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Effect of L-Azetidine-2-carboxylic Acid on Glycosylations of Collagen in Chick-Embryo Tendon Cells

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The glycosylations of hydroxylysine during collagen biosynthesis in isolated chick-embryo tendon cells were studied by using pulse–chase labelling experiments with [^{14}C]-lysine. The hydroxylation of lysine and the glycosylations of hydroxylysine continued after a 5 min pulse label for up to about 10 min during the chase period. These data differ from those obtained previously in isolated chick-embryo cartilage cells, in which, after a similar 5 min pulse label, these reactions continued during the chase period for up to about 20 min. The collagen synthesized by the isolated chick-embryo tendon cells differed markedly from the type I collagen of adult tissues in its degree of hydroxylation of lysine residues and glycosylations of hydroxylysine residues. When the isolated tendon cells were incubated in the presence of L-azetidine-2-carboxylic acid, the degree of glycosylations of hydroxylysine during the first 10 min of the chase period was identical with that in cells incubated without this compound. However, the glycosylations continued in the presence of azetidine-2-carboxylic acid for at least 60 min, whereas no additional glycosylations took place in the control cells after the 10 min time-point. As a consequence, the collagen synthesized in the presence of this compound contained more carbohydrate than did the collagen synthesized by the control cells. Additional experiments indicated that azetidine-2-carboxylic acid did not increase the collagen glycosyltransferase activities in the tendon cells or the rate of glycosylation reactions when added directly to the enzyme incubation mixture. Control experiments with colchicine indicated that the delay in the rate of collagen secretion, which was observed in the presence of azetidine-2-carboxylic acid, did not in itself affect the degree of glycosylations of collagen. The results thus suggest that the increased glycosylations were due to inhibition of the collagen triple-helix formation, which is known to occur in the presence of azetidine-2-carboxylic acid.

The hydroxylysine, galactosylhydroxylysine and glucosylgalactosylhydroxylysine of collagen are synthesized as post-translational modifications of the procollagen polypeptide chains catalysed by the enzymes lysine hydroxylase, collagen galactosyltransferase and collagen glucosyltransferase (for reviews on collagen biosynthesis see Bornstein, 1974; Martin *et al.*, 1975; Prockop *et al.*, 1976; Kivirikko & Risteli, 1976). All these reactions are initiated while the polypeptide chains are growing on the ribosomes, and are continued after the release of completed polypeptide chains from the ribosomes into the cisternae of the endoplasmic reticulum (Uitto & Prockop, 1974*a*; Harwood *et al.*, 1975*a*,*b*; Brownell & Veis, 1975; Oikarinen *et al.*, 1976).

Studies on the properties of partially purified lysine hydroxylase (Kivirikko *et al.*, 1973; Ryhänen & Kivirikko, 1974*b*), collagen galactosyltransferase (Risteli *et al.*, 1976) and collagen glucosyltransferase (Myllylä *et al.*, 1975*b*) have indicated that the conformation of the polypeptide substrate has a marked effect on the reactions catalysed by these enzymes *in vitro*. Although the enzymes readily catalyse the reactions with denatured collagen polypeptide chains, they do not catalyse reactions with triple-helical native molecules. Recent data on the time-course of the hydroxylation of lysine residues and glycosylation of hydroxylysine residues, in isolated chick-embryo cartilage cells, were consistent with the suggestion that these reactions are not continued after the formation of the triple helix, even in intact cells (Oikarinen *et al.*, 1976). Certain other experiments also suggest the inhibition of the hydroxylations of proline and lysine residues by the triple-helical conformation of the substrate in intact cells (Jimenez *et al.*, 1974; Uitto & Prockop, 1974*b*).

In the present work, the effect of substrate conformation on the glycosylation reactions in collagen biosynthesis was studied further by incubating isolated chick-embryo tendon cells with L-azetidine-2-carboxylic acid. This compound acts as an analogue of proline and becomes incorporated into the procollagen polypeptide chains. Experiments with several proline analogues have demonstrated that

procollagen polypeptide chains containing the analogue do not form triple-helical molecules, and it has also been demonstrated that the rate of secretion of such chains from the cells is decreased (see Uitto & Prockop, 1974c; Jimenez & Rosenbloom, 1974; Uitto *et al.*, 1975; Prockop *et al.*, 1976). Control experiments were carried out by incubating the cells with colchicine. This compound likewise decreases the rate of collagen secretion, but is not known to affect the rate of triple-helix formation (Dehm & Prockop, 1972; Diegelmann & Peterkofsky, 1972; Ehrlich *et al.*, 1974; Olsen & Prockop, 1974; Harwood *et al.*, 1976).

