# On the Mechanism of Formation of Desmosine and Isodesmosine Cross-Links of Elastin

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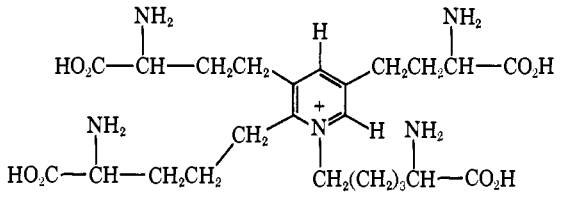
**Abstract:** A mechanism is proposed for the spontaneous formation of desmosine and isodesmosine cross-links which characterize the structural protein elastin. The mechanism is supported by studies with model compounds. These studies show that aliphatic aldimines readily react with aliphatic aldehydes, at room temperature, to yield 1,2-dihydropyridines. The dihydropyridines are readily oxidized by $O_2$ (and other agents) to yield the corresponding pyridinium ions which are analogs of the elastin cross-link, isodesmosine.

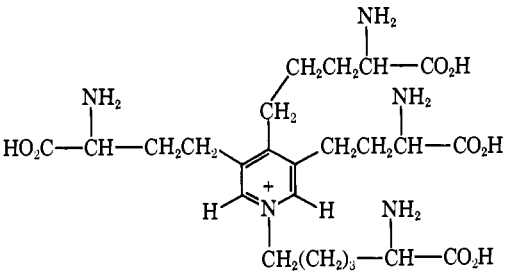
Elastin is a protein which possesses unique elasticity and tensile strength. The fibers which distinguish the protein are found mainly in connective tissues such as large blood vessels and ligaments. In general, elastomers are characterized by flexible, randomly coiled polymer chains, joined together by cross-links into an extensible, three-dimensional network. It has been shown[1] that elastin contains two novel amino acids, desmosine and isodesmosine (Scheme I), which are believed to be chiefly responsible for cross-linking the polypeptide chains of elastin to form insoluble, elastic fibers.

As their structures suggest, and studies[2-5] with radioactively labeled lysine confirm, desmosine and isodesmosine are each derived from four residues of lysine.

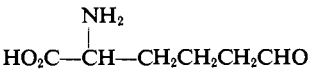
**Scheme I.** Lysine-Derived Amino Acid Residues Found in Elastin



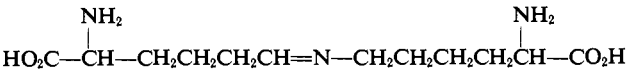
isodesmosine



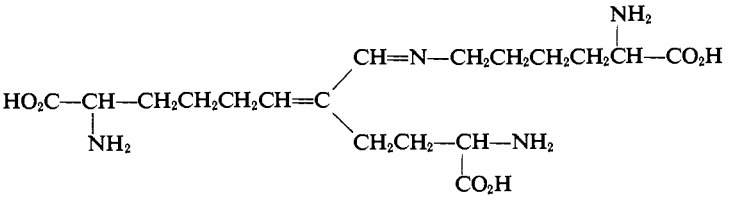
desmosine



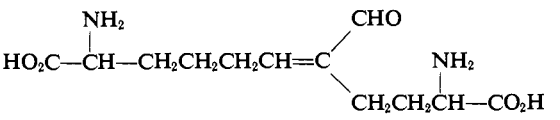
I, allysine (δ-semialdehyde of α-aminoadipic acid[a]



II, $\Delta^{7,8}$-dehydrolysinonorleucine (imine from allysine and lysine)[b]



III, $\Delta^{7,8}$-dehydromerodesmosine (α,β-unsaturated imine)[c]



IV, dehydrated aldol condensation product of allysine[d]

[a] E. J. Miller, S. R. Pinnell, G. R. Martin, and E. Schiffmann, *Biochem. Biophys. Res. Commun.*, **26**, 132 (1967). [b] R. W. Lent and C. Franzblau, *ibid.*, **26**, 132 (1967). [c] B. Starcher, S. M. Partridge, and D. F. Elsden, *Biochemistry*, **6**, 2425 (1967). [d] R. W. Lent, B. Smith, L. Salcedo, B. Faris, and C. Franzblau, *ibid.*, **8**, 2837 (1969).

