8.5), 0.013 M MgCl₂, enzyme preparation containing
1 mg protein, final volume 2.0 ml, 37°C for 30 minutes.

A and C of the latter case, it may be due to the contamination of 5'-isomers that a
small amount of phosphate was observed to be released.

For the adenylic acid isomers, 5'-adenylic acid deaminase from muscle is specific
for 5'-isomer¹⁰. Fig. 9 shows the time-course of ultraviolet absorption at 265 mμ in
0.01 M citrate buffer (pH 6.5). Sample A was not deaminated like 2'-, 3'-AMP
from alkaline digest of nucleic acid.

Paper chromatography of nucleosides obtained from the samples by the phos-
phatase treatment: The samples and 5'-nucleotides were subjected to the phosphatase
treatment in 0.02 M Tris buffer (pH 9.1) with 0.5m M magnesium acetate at 37°C
for 1 hour¹⁷. Paper chromatography of these reaction products revealed that each
Fig. 9 Action of 5'-AMP deaminase on 5'-AMP and the sample A
(Reaction mixture: 0.01 M potassium citrate buffer (pH 6.4), 3.0 ml; enzyme solution, 0.1 ml (1 mg protein); 5’-AMP, 0.208 μmole, AMP from the alkali digest of liver RNA, 0.161 μmole, or the sample A, 0.067 μmole, respectively. Temperature, 20° C.

Table IV Rf values of each nucleoside obtained from the corresponding nucleotide by phosphatase treatment

<table>
<thead>
<tr>
<th>Solvent</th>
<th>A</th>
<th>C</th>
<th>G</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>e</td>
<td>0.38 (0.37)</td>
<td>0.47 (0.43)</td>
<td>0.36 (0.36)</td>
<td>0.67 (0.70)</td>
</tr>
<tr>
<td>f</td>
<td>0.32 (0.33)</td>
<td>0.18 (0.17)</td>
<td>0.04 (0.06)</td>
<td>0.05 (0.10)</td>
</tr>
</tbody>
</table>

N.B. Rf values for the authentic nucleosides are shown in the parenthesis. For the phosphatase treatment and the solvents e and f, see the text.

sample had the same nucleoside as corresponding to 5’-nucleotide (Table IV).

Relationship between the amounts of four 2’- and 3’-nucleotides: It had been confirmed that the characteristic peaks in the acid soluble fraction of the granuloma were due to nucleoside 2’- and 3’-monophosphates. 5’-Isomers appeared also, but, their quantities were rather small and each of the them seemed to vary independently. On the other hand, a definite relationship between the quantities of four nucleoside 2’- and 3’-monophosphates was observed as shown in Table V.

Namely, the relationship of each fraction is as follows: A=U, G=C and A:G=10:16—17, which is also applicable to the amount of radioactivity of each fraction. Supposing that these 2’- and 3’-nucleotides come from some degradation products of RNA, it is reasonable to look for the RNA with such a peculiar base composition in the granuloma. As shown in Table V, the base composition of RNA obtained by the phenol method from the sonicate and from the microsomal and pH 5 fractions of the granuloma is different from that mentioned above. However, ratios of radioactivities
Table V: Molar ratios between each nucleoside 2'-, 3'-monophosphate