Experimental

Materials

L-Azetidine-2-carboxylic acid was purchased from Calbiochem Ltd. (London W.1, U.K.), [^{14}C]lysine from New England Nuclear Corp. (Boston, MA, U.S.A.) and colchicine from Merck (Darmstadt, Germany). Fertilized eggs of white Leghorn chickens were obtained from Siipikarjanhoitajien liitto r.y. (Hämeenlinna, Finland) and were incubated in a moist atmosphere at 37°C until used.

Isolation and incubation of matrix-free cells from tendon

Cells were isolated from leg tendons of 17-day chick embryos by controlled digestion with trypsin (Gibco Corp., Grand Island, NY, U.S.A.) and purified bacterial collagenase (Sigma Chemical Co., Kingston-upon-Thames, U.K.) as described previously (Dehm & Prockop, 1971, 1972). The cells were filtered through lens paper and washed three times with 5ml of a modified Krebs medium (Dehm & Prockop, 1971) containing 10% (w/v) foetal calf serum (Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.). They were then resuspended in the modified Krebs medium for the incubations. In most experiments, 8×10^6–16×10^6 cells were obtained from the tendons of a single embryo. The cells were incubated in a modified Krebs medium containing 10% (w/v) foetal calf serum, with moderate shaking at 37°C (Dehm & Prockop, 1971, 1972). [^{14}C]Lysine was added as indicated in various experiments and, after the incorporation periods indicated, 2.0ml portions of the incubation system (cells plus medium) were quickly pipetted into test tubes containing 0.1 vol. of modified Krebs medium with sufficient cycloheximide and $\alpha\alpha$-bipyridyl to provide a final concentration of 100μg of cycloheximide and 1μmol of $\alpha\alpha$-bipyridyl/ml (Dehm & Prockop, 1971). The samples were centrifuged at 1200*g* for 10min at room temperature (23°C), in order to separate the medium from the cells, and the cell pellet was washed with 1.5ml of modified Krebs medium containing 100μg of cycloheximide and 1μmol of $\alpha\alpha$-bipyridyl/ml. The sample was then re-centrifuged and the wash solution was discarded.

Assays

After incubation with [^{14}C]lysine, the medium and cell fractions were dialysed against running tap water for 24–48h. The samples were evaporated on a steam bath and hydrolysed with 2.0ml of 2M-NaOH in sealed disposable glass ampoules at 105°C for 24h. After hydrolysis the samples were handled as described previously (Myllylä *et al.*, 1975*a*; Oikarinen *et al.*, 1976). A small portion was taken for the assay of total radioactivity in the sample and the rest was assayed in the amino acid analyser.

The amino acid analysis was carried out in a 63cm column (Jeol 5AH amino acid analyser) as described previously (Askenasi, 1973; Oikarinen *et al.*, 1976). Fractions (about 2ml) were collected every 2min and were dissolved in 5ml of Instagel (Packard Instrument Co., La Grange, IL, U.S.A.), and assayed in the liquid-scintillation counter (see Oikarinen *et al.*, 1976).

Assay of collagen galactosyltransferase and glucosyltransferase activities in the tendon cells

Tendon cells were incubated in a modified Krebs medium either without L-azetidine-2-carboxylic acid or with 200μg of this compound/ml for 60min. The cell and medium fractions were separated by centrifugation at 1200*g* for 10min at 4°C and the cells were washed with the homogenization solution without Triton X-100 and re-centrifuged. The cells (1.5×10^7/ml) were then homogenized in a solution containing 0.1M-glycine, 0.1M-NaCl, 50μM-dithiothreitol, 20mM-Tris/HCl buffer (pH7.5 at 4°C) and 0.1% (w/v) Triton X-100 (Risteli & Kivirikko, 1974) with 60 strokes of a Teflon/glass homogenizer. The homogenates were incubated at 4°C for 60min, and then centrifuged at 15000*g* for 30min, at 4°C (Risteli & Kivirikko, 1974). The protein content of the 15000*g* supernatant was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