(1) J. Thomas, D. F. Elsden, and S. M. Partridge, *Nature*, 200, 651 (1963).

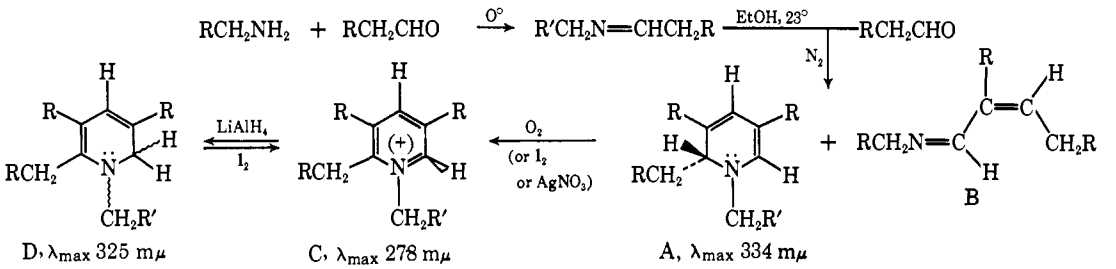
(2) S. M. Partridge, *Fed. Proc.*, **25**, 1023 (1966).

(3) E. J. Miller, G. R. Martin, C. E. Mecca, and K. A. Piez, *J. Biol. Chem.*, **240**, 3623 (1965).

(4) R. A. Anwar and G. Oda, *ibid.*, **241**, 4038 (1966).

(5) R. A. Anwar and G. Oda, *Biochim. Biophys. Acta*, **133**, 151 (1967).

**Scheme II**



R,R′ = $CH_3(CH_2)_2CH_2-$, $CH_3(CH_2)_3CH_2-$; $CH_3CH_2-$, $CH_3CH_2CH_2-$; $CH_3CH_2-$, $CH_3(CH_2)_3CH_2-$; $CH_3CH_2-$, $CH_3O_2C-$; $CH_3(CH_2)_3CH_2-$, $CH_3(CH_2)_3CH_2-$; $CH_3CH_2-$, $Na^+{}^-O_2CCH_2-$; $CH_3(CH_2)_2CH_2-$, CbzNHCH($CO_2CH_3$)$(CH_2)_2CH_2-$

Current evidence indicates that elastin fibers are formed from a polypeptide precursor(s) by a cross-linking process, which apparently involves the oxidative deamination[6] of the ε amino group of certain lysine residues of the precursor(s) to yield the residue allysine, the δ-semialdehyde of (L) α-aminoadipic acid (Scheme I, I). Three residues of allysine could then condense with one lysine residue to yield either desmosine or isodesmosine cross-links. Some workers[7,8] suggested that imines and aldol condensation products (Scheme I) are somehow involved in cross-link formation, but the exact pathway was obscure. In particular, Piez[7] recently pointed out that none of the proposed pathways account for the additional oxidative step which is required by the oxidation state of the cross-links. To account for the formation of these cross-links, we wish to propose the mechanism outlined in Scheme III. This mechanism (see Discussion) features the formation of the stable pyridinium cross-links by the oxidation of *dihydropyridines* which result from the condensation between lysine and lysine-derived residues. Our studies with model systems support this mechanism and are described below.

