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USE OF PLANT EXTRACTS IN SEROLOGICAL STUDIES OF FISH

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ABSTRACT

Seed and bark extracts representing 71 legume species were tested serologically against numerous individuals of the genus *Oncorhynchus* and a limited number of individuals from a broad taxonomic range of fish species. Many of these extracts demonstrated distinct individual variations in certain fish species. The possibility of distinguishing closely related fish species and their hybrids with some of the extracts was demonstrated. Red blood cells must be fresh when these extracts are used with *Oncorhynchus* species because marked alterations of reactions in this genus were found with aged red blood cells.

Eight of the extracts precipitated components of fish sera. Reaction of chinook salmon serum with *Robinia pseudoacacia* bark extract demonstrated significant variability in reactive frequency when tested against groups of individuals from Washington and Alaska.

INTRODUCTION

Identification of subpopulations or races within a species of fish is a problem of vital interest in fisheries management. The expense and physical impairment involved with tagging and marking programs have led to a search for natural characteristics which, if conveniently detectable, could be valuable in studying such populations.

One type of character that is being widely applied is the intraspecific variability of erythrocyte antigens or blood types. Whenever studied, the specificity of antigens has been found to be genetically controlled, thus making blood types highly useful as population markers within a species. Extensive studies of blood types in higher vertebrates have been made, especially concerning humans, cattle, and chickens. These studies were successful and stimulated research on erythrocyte variability in fish. Results of this research showed serological differences in sockeye salmon populations (Ridgway, Cushing, and Durall, 1958), identified two herring populations off Maine (Sindermann and Mairs, 1959), and detected individual differences in erythrocyte antigens of four species of Pacific salmon (Ridgway and Klontz, 1960).

Immune and natural antibodies of man and other animals are the chief sources of blood typing reagents. Although it has been known for over 60 years that plant extracts will agglutinate red blood cells of certain vertebrates, the fact that they would detect individual differences was discovered and utilized only in the past 15 years (Renkonen, 1948; Boyd and Reguera, 1949). Extracts of the seeds of

the family Leguminosae have been used in most work in this field. Extracts of many seeds have been tested against many warm-blooded vertebrates; however, tests for intraspecific variability have been most extensively directed toward the erythrocytes of man (Bird, 1951, 1952; Mäkëla, 1957). To the authors' knowledge, reports on the use of legume bark extracts are limited to the work of Lau (see Mäkëla, 1957), who found that *Robinia pseudoacacia* bark extract agglutinated various mammalian erythrocytes.

This paper presented the results of a preliminary search for reagents capable of demonstrating blood types in Pacific salmon. Its main purpose is to reveal the potentialities of plant extracts as fish blood typing reagents. Preliminary results of individual differences shown by precipitation reactions of fish sera with certain extracts are also discussed.

METHODS AND MATERIALS

Three methods were used to collect fish blood samples. The usual technique was to sever the caudal artery with a sharp knife or razor. Where later use of the fish depended on a minimum of visible mutilation, two other techniques were used--the gill arches on one side were severed and the blood drained through the mouth, or the fish was bled by cardiac puncture. Cells to be tested were washed three times in a modified Alsever's solution and brought to a 2 percent suspension. Blood cells stored up to 15 days after bleeding were used for testing, providing they were not excessively hemolized. The majority of the testing was done on material less than a week after collection.

Extracts of plant materials were made in 1 percent saline solution. Seeds were first ground by mechanical grinder or mortar and pestle. A paper cutter was found to be an excellent tool for cutting bark. One gram of seed or bark was mixed with either 4 or 9 cc. of 1 percent saline, depending on whether the extracts were to be tested for precipitating properties or were to be used solely for agglutination tests. In the latter case the 9 cc. dilution was used. The suspensions were incubated for 2 hours at 37° C. and overnight in the refrigerator. Centrifugation at 3,000 r.p.m. for 20 minutes yielded a supernatant fluid essentially free of particulate matter. This stock extract was used as a base for all testing and dilutions.

Agglutination tests were performed in 10 x 75 mm. tubes using 0.1 cc. of cell suspension and an equal amount of stock extract or extract dilution. Precipitation tests utilized the agar double diffusion technique of Ouchterlony (1958).

