Amino Acid Metabolism of Experimental Granulation Tissue in vitro

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1. The intracellular volume in granulation tissue was about 15% of the total urea space. 2. The experimental granuloma has a greater ability to retain amino acids during the proliferation phase than later during the synthesis of collagen. 3. The synthesis of collagen and other proteins by granulation tissue is related to the concentrations of proline and glutamic acid in the medium. 4. The rate of synthesis of proline from glutamic acid in granulation-tissue slices is greatest during collagen synthesis. It is enhanced by lactate. 5. Extracellular cations influence the synthesis of collagen and ouabain is inhibitory. Synthesis of other proteins is less sensitive in this respect. 6. It is suggested that the synthesis of collagen is related to the supply of certain amino acids, especially proline, and hence to the function of the cell wall.

Three metabolic phases can be distinguished in the development of experimental granulation tissue: cellular proliferation, the mature phase when the tissue is capable of synthesizing collagen and, finally, involution to a scar (Lampiaho & Kulonen, 1967; Kulonen, 1970). Studies carried out with granulation tissue in vitro may have advantages for some purposes over those carried out in fibroblast cultures in that both contacts between the cells and also between the latter and the intercellular matter are intact. There is, of course, the disadvantage that the cell population is less homogeneous.

The rate and extent of collagen synthesis depend on several factors which may include the extracellular supply and intracellular synthesis of the constituent amino acids, post-translational events such as cleavage of the original peptide chains, hydroxylation and glycosylation and, finally, the dislocation and secretion of collagen molecules from the cell (Ehrlich & Bornstein, 1972).

The rates of transport and metabolism of amino acids and consequently their contents differ markedly from one tissue to another (Christensen, 1964). The present studies were aimed at an examination of some of the effects of the amino acid supply on the regulation of collagen synthesis. Some of the results have been presented in a preliminary form (Aalto et al., 1970; Kulonen et al., 1971).

**Experimental**

The methods are based on the work of Green & Lowther (1959) and on our previous experiments (Lampiaho & Kulonen, 1967).

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**Granulation tissue slices and incubation conditions**

*Induction of granulation tissue and preparation of slices.* Male albino rats of the Wistar strain (about 2 months old; 150–210 g) were used. The animals were kept on a standard diet (from Hankkija, Helsinki, Finland) supplemented with vegetables.

Growth of the granulomas was induced by pieces of viscose–cellulose sponge (wet size 10 mm × 10 mm × 20 mm; dry wt. 70–80 mg; made by Säteri Oy, Valkeakoski, Finland), which were sterilized by boiling in 0.9% NaCl for 30 min. Four to six pieces were implanted symmetrically under the dorsal skin of each rat (Viljanto & Kulonen, 1962; Viljanto, 1964).

After an implantation period of 5–50 days, the rats were killed by a blow on the neck, the sponges with ingrown granulation tissue were removed, the surrounding capsules dissected off and the granulomas immersed in ice-cold 0.9% NaCl. The granulomas were cut with a Stadie–Riggs microtome into slices 0.5 mm thick. Each slice was cut into four equal parts and the pieces were pooled at random to try to balance the differences between the sites of implantation and between individual rats. Infected granulomas were discarded.

*Incubation and treatment of the slices.* Incubations of the slices in vitro were started 1 h after the animals had been killed. The standard medium was a Krebs–Ringer phosphate solution at pH 7.4 (Umbreit et al., 1957) containing 22.4 mM-glucose and, in most cases, 2.87 mM-proline (non-radioactive), but no other added amino acids unless stated otherwise. No antibiotics were added. The gas phase was air. Each incubation sample consisted of about 300–400 mg of wet granuloma slices (dry wt. 70–100 mg) in 3–5 ml of medium.
in a 25ml Erlenmeyer flask, which was shaken in a
Gallenkamp incubator set at 180 strokes/min and
37°C. After a 30 min preincubation, 10–100 μCi of
L-[3H]proline (TRA.82; all labelled compounds were
from The Radiocchemical Centre, Amersham, Bucks.,
U.K.) was added and the incubation usually con-
tinued for a further 3h. The flasks were then cooled
in crushed ice and the slices processed immediately.
They were washed four times with 5 ml of ethanol,
four times with 5 ml of ethanol–diethyl ether (1:2,
v/v) and four times with 5 ml of diethyl ether, dried
at 37°C overnight and weighed. Small deviations
from this procedure occurred during the course of the
work, but each series of experiments contained an
appropriate control. All the results presented are
averages of at least duplicate determinations.