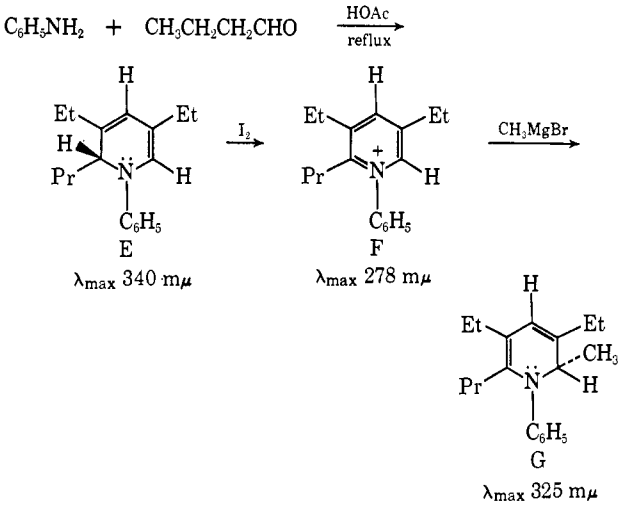
## Results

Several aliphatic aldimines (analogs of $\Delta^{7,8}$-dehydrolysinonorleucine, Scheme I) were prepared from aldehydes (analogs of allysine) and amines (analogs of lysine), as described in the Experimental Section. These aldimines were found to react spontaneously, at room temperature, with a variety of aldehydes (analogs of allysine) to form 1,2,3,5-tetrasubstituted 1,2-dihydropyridines, which were readily oxidized to 1,2,3,5-tetrasubstituted pyridinium compounds (analogs of isodesmosine). This oxidation could be effected by $O_2$ (a possible *in vivo* oxidizing agent) as well as $I_2$ and $AgNO_3$.

These reactions are summarized in Scheme II.

The interconversion A → C → D (Scheme II) was useful in characterizing the products A and C, as well as in demonstrating the ready oxidation of 1,2-dihydropyridines (A) to pyridinium compounds (C), which is a fundamental assumption of our mechanism (Scheme III).

In agreement with the work of Craig, *et al.*,[9] the absorption maxima of the 1,2-dihydropyridines (A) were higher than those of the 1,6-dihydropyridines (D) obtained by reduction ($LiAlH_4$) of the pyridinium compounds (C). These workers synthesized the dihydropyridine, E, by refluxing aniline with butyraldehyde and acetic acid. They assigned the 1,4-dihydropyridine



structure to E and G on the basis of the $\lambda_{max}$ at 340 and 325 mμ, respectively. However, Saunders and Gold[10] later showed that N-phenyl-1,4-dihydropyridine absorbed maximally at 286 mμ, while N-phenyl-1,2-dihydropyridine absorbed at 350 mμ. Therefore, E and G are 1,2- and 1,6-dihydropyridine, respectively. Examination of CPK space-filling models indicates that the steric interaction between substituents on ring positions 1 and 2 would be greater for the 1,6-dihydropyridines than for the 1,2-dihydropyridines. Hence, steric inhibition of resonance could explain the lower absorption maxima of 1,6-dihydropyridines with respect to 1,2-dihydropyridines. It should be mentioned that both $LiAlH_4$ and $CH_3MgBr$ would be expected to add preferentially to the least-hindered 6 position of the pyridinium ring, yielding 1,6-dihydropyridine.

The nmr spectra of the 1,2-dihydropyridines (A) were also consistent with the assigned structure, having the expected resonance peaks ($\delta_{CCl_4}^{TMS}$) at 5.8 ppm (1 olefinic proton, adjacent to N), 5.2 ppm (one olefinic proton), 4.4 ppm (1 proton, triplet, adjacent to C=C and to N), and 3.2 ppm (2 methylene protons, triplet, adjacent to N).

(6) S. R. Pinnell and G. R. Martin, *Proc. Nat. Acad. Sci. U. S.*, **61**, 708 (1968).

