ISOLATION AND PARTIAL CHARACTERIZATION OF NUCLEOSIDES AND NUCLEOTIDES FROM HONEYBEE VENOM AND THREE SCORPION VENOMS

By

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Bachelor of Science

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Riverside, California

1974

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
MASTER OF SCIENCE
May, 1977

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ACKNOWLEDGMENTS

I would like to extend special appreciation to my major adviser, Dr. George V. Odell, for his guidance and support. Appreciation is also expressed to Dr. Ta-Hsiu Liao and Dr. Earl D. Mitchell for their assistance as advisory committee members.

I would also like to thank Dr. Tak Chan and Dr. Ralph Reed for sharing their knowledge and experience with me. Their help was invaluable.

Diana Upp, my wife, has been a valuable source of help and ideas throughout this study. Most important though was the patience, encouragement and understanding she supported me with. For all these things, I can't thank her enough.

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CHAPTER I

INTRODUCTION

The stings of bees and scorpions can pose serious medical problems for the persons who become their victims. Due to the minute amount of venom injected in an individual bee sting, the primary medical consequence is due not to the toxicity of the venom, but rather due to anaphalactic reactions in hypersensitive individuals. Recently with the multiple stings inflicted by the "killer" bees in South America there has been an increased interest in the toxic effects of the venom. Another interest in bee venom has been it's reported effect in the relief of arthritic symptoms.

Scorpions are well known in the tropical regions of the world. In a few countries scorpionism is as large a health problem as snake bite. Scorpion stings involve enough venom for the direct toxic effects to be important. Fatalities from scorpion stings in adults are relatively rare (approximately 1%), but children are considerably more susceptible.

Nucleotides and nucleosides have been found in a few venoms. They have also been found, in one case, to have a synergistic effect with the toxins. There have been no reports as yet concerning the nucleoside or nucleotide content of either scorpion or honeybee venom. This thesis reports an attempt to isolate and characterize nucleosides and nucleotides in these venoms.

CHAPTER II

LITERATURE REVIEW

The genera of scorpion which are most important medically are: Centruroides (North and Central America), Tityus (South America), Buthus (North Africa and Asia), Androdoctus (North Africa and India), Parabutus (South Africa). The most poisonous species, which belong to the Buthidae family, have venoms with an LD_{50} in rodents of about 1 to 20 mg/kg. Man is thought to be somewhat more susceptible than mice. Average yields of dry venom from Tityus serrulatus and T. bahiensis by electrical stimulation were 0.62 and 0.39 mg, respectively (1).

The scorpion sting results in severe local burning pain which is sometimes accompanied by edema of the affected area. The major features of the envenomization are systemic. Constant symptoms are sialorria, rhinorhea, lachrymation, pronounced sudoresis, pallor, muscular twitchings, convulsions, and opisthotonus. Tachycardia, hypertension, glycosuria and hyperglycemia have also been observed. Death is due to respiratory paralysis (1, 2). These symptoms vary little from species to species, suggesting a biochemical similarity in the various venoms.

Symptoms of scorpion poisoning resemble those of parasympathetic-comimetic substances (muscarine, acetylcholine, pilocarpine, eserine) and sympatheticomimetic compounds (adrenaline). Most of the effects

can be explained by the selective action on the sympathetic and parasympathetic autonomic centers in the hypothalamus (2).

As with most venoms, scorpion venom is a complex mixture of substances several of which contribute to toxicity. Rochat et al. (3) found eleven neurotoxins in the venoms of three scorpions; three in Androctonus australis hector, three in Buthus occitanus tuntanus, and five in Leiurus quinquestriatus quinquestriatus. The toxins all had molecular weights of about 7,000 and high isoelectric pH's. Amino acid sequences were determined for four of the toxins, I and II of Androctonus australis, toxin I of Buthus occitanus tuntanus, and toxin III of Leirus quinquestriatus quinquestriatus. These proteins are all single chains crosslinked by four disulfide bridges, and have similar amino acid compositions and sequence.

McIntosh and Watt (4) have reported that venom from Centruroides sculpturatus contains at least 12 different proteins plus other components, e.g., mucus and small molecules. At last eight different polypeptide toxins which are toxic in varying degrees to vertebrates and invertebrates have been found. Four of these toxins have been characterized. They have been found to be a single chain with four disulfide bridges and amino acid composition similar to those described by Miranda (3).

Scorpion venom is relatively free of enzymatic activity, in contrast to spider and snake venoms. Ibrahim (5) has found Phospholipase A activity in two North African scorpions. Significant hyaluronidase activity was reported in two Brazilian scorpion venoms by Diniz and Goncalves (6). Russell et al. (7) have reported acetylcholinesterase activity in Vejovis spinigerus. Wright et al. (8) have reported

hyaluronidase and alkaline phosphotase in Palmaneus gravimanus.

Some of the low molecular weight components of scorpion venom reported have been histamine, serotonin, 5 hydroxytryptophan, tryptophan, and tryptamine (7, 9, 10, 11).

The composition of bee venom is reasonably well known. A number of biologically active compounds have been characterized (Table I).

TABLE I

COMPOSITION OF HONEYBEE VENOM*

Components	% of Dried Venom[a] (approx.)	Reference
Free amino acids (19)	1	Nelson and O'Connor (1968)
Histamine	1	Markovic and Rexova (1963)
Dopamine	?	Owen (1971)
Norepinephrine	1	Owen (1971)
Small peptides	15	
Seven di- and tripeptides	?	Rexova and Markovic (1963)
Histamine-peptides	2	
Mellitins	50	Habermann (1972)
(three compounds)		Jentsch (1972)
Apamin	2	Habermann (1972)
MCD-peptide	2	Habermann (1972)
Minimine	?	Lowry et al. (1971)
Phospholipase A	12	Munjal and Elliot (1972)
Hyaluronidase	2	Barker et al. (1967)

[a]The natural venom is 88% water, by weight (O'Connor et al., 1967), and contains a number of volatile compounds (Gunnison, 1966).

*From Peck and O'Connor (12).

Bee venom hyaluronidase has a molecular weight in excess of 20,000 and is a minor antigenic component. This enzyme spreads the venom within the tissues by breaking down the hyaluronic acid polymer which serves as intercellular cement (13).

Phospholipase A has a powerful indirect hemolytic activity due to it's ability to release lysolecithin, which in turn lyses the cells. In test animals the enzyme produces a dramatic loss of arterial pressure and respiratory paralysis (14). The bee venom enzyme has been well characterized. It has 183 amino acids and a molecular weight of about 18,000 (15). It is probably the main protein involved in hypersensitivity to bee stings (16).

The major peptides in bee venom are mellitins. Mellitin was originally thought to be a single peptide; however Jentsch (17) has reported that mellitin consists of at least three closely related compounds, all having strong direct hemolytic activity. Two of these have since been completely characterized and synthetically produced (18).

A second peptide, apamin, having only 18 residues, is the smallest known neurotoxin peptide (20). Injection of apamin in mice causes extreme uncoordinated hypermotility. A lethal dose causes tonic convulsions and respiratory failure. The site of action has been localized in the spinal cord (20, 21). Apamin appears mainly to augment polysynaptic reflexes and to render excitatory polysynaptic pathways more effective than inhibitory polysynaptic mechanisms.

Phospholipase A, mellitin, and another peptide called mast cell degranulating (MCD) peptide, release histamine from the host organism. While the MCD peptide comprises only a small portion of the

venom, it still causes a large share of the inflammatory response since it is several times as active as mellitin in releasing histamine from host tissue (19).

Lowry (22) has reported another peptide called minimine. When injected into Drosophila melanogaster larva, much smaller flies are produced (approximately ¼ normal). Progeny of these flies are of normal size. It is thought that minimine might be another phospholipase A activity.

Some of the smaller molecules which have been reported in bee venom are: dopamine and norepinephrine, free amino acids, and histaminepeptides (23, 24, 12).

Nucleotides and nucleosides have been reported in several venom sources. Fischer and Dorfel (25) have reported adenosine in the venom of two species of snake, Bitis aritans and Dendroaspis virdis. Adenosine was also reported by Doery (26) in some Australian snake venoms. Wei and Lee (27) identified guanosine in the venom of Bungaris multicinctus. Lo and Chin (28) found the non-protein fraction of Naja naja (cobra) venom contains guanosine, adenosine and inosine.

Recently Geren et al. (29) have isolated inosine and "guanine like" moities from the venom of Loxosceles reclusa. Chan et al. (30) have found tarantula venom to contain up to 5.7% ATP by weight. Studies were also done which indicated that this ATP has a synergistic effect with the venom's major toxin, a necrotoxin. The necrotoxin's toxicity was enhanced by a factor of 3.2 by the addition of ATP.

Wright et al. (8) have also reported the possible presence of nucleosides or nucleotides in the low molecular weight fraction of venom from the scorpion, Palimneus gravimanus. Linden and Raftory

(31) have reported that Tityus serrulatus venom stimulates Na^+ uptake in neuroblastoma cells. If nucleotides were present in Tityus venom they could have a synergistic effect similar to that in the tarantula venom.

CHAPTER III

MATERIALS AND METHODS

A. Sample Sources

Samples used were lyophilized venoms: Apis mellifera, Leiurus quinquestriatus, and Tityus serralatus purchased from Sigma; and Vejovis spinigerus, from Mr. Lorin Honetschlager of Mesa, Arizona. Venom was resuspended in H_2O and used whole.

B. Column Chromatography

Gel filtration chromatography was on a 114 X 1 cm column packed with Sephadex G-25 (Pharmacia). Venoms resuspended in H_2O were applied to the column, and 0.02M NH_4CO_3 buffer was used as the eluent. Fractions of two ml per tube, at a flow rate of 20 ml/hr were collected and monitored at 280 and 260 nm.

Ion exchange chromatography on a Dowex 1 X 8 (BioRad) formate form column (0.6 X 20 cm) was used for the separation of low molecular weight components. Fractions of 1 ml per tube were collected at a flow rate of 1 ml per 3.5 minutes. The elution gradient was of the two reservoir constant mixing type with 15 ml H_2O in the lower reservoir. The reservoir was filled initially with 15 ml H_2O and replaced sequentially with the following solutions: 20 ml 2N formic acid, 20 ml 4N formic acid, 20 ml 4N formic acid + 0.4N ammonium formate, 20 ml

4N formic acid + 0.5N ammonium formate, 20 ml 4N formic acid + 0.6N ammonium formate, 20 ml formic acid + 0.7N ammonium formate, 20 ml 4N formic acid + 0.8N ammonium formate, 4N formic acid + 1.0N ammonium formate. Fractions were monitored at 260 nm.

