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 redox balance, and also 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 25 ml Erlenmeyer flask, which was shaken in a Gallenkamp incubator set at 180 strokes/min and 37°C. After a 30 min preincubation, 10–100 μ Ci of L-[³H]proline (TRA.82; all labelled compounds were from The Radiochemical Centre, Amersham, Bucks., U.K.) was added and the incubation usually continued for a further 3 h. 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 simultaneous incorporation of proline and glutamic acid (Fig. 3) the concentration of glucose was 11.2 mM. To each 3 ml of the medium, 10 μ Ci of L-[³H]proline and 2 μ Ci 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-hydroxyethyl)piperazine-*N'*-yl)ethanesulphonic acid (Hepes) instead of phosphate (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 & Lowther, 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 centrifugation and the supernatants passed through a column (1.6 cm $\times$ 1.0 cm) of Dowex 2 (X8; Cl[−] form; 100–200 mesh) to remove picric acid. The adsorbed solutes 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 & Lowther, 1959).

Variations of the experimental technique. Incorporation 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

(Niinikoski, 1969; Niinikoski & Kulonen, 1970). However, all the incorporation results presented here were obtained under the conditions described above, although Hepes-buffered media were used in the determinations 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, 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 radioactive 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 washings were added to the gelatine solution, which was usually dialysed 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 M-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 μ l portion of the 8 ml sample of unfractionated 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)benzene 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

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 μ Ci of [3 H]proline or 0.5 μ Ci of hydroxy-[14 C]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 $\times$ 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 (Prockop & 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 cm³ 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 (Hurtych & 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

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 $\pm$ s.e.m. for the numbers of duplicate estimations given in parentheses.

Time after implantation (days)	Urea space (ml/g wet wt.)	Raffinose space (ml/g wet wt.)	Difference (ml/g wet wt.)
5–6 (5)	0.954 ± 0.057	0.878 ± 0.034	0.076
10 (4)	1.048 ± 0.033	0.933 ± 0.032	0.115
14–19 (12)	0.710 ± 0.021	0.623 ± 0.014	0.087
23–26 (4)	0.735 ± 0.024	0.590 ± 0.041	0.145

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 Hepes 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).

Amino acid	No. of determinations	7-day (proliferating) granuloma			16-day (mature) granuloma				Rat plasma (mm)
		Medium for all samples (mm)	Slices		Medium for all samples (mm)	Slices			
			Standard medium (μmol/g)	Proline added to medium (μmol/g)		Standard medium (μmol/g)	Proline added to medium (μmol/g)		
Aspartic acid	...	3	1	2	5	3	2	—	
Threonine	0.03 (0.02-0.04)	0.29	0.16 (0.15-0.17)	0.05 (0.05-0.06)	0.15 (0.14-0.15)	0.11 (0.10-0.13)	0.02		
Serine	0.05 (0.04-0.08)	0.51	0.32 (0.28-0.35)	0.11 (0.10-0.12)	0.31 (0.28-0.35)	0.23 (0.20-0.27)	0.27		
Glutamic acid	0.08 (0.06-0.12)	0.94	0.51 (0.45-0.56)	0.14 (0.11-0.18)	0.48 (0.35-0.67)	0.36 (0.32-0.41)	0.33		
Proline	0.10 (0.09-0.11)*	0.55	0.38 (0.33-0.42)	0.21 (0.19-0.22)	0.44 (0.41-0.50)	0.37 (0.32-0.43)	0.50		
Glycine	0.12	0.36	1.68 (1.46-1.89)	0.12 (0.11-0.12)†	0.35 (0.32-0.38)	1.62 (1.43-1.80)	0.16		
Alanine	0.07 (0.07-0.08)*	1.00	0.52 (0.43-0.61)	0.18 (0.17-0.19)	0.53 (0.49-0.60)	0.44 (0.39-0.49)	0.29		
Cysteine	0.07 (0.06-0.09)*	—	0.23	0.20 (0.18-0.22)	0.56 (0.51-0.65)	0.43 (0.38-0.48)	0.31		
Valine	0.02 (0.01-0.02)*	—	—	0.02 (0.01-0.05)	0.08 (0.04-0.10)	0.06 (0.05-0.07)	—		
Methionine	0.04 (0.03-0.06)	0.62	0.30 (0.27-0.33)	0.17 (0.09-0.12)	0.30 (0.26-0.39)	0.22 (0.20-0.28)	0.18		
Isoleucine	0.01 (0-0.01)	0.07	0.04 (0.04-0.04)	0.01 (0.01-0.02)	0.04 (0.03-0.05)*	0.03	0.06		
Leucine	0.03 (0.02-0.04)	0.33	0.16 (0.15-0.17)	0.06 (0.05-0.07)	0.18 (0.15-0.25)	0.14 (0.11-0.17)	0.09		
Tyrosine	0.05 (0.04-0.07)	0.79	0.40 (0.39-0.41)	0.12 (0.10-0.14)	0.42 (0.33-0.56)	0.33 (0.25-0.40)	0.12		
Phenylalanine	0.03 (0.02-0.03)	0.24	0.21 (0.18-0.24)	0.04 (0.03-0.04)	0.12 (0.08-0.16)	0.10 (0.07-0.12)	0.08		
Lysine	0.02 (0.01-0.03)	0.24	0.18 (0.15-0.21)	0.05 (0.04-0.06)	0.16 (0.12-0.22)	0.12 (0.09-0.15)	0.05		
Histidine	0.07 (0.05-0.09)	—	0.41	0.12 (0.11-0.14)	0.34 (0.27-0.44)	0.26 (0.22-0.29)	0.67		
Arginine	0.02 (0.01-0.03)	0.18	0.08	0.06 (0.03-0.04)	0.10 (0.07-0.13)	0.07 (0.05-0.08)	0.06		
	—	—	0.10	0.02 (0.01-0.03)	0.07 (0.07-0.08)	0.08 (0.06-0.11)	0.12		

* Two determinations.

