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Neutral Sites for Calcium Ion Binding to Elastin and Collagen: A Charge Neutralization Theory for Calcification and Its Relationship to Atherosclerosis

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ABSTRACT **Neutral, uncharged binding sites for calcium ions are proposed for elastin and collagen. The sites utilize, particularly from a conformational viewpoint, the most striking feature of the amino acid composition, that is, the high glycine content. Glycines favor the formation of β-turns and associated conformations that are known, from studies on ion-transporting antibiotics, to interact with cations. By analogy with certain antibiotics, which are uncharged polypeptides and depsipeptides that bind cations by coordination with neutral acyl oxygens, it is proposed that calcium-ion binding also utilizes uncharged coordinating groups, i.e., neutral sites, in the protein matrix. The protein matrix, which becomes positively charged by virtue of the bound calcium ions, attracts neutralizing phosphate and carbonate ions, which then allow further calcium ion binding. The driving force is, therefore, the affinity of calcium ions for the neutral nucleation sites.**

The charge neutralization theory of calcification suggests a fundamental role of organic anions, for example sulfated mucopolysaccharides, in regulating bone formation and in retardation of atherosclerosis. The proposed mechanism contains elements that tend to unify several theories on the pathogenesis of atherosclerosis.

Elastin and collagen, particularly the former, exhibit a high affinity for calcium ions. There is, in fact, substantive argument, for example, that calcification of elastin is the initial process in the onset of the chronic disease arteriosclerosis; and the initiation of calcification is obviously an important process in bone and tooth formation as well as tendon insertion into bone and the emplacement of teeth. The significance of the initiation of calcification was emphasized by Urist (1) in his review of Selye's theory of calciphylaxis (2) when he stated that "the nature of the local mechanism of calcification is one of the most important unsolved problems of biochemistry." Several systematic and excellent studies have been carried out with the purpose of defining the site of initial calcium ion binding. In this connection sulfhydryl groups (3), and carboxyl groups associated with threonine hydroxyls (4) and with amino groups (5), have been suggested, although the percentages of these amino acid residues are extremely low in elastin.

The amino acid composition of bovine elastin has been reported by Petruska and Sandberg (6) (see Table 1). 33% of all the residues are glycine, another 65% are hydrophobic, and only 2% contain functional side chains, specifically 0.6% Thr, 0.9% Tyr, and 0.6% Glx with no Asx, Cys, or remaining lysine. These results are similar to others on elastin and collagen (7). Thus, one is hard pressed to explain an unusually high affinity for calcium by implicating residues that occur in unusually low amounts. In the present report, the hypothesis is advanced that it is, rather, the unusually high glycine content (8), coupled with the presence of residues containing bulky side chains, which makes probable some specific conformations with high affinity for cations. Furthermore, there are beautiful model systems from which several cation-binding conformations based on the amino acid composition of elastin can be deduced.

TABLE 1. *Amino acid composition of bovine elastin (ref. 6)*

Amino acid	%
Gly	33
Ala	24
Pro	13
Val	15
Ile	3
Leu	7
Phe	3
Thr	0.6
Tyr	0.9
Glx	0.6

TABLE 2. *Optical isomeric sequences in antibiotics*

Model structure	Sequence
Valinomycin*	$\overline{\perp(\text{-L-L-D-D-})_3\perp}$
Enniatins†	$\overline{\perp\text{L-D-L-D-L-D}\perp}$
Gramicidin A‡	$\text{L-Gly-L-D-(L-D)}_5\text{-L}$
Gramicidin S§	$\overline{\perp(\text{-L-L-L-D-L-Pro-})_2\perp}$

* Shemyakin, M. M., E. I. Vinogradova, M. Y. Feigina, N. A. Aldonova, N. F. Loginova, I. D. Ryabova, and I. A. Pavlenko, *Experientia*, **21**, 548 (1965).

† See ref. 11.

‡ Sarges, R., and B. Witkop, *J. Amer. Chem. Soc.*, **86**, 1861 (1964).

