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EFFECT OF CHELATING AGENTS ON THE BIOSYNTHESIS OF COLLAGEN

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Abstract. Different doses of various chelating agents have been assayed to elucidate 1) the incorporation of (^{14}C)Proline and (^{14}C)Lysine, 2) the biosynthesis of (^{14}C)Hydroxyproline and (^{14}C)Hydroxylysine and 3) the glycosylation of (^{14}C)Hydroxylysine by 10-day-old chick embryo tibiae. According to their effect, the chelators can be classified into three main groups: 1) showing decreased hydroxylation of the precursor amino acids without major effect on their incorporation in undialysable material; 2) showing inhibited uptake and hydroxylation of the precursor amino acids according to the dose. Effects were recorded even at the 0.5 mM dose; 3) showing no effect with doses lower than 5 mM. The various chelators did not influence the uptake of (^{14}C)Pro and (^{14}C)Lys to the same extent. The inhibition of the glycosylation step may be related to the parallel decrease of the biosynthesis of (^{14}C)Hyl. The differences observed suggest that, besides their metal sequestering properties, the chelators may act by other mechanisms as well. Hydralazine, markedly decreased collagen biosynthesis, as well as the incorporation of (^{14}C)D-glucosamine into glyco and/or mucoproteins.

Inhibition of collagen biosynthesis has been considered as a means of avoiding excessive collagen formation, as observed for example, in scleroderma (12, 17, 29).

According to Rukavina et al. (29), scleroderma may represent either a trace metal deficiency, abnormal metal excess, or the dependence of metallo-enzyme systems upon chelation. Various laboratories have demonstrated that collagen biosynthesis proceeds in sequential steps (1, 20, 28) as follows:

Step 1: Biosynthesis of protocollagen (proline- and lysine-rich polypeptide precursor) taking place in ribosomes.

Step 2: Hydroxylation of certain proline and lysine residues of protocollagen to hydroxyproline and hydroxylysine by protocollagen-proline and protocollagen-lysine hydroxylases (PPH, PLH), respectively. Cofactors required are Fe^{++} ions, α-ketoglutarate, ascorbic acid and atmospheric O_2.

Step 3: Galactosylation of certain hydroxylysine residues to galactosyl-*o*-hydroxylysine by collagen: galactosyl-transferase. Cofactors required are: Mn^{++} ions and uridine-diphosphate galactose (UDPGal).

Step 4: Glucosylation of certain galactosyl-*o*-hydroxylysine residues by collagen: glucosyl transferase. Cofactors required are Mn^{++} and uridine-diphosphate glucose (UDPGlu).

Steps 3 or 4 are essential for the extrusion of collagen.

It is worth noting that glycosylated hydroxylysine (Hyl) represents approximately 1/3 of the total Hyl in human skin and bone, although the relative ratio of glucosyl-galactosyl-hydroxylysine to galactosyl-hydroxylysine is approximately 2 in the former and 0.4 in the latter tissue (25). Substances capable of inhibiting the incorporation of proline (Pro) and lysine (Lys) or of chelating Fe^{++} and or Mn^{++} ions should be expected to inhibit collagen biosynthesis. Deoxyglucose can inhibit glycosylation of fully hydroxylated macromolecules (1). Unhydroxylated or unglycosylated macromolecules are not extruded from the cells (1, 14). The abnormal macromolecules synthesized and the lack of their extrusion have been suggested to slow down the synthesis of protocollagen growing on ribosomes (6). Injection "in ovo", or organ cultures performed in the presence of azetidine-carboxylic acid (a proline analogue), which is incorporated into the precursor polypeptide instead of proline, inhibits the hydroxylation and, consequently, the glycosylation steps (19, 20).

Studies have been performed on the effect of various chelating agents on the biosynthesis of hydroxyproline (Hyp) (6, 7, 11, 13, 14, 18, 21, 31). Only $\alpha\alpha'$-dipyridyl has been studied in parallel experiments on the biosynthesis of both amino

acids characteristic of collagen (1). As some differences in the behaviour of PPH and PLH have been reported (32), chelating agents known to affect proline hydroxylation were studied with a view to their possible effect on lysine hydroxylation. In addition to the known chelators, some substances of more or less common medical use, known to have certain chelating properties, were assayed. The purpose of this study was to find inhibitors of collagen biosynthesis and, thus, drugs which might be useful in the treatment of scleroderma.

CHEMICALS

Diethyldithiocarbomate (sodium salt); αα′-dipyridyl; 1–10 phenanthroline; 8-hydroxyquinoline; ethylenediamine tetraacetic acid (disodium salt, EDTA); hydralazine; procainamide and chlorpromazine were products of E. Merck, A. G., Darmstadt. D-dimethylcysteine and *N*-acetyl-DL-penicillamine were products of Sigma Chem. Corp., while tetracycline chloride was obtained from Pfizer Corp.

