

THE MAJOR HEAT STABLE ALLERGEN OF SHRIMP

DONALD R. HOFFMAN, Ph.D., EUGENE D. DAY, JR., B.A., AND
JEFFREY S. MILLER, M.S.

Serum from a patient who had experienced several episodes of anaphylaxis after eating shrimp was used to monitor the isolation of a major allergen from shrimp, which was then tested with sera from 10 other shrimp-reactive patients. The allergen was isolated from a fresh extract of boiled shrimp by gel filtration and from raw shrimp by gel filtration and agarose electrophoresis. The purity of the allergen was established by crossed immunoelectrophoresis against rabbit and guinea pig antisera; it gave only a single peak in each case. The native allergen was excluded on Sephadex G-200 but showed a single band at 38000d in SDS-PAGE with or without reduction. The isoelectric point was about 4.5. The allergen contained about 4% carbohydrate and only 7 Tyr and 9 Phe out of 341 amino acid residues. Using the RAST with 11 allergic sera, the allergen gave a correlation coefficient of 0.98 with whole cooked shrimp extract and of 0.66 with raw shrimp extract. The allergen was a potent inhibitor of RAST to both cooked and raw shrimp. A second, weaker allergen of 21000d chain size was isolated from raw shrimp in highly purified form.

IN RECENT YEARS many investigators have begun to apply modern laboratory techniques to the study of food allergies.¹⁻¹¹ In addition to the definitive method of diagnosis, repeated challenge with disguised allergen after a period of elimination, which is time consuming and dangerous, particularly if the reaction is life threatening, the radioallergosorbent test,^{3,4,6,9,10,15} prick skin test^{7,8,11,14} and histamine release from leukocytes^{7,8,14} have been investigated. All of these tests have both advantages and disadvantages. One of the most serious problems with all of these techniques is that they measure the presence of an IgE antibody and not its clinical significance. For example, many individuals are RAST and skin test positive to cereal grains but ingest them routinely with no adverse effects.^{5,12} Some of these individuals are sensitive to cereals by inhalation but this hypothesis has not been verified for most patients with IgE against cereals.⁵ Some reactions to foods involve severe respiratory distress, vascular collapse and can lead to death.^{1,3,4,6,14,19} In these cases the use of *in vivo* tests is

contraindicated. When used with highly purified relevant allergens, the RAST test was shown to be both highly specific and highly sensitive.³

Purified allergens from a few very common foods, e.g., lactoglobulin, lactalbumin and casein from cow's milk and ovalbumin and ovomucoid from hen's eggs have been available for many years.¹ In recent years several investigators have isolated allergens from several other foods. The best known and most highly purified and characterized is allergen M from cod as described by Aas and his co-workers.^{3,12,13} Some work has been performed with cereal grains,⁵ soybean,¹⁶ peanut¹⁸ and sesame seed.¹⁹ With the exception of cod all of the allergens are enriched fractions and the activity appears to be somewhat heterogeneous.

Crustaceans are traditionally considered to be highly allergenic foods.^{2,4,14} Since we had several patients who had experienced severe anaphylactic reactions upon eating shrimp and also several who had urticarial reactions to contact with shrimp, we decided to attempt to isolate allergens from shrimp. In the United States shrimp are almost always cooked before consumption, so we were most interested in any allergens that were stable to cooking. However, the less stable allergens could also be important in the contact urticaria and traces might still remain after cooking.²⁰

Doctor Hoffman is Associate Professor, Department of Pathology and Laboratory Medicine, East Carolina University School of Medicine, Greenville, North Carolina.

Eugene D. Day and Jeffrey S. Miller are Medical Student Summer Research Participants, Department of Pathology and Laboratory Medicine, East Carolina University School of Medicine, Greenville, North Carolina.