In order to assay the galactosyltransferase activity, 20μl (10μg of protein) of the 15000*g* supernatant was incubated with shaking, for 60min at 37°C, in a final volume of 100μl containing 40mg of gelatinized collagen substrate/ml, 30μM-UDP-[^{14}C]galactose [(New England Nuclear Corp.) diluted with the unlabelled compound (Sigma) to a final specific radioactivity of 8.9Ci/mol], 10mM-$MnCl_2$ and 50mM-Tris/HCl buffer, adjusted to pH7.4 at 20°C (Myllylä *et al.*, 1975*a*). The glucosyltransferase activity was assayed in 100μl of a similar mixture, except that 60μM-UDP-[^{14}C]glucose [New England Nuclear, diluted with the unlabelled compound (Sigma) to a final specific radioactivity of 2.9Ci/mol] was used (Myllylä *et al.*, 1975*a*). The products of the reactions

were then assayed as described by Myllylä *et al.* (1975*a*).

All ^{14}C-radioactivity counting was performed in a Wallac liquid-scintillation spectrometer with an efficiency of 80% and a background of 25 c.p.m., by using scintillants reported in the assay procedures (Myllylä *et al.*, 1975*a*; Oikarinen *et al.*, 1976).

Results

Hydroxylation of [^{14}C]lysine and formation of galactosylhydroxy[^{14}C]lysine and glucosylgalactosylhydroxy-[^{14}C]lysine by the tendon cells

Tendon cells (1.5×10^8) were pulsed with 50 μCi of [^{14}C]lysine for 5 min, and the pulse was stopped by adding 3 mg of unlabelled lysine. Small samples were taken after the incubation times indicated, and at each time-point total non-diffusible radioactivity and hydroxy[^{14}C]lysine and its glycosides were assayed. The total radioactivity did not increase significantly during the chase period (Fig. 1*a*). The hydroxylation of [^{14}C]lysine increased during the chase period for about 10 min, and was relatively constant thereafter. The galactosylation and glucosylation of hydroxy-[^{14}C]lysine increased likewise (Fig. 1*b*).

Effect of azetidine-2-carboxylic acid on the incorporation of [^{14}C]lysine

Tendon cells (2.4×10^8) were pulsed with 40 μCi of [^{14}C]lysine, with or without azetidine-2-carboxylic acid for 5 min, and the pulse was stopped by adding 3 mg of unlabelled lysine. The samples were then handled as described above. The total incorporation of [^{14}C]lysine decreased by about 20% with 200 μg of azetidine-2-carboxylic acid/ml, and after a chase period of 120 min most of the radioactivity was found in the cellular fraction, whereas in the control cells most of the radioactivity was in the medium at this time-point (Fig. 2).

Effect of azetidine-2-carboxylic acid on the glycosylations of hydroxyl[^{14}C]lysine

The hydroxylation of lysine and the glycosylations of hydroxylysine were studied in the experiment described in the preceding paragraph, by assaying the radioactivity in hydroxylysine, galactosylhydroxylysine and glucosylgalactosylhydroxylysine. The values found at each time-point for the total system (cells plus medium) are shown in Fig. 3. The addition of azetidine-2-carboxylic acid did not significantly affect the relative amount of hydroxylysine expressed

