DIFFERENTIATION OF FRESH HADDOCK FILLETS FROM COD BY THE PRECIPITATION TEST.

G. A. McCURDY,

Pathological Department, Dalhousie University, Halifax, N. S.

and

V. D. VLADYKOV,

Atlantic Biological Station, St. Andrews, N. B.

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ABSTRACT.

Anti-haddock and anti-cod sera were obtained by injecting rabbits with saline extracts prepared from the fresh fillets of these fishes. Using precipitation technique with both antisera diluted 1:10, no distinction between cod and haddock antigens was observed. On repeating the tests with the antisera diluted 1:50 the antigens were differentiated from each other. Control tests using normal rabbit serum in dilutions of 1:10 and 1:25 titrated against cod and haddock antigens did not give any precipitation in antigen dilutions of 1:25 to 1:1000. The following antigens were also tested against anti-haddock and anti-cod sera (diluted 1:10): pollock, halibut, witch, hake and skate. Precipitation occurred with all five antigens; the first two antigens were rich in dissolved protein and gave heavy precipitates, the latter three contained less dissolved protein and the precipitates were correspondingly smaller.

During recent years, with advance of refrigeration, more fish are being placed upon the market in fresh condition, especially in the shape of "fillets". Haddock (*Melanogrammus aeglefinus*) fillets have a particularly wide reputation. Unfortunately substitution by fillets made from other species of fish as, for instance, cod (*Gadus callarias*) or hake (*Urophycis chuss*) for haddock fillets occurs.

Hake fillets are usually quite easily recognized by a fish handler by a simple manipulation. When a haddock fillet is bent, it breaks, while the hake fillet, being much more elastic, does not. On the other hand, there is practically no external distinction between fillets made from cod and haddock. Some dealers, however, claim they can recognize about 25% of the fillets in question, using as a criterion a few ribs left in the fillets. Haddock has stouter ribs, while

cod possesses softer ones. Of course, in some cases fillets of these two species may be recognized by their size, cod being much larger. Size alone very seldom counts, because, as a rule, fillets for falsification are cut only from smaller specimens of cod ("market cod").

With a view to finding a distinction between the flesh of the cod and haddock, precipitation tests were carried out. Unfortunately, the authors had very little time at their disposal for this study, and, as the work was undertaken primarily for practical purposes, only general observations were made. It is realized, however, that many important details have been omitted, and hence this article contains only results of a preliminary nature.

The present investigation was carried on in the Pathological Department of Dalhousie University, Halifax, N. S. The authors have the pleasure of expressing thanks to Professor R. P. Smith for the kind permission to use the laboratory facilities for this investigation, to the Biological Board of Canada for permission to devote certain time of one of the authors to this study, and to Mr. W. H. Boutilier, Vice-President of the Maritime-National Fish Co., Limited, Halifax, N. S., for kindly supplying fillets of different kinds of fish for this work.

The precipitation test discovered by Rudolph Kraus in 1897[1] is now widely used not only in medicine as a diagnostic agent, but also for technical purposes; e.g., in the detection of meat adulteration (Uhlenhuth[2]). This test also plays an important role in biology in tracing genealogical relationships between different animal groups. The extensive investigations of Nuttall[3a] may be mentioned in this connection. He tested the blood of more than 500 animal species with about 30 immune sera, making not fewer than 16,000 experiments. In his experiments on fishes he did not use antisera obtained from animals sensitized to fish blood, but only em-

[1]Kraus, R. *Wien. Klin. Wschr.*, **10**, 431 (1897).

[2]Uhlenhuth, P. *Deuts. Med. Wschr.*, **26**, 734 (1900).

[3] a. Nuttall, G. H. F. *"Blood Immunity and Blood Relationship"*, Cambridge University Press. (**1904**). b. ibid. p. 61.

ployed antisera from other vertebrate groups, and also from lobster, using fish blood as the antigen; all these tests were negative.