§ Battersby, A. R., and L. C. Craig, *J. Amer. Chem. Soc.*, **73**, 1887 (1951).

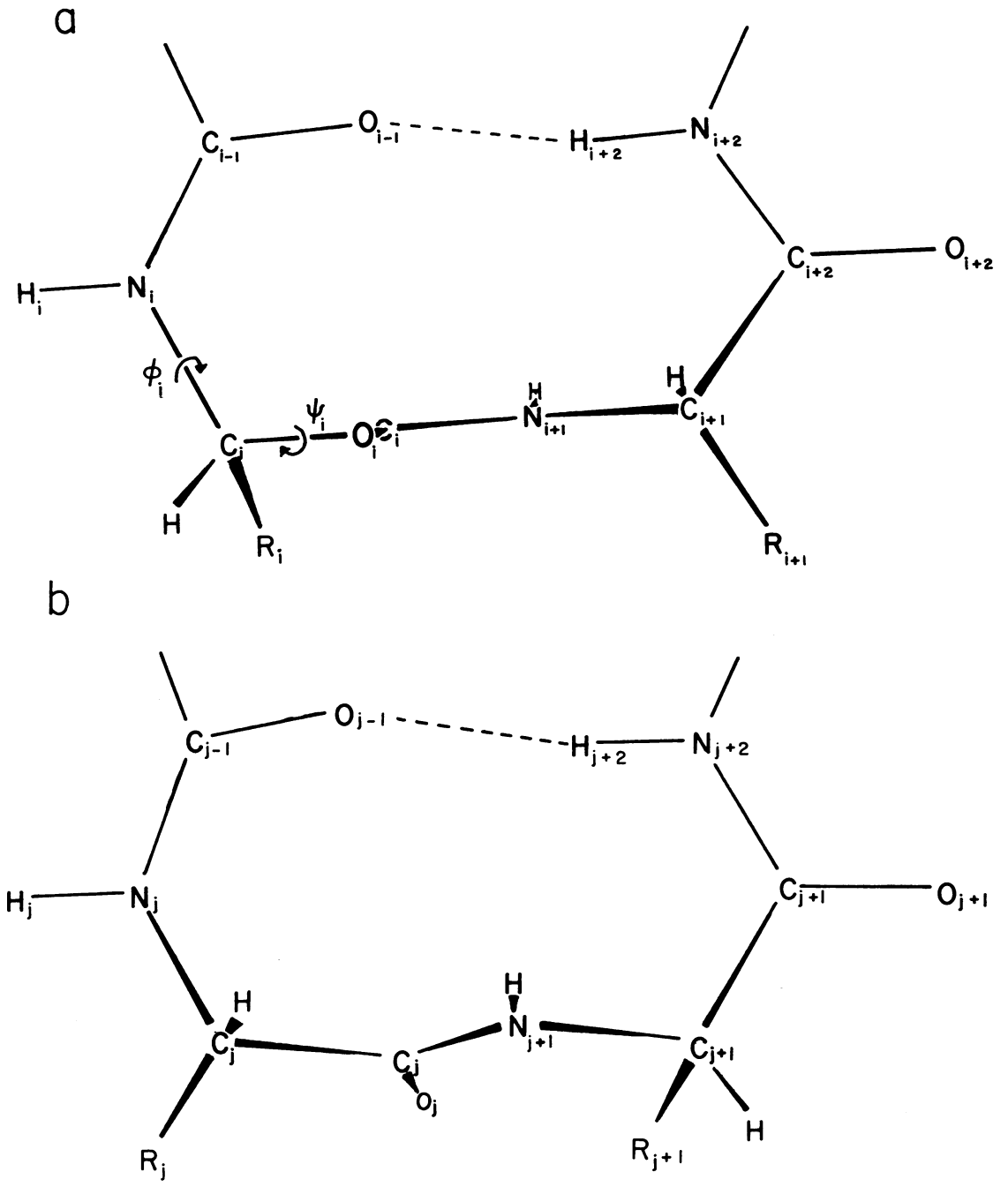

FIG. 1. β-turns plotted from the coordinates of Geddes *et al.* (16). Residues i and $j + 1$ are glycines, i.e., $R_i = R_{j+1} = H$. The peptide moiety formed from i and $i + 1$ in (*a*) and from j and $j + 1$ in (*b*) is referred to as the end peptide of the β-turn. The end-peptide oxygen juts out from the mean plane defined by the polypeptide backbone atoms. The corner occupied by glycine determines the direction in which the C–O points. In both cases the oxygen of the end peptide is well exposed for coordination with cations. (From ref. 15.)