Experimental modifications of the medium. The
effects of added amino acids were determined in
media that contained 2.87 mM-proline (unlabelled)
and 1–10 mM of the amino acid in question. For the
elucidation of the effects of extracellular proline and
glutamic acid, the slices were preincubated without
added amino acids and then transferred to flasks that
contained various concentrations of labelled proline
or glutamic acid of constant specific radioactivity
(Figs. 1 and 2). In experiments to study the simul-
taneous incorporation of proline and glutamic acid
(Fig. 3) the concentration of glucose was 11.2 mM. To
each 3 ml of the medium, 10 μCi of L-[3H]proline and
2 μCi of L-[14C]glutamic acid (CFB.10) were added.

Intra- and extra-cellular spaces. The slices of
granulation tissue were incubated for 1 h in a Krebs–
Ringer medium (Umbreit et al., 1957), which was
buffered with 20 mM-2-(N-2-hydroxyethylpiperazine-
N'-yI)ethanesulphonic acid (Hepes) instead of phos-
phate (Uitto, 1970) (Calbiochem, San Diego, Calif.
92112, U.S.A.), pH 7.4, and contained 22.4 mM-
glucose, 2.87 mM-proline, 6.7 mM-rafﬁnose and 10 mM-
urea (Green & Lwther, 1959). The slices were
wiped dry, weighed and homogenized in water. An
equal volume of a saturated aqueous solution of
picric acid was added to both the homogenate and
the medium. The precipitates were removed by centri-
fugation and the supernatants passed through a column
(1.6 cm × 1.0 cm) of Dowex 2 (X8; Cl– form; 100–200 mesh) to remove picric acid. The adsorbed
solutcs were eluted with 0.02 M-HCl. The urea and
raffinose spaces were calculated from the respective
concentrations in the medium and the total contents
in the slices (Green & Lwther, 1959).

Variations of the experimental technique. Incorpora-
tion of labelled proline was no better in Eagle’s
minimal essential medium (Eagle, 1959) than it was
in Krebs–Ringer phosphate medium. During the
work it was found that the incorporation is twofold
greater in Hepes-buffered media (cf. Uitto, 1970) and
that a higher concentration of O2 in the gas phase
also enhances the incorporation considerably
However, all the incorporation results presented here
were obtained under the conditions described above,
although Hepes-buffered media were used in the deter-
minations of urea and raffinose spaces and in the
experiments described in Table 2.

When the initial pH of the Krebs–Ringer phosphate
medium was either increased to 8.0 or decreased to
6.8, the incorporation of proline as hydroxyproline
was lowered by 50–55%. During a 2 h incubation, the
pH of the Krebs–Ringer phosphate medium decreased
from the initial value of 7.2 to 6.5. In the Krebs-
Ringer bicarbonate medium (Umbreit et al., 1957)
[with CO2 + O2 (5:95) as the gas phase], in Eagle’s
minimal essential medium (with CO2 + O2) and in
20 mM-Hepes-buffered media, the change was smaller,
up to pH 6.8–6.9.

When the content of sliced tissue in the medium
was increased about twofold, the total incorporation
of [3H]proline into non-collagenous protein did not
increase proportionally and the synthesis of collagen
was affected even more.

Protein synthesis occurred mainly during the first
3 h, as shown by measurements of activity at 1 h
intervals, and little could be gained by prolonging
the incubation. A lag period of 30–60 min was
occasionally observed in the accumulation of radio-
active hydroxyproline.