C. Thin Layer Chromatography

Thin layer chromatography was conducted on 20 X 20 cm glass thin layer plates prepared by mixing 15 grams of MN300 cellulose (Machery, Nagel & Co., Germany), 6 grams of Silica Gel G (Brinkman & Co.), and 120 ml H_2O, blending 60 seconds in a Waring blender and spreading a uniform layer of 0.75 mm thick over the plates. The plates were air dried, and activated for two hours at 110°C before use. The solvent system employed contained n-butanol, acetic acid, acetone, 5% aqueous ammonium hydroxide and H_2O (7:3:5:3:4 v/v). The nucleotide components were detected by quenching, when viewed under a short wavelength ultraviolet light.

Ion exchange thin layer chromatography was conducted on commercially prepared poly-(ethyleneimine)-cellulose plates (Macher, Nagel & Co., Germany) (33). The solvent employed was 1 M LiCl. Components were again visualized by ultraviolet quenching.

D. Amino Acid Analysis

Amino acid analyses were obtained using an instrument constructed by Dr. Ta-Hsiu Liao of the Biochemistry Department of Oklahoma State University. The instrument was an adaptation of the design of Spackman, Stein and Moore (33) using small ion exchange columns (34) to detect amino acids in the nanomolar range.

E. Ultraviolet Absorption Spectroscopy

Ultraviolet absorption spectra were obtained with a Cary Model 14 Recording Spectrophotometer. Spectra were obtained at pH-1 and pH-7 using 0.1N HCl and 0.02M sodium phosphate buffers, respectively.

CHAPTER IV

RESULTS AND DISCUSSION

A. General Procedure

Four venoms were investigated to determine their nucleotide content. The amount of venom used was determined by the availability of the venom. The isolation procedure was similar in all four cases and involved an initial separation on a G-25 column, followed by fractionation on a Dowex 1 X 8 column. The resulting fractions were further chromatographed on a silica gel--cellulose thin layer system. The components thus isolated were then eluted and identification attempted by virtue of their ultraviolet spectra.

B. Apis mellifera

Fifty-one mg of Apis mellifera venom was applied to the G-25 column (Fig. 1). Two major peaks, centered around tubes 45 and 115 were found. Neither of these indicated the presence of nucleotides judging from the A_{280}--A_{260} ratios. Tubes 50-150 were pooled, lyophylized, resuspended and applied to a Dowex 1 X 8 column. Previous studies had shown standard common nucleotides to elute between tubes 50 and 150. The Dowex column yielded several peaks as indicated in Figure 2. Figure 3 has been provided to allow comparison of peak retention times with those of known compounds.

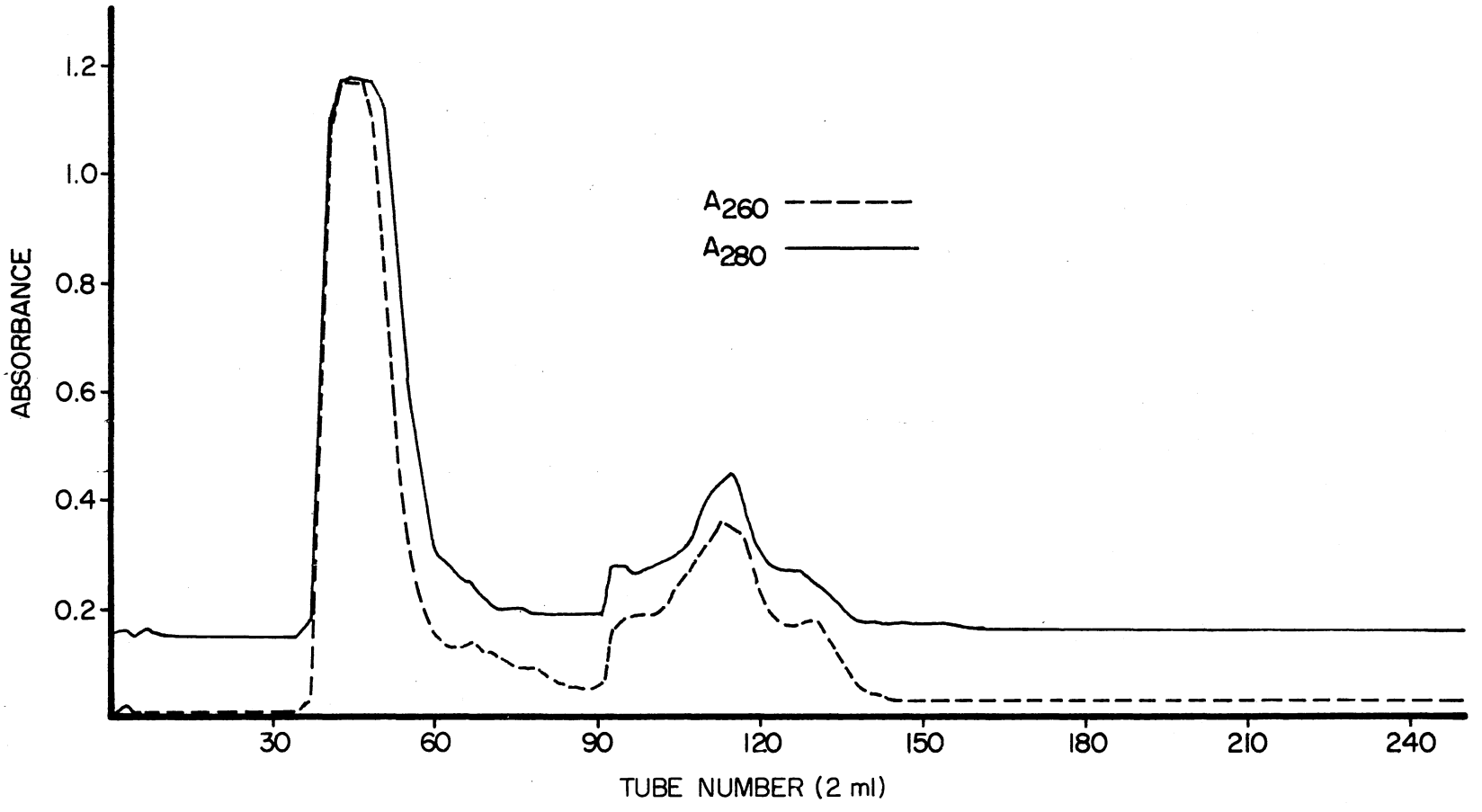

Figure 1. Sephadex G-25 Chromatogram of Whole Resuspended Apis mellifera Venom

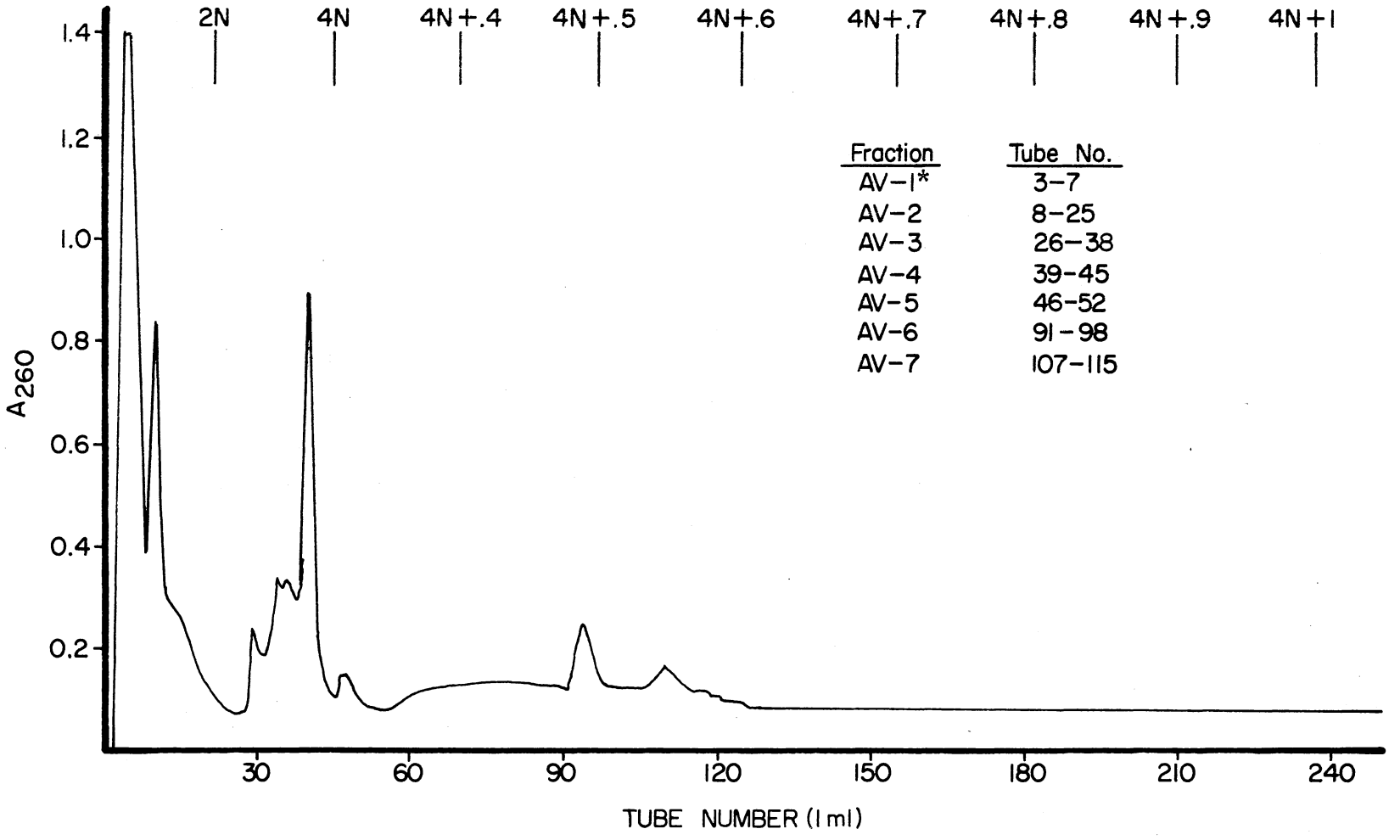

Figure 2. Dowex 1 X 8 Chromatogram of Low Molecular Weight Fraction from Apis mellifera (See p. 8)
*AV-1, 2, 3, ..., 7 denote fraction pooled from column.