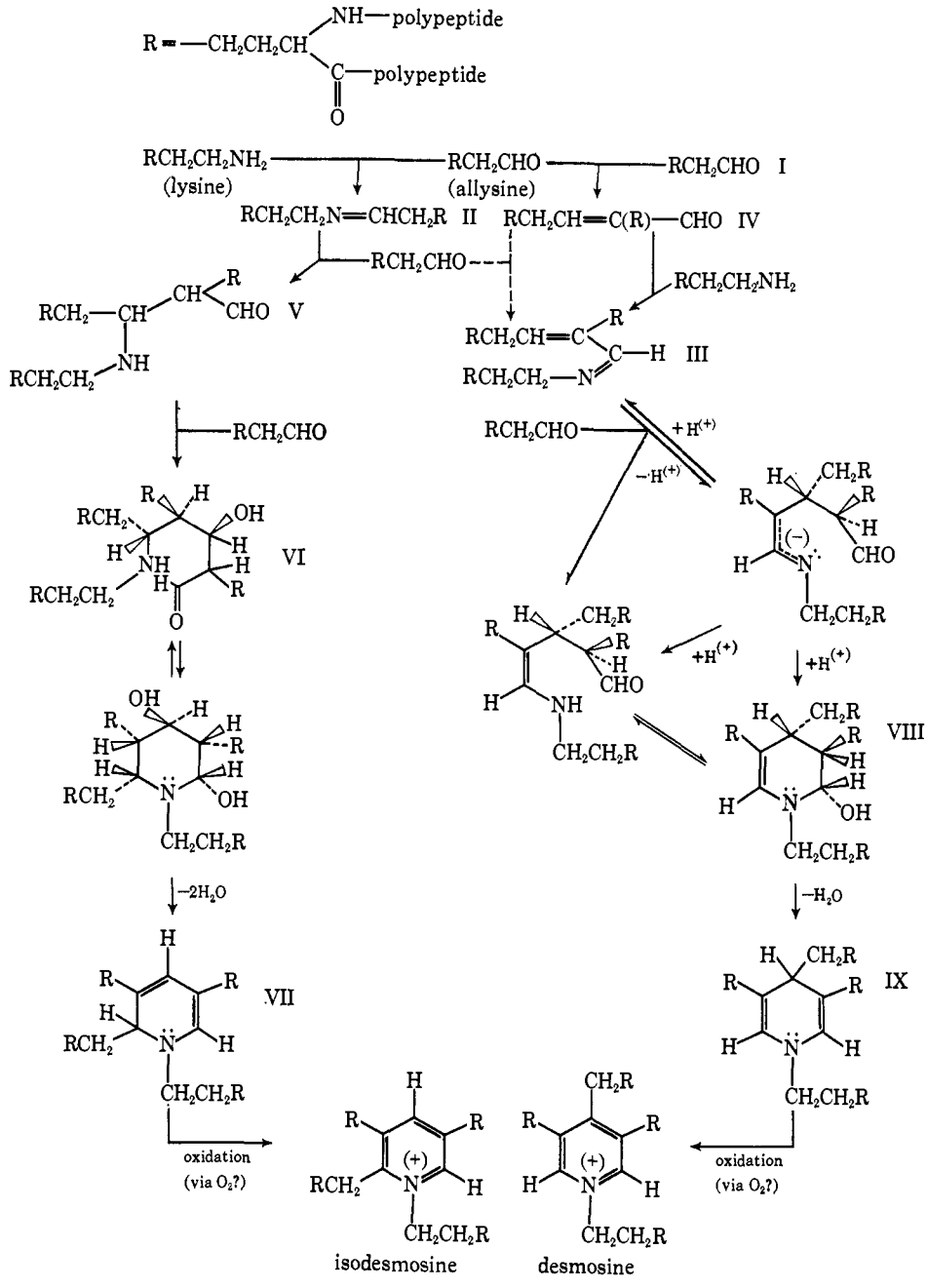
(7) K. A. Piez, *Annu. Rev. Biochem.*, **37**, 547 (1968).

(8) S. M. Partridge, "Fibrous Proteins," W. G. Crewther, Ed., Butterworths, London, 1968, p 246.

(9) D. Craig, L. Schaefgen, and W. P. Tyler, *J. Amer. Chem. Soc.*, **70**, 1624 (1948).

(10) M. Saunders and E. H. Gold, *J. Org. Chem.*, **27**, 1439 (1962). These authors unambiguously assigned the 1,2- and 1,4-dihydropyridine structures to the species with $\lambda_{max}$ at 350 and 286 mμ, respectively, on the basis of nmr and chemical studies.

**Scheme III.** Mechanism of Cross-link Formation




The oxidation of 1,2-dihydropyridines to pyridinium salts with $I_2$ was instantaneous in neutral or alkaline ethanol. However, the titrations with $I_2$ could be followed spectrophotometrically, and the system of absorption curves so obtained had a tight isosbestic point at 298 mμ (indicative of the direct abstraction of hydride by $I_2$). Oxidation with $O_2$ was much slower.

