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PREVENTION OF EXPERIMENTAL IMMUN-ARTERIOSCLEROSIS BY CALCITONIN

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Abstract—Typical sclerotic lesions in the rabbit aorta can be induced by immunisation with κ-elastin [15]. These lesions include fragmentation of the elastic fibers and diffuse calcium deposition, accompanied by an increase of the 5M-guanidinium Cl-soluble (structural glycoprotein-containing) fraction of the media, increase of the hexose and hydroxyproline (collagen) content of the 1M $MgCl_2$-soluble aorta extract (soluble protein and glycoprotein fraction) and guanidinium-soluble hydroxyproline (polymeric collagen). In animals treated simultaneously with calcitonin, the morphological and most of the biochemical modifications produced by κ-elastin immunisation were not observed or lessened. There was much less fragmentation of the elastic lamellae and calcium deposition in the media. Calcitonin administration abolished the increases of the guanidinium-soluble protein fraction, of the glycoproteins in the $MgCl_2$-extracts and of the hydroxyproline (collagen) content in the $MgCl_2$ and guanidinium extracts. Calcitonin alone produced some modifications in the chemical composition of the aorta, such as a decrease in its elastin content, an increase in the hexose and hexosamine content of all extracts. The distribution of calcium salts in the aorta extracts was also modified by calcitonin administration: the calcium content of the $MgCl_2$ and guanidinium-extracts decreased and that of the final residue (elastin) increased. The results indicate that calcitonin influences the biosynthetic activity of the smooth muscle cells of the media and exerts a protective action against the biochemical and morphological modifications produced by immun-arteriosclerosis.

Some demineralization of spine and arterial calcification are frequent findings on roentgenographic examinations of elderly people. The question arises as to whether these calcium movements are associated with one another and share a common pathogenesis. The main disturbances of calcium metabolism found in osteoporosis are a decreased rate of bone formation and of calcium absorption in the gut and a relative increase in bone resorption and urinary calcium output. These disturbances can be corrected by long term administration of "low" doses of calcitonin, i.e. by substitutive therapy. This finding leads to the consideration of post-menopausal and senile osteroporosis as conditioned by a decrease of calcitonin production with age [1]. As very little is known with respect to the role of endocrine factors in the regulation of calcium metabolism in arterial wall (such as the effect of calcitonin on the development of the arteriosclerotic process), it seemed of interest to study the effect of calcitonin administration on the deposition of calcium salts in cells and matrix macromolecules of the aorta, which is indeed one of the important biochemical alterations observed with aging and in arteriosclerosis [2]. Two types of calcium deposition can be distinguished in the large blood vessels: a diffuse one, concerning mainly elastic fibers and a localised one, observable microscopically, involving the formation of crystalline or amorphous calcium salt accumulations in cells and in atherosclerotic plaques [3–5]. Diffuse calcium deposits in elastic fibers is a continuous process, increasing steadily during the whole life span [3, 6, 7]. Recent results indicate that the elastin peptide chains can react with Ca^{2+}-ions to form strong complexes [8, 9]. Calcium and lipid (cholesterol) deposition is a mutually potentiated process [10] which exposes the elastic fibers to faster attack by elastases [11, 12]. The factors influencing calcium salt depositions are unknown although the acidic (structural) glycoprotein components may play a role as nucleation centers [13, 14]. In experimental arteriosclerosis, the relative intensity of calcium salt and of lipid deposition varies widely according to the technique used for its induction. Immunisation of rabbits with κ-elastin in complete Freund's adjuvant induces the formation of predominantly sclerotic calcified lesions [15]. This model therefore appeared particularly suitable for investigating the effect of calcitonin on the composition and reactivity of the arterial wall.