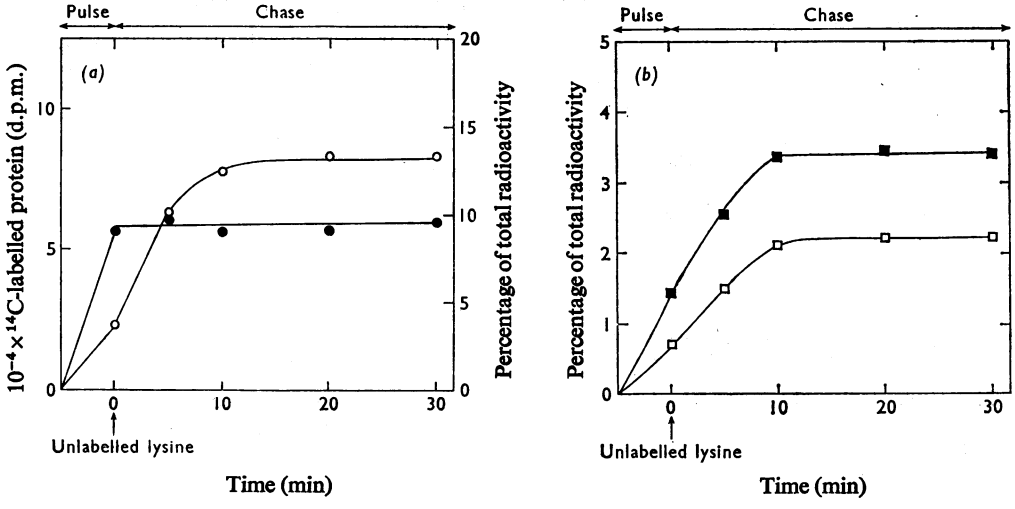

Fig. 1. *Incorporation and hydroxylation of [^{14}C]lysine and glycosylations of hydroxy[^{14}C]lysine when studied by a pulse–chase experiment in the tendon cells*

Tendon cells (1.5×10^8) were incubated in 12 ml of a modified Krebs medium containing 10% foetal calf serum as described in the Experimental section. The cells were pulsed with 50 μCi of [^{14}C]lysine for 5 min, and the pulse was stopped by adding 1 ml of a modified Krebs medium containing 3 mg of unlabelled lysine. At each time-point, 2 ml of the incubation system (cells plus medium) was taken and analysed for total non-diffusible radioactivity, and for substituted and non-substituted hydroxy[^{14}C]lysine as described in the Experimental section. (*a*) Total non-diffusible radioactivity in the cells plus medium (●); total non-diffusible hydroxy[^{14}C]lysine in the cells plus medium, given as percentage of total non-diffusible radioactivity (○). (*b*) Total non-diffusible galactosylhydroxy[^{14}C]lysine in the cells plus medium, given as the sum of galactosylhydroxy[^{14}C]lysine and glucosylgalactosylhydroxy[^{14}C]lysine and expressed as percentage of total radioactivity (■); total non-diffusible glucosylgalactosylhydroxy[^{14}C]lysine in the cells plus medium, expressed as percentage of total radioactivity (□).

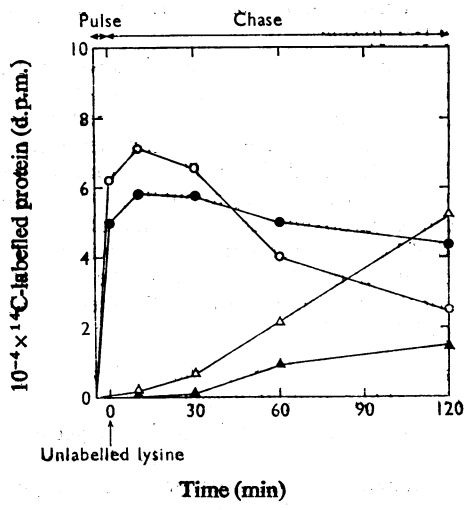

Fig. 2. *Effect of azetidine-2-carboxylic acid on the incorporation of [^{14}C]lysine by the tendon cells*

Tendon cells (2.4×10^8) were incubated in 12ml of a modified Krebs medium containing 10% foetal calf serum for 5min, with or without the addition of 200μg of azetidine-2-carboxylic acid/ml. The cells were then pulsed with 40μCi of [^{14}C]lysine for 5min, and the pulse was stopped by adding 1ml of a modified Krebs medium containing 3mg of unlabelled lysine. At each time-point, 2.0ml portions of the incubation system (cells plus medium) were taken and analysed for total non-diffusible radioactivity, and for substituted and non-substituted hydroxy[^{14}C]lysine in the cells and medium, as described in the Experimental section. ○, △, Control incubation; ●, ▲, incubation with azetidine-2-carboxylic acid. Total non-diffusible radioactivity in the medium (△, ▲); total non-diffusible radioactivity in the cells (○, ●).

as a percentage of total radioactivity (Fig. 3*a*). The glycosylations of hydroxylysine occurred similarly in the presence or the absence of azetidine-2-carboxylic acid, for the first 10min during the chase period, but little further increase in the glycosylations was found in the control cells after this time-point, whereas the reactions continued in the presence of azetidine-2-carboxylic acid for at least 60min (Fig. 3*b*).