Materials and Methods

Patients and Sera—Serum was collected from 10 individuals who experienced reactions to shrimp every time they ingested it; several also reacted to contact. The types of reactions are given for each patient in Table I. All of the sera were RAST positive and those patients tested were skin test positive. Sera were stored frozen until used. Serum from patient 1149 was used to follow purification schemes. This patient had his last episode of anaphylaxis seated beside a physician in a restaurant.

Shrimp Extracts—Fresh shrimp were purchased locally. Extracts were made using a blender with 10 times the weight of borate buffered saline, pH 8.0. The extracts were centrifuged and filtered through an 0.45 micron membrane filter until clear. Extracts were prepared from raw shrimp bodies, shells and cooked shrimp bodies. Shrimp were cooked by boiling for five minutes in the shell. After cooling the shells were removed and the bodies extracted in the blender. An almost identical extract could be prepared by heating the raw body extract in boiling water for 10–15 minutes and centrifuging.

RAST—The radioallergosorbent test was performed as previously described by our laboratory.²¹ All reagents were prepared as described for venom and the same quality control criteria were used. RAST inhibition experiments were performed as we have described recently.²²

Gel Filtration—Gel filtration was performed on 1.6 × 95 cm columns of Sephadex G-75 and G-200 as previously described²³ except that the eluant was borate buffered saline pH 8.0. Columns were calibrated with known protein standards.

Amino Acid Analysis—The amino acid analysis of the purified allergens was carried out on a Beckman analyzer using ninhydrin detection with automatic integration and calculation.²⁴ Results given are the average of three hydrolyses and analyses.

Protein and Carbohydrate Assays—Total protein was determined by the Lowry method.²⁵ Assays of purified proteins were corrected to the bovine serum albumin standard by using the ratio of tyrosine residues obtained from amino acid analysis. Carbohydrate was determined as hexose using the orcinol reaction²⁶ with a glucose standard.

Polyacrylamide Gel Electrophoresis—PAGE of undenatured proteins was performed using the gel formulation of Ziegler and Hengartner²⁷ for large proteins. The buffer was changed to 0.188M Tris and 0.188M glycine. Proteins were stained with Coomassie blue G-250. Sodium dodecylsulfate PAGE was performed on samples that were heated in a boiling water bath with SDS with and without dithiothreitol added to reduce disulfide bonds. Electrophoresis was in 7.5% gels using a buffer consisting of 24.8g Tris, 11.7ml glacial acetic acid and 1g of SDS per liter. Gels were run and processed using the instructions provided by Bio-Rad Laboratories with their

Bio-Phore gels. A series of five standard proteins were included in each run.

Isoelectric Focusing—Proteins were focused on Ampholine PAG Plates, purchased from LKB Instruments, according to the manufacturer's instructions. The pH gradient was measured with a flat surface electrode with the plate on the cooling surface. Proteins were stained with Coomassie Blue R-250 and Crocein scarlet.

Crossed Immunoelectrophoresis—CIEP was performed as described by Weeke.²⁸ The patterns were stained with Coomassie Blue G-250. Antisera were produced in rabbits by immunization with whole shrimp extract emulsified in Freund's complete adjuvant as we previously described.²³ Antisera to purified antigens were produced in both rabbits and guinea pigs by immunization in Freund's complete adjuvant. The same buffer was used for agarose electrophoresis.

Dialysis—Extracts were dialyzed using Spectrapor 1 tubing and concentrated using Amicon PM-10 membranes. The dialysis cut-off was 6–8000 daltons.

Results

Extracts of raw shrimp bodies, raw shrimp shells and cooked shrimp bodies were prepared and analyzed by crossed immunoelectrophoresis (CIEP). Allergic activity was measured by the radioallergosorbent test (RAST). The RAST results for both cooked and raw shrimp extracts are presented in Table I along with a short description of the patient's reactions. All 11 sera were RAST positive to both raw and cooked shrimp extracts. Results with shell were similar to raw extract. CIEP of the three extracts are shown in Figure 1. The raw and shell extracts each contain a large number of distinguishable antigens, many of which appear to be similar. On the other hand the cooked extract shows a much simpler pattern with only a few major components. A similar pattern was produced by the supernatant of raw extract after it was heated in a boiling water bath for 10 minutes.