Acyl oxygens of peptide and ester moieties have been found to provide excellent coordination for alkali metal ions. [In this connection it may be noted that the binding constant of elastin for Na^+ has been reported to be greater than that for Ca^{++} (5).] Thus, valinomycin (9, 10), enniatins (11, 12), gramicidin A (13), and nonactins (14) all utilize acyl oxygens in coordination of alkali and alkaline earth metal ions. These antibiotics are neutral molecules with high affinities for cations. Common in these structures is the presence of D residues. It is, interestingly, the sequential order in which the L and D residues exist that is a dominant feature for determining the backbone conformation. Table 2 indicates the optical isomeric sequences for valinomycin, enniatins, gramicidin A, and gramicidin S.

As seen in the gramicidin A sequence, glycine can replace D residues. An important conformational aspect of glycine residues is that they can effect the insertion of near right angle turns in the polypeptide backbone (15). In development of our conformational analysis of antibiotics it was argued that glycines adjacent to residues with bulky side chains insert β-turns (16) (see Fig. 1) and that D residues in place of glycines enhance this effect (15). We now return to glycine, having noted the dramatic effect of D residues in many model poly-

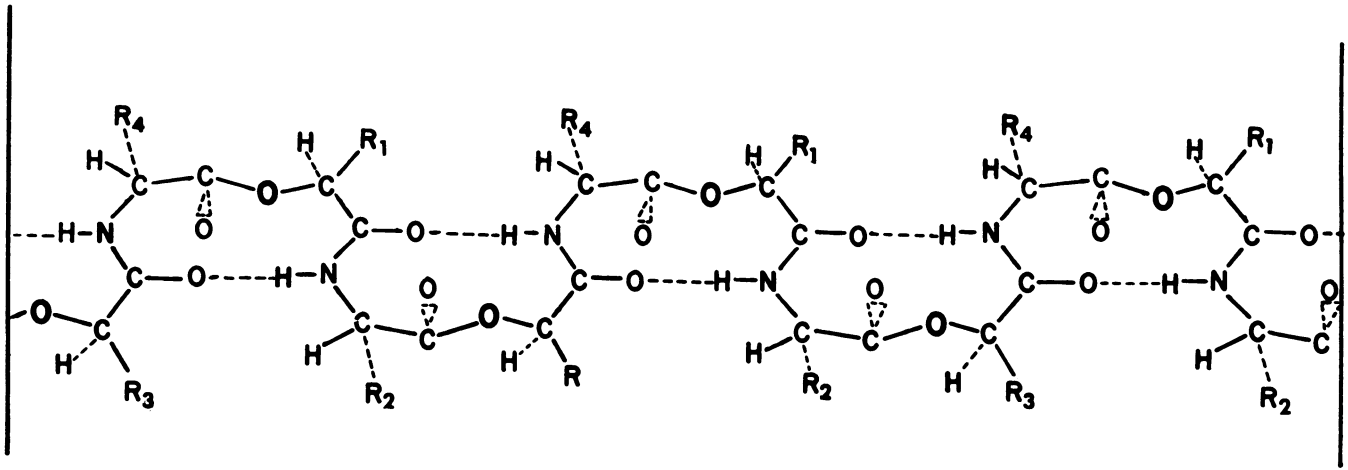

FIG. 2. Secondary structure of valinomycin when complexed with cations. Residues 1 and 2 are of the L configuration and 3 and 4 are of the D configuration. The structure is seen as a series of β-turns. (From refs. 9 and 15.)