MATERIALS AND METHODS

White rabbits (Blancs Bouscat) 2 months old (2–3 kg) of both sexes were used. They were divided in four groups. Group I (4 animals) was used as control and received only a normal commercial rabbit diet (U.A.R. No. 112). Group II (10 animals) received biweekly i.m. injections of 5 mg of κ-elastin in complete Freund's adjuvant, during 4 weeks. Group III (10 animals, 8 survived) received the same treatment as group II and in addition two daily i.m. injections of 0.16 M.R.C. units of purified porcine calcitonin. Group IV (6 animals) received only the calcitonin injections with no elastin. κ-elastin was prepared from

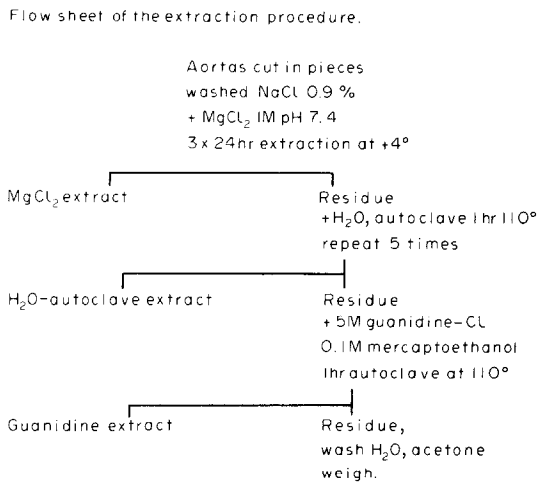

Fig. 1. Flow sheet of the extraction procedure used. For details see Methods.

porcine aorta and from bovine ligamentum nuchae, as described [16]. Extractive porcine calcitonin was purified by Roussel-Uclaf Laboratories.

At the end of the experimental period, the animals were sacrificed, the aortas excised and examined both macroscopically and microscopically with histochemical techniques (Van Kossa stain for calcium, Veigert's resorcinfuchsine and orcein for elastin and Masson's Trichrom for collagen) and submitted to a sequential extraction procedure.

Extraction of aortas. After washing in saline to free the aortas from blood, the aortas were cut in small (a few mm^2) fragments, soaked in 1M $MgCl_2$ pH 7.4 (about 10 ml per g wet weight), homogenized in the cold and shaken at +4° for 14 hr. After centrifugation this $MgCl_2$-extraction was repeated twice. The residue was washed in water, suspended in about 10 vol. of distilled water and autoclaved 5 times at 110° for 1 hr. The autoclaved residue was suspended in 5 M guanidinium chloride–0.1 M mercaptoethanol and autoclaved again at 110° for 1 hr. The final residue was washed in water and acetone, dried at 37° and weighed. Figure 1 shows the flow sheet of this extraction procedure. All the extracts ($MgCl_2$, H_2O-autoclave and guanidine–mercaptoethanol) were dialysed exhaustively against running tap water and distilled water.

Chemical determinations. The determination of proteins, hexoses, hexosamines, uronic acid and hydroxyproline were carried out as previously described [5, 17]. Serum and tissue calcium were determined by atomic absorption spectrophotometry. Cross-link-amino-acids of elastin (desmosine, lysino-norleucine) were determined by high voltage electrophoresis [18].

RESULTS

1. *Action of calcitonin on the arteriosclerotic lesions.* Table 1 gives the severity of the lesions produced by immunisation with κ-elastin in the rabbit aortas. The unimmunised group (control group No. I) showed only scarce and light lesions known to be present in several rabbit strains and occurring "spontaneously" [4]. These lesions did not exceed the fatty streak state and did not resemble the lesions produced by immunisation with elastin. The aortas of rabbits from group II, immunised with κ-elastin were covered with indurated, calcified plaques, similar to those described earlier in elastin-immunised rabbits [15]. Aortas from rabbits in group III which received calcitonin during the immunisation period, also had similar lesions to those seen in group II aortas, but they were much less severe, much less frequent and covered a significantly smaller surface of the aorta than in group II animals. A distinct protective effect was discernible by the macroscopic inspection of the aortas and was confirmed by the histological findings. Aortas of group IV animals, treated with calcitonin alone were similar to those of the control group.