Following Nuttall's work several other investigations were carried on in order to establish more definitely animal relationships. Boyden[4a], using rabbit and chicken antisera, tried to obtain more accurate results by standardizing the antigens. His work was practically limited to domestic animals and human blood. Precipitation technique in the case of various mammal groups was further elaborated by Wolfe[5] who used a "short method" (3 or 4 injections) in producing antisera. He succeeded in obtaining titers as high as 1:102,400 in the case of anti-mink and anti-fitch sera.

After injections of rabbits with the blood sera of domestic and wild ducks and geese Sasaki[6] was able to distinguish by precipitation tests between different genera. In the case of closely related species, as the wild duck (*Anas boschas*) and its domestic form (*A. domestica erecta*), the distinction by precipitation tests was not so clearly shown. Hicks and Little[7a] demonstrated by precipitation tests the serological identity of two species of mice (*Mus musculus* and *M. faeroensis*), while the Japanese Waltzer and M. *bactrianus* must be included in another group.

Erhardt[8] studied the precipitation tests in the case of invertebrate animals. His experiments showed quite clearly that rabbit antisera reacted not only with the homologous antigens but also with antigens of other species belonging to the same order.

With regard to the work dealing with fishes, the following authors may be mentioned. Tchistovitch[9], who injected eel

[4] a. Boyden, A. A. *Biol. Bull.* (*Woods Hole*), **50**, 73-107 (1926).
b. ibid. pp. 84-88. c. ibid. p. 95, 101.
[5] Wolfe, H. R. *J. Immunol.*, **29**, 1-12 (1935).
[6] Sasaki, K. *Jour. Dept. Agric. Kyushu. Imp. Univ. Fukuoka*, Japan, (**1928**).
[7] a. Hicks, R. A. and Little, C. C. *Genetics*, **16**, 397-421 (1931).
b. ibid. p. 416.
[8] Erhardt, A. *Z. Immun. forsch.*, **60**, 156-166 (1929).
[9] Tchistovitch, Th. *Ann. Inst. Pasteur*, **13**, 406 (1899).

blood into rabbits and guinea pigs, obtained a precipitate when these antisera were mixed with eel blood. Kodama[10], using precipitation and complement fixation tests, studied the differences between the roe of fishes belonging to the sturgeon, salmon, carp and herring families.

Only a very brief description of his technique was given, which is as follows: one gm. of roe was mixed with 10cc. physiological saline and, after storing overnight, the extract was diluted 1:10. From 2 to 4 cc. of the diluted extract was injected into a rabbit every 5 days. Seven days after the last injection the animals were bled, and the antisera obtained. His results showed that antisera obtained for one species gave a precipitate when mixed not only with the homologous antigen but also with antigens derived from the roe of different species belonging to the same family. Slight reactions were obtained in some cases with antigens obtained from roe of other families. Complement fixation tests gave parallel results.

More recently Masuda[11] studied the properties of fish roe and found that marked racial and organ specificity of fish roe protein was shown by the complement fixation and precipitation reactions. Unfortunately, this paper was not available, and further details are lacking.

Throughout the whole period of the present investigation the technique employed was not uniform. During the first part, from June to December, 1935, the rabbits were immunized with diluted (1:20) fillet extracts. The antisera thus obtained failed to show precipitation even with the homologous antigens. In order to obtain satisfactory antisera, a more concentrated (1:4) fillet extract was employed for injections during the second part of this study.

As the method employed very often affects the results (according to Wolfe[5]) a detailed description of the preparation of the antigens and antisera is given.

[10]Kodama, H. *Arch. f. Hygien*, **78**, 247-259 (1913).

[11]Masuda, Y. *Japan Z. Mikrobiol. Path.*, **27**, 366-383; ibid. 423-437 (1933).

Preparation of Antigen.

The antigen used in the first two series of injections (from June to December, 1935) were prepared as follows. One part of fresh fillet, i.e., prepared from fish caught and stored two to six days on ice at 0°C., was minced, then emulsified with four parts of saline (0.85%). The suspension was allowed to remain in the refrigerator (0° to 5°C.) for about 18 hours, and filtered first of all through gauze, and then through a Seitz filter. The filtrate thus prepared was a clear limpid fluid. The filtrate prepared from cod or haddock will give a faint opalescence in dilutions of 1:20 when heated to boiling and a drop of dilute nitric acid added. The pH of the cod extract was 7.1, that of the haddock 6.8. Accurate analyses of the protein content of the different antigens were not made.