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>C</th>
<th>G</th>
<th>U</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid soluble fraction of the granuloma</td>
<td>10.0</td>
<td>16.6</td>
<td>16.3</td>
<td>10.6</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>15.4</td>
<td>14.4</td>
<td>9.6</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>16.1</td>
<td>16.2</td>
<td>10.4</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>16.8</td>
<td>16.9</td>
<td>10.1</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>17.1</td>
<td>17.1</td>
<td>9.8</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>17.5</td>
<td>17.8</td>
<td>10.6</td>
</tr>
<tr>
<td>Total RNA of the granuloma</td>
<td>(10.0)</td>
<td>(16.1)</td>
<td>(15.6)</td>
<td>(9.1)</td>
</tr>
<tr>
<td>Microsomal RNA of the granuloma 1)</td>
<td>10.0</td>
<td>15.8</td>
<td>20.0</td>
<td>10.3</td>
</tr>
<tr>
<td>RNA of pH 5 fraction 1)</td>
<td>10.0</td>
<td>17.3</td>
<td>21.7</td>
<td>11.0</td>
</tr>
<tr>
<td>RNA of rat liver 2)</td>
<td>10.0</td>
<td>13.6</td>
<td>18.1</td>
<td>11.5</td>
</tr>
<tr>
<td>Nucleotides from rat liver RNA digested by granuloma sonicate 3)</td>
<td>10.0</td>
<td>12.4</td>
<td>17.4</td>
<td>11.3</td>
</tr>
<tr>
<td>Base components of DNA from the granuloma 4)</td>
<td>10.0</td>
<td>10.9</td>
<td>8.3</td>
<td>12.6</td>
</tr>
</tbody>
</table>

N.B. 1) Ratios between the radioactivities of each nucleoside 2', 3'-monophosphate are shown in the parenthesis.
2) RNA was extracted by the phenol method, digested in 0.5 N NaOH, 37°C for 16 hours and analysed by the use of ion exchange column chromatography.
3) About 10 mg of the same rat liver RNA sample mentioned above was dissolved in 2 ml-portion of Krebs-Ringer phosphate solution (pH 7.4) and incubated with 1 ml-portion of the 105,000 x g supernatant of the granuloma sonicate at 37°C for 60 minutes. The sonicate was obtained from the granuloma of 1.5 g wet weight which was subjected to the sonication, 9 KC for 20 minutes in 10 ml-portion of phosphate buffer. The reaction was stopped by adding TCA and the acid soluble fraction was analysed after the ether extraction.
4) Sediment of the sonicate in 800 x g for 10 minutes was subjected to the phenol extraction and the residue was extracted with M NaCl containing 0.5% sodium lauryl sulphate. Ethanol was added to the extract after the washing by ether, and the precipitate obtained was treated with diluted alkali to remove contaminating RNA. This substance showed the positive diphenslamine reaction and the negative orcinol reaction. This was decomposed to the base components with the treatment by 99% formic acid at 175°C for 35 minutes. Paper chromatography using the solvent e and f was carried out for the separation and identification of these base components. Each spot was eluted and identified, and its amount calculated by its ultraviolet absorption spectrum.

of four nucleotides obtained from RNA of the sonicate show rather in good accordance with those obtained from acid soluble fraction, when ³²P has been administered in a granuloma-bearing rat 3 hours before the removal. The incorporation of ³²P in the microsomal and pH 5 fractions was too low to be analysed. If the 2'- and 3'-nucleotides come from a special RNA, an enzyme system in the granuloma should split RNA into mononucleotides which also should be recovered in the same base ratio. To
certify this point, the following experiment was carried out. Purified RNA obtained from rat liver by the phenol extraction was incubated with a small amount of sonicate of the granuloma. The molecular ratio of four mononucleotides in the acid soluble fraction agreed fairly well with the base composition of the RNA, while any di- or tri-nucleotides did not appear in the acid soluble fraction (Table V). The four bases of DNA of the granuloma were separated by paper chromatography and estimated spectrophotometrically after the elution. Although this was only semi-quantitative, the base ratio seemed to be markedly different from that in the acid soluble fraction.