Table 1. Macroscopically observable arteriosclerotic lesions in rabbit aortas in the four groups of rabbits (see Methods)

Group I		Group II		Group III		Group IV	
no. of rabbits	lesion	no. of rabbits	lesion	no. of rabbits	lesion	no. of rabbits	lesion
74	X	91	XXXX	49	XX	79	XX
57	XX	81	XX	23	X	59	X
85	X(X)	73	XX	35	X(X)	60	0
33	XX	41	XXX	30	0	66	XXX j.
		42	XXX	25	0	68	XX
		72	XXX	31	XX	62	XX
		71	XXX	36	X		
		70	XXX	24	X		
				21	X		
				34	X		
*Average degree of lesion	1.7		= 3.0		= 1.1		= 1.6

* Average value of crosses per animal in groups.
0 no alteration.
X light fatty streaks.
XX fatty plaques.
XXX large calcified plaques.
XXXX large calcified plaques with ulcerations.

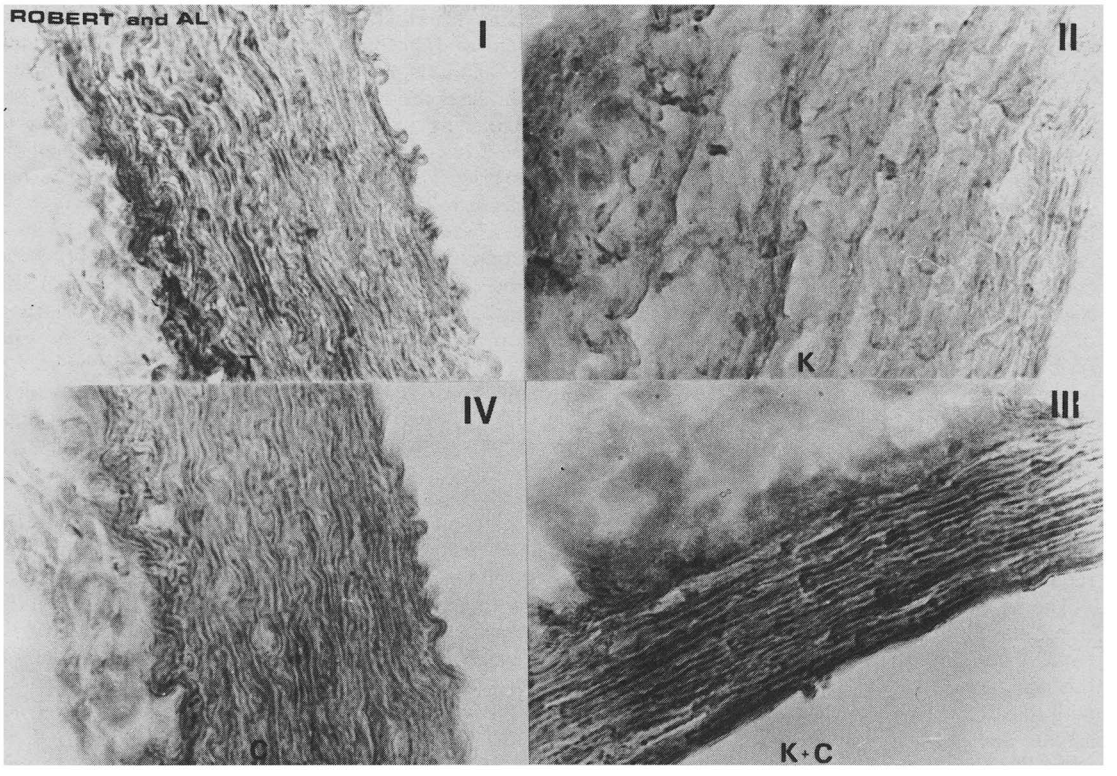

Fig. 2. Histological section of the aortas of rabbits of group I (controls), II, (K-elastin treated), III (K-elastin and calcitonin treated) and IV (calcitonin treated). Cryostat sections, stained with orcein, ×250. Notice the disorganisation of the elastic lamellae in group II aorta and the good conservation of the elastic structures in group III aorta.

