DISTINGUISHING TUNA SPECIES BY IMMUNOCHEMICAL METHODS

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ABSTRACT

Interspecies differences in the sera of adult tuna (albacore, *Thunnus alalunga;* yellowfin, *Thunnus albacares*; bigeye tuna, *Thunnus obesus:* skipjack, *Katsuwonus pelamis;* little tunny (wavyback skipjack), *Euthynnus yaito;* and dogtooth tuna, *Gymnosarda nuda*) were demonstrated by double diffusion precipitation with antisera produced in rabbits. Studies of the soluble antigens extractable from the flesh of tuna were complicated by spontaneous precipitation. It was possible, however, to distinguish skipjack tuna from albacore and yellowfin by means of immunodiffusion of extractable tissue antigens.

The tunas, an economically and biologically major group of marine fishes, have been the subject of considerable study by fishery scientists. One of the most perplexing problems in these studies has been the identification of larval forms of these fishes. Work on various aspects of this problem using morphological and paper chromatographic methods has been presented by Walter M. Matsumoto (1958, 1959, and 1960), who has discussed at considerable length the ultimate objectives and the difficulties of such research.

Because of difficulties in application of morphological methods, due partly to the lack of known juvenile materials from some of the species, an independent approach to this problem was needed to supplement and check the results of the morphological research. At the suggestion of Albert Tester we made a preliminary study of the applicability of some immunochemical methods we have used extensively in our salmon investigations. Since our time and facilities were largely committed to salmon research, our tuna studies were undertaken on a restricted scale and with limited objectives. These objectives were to determine whether differences could be detected in the serum antigens of adult tuna which were characteristic of the species. If these objectives could be accomplished, it was considered that it would be reasonable to use appropriate immunological methods for attacking the much more difficult problems involved in identification of the larval forms.

Our preliminary studies, which were carried out over a considerable period as material and time became available, indicated that there were detectable interspecific differences in the serum antigens of adult tuna. Financial support in 1960 from the Bureau's Biological Laboratory, Honolulu, enabled us to make a more complete study of many of the antisera we had prepared and also to make a preliminary study of the soluble antigens of adult tuna flesh.

This paper describes our studies on interspecific differences in the serum antigens of adult tuna. Similar studies on albacore, bigeye, and yellowfin tunas, reaching essentially the same conclusions, have been reported by Suzuki and Morio (1959). Charles Matsumoto had the primary responsibility for our soluble flesh antigen studies and is preparing a manuscript describing them; consequently, only a summary of the most important aspects of our research on these anti-

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gens is presented. Finally, I have made recommendations concerning the direction that future immunochemical or serological research on this problem should take, in light of the results we and others have obtained, and taking into account certain other important factors.

BACKGROUND

The use of immunological methods for the identification of species is based on the specificity of antigen-antibody reaction. Knowledge of this specificity is general, since we know that immunity for one disease will not protect us when we are exposed to another.

Nuttall (1904) established that animal species can be distinguished by the specificity of characteristic antigens present in their serum. He also established that the degree of overlapping or cross reactivity in the specificity of the serum antigens of animals closely paralleled their taxonomic relationships. Many immunologists have subsequently used and extended these findings. Probably the most important studies are those of Irwin (1947) and his associates. They studied the antigenic constitution of the sera of species, species hybrids, and backcross hybrids of pigeons and doves, and demonstrated that the specificites of individual serum antigens were under direct genetic control. Thus, antigenic differences that we find between species can be considered direct consequences of the genetic differences between species.

METHODS AND MATERIALS

THE DOUBLE DIFFUSION PRECIPITIN ANALYSIS METHOD

Throughout this research we have used a modification of the Ouchterlony method of double diffusion precipitin analysis, exactly as described in another paper from this laboratory (Ridgway, Klontz, and Matsumoto, 1961). Since we described this method completely in that paper and it has been fully treated elsewhere in the literature (Ouchterlony, 1958) only a brief description will be given here. The method consists of allowing an antigen solution and an antiserum to diffuse toward each other through a semisolid medium containing a suitable concentration of saline or buffer and a preservative.[1] Where the molecules of a given antigen meet their specific antibodies in an optimal ratio of concentrations, a line of precipitation will form. Since the position of this line is dependent on the diffusion rates and initial concentrations of the antigen and its specific antibody and since these will vary between separate components, a separate line can form for each antigen-antibody system present. When two antigen solutions diffuse side by side toward a single antiserum the lines for identical antigens will fuse while those for unrelated antigens will cross.