The first separation method used to attempt to purify shrimp allergens was gel filtration. Profiles of both raw

Table I. Patients Allergic to Shrimp and RAST Results to Shrimp and Shrimp Allergens.

Patient	Reaction	RAST in percent binding minus non-specific			
		Cooked	Raw	21000d, I	Heat-stable, II
234	Urticaria Angioedema	21.4	12.6	2.2	36.4
351	Urticaria	11.1	21.1	2.2	11.8
508	Urticaria	1.8	2.7	0	0.6
632	Eczema flare	3.4	13.1	0	3.9
800	Eczema flare	1.3	2.4	1.2	1.3
822	Anaphylaxis	21.0	30.3	0	27.9
860	Eosinophilic Granuloma	1.4	2.4	0	0.9
1415	Eczema flare	7.7	10.0	2.5	9.4
2053	Angioedema	7.9	20.0	1.3	9.4
2320	Rash	7.9	17.9	2.6	7.5
1149	Anaphylaxis	27.0	26.5	14.0	29.0

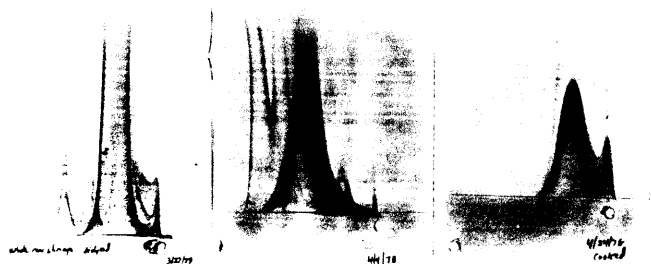


Figure 1. Crossed immunoelectrophoresis of shrimp extracts using pools of rabbit antisera. The plate on the left is dialyzed whole shrimp extract, the center plate is raw shrimp shell extract and the right plate is cooked whole shrimp extract.

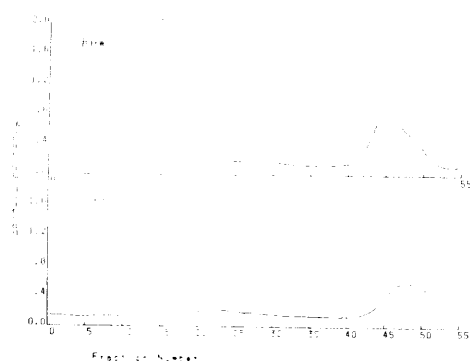


Figure 2. Gel filtration on Sephadex G-75 of extracts of raw and cooked shrimp. The column was 1.6×95 cm; elution was with borate buffered saline, pH 8.0.

and cooked extracts are shown in Figure 2. The raw extract gave three major and two minor peaks and the cooked extract separated into two major and one minor peak. The G-75 fractions from each extract were concentrated and tested by CIEP. Some of these results are shown in Figure 3. The fractions making up peak I from both the raw and shell extract show a large number of components. The second peak, however, consists almost entirely of a single antigen, which we named antigen I. The CIEP analysis of the two peaks of the G-75 separation of the cooked extract are shown in Figure 4. In this case the first peak appears to be a single antigen, which we named antigen II, and the second peak contains at least one additional antigen. Further attempts to isolate other antigens from the shrimp by DEAE chromatography, reduction and alkylation and acid precipitation were not successful. Antigens I and II were purified to immunochemical homogeneity by repeated gel filtration.