peptide and depsipeptide systems. The β-turns depicted in Fig. 1 show the polypeptide backbone to have a mean plane close to that of the paper, with the plane of the end peptide moiety [formed from residues i and $i + 1$ in (*a*) and from j and $j + 1$ in (*b*)] perpendicular to the plane of the paper. When the sequence is a glycine followed by an L-residue such as L-Val with its bulky side chain, then the β-turn can be a stable conformation. When the L residue, with the bulkier side chain, precedes glycine in the sequence, then the β-turn (*b*) can be a stable conformation. In each case the bulky *R*-group can be in the sterically favored equatorial position. The significant feature of a β-turn, from the standpoint of a cationic binding site, is the accessibility for interaction of the end peptide oxygen. Acyl (carbonyl) oxygens from a glycine tripeptide have been shown in crystal structure to directly coordinate calcium (17). In discussion of the crystallographic work it was noted that the calcium ion is capable of holding six to nine coordinating oxygens. The ability of the calcium ion to accommodate irregular crystal fields as well as its valency of two can be considered important factors in its affinity for elastin and in its capacity to initiate crystallization.

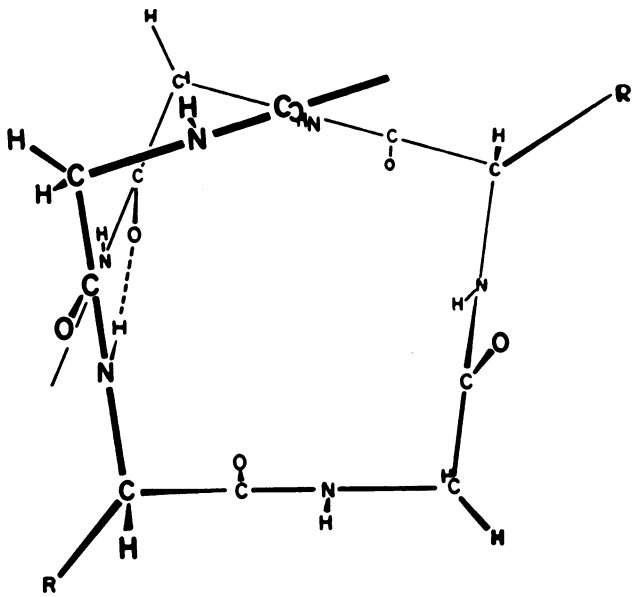

FIG. 3. Helix axis perspective of a left-handed $\pi_{(L,D)}$ helix. Note that in each turn there are two and a fraction C–O moieties pointing up, and two and a fraction C–O moieties pointing down. This suggests that separation of the two turns would allow ready coordination of an inserted cation.

PROBABLE GLYCINE-CONTAINING SEQUENCES AND ASSOCIATED NEUTRAL CATION-BINDING CONFORMATIONS

(*a*) -L-L-Gly-Gly-L-L-. The presence of adjacent glycine residues would allow a conformation that is equivalent to one-half of a valinomycin molecule, with acyl oxygens in position to provide three of six coordinations for an octahedral field. The secondary structure of valinomycin is given in Fig. 2 as a series of β-turns. The structure completes its cyclization by coiling on a center of curvature below the plane, thereby directing six acyl oxygens inward to hold the cation in an octahedral field (9, 10). Adding the sequence Gly-Gly-L-L to the above sequence could conceivably introduce into the elastin polypeptide chain a valinomycin-type structure with a tight binding capacity.

(*b*) -L-Gly-L-Gly-L-. This sequence allows one turn of a $\pi_{(L,D)}$ helix as has been proposed for gramicidin A (13) (Fig. 3). A cation can be inserted between two turns of the helix with the result of an approximate tetrahedral field provided by the peptide oxygens. This is possible with relaxations in conformation of a helix in which there are approximately 4.4 residues per turn and in which adjacent C–O moieties point toward opposite ends of the helix. It is also possible for the ion-relaxed $\pi_{(L,D)}$ helix to provide five, and to relax further to provide six, coordinations for a flattened octahedral field. The enniatins provide an example of a flattened octahedral field which is possible with the addition of a glycine residue to the alternating sequence above (see Fig. 4). This sequence is found in porcine calcitonin.