Thus we have a system which allows us to separate out and study the reactions of individual antigens in a complex mixture and to compare the antigenic composition of one preparation with that of other preparations.

Photographic records of all of our tests were made as described in a previous publication (Klontz, Ridgway, and Wilson, 1960).

PRODUCTION OF ANTISERA

Antisera were prepared in rabbits in two different ways. One method consisted of emulsifying 2.5 ml. of pooled sera from several individuals of a given tuna species with 2.5 ml. Difco complete Freund's adjuvant and injecting the emulsion in several regional lymph node sites. After 15 days the rabbits were bled on 3 successive days. After a rest of 1 month, the animals were restimulated by two intraperitoneal injections of 0.25 ml. of serum and bled 4, 5, and 6 days later.

The second method used to produce antisera consisted of giving five to nine intraperitoneal injections of 0.25 to 0.5 ml. of pooled serum and bleeding on the fifth, sixth, and seventh days after the last injection. After 2 to 3 weeks rest, the rabbits were restimulated by two intraperitoneal injections of 0.25 ml. pooled serum and bled 4, 5, and 6 days later. The best antisera were obtained with the latter method. We also prepared several antisera in chickens by giving three intraperitoneal injections of 0.25 ml. pooled albacore serum at 4-day intervals and bleeding 5 days after the last injection. Although potent antibodies were detectable by the usual precipitin method we were unable to obtain useful results with these antisera in double diffusion tests.

[1] The medium we use has the following composition: Difco agar 1.5 g., sodium chloride 0.72 g., sodium citrate 0.6 g., "Merthiolate" Lilly 0.01 g., trypan blue 0.01 g., distilled water to make 100 ml. Adjust pH to 6.7 with hydrochloric acid.

TUNA SERUM SAMPLES

The samples of tuna serum were obtained for this study by the biologists of the Bureau of Commercial Fisheries Biological Laboratory, Honolulu, Hawaii.

Table 1 gives a list of these samples. Since we have found little evidence for individual differences, information about the individual samples is not included. Information on place and date of capture, sex, and size are on file in the Bureau of Commerical Fisheries Biological Laboratory, Seattle, Wash.

TABLE 1.—*Samples of sera from adult tuna*

Species		Number of individual samples	Vessel	Year collected
Common name	Scientific name			
Albacore	*Thunnus alalunga*	11	*John R. Manning*	1957
Yellowfin	*Thunnus albacares*	8	*Charles H. Gilbert*	1957
		1	*Charles H. Gilbert*	1959
Bigeye	*Thunnus obesus*	8	*Charles H. Gilbert*	1958
		2	*Hugh M. Smith*	1959
Skipjack	*Katsuwonus pelamis*	16	*Charles H. Gilbert*	1957
		19	*John R. Manning*	1957
Little tunny	*Euthynnus yaito*	2	*Charles H. Gilbert*	1957
		2	*John R. Manning*	1957
Dogtooth tuna	*Gymnosarda nuda*	2	*Charles H. Gilbert*	1957

TABLE 2.—*The distinction of species of adult tuna by means of double diffusion precipitation analysis of their sera with antisera prepared in rabbits*

Antiserum	Serum (tuna species)	Number of precipitation lines	Number of distinctive lines
Antialbacore	Albacore	7	
	Yellowfin	4	3
	Bigeye	5	2
	Skipjack	4	3
	Euthynnus	3	4
	Dogtooth	2	5
Antibigeye	Bigeye	11	
	Albacore	8	3
	Yellowfin	8	3
	Skipjack	5	6
	Euthynnus	5	6
	Dogtooth	3	8
Antiyellowfin	Yellowfin	9	
	Albacore	8	1
	Bigeye	8	1
	Skipjack	5	4
	Euthynnus	5	4
	Dogtooth	3	6
Antiskipjack	Skipjack	3	
	Albacore	2	1
	Yellowfin	2	1
	Bigeye	2	1
	Euthynnus	2	1
	Dogtooth	1	2
Anti-*Euthynnus*	Euthynnus	6	
	Albacore	5	1
	Yellowfin	5	1
	Bigeye	5	1
	Skipjack	5	1
	Dogtooth	5	1
Antidogtooth	Dogtooth	11	
	Albacore	4	7
	Yellowfin	4	7
	Bigeye	6	5
	Skipjack	4	7
	Euthynnus	4	7

Samples were taken by cutting the isthmus of tuna, collecting the spurting blood in bottles or bowls, allowing it to clot for 1 to 2 hours at air temperature and up to 24 hours under refrigeration. The serum was decanted from the clotted blood and clarified by centrifugation. Serum samples were then frozen and stored at −15° C. to −30° C. At our laboratory, samples being used were separated into small aliquots to avoid repeated thawing and freezing. Most samples were in excellent condition when received and have remained stable under frozen storage.

