Acknowledgments. The BTI group thanks Z. Kratky, B. Bradford for help in the initial phase of this work; J. Westa, D.J. Burgess, M.B. Stimmel for excellent technical assistance; Peter Demou, Yale University, for obtaining some of the NMR spectra; T. Wachs, Cornell University, and David Fuller, Cornell University, for obtaining the CI mass spectra; R.P. Scheffer, Michigan State University, and H.E. Wheeler, University of Kentucky, for providing the strains of *C. victoriae*. Supported in part by grants from U.S. Department of Agriculture (82-CRCR-1-11-49) and from U.S. National Science Foundation (8314357) to V.M. The Bruker WM-500 NMR spectrometer, NSF facility at Yale University is supported in part by a grant from the U.S. National Science Foundation (CHE-791620).
The ETH group thanks R. Brunner for preparing the diastereomeric racemates of the two hydroxyamino acids. Financial support from Sandoz AG to D.A. is gratefully acknowledged.
Abbreviations used: FAB, fast atom bombardment; CI, chemical ionization; TFA, trifluoracetic acid; THF, tetrahydrofuran; HPLC, high performance liquid chromatography; TLC, thin layer chromatography.

* Signals at δ 6.15, 1H, s (C–6–H); δ 3.85, 1H, dd, $J_1 = 9$, $J_2 = 4.5$ (C–2–H; δ 2.75, 1H, dd, $J_1 = 15$, $J_2 = 4.5$ and δ 2.6, 1H, dd, $J_1 = 15$, $J_2 = 9$ (C–3–H_2); δ 1.89, 3H, s (C–5–H_3).

** We thank Proff. Meinwald and Gloer for informing us that they have independently carried out hydrolysis studies with the HV toxin isolated by Walton and Earle[10] and have identified the structures of three of the fragments as 5,5-dichloroleucine, erythro-β-hydroxyleucine and a β-hydroxylysine of nonspecified relative configuration.

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Studies on the fungal phytotoxin victorin: structures of three novel amino acids from the acid hydrolyzate

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Summary. The host-selective phytotoxin victorin, produced by the fungus *Cochliobolus victoriae*, was found to be at least partially peptidic in nature, and did not contain victoxinine. The exact mass of the M-H ion was measured by FABMS as 795.1877. Derivatives of three major acid hydrolysis products were isolated. The structures of the corresponding amino acids were assigned as 2S,3R-3-hydroxyleucine, 5,5-dichloroleucine, and 3-hydroxylysine. A fourth hydrolysis product was also isolated and partially characterized. Tritiated leucine, but not ^{35}S, was incorporated into victorin by the fungus in vivo.
Key words. Cochliobolus victoriae; Helminthosporium; Bipolaris; Avena.

Fungi which produce toxins are responsible for many important crop diseases. Some of these toxins exhibit host-selectivity, that is, they affect only particular varieties or genotypes of a plant species. Such specificity suggests that structural knowledge of these toxins could lead to better understanding of the fundamental mechanisms of plant pathogenesis.

Victorin, also known as HV-toxin, was the first host-selective toxin discovered and is the most toxic and most selective of the twenty or so currently known[3,4]. Victorin is produced by the plant pathogenic fungus *Cochliobolus victoriae* Nelson (imperfect stage *Helminthosporium victoriae* Meehan and Murphy or *Bipolaris victoriae* Shoem.) which parasitizes certain cultivars of oat, *Avena*

sativa. We report here our studies on the structure of victorin, which support data obtained independently and recently presented by other workers[5,6].

Results and discussion

Victorin was purified by high-performance liquid chromatography (HPLC) as described previously[7]. It was isolated as a pale yellow amorphous solid which was soluble in methanol, ethanol, DMSO, and water. It gave a purple color with ninhydrin, suggesting the presence of at least one primary amino group. Its UV spectrum in methanol contained an absorption maximum at 243 nm ($\varepsilon = 6500$). The IR spectrum contained amide carbonyl absorptions, and the 300-MHz ^{1}H-NMR spectrum in D_2O showed signals suggestive of a small peptide. Analysis by electron impact mass spectrometry (EIMS) failed to provide any useful information. Fast atom bombardment (FAB) mass spectral analysis showed a number of ions in the range from m/z 750 to 1000. All of these major ions were associated with large M + 2 and M + 4 isotope peaks, implying the presence of a number of halogen atoms. Changing the matrix from glycerol to thioglycerol gave a set of completely different ions in this mass range. The positive- and negative-ion spectra were also quite different from each other in each matrix. Comparison of the data led to the conclusion that multiple adducts and dehydrated adducts of the molecular ion with molecules of the matrices were being formed. Both negative-ion spectra did show an ion at m/z 795 which was assigned as the M-H ion, since all the other ions at higher mass could be ascribed to adducts or dehydrated adducts of this species with glycerol or thioglycerol. The exact mass of this ion was measured by high resolution FABMS as 795.1877 daltons. Unfortunately, high background hindered interpretation of the isotope clusters and the number of halogen atoms present was not conclusively determined, though the presence of bromine was excluded.