Analytical methods
Radioactivity of proteins in the incubated slices.
Collagen in the slices was gelatinized in 10 ml of water
at 130°C for 3 h in screw-capped tubes and the residue
was washed twice with 10 ml of hot water. The wash-
ings were added to the gelatin solution, which was
usually dialyzed against running tap water. Less than
10% of the collagen remained in the insoluble
residue. Both the gelatines and the residues were
hydrolysed in 6 N-HCl at 130°C for 3 h and the
hydrolysates evaporated on a boiling-water bath. For
the determination of the total radioactivity of a
protein sample, a 200 μl portion of the 8 ml sample
of unfractioinated hydrolysate was pipetted directly
into 10 ml of scintillation solvent [6 ml of ethylene
glycol monomethyl ether and 10 ml of scintillation
liquid containing 15 g of 2,5-diphenyloxazole and
50 mg of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benz-
enz in 1000 ml of distilled toluene]. The radioactivities
were measured with a Packard Tri-Carb liquid-
scintillation spectrometer model 3320. At least 1000
disintegrations were counted. The counting efficiency
was about 90% for 14C and 38% for 3H, except in
systems containing methyl Cellosolve where it was
24% (total protein fractions).

Radioactivity of hydroxyproline. The radioactivity
of hydroxyproline was determined by the procedure
of Juva & Prockop (1966). The hydroxyproline
radioactivities were calculated per weight of dry
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tissue, which reflects the volume of tissue, because the sponge accounts for the greater part of the dry weight (Viljanto, 1964). No attempt was made to calculate the absolute number of disintegrations (Juva, 1968). Most of the results are presented as percentages of the results obtained in the control experiments.

To determine to what extent proline and hydroxyproline were separated during the analytical procedure, pilot experiments were carried out in which either $2\mu$ Ci of [1H]proline or $0.5\mu$ Ci of hydroxy-[14C]proline was added to the unlabelled hydrolysates of gelatine. After the first oxidation step at room temperature, the first toluene extract contained 52.2–63.2 % of the added proline and 5.3–5.4 % of the added hydroxyproline (two determinations). The second toluene extract of the same hydrolysate contained 21.1–24.9 % of the proline and 0.9 % of the hydroxyproline. By three consecutive extractions, 84.1–98.2 % of the total proline was collected but also 6.3–6.8 % of the added hydroxyproline. When the toluene extraction was repeated after boiling the mixture to convert hydroxyproline into pyrrole, only 0.04 % of the original proline was found in the eluate, but 38.9 % of the original hydroxyproline was found. The latter percentage could be increased by 0.2–0.8 % by repeated washing of the silica-gel column, [1.5 g of silicic acid in a column (1.0 cm x 30 cm)], but by an additional 6 % by repeated extraction with toluene. The yield of pyrrole from non-labelled hydroxyproline, determined independently by using Ehrlich's reagent (Procop & Udenfriend, 1960), was 48–50 %. This was checked in each experimental series and found to be constant.

**Amino acids.** Automatic equipment built as described by Spackman et al. (1958) was used (Pikkarainen, 1968).

**Raffinose and urea.** Urea was determined according to Coulombe & Favreau (1963) and raffinose as described by Roe et al. (1949).

**Results**

**Extra- and intra-cellular spaces**

The results in Table 1 show that the cell volume estimated from the difference between the urea and raffinose spaces is from the tenth day onwards about 10–15 % of the whole-tissue volume and about 20 % of the urea space, in close agreement with the results of Green & Lowther (1959). About 30 % of the volume of full-grown granulation tissue is not penetrated by urea. Every cell in granulation tissue on average has an extracellular domain which is filled with secreted material and which is not invaded by other cells.

**Amino acids in the slices and the medium**

As may be deduced from the results in Table 2, there is a leakage of amino acids from the slices into the medium. This might explain why additions of amino acids, with the notable exceptions of proline and glutamic acid, to the medium do not have significant effects in short-term experiments. After a standard incubation with radioactive proline, it was found that the total radioactivity of the slices from 2 cm$^3$ of a 10-day granuloma was 26400 (25100–27800) c.p.m., and that of the slices of 17-day granuloma 16100 (15300–16500) c.p.m., whereas the total radioactivities of the media were 14600 (14200–15600) c.p.m./ml and 16500 (15800–17500) c.p.m./ml, respectively. These results support the conclusion that the younger granulation tissue has a greater capacity to concentrate amino acids. No attempt was made to calculate the exact values of the intra-/extra-cellular ratios on the basis of the raffinose and urea spaces.

