

IMMUNE SERA IN THE STUDY OF FOOD ANTIGENS

I. FISH

SHELDON G. COHEN, M.D.,* WILKES-BARRE, PA.
WITH THE TECHNICAL ASSISTANCE OF JOHN SABA**

INTRODUCTION

TECHNICAL difficulties experienced in attempting to isolate purified antigenic fractions from foodstuffs have hindered studies of possible biochemical and immunologic identity between food allergens of man and precipitin-producing antigens of experimental animals. Although the reagin of natural allergic man and the serum precipitin of the immunized rabbit differ in physicochemical properties and characteristic biologic actions, they do possess a common denominator of specific reactivity with the same antigen. Thus, any in vitro experimental study involving a soluble food antigen-precipitating antibody system may offer only indirect evidence of the potential role of the particular food as an allergen in man. However, in view of the specificity of action of the in vitro precipitin test, we have attempted to utilize this objective experimental tool in studying some problems related to food allergens that could not be resolved satisfactorily by either direct or indirect intracutaneous tests alone.

The general experimental approach revolved around the ability of immune serum, through its content of precipitating antibodies, to react with specific antigenic counterparts. Antisera from rabbits immunized to fish extracts were thus employed in an in vitro detection of antigenic components of fish-derivative preparations.

EXPERIMENTAL PROCEDURES AND RESULTS

Male rabbits, varying in weight between 2.5 and 3 kilograms, were utilized in active immunization procedures for the production of specific antisera to fish antigens. The immunizing preparations consisted of fluid glycerosaline extracts† of cod (genus *Gadus*) and halibut (genus *Hippoglossus*). Each rabbit received a total of 12 mg. of protein nitrogen of either halibut or cod fish extract through a series of twelve equally divided doses given by intravenous injections over a period of twenty-one days. Antisera were prepared from blood taken by cardiac puncture seven days after the last injection.

From the Department of Biology, Wilkes College.

Presented in part at the fifteenth annual meeting of the American Academy of Allergy, Chicago, Illinois, Feb. 9 to 11, 1959.

This study was sponsored by the Committee on Food Allergy of the Research Council, American Academy of Allergy.

Received for publication Dec. 31, 1958.

*Associate Professor of Biological Research.

**Student Laboratory Assistant.

†Prepared and supplied through the courtesy of Margaret Strauss, Director of the Allergy Laboratory, University Hospital, New York University-Bellevue Medical Center.

Pooled sera showing equivalent high titers to cod extract and similarly pooled antisera to halibut extract were subsequently utilized in precipitin test procedures.

Qualitative antigenic analyses of fish extracts and fish-derivative preparations were carried out by precipitin test techniques in agar-filled Petri dishes, according to the double diffusion method described by Ouchterlony.¹ The soluble fish preparation and the antiserum to fish extract were placed in oppositely located wells within the agar to allow for their interreaction while diffusing through the gel. Since each band of precipitate represented an independent antigen-antibody system, these reactions permitted the resolution of reactants into individual components. Reactions of identity and those of nonidentity between individual antigenic constituents of soluble fish preparations were determined by allowing each to diffuse from a parallel position within the agar against a common reference antiserum to the fish extract.

The antisera employed in all subsequently described experiments possessed the following characteristics: (1) antiserum to cod contained six immunologically distinct reactive antibody components, and (2) antiserum to halibut contained five such components.

Fish-Liver Oils.—Commercially available cod-liver oils and halibut-liver oils, utilized as sources of vitamins A and D, were studied for possible fish antigen content. Random lots of cod-liver oil and Haliver oil from different pharmaceutical houses were obtained on the open market, and each was utilized in two different types of experiments.

Series of rabbits were injected with either one of the five cod-liver oil preparations or one of the two Haliver oil preparations. Each animal received 1 c.c. of the oil twice weekly via alternating intramuscular and intraperitoneal injections. After the third week, and at weekly intervals thereafter, each rabbit was bled from the central artery of the ear and the serum was separated and studied for possible antibody content to the homologous fish extract. These semiweekly injections and weekly bleedings were continued over a sixteen-week period. In vitro tests were carried out with each weekly sample of rabbit sera and a fish extract of proved antigenicity (halibut extract containing six distinct antigens and codfish extract with five individual antigenic constituents). In no instance was the development of an antigen-antibody precipitate noted.

An oil-in-water emulsion was prepared from each lot of cod-liver oil and Haliver oil in amounts equal to those employed in immunization procedures. After repeated agitation over a three- to four-day period, the oil and water were allowed to separate in layers and were individually collected. The aqueous fraction of each preparation was employed in agar-gel diffusion precipitin tests with antisera to fish extracts. The results are shown in Table I.