Analysis of victorin by carbon-13 NMR spectroscopy (D_2O) showed the presence of at least 25 carbons, including at least 5 carboxyl or amide carbonyl carbons, but did not give a conclusive carbon count due to small sample size and the presence of a number of small peaks. These could be explained by the presence of two or more forms of victorin which have slightly different C-13 NMR spectra. Carbon signals from the different forms which did not overlap would give rise to two (or more) signals with fractions of the expected intensity. This suggests that victorin exists in solution in at least two forms which differ by tautomerization, in the degree of protonation, or in some similar way. Additional signals were present at 114 and 163 ppm, which suggested that trifluoroacetic acid (TFA) used in the isolation procedure combines with victorin to form a TFA salt.

Acid hydrolysis of victorin (0.5 mg; 6 N hydrochloric acid, 110 °C, 24 h) gave a mixture of ninhydrin-positive products. Amino acid analysis gave several peaks, but none corresponded to any of the more common amino acids. A sample of the hydrolyzate was treated with butanolic hydrogen chloride, followed by trifluoroacetic anhydride, to give a mixture of trifluoroacetyl *n*-butyl esters (TFA *n*-butyl esters) of the amino acids present. Analysis of this mixture by gas chromatography (GC)[8], followed by EIMS and chemical ionization mass spectrometry (CIMS) showed the presence of three major volatile components. The first gave a GC/CIMS M + H ion at m/z 396 and major GC/EIMS ions at M-114 (loss of TFA) and M-101 (loss of a carboxybutyl group). These ions suggested that this component was a derivatized hydroxyleucine, but the location of the side-chain hydroxyl group could not be assigned by MS.

The second component gave an M + H ion at m/z 352 by GC/CIMS. The EI mass spectrum contained an M-101 peak and also revealed the presence of two chlorine atoms in the molecule, as evidenced by isotope peak ratios as well as by two consecutive losses of HCl. These data suggested that this component is an α-amino acid derivative with a $C_4H_7Cl_2$ side-chain. Again, however, the fragmentation patterns were not sufficient to allow assignment of the connectivity of the side-chain. A much smaller GC peak (< 5%) eluted just before this component. Comparison of its mass spectrum to those of the dichloro-amino acid derivative clearly showed that this minor component was its N-methylated analog. Thus, it appears that the victorin preparation described here is actually a mixture of two or more homologs, but containing one major component.

The third major amino acid derivative gave a M + H ion at m/z 507 by GC/CIMS, suggesting an even number of nitrogen atoms. The GC/EI mass spectrum showed major ions at M-114 (loss of TFA) and M-101 (loss of a carboxybutyl group). Other fragments, coupled with knowledge of the molecular weight, led us to the assignment of this component as a hydroxylysine derivative. Again, the exact structure of this compound could not be assigned from mass spectral data alone.

Efforts were undertaken to isolate samples of these amino acid derivatives for ^{1}H-NMR analysis. A 2-mg sample of slightly impure victorin was subjected to acid hydrolysis and derivatized as described above, using methanolic HCl in place of butanolic HCl, so that the NMR spectra would not contain carboxybutyl group multiplets. The resulting mixture was subjected to semipreparative GC, and 150–300 μg of each component was collected. The 300-MHz ^{1}H-NMR spectrum ($CDCl_3$)[9] of the first component contained doublets for two diastereotopic methyl groups (0.97 and 1.11 ppm) which were coupled to a methine proton (2.18 ppm; m). This proton was also coupled to a downfield-shifted methine proton (5.04 ppm; dd) which was, in turn, coupled to the α-proton (4.97 ppm; dd). The α-proton was recognized by its coupling to the amide N-H resonance at 7.06 ppm. A complete set of decoupling experiments corroborated these assignments, and the structure of this compound was assigned as N-O-*bis* trifluoroacetyl-3-hydroxyleucine methyl ester (**1**). Coinjection with authentic derivatized samples of commercially available *threo*- and *erythro*-3-hydroxyleucine demonstrated that it is an *erythro* isomer. The underivatized 3-hydroxyleucine (**2**) from victorin was decomposed by L-amino acid oxidase, but not by D-amino acid oxidase, suggesting that it is the 2S,3R-enantiomer.

