Inhibition of prolyl hydroxylation and procollagen processing in chick-embryo calvaria by a derivative of pyridine-2,4-dicarboxylate

Characterization of the diethyl ester as a proinhibitor*

Georg TSCHANK,[†] Dietrich G. BROCKS,[‡] Klaus ENGELBART,[‡] Jürgen MOHR,[§] Ekkehard BAADER,[‡] Volkmar GÜNZLER,^{‡¶} and Hartmut M. HANAUSKE-ABEL^{||¶}

[†]Institute of Toxicology and [§]Institute of Organic Chemistry, Johannes Gutenberg University, Mainz, Federal Republic of Germany, [‡]Hoechst Aktiengesellschaft, Frankfurt/Main, Federal Republic of Germany, and ^{||}Department of Pediatrics, Rush Medical Center, Chicago, IL 60612, U.S.A.

The biochemical and morphological consequences of procollagen prolyl 4-hydroxylase inhibition by pyridine-2,4-dicarboxylic acid (2,4-PDCA) and its diethyl ester (diethyl-2,4-PDC) were studied in chick-embryo calvaria, which predominantly synthesize type I collagen. Half-maximal inhibition of tissue hydroxyproline formation required 650 μM -2,4-PDCA, whereas the K_i with respect to chicken prolyl 4-hydroxylase *in vitro* was 2 μM . In contrast, half-maximal inhibition was caused by 10 μM -diethyl-2,4-PDC in the intact calvaria, although chicken prolyl 4-hydroxylase *in vitro* was not inhibited even at 1 mM. The collagenous material produced in the presence of diethyl-2,4-PDC showed an altered 'melting' profile and a lowering of the transition temperature by 10 °C, indicating misalignment and thermal instability of its triple-helical structure. Amount and electrophoretic mobility of procollagen type I chains were increased in a dose-dependent manner. The amounts of partially processed species and α -chains were decreased, without change in mobility. This marked effect on procollagen–collagen conversion in the intact calvaria suggests that the underhydroxylated collagenous material generated in the presence of diethyl-2,4-PDC is resistant to or acts as endogenous secondary inhibitor of type I procollagen N-proteinase. Electron microscopy of treated calvaria cells showed dilated rough endoplasmic reticulum and numerous phagolysosomes, indicating intracellular retention and lysosomal degradation of the newly synthesized underhydroxylated collagenous material. In summary, these results identify 2,4-PDCA and diethyl-2,4-PDC as the first prolyl 4-hydroxylase-directed inhibitor/proinhibitor pair that affects intra- and extra-cellular events during collagen formation.

INTRODUCTION

Recently, the structural isomers of pyridinedicarboxylate have received much interest as inhibitors of collagen hydroxylation [1–5]. By acting as 2-oxoglutarate analogues, they interfere with the activity of prolyl 4-hydroxylase (EC 1.14.11.2), prolyl 3-hydroxylase (EC 1.14.11.7) and lysyl hydroxylase (EC 1.14.11.4) [6,7]. Structure–activity studies revealed that the pyridine-2-carboxylate moiety, by imparting metal-ion-binding properties [8–10], selects for the metal-ion-dependent 2-oxoglutarate-binding site of the collagen hydroxylase while disfavours interaction with 2-oxoglutarate dehydrogenase (EC 1.2.4.2) [7] of the citric acid cycle, an enzyme that shows metal-ion-independent 2-oxoglutarate binding [11].

Prolyl 4-hydroxylase activity is crucial for conformation and secretion of interstitial collagen [12]. Pyridine-2,4-dicarboxylic acid (2,4-PDCA) is a less effective inhibitor of purified chicken prolyl 4-hydroxylase than the 2,5-isomer; the K_i values are 2.1 and 0.8 μM respectively [6]. Surprisingly, 2,4-PDCA was distinctly more potent than 2,5-PDCA in cell culture [13]. Both compounds did not compromise viability or general protein synthesis and did not produce degenerative ultrastructural changes even in millimolar concentrations. Such high concentrations, required to suppress cellular hydroxyprolyl formation, were attributed primarily to the minimal lipophilicity of these dianionic molecules

[13]. Prolyl 4-hydroxylase is shielded by two lipid barriers, the cytoplasmic and the rough-surfaced endoplasmic membranes [12]. To dissect the individual contributions of each membrane barrier, the inhibitory effects of both isomers were studied in a microsomal prolyl 4-hydroxylase system isolated from chick-embryo calvaria [14]. The endoplasmic membrane did not restrict access of 2,4-PDCA to the enzyme, but impeded access of 2,5-PDCA. This exclusion of the 2,5-isomer explained the discrepancy between the kinetic [6] and the biosynthetic [13] determination of the two isomers' potency. It also led to the suggestion that a 2,4-PDCA-specific transport system exists in the endoplasmic membrane [14]. The study identified the cytoplasmic membrane as the major barrier for the access of the polar 2,4-PDCA to prolyl 4-hydroxylase.