(*c*) -L-L-Gly-L-L-Gly-. This sequence compares to a β-type structure of a cyclic hexapeptide with two β-turns in which the end peptide oxygens are directed in a manner favorable for ion binding (15). In this case both end C–O groups point in the same direction, that is, they are on the same side

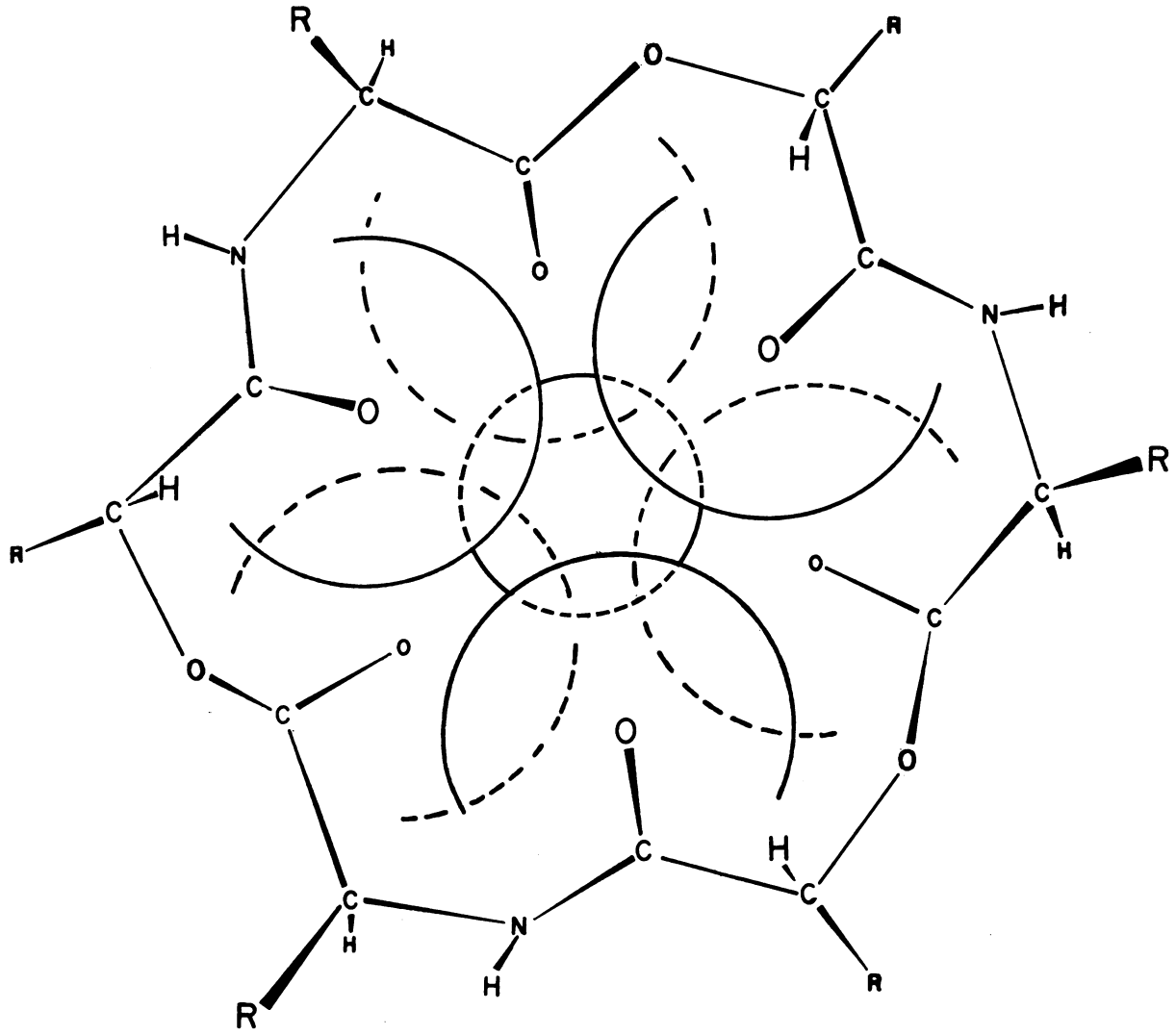

FIG. 4. The enniatin cation complex, showing the cation to be held centrally by an octahedral field of six acyl oxygens (11).

and perpendicular to the gross plane defined by the hexapeptide.