METHODS

Tibiae from 10-day-old chick embryos were dissected under the microscope. Tissues were then preincubated in a medium containing glucose, inorganic salts and phosphate buffer (15) for 1 hour at 37°C. The preincubation was continued for 30 minutes in the presence of the substances assayed. Tissues were then incubated with 5 μCi of (^{14}C)*L*-proline or (^{14}C)*L*-lysine for 2 hours at 37°C in parallel experiments. At the end of the incubation period the tibiae were homogenized and the homogenates were dialysed exhaustively against running tap water.

In order to assay (^{14}C)hydroxyproline or total (^{14}C)-hydroxylysine, aliquots of the dialysed homogenates were submitted to acid hydrolysis with 6 N HCl at 120°C overnight. HCl was then evaporated under reduced pressure. (^{14}C)Hyp was assayed according to Juva & Prockop (16). (^{14}C)Hyl was assayed according to Blumenkrantz & Prockop (2) in the following way. Total (^{14}C) Hyl was assayed on an aliquot of the acid-hydrolysed sample. Unglycosylated (^{14}C)Hyl was determined on an unhydrolysed aliquot. Glycosylated (^{14}C)Hyl was determined by difference between total (^{14}C) and unglycosylated (^{14}C)Hyl. Total uptakes of (^{14}C)Pro, and (^{14}C)Lys were determined on aliquots of the (^{14}C)Pro and (^{14}C)Lys labelled undialysable samples, respectively.

The effect of various doses of different chelators was studied in relation to uptake of (^{14}C)Lys and (^{14}C)Pro and their hydroxylation. Glycosylation of (^{14}C)Hyl was also studied.

Results were calculated as dpm per bone μCi per hour of incubation and were expressed as percentage of control values. Controls were run without addition of the chelators.

RESULTS

Based upon the effect of the chelators assayed on 1) total (^{14}C) uptake, 2) biosynthesis of hydroxyproline and hydroxylysine, and 3) glycosylation of hydroxylysine, they could be classified in 3 main groups (Table I).

Group 1. The main effect observed was the inhibition of hydroxylation by (^{14}C)Pro and (^{14}C)-Lys. Glycosylation of (^{14}C)Hyl was correspondingly inhibited. αα′-dipyridyl and 8-hydroxyquinoline belong to this group, the former being the strongest inhibitor at the same dosage.

Group 2. Inhibition of (^{14}C)Pro and (^{14}C)-Lys incorporation in relation to the concentration of the chelators in the medium was observed. However, their effect on the hydroxylation and glycosylation steps was even stronger. 1-10 phenanthroline, Na-diethyldithiocarbamate, chlorpromazine, tetracycline chloride, hydralazine, and procainamide belong to this group. Na-diethyldithiocarbamate which showed a divergent behaviour in relation to (^{14}C)Pro and (^{14}C)Lys uptake and their hydroxylations, respectively, had a remarkable effect on (^{14}C)Pro and (^{14}C)Hyp. Glycosylation of (^{14}C)Hyl, although decreased, followed patterns parallel with the incorporation of (^{14}C)Lys and its hydroxylation.

Group 3. (Comprising D-penicillamine, *N*-acetyl D,*L*-penicillamine and EDTA). As no effect on the parameters studied was observed till a 2.5 mM concentration was reached, the effect of the two former chelators was studied in a broader concentration range. D-penicillamine and *N*-acetyl D,*L*-penicillamine inhibited the incorporation of (^{14}C)Pro and (^{14}C)Lys and their hydroxylation, in relation to the dose, when this was above 5 mM.

DISCUSSION

The results show that the various chelators do not affect incorporation and hydroxylation of (^{14}C)-Pro and (^{14}C)Lys to exactly the same extent. The differences observed may be related to the fact that, besides their metal sequestering capacity, they may act by other mechanisms as well, i.e.