Fish Emanations.—

Boiled fish: Small pieces of fresh raw cod were placed in a distilling flask containing water, and the contents of the flask were heated to boiling temperatures. The resultant steam was led off directly through glass tubing into a water-cooled condensor, and the distillate was collected. The same procedure was repeated for raw halibut.

Fried fish: The above-described apparatus was modified to collect the distillates of fish-in-oil fumes of fresh raw cod and of halibut frying in hydrogenated cooking fats. Oil-in-water emulsions of the distillate were prepared and thoroughly agitated, and the aqueous fraction was separated and collected.

Raw fish odors: Raw ground cod was placed in refrigerated empty sterile filtering flasks. Rubber stoppers were used to close off the openings at the side arms and mouths of the flasks. At periodic intervals over five days the flasks

TABLE I

AQUEOUS EXTRACT	ANTISERUM TO:	NUMBER OF PRECIPITIN BANDS
Cod-liver oil	Codfish	
Brand A	Codfish	1-2
Brand B	Codfish	1-3
Brand C	Codfish	0
Brand D	Codfish	2-5
Brand E	Codfish	0
Halibut-liver oil	Halibut	
Brand A	Halibut	2
Brand B	Halibut	0-1

were removed from the refrigerator and the air within the flask was aspirated by means of a vacuum pump through the side arms. The fish odor-laden air was then led off through tubing and bubbled directly into the buffered saline contents of sterile vials. Similar procedures were carried out with fresh ground raw halibut.

Precipitin tests: The steam distillates, aqueous extracts of the oil distillates, and saline solutions of raw and decomposing fish emanations of both cod and halibut were employed in agar-gel diffusion tests with high-titer antisera to cod and halibut, respectively. In no instance was a visible band of precipitate noted within the agar.

TABLE II

TEST PHARMACEUTICAL	ANTISERUM TO:	NUMBER OF PRECIPITIN BANDS
Protamine sulfate	Cod	1-2
Protamine sulfate	Halibut	2
Sodium morrhuate	Cod	5
Sodium morrhuate	Halibut	5

Fish-Derivative Pharmacologic Agents.—Solutions of protamine and sodium morrhuate injectable pharmaceutical preparations obtained on the open market were employed in agar-gel diffusion precipitin tests with antisera to cod and to halibut. The results are shown in Table II.

Fish Roe.—An extract was prepared from the roe of yellow pike (*Stizostedion vitreum*), utilizing buffered saline according to standard methods of extraction. Antisera to fish roe were prepared according to the immunization techniques previously described for the production of fish antisera. Agar-gel

diffusion precipitin tests were carried out with fish roe extract, homologous antisera, and the antigenic preparations of chicken egg yolk and egg white and their antisera, the preparation and properties of which were described in a previous report.²

The interreaction of antiserum to fish roe and homologous fish roe extract resulted in the production of two bands of precipitate. However, in tests employing (1) antiserum to fish roe with chicken egg white and egg yolk and (2) fish roe extract with the antisera to chicken egg white and egg yolk, no precipitin bands could be noted.

Fish Extracts.—Randomly selected samples of cod and halibut fluid extracts prepared in different laboratories for purposes of skin testing were employed in agar-gel diffusion tests with the antisera to cod and to halibut. The results are summarized in Table III.

TABLE III

ANTISERUM TO:	EXTRACT	NUMBER OF PRECIPITIN BANDS			
		A	B	C	D
Cod	Cod	5-6	0-3	3-4	4-5
Halibut	Halibut	3-5	0	4	

COMMENT

None of the commercially available lots of cod- and halibut-liver oils employed were found to be capable, by these techniques, of inducing antibody formation to the identifiable antigens of homologous fish-muscle extracts. If the ability of an animal-derived oil to induce precipitating antibody in the rabbit were accepted as the sole requirement of its protein content, one would be tempted to infer that these fish-liver oils were protein-free. By aqueous treatment of the oil, however, sufficient fish protein could be extracted and concentrated to react with specific antibody components of high-titer antifish sera. Even though the total amount present was apparently below the quantitative critical level required for precipitin production, its antigenic potential in animals already sensitized must be considered.

The fact that fish-liver oils may contain protein fragments and carbohydrates and their derivatives is taken into account in the processing of these materials.³ One of the many variables determining the composition of the final product is the extent of preliminary damage to the native oil-containing fish tissue. Hydrolysis and degradation of certain proteins increase their solubility and dispersibility in oil,³ and fish oils in contact with decomposing fish proteins can absorb and chemically combine with appreciable quantities of nitrogenous compounds.⁴ Since the greater part of such constituents is soluble in oil only in anhydrous form, the greater part of the phosphatides, proteins and protein fragments, resinous and mucilaginous materials, and hydrocarbons may be removed by hydration or by alkali treatment. Thus, the second variable in determining the final composition of the oil product is directly related to the

efficacy of the "degumming process." It is, therefore, understandable that different lots of fish-liver oils can vary widely in both quantitative and qualitative protein content.