(*d*) -L-Gly-L-Gly-L-L-. This is a sequence which also resembles a β-type structure for a cyclic hexapeptide, but the end peptides capable of coordination have oxygens on opposite sides of the mean plane.

(*e*) -Gly-L-Pro-. The sequence D-L-Pro, which is repeated twice in gramicidin S, inserts two very stable β-turns (18, 19). Gramicidin S is a cyclic decapeptide antibiotic also known to transport cations across lipid bilayers. The Gly-L-Pro sequence is probably due to the 13% L-Pro residues in elastin. This pairing of residues greatly favors β-turn formation.

Thus, probable sequences in elastin can be related to model structures with cation-binding capacities. It should also be apparent that, if the sequences repeat, regions of regular structures can be expected. Such structures may be involved in the elastic properties of elastin. Ordered collagen, by its triple-stranded nature, cannot form the structures, but the unravelled ends or telopeptides of collagen could readily do so. This may be relevant to tendon insertion into bone and to tooth emplacement.

Measurements of circular dichroism suggest (20) that solubilized elastin undergoes a marked conformational change on coacervation. Similar, but less marked, changes occur on addition of ethanol to aqueous solutions (21). Recent studies in this laboratory, to be reported elsewhere, show that the same conformational change occurs in trifluoroethanol containing 2.5% water and in trifluoroethanol containing 2.5% water plus 4% trifluoroacetic acid. Addition of calcium chloride to these systems results in a complete reversion to the aqueous circular dichroism pattern. That this reversal of conformation brought about by calcium ion occurs both in the presence and absence of the strong organic acid, trifluoroacetic acid, strongly supports the contention that the cationic binding sites are neutral, since in the acid solution the side chains are either neutral or positively charged.

CHARGE NEUTRALIZATION THEORY OF CALCIFICATION

That the binding sites for calcium ion are neutral has interesting implications. The affinity of calcium ion for neutral binding sites in elastin means that local areas on the elastin fiber will become positively charged. Space-charge saturation limits the number and proximity of ions bound to neutral sites on elastin; however, such positive calcium-ion-containing loci will attract charge-neutralizing ions such as phosphate

and carbonate. Once a given site is neutralized, adjacent loci can bind calcium ions. These in turn are neutralized, etc., until the crystallization process is initiated. Favorable juxtaposition of binding sites would, of course, facilitate crystal growth by providing a sterically correct nucleation site for crystallization. Accordingly, the protein provides a neutral matrix for holding positive calcium ions, and the driving force for calcification is the affinity of calcium for the neutral binding sites. Binding of calcium ions to the backbone of a single peptide chain and particularly an ion shared by two chains would rigidify the structure and result in loss of elasticity. Also, the charge repulsion between the bound calcium ions could result in extension of the elastin such that calcification may be expected to occur in regions where elastin appears to be extended.

The charge neutralization theory suggests that in arteries and cartilage there should be a natural biological mechanism to impede calcification of elastin, since the binding of calcium ion could be expected to be a chronic process, becoming progressively greater with the age of the elastin. Anionic species other than inorganic phosphate or carbonate are required that would effectively neutralize charge without initiating calcification. An obvious candidate would be the sulfated mucopolysaccharides, which are known to increase in concentration in arteries with age and to be present at high concentrations in cartilage. According to this assumption, for example, regions of the aorta where calcification is becoming prevalent would also be regions deficient in sulfated mucopolysaccharides. Such areas would be prime regions for blood clotting. When sulfated mucopolysaccharides are deficient, lipids and particularly cholesterol could also become associated with the hydrophobic, but positively charged, protein matrix. This sets the stage for a milieu or gruel of blood clot, lipids, and cholesterol and the eventual atherosclerotic plaque. In cartilage the presence or absence of sulfated mucopolysaccharides could be the regulatory mechanism for calcification or bone formation and growth.