Antigens I and II were assayed for allergen activity by direct RAST. The results are shown in Table I. All of the 11 sera reacted strongly with antigen II and seven of the eleven reacted with antigen I. The data in Table I were analyzed for correlation between the antigens and extracts; the results are given in Table II. The highest correlation coefficient was found between cooked shrimp extract and antigen II. The lowest correlation was between raw shrimp and antigen I, only slightly less than that between the two antigens. The cooked shrimp extract also showed a low correlation with antigen I. The relationship of allergenic activity was further investigated by inhibition of RAST as is shown in Figure 5. Inhibition of RAST to raw shrimp extract by antigen II (heat stable allergen) was performed with four individual sera. The RAST was completely inhibited by antigen II with sera 822 and 2053 and significant inhibition was seen for sera 1149 and 234. In a similar experiment with antigen II inhibiting binding to cooked shrimp extract 92% inhibition was found with serum 1149, 86% with 2053 but only 16% with 234, much less than was seen for inhibition of raw shrimp binding with that serum. Both direct and inhibition RAST show strong allergen activity for antigen II, the heat stable allergen.

Before the RAST studies were undertaken the purity and homogeneity of the two antigen preparations was assessed by immunizing several rabbits and guinea pigs with each preparation and using these sera in CIEP to verify that only one arc was found. The two purified antigens were then subjected to a variety of physicochemical analyses. The results are summarized in Table III. Amino acid analyses of both antigens are given in Table IV. Antigen I appears to be a dimer of two polypeptide chains of molecular weight 21000 with an isoelectric point of 4.75–5.0. The amino acid composition is unremarkable. Antigen I chains are apparently non-covalently associated in the native molecule, since they separate in PAGE and SDS-PAGE without reduction. Antigen II, the heat stable antigen, has a native molecular weight of over 200,000, possibly due to heat aggregation. This antigen is difficult to isolate from unheated

MAJOR HEAT STABLE ALLERGEN OF SHRIMP—HOFFMAN ET AL

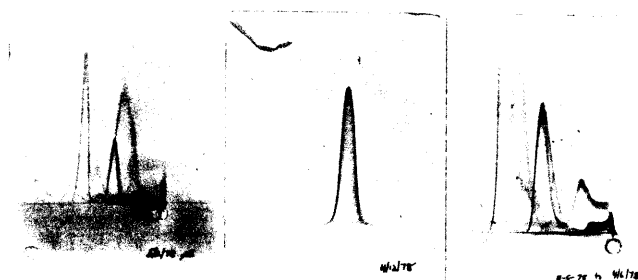


Figure 3. Crossed immunoelectrophoresis of G-75 gel filtration fractions of raw shrimp extracts. The left pattern is raw shrimp body extract peak I, the center plate is raw shrimp body peak II and the right pattern is raw shell extract peak I.

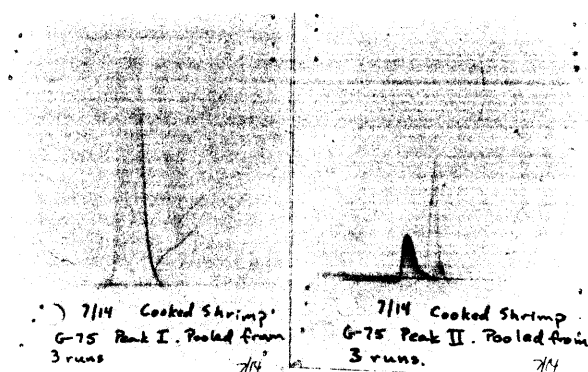


Figure 4. Crossed immunoelectrophoresis of G-75 gel filtration fractions of cooked shrimp extracts. The left plate is peak I after rechromatography and the right plate is peak II.

Table II. Correlation of RAST with Shrimp Allergens and Whole Shrimp Extracts for 10 Allergic Patient Sera.

Pair	Correlation coefficient r	Slope
Cooked—raw	0.76	0.64
Cooked—Antigen I	0.52	1.04
Cooked—Antigen II	0.98	0.63
Raw—Antigen I	0.44	1.06
Raw—Antigen II	0.66	0.51
Antigen I—Antigen II	0.48	0.15

shrimp extract. The molecular weight agreed quite well by the various methods giving a chain size of 38000 with 4% carbohydrate. Again the chains are non-covalently associated, since reduction with dithiothreitol does not further decrease chain size. The isoelectric point is 5.4–5.8 and the amino acid composition is somewhat unusual in the low fraction of tyrosine and phenylalanine. Tryptophane was not measured.