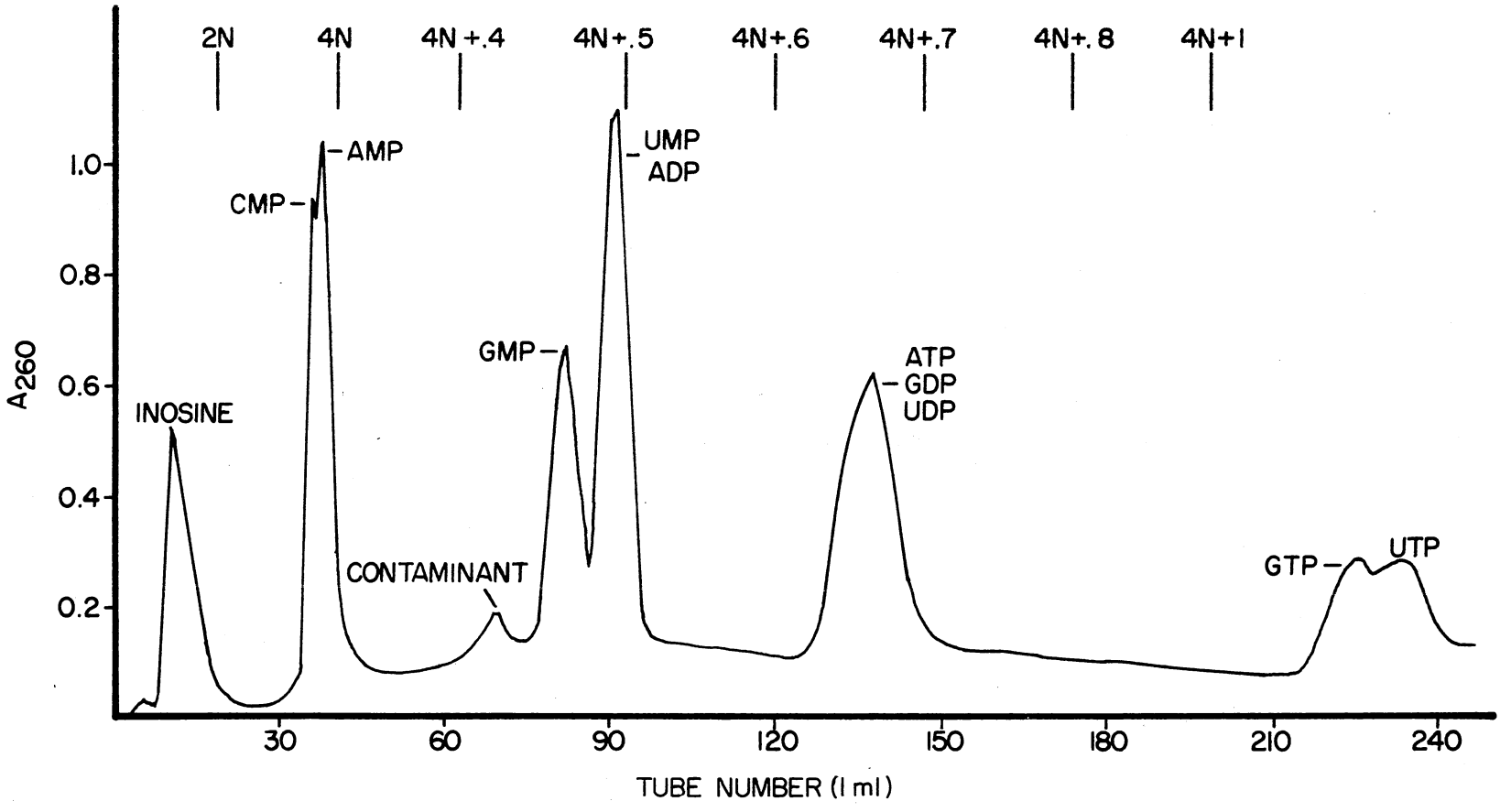

Figure 3. Dowex 1 X 8 Chromatogram of Standard Nucleotides (100 µg each) (See p. 8)

Fractions were pooled, lyophilized, then spotted on silica gel--cellulose thin layer plates and developed in n-butanol, acetone, acetone, acetic acid, 5% aqueous NaOH, H_2O (7:3:5:3:4) solvent. The fractions which displayed ultraviolet quenching components were AV-2, with two components (AV-2 denotes fraction two of Apis venom); AV-3, one; AV-4, one; and AV-6 with one (Fig. 4). Throughout the studies, Rf's in these thin layer chromatograms were quite variable. This was due to the moisture absorbed from the air during the long time it took to spot the plates. The order in which the standards eluted remained constant.

The ultraviolet quenching components were then removed from the plate along with the solid support and eluted with H_2O. The material was then lyophilized and resuspended in the appropriate buffer for ultraviolet absorption spectra. The ultraviolet spectra were made at pH-1 and pH-7 using 0.1N HCl and 0.02M sodium phosphate, respectively, as buffers.

The ultraviolet quenching components of bee venom separated by TLC which had spectra similar to those of nucleotides were the bottom component of AV-2 (Fig. 5), AV-3 (Fig. 6), and AV-4 (Fig. 7). The spectra illustrated in the figures have been adjusted to best show shifts due to pH change, exact values from the original spectra are in Table II. Standard spectra were made for eight common bases and monophosphates, plus inosine, seratonin, tryptamine, tryptophan, and 5-hydroxytryptophan. The spectral data obtained for the standards were in close agreement with published data (35). None of the standard spectra matched the spectra of the bee venom components.

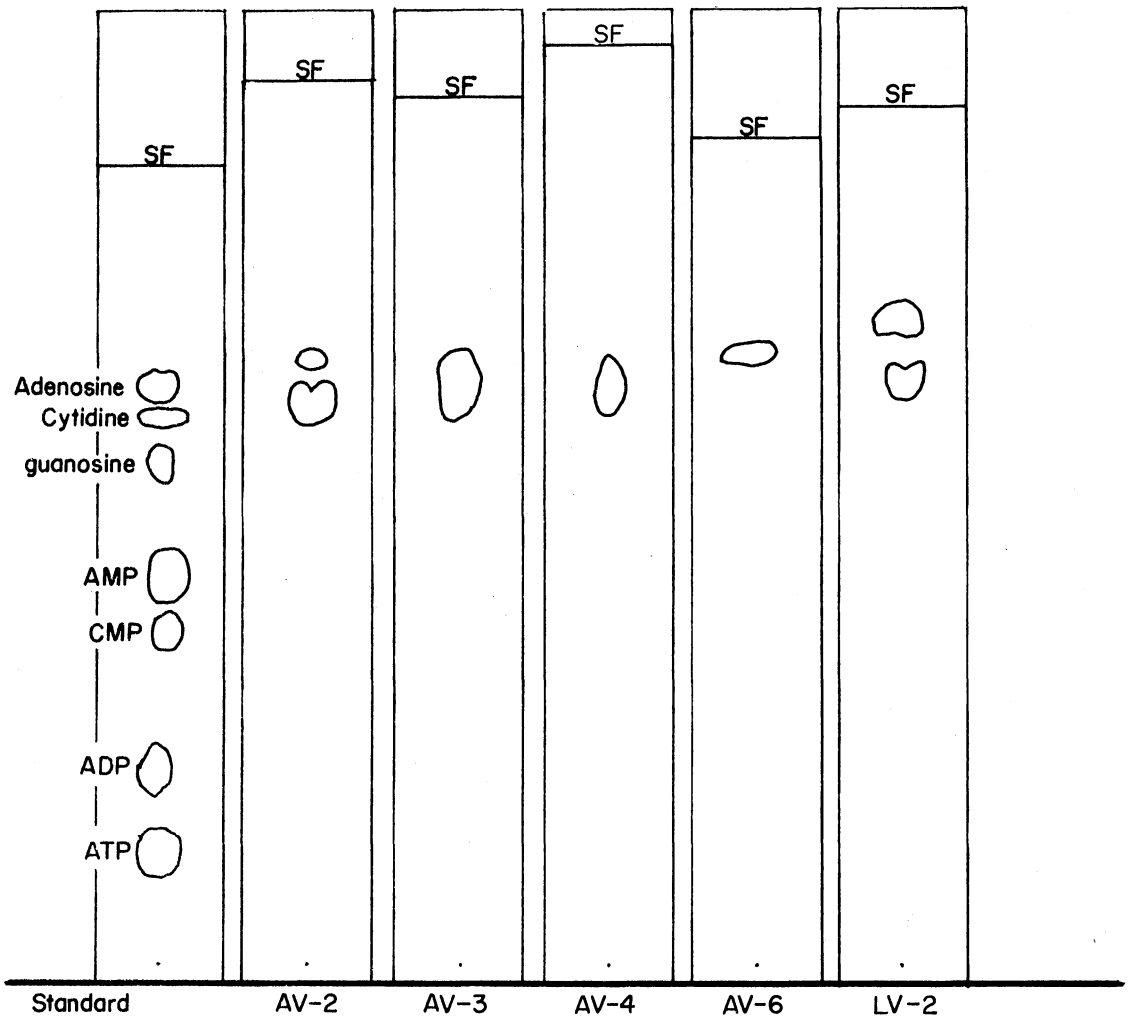

Figure 4. Thin-Layer Chromatograms of Dowex Fractions from Apis mellifera and Leiurus quinquestriatus Venom (Outlines delineate ultraviolet quenching components.)