2. *Histological findings.* Sections from the cross of the aorta from the four groups of animals were compared using specific elastin and calcium stains (see Methods and Fig. 2). In group II (κ-elastin treatment), severe alterations were present in the elastic fibers (fragmentation and lysis especially in the inner third part of the media) giving a fenestrated appearance to the media. Collagen stains more intensely than in controls. Heavy calcium deposits could be detected by the Van Kossa stain, mainly where elastic fibers appeared severely altered (details of the morphological studies will be published separately [20]. In group III (κ-elastin and calcitonin treatment) slight fragmentation of elastic fibers is present without an increase in collagen staining; calcium staining of the elastic fibers is slight (see Fig. 2). No alteration of elastic fibers and no calcium deposition could be detected in the control (group I) aortas. These results suggest a protective effect of calcitonin against the elastin induced aorta-alterations.

Table 2. Chemical composition of the extracts of aortas obtained by method shown on Fig. 1 from the four groups of rabbits (see Methods)

Group	Treatment	Extract	Proteins	Hydroxyproline	Hexoses	Hexosamines
I	Control	$MgCl_2$	32.7 ± 7.7	0.45 ± 0.15	7.95 ± 0.55	4.3 ± 0.15
		Autoclave	62.2 ± 18.2	10.20 ± 0.55	2.70 ± 1.10	1.4 ± 0.34
		Guanidine	5.5 ± 4.0	1.20 ± 0.87	8.50 ± 1.15	5.4 ± 0.5
		Residue	5.06 ± 0.72	0.84		
II	κ-elastin	$MgCl_2$	30.7 ± 8.6	1.6 ± 0.22	14.4 ± 3.7	3.4 ± 0.6
		Autoclave	55.7 ± 7.2	8.7 ± 1.24	3.01 ± 0.60	1.3 ± 0.37
		Guanidine	8.4 ± 2.9	2.6 ± 0.85	6.8 ± 2.0	4.4 ± 1.3
		Residue	3.33 ± 1.09	0.65		
III	κ-Elastin and calcitonin	$MgCl_2$	29.0 ± 10.0	0.75 ± 0.28	7.8 ± 1.7	4.15 ± 1.75
		Guanidine	2.95 ± 0.65	0.60 ± 0.28	9.84 ± 4.00	4.00 ± 1.15
		Residue	2.66 ± 0.90	1.4		
IV	Calcitonin	$MgCl_2$	25.80 ± 7.05	0.60 ± 0.35	12.70 ± 2.30	3.2 ± 0.89
		Autoclave	46.4 ± 6.2	12.3 ± 2.66	4.2 ± 0.3	2.2 ± 0.45
		Guanidine	3.0 ± 0.4	1.2 ± 0.43	13.3 ± 2.5	7.4 ± 1.3
		Residue	1.98 ± 0.70	0.76		

Average values ± S.E.M. are given in terms of mg/g fresh weight for proteins and mg% proteins for the other components. Results significantly different from control group values ($P < 0.001$ to 0.05) are underlined.

3. *Chemical composition of the aorta.* Table 2, shows the protein, hexose, hexosamine and hydroxyproline content of the aorta extracts obtained by the above described procedure. The distribution of proteins between the sequential extracts is somewhat different from that obtained with previously used extraction procedures [5, 17]. The $MgCl_2$ extract contains the salt soluble diffusible macromolecules (soluble collagen and soluble proteoglycan and glycoprotein components) [15, 17]. The H_2O-autoclaved fraction contains mainly polymeric collagen as shown by its high hydroxyproline content. The complete extraction of collagen from aorta by this procedure was described by Partridge [21]. The guanidine extract (obtained also by autoclaving) contains the structural glycoproteins of aorta. A detailed investigation of these components was previously reported [17, 22]. This extract contains also some collagen and/or elastin as suggested by its hydroxyproline content. The very strong interaction of structural glycoproteins with collagen [22] and elastin [23] was previously reported.