RESULTS

The results obtained with the most discriminating sera among those we have prepared are summarized in table 2. For example, when testing our most discriminating antialbacore serum, we find that there are seven distinguishable precipitation lines with albacore serum, four of which cross-react with yellowfin serum, five with bigeye serum, four with skipjack serum, three with *Euthynnus* serum and two with dogtooth serum. Thus, with this serum, the number of lines (antigens) which distinguish albacore from the other species are as follows: Three with yellowfin, two with bigeye, three with skipjack, four with *Euthynnus* and five with dogtooth. Illustrations of tests with antidogtooth and antibigeye sera are presented in figures 1 and 2.

Considerable variability exists among the discriminatory abilities of the various antisera, even when we use the best bleedings. (There is, of course, considerably more variability among the antisera in discriminatory ability when one considers the results obtained with some of the poorer ones.) We have antialbacore, antibigeye, and antidogtooth antisera which possess considerable discriminatory power. On the other hand, the discriminatory qualities of our best antiyellowfin, and antiskipjack and anti-*Euthynnus* antisera are not great. This points up the fact that there is considerable variability between individual rabbit sera, so that one may need to immunize 6 to 20 rabbits in order to produce an antiserum of

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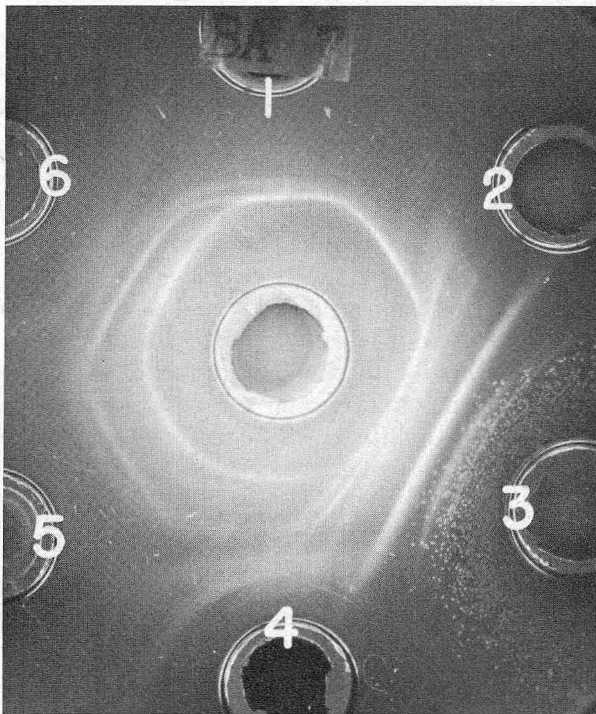

FIGURE 1.—The distinction of dogtooth tuna serum from that of other species. The peripheral wells contain sera of the following species: 1. *Euthynnus*. 2. Skipjack. 3. Dogtooth. 4. Yellowfin. 5. Albacore. 6. Bigeye. Antidogtooth-tuna-serum rabbit serum in center well. The random dots around dogtooth well are due to crystallization of material, which becomes insoluble on dilution, and have no effect on the immunological reaction.