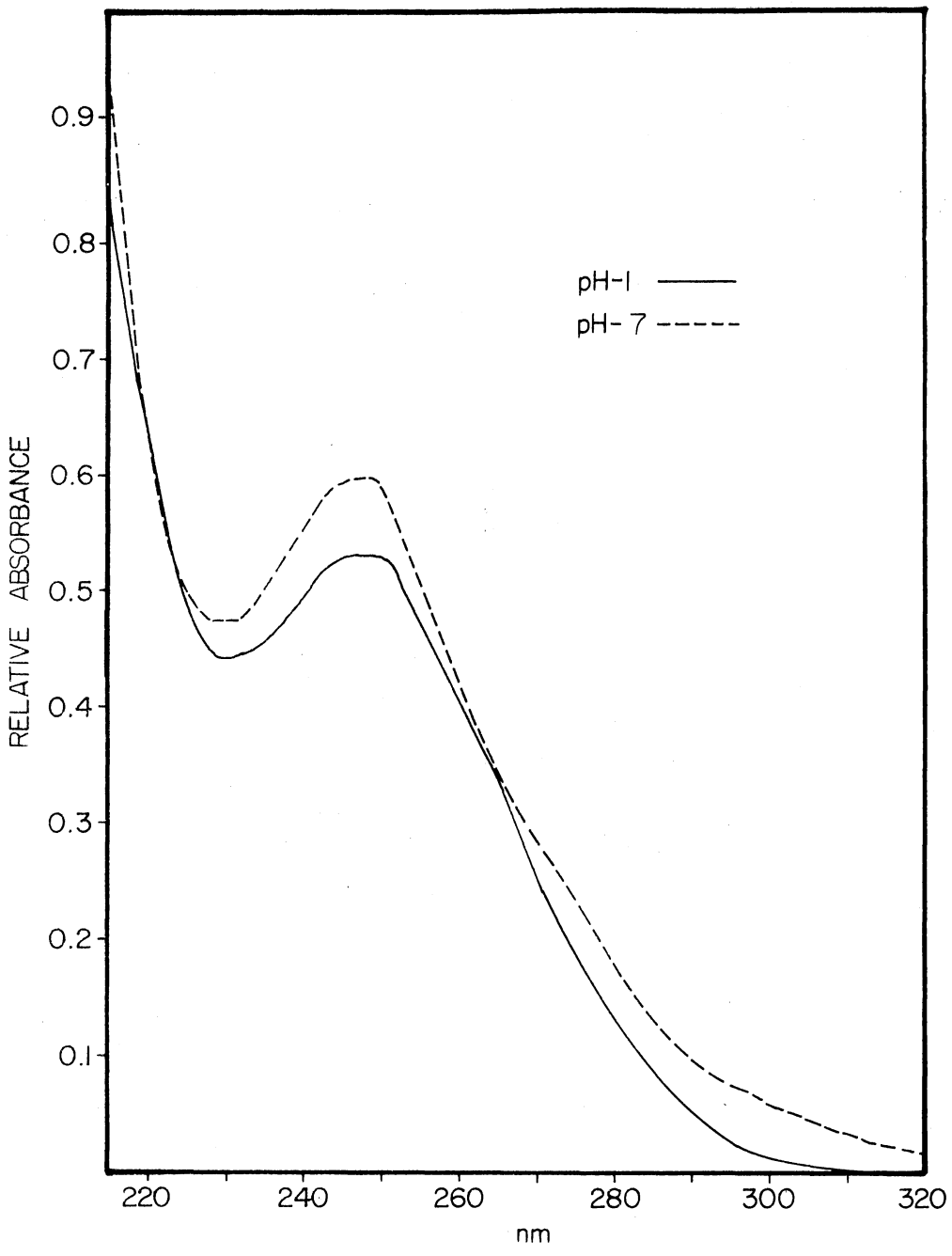

Figure 5. Ultraviolet Spectrum of the Bottom TLC Ultraviolet Quenching Component of Fraction AV-2

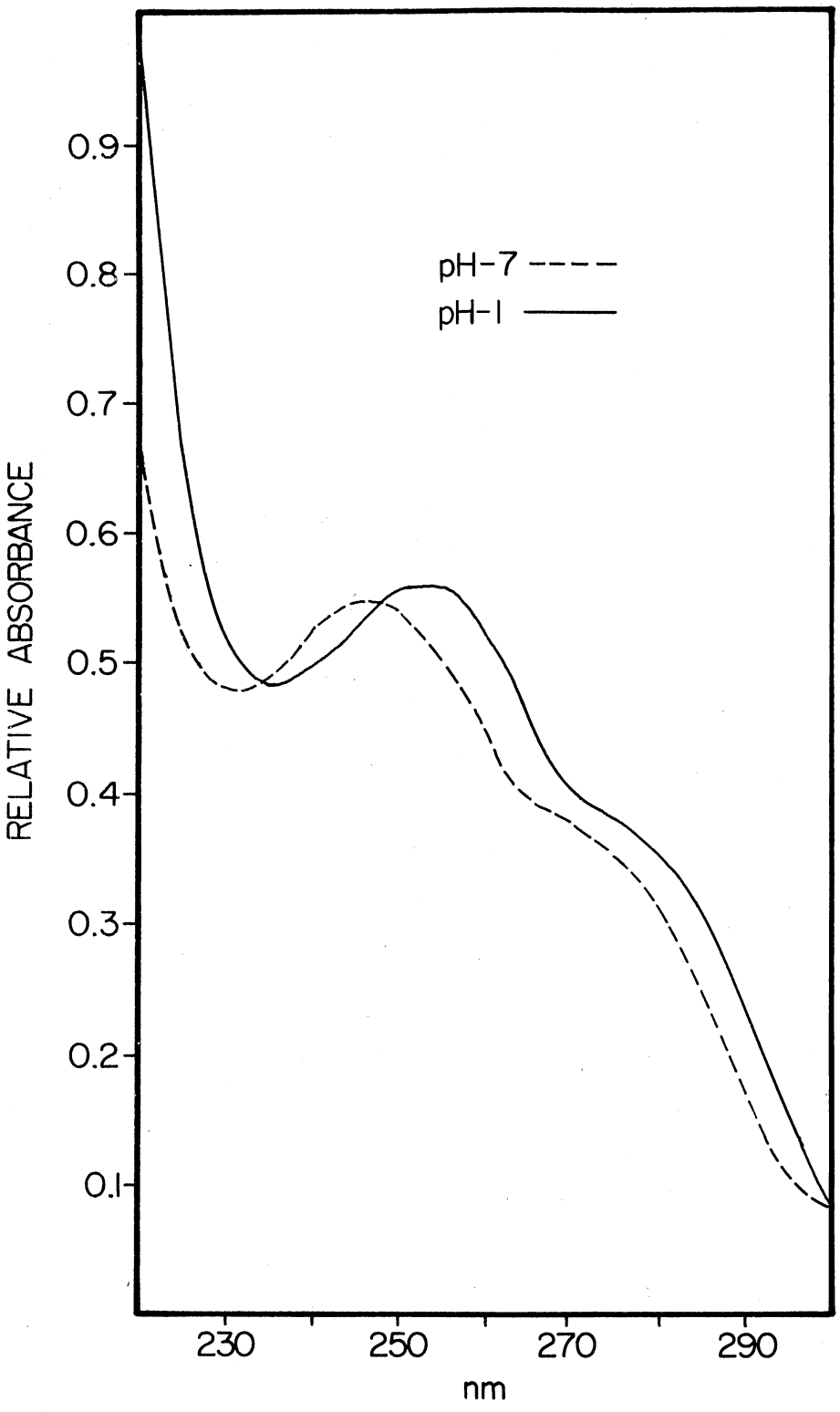

Figure 6. Ultraviolet Absorption Spectrum of Ultraviolet Quenching Component of Fraction AV-3

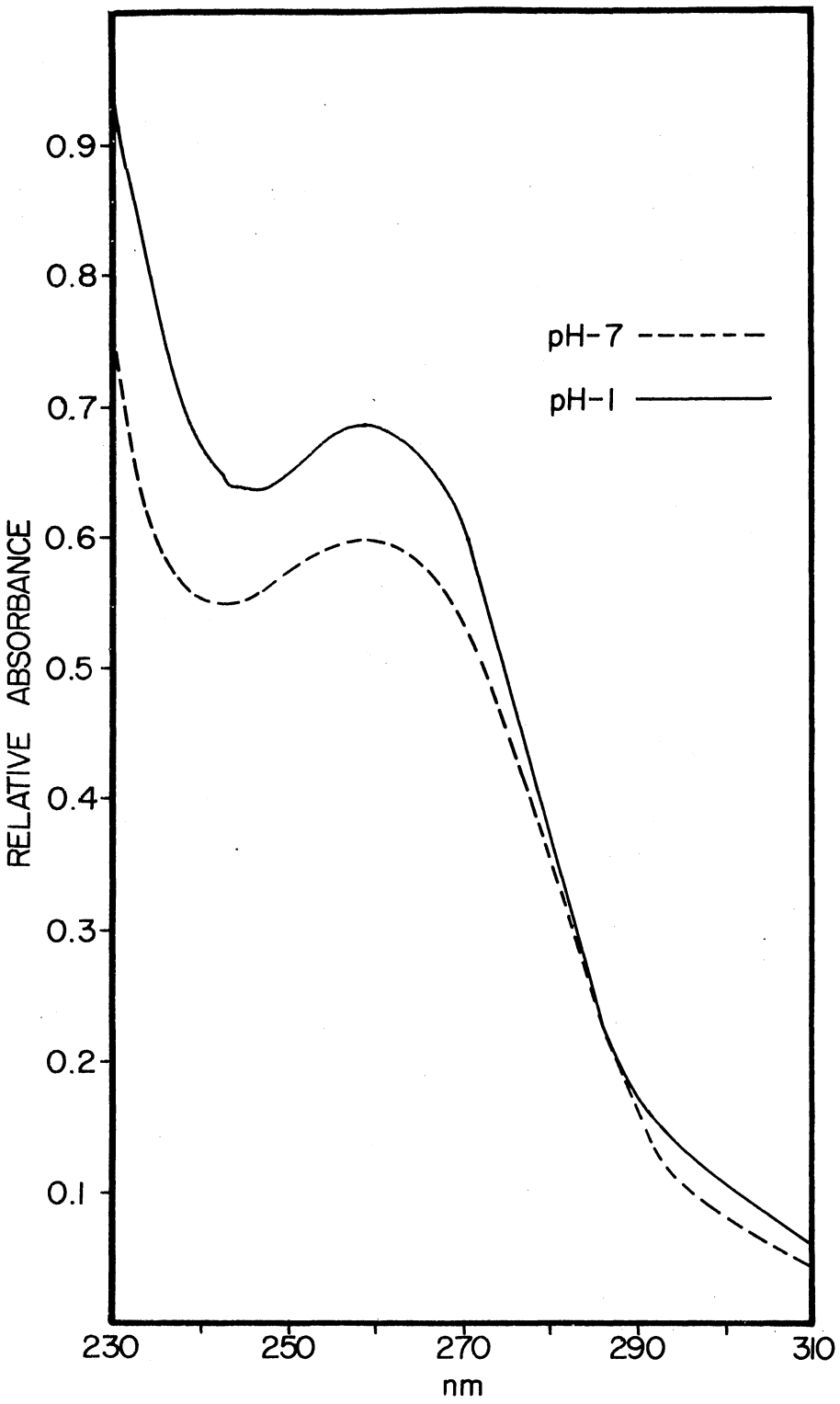

Figure 7. Ultraviolet Absorption Spectra of Ultraviolet Quenching Component of AV-4