To overcome this barrier, we have synthesized the diethyl ester (diethyl-2,4-PDC) as a lipophilic derivative. We anticipated that this compound might act as a transport form of the lipophobic 2,4-PDCA, reconstituting it via intracellular hydrolysis and thus facilitating its access to the intracisternal target enzyme. We now report that diethyl-2,4-PDC, which does not inhibit purified or microsomal chicken prolyl 4-hydroxylase [14], effectively suppresses not only procollagen hydroxylation and secretion, but also the procollagen–collagen conversion in intact chick-embryo calvaria.

Abbreviations used: pro $\alpha 1(I)$ and pro $\alpha 2(I)$, $\alpha 1$ - and $\alpha 2$ -chains respectively of procollagen type I; pC $\alpha 1(I)$, molecules of the $\alpha 1$ -chain of procollagen type I lacking the N-terminal extension peptide; pN $\alpha 1(I)$, molecules of the $\alpha 1$ -chain of procollagen type I lacking the C-terminal extension peptide; 2,4-PDCA and 2,5-PDCA, pyridine-2,4-dicarboxylic acid and pyridine-2,5-dicarboxylic acid respectively; diethyl-2,4-PDC, diethyl pyridine-2,4-dicarboxylate; IC_{50} , concentration of inhibitor yielding 50% inhibition.

* This paper is dedicated to Professor Hansgeorg Gareis on the occasion of his 60th birthday.

¶ Correspondence should be addressed either to Dr. V. Günzler, at Hoechst A.G. H 821, Postfach 80 03 20, D-6230 Frankfurt 80, Federal Republic of Germany, or to Dr. H. M. Hanauske-Abel, at Department of Pediatrics, Rush Medical Center, 1653 West Congress Parkway, Chicago, IL 60612, U.S.A.

MATERIALS AND METHODS

Reagents

2,4-PDCA was purchased from Aldrich, Steinheim, Germany. Trypsin (195 units/mg) and chymotrypsin (67 units/mg) were obtained from Paesel, Frankfurt, Germany. Bacterial collagenase (CLSPA; Worthington) was obtained through Millipore, Neu-Isenburg, Germany. Dulbecco's minimal essential medium and BSA were purchased from Serva, Heidelberg, Germany. L-[U-¹⁴C]Proline and L-[4-³H]proline were obtained from NEN, Dreieich, Germany. L-[U-¹⁴C]Leucine was purchased from Amersham Buchler, Braunschweig, Germany. All other compounds were obtained from Sigma, Munich, Germany, or E. Merck, Darmstadt, Germany.

Synthesis of diethyl-2,4-PDC

The synthesis of diethyl-2,4-PDC followed the published procedure [15]. An 18 g yield of product was obtained from 20 g of 2,4-PDCA (67% yield; m.p. 30–31 °C) [Found: C, 59.12; H, 6.03; N, 6.03. Calc. for C₁₁H₁₃NO₄ (223.23): C, 59.19; H, 5.87; N, 6.27%].

Chick-embryo calvaria cultures and metabolic labelling

Calvaria were removed from 15-day chick embryos and washed for 3 min in Dulbecco's minimal essential medium at 37 °C. They were then transferred into Pyrex tubes (eight calvaria/tube) containing 3 ml of medium supplemented with 2 mM-glutamine, 6 µCi of [U-¹⁴C]proline and various concentrations of 2,4-PDCA or diethyl-2,4-PDC. The samples were incubated for 1.5–6 h at 37 °C to monitor for time-dependent alterations of total proline incorporation and hydroxyproline synthesis. The experiment was terminated by placing the tubes in ice and separation of calvaria from the culture medium. The calvaria were washed once with 3 ml of fresh medium, which was then pooled with the incubation medium. BSA and phenylmethanesulphonyl fluoride were added (final concentrations 1 mg/ml and 6 µg/ml respectively). The calvaria were extracted for 16 h with 25 ml of 0.5 M-acetic acid.

All samples were subsequently dialysed extensively against 0.5 M-acetic acid at 4 °C. Samples of equal volume were withdrawn for SDS/PAGE and the collagen conformation studies described below. The remaining material was freeze-dried, resuspended in 2 ml of 6 M-HCl and hydrolysed at 105 °C for 24 h. After evaporation of the acid, the samples were dissolved in 2 ml of water and used for the determination of proline incorporation and the hydroxyproline synthesis according to a standard procedure [16].

Alternatively, calvaria were incubated with medium containing L-[U-¹⁴C]leucine (2 µCi/ml) as a marker of total protein synthesis. The culture conditions were identical with the procedure described above, and the radioactivity was measured directly after the hydrolysis step.