† Three determinations.

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 $126.2 \pm 17.3\%$ (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 $60.0 \pm 21.2\%$ (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 gelatine 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 [³H]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 $11.5 \mu\text{Ci}/\mu\text{mol}$. Incorporation into: ●, non-collagenous protein; ○, hydroxyproline of gelatinized protein. In Expt. 2 the specific radioactivity of proline was $4.4 \mu\text{Ci}/\mu\text{mol}$. □, Incorporation into hydroxyproline of gelatinized protein.

Fig. 2. Incorporation of [¹⁴C]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 $0.23 \mu\text{Ci}/\mu\text{mol}$. 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 3h.

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

Fig. 3. Simultaneous incorporation of [^3H]proline and [^{14}C]glutamic acid as hydroxyproline into granulation tissue at various phases of the development

Slices from sponge-induced granulation tissue, at indicated intervals after the implantation, were incubated in Krebs-Ringer phosphate medium which contained both [^3H]proline and [^{14}C]glutamate as described in detail in the Experimental section. No other amino acids were added. ●, ○, Incorporation into hydroxyproline of gelatinized protein from the precursors [^3H]proline (●) and [^{14}C]glutamic acid (○) respectively; □, amount of hydroxyproline accumulated ($\mu\text{mol/sample}$ of average dry wt. 57.6mg).

Table 3. Effect of the addition of lactate on the incorporation of labelled glutamic acid to proteins of granulation-tissue slices

For details see the text. The radioactivity of added [^{14}C]glutamate was in the range 0.17–2.0 $\mu\text{Ci/ml}$ of medium. The concentration of lactate was 10mm. N.D., not determined.

Concn. of glutamate (mm)	Incorporation into hydroxyproline (c.p.m./100mg dry wt. of slices)		Incorporation into non-collagenous proteins (c.p.m./100mg dry wt. of slices)	
	Lactate not added	Lactate added	Lactate not added	Lactate added
0.72	870	1220 (+40%)	84600	139100 (+65%)
1.42	1170	1680 (+43%)	114200	157000 (+37%)
1.42	870	1120 (+29%)	76700	78300 (+2%)
2.87	1290	1620 (+25%)	N.D.	N.D.

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 pro-collagen 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 extra-cellular 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

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 22.4mm-glucose and 2.87mm-proline (non-radioactive) as described in the Experimental section. [^3H]Proline was added as the precursor and ouabain (strophantigin G, Sandoz, Basle, Switzerland) was added to the experimental samples.

	Concn. of ouabain (mm)	Incorporation into hydroxyproline (c.p.m./100mg dry wt. of slices)		Incorporation into non-collagenous proteins (c.p.m./100mg dry wt. of slices)		Radioactivity ratio of hydroxyproline non-collagenous proteins	
		Control	Experiment	Control	Experiment	Control	Experiment
7-day (proliferating) granulomas	0.1	1270	1180 (−7%)	14770	20540 (+39%)	0.086	0.056
	0.1	5180	3380 (−35%)	90600	65700 (−27%)	0.057	0.051
	1.0	1270	190 (−85%)	14770	4790 (−27%)	0.086	0.038
21–32-day (mature) granulomas	0.1	8420	5380 (−36%)	98500	93700 (−5%)	0.085	0.058
	0.1	22890	15920 (−30%)	288500	269200 (−7%)	0.079	0.059
	1.0	9630	3970 (−59%)	—	—	—	—

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.

	[Na^+] (mm)	[K^+] (mm)	Incorporation into hydroxyproline (c.p.m./100mg dry wt. of slices)	Incorporation into non-collagenous proteins (c.p.m./100mg dry wt. of slices)	Radioactivity ratio of hydroxyproline non-collagenous proteins
7-day (proliferating) granulomas	125	5	3459	41400	0.083
	0	125	643	12600	0.051
	125	0	439	21500	0.021
18–25-day (mature) granulomas	119	6	16670	309000	0.054
	120	5	26400	289000	0.091
	120	5	20900	266000	0.079
	120	5	13170	243000	0.054
	121	4	18400	218000	0.084
	120	10	16840	285000	0.059
	124	1	12280	226000	0.054
	0	120	5850	204000	0.029
	0	125	1353	75000	0.018
	0	125	7230	189000	0.038
	0	5	6210	172000	0.036
	120	0	7490	273000	0.027
	125	0	4220	145000	0.029

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 0.16 mM, according to Christensen & Cullen (1968) or 0.11 mM (Bethel *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 0.15 mM. 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 2 h 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 & Sriver, 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), Eavenson & 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; Kershenovich *et al.*, 1970). The increased formation of proline after the shift in the redox balance resulting from the catabolism of ethanol (Häkkinen & 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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