TABLE II

SUMMARY OF ULTRAVIOLET ABSORPTION SPECTRA DATA OF VENOM COMPONENTS

Component*		λmax (nm)	λmax (nm)	Absorbance			Ratio A_{250}/A_{260}	Ratio A_{280}/A_{260}
				250 nm	260 nm	280 nm		
AV-2 Bottom	pH-1	247	230	1.05	0.80	0.30	1.31	0.38
	pH-7	247	230	0.96	0.85	0.34	1.13	0.40
AV-3	pH-1	253	235	0.61	0.58	0.41	1.05	0.71
	pH-7	247	232	0.89	0.70	0.47	1.27	0.67
AV-4	pH-1	257	244	0.65	0.69	0.37	0.94	0.54
	pH-7	258	242	0.57	0.60	0.34	0.95	0.57
LV-2 Top	pH-1	256	232	1.46	1.00	0.10	1.46	1.00
	pH-7	258	229	1.02	1.22	0.28	0.84	0.23
VV-2	pH-1	272	252	0.46	0.49	0.52	0.94	1.06
	pH-7	273	257	0.52	0.46	0.55	1.13	1.20

*AV-2 Bottom, AV-3, AV-4 denote component from A. mellifera; LV-2 Top, a component of L. quinquestriatus; VV-2, a component of V. spinigerus.

The spectrum of the component from AV-3 has a shoulder near 270 nm, which is very similar to that of guanoid compounds. The minima of the AV-3 component, however, differs significantly from those of guanoid compounds. This is the only venom component to display an apparent shift in the wavelength of the maxima between pH-1 and pH-7.

C. Leiurus quinquestriatus

Twenty-five mg of Leuirus quinquestriatus venom were applied to the G-25 column (Fig. 8). The peak centered at tube 124 appeared to be an excellent possibility for being a nucleotide due to it's high A_{260}--A_{280} ratio. Tubes 50-150 were pooled and lyophilized for further chromatography on Dowex 1 X 8. The Dowex column yielded the elution profile in Figure 9. These fractions were then spotted and developed on the same silica gel--cellulose thin layer system as was the bee venom. Thin layer chromatography showed fraction LV-2 to have two ultraviolet quenching components (Fig. 4). These components were eluted, lyophilized and resuspended in the appropriate buffer for ultraviolet analysis. Only the faster migrating component had a nucleotide like spectrum (Fig. 10). The spectrum of this compound closely resembles the spectrum of NAD and NADP. Neither NAD nor NADP, however, chromatographed with the unknown on the silica gel--cellulose thin layer system.

D. Tityus serrulatus

Fifty mg of Tityus serrulatus venom was applied to the G-25 column (Fig. 11). As before, tubes 50-150 were pooled, lyophilized and resuspended in preparation for chromatography on Dowex 1 X 8. Figure

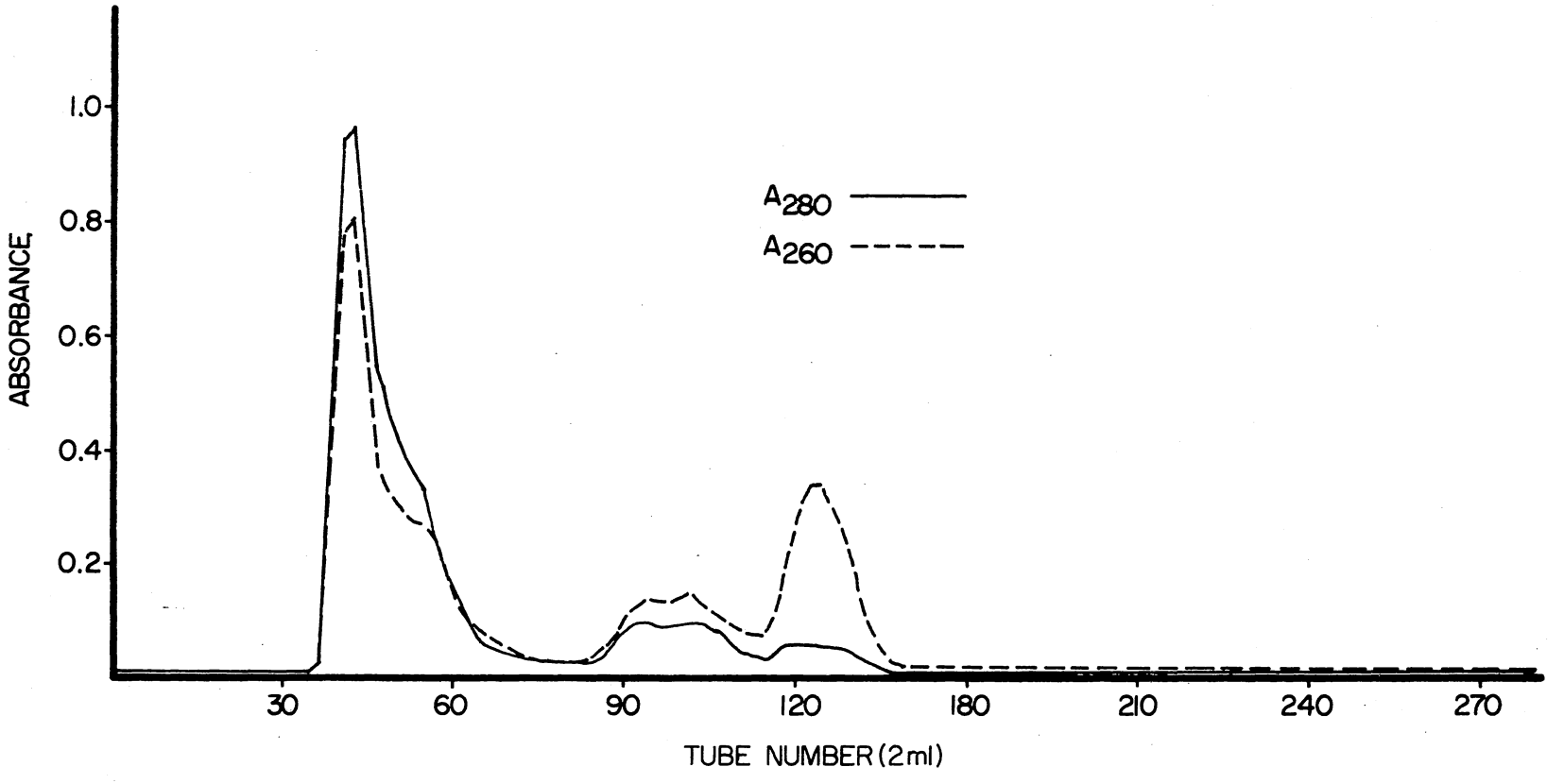

Figure 8. Sephadex G-25 Chromatogram of Whole Resuspended Leiurus quinquestriatus Venom

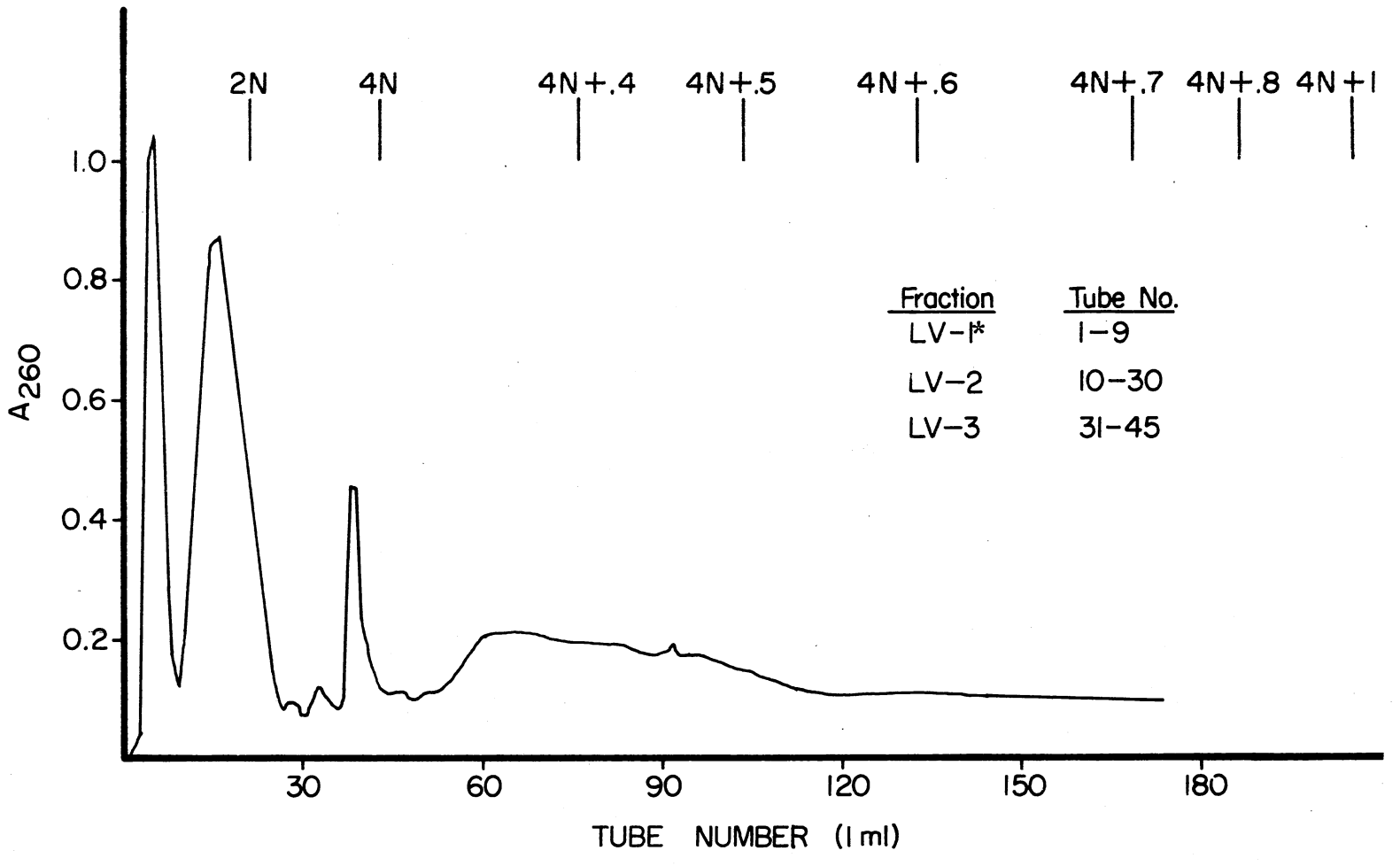

Figure 9. Dowex 1 X 8 Chromatogram of Low Molecular Weight Fraction From Leiurus quinquestriatus
(See p. 8) *LV-1, 2, 3 denote fractions pooled from column.