Some free amino acids in the slices may result from catabolism of the proteins (Hurych & Chvapil, 1965; Crawhall & Davis, 1971). The addition of proline to the medium moderately decreased the concentrations of other amino acids in the slices. The ratio of the amino acid concentrations (proline, histidine and arginine excepted) in the slices of 16-day granulomas kept in the medium containing proline and in the normal medium was 0.78 ± 0.03 (s.d.) and the same ratio in the slices of 7-day granulomas was slightly less, 0.61 ± 0.13. The ratios of the concentrations of amino acids (proline excepted) in the slices (no addition) and in the medium were 11.2 ± 2.8 and 3.0 ± 0.5 for the 7-day and 16-day granulomas, respectively. Those "concentrating

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**Table 1. Urea and raffinose spaces in the sponge-induced granulation tissue**

The conditions of the assay are explained in the text. Results are means ± s.e.m. for the numbers of duplicate estimations given in parentheses.

<table>
<thead>
<tr>
<th>Time after implantation (days)</th>
<th>Urea space (ml/g wet wt.)</th>
<th>Raffinose space (ml/g wet wt.)</th>
<th>Difference (ml/g wet wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5–6 (5)</td>
<td>0.954 ± 0.057</td>
<td>0.878 ± 0.034</td>
<td>0.076</td>
</tr>
<tr>
<td>10 (4)</td>
<td>1.048 ± 0.033</td>
<td>0.933 ± 0.032</td>
<td>0.115</td>
</tr>
<tr>
<td>14–19 (12)</td>
<td>0.710 ± 0.021</td>
<td>0.623 ± 0.014</td>
<td>0.087</td>
</tr>
<tr>
<td>23–26 (4)</td>
<td>0.735 ± 0.024</td>
<td>0.590 ± 0.041</td>
<td>0.145</td>
</tr>
</tbody>
</table>
Table 2. Amino acid concentrations of incubated granulation-tissue slices and medium