Antigen I and antigen II were distinct by CIEP with

sera from multiple individuals of two species (rabbit and guinea pig), PAGE, SDS-PAGE, gel filtration, isoelectric point, carbohydrate analysis and amino acid analysis. In addition, antigen II was a strong allergen for humans clinically allergic to shrimp, while antigen I was a weak allergen. Antigen II was reactive with sera from all 11 shrimp allergic patients studied and was a potent inhibitor of RAST to cooked and raw shrimp. RAST to antigen II gave a correlation of 0.98 with RAST to cooked shrimp. Antigen II was relatively stable to cooking or heating in boiling water; this property was used to isolate it. Both allergens were found in raw shrimp body and shell extracts. There was only a trace of antigen I detectable in cooked shrimp extract. Antigen II appears to be a major allergen of shrimp; its heat stability indicates that it may be the major clinically relevant allergen of shrimp.

Discussion

The isolation, purification and partial characterization

MAJOR HEAT STABLE ALLERGEN OF SHRIMP—HOFFMAN ET AL

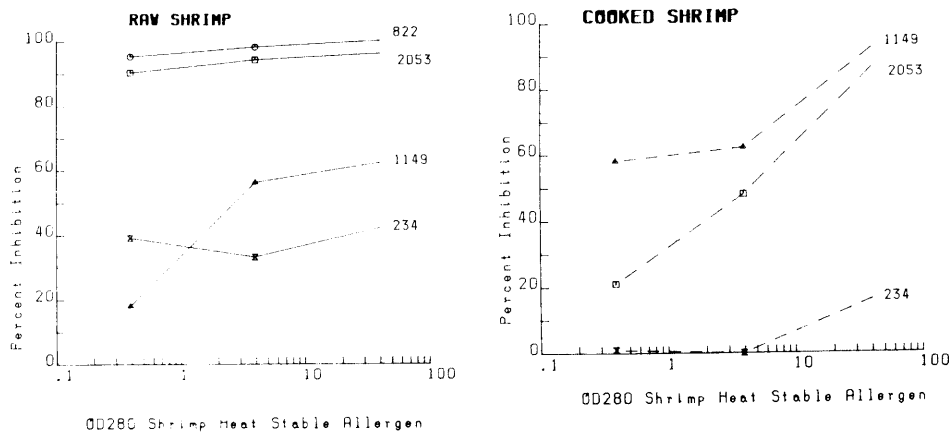


Figure 5. Inhibition of the radioallergosorbent test by shrimp heat stable allergen (antigen II) of binding to raw shrimp extract discs on the left and cooked shrimp extract discs on the right.

Table III. Physico-chemical Properties of Shrimp Antigens I and II.

	Antigen I	Antigen II
Molecular weight (gel filtration)	45000	over 200000
Molecular weight (PAGE)	21000	38000
Molecular weight (SDS-PAGE-reduced)	21000	37500
Isoelectric point	4.75-5.0 (3 bands)	5.4-5.8 (not resolved)
Percent carbohydrate	0.5	4.0
Molecular weight (amino acid anal.)	20400	36800
Molecular weight (Incl. CHO)	20500	38300

of two antigens having allergenic activity from shrimp have been described in this paper. One of these antigens, antigen II, is heat stable, gives a RAST correlation of 0.98 with cooked shrimp, is a potent inhibitor of RAST to cooked and raw shrimp, and is one of only a few shrimp proteins stable to boiling water temperature. Since shrimp are rarely, if ever, eaten raw in this country, antigen II appears to be the major allergen since there are only a few other components in the cooked shrimp extract and they have much less RAST activity (data not shown). Antigen I has RAST activity with seven of the 11 sera from allergic patients but it is weaker than antigen II, and antigen I is not heat stable. Physicochemical and chemical analyses confirm the results of immunologic studies showing that the two antigens are not related.