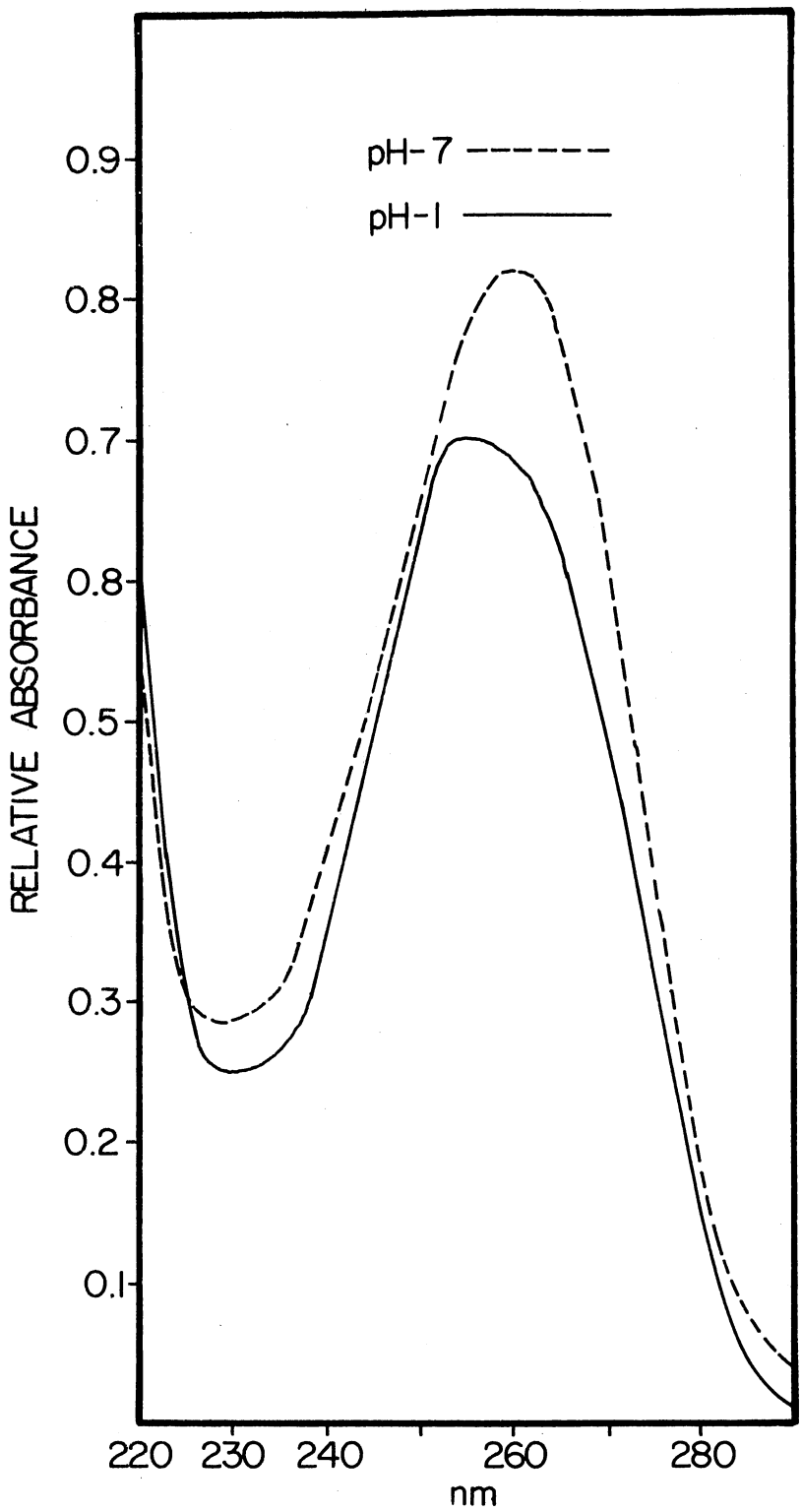

Figure 10. Ultraviolet Absorption Spectrum of Fastest Migrating Ultraviolet Component From Fraction LV-2

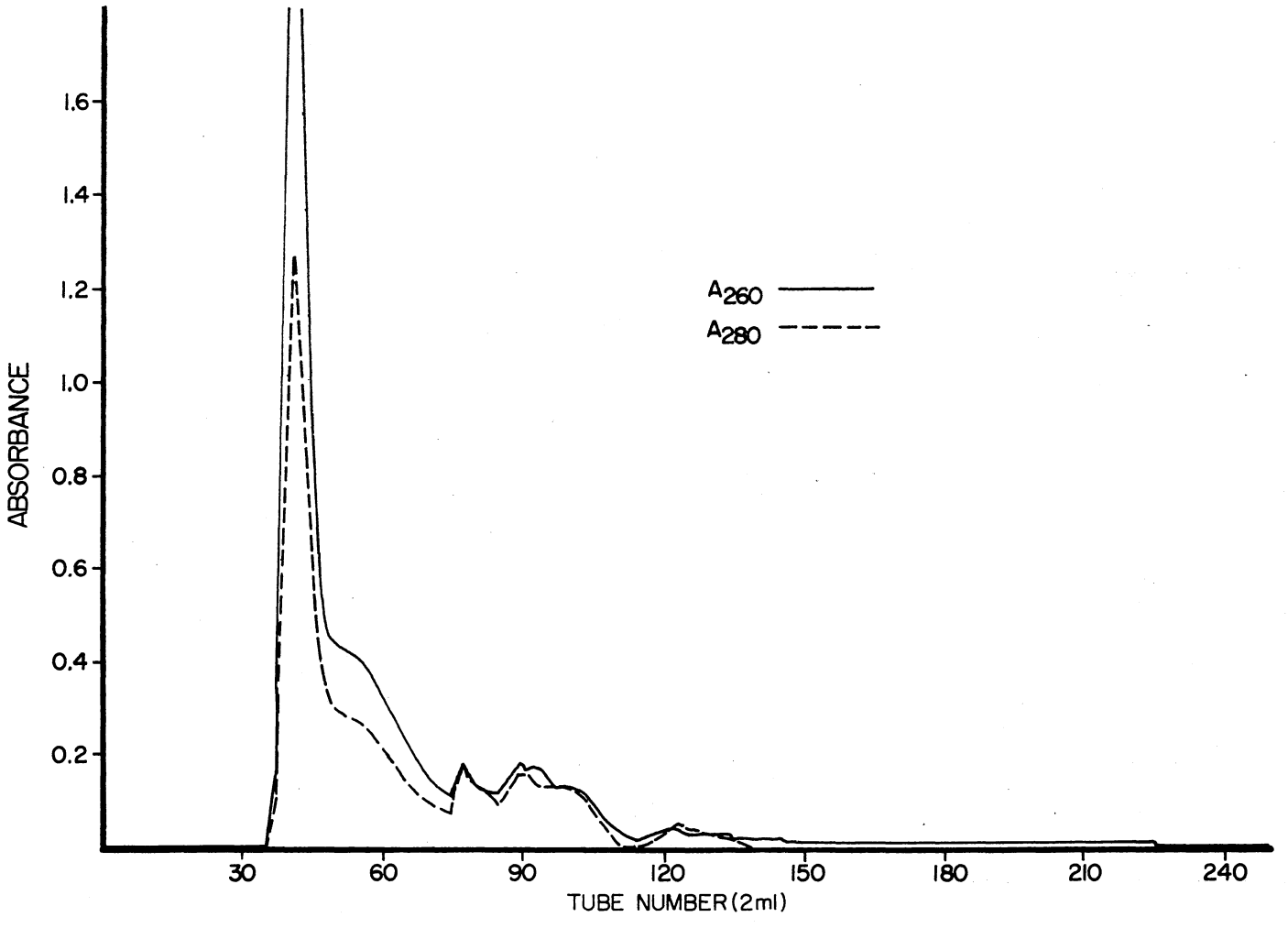

Figure 11. Sephadex G-25 Chromatogram of Whole Resuspended Tityus serrulatus Venom

12 shows the elution profile of this G-25 fraction on Dowex 1 X 8. Four fractions were pooled, lyophilized, and resuspended. Further chromatography of these fractions on the silica gel--cellulose thin layer system yielded two ultraviolet quenching components (Fig. 13). Neither of these components had spectra resembling those of nucleotides.

E. Vejovis spinirgerus

The venom from Vejovis spinirgerus was treated differently due to the small amount available. Only 7.0 mg could be obtained, therefore, in order to reduce losses the resuspended venom was chromatographed on the Dowex 1 X 8 directly (Fig. 14), deleting the G-25 column. When the pooled fractions were chromatographed on the silica gel--cellulose thin layer system as with previous venoms, two quenching components were detected; one in VV-2, and one in VV-3 (Fig. 13).

Only the component from VV-2 has a nucleotide like spectrum. The spectrum has a small plateau or step at 285 nm, suggesting a tryptophan containing compound (Fig. 15). The component is not, however, free tryptophan since the spectrum of the unknown doesn't match that of a tryptophan standard. Free amino analysis of the sample also revealed no free tryptophan. This does not eliminate the possibility of a tryptophan containing peptide in this fraction. Lack of sample prevented amino acid analysis of a hydrolysate of the fraction to investigate this possibility.