The uv spectra of the model pyridinium salts (C) were essentially identical with the spectrum of isodesmosine, with absorption maximum at 278 mμ ($E_{EtOH}$ = 8000) and a shoulder near 289 mμ. The spectra are quite characteristic of 1,2,3,5-tetraalkylpyridinium compounds in general (*e.g.*, compound F). The nmr spectra of the pyridinium salts in $CCl_4$ revealed the expected features, namely two aromatic singlets at δ 8.6 ($H_\alpha$) and 8.0 ppm ($H_\gamma$), a methylene triplet at 4.7 ppm (2 protons, $-CH_2N$), and six benzyl protons (envelope) at 2.9 ppm. These values are comparable to those reported for isodesmosine[11] itself. The exact aromatic band positions were solvent dependent for all pyridinium compounds studied. The nature of the anion and the concentration of the pyridinium salt also affected the position of the aromatic bands (especially $H_\alpha$).

In further support of the assigned structures, the ir spectra revealed a moderately strong aromatic C=C stretching band at 1628 $cm^{-1}$ (*cf.* 1638 $cm^{-1}$ for N-methylpyridinium iodide).

As expected,[12] the pyridinium salts were reduced with $LiAlH_4$ (by reflux in dry THF) to yield a species classified (see above) as a 1,6-dihydropyridine (D, Scheme II) on the basis of its uv spectrum (325 mμ) and ready oxidation back to the starting pyridinium salts.

(11) G. R. Bedford and A. K. Katritzky, *Nature*, 200, 652 (1963).
(12) H. O. House, "Modern Synthetic Reactions," W. A. Benjamin, Inc., New York, N. Y., 1965, p 40.

When the dihydropyridine reaction mixture (from butyraldehyde and N-butylidenebutylamine) was examined by vpc (10% E6Sx on Gas Chrome P, 5 ft × 1/4 in.; 150°, 40 psi, $N_2$), a second major product was detected. This compound was identified as N-(2-ethyl-3-propyl)-2-propenylidenebutylamine (B, Scheme II) by comparison with the authentic compound synthesized by a modification of the method of Emerson, *et al.*[13] Since this synthesis did not proceed in our hands without acid catalysis, it is possible that Emerson, *et al.*, used butyraldehyde contaminated with butyric acid. The spectra of both products were identical. Both exhibited the expected ir bands at 6.07 (conjugated C=C) and 6.11 $\mu$ (conjugated C=N), as well as uv maxima at 230 m$\mu$ ($E$ = 20,000), which shifted to 259 m$\mu$ upon acidification of the ethanol solution (imine protonation). The nmr spectra in $CCl_4$ showed bands at $\delta$ 6.4 (t, 1 olefinic proton), 7.5 (s, 1 proton on the C=N carbon), 3.3 (t, 2 methylene protons adjacent to N), and 2.2 ppm (m, 4 methylene protons adjacent to C=C).

Reaction of the imine with $NaBH_4$ reduced the C=N bond, thereby destroying the absorption band at 230 m$\mu$, the ir bands at 6.07 and 6.11 $\mu$, and the nmr band at $\delta$ 7.5 ppm, as expected for a compound of the assigned structure.

## Kinetic Results

The initial rates of 1,2-dihydropyridine formation (V) were found to vary in direct proportion to the imine concentration and to the square of the hexanal concentration (over the 0.15–0.25 $M$ imine concentration range and a 1.0–3.0 $M$ hexanal concentration range studied), *i.e.*, $V = k[\text{imine}][\text{hexanol}]^2$.

Initial rates were determined from plots of the observed 1,2-dihydropyridine concentrations against time, which were linear for the first 5–10 min of the reaction. The third-order rate constant, $k$, was estimated to be $2.2 \times 10^{-2}$ ($\pm 0.001$) $\text{mol}^{-2}$ $\text{l.}^2$ min from the variation of initial rates with imine and hexanal concentrations.

## Discussion

The model system demonstrates the ready reaction of allysine analogs (hexanal in particular) with aliphatic aldimines (analogs of $\Delta^{7,8}$-dehydrolysinonorleucine) to yield easily oxidized 1,2-dihydropyridines, as well as $\alpha,\beta$-unsaturated imines (analogs of the $\Delta^{7,8}$-dehydromerodesmosine of elastin). Hence, they lend support to our proposed cross-linking mechanism.