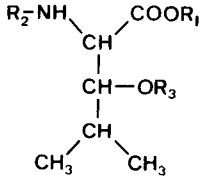
A complete set of ^{1}H-NMR decoupling experiments[9] was also performed on the second component. Irradiation of either a methyl doublet at 1.23 ppm or a downfield-shifted methine doublet at 5.77 ppm simplified a methine

multiplet at 2.15 ppm. Irradiation of this multiplet collapsed the doublets at 1.23 and 5.77 ppm to singlets, and changed two broadened doublets of doublets at 1.97 and 2.09 ppm. Irradiation of either of these one-proton signals collapsed the other to a broadened doublet, simplified the methine at 2.15 ppm, and changed a multiplet at 4.70. The multiplet at 4.70 was found to be coupled to a broad amide N-H doublet at 6.75 ppm, as well as to the two signals at 1.97 and 2.09 ppm. These results, along with the mass spectral data, indicated that this component must be N-trifluoroacetyl-5,5-dichloroleucine methyl ester (**3**). The occurrence of the corresponding free amino acid (**4**) has not been previously reported. Based on this information, the homologous amino acid derivative described earlier was assigned as N-trifluoroacetyl-N-methyl-5,5-dichloroleucine methyl ester (**5**). The corresponding amino acyl unit has recently been reported as a structural unit of a minor metabolite of a marine sponge[10].

For the third GC component, irradiation of a broad amide resonance at 6.44 ppm simplified a two-proton signal at 3.42 ppm[9]. Irradiation of this apparent methylene signal sharpened the resonance of 6.44 ppm to a broadened singlet, and changed a four-proton multiplet at 1.74 ppm. This multiplet was ascribed to two overlapping methylene signals. Irradiation of this multiplet simplified the signal at 3.42 ppm and collapsed a methine multiplet at 5.62 ppm to a doublet. Irradiation of this resonance changed the multiplet at 1.74 ppm and collapsed a one-proton doublet of doublets at 4.92 ppm to a doublet. This proton was also shown to be coupled to an amide N-H doublet at 6.96 ppm. Combining this information with the mass spectral data discussed above led to the characterization of this component as N,N′,O-*tris*-trifluoroacetyl-3-hydroxylysine methyl ester (**6**). The corresponding amino acid **7** has not been previously reported as a natural product[11].

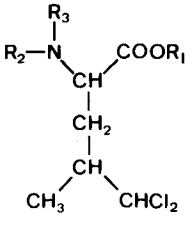
Totalling the molecular formulae of the three amino acyl residues now shown to be present in victorin gives $C_{18}H_{36}N_4O_5Cl_2$, which accounts for over half of the total molecular weight. There was no evidence for any additional major components in the GC trace of the derivatized amino acid mixture. ^{1}H-NMR analysis of a sample of the hydrolyzate itself, however, did reveal the presence of (at least) one other component. Knowing the structures of the three amino acids described above allowed assignment of their signals in the ^{1}H-NMR spectrum of the hydrolyzate. After accounting for all of the resonances expected for the known amino acids, a few multiplets remained. Two of these multiplets were shown by decoupling experiments to comprise the two spin systems depicted in figure 1. Corresponding multiplets were clearly present in the ^{1}H-NMR spectrum of victorin, and the assignment of these to a missing structural fragment allowed the assignment of nearly all of the other resonances in the spectrum to the known amino acyl units. Once these correlations were established, it became apparent from chemical shift comparisons that the hydroxyl groups of the hydroxyleucyl and hydroxylysyl units and the 6-amino group of the hydroxylysyl unit are not acylated in the intact peptide.