The extent of prolyl hydroxylation in collagenous peptides synthesized *de novo* was determined by dual-labelling the calvaria with [4-³H]proline (10 µCi/ml) and [U-¹⁴C]proline (2 µCi/ml) for 3 h under the conditions described above and measuring the changes in the isotope ratio of a collagenase digest as described before [17]. The percentage of proline hydroxylation was calculated by using the formula:

$$1 - \frac{(^3\text{H}/^{14}\text{C} \text{ ratio in collagenase digest} / \text{initial } ^3\text{H}/^{14}\text{C} \text{ ratio})}{\text{Fraction of total tritium in 4-trans position of proline}} \times 100$$

Collagen conformation studies

To determine the hydroxyprolyl-dependent stability of the collagen triple helix, which imparts resistance to proteolysis,

the temperature-dependent susceptibility of the synthesized collagenous material to tryptic digestion [18] was studied. Samples representing 5000 d.p.m. of dialysed supernatant or calvaria extract were incubated in 40 M-NaCl/0.1 M-Tris/HCl buffer, pH 7.4, for 15 min at temperatures between 25 and 45 °C (total volume 800 µl). After quenching to 0 °C, 200 µl of a mixture containing 1 mg of both trypsin and chymotrypsin per ml was added. The samples were digested for 15 min at room temperature. Then 100 µl of BSA solution (1 mg/ml) was added, and the proteinase-resistant radioactivity, consisting of triple-helical collagen, was precipitated with 100 µl of 100% (w/v) trichloroacetic acid. The sample was transferred in total to a filter paper of 2.5 cm diameter (Schleicher und Schüll OE 67). The digested material was removed by repeated washing with cold 5% trichloroacetic acid and methanol. The filters were dried, then transferred to 20 ml liquid-scintillation vials, and the radioactivity of the undigested materials was determined.

SDS/PAGE

Freeze-dried samples were reconstituted with 10 mM-Tris/HCl buffer, pH 8, containing 1 mM-EDTA, 1% SDS and 5% (v/v) 2-mercaptoethanol and denatured at 95 °C for 10 min. A protein sample containing 20000–50000 d.p.m. of [¹⁴C]proline was run on a 1.5 mm × 150 mm linear-gradient SDS/PAGE gel (5–10%) [19]. After fixation in methanol/acetic acid/water (3:1:6, by vol.) the gels were processed for autoradiography according to the Bonner–Laskey method [20]. The dried films were exposed to Kodak X-Omat films at –70 °C. The individual species were identified by the use of unlabelled marker proteins and on the basis of a 2:1 ratio of type I component α-chains in agreement with previously published data [21–23]. Densitometric quantification was kindly performed by Dr. G. Berscheid, Hoechst A.G., with an LKB UltroScan XL instrument.

Electron microscopy

Calvaria from 12-day chick embryos were incubated in the presence or in the absence of 25 µM-diethyl-2,4-PDC for 4.5 h as described above. After fixation in 2.5% (v/v) glutaraldehyde buffered in 0.1 M-phosphate, the calvaria were cut into pieces of approx. 1 mm × 1 mm, and the fixation was continued in 1% osmic acid in the same buffer. The samples were then embedded in Epon resin. Ultra-thin sections were contrasted with uranyl acetate and lead citrate.

Assay for prolyl 4-hydroxylase and lysyl oxidase

Standard procedures were used in the purification of prolyl 4-hydroxylase from chick embryos [24,25], preparation of [4-³H]-proline-labelled protocollagen substrate and the assay of enzyme activity [26]. Lysyl oxidase activity was measured with a human recombinant tropoelastin substrate labelled with [4,5-³H]lysine [27]. In both the prolyl 4-hydroxylase and the lysyl oxidase assays, the release of tritiated water from the respective protein substrates was determined.

Partition ratios

Solutions (1 mM) of 2,4-PDCA or diethyl-2,4-PDC in 50 mM-phosphate buffer, pH 7.4, were added to equal volumes of octanol. After vigorous shaking, the u.v. absorbance (280 nm) of the aqueous phase was determined. The procedure was repeated until no further change of absorbance occurred. The partition ratio (octanol/phosphate buffer) was then calculated according to the formula:

$$\text{Partition ratio} = \frac{A_{(o)} - A_{(n)}}{A_{(n)}}$$

$A_{(o)}$ being the initial absorbance of the aqueous phase and $A_{(n)}$ the final value.

RESULTS

Partition ratios of 2,4-PDCA and diethyl-2,4-PDC

The oil/water partition ratio in the system octan-1-ol/50 mM-phosphate buffer, pH 7.4, was determined to be 0.003 for 2,4-PDCA, indicating that at neutral pH the dianionic species lacks appreciable lipophilicity. In contrast, diethyl-2,4-PDC showed an oil/water partition ratio of 45 under the same experimental conditions, and thus is almost completely dissolved in the lipid phase.

Enzyme assays

Diethyl-2,4-PDC did not inhibit purified chicken prolyl 4-hydroxylase at concentrations up to 1 mM. Lysyl oxidase activity in chick-embryo calvaria and medium, measured after 24 h and 48 h of serum-free incubation in Dulbecco's minimal essential medium with either 2,4-PDCA or diethyl-2,4-PDC at concentrations up to 1 mM, was unaffected (H. M. Hanauske-Abel, D. Bedell-Hogan & H. Kagan, unpublished work).