Since the ratio of radioactivity in hydroxylysine to total radioactivity may be affected either by the degree of hydroxylation of lysine residues in collagen, or by the relative amount of collagen synthesis compared with total protein synthesis, an attempt was made to estimate the degree of hydroxylation of lysine residues in the collagen itself. A sample of the radioactive protein synthesized either by the control cells (54×10^3 d.p.m.) or in the presence of azetidine-2-carboxylic acid (64×10^3 d.p.m.) was digested with 0.5mg of highly purified bacterial collagenase (Sigma) for 24h at 37°C, in a solution consisting of 0.02M-NH_4HCO_3 and 0.1mM-$CaCl_2$, pH7.5. Diffusible peptides were then assayed for total radioactivity in hydroxylysine. The results indicated that the ratio of hydroxy[^{14}C]lysine to total radioactivity in diffusible peptides, at 120min during the chase period, was about 0.46 in the protein synthesized by the control cells and about 0.52 in the presence of azetidine-2-carboxylic acid. On the basis of this value, and the degrees of galactosylation of hydroxylysine and glucosylation of galactosylhydroxylysine, it was possible to calculate the values shown in Table 1.

Effect of azetidine-2-carboxylic acid on the collagen glycosyltransferase activities

Tendon cells (3×10^7) were incubated for 60min

Table 1. *Hydroxylation of lysine and glycosylations of hydroxylysine in the collagen synthesized by isolated chick-embryo tendon cells, in the presence or absence of azetidine-2-carboxylic acid*

The sum of lysine plus hydroxylysine was assumed to be 35 residues per 1000 residues, as reported for collagen synthesized by chick-embryo tendon cells (Berg & Prockop, 1973). The degree of hydroxylation of lysine residues was estimated after collagenase digestion as described in the text, and the degrees of glycosylations of hydroxylysine were calculated from the relative radioactivities in free hydroxylysine, galactosylhydroxylysine and glucosylgalactosylhydroxylysine. The values for citrate-soluble rat skin collagen were taken from the data of Spiro (1969), assuming the value of 35 residues per 1000 residues for the sum of lysine plus hydroxylysine as consistent with several reports on amino acid analysis for type I collagen (Gallop & Paz, 1975).

Amino acid	Citrate-soluble rat skin collagen (residues/1000)	Collagen synthesized by the tendon cells: Control (residues/1000)	Collagen synthesized by the tendon cells: +Azetidine-2-carboxylic acid (residues/1000)
Total hydroxylysine	7.6	16	18
Substituted hydroxylysine	1.3	5.2	9.7
Monosaccharide	0.5	1.8	3.0
Disaccharide	0.8	3.4	6.7
Lysine	27.4	19	17
Hydroxylysine plus lysine	35	35	35

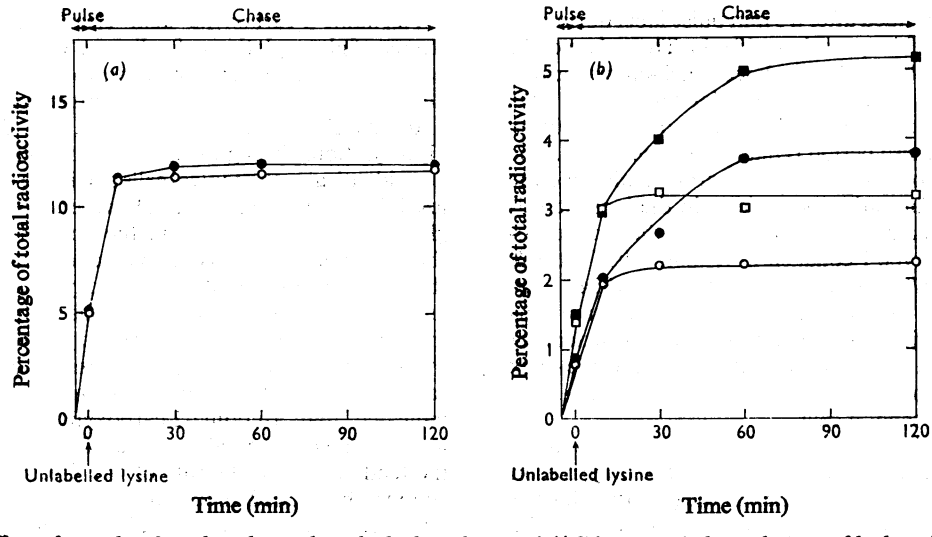

Fig. 3. *Effect of azetidine-2-carboxylic acid on the hydroxylation of [^{14}C]lysine and glycosylations of hydroxy[^{14}C]lysine by the tendon cells*