The standard extract (one part muscle and four parts saline) was diluted 1:4 with 0.85% NaCl solution and kept in cold storage room at -12°C. Immediately after thawing the extract was limpid, but if kept at room temperature for five to eight hours it became opalescent. It was also found that storage for several days at 0° to 5°C. did not prevent the solution from becoming opalescent.

The antigen used in the third series of injections (from January 4 to February 14, 1936) and in the subsequent titration experiments was prepared as follows. The fillets were minced and emulsified with five parts of saline. The extract thus obtained was filtered through a Seitz filter, and was not subjected to further dilution. The antigens of this series were freshly prepared each week, part was injected into the rabbits at once and the remainder stored in the refrigerator (0° to 5°C.) for four days when it was used for the second injection.

Experimental Animals.

Four six-months old Chinchilla rabbits, three males and one female, weighing 1-1.5 kgms. were used for this experiment. Two (RC1, RC2) were injected with the cod extract, and two (RH1, RH2) with the haddock.

Preparation of Antiserum.

The method used in preparing the antisera was that outlined by Kolmer[12] with slight modifications.

The first series of injections was started on June 21, 1935, and was carried out as follows: 3 cc. of the diluted antigen (1:20) were injected into the marginal ear vein of the rabbit at three-day intervals, these were followed by three 10 cc. intra-peritoneal injections given at six-day intervals. A period of three months was allowed to elapse and the following series of injections given at five-day intervals 1, 2, 3, 3, 5, 5 and 3 cc. Two weeks more were allowed to elapse and 5 cc. of serum were obtained from each rabbit, and a preliminary estimation of the strength of the antisera was made by precipitation tests (not shown in tables). The tests failed to show the presence of precipitins.

Three months were again allowed to elapse and a third series of injections was commenced on January 4, 1936, the antigen used in this series being the undiluted one. The injections were given at four and three-day intervals, respectively, as follows: 3 and 8 cc. intravenously and injections of 5, 10 and 5 cc. intra-peritoneally. On February 14, 1936, samples of serum were withdrawn from each animal and their titers estimated. The results of these titrations are given in Table 1. On February 19, rabbits RH1 and RC2, were bled and 35 cc. of serum were obtained from each animal. The sera were stored in test tubes in the refrigerator (0° to 5°C.).

The precipitation tests were carried out in ordinary Kahn tubes. In each titration 1 cc. of antiserum was added to an equal volume of antigen. The strength of the antiserum was kept constant throughout the individual experiments, while the concentration of the antigen was varied (Tables 1-6). The method of serial dilutions was used to obtain the different antigen strengths.

[12]Kolmer, J. A. "*A Practical Text-book of Infection, Immunity and Biologic Therapy*", Third Ed., W. B. Saunders Co. (1924).

TABLE 1.

Preliminary Precipitation Tests Made February 16-17, 1936.

Antiserum 1:10 dilution	**Rabbit H_1 Haddock**			**Rabbit H_2 Haddock**			**Rabbit C_1 Cod**			**Rabbit C_2 Cod**		
Antigen dilution	After 45 min.	After 9 hrs.	After 18 hrs.	After 45 min.	After 9 hrs.	After 18 hrs.	After 45 min.	After 9 hrs.	After 18 hrs.	After 45 min.	After 9 hrs.	After 18 hrs.
1/10	pp	PP	PP	pp	PP	PP	p(?)	PP	PP	pP	PP	PP
1/20	pp	PP	PP	pp	PP	PP	p(?)	Pp	Pp	p(?)	PP	PP
1/40	pp(?)	PP	PP	pp	PP	PP	p(?)	p	p	(?)	Pp	Pp
1/80	p	Pp	Pp	p	p	p	(?)	Op	Op	(?)	Op	Pp
1/160	p(?)	p	p	p(?)	p	p	L	L	L	L(?)	L(?)	Op
1/320	(?)	p	p	p(?)	L	L	L	L	L	L	L	L
1/640	L	L	L	(?)	L	L	L	L	L	L	L	L
1/1280	L	L	L	L	L	L	L	L	L	L	L	L
1/2560	L	L	L	L	L	L	L	L	L	L	L	L

P—Large flocculi
p—Small flocculi
p_1—Very Small flocculi
O—Opalescent
L—Limpid
(?)—Uncertain

Results.