As mentioned in the introduction, the initial, enzymatic event in the cross-linking reaction is believed to be the *oxidative deamination*[6] of certain lysine residues to yield allysine (aldehyde) residues. According to our model, these allysine residues can yield isodesmosine by spontaneous (not enzyme catalyzed) reaction with allysine residues, as indicated in Scheme III.

Condensation between allysine and an appropriately oriented lysine residue would yield an imine ($\Delta^{7,8}$-dehydrolysinonorleucine, II, Scheme I, and Scheme III) which could in turn condense with another allysine residue to yield the amino aldehyde, V. Aldol condensation of this species, V, with a third allysine residue would yield a species, VI, which could easily cyclize and dehydrate to form 1,2-dihydroisodesmosine, VII. This species, in turn, could be oxidized (quite possibly by $O_2$ from the plentiful blood vessels of the tissue) to form the stable isodesmosine cross-link.

Starcher, *et al.*,[14] have recently demonstrated the probable existence of dehydromerodesmosine, III, in elastin, and have suggested that this unsaturated imine (likely $\alpha,\beta$-unsaturated imine) is a precursor of desmosine. Our model studies show that this species can readily arise from aldol attack of imine on aldehyde, but the alternate pathway[7] (lysine reacting with the dehydrated aldol condensation product of 2 allysines, IV) is equally plausible for the *in vivo* system. Indeed, both pathways to the $\alpha,\beta$-unsaturated imine, III, could be operative *in vivo*.

Michael addition of an aldehyde to $\alpha,\beta$-unsaturated imines and subsequent formation of 1,3,4,5-tetrasubstituted 1,4-dihydropyridines were not observed under the conditions of our model system.[15] However, proper steric disposition of these residues *in the protein* could favor the (reversible) Michael addition (I and III). Subsequent to cyclization, dehydration of the Michael adduct, VIII, to 1,4-dihydrodesmosine, IX, and oxidation of this species to yield the stable desmosine cross-links would pull the Michael reaction to completion. Although the mechanism for desmosine formation is proposed tentatively, it is strengthened by analogy to the isodesmosine mechanism, which is strongly supported by the model system (structural and kinetic data).

The above model accounts for all the lysine-derived residues found in elastin (including lysinonorleucine, a trace component arising from reduction of the imine group of II). Both desmosine and isodesmosine pathways lead to an easily oxidized dihydropyridine species, a feature which would clarify the nature of the previously obscure[7] second oxidative step. The hexanal–hexylamine system is an especially appropriate model, since the examination of space-filling (CPK) models shows that the steric situation around the functional groups and the ring is virtually identical with that for allysine and lysine.

A fundamental assumption of the model is that after the oxidative deamination of the lysine residues, the reactions are spontaneous and do not require catalysis by enzymes. During the preparation of this manuscript, a paper by Barnes, *et al.*,[16] appeared. These workers obtained a sample of aortic elastin from guinea pigs 5 days after they had been fed [4,5-$^3$H]-lysine. Soluble elastin, obtained from insoluble elastin by treatment with boiling oxalic acid, contained desmosine and isodesmosine with specific activities of 875 and 918 cpm/$\mu$mol, respectively. Upon incubation of this soluble elastin for 1 day, the specific activities rose to 1726 and 1724 cpm/$\mu$mol for desmosine and isodesmosine, respectively. Apparently, in this soluble, enzyme-free elastin, as many cross-links formed in 1 day as formed in the living tissue in 5 days. As assumed in our model of cross-link formation, the generation of desmosine and isodesmosine from lysine and lysine-derived residues is necessarily nonenzymatic in this *in vitro* system.

(13) W. S. Emerson, S. M. Hesse, and F. C. Uhle, *J. Amer. Chem. Soc.*, **63**, 872 (1941).

(14) See Scheme I, footnote *c*.

(15) Side reactions were too fast.

(16) M. J. Barnes, B. J. Constable, and E. Kodicek, *Biochem. J.*, **113**, 387 (1969).