Accordingly, the charge neutralization theory of calcification contains aspects that tend to unify several theories on the pathogenesis of atherosclerosis, these being the calcification of elastin and collagen and the lipid filtration and thrombogenic theories. As noted above, the positively charged, yet hydrophobic, elastin would attract cholesterol and negatively charged lipids. Also, there is analogy with protein blood-clotting factors that require calcium ion and are inhibited by heparin and that have bound calcium ions. Conceivably, elastin charged with calcium ion and unprotected by sulfated mucopolysaccharides could directly fill the role of a blood-clotting factor.

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1. Urist, M. R., *Science*, **137**, 120 (1962).
2. Selye, H., *Calciphylaxis* (University of Chicago Press, Chicago, Ill., 1962).
3. Schiffmann, E., B. A. Corcoran, and G. R. Martin, *Arch. Biochem. Biophys.*, **115**, 87 (1966).
4. Hall, D. A., *Biochem. J.*, **59**, 459 (1955).
5. Molinari-Tosatti, M. P., L. Galzigna, V. Moret, and L. Gotte, *Calcif. Tissue Res.*, **2**, Suppl., 88 (1968).
6. Petruska, J. A., and L. B. Sandberg, *Biochem. Biophys. Res. Commun.*, **33**, 222 (1968).
7. Franzblau, C., and R. W. Lent, *Brookhaven Symposia in Biology*, **21**, 358 (1969).
8. Glycine is also the most prevalent amino acid in enamel protein. See, for example, Burrows, L. R., in *Tooth Enamel: Its Composition Properties and Fundamental Structure*, ed. M. V. Stack and R. W. Fearnhead (John Wright and Sons Ltd., Bristol, England, 1965), p. 59.
9. Ohnishi, M., and D. W. Urry, *Science*, **168**, 1091 (1970).
10. Pinkerton, M., L. K. Steinrauf, and P. Dawkins, *Biochem. Biophys. Res. Commun.*, **35**, 512 (1962).
11. Ovchinnikov, Yu. A., V. T. Ivanov, A. V. Evstratov, V. F. Bystrov, N. D. Abdulaev, E. M. Popov, G. M. Lipkind, S. F. Arkhipova, E. S. Efremov, and M. M. Shemyakin, *Biochem. Biophys. Res. Commun.*, **37**, 668 (1969).
12. Dobler, M., J. D. Dunitz, and J. Krajewski, *J. Mol. Biol.*, **42**, 603 (1969).
13. Urry, D. W., *Proc. Nat. Acad. Sci. USA*, **68**, 672 (1971).
14. Kilbourn, B. T., J. D. Dunitz, L. A. R. Pioda, and W. Simon, *J. Mol. Biol.*, **30**, 559 (1967).
15. Urry, D. W., and M. Ohnishi in *Spectroscopic Approaches to Biomolecular Conformation*, ed. D. W. Urry (American Medical Association Press, Chicago, Ill., 1970), p. 263.
16. Geddes, A. J., K. D. Parker, E. D. T. Atkins, and E. Beighton, *J. Mol. Biol.*, **32**, 343 (1968).
17. van der Helm, D., and T. V. Willoughby, *Acta Crystallogr.*, **B25**, 3217 (1969).
18. Stern, A., W. Gibbons, and L. C. Craig, *Proc. Nat. Acad. Sci. USA*, **61**, 734 (1968).
19. Ohnishi, M., and D. W. Urry, *Biochem. Biophys. Res. Commun.*, **36**, 194 (1969).
20. Urry, D. W., B. Starcher, and S. M. Partridge, *Nature*, **222**, 795 (1969).
21. Mammi, M., L. Gotte, and G. Pezzin, *Nature*, **220**, 371 (1968).