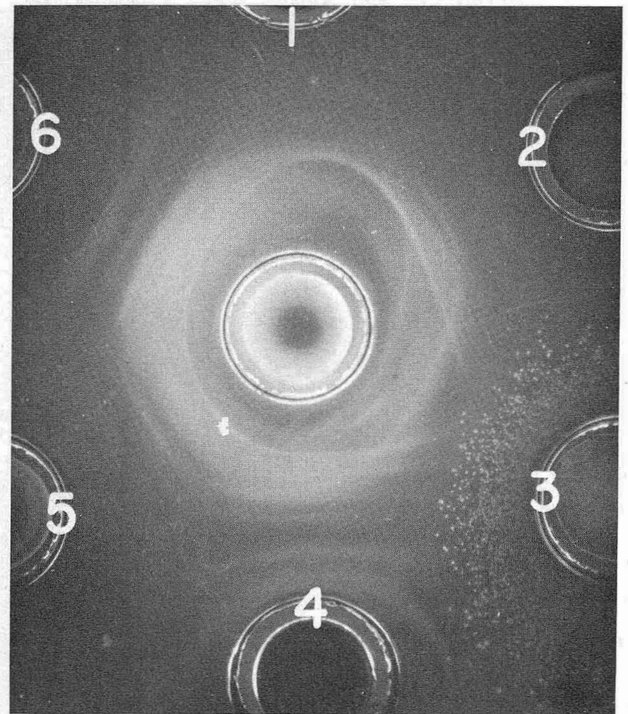

FIGURE 2.—Distinction of bigeye tuna serum from that of other species. The peripheral wells contain sera of the following species: 1. *Euthynnus*. 2. Skipjack. 3. Dogtooth. 4. Yellowfin. 5. Albacore. 6. Bigeye. Antibigeye-tuna-serum rabbit serum in center well.

the specificity necessary to make a particular distinction. We have noted this in some of our other work (Ridgway, Klontz, and Matsumoto, 1961), and this variability among the responses of several rabbits to the same antigenic stimulus has been pointed out by other immunologists.

That tuna species can be distinguished by the antigenic constitution of their sera, as summarized in table 2, has been confirmed by several replications. In the case of the albacore, yellowfin, and bigeye, distinctions were also made by absorption experiments. These absorptions were carried out both by the specific inhibition or intragel absorption method of Bjorklünd (1952), which involves incorporating the absorbing tuna serum in the agar medium at a concentration of 20 percent; and by placing the absorbing serum in the center well for an interval of 10 to 20 minutes before adding the antiserum. All absorption experiments were carried out using the micromethod of agar diffusion (Ridgway, Klontz, and Matsumoto, 1961). In this method, 2 ml. of hot agar medium is pipetted on the unfrosted portion of a 25×75 mm. microscope slide which has been cleaned with alcohol. The antigen and antiserum wells are cut out of the agar using stainless steel punches of appropriate diameter. In these experiments summarized in table 3, 10 to 20 microliters of tuna sera were placed in the respective wells, the absorbing serum was placed in the central antiserum well also. After a short interval, 20 microliters of antiserum were placed in the center well and diffusion allowed to proceed for 4 hours at 37° C. and overnight in the refrigerator. It is evident from the results presented in table 3 that the closely related species bigeye, yellowfin, and albacore do possess species-specific antigenic factors as part of the constitution of their serum proteins, and that these factors can be detected with immune rabbit sera.

TABLE 3.—*Distinctive reactions of sera of species of adult tuna after removal of cross-reactive antibodies by absorption*

Antiserum	Absorbing serum	Test serum	Reaction (number of precipitation lines)
Antialbacore	Bigeye	Albacore	1
Do	do	Yellowfin	1
Do	do	Bigeye	0
Do	Yellowfin	Albacore	1
Do	do	Yellowfin	0
Do	do	Bigeye	0
Antiyellowfin	Albacore	Yellowfin	1
Do	do	Albacore	0
Do	do	Bigeye	1
Do	Bigeye	Yellowfin	2
Do	do	Albacore	1
Do	do	Bigeye	0
Antibigeye	Albacore	Bigeye	2
Do	do	Albacore	0
Do	do	Yellowfin	1
Do	Yellowfin	Bigeye	2
Do	do	Albacore	1
Do	do	Yellowfin	0

SOLUBLE TISSUE ANTIGEN STUDIES

Encouraged by the results obtained from our studies of serum antigens we undertook some preliminary studies on the soluble antigens of muscle tissue. We felt that such antigens would be more useful for the identification of larval forms since muscle obviously makes up a larger portion of the animal than does blood serum.

In order to adapt the double diffusion precipitin analysis method to the detection and study of soluble tissue antigens of tuna flesh, and characteristic differences in them between species, several problems had to be investigated first. These problems included the development of methods for extraction of soluble antigens from tuna muscle tissue, stabilization of these extracts, and the production of potent and specific antisera.

The results of these studies can be summarized as follows: Extraction of muscle tissue with neutral physiological saline resulted in quite dilute and unstable solutions from which most of the dissolved protein precipitated spontaneously, even on overnight storage at 0° C. to 4° C. The use of alkaline saline solutions (pH 10) increased the efficiency of extraction and the stability of the solutions. From the standpoint of efficiency of extraction and stability of the resulting solution, however, the best extracting medium was found to be 50 ml. glycerol, 1 g. NaCl, and water to make 100 ml. The spontaneous precipitation which occurred in muscle tissue extracts was increased by increasing temperatures or by freezing and thawing. It was not significantly inhibited by heavy metal ions indicating that it may be due to causes other than the actions of proteolytic enzymes.