The final residue contains a highly reticulated portion of insoluble elastin as indicated by its desmosine content (see Fig. 4). The hydroxyproline content of this residue (0.65 to 1.4%, see Table 2) is in agreement with that reported for purified elastin [21]. The hexosamine and hexose contents reflect the glycoprotein and glycosaminoglycan contents of the extracts. As expected from previous work carried out with a somewhat different extraction procedure [15, 17, 22, 24], most of the glycan-containing components are in the $MgCl_2$-extract and in the guanidine extract. The hexose and hexosamine content of the autoclaved extracts reflect the glycosaminoglycans and glycoproteins strongly associated with polymeric collagen and not extractable even at high ionic strengths. These glycoprotein-glycosaminoglycan-collagen complexes appear to result from non covalent interaction between these components [22]. The distributions of chemical components of the vessel wall in the successive extracts as shown in Table 2 are sufficiently reproducible to be used for the characterisation of modifications produced by treatment with κ-elastin and calcitonin.

Several differences can be noticed between the chemical composition of the aorta from the treated and untreated rabbits. Immunisation with κ-elastin (group II) produced a strong increase in the hexose content of the $MgCl_2$ extract, an increase in the protein and hexose contents of the guanidine extract (structural glycoprotein fraction) and a decrease in the final residue (highly cross-linked fraction of elastin). The hydroxyproline content increased in the $MgCl_2$-extract (soluble collagen) and in the guanidine extract. The hexosamine content of the $MgCl_2$-extract decreased slightly.

These modifications were absent or much attenuated in the calcitonin treated group III. When the composition of group III aortas is compared to that of group II, the results turn out to be significantly different (close to normal levels) in the calcitonin treated group III extracts. This can be seen on the glycoprotein (hexose, hexosamine) content of the $MgCl_2$-extract and the protein content of the guanidine extract, both being close to control values. The polymeric elastin content (protein content of residues, see Table 2) is however significantly lower than in the control group.

Calcitonin treatment alone produced modifications in the hexose and hexosamine content of all extracts, without morphological alterations (see Fig. 2). The polymeric elastin content was especially low in this group and the hexose values higher than in the controls. It appears that calcitonin administration can influence the distribution of the glycosaminoglycans and glycoproteins in the aorta.

Figure 3 shows that if the hydroxyproline content of the $MgCl_2$-extracts is expressed as soluble collagen, the increase is strong in the immunised group II and only very moderate in the calcitonin treated group III animals. The results of uronic acid determinations are similar but less striking. It appears that calcitonin treatment prevented most of the modifications of distribution of chemical components produced in the aortas by κ-elastin immunisation. The desmosine content of the final residue (polymeric elastin) confirmed the above findings (see Fig. 4). The desmosine content of elastin is severely reduced in the κ-elastin group and only slightly in the calcitonin treated group. Calcitonin treatment alone also elicited a slight loss of desmosine.

Table 3 shows the distribution of calcium in the extracts of group II and III animals. The total calcium content recovered in the three fractions studied is remarkably constant. The distribution of calcium is however quite different in these two groups. Calcitonin treatment decreased the calcium content of the