Fig. 1. Effect of increasing concentrations of diethyl-2,4-PDC on hydroxyproline synthesis in chick-embryo calvaria after metabolic labelling with [^{14}C]proline

The means for eight diethyl-2,4-PDC-treated calvaria incubated for 1.5 h (●), 3 h (○), 4.5 h (▲) and 6 h (△) are shown. The hydroxyproline synthesis in controls, expressed as mean Hyp/(Hyp+Pro) ratio of 16 calvaria, was 0.286, 0.265, 0.324 and 0.318 after 1.5 h, 3 h, 4.5 h and 6 h incubation respectively.

Effects of 2,4-PDCA and diethyl-2,4-PDC on hydroxyproline synthesis and total protein biosynthesis

The dose-response study of 2,4-PDCA revealed an IC_{50} of 650 μM after incubation for 2.5 h. This is of the same order of magnitude as the IC_{50} of the agent in human fibroblasts (2200 μM) [13]. Variation in the duration of the incubation period between 1.5 and 6 h resulted in no significant alteration of the dose-response relation. Diethyl-2,4-PDC produced 50% inhibition of hydroxyproline synthesis at 10–20 μM , and $14.6 \pm 12.4\%$ inhibition at 1 μM (Fig. 1). Correspondingly, the hydroxyproline content of the collagenase-digestible material was decreased in a dose-dependent manner (Table 1).

Total proline incorporation, which was used as a marker of total protein biosynthesis, was not affected in this study (Table 2). In additional experiments, the incorporation of [^{14}C]proline and [^{14}C]leucine remained unchanged by diethyl-2,4-PDC at concentrations up to 0.5 mM and 1 mM respectively.

Biochemical and biophysical consequences of the deficient collagen hydroxylation

To assess the effects of the underhydroxylation, we studied the electrophoretic mobility of the collagenous material synthesized in the presence of diethyl-2,4-PDC and, as an indicator of its conformational state, its susceptibility to proteolytic degradation.

Table 2. Total incorporation of radioactivity in chick-embryo calvaria cultures in the presence of various concentrations of diethyl-2,4-PDC

Chick-embryo calvaria were incubated in the presence of [^{14}C]proline and various concentrations of diethyl-2,4-PDC for 1.5–6 h as described in the Materials and methods section. The total incorporation of radioactivity (proline + hydroxyproline) was measured from a hydrolysed sample of pooled material from eight calvaria in the treated samples, and 16 calvaria in the controls.

Concn. of diethyl-2,4-PDC (μM)	Duration of incubation ...	$10^{-3} \times$ Total incorporation of radioactivity (d.p.m./calvaria)			
		1.5 h	3.0 h	4.5 h	6.0 h
0		94.7	163.9	254.2	232.8
1		104.1	194.6	247.5	252.2
5		—	190.1	265.4	249.2
10		94.1	171.4	279.7	285.1
50		87.5	186.2	201.5	277.0

Table 1. Effects of diethyl-2,4-PDC on prolyl hydroxylation, electrophoretic migration and processing of procollagen type I in chick-embryo calvaria

Chick-embryo calvaria were incubated in the presence of [$4\text{-}^3\text{H}$]proline and [^{14}C]proline and various concentrations of diethyl-2,4-PDC as outlined in the Materials and methods section. The extent of prolyl hydroxylation in newly synthesized acetic acid-extractable material was calculated as described previously [17]. The migration difference refers to the electrophoretic position of the procollagen $\alpha 1$ - and $\alpha 2$ -chains, extracted from calvaria treated with diethyl-2,4-PDC, relative to those extracted from control calvaria. Proportion of pC $\alpha 1$ collagen refers to the percentage amount of this species in the total collagenous material. Abbreviation: N.D., not determined.

Concn. of diethyl-2,4-PDC (μM)	Extent of proline hydroxylation in acetic acid-soluble material (%)		Migration difference (mm)		Proportion of pC $\alpha 1$ collagen (%)
	From calvaria	From medium	Pro $\alpha 1$	Pro $\alpha 2$	
0	37.5	42.6	0.00	0.00	12.3
1	N.D.	N.D.	0.08	0.00	10.2
5	25.4	27.0	−0.04	0.48	4.6
10	18.4	11.3	0.68	1.00	7.0
50	11.3	15.0	1.28	1.32	2.8

Fig. 2. Electrophoretic analysis of acid-soluble procollagen type I produced by chick-embryo calvaria in the presence and in the absence of diethyl-2,4-PDC

Concentrations of inhibitor were 0 μM (lanes 1 and 7), 50 μM (lanes 2 and 3), 10 μM (lane 4), 5 μM (lane 5) and 1 μM (lane 6). Identification of the position of the procollagen chains, the pC species and the collagen α -chains agrees with references [21–23].

Fig. 3. 'Melting' profiles of collagenous material obtained from chick-embryo calvaria and medium of cultures incubated in the presence of [^{14}C]proline without (○) and with (●) 25 μM -diethyl-2,4-PDC

Helix stability was probed by digestion of a 5000 d.p.m. portion of acid-soluble material with trypsin + chymotrypsin as described in the Materials and methods section.