Three methods for the production of antisera were tried; intraperitoneal injection of several doses of untreated extract, intramuscular injection of alum precipitated extract, and the incorporation of the extract in Freund's adjuvant for an initial subcutaneous stimulation, followed by several intraperitoneal injections of the untreated extract after 2 to 3 weeks. The last method gave the most potent antisera.

Spontaneous precipitation of the extracts occurred during the diffusion tests which complicated their interpretation. Nevertheless we did find that skipjack differed from yellowfin, albacore, and bigeye by at least one tissue antigen detectable when the tests were run at 4° C. The soluble tissue antigens of yellowfin, albacore, and bigeye were not distinguishable using the antisera and methods we developed, but they did possess a character missing from the skipjack extracts.

The results of our preliminary studies also indicated that the soluble proteins of fish muscle tissue are antigenically quite distinct from the serum proteins of the same species. The few weak reactions obtained on testing tissue extracts with antiserum sera may be attributable to the presence of small amounts of blood and lymph in the tissues. In addition, the degree of cross-reactivity among taxonomic groupings of fish is apparently greater in the case of soluble muscle proteins than in the case of serum proteins. These findings may be of interest from the standpoints of ontogeny and evolution, but they increase the difficulties involved in the utilization of soluble tissue antigens for the distinction of species.

DISCUSSION

The demonstration of interspecific differences in serum protein antigens of tuna with only a small-scale study indicates that there are definite possibilities in the application of immunochemical methods to the problem of identifying the species of larval forms of tuna. This is, of course, dependent on the development of species specificity in antigenic constitution early in embryological development. There are ultramicro precipitin methods available for the study of soluble antigens, for example the double diffusion method can be carried out on cellulose acetate films as reported by Consden and Kohn (1959), with as little as

0.001 ml. of serum. With potent and specific antisera the usual ring test can detect even smaller amounts of antigen. A larger scale program utilizing a great many more rabbits could probably develop such potent and specific antisera.

Nevertheless, it seemed more reasonable to study the soluble antigens of muscle tissue since muscle makes up a much larger portion of the animal than does serum. However, our finding that the soluble antigens of tissue appear to be less distinct than those of serum, coupled with the technical problems involved in preventing spontaneous precipitation of tissue antigens, indicates that soluble tissue antigens may not be the best material for use in the distinction of tuna species. More study of these antigens is warranted, especially the investigation of other methods of immunization in order to produce more discriminating antisera, and the examination of methods for extracting only the more stable antigens.

An even more promising approach is the use of insoluble cellular antigens. There is evidence from the studies of Cushing (1956) and Suzuki, Shimizu, and Morio (1958) that interspecific differences in the red cell antigens of tuna exist. The production of species-specific hemagglutinating antisera is also readily accomplished because of the relative ease of absorption with red cells. On the other hand, removal of cross-reacting precipitins by absorption is difficult to accomplish satisfactorily. The presence of species-specific red cell antigens in other tissues of the larval tuna would, of course, be required for the application of this method. In human beings and certain other mammals red cell antigens have been demonstrated to be present in other tissues. The techniques available for testing for these antigens in tissues include the mixed agglutination method which has been used to demonstrate the presence of A and B antigens in human epithelial cells by Coombs, Bedford, and Rouillard (1956) and the fluorescent antibody techniques of Coons (1954) which are so sensitive that the presence of antigens on individual cells is demonstrable.

Of course, suitable preservation methods will have to be developed for the maintenance of the antigens of larvae from the time the larvae are collected until they can be sorted from the plankton and tested.

Undoubtedly, the way is open for greater application of the ultrasensitive methods of immunochemistry and blood-group serology to problems of fishery biology. The important thing to be recognized is that these methods are not in the nature of "magic wands", but are developed and applied through knowledge and painstaking experimentation.

SUMMARY

Through the application of the Ouchterlony method of diffusion precipitin analysis, with rabbit immune sera, the presence of species-specific differences in serum antigens of adult tuna was demonstrated. The existence of these differences was confirmed by absorption methods.

In studies on soluble antigens of the muscle tissue of tuna, evidence was obtained for distinguishing skipjack from albacore, yellowfin, and bigeye tuna.

No characteristic differences in their soluble tissue antigens were found which allowed the mutual distinction of the latter three species. In the study of soluble tissue antigens, technical problems involving extraction media, stability of extracts, and production of potent antisera were encountered and preliminary methods for their solution developed.

The course which further developments in these and allied fields might take resulting in possible distinction of larval forms was discussed.

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