On February 16-17, 1936, the strengths of the different antisera which were diluted 1:10 were tested. In Table 1 the titers of antisera C_1, C_2, H_1 and H_2 are given; these were 1:80, 1:160, 1:320 and 1:160 respectively. It will be noticed that the readings made after 45 minutes are practically identical with those made after 9 and 18 hours; the latter two readings however were more sharply defined.

The reactions of the stronger antisera C_2 and H_1 were then further investigated not only with their homologous antigens but also with heterologous ones from the following fishes: pollock (*Pollachius virens*), cusk (*Brosme brosme*), hake (*Urophycis chuss*), halibut (*Hippoglossus hippoglossus*), witch (*Glyptocephalus cynoglossus*) and skate (*Raja laevis*). The results of these tests appear in Tables 2 and 3. It is seen that with antiserum H_1 marked precipitation was obtained, not only with haddock antigen and that of closely related genera[13] as cod, pollock and cusk, but also with halibut. The reaction with hake and with witch was not so strong, and that with skate was extremely weak. The results with antiserum C_2 (Table 3) are practically identical, though slightly weaker than those of antiserum H_1. Only with pollock antigen did antiserum C_2 give precipitation with large flocculi; with the remaining antigens only small flocculi were present.

An additional experiment was made on March 14-15, using the following two dilutions of antisera, 1:10 and 1:50.

[13]In order to inform readers not familiar with fish systematics, the taxonomic relations between different fishes mentioned throughout this paper are briefly outlined. Haddock, cod, pollock, cusk and hake represent different genera of the Cod family (*Gadidae*). The first three mentioned genera are much more closely related to each other than to the remaining two genera. Cusk and hake on the other hand are more closely related to each other than to the former three. The Cod family constitutes part of a larger taxonomic unit, the order of the soft-rayed fishes (*Anacanthini*). The halibut and witch belong to two closely related families, the Halibut family (*Hippoglossidae*), and the Flounder family (*Pleuronectidae*). Both these families belong to another order, viz., the Flat-fishes (*Heterosomata*). The above two orders are included in the class of true fishes (*Pisces*). The skate belongs to another class of fishes comprising sharks and skates (*Elasmobranchii*). This classification is that of D. S. Jordan, B. W. Evermann and H. W. Clark. *Rep. U. S. Comm. Fish., Part II*, 1-670, (1928).

TABLE 2.

Precipitation Tests with Antiserum from Rabbit H_1 (in dilution 1:10) and Different Antigens, Made February 22-23, 1936. (Readings were made after 18 hours).

Antigen dilution	Haddock	Cod	Pollock	Cusk	Hake	Halibut	Witch	Skate
1/25	PP	PP	PP	PP	pp	pp	pp	p
1/50	PP	PP	PP	PP	pp	pp	pp	L
1/100	PP	pp	pp	pp	p	pp	p	L
1/500	p	L	p	p	L	pp	L	L
1/1000	L	L	L	L	L	p	L	L
Antigen (1/25) control	O	O	OO	O	L	O	L	L

TABLE 3.

Precipitation Tests with Antiserum from Rabbit C_2 (in dilution 1:10) and Different Antigens, Made February 22-23. 1936. (Readings were made after 18 hours).

Antigen dilution	Haddock	Cod	Pollock	Cusk	Hake	Halibut	Witch	Skate
1/25	pp	pp	PP	p	pp	pp	p	pı
1/50	pp	pp	PP	p	p	pp	p	L
1/100	pp	p	pp	p	p(?)	p	p	L
1/500	p	p	p	p	L	p	L	L
1/1000	L	L	L	L	L	p	L	L
Antigen (1/25) control	O	O	OO	O	L	O	L	L

Antiserum H_1 1:10 gave practically the same precipitation with either cod or haddock antigens. However, the same antiserum in a dilution of 1:50 showed a marked difference. In the case of haddock antigen, precipitation was noticed up to 1:1000 whereas with cod antigen only a very slight precipitation occurred in 1:500 dilution.