F. Free Amino Acid Analysis

Free amino acid analysis of the venom components having nucleotide like spectra detected few amino acids, and those only at low

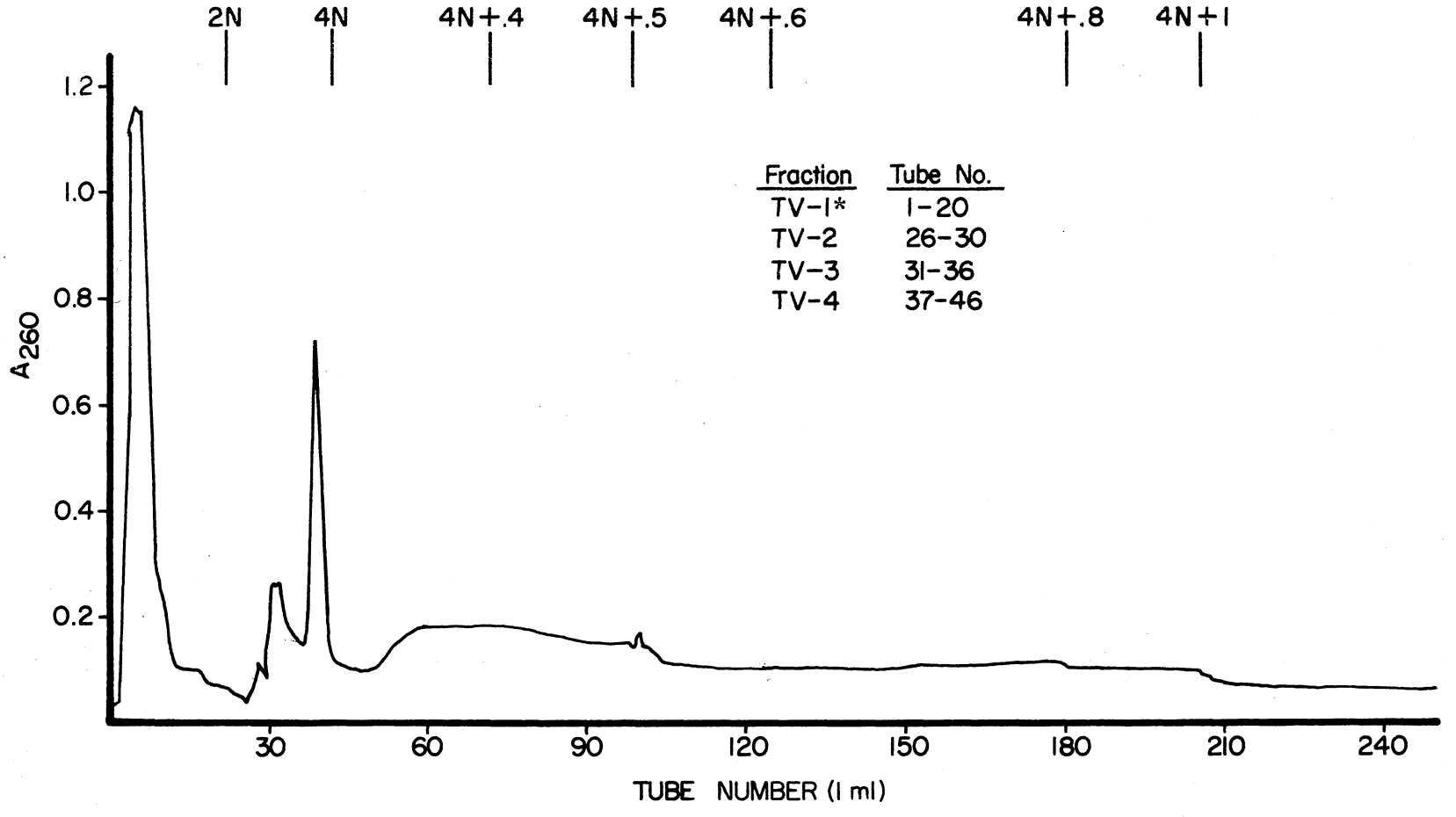

Figure 12. Dowex 1 X 8 Chromatogram of Low Molecular Weight Fraction From Tityus serrulatus (See p. 8)
*TV-1, 2, 3, 4 denote fractions pooled from column.

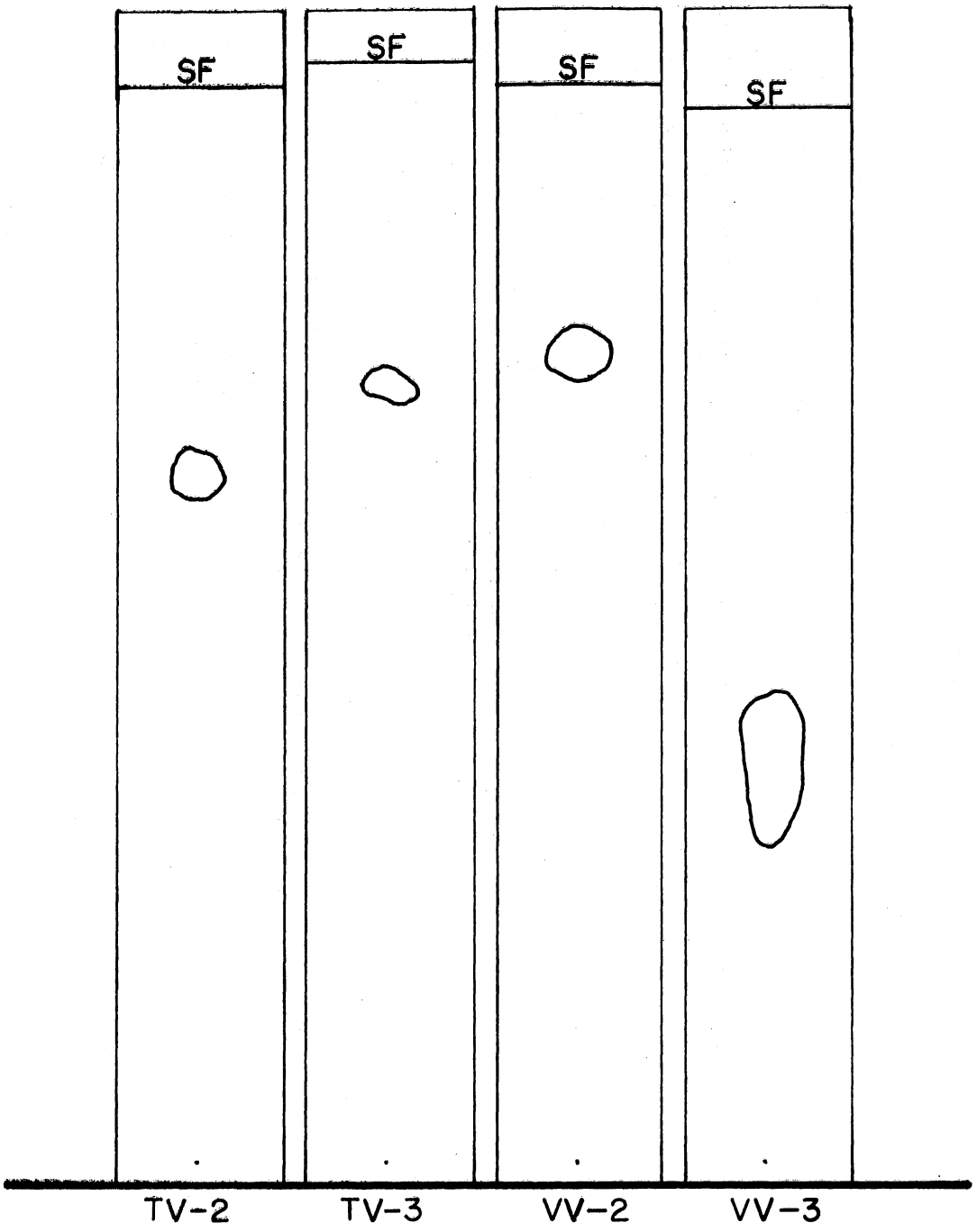

Figure 13. Thin-Layer Chromatograms of Tityus serrulatus and Vejovis spinigerus Fractions From Dowex 1 X 8 Columns (Outlines delineate ultraviolet quenching components.)