The values are those found after a 1 h incubation in Krebs–Ringer Hapes medium (see the text) and are given as means and ranges. When no range is given, results are for one determination only. When indicated, 2.87 mm-proline was added to the medium. The values for rat plasma are taken from Christensen & Cullen (1968).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>No. of determinations</th>
<th>Medium for all samples (mm)</th>
<th>Standard medium (µmol/g)</th>
<th>Proline added to medium (µmol/g)</th>
<th>Medium for all samples (mm)</th>
<th>Standard medium (µmol/g)</th>
<th>Proline added to medium (µmol/g)</th>
<th>Rat plasma (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>0.03 (0.02–0.04)</td>
<td>0.29</td>
<td>0.16 (0.15–0.17)</td>
<td>0.05 (0.05–0.06)</td>
<td>0.15 (0.14–0.15)</td>
<td>0.11 (0.10–0.13)</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>0.05 (0.04–0.08)</td>
<td>0.51</td>
<td>0.32 (0.28–0.35)</td>
<td>0.11 (0.10–0.12)</td>
<td>0.31 (0.28–0.35)</td>
<td>0.23 (0.20–0.27)</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>0.08 (0.06–0.12)</td>
<td>0.94</td>
<td>0.51 (0.45–0.56)</td>
<td>0.14 (0.11–0.18)</td>
<td>0.48 (0.35–0.67)</td>
<td>0.36 (0.32–0.41)</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.10 (0.09–0.11)*</td>
<td>0.55</td>
<td>0.38 (0.33–0.42)</td>
<td>0.21 (0.19–0.22)</td>
<td>0.44 (0.41–0.50)</td>
<td>0.37 (0.32–0.43)</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>0.12</td>
<td>0.36</td>
<td>1.68 (1.46–1.89)</td>
<td>0.12 (0.11–0.12)†</td>
<td>0.35 (0.32–0.38)</td>
<td>1.62 (1.43–1.80)</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>0.07 (0.07–0.08)*</td>
<td>1.00</td>
<td>0.52 (0.43–0.61)</td>
<td>0.18 (0.17–0.19)</td>
<td>0.53 (0.49–0.60)</td>
<td>0.44 (0.39–0.49)</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>0.07 (0.06–0.09)*</td>
<td></td>
<td>0.23</td>
<td>0.20 (0.18–0.22)</td>
<td>0.56 (0.51–0.65)</td>
<td>0.43 (0.38–0.48)</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.02 (0.01–0.02)*</td>
<td></td>
<td></td>
<td>0.02 (0.01–0.05)</td>
<td>0.08 (0.04–0.10)</td>
<td>0.06 (0.05–0.07)</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>0.04 (0.03–0.06)</td>
<td>0.62</td>
<td>0.30 (0.27–0.33)</td>
<td>0.17 (0.09–0.12)</td>
<td>0.30 (0.26–0.39)</td>
<td>0.22 (0.20–0.28)</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>0.01 (0–0.01)</td>
<td>0.07</td>
<td>0.04 (0.04–0.04)</td>
<td>0.01 (0.01–0.02)</td>
<td>0.04 (0.03–0.05)*</td>
<td>0.03</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.03 (0.02–0.04)</td>
<td>0.33</td>
<td>0.16 (0.15–0.17)</td>
<td>0.06 (0.05–0.07)</td>
<td>0.18 (0.15–0.25)</td>
<td>0.14 (0.11–0.17)</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>0.05 (0.04–0.07)</td>
<td>0.79</td>
<td>0.40 (0.39–0.41)</td>
<td>0.12 (0.10–0.14)</td>
<td>0.42 (0.33–0.56)</td>
<td>0.33 (0.25–0.40)</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.03 (0.02–0.03)</td>
<td>0.24</td>
<td>0.21 (0.18–0.24)</td>
<td>0.04 (0.03–0.04)</td>
<td>0.12 (0.08–0.16)</td>
<td>0.10 (0.07–0.12)</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.02 (0.01–0.03)</td>
<td>0.24</td>
<td>0.18 (0.15–0.21)</td>
<td>0.05 (0.04–0.06)</td>
<td>0.16 (0.12–0.22)</td>
<td>0.12 (0.09–0.15)</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>0.07 (0.05–0.09)</td>
<td></td>
<td>0.41</td>
<td>0.12 (0.11–0.14)</td>
<td>0.34 (0.27–0.44)</td>
<td>0.26 (0.22–0.29)</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>0.02 (0.01–0.03)</td>
<td>0.18</td>
<td>0.08</td>
<td>0.06 (0.03–0.04)</td>
<td>0.10 (0.07–0.13)</td>
<td>0.07 (0.05–0.08)</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>–</td>
<td></td>
<td>0.10</td>
<td>0.02 (0.01–0.03)</td>
<td>0.07 (0.07–0.08)</td>
<td>0.08 (0.06–0.11)</td>
<td>0.12</td>
<td></td>
</tr>
</tbody>
</table>

* Two determinations.
† Three determinations.
AMINO ACIDS IN GRANULATION TISSUE

ratios' are low for proline and glutamic acid in the 7-day granulomas and for glutamic acid also in the 16-day granulomas. The media contained on average 2.3±0.75 times as much amino acids when the slices were from 16-day granulomas than when the slices were from 7-day granulomas. The slices from the older granulomas contained less free amino acids, but the difference can be accounted for to a large extent by the smaller water-penetrable volume.

Extracellular concentrations of amino acids and the synthesis of collagen

When leucine (1 mM) was present in the medium, the amount of hydroxyproline formed was 126.2±17.3 % (S.E.M., n = 5) of the amount formed in control experiments. On the other hand, phenylalanine (1 mM) decreased the incorporation of proline as hydroxyproline to 60.0±21.2 % (S.E.M., n = 4). However, neither of these results is statistically significant.