Antiserum C_2 in 1:50 dilution showed a difference between cod and haddock antigens. With cod antigen up to 1:1000 precipitation was observed, while with haddock antigen precipitation did not occur in a dilution greater than 1:50.

In order to exclude the possible influence of rabbit serum by itself in precipitation reactions a final control test was made in which the reactions of antiserum H_1 and normal rabbit serum were compared (Tables 5 and 6). Two dilutions of sera were tried, 1:10 and 1:25. From these tables it is clearly seen that, in the case of normal serum in the dilutions tried, no precipitation was observed with either cod or haddock antigens, while antiserum H_1 reacted as usual positively in both dilutions.

Discussion.

By comparing the reactions of antisera C_2 and H_1 with haddock and cod antigens, it will be seen that certain discrepancies apparently are present. Thus, for example, antiserum H_1 in a dilution of 1:10 tested against haddock antigens, prepared from different fillets (specimens), gave the following titers: 1:500 (Table 2), 1:1000 (Table 4) and 1:100 (Table 5). These differences are most probably explained in light of Boyden's work[4b] in that the titer of an antiserum varies directly with the concentration of soluble protein in the antigen used.

Although the technique of the preparation of the antigens used by us was uniform, the amount of protein extracted may have varied considerably in the individual specimens. Thus, the protein concentration of the haddock antigen in a dilution of 1:100 (Table 5) may be equal to that of the haddock antigen diluted to 1:1000 referred to in Table 4. This is substantiated by the fact that haddock flesh during and immediately after spawning is poorer in protein than that of fish taken in winter months (Vladykov[14]). Hence, in experiments made in February and March (Tables 1-4) the fillets were

[14]Vladykov, V. D. *Jour. Biol. Board Can.* in press.

TABLE 4.

The Precipitation Test with Antiserum in Different Dilutions, March 14-15, 1935. (Readings were made after 18 hours).

Antiserum	Rabbit H_1: 1/10		Rabbit H_1: 1/50		Rabbit C_2: 1/10		Rabbit C_2: 1/50	
Antigen dilution	Haddock	Cod	Haddock	Cod	Haddock	Cod	Haddock	Cod
1/25	PP	P	PP	pp	PP	PP	p	pp
1/50	PPP	PPP	P	p	p	pp	p	p_1
1/100	PPP	PP	P	p	L	pp	L	p_1
1/500	pp	pp	p	p_1	L	p	L	p_1
1/1000	p	p_1	p	L	L	p	L	(?)
Antigen (1/25) control	p_1p_1	p_1p_1	p_1p_1	p_1p_1	p_1p_1	p_1p_1	p_1p_1	p_1p_1
Antiserum control	L		L		L		L	

no doubt richer in protein than those used in experiments performed during April (Tables 5-6) when the haddock were "spent". These remarks are applicable to cod antigens also.

As the main object of this work was to find by precipitation tests a distinction between cod and haddock fillets and, since both these species are closely related from the taxonomic standpoint, a distinction between them by serological methods involves certain difficulties (Landsteiner[15a]). Moreover, according to Johnston[16], protein solubility of both is practically identical.

In the experiments (Tables 2-4) when the protein concentration in the antigen used was presumably high, both antisera H_1 and C_2 (in dilution 1:10) showed practically the same precipitation with either cod or haddock antigens. With antisera diluted 1:50 (Table 4) a certain specificity was noted. Antiserum H_1 gave a marked precipitation with the homologous antigen up to 1:1000, whereas with cod antigen slight precipitation was observed in dilutions up to 1:500. Antiserum C_2 with haddock antigen only showed precipitation in dilution of 1:25 and 1:50, whereas with homologous antigen precipitation was noticed up to 1:500. Unfortunately the small quantity of antisera remaining did not permit further observations.