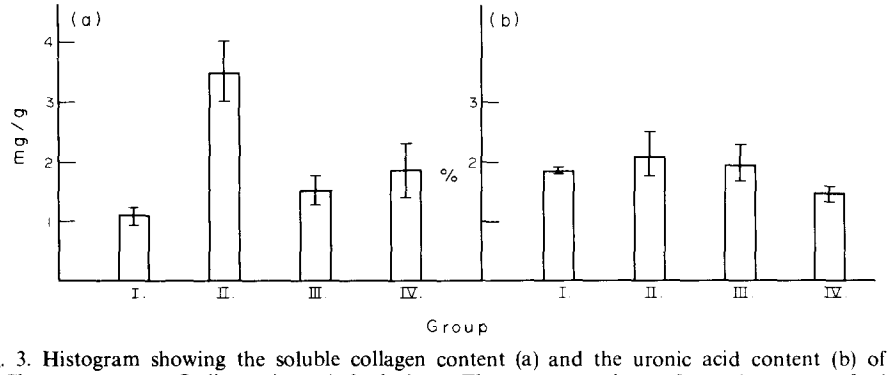

Fig. 3. Histogram showing the soluble collagen content (a) and the uronic acid content (b) of the $MgCl_2$-aorta extracts. Ordinates in mg/g fresh tissue. The roman numbers refer to the groups of rabbits (see Methods). The bars on top of the columns represent the standard error (σ).