The electrophoretic pattern of the acid-extractable material synthesized *de novo* revealed that the chick-embryo calvaria produced type I collagen. The procollagen α 1- and α 2-chains, the pC intermediates and the α 1- and α 2-chains were shown to be present in the material extracted from control calvaria. Densitometry showed that about 65% of the material consisted of completely processed α -chains (Fig. 2).

The application of diethyl-2,4-PDC resulted in characteristic changes of this electrophoretic pattern (Fig. 2). At 50 μM -diethyl-2,4-PDC the total amount of acid-extractable collagen was dose-dependently decreased to 37% of the control value. The amount of completely processed collagen α -chains was decreased by 17% and 78% at 1 μM and 50 μM respectively. The pC α 1 species was particularly affected (Table 1). In contrast, the proportion of procollagen α 1- and α 2-chains in the total collagenous material was increased from 15% in the controls to 55% at 50 μM . In addition, an increased mobility of these species was found (Table 1). The α -chains and the pC collagen did not show an increased mobility. Similar effects, though not as pronounced, were observed in collagenous material obtained from the culture medium (results not shown).

Relying on the trypsin + chymotrypsin technique [18,28], the midpoint of the thermal transition (T_m) was determined to be

40 $^{\circ}\text{C}$ in the acid-extractable collagenous material from calvaria and 41 $^{\circ}\text{C}$ in material obtained from medium of untreated controls (Fig. 3). Both thermal-transition curves showed a steep profile, indicating the presence of a homogeneous population of fully aligned triple-helical collagen molecules [28,29]. Incubation with 25 μM -diethyl-2,4-PDC for 3 h lowered the T_m of the material synthesized *de novo* to 29 $^{\circ}\text{C}$ both in the calvaria and in the supernatant (Fig. 3). Such a lowering of T_m has previously been shown to reflect significant hydroxyprolyl deficiency [18,28]. Diethyl-2,4-PDC not only shifted the thermal-transition curve to lower temperatures, but also decreased its steepness. Complete helix-to-coil transition occurred in an interval of 5 $^{\circ}\text{C}$ in the controls, but in an interval of 10 $^{\circ}\text{C}$ in the treated samples. Such a change of the thermal-transition curve shape was shown to result from sample heterogeneity due to misaligned and partially renatured collagenous molecules [18,29]. At the physiological temperature of 37 $^{\circ}\text{C}$, all the acid-soluble collagenous material synthesized in the presence of diethyl-2,4-PDC is in a proteinase-susceptible state (Fig. 3).

Morphological consequences of deficient collagen hydroxylation

In order to assess morphological indicators of disturbed collagen synthesis within the cells of the calvarial tissue, we studied their ultrastructural appearance in the absence and in the presence of diethyl-2,4-PDC. In the controls, the rough endoplasmic reticulum, the intracellular site of the post-translational modification of collagen, appeared as an assembly of parallel membranes enclosing a lumen of remarkably uniform width. This lamellar structure almost completely disappeared on incubation with 25 μM -diethyl-2,4-PDC for 4.5 h. This treatment generated a diffuse distribution of rounded individual organelles with diameters up to approx. 1 μm and ribosomes still present at the cytoplasmic surface (Fig. 4a). The parallel-sheet structure of the rough endoplasmic reticulum was found to be continuous with areas of vesicular dilation (Fig. 4b). Exposure to diethyl-2,4-PDC also led to the formation of membrane-bounded vesicular elements containing electron-dense material of diverse morphology (Fig. 4a, arrows). These structurally prominent elements were induced by exposure to diethyl-2,4-PDC, and closely resemble phagolysosomes. Exposure to diethyl-2,4-PDC was not associated with morphologically discernible signs of damage to or death of cells within the tissue of the calvaria. In particular, this lipophilic compound did not lead to interruption of cytoplasmic membrane continuity, did not produce degenerative changes in the cytosol or the nuclei, and did not affect mitochondrial structure.

DISCUSSION

The findings presented here suggest that diethyl-2,4-PDC, a derivative of a prolyl 4-hydroxylase inhibitor that itself does not affect the purified or microsomal chicken enzyme [14], specifically suppresses hydroxyprolyl formation and collagen biosynthesis in chick-embryo calvaria in a dose-dependent manner. A time-dependence reflecting the obligatory formation of an inhibitory molecule from diethyl-2,4-PDC could not be demonstrated under the experimental conditions employed. This may indicate that the required activation of diethyl-2,4-PDC occurs more rapidly than the metabolic labelling of protein used in this study to detect inhibitory effects. As microsomal preparations isolated from chicken bone do not show any diethyl-2,4-PDC-dependent suppression of hydroxyprolyl formation [14], the generation of the inhibiting agent should occur outside the endoplasmic reticulum, but inside the intact calvaria cells. We suggest that 2,4-PDCA, generated in the cytoplasm from the inactive diethyl-2,4-PDC, mediates the observed suppression of collagen

Fig. 4. Morphological appearance of chick-embryo calvaria cells of a culture treated with 25 μ M-diethyl-2,4-PDC