Allergic-type reactions to ingested fish-liver oils have been reported.⁵⁻⁷ Whether all such instances represent true hypersensitivity reactions to constituent fish proteins or to an "oil antigen," or perhaps even an untoward reaction to one of the many variable unidentified organic contaminants, is not apparent. The fact that only trace amounts of fish protein were demonstrated in some lots of cod-liver and Haliver oils may help to interpret the clinical observation of Feinberg⁶ that instances of fish-oil allergy apparently occur only in fish-sensitive persons with extreme sensitiveness and that the average fish-sensitive patient may tolerate fish oils in average small doses.

Along with the inconstant presence or absence of fish protein in fish-liver oil, these precipitin tests also demonstrated the variability in number of individual antigens in the final product. Thus, along with a quantitative factor, perhaps another important determinant of allergic reactivity for any fish-liver oil may be the variable presence of that particular protein fraction of fish for which the recipient possesses reagins and clinical sensitivity.

The failure of any of the steam or fried distillates or soluble preparations of the emanations of raw fish to react visibly with specific antisera to the fish suggests that none of these materials contained identifiable antigenic fish fractions. In view of the high molecular weight of fish-protein molecules, it is not surprising that they failed to undergo processes of volatilization after aqueous solution or to be carried off adsorbed to hot oil molecules without prior denaturization or degradation. The question then arises as to what explanation may be placed upon those reported clinical observations in which acute allergic symptoms have appeared in patients merely exposed to the odor of raw or cooked fish. Heretofore, these have been attributed to a volatile fraction of fish antigen.⁶⁻⁸ Furthermore, according to Tuft,⁹ the denaturization of fish through cooking may reduce the skin-test activity and nitrogen content of the allergenic extract, but this may be insufficient to be clinically important. A suitable explanation for this finding, therefore, can be only a matter for speculation. Among suggested possibilities, the following are evident: (1) The products of hydrolysis of the proteins of frying or cooking fish, such as ketones and aldehydes, may be nonspecifically irritating to an allergic respiratory mucosa. (2) Only the undenatured protein molecule will meet the antigenic requirements for specific in vitro precipitin reactions with animal antisera, whereas fragments of the molecule existing as polypeptides may serve as degraded antigens of lesser molecular weight or in some haptenic fashion as specific food allergens in the highly reaginic-sensitive human being.

Protamine sulfate is described as any one of a group of simple proteins or "protein-like" substances occurring in combination with nucleic acid in the sperm of fish. It is a commonly employed pharmacologic agent for antiheparin action and for the conversion of insulin into a long-acting preparation. Apparently, reported local sensitivity reactions to injected protamine¹⁰ and to

salmine of protamine zinc insulin¹¹ are rare. The possibility that some reactions following insulin therapy may be due to the added protamine has been considered and investigated,¹¹⁻¹² and it has generally been felt that protamine is devoid of allergenic properties.⁶ However, the fact that antigenic fish constituents can be identified by these techniques suggests potential allergenicity in fish-sensitive patients.

Sodium morrhuate, the sodium soap of the fatty acids of cod-liver oil, is employed in aqueous solution as a sclerosing agent for varicose veins. Occasional reactions of the hypersensitivity type, such as urticaria and even shock, have been reported.¹³⁻¹⁵ Here again, the identification of component fish antigens suggests their possible allergenic role.

The known components of chicken egg yolk¹⁶ include lipovitellin, livetin, lipovitellenin, phosvitin, vitellin, and vitellenin. Of these, lipovitellin (ichthulin), livetin (thuetin), and phosvitin have been identified in some fish roe.¹⁷ The question then arises as to the possibility of cross-antigenicity between these three biochemically related yolk proteins of diverse species origin. However, by immunologic techniques we have been able to note only two distinct antigenic components for fish roe, and these do not appear to possess any immunologic identity with the demonstrated antigenic fractions of the chicken egg.

The results of these serologic tests point out the wide variation in content of precipitin-producing antigens that may exist between species-identical fish extracts. All were submitted as skin-test-active extracts, and presumably they were prepared by similar laboratory techniques. It has not been determined whether these differences can be related to such factors as minor variations in technique of extraction, the state of the fish sources (for instance, frozen or fresh), the length of time elapsed and the efficiency of preliminary refrigeration before extraction was accomplished, the method and duration of storage, and the use of preservatives in the extract. It is also not apparent whether or not such variations in qualitative antigenic content might explain many of the inconstant results observed in skin tests with fish extracts that often confuse clinical interpretation.