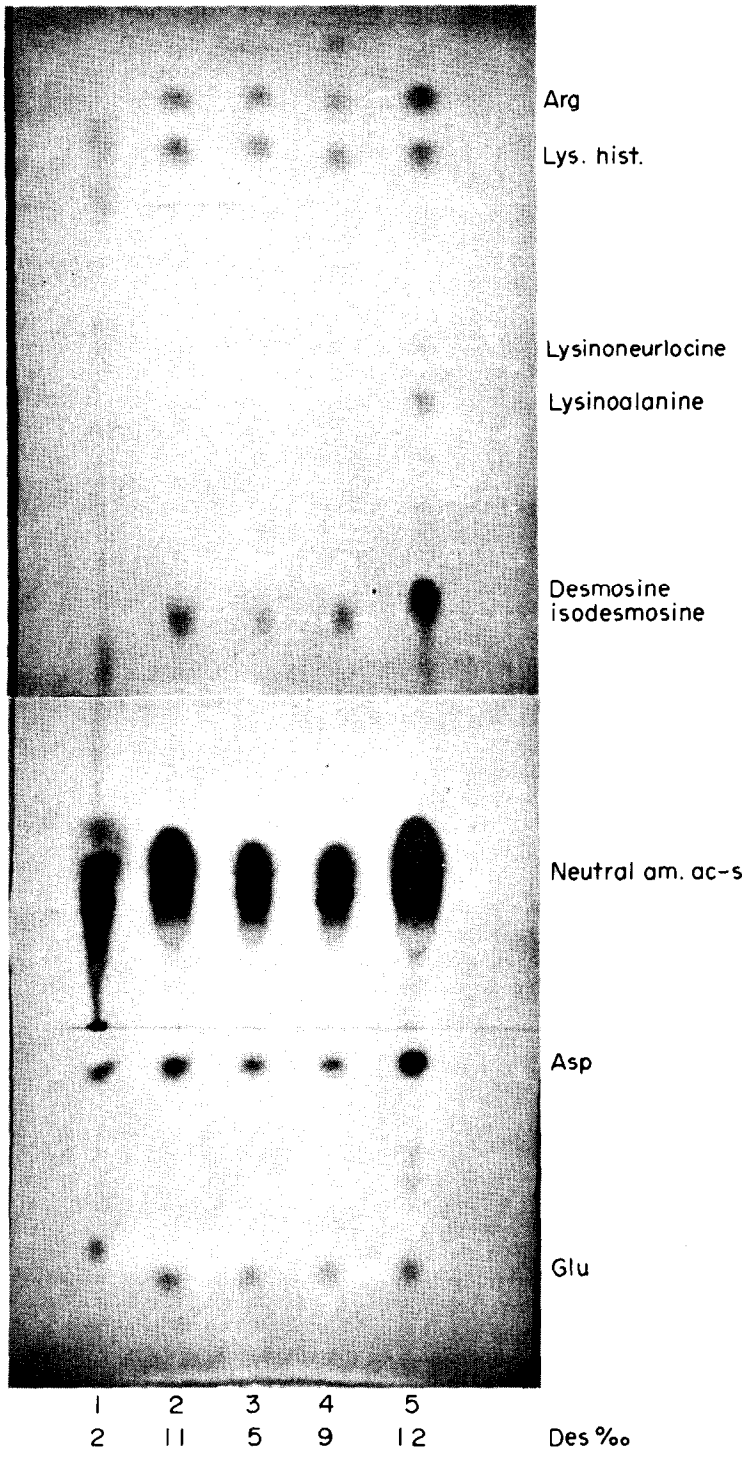

Fig. 4. High voltage electrophoretogram for desmosine-determinations in the final residues (crosslinked elastin) from the four groups of rabbits. 1: group II (K-elastin); 2: group I (controls); 3: group IV (calcitonin); 4: group III (K-elastin and calcitonin); 5: elastin hydrolysat. Electrophoresis carried out in pyridine-acetate buffer pH 3.8 as described [18]. The position of the different aminoacids is indicated in the right margin. The figures below the photo indicate relative desmosine content in lysine equivalents (res. pro 1000 res). No desmosine is detectable in group II aortas; desmosine is present in calcitonin treated aortas in somewhat lower amount than in untreated controls.

Table 3. Calcium content of the aorta extracts from group II (κ-elastin treated) and group III (κ-elastin and calcitonin treated rabbits).

Group	$MgCl_2$ extract	Guanidine extract	Final residue (elastin)	Total of three extracts
III				
κ-Elastin + calcitonin	2.84 ± 0.66	2.87 ± 0.60	5.89 ± 0.89	11.60
II				
κ-Elastin	3.33 ± 0.38	3.83 ± 0.52	3.96 ± 0.62	11.12

Results are given in mg/g fresh weight, average ± S.E.M. The values for all three extracts are significantly different for the two groups (P between 0.001 and 0.05).

$MgCl_2$ and guanidine extracts to a marginally significant level and significantly increased the calcium content of the cross-linked elastin fraction.

DISCUSSION

The reported experiments suggest that calcitonin acts upon the aorta which becomes less susceptible to the alterations produced by the immunisation with κ-elastin. The chemical and morphological changes produced by this immunisation are quite characteristic and not dissimilar from those seen in human arteriosclerosis [5, 15]. The increases of soluble and insoluble (structural) glycoproteins, soluble collagen and the decrease of highly reticulated elastin [5, 25] were demonstrated in human arteriosclerotic aortas. Most of these disturbances were prevented by simultaneous treatment with calcitonin. Calcitonin treatment alone produced some chemical modifications, such as an increase in the calcium content and a decrease in the desmosine content of elastic fibers; this suggests that this protective action may reside in a modification of the metabolism or reactivity of smooth muscle cells. On the basis of available data it cannot be decided if the observed effect is a direct one due to the action of calcitonin on the smooth muscle cells of the aorta or to an indirect one mediated by a hormonal, nervous or other relay.

There is a good agreement between the morphological changes and biochemical findings within each experimental group. Histochemical studies (to be reported elsewhere [20]) show a considerable decrease of the van Kossa stainable calcium in the calcitonin treated aortas. This is in keeping with the observation (see Table 3) that $MgCl_2$ and guanidine extractable $CaCl_2$ decreased in the calcitonin treated group. The calcium diffusely fixed in elastin, which increases after calcitonin administration is not detected by this method [20]. The increased fixation of calcium within the elastic fibers is not dissimilar to the increased fixation of calcium on the organic macromolecular matrix of bones observed after calcitonin administration [1]. It can be concluded therefore that calcitonin administration at low doses (ca 0.1 M.R.C. unit/kg) is largely able to prevent the changes due to κ-elastin immunisation. This observation suggests that calcitonin secretion may be one of the factors influencing the alterations of the elastic fibers during arteriosclerosis and aging. Therefore, a decrease in calcitonin production may lead to osteoporosis and to the deposition of calcium salts in the cells and interfibrillar matrix of the arteria. In the present study calcitonin was administered during the immunisation procedure. This leaves the question open as to a possible therapeutic role for calcitonin in the management of arteriosclerosis. Further experiments are clearly required.