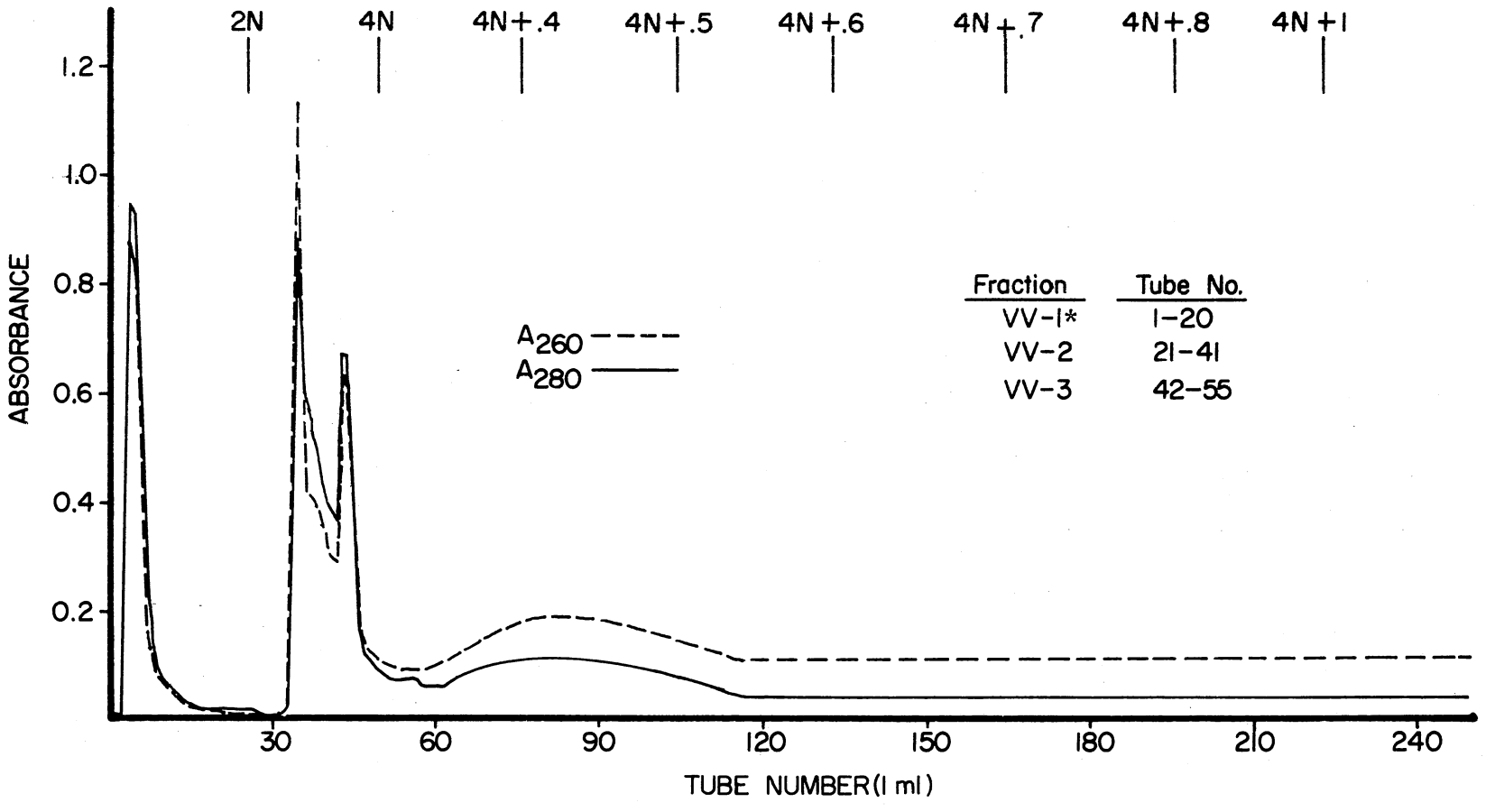

Figure 14. Dowex 1 X 8 Chromatogram of Whole Resuspended Vejovis spinigerus Venom (See p. 8)
*VV-1, 2, 3 denote fractions pooled from column.

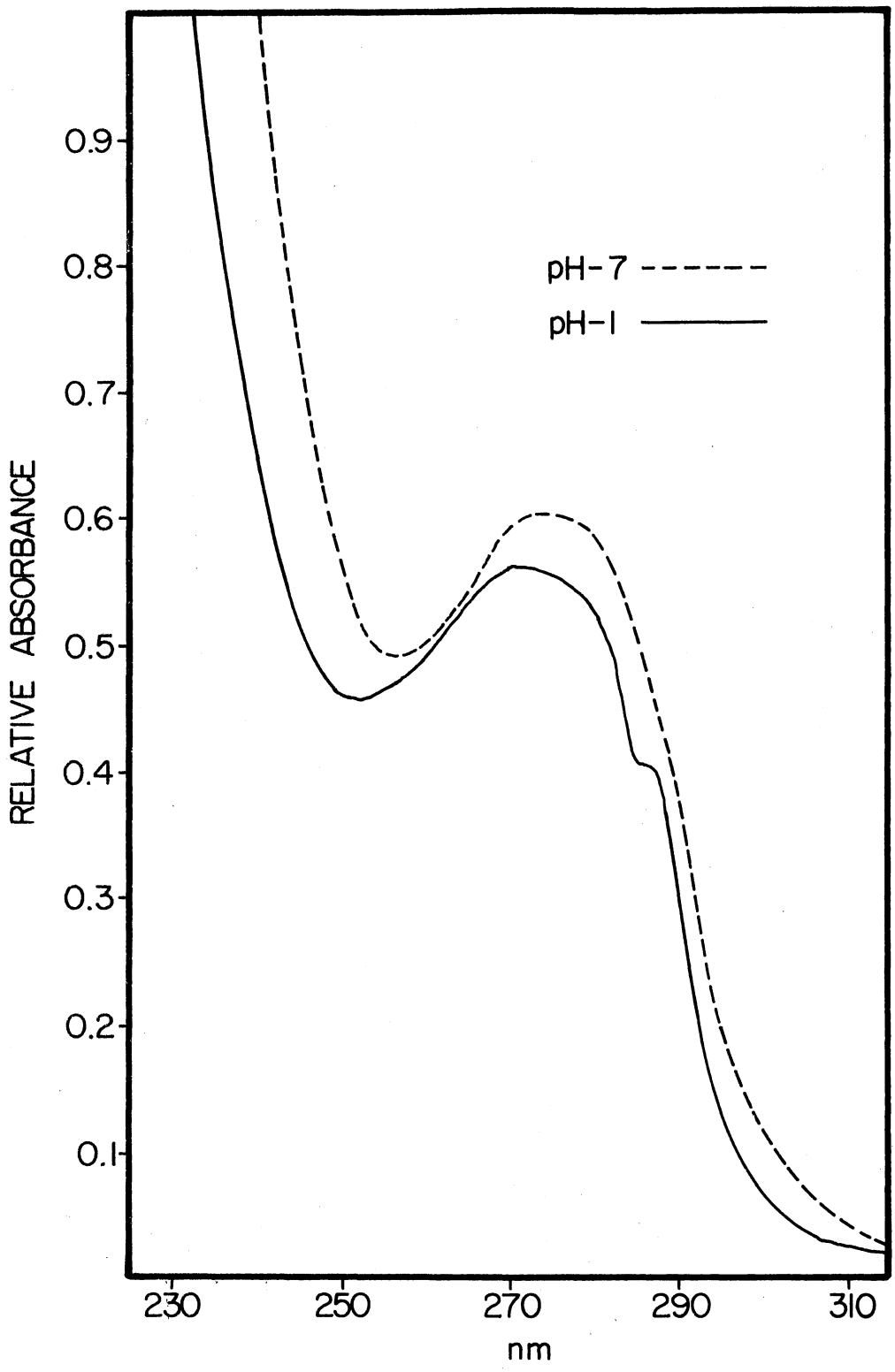

Figure 15. Ultraviolet Absorption Spectrum of Ultraviolet Quenching Component of Fraction VV-2

levels. No tryptophan was found in any sample, and only an insignificant amount of tyrosine in fraction VV-2. This makes it unlikely that there was any distortion of the ultraviolet spectra due to the presence of aromatic amino acids. As stated before, though, this does not eliminate the possibility of distortion due to a peptide. Again, shortage of sample made acid hydrolysis impractical.

G. PEI Thin Layer Chromatography

In a final attempt to determine if there was any material present which would perturb the ultraviolet spectra of the venom components, these components were chromatographed on a poly-(ethyleneimine)-cellulose thin layer system. This system, with 1M LiC as the solvent, yields reasonable separation of nucleotide bases and monophosphates, with good sensitivity. In all five cases only one quenching component was detectable in each fraction.

H. Standardization

As a standard, 200 µg of AMP was applied to the G-25 column and then passed through the rest of the procedure, just as the venom samples had been. The ultraviolet spectrum of the resulting product was made, and was found to be the same as that for standard AMP. This indicated that nucleotides, if present in the venom would remain unchanged and exhibit the same spectral qualities as before fractionation. Approximately 50.1 µg of the original 200 µg were recovered at the end of the purification schedule, giving a recovery of 25%.

I. Sensitivity of Detection

The least sensitive step of the fractionation procedure is the Dowex 1 X 8 step. To definitely detect a peak through the background approximately 20 µg must be applied to the column. Judging from the recoveries of the rest of the fractionation procedure there would be about 5 µg of sample recovered for ultraviolet analysis, when starting with 20 µg on the Dowex column. Five micrograms should be adequate for detection using the Cary 14. Recoveries from the G-25 column were approximately 85%; therefore, 22 µg applied to the G-25 column should be detected by the ultraviolet spectrophotometer. Assuming 5 µg as the threshold of detection it can be concluded that if nucleotides were present in the venoms they would have to be below the following levels: 0.05% (dry weight) in Apis mellifera venom, 0.05% in Leiurus quinquestriatus venom, 0.1% in Tityus serrulatus venom, and 0.3% in Vejovis spinirgerus venom, or they would have been detected. Contrasting these low nucleotide levels with the five percent ATP present in tarantula venom, one might reasonably conclude that nucleotides in honeybee and scorpion venoms have little or no function in the action of the toxins.

CHAPTER V

SUMMARY

The venoms of the common honeybee (Apis mellifera) and three scorpions (Leiurus quinquestriatus, Tityus serrulatus, and Vejovis spinigerus) were investigated for the presence of nucleotides. The fractionation procedure involved first, separation of the low molecular weight fraction on a Sephadex G-25 column. The tubes were pooled, lyophilized, resuspended and then further fractionated on a Dowex 1 X 8 formate form column, which was eluted with formic acid and increasing concentrations of ammonium formate. After being pooled, lyophilized, and resuspended again, the fractions were chromatographed on a silica gel--cellulose thin layer system. The solvent system was n-butanol, acetone, acetic acid, 5% aqueous NaOH, H_2O (7:3:5:3:4 v/v). The ultraviolet quenching components were then removed, eluted from the solid support, lyophilized and resuspended in the appropriate buffer for ultraviolet analysis. The ultraviolet spectra were obtained at pH-1 and pH-7 using 0.1M HCl and 0.02M sodium phosphate buffers, respectively.

Components with nucleotide-like ultraviolet absorption spectra were found in Dowex fractions two, three, and four of Apis mellifera, Dowex fraction two of Leiurus quinquestriatus, and Dowex fraction two from Vejovis spinigerus. None of the components could be identified by comparison of their ultraviolet spectra to those of standards. Standard spectra were made for eight common bases and monophosphates,

plus inosine, serotonin, tryptophan, tryptamine, and 5-hydroxytryptophan.

Amino acid analysis was conducted on the five components with nucleotide like spectra. None were found to contain sufficient amounts of aromatic amino acids to cause distortion of the ultraviolet absorption spectra. This does not, however, eliminate the possibility of nucleotides being present.

The five components with nucleotide-like ultraviolet spectra were chromatographed on poly-(ethyleneimine)-cellulose thin layer system (1M LiCl as solvent). None were found to have more than one ultraviolet quenching component.

It was estimated that amounts of nucleotide in excess of 0.05% (dry weight) in Apis mellifera venom, 0.05% in Leiurus quinquestriatus, 0.1% in Tityus serrulatus venom, and 0.3% in Vejovis spinigerus would have been detected by the methods used in this study. These estimates are based on recoveries and detection sensitivities through the fractionation procedure. Since no nucleotides were detected one must conclude that if nucleotides are present they would be at concentrations less than those specified above.

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