The experiment is the same as in Fig. 2. ○, □, Control incubation; ●, ■, incubation with azetidine-2-carboxylic acid. (*a*) Total non-diffusible hydroxy[^{14}C]lysine in the cells plus medium given as percentage of total radioactivity (○, ●); (*b*) total non-diffusible galactosylhydroxy[^{14}C]lysine in the cells plus medium given as the sum of galactosylhydroxy[^{14}C]lysine and glucosylgalactosylhydroxy[^{14}C]lysine, and expressed as percentage of total radioactivity (□, ■); total non-diffusible glucosylgalactosylhydroxy[^{14}C]lysine in the cells plus medium, expressed as percentage of total radioactivity (○, ●).

Table 2. *Effect of azetidine-2-carboxylic acid on the collagen galactosyltransferase and collagen glucosyltransferase activities in the 15000g supernatant of tendon cell homogenates*

Tendon cells (3×10^7) were incubated in 4ml of a modified Krebs medium containing 10% (w/v) foetal calf serum, without azetidine-2-carboxylic acid or with 200μg of this compound/ml, for 60min. The activities of collagen galactosyltransferase and collagen glucosyltransferase were assayed in the 15000*g* supernatant as described in the Experimental section. Values are the means of duplicate samples from one experiment.

Cells	Galactosyltransferase activity (d.p.m./100μg of supernatant protein)	Glucosyltransferase activity (d.p.m./100μg of supernatant protein)
Control	3040	3580
Azetidine	3100	3330

with or without azetidine-2-carboxylic acid, and the galactosyltransferase and glucosyltransferase activities were assayed as described in the Experimental section. Azetidine-2-carboxylic acid had no effect on the collagen glycosyltransferase activities of cells under these conditions (Table 2). Additional experiments indicated that this compound also had no effect on collagen glycosyltransferase activities, when added in a concentration of 200μg/ml directly to the enzyme incubation mixture.

Effect of colchicine on the glycosylations of hydroxy-[^{14}C]lysine

Tendon cells (3×10^7) were preincubated for 20min with 1μM-colchicine or without this compound, and were then pulse-labelled for 10min with 4μCi of [^{14}C]lysine. After a chase period of 120min the cells and medium were separated as described in the Experimental section. At this time-point only about 16% of the total radioactivity was in the cells in the absence of colchicine, whereas in the presence of this compound about 48% of the total radioactivity was in the cells. The degree of total galactosylation of hydroxylysine (galactosylhydroxylysine plus glucosylgalactosylhydroxylysine) expressed as the percentage of total hydroxy[^{14}C]lysine was 32.2% in the control cells and 31.8% in the cells incubated with colchicine. The corresponding degree of glucosylation of hydroxylysine was 24.0 and 23.2%.

Discussion

The present results indicate that in isolated chick-embryo tendon cells the hydroxylation of lysine residues and the glycosylations of hydroxylysine

residues continued after a 5 min pulse label during the chase period for up to about 10 min. These data differ from those obtained in isolated chick-embryo cartilage cells, in which after a similar 5 min pulse label these reactions continued for up to about 20 min during the chase period (Oikarinen *et al.*, 1976). The disulphide bonding between the pro-α chains and the triple-helix formation occur later in the biosynthesis of type II collagen in the cartilage cells than in the biosynthesis of type I collagen in tendon cells (Uitto & Prockop, 1973, 1974*d*; Schofield *et al.*, 1974; Harwood *et al.*, 1975*a*), and the present data are thus consistent with the suggestion that the extent of glycosylation reactions is limited by the triple-helix formation both in cartilage cells (Oikarinen *et al.*, 1976) and in tendon cells.