(a) The rough endoplasmic reticulum is dilated because of accumulated collagenous material. Ribosomes are still present. Transition stages between the normal tubular and dilated appearance are observed (arrows). (b) The number of phagolysosomes (arrows) as a morphological correlate of collagen degradation is increased. Other cellular structures, particularly mitochondria, appear to be unaffected. The bar in the lower left-hand corner indicates 0.5 μ m.

hydroxylation. The concept that diethyl-2,4-PDC functions as the bioactivatable transport form for 2,4-PDCA, and that these two compounds represent a proinhibitor/inhibitor pair, is also supported by the remarkably similar concentrations required for half-maximal inhibition of the purified chicken enzyme (2 μ M-

2,4-PDCA) [6], the microsomal enzyme prepared from chicken bone (5 μ M-2,4-PDCA) [14] and the enzyme in cells of intact chick-embryo calvaria (10 μ M-diethyl-2,4-PDC).

Diethyl-2,4-PDC is on a molar basis 65 times more potent than 2,4-PDCA and at least 10 times more potent than the widely

Fig. 5. Activation of the proinhibitor diethyl-2,4-PDC and effects of the inhibitor 2,4-PDCA on collagen biosynthesis in the chick-embryo calvaria system, conceptualized in accord with the findings presented here and the previously published data from different laboratories [1,2,6,13,14,43]

Black arrows, mode of action; hatched arrows, consequences; A, structurally non-specific permeation of the cytoplasmic membrane by the lipophilic proinhibitor; B, structurally specific permeation of the endoplasmic membrane by the hydrophilic inhibitor, facilitated by the 2,4-PDCA-selective-uptake mechanism. The active prolyl 4-hydroxylase inhibitor 2,4-PDCA, itself efficiently excluded from the intracellular space by the cytoplasmic membrane, is enzymically generated in the cytosol from its inactive but lipophilic transport form diethyl-2,4-PDC. 2,4-PDCA then enters the intracisternal compartment via a structure-selective element in the endoplasmic membrane. A physiological ligand for the 2,4-PDCA-selective-uptake mechanism in the endoplasmic membrane has not yet been identified. 2,4-PDCA, attaching to the co-substrate-binding site of prolyl 4-hydroxylase as a non-utilizable 2-oxoglutarate analogue (inset), arrests the enzyme's catalytic cycle before the formation of the ferryl ion, the reactive species effecting prolyl hydroxylation. The suppression of collagen hydroxylation leads to intracisternal accumulation of denatured non-helical collagenous material and produces the globular transformation of the rough endoplasmic reticulum (RER) (right). The intracisternal denatured collagenous material is transferred to the lysosomes for proteolytic degradation. A small proportion may be released. In the pericellular space, it should be resistant to type I procollagen N-proteinase, which requires native substrate conformation [21] and, because of this enzyme's sensitivity to denatured procollagen fragments [39], may locally suppress its activity by acting as a secondary endogenous inhibitor. The N-terminally unprocessed collagen precursors, if triple-helical, interfere with fibril growth [42]. In a tissue containing both diethyl-2,4-PDC-sensitive and -insensitive cells, the latter secreting fully hydroxylated triple-helical procollagen, the defective procollagen N-proteinase activity could lead to the disturbance of fibrillogenesis.

used non-specific inhibitors of cellular collagen hydroxylation, such as metal-ion chelators [13] or proline analogues [30,31]. In contrast with these agents, diethyl-2,4-PDC produced a dissociation of total amino acid incorporation and prolyl hydroxylation. A similarly selective effect has previously been demonstrated only for 2,4-PDCA [13]. Ultrastructurally, the pattern of a generalized dilation of the rough endoplasmic reticulum induced by diethyl-2,4-PDC, with other cellular elements morphologically unaffected, is identical with that de-

scribed for 2,4-PDCA [13]. These experimental findings are compatible with our concept (Fig. 5) that the suppressive effect of diethyl-2,4-PDC on collagen hydroxylation is mediated through 2,4-PDCA, generated by cytoplasmic proinhibitor cleavage within the intact chick-embryo calvaria cells.

Our results with the diethyl ester of 2,4-PDCA differ in this important aspect from those reported for the ethyl ester of another prolyl 4-hydroxylase inhibitor, 3,4-dihydroxybenzoic acid. This agent, with a K_i value of $5 \mu\text{M}$ [33], produced half-