The compound containing the spin system in figure 1 was eventually isolated in μg quantities by HPLC fractionation of the hydrolyzate[12], but the polarity of this component and sample limitations have thus far precluded its identification. It is responsible for the UV activity of victorin (λ_{max} 266 nm, ε approximately 5000), and this facilitated its isolation. It gave a blue color with ninhydrin and was soluble in water and methanol. Treatment with methanolic HCl followed by TFA anhydride gave a less polar, ninhydrin-negative product. At pH values less than 7, the λ_{max} of this derivative (or the underivatized component) dropped off rapidly, levelling off at pH 2 (λ_{max} 250 nm). A plot of wavelength versus pH suggested a pK of about 4.8 for the functional group associated with this variability. Since any free carboxyl or simple acylable groups should have been blocked by the derivatization conditions, this functionality could not be any such group. This is consistent with the polarity and lack of



1 $R_1 = CH_3$; $R_2 = COCF_3$; $R_3 = COCF_3$

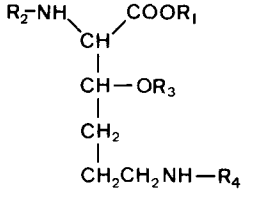
2 $R_1 = H$; $R_2 = H$; $R_3 = H$



3 $R_1 = CH_3$; $R_2 = COCF_3$; $R_3 = H$

4 $R_1 = H$; $R_2 = H$; $R_3 = H$

5 $R_1 = CH_3$; $R_2 = COCF_3$; $R_3 = CH_3$



6 $R_1 = CH_3$; $R_2 = COCF_3$; $R_3 = COCF_3$; $R_4 = COCF_3$

7 $R_1 = H$; $R_2 = H$; $R_3 = H$; $R_4 = H$

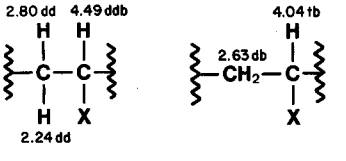


Figure 1. Partial proton NMR data on the unidentified chromophore in victorin.

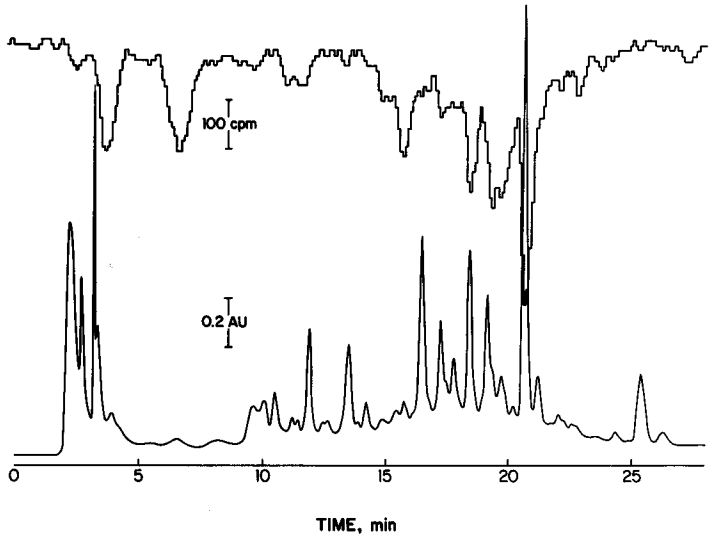


Figure 2. Analysis by HPLC of a sample of partially purified culture filtrate of *C. victoriae*, showing radioactivity (upper trace) and OD_{254} (lower trace). Four days after inoculation of one 1-l flask containing 125 ml modified Fries medium[7] plus 0.5 g oat flakes, 50 µCi of sterile L-[4,5-^{3}H]leucine (Amersham) were added, and the flask incubated an additional 5 days. The flasks were kept at 21 °C on an open laboratory bench throughout the experiment. The culture filtrates were collected by filtration through cheesecloth and concentrated to approximately 20 ml by rotary flash evaporation at 40 °C. Methanol (100 ml) was added and the culture filtrates placed at 4 °C overnight, then filtered (0.2 µm membrane), flash evaporated, and refiltered (0.2 µm). An aliquot was injected onto the HPLC (Beckman Model 332, Whatman PX5 10/25 ODS 3 column), eluted with a 0% B to 60% B (solvent A: 0.1% TFA in H_2O; solvent B: 0.1% TFA in acetonitrile) linear gradient in 30 min, flow rate 1.5 ml/min, and monitored for UV absorption (254 nm, Beckman Model 160) and radioactivity (Radiomatic HP in-line scintillation counter, scintillant mixture flow rate 4 ml/min). The major peak of victorin biological activity elutes at 21 min.