Table I. *Effect of various chelating agents on uptake of* (^{14}C)*Pro and* (^{14}C)*Lys and their hydroxylation on the glycosylation of* (^{14}C)*Hyl by 10-day-old chick embryo tibiae*

Results were calculated as dpm/bone/μCi/hr incubation and expressed as % of controls
Controls were run without the addition of chelating substances

Chelating agent tested	Conc. in medium (mM)	Total uptake		Collagen biosynthesis			
		(^{14}C)Pro	(^{14}C)Lys	(^{14}C)Hyp	Total (^{14}C)Hyl	(^{14}C)Glyc. Hyl	(^{14}C)GlNH$_2$
Group 1							
αα′-Dipyridyl	0.5	107	102	0.61	3	0	
	1.0	94	100	0.14	3	0	
	2.5	91	98	0.17	3	0	
8-Hydroxyquinoline	0.5	87	100	3.06	19	13	
	1.0	87	99	0.9	3	0	
	2.5	60	99	0.35	2	0	
Group 2							
1.10-Phenanthroline	0.5	78	97	4.43	3.8	0	
	1.0	55	73	4.04	3.1	0	
	2.5	18	33	2.53	1.6	0	
Na-Diethyldithio-carbamate	0.5	38	30	27	29	24	
	1.0	16	30	18	26	23	
	2.5	16	30	12	24	17	
Chlorpromazine	0.018	92	80	82	101	96	
	0.055	41	37	33	30	28	
	0.27	2.3	1.32	4	3	0	
Hydralazine	2.4	67	66	7	7	5.6	84
	4.8	52	39	6	2	1.3	66
	7.2	39	25	2	2	0.9	58
	12.0	5	10	2	2	0.3	28
Procainamide	2.4	58	79	56	76	70	
	4.8	53	66	55	71	71	
	12.0	35	51	35	48	47	
Tetracycline chloride	0.5	42	73	91	49	48	
	1.0	31	65	88	40	39	
	2.5	16	38	40	24	23	
Group 3							
EDTA	0.5	108	96	104	100	100	
	1.0	102	81	102	100	100	
	2.0	100	57	100	100	100	
	2.5	100	45	100	94	92	
D-Penicillamine	0.5	92	95	95	102	100	
	1.0	91	78	90	102	103	
	2.5	91	76	89	77	74	
	5.0	98	90	87	52	51	
	10.0	95	90	50	43	41	
	20.0	22	88	26	25	25	
N-acetyl-DL-Penicilla-mine	0.5	94	84	82	106	97	
	1.0	88	79	80			
	2.5	83	79	77	103	100	
	5.0	78	86	84	47	47	
	20.0	19	65	15	28	27	

competition for a common carrier system for transport, oxido-reduction mechanisms, etc. No chelator was able to inhibit glycosylation of hydroxylysine only. Rifkin et al. (27) have shown that αα′-dipyridyl decreased the uptake of inorganic phosphate by mitochondria of kidney and liver of the rat. A marked drop (36%) in the P/O ratio, indicating an uncoupling of oxidative phosphorylation and alteration of mitochondrial metabolism, possibly by sequestering essen-

tial divalent cations other than ferrous ions, was suggested (27). Paine et al. (23) did not observe any inhibition in the uptake of glycine by Ehrlich cells when various chelators were added simultaneously with the amino acid. The authors concluded that metal chelation is not involved in the primary reaction between amino acid and transport carrier and that there is no competition between chelators and glycine for the carrier system.

Glycosylation of hydroxylysine followed the same pattern as that of the hydroxylation of lysine.

It is worth noting that substances of common medical use as hydralazine, procainamide and chlorpromazine affected collagen biosynthesis to a considerable extent. Hydralazine also affected the biosynthesis of glyco and/or mucoproteins as appears from the decreased incorporation of (^{14}C)D-glucosamine into undialysable material.

Hydralazine has been reported to be capable of chelating Fe^{++} and Mn^{++} ions (24). Decreased turnover of Mn^{++} has been reported in one patient with the hydralazine syndrome and in 7 patients with rheumatoid arthritis (8). Hydralazine can also bind α-ketoglutarate, another cofactor required for protocollagen hydroxylation (24). The decreased collagen biosynthesis effected by hydralazine is well explained by the mentioned chelating and binding effects. It should be emphasized that substances reported to produce the lupus erythematosus syndrome, such as hydralazine and procainamide (24, 30), can reduce collagen biosynthesis. The effect of chlorpromazine, reducing the biosynthesis of collagen and other proteins in a parallel pattern should be considered in view of the fact that chlorpromazine has been reported to chelate Mn^{++} ions (3). Chlorpromazine is able to produce a direct toxic effect on proliferating cells and retards growth and development of fertilized chicken embryos. An inhibitory effect on cell division or DNA-synthesis has also been reported (4, 5, 9, 22, 26).

The differences observed in the uptake and hydroxylation of (^{14}C)Pro and (^{14}C)Lys under the effect of various chelators and especially Na-diethyldithiocarbamate stress the need of studying both amino acids in relation to collagen research. Glycosylation as the final step in collagen formation related to the extrusion of the collagen molecule, belongs to the important processes which must be revealed to obtain the desirable information of the synthetic procedure.

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