The results described in Figs. 1 and 2 show the significance of the extracellular concentrations of proline and glutamic acid, respectively. The extent of synthesis of proteins, including collagen, increases with the extracellular concentrations of some amino acids, markedly for proline and to a lesser extent for glutamic acid. An inflexion can be observed in both curves. Additional experiments were carried out to find out whether the curve is strictly horizontal at higher proline concentrations, but there was always some concentration-dependence of the collagen synthesis. Non-specific adsorption of labelled proline cannot be altogether excluded. The low capacity of the granulation tissue to concentrate glutamic acid may explain the importance of its extracellular concentration. When 1 mM-glutamic acid or a gelatin hydrolysate calculated to be 1 mM in proline was included in the medium which contained 2.87 mM-proline (labelled), the radioactivity decreased to 65–75 % of the original value. This decrease may be accounted for by dilution.

Attempts were made to modify the rate of collagen synthesis in slices of granulation tissue by adding homogenates and extracts from various tissues. Several tissues retarded the incorporation, partly by dilution of the precursor proline. A homogenate from 18–80-day granulomas that sedimented at 100000g but not at 600g was especially active.

Fig. 1. Incorporation of \[^{3}H\]proline into granulation tissue at the indicated concentrations of proline in the medium

Slices from mature (16–21-day) sponge-induced granulation tissue were incubated as described in detail in the Experimental section. In Expt. 1 the specific radioactivity of proline was 11.5 \(\mu\)Ci/\(\mu\)mol. Incorporation into: ●, non-collagenous protein; ○, hydroxyproline of gelatinized protein. In Expt. 2 the specific radioactivity of proline was 4.4 \(\mu\)Ci/\(\mu\)mol. □, Incorporation into hydroxyproline of gelatinized protein.

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Fig. 2. Incorporation of \[^{14}C\]glutamic acid into granulation tissue at the indicated concentrations of glutamic acid in the medium

Slices from mature (16–21-day) sponge-induced granulation tissue were incubated as described in detail in the Experimental section. The specific radioactivity of glutamic acid was 0.23 \(\mu\)Ci/\(\mu\)mol. Incorporation into: ●, non-collagenous protein; △, gelatinized protein; ○, hydroxyproline of gelatinized protein.
Appreciable amounts of free proline were liberated from this subcellular fraction to a Krebs–Ringer phosphate medium at 37°C in 3 h.

The fibroblast can convert glutamic acid into proline, especially during collagen synthesis (Fig. 3). This reaction involves a reduction step. A change in

\[
\text{Concn. of glutamate (mM)} \quad \text{Incorporation into hydroxyproline (c.p.m./100mg dry wt. of slices)} \quad \text{Incorporation into non-collagenous proteins (c.p.m./100mg dry wt. of slices)}
\]

<table>
<thead>
<tr>
<th>Conc. of glutamate (mM)</th>
<th>Lactate not added</th>
<th>Lactate added</th>
<th>Lactate not added</th>
<th>Lactate added</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.72</td>
<td>870</td>
<td>1220 (+40%)</td>
<td>84600</td>
<td>139100 (+65%)</td>
</tr>
<tr>
<td>1.42</td>
<td>1170</td>
<td>1680 (+43%)</td>
<td>114200</td>
<td>157000 (+37%)</td>
</tr>
<tr>
<td>1.42</td>
<td>870</td>
<td>1120 (+29%)</td>
<td>76700</td>
<td>78300 (+2%)</td>
</tr>
<tr>
<td>2.87</td>
<td>1290</td>
<td>1620 (+25%)</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>
the redox balance produced by an addition of lactate seems to increase the rate of synthesis of collagen from glutamic acid (Table 3). Green & Goldberg (1964) have previously demonstrated that lactate promoted the synthesis of collagen in cell culture. An enhancing effect of lactate on the activity of protocollagen proline hydroxylase in cultured fibroblasts has been reported by Comstock & Udenfriend (1970).

**Active transport and the synthesis of collagen**

The present findings on the importance of extracellular concentrations of certain amino acids and on the differences in their concentrations during the development of granulation tissue prompted experiments on the active transport, which was modified either by the addition of ouabain or by changes in

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**Table 4. Effect of ouabain on the incorporation of proline into proteins of granulation tissue**

Slices of sponge-induced granulation tissue were incubated in Krebs–Ringer phosphate medium which contained 22.4 mM-glucose and 2.87 mM-proline (non-radioactive) as described in the Experimental section. [3H]Proline was added as the precursor and ouabain (strophanthin G, Sandoz, Basle, Switzerland) was added to the experimental samples.