However, it seems likely that the difference in the rate of triple-helix formation is not the only reason for the difference in the extent of the glycosylations of collagen between these cell types. About 65% of the hydroxylysine residues were glycosylated in the collagen secreted by the isolated cartilage cells (Oikarinen *et al.*, 1976), whereas only about 32% were glycosylated in the collagen secreted by the tendon cells in the present study. The difference is clearly larger than the extent of additional glycosylations which took place between 10 and 20 min of the chase period in the cartilage cells (Oikarinen *et al.*, 1976). It has been reported that the activities of the two collagen glycosyltransferases are about 35–40% higher in the cartilage cells than in the tendon cells (Harwood *et al.*, 1975*b*), and it thus seems likely that this difference contributed to the difference in the extent of glycosylations of the two collagens.

The present results indicate that the collagen synthesized by isolated chick-embryo tendon cells differs markedly from the type I collagen isolated from adult tissues in its degree of hydroxylation of lysine residues and glycosylations of hydroxylysine residues. A similar high degree of hydroxylation of lysine residues, in the collagen synthesized by isolated chick-embryo tendon cells, was previously reported for highly purified procollagen from which the *N*- and *C*-terminal extensions had been removed by digestion with proteolytic enzymes (Berg & Prockop, 1973). A high degree of hydroxylation of lysine residues seems to be a common feature for type I collagens isolated from various embryonic tissues (Barnes *et al.*, 1974), and the degree of glycosylations of hydroxylysine residues has also been reported to vary with age (Murai *et al.*, 1975). The reasons for these changes are not known in detail, but a very high lysine hydroxylase activity has been found in foetal human skin compared with adult human skin (Anttinen *et al.*, 1973), and considerable differences were reported in lysine hydroxylase activity in chick embryos between various tissues and in the same tissue as a function of age (Ryhänen & Kivirikko, 1974*a*).

Azetidine-2-carboxylic acid clearly affected the degree of glycosylations of collagen. When the values were expressed either in relation to the total radioactivity (Fig. 3*b*) or as a percentage of the total hydroxylysine (results not shown), the degrees of glycosylations during the first 10 min of chase period were identical in the control cells and in the presence of azetidine-2-carboxylic acid. However, no additional glycosylations took place in the control cells after this time-point, whereas these reactions continued in the presence of azetidine-2-carboxylic acid for at least 1 h. As a consequence, the collagen synthesized in the presence of azetidine-2-carboxylic acid differed from that synthesized by the control cells in its carbohydrate content (Table 1). Control experiments indicated that azetidine-2-carboxylic acid did not increase the collagen glycosyltransferase activities in the tendon cells or the rate of the glycosylation reactions when added directly to the enzyme incubation mixture. It was further shown, in control experiments with colchicine, that a delay in the rate of collagen secretion itself did not affect the degree of glycosylations of collagen. It therefore seems that the increased glycosylations were due to an inhibition of triple-helix formation by azetidine-2-carboxylic acid (see the introduction). The results support the previous suggestion that the triple-helix formation prevents additional glycosylations of hydroxylysine residues in the biosynthesis of collagen (Myllylä *et al.*, 1975*b*; Risteli *et al.*, 1976; Oikarinen *et al.*, 1976).

The extent of the hydroxylation of lysine residues has also been found to be limited by the triple-helix formation (Kivirikko *et al.*, 1973; Ryhänen & Kivirikko, 1974*b*; Uitto & Prockop, 1974*b*). However, no definite increase was found in the extent of the hydroxylation of lysine residues in the presence of azetidine-2-carboxylic acid, although a slight increasing tendency was observed (Table 1). This finding is probably explained by the high degree of hydroxylation of lysine residues in the collagen synthesized by the tendon cells in the absence of azetidine-2-carboxylic acid. In the presence of this compound more than half of the lysine residues were hydroxylated. Lysine hydroxylase presumably hydroxylates lysine residues only in the 'Y positions' of repeating -X-Y-Gly- sequences of collagen (Kivirikko *et al.*, 1972, 1973), and thus the theoretical maximum value is determined by the distribution of lysine residues between the 'X' and 'Y positions'. Further, even the lysine residues which are present in the 'Y positions' of certain sequences can only become poorly hydroxylated (Kivirikko *et al.*, 1972, 1973). It therefore seems possible that the degree of hydroxylation noted in the absence of azetidine-2-carboxylic acid represents almost the maximum value for type I collagen, which would explain the failure to find a larger increase in the presence of azetidine-2-carboxylic acid.

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