In the evaluation of any hypersensitivity reaction, consideration must always be given to the quantitative factor of antigen content in relationship to the existing degree of clinical sensitivity. Such quantitative variations in antigen content common to different species of fish have been demonstrated by Tuft and Blumstein,⁸ utilizing passive transfer neutralization techniques with fish-reaginic sera. However, the results of this study also suggest another factor for consideration, namely, the wide variability in qualitative content of multiple antigenic fractions in fish-derivative preparations. It is possible, therefore, that one important determinant of skin-test activity of a fish extract or clinical allergic reactivity for a food or pharmaceutical of fish origin may be the variable presence of that particular protein fraction of fish for which the recipient possesses reagins and clinical sensitivity, respectively. Proof of this, however, must await the results of clinical correlation and a clearer understanding of the exact relationships of precipitin-producing antigens to food allergens. The

importance of these unresolved points as subjects for further investigation is self-evident. Such questions are, therefore, under current consideration and study by the Committee on Food Allergy of the Research Council.

SUMMARY

1. Sera from specifically immunized rabbits were employed in agar-gel diffusion precipitin test techniques in the qualitative antigenic analyses of fish extracts and fish-derivative preparations.

2. Fish antigens were demonstrated in some samples of cod- and halibut-liver oils, with wide variations in components existing among different lots of these materials.

3. Antigenic fish constituents could not be detected in the emanations of raw, fried, or boiled cod and halibut.

4. Fish antigens were identified in protamine and sodium morrhuate.

5. The antigenic components of fish roe did not demonstrate cross-antigenicity with egg proteins of chicken origin.

6. A wide qualitative variation among individual component antigens of fish muscle was demonstrated in homologous species skin-testing extracts of different laboratory sources.

REFERENCES

1. Ouchterlony, Ö.: Antigen-Antibody Reaction in Gels. IV. Types of Reactions in Co-Ordinated Systems of Diffusion, *Acta. path. et microbiol. scandinav.* 32: 231, 1953.
2. Cohen, S. G., and Mines, S. C.: Variations in Egg White and Egg Yolk Components of Virus and Rickettsial Vaccines, *J. ALLERGY* 29: 479, 1958.
3. Bailey, A. E.: Industrial Oil and Fat Processes, New York, 1945, Interscience Publishers, Inc., pp. 22-25, 501.
4. Davies, W. L., and Gill, E.: *J. Soc. Chem. Ind.* 55: 141, 1936. (Cited by Bailey.²)
5. Balyeat, R. M., and Bowen, R.: Cod Liver Oil Sensitivity in Children, *Am. J. Dis. Child.* 47: 529, 1934.
6. Feinberg, S. M.: Allergy in Practice, ed. 2, Chicago, 1946, Year Book Publishers, Inc., pp. 320-321, 353.
7. Hoffman, S. J., and Rattner, H.: Infantile Eczema From Cod Liver Oil, *J. A. M. A.* 107: 494, 1936.
8. Tuft, L., and Blumstein, G.: Studies in Food Allergy, *J. ALLERGY* 17: 329, 1946.
9. Tuft, L.: Clinical Allergy, ed. 2, Philadelphia, 1949, Lea & Febiger, p. 176.
10. Eli Lilly and Company: Product Information.
11. Alexander, H. L.: Reactions With Drug Therapy, Philadelphia, 1955, W. B. Saunders Company, p. 205.
12. Kern, R. A., and Langner, P. H., Jr.: Protamine and Allergy; Nature of the Local Reactions and Injections of Protamine Zinc Insulin; Induction of Sensitivity to Insulin by Injections of Protamine Zinc Insulin, *J. A. M. A.* 113: 198, 1939.
13. Zimmerman, L.: Allergic-Like Reactions From Sodium Morrhuate in Obliteration of Varicose Veins, *J. A. M. A.* 102: 1216, 1934.
14. Praver, L. L., and Becker, S. W.: Sensitization Phenomena Following the Use of Sodium Morrhuate for Chemical Obliteration of Varicose Veins, *J. A. M. A.* 104: 947, 1935.
15. Simmons, N. J.: Anaphylaxis to Sodium Morrhuate Following Injection of Internal Hemorrhoids, *New England J. Med.* 218: 527, 1938.
16. Warner, R. C.: Egg Proteins. In Neurath, H., and Bailey, K. (editors): The Proteins, New York, 1954, Academic Press, Inc., vol. II, pp. 435-485.
17. Young, E. G., and Phinney, J. L.: On the Fractionation of the Proteins of Egg Yolk, *J. Biol. Chem.* 193: 73, 1951.