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REFERENCES

1. G. Milhaud, J. N. Talbot and G. Coutris, *Biomedicine* **23,** 223 (1975).
2. L. Robert, A. Kadar and B. Robert, in *Advances in Experimental Medical Biology* **43** (Eds W. D. Wagner and T. B. Clarkson) p. 85. Plenum Press, New York (1974).
3. A. I. Lansing, *The Arterial Wall.* Williams & Wilkins, Baltimore (1959).
4. Y. Grosgogeat and J. Lenegre, in *Le Rôle de la Paroi Artérielle dans l'Athérogénèse* (Ed. C.N.R.S.) p. 241. Paris (1968).
5. J. Ouzilou, A. M. Robert, L. Robert, H. Bouissou and M. T. Pierragi, *Paroi Artérielle* **1,** 105 (1973).
6. Shiu Yeh Yu, in *Advances in Experimental Medical Biology* **43** (Eds W. D. Wagner and T. B. Clarkson) p. 403. Plenum Press, New York (1974).
7. Shiu Yeh Yu and H. J. Blumenthal, *J. Geront.* **18,** 119 (1963).
8. D. W. Urry, in *Advances in Experimental Medical Biology* **43** (Eds W. D. Wagner and T. B. Clarkson) p. 211. Plenum Press, New York (1974).
9. D. W. Urry, M. M. Long, C. F. Hendrix and K. Okamoto, *Biochemistry* **15,** 4089 (1976).
10. W. Hornebeck and S. M. Partridge, *Eur. J. Biochem.* **51,** 73 (1975).
11. B. Robert, L. Robert and A. M. Robert, *Path. Biol.* **22,** 661 (1974).
12. H. M. Kagan, G. D. Crombie, R. E. Jordan, W. Lewis and C. Franzblau, *Biochemistry* **11,** 3412 (1972).
13. L. Gotte, A. Serafini-Fracassini and V. Morel, *J. Atherosclerosis Res.* **3,** 244 (1963).
14. L. Robert, Y. Grosgogeat, A. M. Robert and B. Robert, *Israel J. Med.* **7,** 431 (1971).
15. A. M. Robert, Y. Grosgogeat, V. Reverdy, B. Robert and L. Robert, *Atherosclerosis* **13,** 427 (1971).
16. L. Robert and N. Poullain, *Bull. Soc. Chim. biol.* **45,** 1317 (1963).
17. M. Moczar and L. Robert, *Atherosclerosis* **11,** 7 (1970).
18. E. Moczar, B. Robert and L. Robert, *Analyt. Biochem.* **45,** 422 (1972).
19. J. Renais, P. Hadjisky and L. Scebat, in *Advances in Experimental Medical Biology* **43** (Eds W. D. Wagner and T. B. Clarkson). p. 335. Plenum Press, New York (1974).
20. A. M. Robert, G. Godeau, D. Brechemier, W. Hornebeck, G. Milhaud and L. Robert, submitted for publication.

21. S. M. Partridge, in *Biochemistry and Molecular Biology of Intercellular Matrix* (Ed. E. A. Balazs) Vol. 1, p. 593. Academic Press, New York (1969).
22. M. Moczar, E. Moczar and L. Robert, *Biochimie* **59**(2), 141–152 (1971).
23. A. Kadar, B. Robert and L. Robert, *Path. Biol.* **21**, 80 (1973).
24. M. Moczar, L. Robert and E. Moczar, *Atherosclerosis* **12**, 31 (1970).
25. Y. Moschetto, M. Debacker, O. Pizieux, H. Bouissou, M. T. Pieraggi and M. Julian, *Paroi Artérielle* **2**, 161 (1974).