<table>
<thead>
<tr>
<th>Conc. of ouabain (mM)</th>
<th>7-day (proliferating) granulomas</th>
<th>21–32-day (mature) granulomas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Experiment</td>
</tr>
<tr>
<td>0.1</td>
<td>1270</td>
<td>1180 (−7%)</td>
</tr>
<tr>
<td>0.1</td>
<td>5180</td>
<td>3380 (−35%)</td>
</tr>
<tr>
<td>1.0</td>
<td>1270</td>
<td>190 (−85%)</td>
</tr>
<tr>
<td>0.1</td>
<td>8420</td>
<td>5380 (−36%)</td>
</tr>
<tr>
<td>0.1</td>
<td>22890</td>
<td>15920 (−30%)</td>
</tr>
<tr>
<td>1.0</td>
<td>9630</td>
<td>3970 (−59%)</td>
</tr>
</tbody>
</table>

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**Table 5. Effects of Na+ and K+ concentrations in the medium on the incorporation of proline into proteins of granulation tissue**

Granulation-tissue slices were incubated as described in the legend of Table 4 and in the Experimental section. The initial concentrations of Na+ and K+ in the medium are indicated.

<table>
<thead>
<tr>
<th>[Na+] (mM)</th>
<th>[K+] (mM)</th>
<th>7-day (proliferating) granulomas</th>
<th>18–25-day (mature) granulomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>125</td>
<td>5</td>
<td>3459 (−30%)</td>
<td>16670 (−36%)</td>
</tr>
<tr>
<td>0</td>
<td>125</td>
<td>643 (−30%)</td>
<td>26400 (−30%)</td>
</tr>
<tr>
<td>125</td>
<td>0</td>
<td>439 (−30%)</td>
<td>20900 (−30%)</td>
</tr>
<tr>
<td>119</td>
<td>6</td>
<td>16670 (−30%)</td>
<td>13170 (−30%)</td>
</tr>
<tr>
<td>120</td>
<td>5</td>
<td>26400 (−30%)</td>
<td>18400 (−30%)</td>
</tr>
<tr>
<td>120</td>
<td>5</td>
<td>20900 (−30%)</td>
<td>24300 (−30%)</td>
</tr>
<tr>
<td>121</td>
<td>4</td>
<td>18400 (−30%)</td>
<td>21800 (−30%)</td>
</tr>
<tr>
<td>120</td>
<td>10</td>
<td>16840 (−30%)</td>
<td>22600 (−30%)</td>
</tr>
<tr>
<td>124</td>
<td>1</td>
<td>12280 (−30%)</td>
<td>22600 (−30%)</td>
</tr>
<tr>
<td>0</td>
<td>120</td>
<td>5850 (−30%)</td>
<td>20400 (−30%)</td>
</tr>
<tr>
<td>0</td>
<td>125</td>
<td>1353 (−30%)</td>
<td>75000 (−30%)</td>
</tr>
<tr>
<td>0</td>
<td>125</td>
<td>7230 (−30%)</td>
<td>189000 (−30%)</td>
</tr>
<tr>
<td>0</td>
<td>5</td>
<td>6210 (−30%)</td>
<td>172000 (−30%)</td>
</tr>
<tr>
<td>120</td>
<td>0</td>
<td>7490 (−30%)</td>
<td>273000 (−30%)</td>
</tr>
<tr>
<td>125</td>
<td>0</td>
<td>4220 (−30%)</td>
<td>145000 (−30%)</td>
</tr>
</tbody>
</table>

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the extracellular concentrations of Na\(^+\) and K\(^+\). The results in Table 4 show that incorporation of proline into proteins of granulation tissue is sensitive to ouabain. The synthesis of collagen is affected more than is that of the other proteins. Similar conclusions about the special sensitivity of collagen synthesis to surface conditions of cells can be drawn from the effects of different concentrations of electrolytes (Table 5). The average ratio of hydroxyproline/non-collagenous proteins in mature granulomas was about 0.068 (0.054–0.091) in the presence of both Na\(^+\) and K\(^+\) but about 0.030 (0.018–0.038) in the absence of either cation.