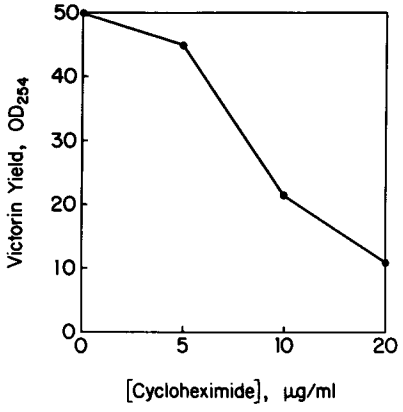


Figure 3. Inhibition of victorin biosynthesis by the protein-synthesis inhibitor cycloheximide. L-[4,5-^{3}H]leucine (40 µCi) and cycloheximide at the indicated concentrations were added together 4 days after inoculation, and the culture filtrates collected, partially purified, and analyzed by HPLC as in the legend to figure 2. Quantitation of victorin was based on height of the peak eluting at 21 min (fig. 2).

volatility of this derivative. Even after derivatization, it could not be analyzed by gas chromatography and was soluble only in water, methanol, and DMSO.

Several early studies on victorin suggested that it contains the seventeen-carbon alkaloid, victoxinine[13]. We were able to isolate victoxinine from culture filtrates of *C. victoriae* by solvent extraction[14] and HPLC, but were unable to find it as a component of victorin.

To obtain further evidence that these structural conclusions were correct, experiments were done in which possible metabolic precursors of victorin were fed in vivo to *C. victoriae*. Although ^{35}S (as $^{35}SO_4^{-2}$) was not incorporated into victorin, L-[4,5-^{3}H]leucine was (fig. 2). Tritiated leucine was incorporated not only into the peak at 21-min elution time corresponding to the major peak of victorin biological activity and UV absorbance, but also into two peaks at slightly earlier retention time, corresponding to minor peaks of UV absorption and biological activity[7]. On the basis of experiments in which the total leucine incorporation was low, for example, when the flasks were harvested only one or two days after addition of ^{3}H-leucine, or when cycloheximide was added (see below), the peak eluting at 7-min was tentatively assigned to residual free ^{3}H-leucine. The identities of the other peaks of radioactivity are unknown.

In one experiment 5 mCi of L-[4,5-^{3}H]leucine was added to a single 1-l flask containing 125 ml medium, 4 days after inoculation with *C. victoriae*. The culture filtrate was collected on day 9, and the major victorin species purified by HPLC (see legend to fig. 2). The specific activity (molarity calculated by UV absorption, radioactivity by scintillation counting) was 5.3 mCi/mmole. Of the total radioactivity added, 0.12% was found in the final purified victorin.

In an attempt to increase the percentage incorporation of ^{3}H-leucine into victorin, we tested the effect of cycloheximide on victorin synthesis. The rationale for this was that some cyclic peptides are known to be synthesized extraribosomally[15], so that blocking ribosomal protein synthesis with cycloheximide might increase the proportion of exogenous ^{3}H-leucine incorporated into a secondary peptide product such as victorin. We found, however, that concentrations of cycloheximide above 5 µg/ml caused inhibition of both mycelial growth (data not shown) and victorin synthesis (fig. 3). Specific activity of the victorin produced was similar (±15%) at all cycloheximide concentrations tested. Qualitatively similar results were obtained if ^{3}H-leucine and/or cycloheximide were added 5 days after inoculation and/or the culture filtrates were harvested 8 or 10 days after inoculation. No evidence of turnover of victorin was found[16].