Many patients not only have reactions on ingestion of shrimp but also develop contact urticaria from handling shrimp or even packages that have contained shrimp. Patient 1149, used extensively in this study, worked in a supermarket and he developed contact urticaria when-

Table IV. Amino Acid Analysis of Shrimp Allergens.

	I	II
Asp	28	58
Thr	6	12
Ser	6	15
Glu	23	61
Pro	4	6
Gly	15	20
Ala	17	31
Cys	2	2
Val	14	19
Met	3	9
Ile	9	12
Leu	13	30
Tyr	12	7
Phe	12	9
His	2	4
Lys	14	27
Arg	9	19
	189	341
M.W.	20412	36828
CHO	0.5%	4.0%

Results are the average of three hydrolyses of each antigen and are rounded off to the nearest integer.

ever he handled packages of shrimp, lobster or crab. He had anaphylaxis requiring immediate administration of epinephrine whenever he ingested any of these crustaceans. Some of the allergens that are not heat stable may be important in this type of reaction. In addition traces of undenatured allergens may remain after cooking and could be important in many cases.

The major allergen from codfish, allergen M, is a parvalbumin with pI 4.75 and molecular weight 15,500²⁸ and an unusual amino acid composition high in Phe and low in Tyr and Trp. Antigen II from shrimp is much

larger, has pI around 5.6 and is low in both Phe and Tyr. Both antigens are heat stable.

Antigen II appears to be the major ingestion allergen of shrimp since it is closely correlated with RAST to whole cooked shrimp, reactive with all allergic patients tested, a good inhibitor of RAST to both cooked and raw shrimp and heat stable. In these ways it is quite analogous to cod allergen M. Although many of the patients also report reactions from crab and lobster, we have not as yet tested the cross-reactivity. However, we have sera that react to only one of the crustaceans by RAST. Antigen II may have both specific and shared allergenic determinants. At present we recommend testing with all of the appropriate extracts. Shrimp shows no relation to sensitivity to bivalves such as clam and oyster or to sensitivity to codfish.

Acknowledgments

The authors thank the several physicians who provided us with sera from their shrimp allergic patients and particularly Dr. Robert Townley, who provided patient 1149. Excellent secretarial assistance was provided by Ms. Cathy Everette and Mrs. Jo Gillin.