As Piez[7] has recently pointed out, it is very difficult to visualize how an enzyme[17] could function within the dense network of the elastin fiber. It seems likely that *in vivo*, the rate-limiting step(s) would be the disposition of the reactive lysine and lysine-derived side-chain functional groups. The rapid formation of pyridinium compounds in both our model system and in the soluble elastin[16] certainly indicates that once the relevant groups are in appropriate positions, the cross-links form readily. In any case, these studies show that a non-enzymatic pathway is available for the spontaneous generation of elastin cross-linkages.

## Experimental Section

All aldehydes and amines used were fresh reagent grade chemicals and were not further purified. Infrared spectra were recorded in $CHCl_3$ or thin films on a Perkin-Elmer Model 257 spectrophotometer. Ultraviolet spectra were recorded in ethanol on a Unicam Model SP800 or a Cary 14 recording spectrophotometer. Nmr spectra were recorded in $CCl_4$ (TMS internal standard) on a Varian A60 spectrometer.

(i) Aldimines from simple, monofunctional amines and aldehydes were synthesized by the method of Tiollais[18] and distilled for immediate use: ir 6.01 ± 0.02 $\mu$ (C=N).

(ii) **N-Butylideneglycine Ethyl Ester.** Glycine ethyl ester (0.10 mol) was dissolved in 20 ml of acetonitrile, and the solution cooled in an ice bath. Butyraldehyde (0.10 mol, 9.4 ml) was added dropwise to the cooled, magnetically stirred amine solution over a period of 1 hr. By this time, the mixture consisted of two phases. The lower phase (1.4 ml of $H_2O$) was removed, and the upper phase stirred for 10 min with BaO to remove further traces of $H_2O$ and complete dehydration of the intermediate amino alcohol.

*In vacuo* removal of acetonitrile left a yellowish oil, which was used fresh in the dihydropyridine synthesis: ir 5.74 (C=O) and 6.01 $\mu$ (C=N).

(iii) **N-Butylidene-β-alanine.** The sodium salt of β-alanine (0.10 mol) was dissolved in 25 ml of absolute ethanol, and the solution cooled to 10° in a water bath. Butanal (0.1 mol, 8.4 ml) was added dropwise to the cooled, magnetically stirred solution over a period of 1 hr, and the yellow solution stirred for 10 min with BaO to force dehydration of the intermediate amino alcohol. The BaO–$Ba(OH)_2$ was removed by centrifugation and the BaO treatment repeated. The aldimine was used fresh (as the ethanol solution) in the dihydropyridine synthesis: ir, 6.01 $\mu$ (imine).

(iv) **N-Hexylidene-(N-Cbz)-lysine Methyl Ester.** This imine was prepared from N-Cbz-(L)-lysine methyl ester (5 mmol) and hexanal (5 mmol) by the procedure outlined (iii) for N-butylidene-β-alanine, and was used fresh.

**Dihydropyridine Synthesis.** The method used for all dihydropyridine preparations was as follows. To a 1 *M* solution of aldimine in absolute ethanol, cooled to 0° in an ice–water bath, enough aldehyde was added, all at once, to give a 1.5–2.0 *M* solution. The round-bottomed reaction flask was quickly flushed with $N_2$, and stirred under $N_2$, at room temperature (water bath), for 3–4 hr. By this time, the uv absorption for reaction mixture aliquots (diluted suitably) had reached a maximum at 330–334 m$\mu$. The species absorbing at 330–334 m$\mu$ were shown to be 1,2,3,5-tetrasubstituted 1,2-dihydropyridines.

**Oxidation of Dihydropyridine to Pyridinium.** After 4 hr of stirring, the dihydropyridine reaction vessel was flushed out with $O_2$, and connected to an $O_2$ tank, and the stirring was continued. Samples removed periodically revealed a progressive decrease in the absorbance at 330–334 m$\mu$, and a corresponding increase in absorbance at 278 m$\mu$, due to the oxidation of dihydropyridine to a pyridinium compound.

The oxidation could be conveniently followed by transferring an aliquot of dihydropyridine solution to a cuvette containing ethanol saturated with $O_2$, and continuously scanning the spectra over the 270–340 m$\mu$ region.