DISCUSSION

Though the analyses of the acid soluble fraction in various tissues have been reported by many authors, little is known about the presence of nucleoside 2'- and 3'-monophosphates; it has only been reported about 3'-AMP and 2'-(3')UMP in the milk by Deutsch et al.18, and about 3'-UMP in the human milk by Kobata et al.19) Deutsch supposed that 3'-AMP might come from 3'-phosphoadenosine-5'-phosphosulfate. In the granuloma induced by the agar implantation, 2'- and 3'-nucleotides appeared more remarkably in the amount, than the other 5'-nucleotides with a definite quantitative relationship between each of them. Cold and the scorbutic conditions diminished these 2',3'-nucleotides. Incubation of the granuloma in vitro also decreased the nucleotides in spite of the increase in uric acid and unadsorbable fraction containing bases and nucleosides. Generally, phosphate at the α-position of nucleotide is considered to be metabolically fairly stable. However, in the granuloma, a considerably large amount of incorporation into 5'-nucleotides was shown 1 hours after P32-administration, followed by the marked incorporation into 2',3'-nucleotides 4 to 6 hours thereafter. This was confirmed by the action of intestinal phosphatase and 3'-nucleotidase which release the phosphate incorporated in the nucleotide. On the other hand, the radioactive phosphate was incorporated into the RNA fraction 3 hours after the injection, in which the ratios of the radioactivities of each nucleotides fairly agreed with the ratios obtained in the acid soluble fraction. From these facts, it is suggested that there exists certain special, metabolically active RNA in the granuloma along with its degradation products, 2'- and 3'-nucleotides in the equilibrated state. These characteristics, namely, metabolic activity and nucleotide composition, seem to resemble those of the so-called "messenger RNA". As reported in the bacterial system, the messenger RNA was defined as having the same nucleotide composition as that of DNA.

However, the messenger RNAs in the mammalian tissues have generally no such direct relationship to the DNA. Moreover, the "puff formation" is observed in a chromosome of salivary gland of fly, which may suggest that a part of DNA can be only active to transfer its genetic information to the messenger RNA in some stages of cell development. Recently, Butenandt reported that some steroids stimulated the puff formation in a chromosome of *Bombix mori* when the worm is going to form a pupa.

It has been well known that some kinds of steroid hormones, cortisone and its derivatives, affect the biosynthesis of the protein and nucleic acid. Especially, these hormones are considered to be an antiphlogistic and inhibitory reagent against the connective tissue formation.

As reported in this paper, formation of the characteristic 2'- and 3'-nucleotides in the granuloma were enhanced by the conditions such as the cold, ascorbic acid deficiency and steroid hormone administration. Therefore, we would like to suppose the occurrence of 2'- and 3'-nucleotides to be related with certain activity of the granuloma, especially the collagen synthesing activity.

In the investigation on the constituent amino acids and DNAs of various species of bacteria, Sueoka found that an amount of some amino acids (glycine, proline, alanine and arginine) in the bacterial protein were proportional to the G-C content in the DNA, while isoleucine, lysine, asparagine, and aspartic acid, glutamine, glutamic acid, tyrosine and phenylalanine showed the negative correlation coefficient to the G-C content. Nierenberg et al and Ochoa et al determined recently the "nucleotide code" based upon the fact that polyuridylic acid acted as the "messenger RNA" for the synthesis of polyphenylalanine, and polycytidylic acid for that of polyproline. These codes seem to agree with the Sueoka's data in their G-C contents.

Collagen contains the amino acids such as glycine and proline, which show a positive correlation with the G-C content much more than the others, being adversely proportional to the G-C content.

If metabolically active RNA has a base ratio which agrees with that of 2'- and 3'-nucleotides in the acid soluble fraction, its G-C content would be calculated as more than 60%, and therefore, it is possible to suppose that this RNA may play a role in determining the amino acid sequence of collagen. To clarify this point, studies on the collagen biosynthesis *in vitro* are now being carried on.

---


SUMMARY

1. The acid soluble fraction of the agar-induced granuloma contained four nucleoside 2'- and 3'-monophosphates which were proved as 2'-, 3'-adenylic, 2'-,(3') cytidylic, 2'-,3'-guanylic and 2'-,3'-uridylic acids by the use of the chromatographic, analytical chemical, and the enzymatic procedures.

2. The radioactive phosphate injected intraperitoneally was incorporated into these nucleotides 4 to 6 hours after the administration.

3. The quantitative relationship of these nucleotides was represented by the formulae; A=U, G=C and A:G=10:16 [17].

4. These 2'- and 3'-nucleotides were reconsidered as degradation products of certain special RNA which should contain guanine and cytosine more than 60% of its total bases. The relationship between this RNA and the amino acid composition of collagen was discussed.

REFERENCES