References

- Goldstein GB and Heiner DH: Clinical and immunological perspectives in food sensitivity. *J Allerg* 46: 270, 1970.
- Haddad ZH and Korotzer JL: Immediate hypersensitivity reactions to food antigens. *J Allerg & Clin Immunol* 49: 210, 1972.
- Aas K and Lundkvist U: The radioallergosorbent test with a purified allergen from codfish. *Clin Allerg* 3: 255, 1973.
- Hoffman DR and Haddad ZH: Diagnosis of IgE mediated reactions to food antigens by radioimmunoassay. *J Allerg & Clin Immunol* 58: 165, 1974.
- Hoffman DR: The specificities of human IgE antibodies combining with cereal grains. *Immunochem* 12: 535, 1975.
- Gillespie DN, Nakajima S and Gleich GJ: Detection of allergy to nuts by the radioallergosorbent test. *J Allerg & Clin Immunol* 57: 302, 1976.
- May CD: Objective clinical and laboratory studies of immediate hypersensitivity reactions to food in asthmatic children. *J Allerg & Clin Immunol* 58: 500, 1976.
- Chua YY, Bremner K, Lakdawalla N, Llobet JL, Orange RP and Collins-Williams C: *In vivo* and *in vitro* correlates of food allergy. *J Allerg & Clin Immunol* 58: 299, 1976.
- Chua YY, Bremner K, Llobet JL, Kokubu, HL and Collins-Williams C: Diagnosis of food allergy by the radioallergosorbent test. *J Allerg & Clin Immunol* 58: 477, 1976.
- Dannaus A, Johansson SGO, Foucard T and Ohman S: Clinical and immunological aspects of food allergy in childhood. *Acta Pediat Scand* 66: 31, 1977.
- Bock SA, Buckley J, Holst A and May CD: Proper use of skin tests with food extracts in diagnosis of hypersensitivity to food in children. *Clin Allerg* 7: 375, 1977.
- Aas K: The diagnosis of hypersensitivity to ingested foods. *Clin Allerg* 8: 39, 1978.
- Aukrust L, Apold J, Elsayed S and Aas K: Crossed immunoelectrophoretic and crossed radioimmuno-electrophoretic studies employing a model allergen from codfish. *Int Arch Allerg & Appl Immunol* 57: 253, 1978.
- May CD and Bock SA: Adverse reactions to food due to hypersensitivity. In *Allergy: Principles and Practice*, Middleton E Jr, Reed CE and Ellis EF. (Eds.). St. Louis: C. V. Mosby Co., p. 1159, 1978.
- Wraith DG, Merrett J, Roth A, Yman L and Merrett TG: Recognition of food allergic patients and their allergens by the RAST technique and clinical investigation. *Clin Allerg* 9: 25, 1979.
- Moroz LA and Yang WH: Kunitz soybean trypsin inhibitor. *New Eng J Med* 302: 1126, 1980.
- May CD: Food allergy—material and ethereal. *New Eng J Med* 302: 1142, 1980.
- Sachs MI, Jones RT and Yunginger JW: Isolation and partial characterization of a major peanut allergen. *J Allerg & Clin Immunol* 67: 27, 1981.
- Malish D, Glosky MM, Hoffman DR, Ghekiere I and Hawkins JM: Anaphylaxis after sesame seed ingestion. *J Allerg & Clin Immunol* 67: 35, 1981.
- Hoffman DR, Day ED and Miller JS: The major heat-stable allergen of shrimp. *Fed Proc* 38: 1415, 1979.
- Hoffman DR: The use and interpretation of RAST to stinging insect venoms. *Ann Allerg* 42: 224, 1979.
- Hoffman DR: Allergens in Hymenoptera Venom. VI Cross reactivity of human IgE antibodies to the three vespid venoms and between vespid and paper wasp venoms. *Ann Allerg* (in press).
- Hoffman DR, Shipman WH and Babin D: Allergens in Bee Venom. II. Two new high molecular weight specificities. *J Allerg & Clin Immunol* 59: 147, 1977.
- Schroeder WA, Shelton JR, Shelton JB, Cormick J and Jones RT: The amino acid sequence of the gamma chain of human fetal hemoglobin. *Biochem* 2: 992, 1963.
- Lowry OH, Rosebrough NJ, Farr AL and Randall R: Protein measurements with the Folin phenol reagent. *J Biol Chem* 193: 265, 1951.
- Kabat EA: *Kabat and Mayer's Experimental Immunology*. Springfield, Ill.: C. T. Thomas Co. p. 527, 1961.
- Ziegler A and Hengartner H: Sodium dodecyl sulfate electrophoresis of high molecular weight proteins in N,N'-diallyltartardiamide cross linked polyacrylamide slab gels. *Eur J Immunol* 7: 690, 1977.
- Weeke B: Quantitative immunoelectrophoresis. *Scand J Immunol* 22, suppl 1: 15, 1973.
- Elsayed S, Aas K and Christensen T: Partial characterization of homogeneous allergens (cod). *Int Arch Allerg & Appl Immunol* 40: 439, 1971.

Requests for reprints should be addressed to:
Donald R. Hoffman, Ph.D.
Associate Professor
Department of Pathology and Laboratory Medicine
East Carolina University School of Medicine
Greenville, North Carolina 27834

"In science, read by preference, the newest works; in literature, the oldest."
Edward Bulwer-Lytton