The use of diluted antisera was in accordance with Dean's[17] recommendation, who stated that: "if the antiserum is to be used for the differentiation of the homologous serum from the serum of nearly allied species it is important to use the antiserum in the highest dilution which will yield visible precipitates when mixed with dilutions of the homologous protein."

Antigens prepared from pollock, cusk and halibut react with either H_1 or C_2 sera practically to the same titer as haddock and cod antigens (Tables 2 and 3). Both antisera,

[15] a. Landsteiner, Karl, *"The Specificity of Serological Reactions"*, Charles C. Thomas, Baltimore (**1936**).
b. ibid., p. 13.

[16] Johnston, W. W. *Biol. Board Can. MS. Rep.*, No. **29**, 11 pp. (1930)

[17] Dean, H. R. *"A System of Bacteriology"*, Vol. 6, p. 428. His Majesty's Stationery Office, London (**1931**).

TABLE 5.

The Precipitation Test with Antiserum and Normal Rabbit Serum and Haddock Antigen, April 5-6, 1936.

Serum	Rabbit H_1: 1/10		Rabbit H_1: 1/25		Rabbit N: 1/10		Rabbit N: 1/25	
Haddock Antigen	After 6 hrs.	After 24 hrs.	After 6 hrs.	After 24 hrs.	After 6 hrs.	After 24 hrs.	After 6 hrs.	After 24 hrs.
1/25	PP	PP	pp	ppp	L	L	L	L
1/50	pp	PP	p	p	L	L	L	L
1/100	p	pp	L	p	L	L	L	L
1/500	L	L	L	L	L	L	L	L
1/1000	L	L	L	L	L	L	L	L
Antigen (1/25) control	p	p	p	p	p	p	p	p
Serum control	L	L?	L	L	L	L	L	L

TABLE 6.

Precipitation Test with Antiserum and Normal Rabbit Serum and Cod Antigen.

Serum	Rabbit H_1: 1/25		Rabbit N: 1/10		Rabbit N: 1/25	
Cod Antigen	After 6 hrs.	After 24 hrs.	After 6 hrs.	After 24 hrs.	After 6 hrs.	After 24 hrs.
1/25	ppp	ppp	L	L	L	L
1/50	p	pp	L	L	L	L
1/100	L	pp	L	L	L	L
1/500	L	L	L	L	L	L
1/1000	L	L	L	L	L	L
Antigen (1/25) control	p_1(?)	p	p_1(?)	p	p_1(?)	p
Serum control	L	L	L	L	L	L

on the other hand, only gave precipitation with skate antigen in a dilution no greater than 1:25. The reactions with hake and witch antigens are intermediate in position between the above two groups. These variations in titers are believed to be due to differences in the concentration of soluble protein in the various antigens, and are not attributed to zoological relationships. Of course this conclusion must be substantiated by determination of the soluble protein in the antigens used.

In the literature quite often the degree of relationship between the animal species furnishing the antigen and that used for immunization is considered important in precipitation tests. Nuttall[3b] and Boyden[4c], for instance, believed that an animal far removed phylogenetically from the one to be tested is the most desirable as the antibody producer. Sasaki[6] succeeded in distinguishing between different species of ducks by using rabbit antisera, and, even in the case of invertebrates, rabbits were found suitable (Erhardt[8]).

On the other hand Hicks and Little[7b], Landsteiner and many others consider as the best antibody producer an animal quite closely related zoologically to those to be tested. For instance, using rabbit immune serum, different species of mice can be differentiated (Hicks and Little[7c]), while rabbit sera do not show clear distinction between such dissimilar birds as chicken, pigeon and goose. Likewise Kodama[10], using this animal, demonstrated differences between the roes of fishes belonging to different families; these antisera however were not "species specific".

Summarizing the above widely different opinions one can only conclude that rabbits may be used as antibody producers with some measure of success in differentiating between different species of fish, but the animal most suitable can only be discovered by elimination. With this thought in mind, at some future date, it is planned to use as the antibody producer not only rabbits but also certain species of fish, e.g., cod and skate.