maximal inhibition of cellular hydroxyproline formation at 3000 μM [34], whereas its ethyl ester had the same effect at 270 μM [35]. 3,4-Dihydrobenzoic acid and ethyl 3,4-dihydroxybenzoate show identical efficacy with crude prolyl 4-hydroxylase [35] and identical K_i values with purified prolyl 4-hydroxylase (V. Günzler, unpublished work) by virtue of their common *o*-dihydroxybenzene moiety, the only structural requirement for enzyme inhibition [33]. Thus esterification of 3,4-dihydroxybenzoic acid does not give a compound that meets the definition of a proinhibitor [36]. In contrast, esterification of 2,4-PDCA completely abolishes its suppressive effect on purified and microsomal chicken prolyl 4-hydroxylase [14]. Only in intact cells of chick-embryo calvaria does the ester derivative give enzyme inhibition. Therefore it appears that the reconstitution of any inhibitory effect requires an intracellular but extramicrosomal metabolic event, the proinhibitor-inhibitor conversion. The collagenous material produced in the presence of diethyl-2,4-PDC displayed altered biophysical characteristics, which we interpret to result from underhydroxylation. The thermal-transition curve was shifted to lower temperatures. In the medium the interval required for complete helix-to-coil transition was doubled, indicating sample heterogeneity due to misaligned partially refolded molecules with triple-helical segments of various lengths [18,29]. The electrophoretic mobility of procollagen $\alpha 1$ - and $\alpha 2$ -chains was increased in a dose-dependent manner, but the mobility of the pC species and α -chains remained unchanged (Fig. 2). Similar migration abnormalities are known to result from compromised cellular prolyl hydroxylation, as caused by ascorbate deficiency [37] or iron depletion with 2,2'-bipyridyl [38]. These migration differences coincided with the decrease in hydroxyproline content (Table 1).

Hydroxyproline deficiency strikingly affected procollagen-collagen conversion too. The decrease in hydroxyproline content was associated with a proportional increase in procollagen chains and a corresponding decrease in proteolytically processed molecules, particularly pC species (Table 1). This finding is in agreement with the observation that the pC-collagen-forming enzyme, type I procollagen N-proteinase (EC 3.4.24.14), accepts as substrate only triple-helical, i.e. fully hydroxylated, molecules [21] and is inhibited by non-helical, i.e. underhydroxylated, collagen species [39]. This joint decrease renders an independent inhibition of the extracellular type I procollagen N-proteinase unlikely. The IC_{50} for the suppression of pC $\alpha 1(\text{I})$ is about 10 μM -diethyl-2,4-PDC, making this compound the most effective suppressor of pC collagen formation known at present, 3 times more potent than non-peptide [40] and over 100 times more potent than peptide inhibitors [41] of type I procollagen N-proteinase. The presence of *N*-terminally unprocessed helical collagen precursors in the pericellular space, a consequence of functional deficiency of that enzyme, is known to disrupt the self-organization of biomechanically functional collagen fibrils [42].

The altered ultrastructure of the rough endoplasmic reticulum appears to be a consequence of underhydroxylation. Procollagen secretion is known to be delayed if the hydroxyproline-dependent formation of the triple helix is defective [12], and the intracellularly accumulated non-helical and non-functional material is subsequently degraded in the lysosomal compartment [32]. 2,4-PDCA was shown to cause intracellular retention of underhydroxylated collagenous material, associated with dilation of the rough endoplasmic reticulum and the emergence of immunoreactive type I collagen in secondary lysosomes [13,43]. The electron-microscopic findings reported here for diethyl-2,4-PDC also reveal marked dilation of the rough endoplasmic reticulum and lysosomal prominence, without any evidence for further morphological alterations.

2,4-PDCA lends itself to further modifications that give diverse

proinhibitor molecules. Such latent inhibitors [36] may allow tissue direction of the suppressive effect on collagen formation: exclusively that cell population which converts an inactive proinhibitor into the active inhibitor is a target for fibrosuppression. Ultimately such 2,4-PDCA-derived fibrosuppressive agents with tissue-selectivity could offer new approaches to the treatment of localized excessive collagen formation, a characteristic of diseases such as lung fibrosis and liver cirrhosis.

We thank G. Kaule, B. Wölflinger and R. Braun for expert technical assistance, and H. H. Schöne for his critical support. H.M.H.-A. is grateful to Dr. K. I. Kivirikko, Dr. C. S. Anast and Dr. J. F. Crigler, Jr., for advice, and thanks D. Bedell-Hogan and H. Kagan for providing the reagents for the lysyl oxidase assay. G.T., J.M., V.G. and H.M.H.-A. were supported by Grant Ha-1293 from the Deutsche Forschungsgemeinschaft and the Evariste Galois Fund.