The oxidation could also be effected by $I_2$ and $AgNO_3$. The oxidation rate with $I_2$ was too fast to measure (instantaneous). Spectrophotometric titration of dihydropyridine with $I_2$ yielded a system of absorption curves with an *isosbestic* point at 298 m$\mu$.

Pyridinium salt from the preparative scale $O_2$ oxidation of 1-hexyl-2-pentyl-3,5-dibutyl-1,2-dihydropyridine was chromatographed (after vacuum distillation of the solvent) on 100-mesh Mallinkrodt silica gel (mixed with one-half its volume of Celite and activated at 110° for 1 hr). The oxidation reaction mixture was applied in hexane, and after elution of several impurities with ethyl acetate, the pyridinium peak (blue-white fluorescence on the column under a uv lamp) was eluted with absolute ethanol. A yellow oil remained after the evaporation of the solvent.

Since the ir spectrum revealed the presence of considerable carbonyl impurity in the eluted pyridinium compound, the material was dissolved in an equal volume of $CHCl_3$ and repeatedly extracted with saturated aqueous sodium bisulfite until no carbonyl band was detected in the ir. The resultant yellowish oil was converted to the chloride salt by dissolution in ethanol containing excess concentrated HCl. Evaporation of the solvent and repeated evaporation of ethanol solutions yielded a very viscous, slightly yellow oil (mp −26 ± 1°). This was homogeneous on tlc (silica gel, E. Merck HR, extra pure; activated 1 hr, 110°) in a variety of developing solvents: nmr ($CCl_4$) $\delta$ 8.6 (s, 1 aromatic proton), 8.0 (s, 1 aromatic proton), 4.7 (t, $CH_2N$), and 2.9 ppm (m, 6 benzyl protons).

*Anal.* Calcd for $C_{24}H_{44}NCl \cdot {}^3/_2H_2O$: C, 70.60; H, 11.58; N, 3.43; Cl, 8.67; mol wt, 409. Found: C, 70.83, 70.80; H, 11.50, 11.77; N, 3.36, 3.59; Cl, 8.77, 8.38; mol wt, 410 ($Cl^-$ titration); 350–450 (Sephadex G-10 chromatography).

**N-(2-Ethyl-3-propyl)-2-propenylidenebutylamine.**[19] N-Butylidenebutylamine was refluxed with 0.25% (by volume) glacial acetic acid for 3 hr. The reaction mixture was distilled, and the fraction boiling between 200 and 240° was collected. This fraction was redistilled, and the material (63% yield) boiling between 218 and 220° (1-ft Vigreux column) was used for spectral comparison to the α,β-unsaturated imine (B, Scheme II, R, $R^1$ = Et, Pr) formed in the dihydropyridine reaction mixtures.

*Anal.* Calcd for $C_{12}H_{23}N$: C, 79.5; N, 7.74; H, 12.8. Found: C, 79.1; N, 7.76; H, 12.9.

**Kinetic Experiments.** Enough hexanal was added to a N-hexylidenehexylamine (double-distilled) solution in ethanol to give the desired molarity after the volume had been adjusted rapidly with ethanol. The reaction vessel was flushed with $N_2$ and stirred under $N_2$ at room temperature throughout the run. Aliquots were removed at set times with a Hamilton syringe and diluted $^1/_{1000}$ in 10 ml of ethanol containing slightly more than enough $I_2$ to instantly oxidize all the dihydropyridine to the stable pyridinium species. The quenched, oxidized samples were examined (after destruction of excess $I_2$ with $Na_2S_2O_3$) for absorption at 279 m$\mu$ to determine the amount of pyridinium and, hence, dihydropyridine which had formed by the time of sampling. These results were plotted (dihydropyridine concentration *vs.* time) to yield initial rates of dihydropyridine formation.

**Acknowledgment.** We thank the Medical Research Council of Canada for their generous support of this work. One of us (N. R. D.) holds a Medical Research Council Predoctoral Studentship.

(17) Our colleague, Dr. C. S. Hanes, has suggested that elastin itself could provide some catalysis through appropriate acidic and basic residues.

(18) R. Tiollais, *Bull. Soc. Chim. Fr.*, **14**, 708 (1947).

(19) A modification of the method of Emerson, *et al.*[13]