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8 All gas chromatography employed a Varian model 2100 instrument equipped with a flame-ionization detector and a 1/8 inch ID X 6 foot glass column packed with 3% OV-1 on 100/120 Gas-chrom Q. Analytical runs were temperature-programmed from 60 to 260 °C at 10 °C/min using a carrier gas (N_2) flow rate of 30 ml/min. Retention times (min): *erythro*-3-hydroxyleucine TFA *n*-butyl ester, 6.7; TFA methyl ester, 5.1; 5,5-dichloroleucine TFA *n*-butyl ester, 10.1; TFA methyl ester, 8.6; 3-hydroxylysine TFA *n*-butyl ester, 11.6; TFA methyl ester, 10.1. Semipreparative work was performed under the same conditions using a 10:1 flow splitter and a carrier flow rate of 40 ml/min.
9 NMR data were recorded on a Bruker WM-300 FTNMR instrument. Chemical shifts are reported in ppm downfield from TMS. 3-Hydroxyleucine TFA methyl ester (**1**): 7.06 db, $J = 7.5$ Hz (NH); 5.04 dd, $J = 3.3$, 8.6 Hz (H-3); 4.97 dd, $J = 3.3$, 7.5 Hz (H-2); 3.84 s (3H, $COOCH_3$); 2.18 m, $J = 6.7$, 6.8, 8.6 Hz (H-4); 1.11 d, $J = 6.8$ Hz (CH_3); 0.97 d, $J = 6.7$ Hz (CH_3). 5,5-Dichloroleucine TFA methyl ester (**3**): 6.75 db, $J = 8.2$ Hz (NH): 5.77 d, $J = 2.9$ Hz (H-5); 4.70 ddd, $J = 3.1$, 4.0, 8.2 Hz (H-2); 3.81 s ($COOCH_3$); 2.15 m (H-4); 2.09 ddb, $J = 3.1$, 10 Hz (H-3a); 1.97 ddb, $J = 4.0$, 10 Hz (H-3b); 1.23 d, $J = 6.5$ Hz (CH_3). 3-Hydroxylysine TFA methyl ester (**6**): 6.96 db, $J = 8.8$ Hz (2-NH); 6.44 mb (6-NH); 5.62 m (H-3), 4.92 dd, $J = 2.2$, 8.8 Hz (H-2); 3.81 s (3H, $COOCH_3$); 3.42 m (H-6a, H-6b); 1.74 m (4H; 4-CH_2 and 5-CH_2).
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12 HPLC employed a Waters gradient system (two model 6000A pumps, model 660 solvent programmer, and model 440 UV detector) with reversed-phase ODS semipreparative column (10 mm ID × 25 mm) at a flow rate of 3.0 ml/min. Solvent program: 0–100% acetonitrile in water in 20 min with monitoring at 254 nm. Retention times (min): unidentified component, 6.7; TFA methyl ester derivative, 10.1.
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16 We wish to thank Prof. D. Arigoni and his coworkers for informing us that they have independently determined the structures described above, and that they have also completed the assignment of the entire victorin structure.

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Moderate cooling depresses the accumulation and the release of newly synthesized catecholamines in isolated canine saphenous veins[1]

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Summary. Moderate cooling (from 37° to 24 °C) depressed the formation of ^{3}H-dopamine and ^{3}H-norepinephrine from ^{3}H-tyrosine by isolated canine saphenous veins. Cooling reduced the evoked release of newly synthesized catecholamine to the same extent as that of stored norepinephrine. Hence the augmentation by cold of the contractile response to sympathetic nerve stimulation observed in earlier work is not accompanied by an augmentated release of newly synthesized norepinephrine.
Key words. Cooling; canine saphenous vein; newly synthesized norepinephrine; release; electrical stimulation; accumulation.

In the isolated saphenous vein of the dog, moderate cooling augments the contractile responses to norepinephrine[18, 19]. This potentiation is due in part to an instantaneous increase in the affinity of postjunctional alpha-adrenoceptors[10]. Cooling augments the contractions evoked by sympathetic nerve stimulation more than those evoked by exogenous norepinephrine[10, 19], which suggests that the smooth muscle cells sense an increased concentration of the adrenergic transmitter in the junctional cleft. However, moderate cooling markedly decreases the stimulation-evoked overflow of both endogenous and ^{3}H-norepinephrine[9, 20]. Newly synthesized norepinephrine probably is stored in a small neuronal compartment and may contribute differently to the release process than the rest of the endogenous norepinephrine or than ^{3}H-norepinephrine recently taken up by the nerve endings[5, 8, 11–15]. Therefore, the aim of the present study was to investigate the effects of moderate cooling on the accumulation and release of newly synthesized catecholamines in canine saphenous veins.

Materials and methods

Both lateral saphenous veins were taken from mongrel dogs, anesthetized with sodium pentobarbital (30 mg/kg i.v.). The veins were placed in ice-cold modified Krebs-Ringer bicarbonate solution (mmolar composition: NaCl, 118.3; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25.0; glucose, 11.1; Ca EDTA, 0.026; control solution). They were cut into helical strips (5 mm in width) of 1 cm in length for incubation studies and 10 cm in length for superfusion studies.

1. Incubations

All tissues were equilibrated for 2 × 30 min in 2 ml of aerated (95% O_2-5% CO_2) control solution at 37 °C. They were then incubated for 30 min with 3,5-^{3}H-(−)-tyrosine (3.5×10^{-7} M; specific activity 53 Ci/mmole; New England Nuclear) either in 2 ml of control solution or in 2 ml of solution containing 50 mM K^+ (in equimolar re-