REFERENCES

- Hanauske-Abel, H. M. & Günzler, V. (1982) *J. Theor. Biol.* **94**, 421-455
- Hanauske-Abel, H. M. (1983) M.D.-Ph.D. Thesis, Philipps-Universität, Marburg
- Kivirikko, K. I. & Myllylä, R. (1985) *Ann. N.Y. Acad. Sci.* **460**, 187-201
- Kivirikko, K. I. & Majamaa, K. (1985) *Ciba Found. Symp.* **114**, 34-64
- Kivirikko, K. I. & Savolainen, E.-R. (1988) in *Liver Drugs* (Testa, B. & Perrissoud, D., eds.), pp. 194-222, CRC Press, Boca Raton
- Majamaa, K., Hanauske-Abel, H. M., Günzler, V. & Kivirikko, K. I. (1984) *Eur. J. Biochem.* **138**, 239-245
- Majamaa, K., Turpeenniemi-Hujanen, T., Latipää, P., Günzler, V., Hanauske-Abel, H. M., Hassinen, I. & Kivirikko, K. I. (1985) *Biochem. J.* **229**, 127-133
- Martell, A. E. & Smith, R. M. (1974) *Critical Stability Constants*, vol. 1, pp. 367-370, Plenum Press, New York
- Martell, A. E. & Smith, R. M. (1982) *Critical Stability Constants*, vol. 5, p. 129, Plenum Press, New York
- Smith, R. M., & Martell, A. E. (1989) *Critical Stability Constants*, vol. 6, p. 163, Plenum Press, New York
- Reed, L. J. (1974) *Acc. Chem. Res.* **7**, 40-46
- Prockop, D. J., Berg, R. A., Kivirikko, K. I. & Uitto, J. (1976) in *Biochemistry of Collagen* (Ramachandran, G. N. & Reddi, A. H., eds.), pp. 163-273, Plenum Press, New York
- Tschank, G., Raghunath, M., Günzler, V. & Hanauske-Abel, H. M. (1987) *Biochem. J.* **248**, 625-631
- Tschank, G., Hanauske-Abel, H. M. & Peterkofsky, B. (1988) *Arch. Biochem. Biophys.* **261**, 312-323
- Efimovsky, O. & Rumpf, P. (1954) *Bull. Soc. Chim. Fr.* 648-649
- Juva, K. & Prockop, D. J. (1966) *Anal. Biochem.* **15**, 77-83
- Chojkier, M., Peterkofsky, B. & Bateman, J. (1980) *Anal. Biochem.* **108**, 385-393
- Bruckner, P. & Prockop, D. J. (1981) *Anal. Biochem.* **110**, 360-368
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680-685
- Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83-88
- Tanzawa, K., Berger, J. & Prockop, D. J. (1985) *J. Biol. Chem.* **260**, 1120-1126
- Hojima, Y., van der Rest, M. & Prockop, D. J. (1985) *J. Biol. Chem.* **260**, 15996-16003
- Hata, R., Kurata, S. & Shinkai, H. (1988) *Eur. J. Biochem.* **174**, 231-237
- Tuderman, L., Kuutti-Savolainen, E. R. & Kivirikko, K. I. (1975) *Eur. J. Biochem.* **52**, 9-16
- Kedersha, N. L. & Berg, R. A. (1981) *Collagen Relat. Res.* **1**, 345-353
- Peterkofsky, B. & DiBlasio, R. (1975) *Anal. Biochem.* **66**, 279-286
- Rosenbloom, J., Indik, Z., Abrams, W., Bedell-Hogan, D. & Kagan, H. (1990) *FASEB J.* **4**, A2308
- Berg, R. A. & Prockop, D. J. (1973) *Biochem. Biophys. Res. Commun.* **52**, 115-120
- Bächinger, H. P., Bruckner, P., Timpl, R., Prockop, D. J. & Engel, J. (1980) *Eur. J. Biochem.* **106**, 619-632

30. Javitt, J. C. & Prockop, D. J. (1980) in *Biology of Collagen* (Viidik, A. & Vuust, J., eds.), pp. 77–85, Academic Press, New York
31. Elridge, C. F., Bunge, R. P. & Bunge, M. B. (1988) *Exp. Cell Res.* **174**, 491–501
32. Neblock, D. S. & Berg, R. A. (1985) in *Diseases of Connective Tissue* (Uitto, J. & Perejda, A. J., eds.), pp. 233–246, Marcel Dekker, New York
33. Majamaa, K., Günzler, V., Hanauske-Abel, H. M., Myllylä, R. & Kivirikko, K. I. (1986) *J. Biol. Chem.* **261**, 7819–7823
34. Majamaa, K. & Uitto, J. (1986) *Clin. Res.* **34**, 417A
35. Sasaki, T., Majamaa, K. & Uitto, J. (1987) *J. Biol. Chem.* **262**, 9397–9403
36. Sinkula, A. A. & Yalkowski, S. H. (1975) *J. Pharm. Sci.* **64**, 181–210
37. Takehara, K., Grotendorst, G. R., Trojanowska, M. & Leroy, E. C. (1986) *Collagen Relat. Res.* **6**, 455–466
38. Clark, C. C. & Richards, C. F. (1988) *Biochem. J.* **250**, 65–70
39. Tuderman, L. & Prockop, D. J. (1982) *Eur. J. Biochem.* **125**, 545–549
40. Dombrowski, K. E., Sheats, J. E. & Prockop, D. J. (1986) *Biochemistry* **25**, 4302–4309
41. Morikawa, T., Tuderman, L. & Prockop, D. J. (1980) *Biochemistry* **19**, 2646–2650
42. Fleischmajer, R. (1989) *J. Invest. Dermatol.* **89**, 553–554
43. Raghunath, M., Tschank, G., Becker, K., Hanauske-Abel, H. M. & Rumpelt, H. J. (1989) *FASEB J.* **3**, A624

Received 3 September 1990/22 October 1990; accepted 1 November 1990