**Discussion**

The concentration of proline in the blood plasma of mammals is in the critical range for protein synthesis in the fibroblasts in the rat 0.16mM, according to Christensen & Cullen (1968) or 0.11mM (Bethell et al., 1965). The distribution of amino acids between liver and blood as reported by Christensen & Cullen (1968) seems to hold also for mature granulation tissue, where the ratios of the respective concentrations are of the order of 3–4. For proliferating granulation tissue, the ratio is higher still. A large shift from anabolism to catabolism of proteins occurs when an animal passes from foetal life to infancy and then to adulthood. In consequence, the transport of amino acids into the cell is high in a newborn, but declines slowly thereafter (Tews & Harper, 1969).

The significance of proline concentration in the synthesis of collagen in rat calvaria has been studied by Finerman et al. (1967) with analogous results. They found further that the intracellular concentration of proline is proportional to the extracellular concentration. The 'saturation limit' of extracellular proline was 0.15mM. Added 2-aminoisobutyric acid, hydroxyproline and azetidine-2-carboxylic acid led to a decrease in the intracellular concentration of free proline. The administration of azetidine-2-carboxylic acid arrested the accumulation of collagen in chick embryo (Lane et al., 1971a) and the small amount of this analogue of proline incorporated influenced the properties of collagen (Lane et al., 1971b). The rate of proline influx clearly controls the size of the precursor pool available for protein synthesis.

The injection of N-methyl-2-aminoisobutyric acid produced within 2h an aminoaciduria limited to proline and hydroxyproline, and 2-aminoisobutyric acid caused a considerable decrease in the amino acids of the liver (Christensen & Cullen, 1968). The transport of proline is inhibited by 2-aminoisobutyric acid (Mackenzie & Scriver, 1970), although the transport of imino acids is different from that of 2-aminoisobutyric acid in foetal calvaria (Phang et al., 1971). In contrast to the results of experiments reported in the present paper, it should be mentioned that glutamic acid and proline could be individually omitted from the medium without affecting the growth of tibia cultures during a 6-day period (Biggers, 1965). This may point to differences in the requirements for amino acids by granulation tissue, cartilage and bone, but these experiments (Biggers, 1965) were carried out over much longer periods than those described in the present paper.

The relationship between Na\(^+\) concentration and amino acid transport in erythrocytes has been studied by Christensen et al. (1967), Eaveson & Christensen (1967), Wheeler & Christensen (1967) and Thomas & Christensen (1971). Growth can be controlled in the fibroblasts by changing the Na\(^+\)/K\(^+\) molar ratio (optimum 14:1) as found by Kuchler (1967). In adult epiphysis, but not in foetal membranous bone, transport of neutral amino acids is related to the presence of Na\(^+\) (Hahn et al., 1969a,b). The transport of proline is inhibited by ouabain, cyanidine and glycine and by a decrease in the Na\(^+\) concentration in the medium (Mahoney & Rosenberg, 1970). The concentrative transport of amino acids is inhibited by ouabain also in bone (Adamson et al., 1966). The findings presented in Tables 4 and 5 cannot be explained entirely by a disturbed transport of amino acids unless the synthesis of collagen is more sensitive to a shortage of amino acids than is that of other proteins.

The collagen content of the liver correlates with the concentration of free proline and is inversely proportional to that of the free glutamic acid (Rojkind & Diaz de Leon, 1970; Kershenobich et al., 1970). The increased formation of proline after the shift in the redox balance resulting from the catabolism of ethanol (Hääkkinen & Kulonen, 1970) may contribute to liver cirrhosis.

The results of the present experiments justify attempts to influence the formation of collagenous fibres by the fibroblasts, first, by altering the supply and active transport of certain amino acids, especially of proline, secondly, by altering the redox balance and, finally, by altering the functions of the cell wall.

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**References**


Christensen, H. N. (1964) *Mammalian Protein Metab.* 1, 105–124