The extracts are quite stable when stored at -30° C. The reactivity of the extracts was not significantly altered by thawing and refreezing, although a few extracts lost potency after several months of constant use.

Comparable results were obtained using both fresh and dried materials in this study. Only green barks including the cambium layer were collected. Fresh extracts were made, and any bark remaining was dried and stored at room temperature. Extracts made from barks stored up to 8 months have shown little, if any, loss of reactivity when compared with extracts of freshly collected bark.

A quantitative seasonal variation has been noted in reactivity of certain barks. Extracts made from bark collected in the late spring and summer of *Robinia pseudoacacia*, *Wistaria floribunda*, and *Cytisus scoparius* barks have shown far weaker or negative reactions compared to extracts made from the same plant in the fall, winter, or early spring. It appears advisable to collect bark when the plant is in a dormant rather than actively growing state.

STUDY OF PHYTOAGGLUTININS

Many seed and bark extracts were tested for agglutinins (table 2). Extracts were considered promising if they could be used to detect individual variability or differences among species. Although other promising extracts were found, only those listed in tables 3 through 8 were selected for more extensive study. Only a single seed extract of those originally screened is represented for each

genus unless evidence was found that indicated a difference in specificity. When available, a bark extract of the same or a closely related species was used in order to compare specificities.

The data have been divided into six tables for the purpose of clarity. Each table represents a natural, though in some instances broad, grouping of fish species, with the progression being from more to less primitive. American Fisheries Society (1960) was used as the reference for fish classification.

Table 3 represents reactions of the four most primitive species tested. The single ratfish tested showed the only reaction against *Laburnum vulgare* bark and the strongest reaction (where titered) against *Sophora japonica* bark. Indication of individual variation was noted in the reaction of dogfish against *Robinia pseudoacacia* bark extract.

Table 4 includes various members of the order *Clupeiformes*. Four of the extracts detected within species variations in Pacific salmon. The *Cytisus scoparius* bark extracts reacted variably with chum and red salmon; lima bean with pink, red, silver, and chinook salmon; *Robinia pseudoacacia* with chinook salmon; and *Wistaria floribunda* bark with red and silver salmon. The same extracts appear

Table 1.--Explanation of symbols used in tables 2-8.

Symbol	Explanation	Table where used
s	Seed extract tested	2
b	Bark extract tested	2
—	No test made for this combination of reactants	3 - 8
±	Apparent qualitative variation between fish observed in undiluted extract--no titration made	3 - 8
+	Reacted strongly with undiluted extract--no titration made	3 - 8
W	Reacted weakly with undiluted extract--no titration made	3 - 8
0	No reaction with undiluted extract	3 - 8
1-12	Titers are expressed as the negative exponent of 2 of the final dilution at which visible agglutination occurred. (A single number appears where within fish species titer variation did not exceed one serial dilution. Two numbers represent a range of titers where within species variation exceeded one serial dilution).	3 - 8

Table 2.--Seed and bark extracts tested for presence of agglutinins

Plant species	Reacted	Promise	Plant species	Reacted	Promise
Acacia farnesiana (s&b)			*Lathyrus odoratus* (s)		
Albizzia julibrissin (s&b)			*Lathyrus latifolius* (s)	x	
Arachis hypogae (s)	x	x	*Lathyrus littoralus* (s)		
Baptisia australis (s)			*Lens esculenta* (s)		
Baptisia tinctoria (s)			*Lotus coniculatus* (s)		
Campylotropis macrocarpa (s)			*Lotus edulus* (s)		
Campylotropis macrocarpa (b)	x		*Lupinus* (Russel strain) (s)	x	
Canavalia ensiformis (s)			*Lupinus* sp. (s&b)		
Caragana sp. (s)	x		*Lupinus arcticus* (s)		
Caragana arborescens (b)	x		*Maackia amurensis* (s&b)	x	x
Caragana macrophylum (s&b)	x		*Maackia chinensis* (s&b)	x	
Caragana microphylum (b)	x		*Medicago sativa* (s)		
Carmichaelia astonii (b)			*Mimosa pudica* (s)		
Cassia acutifolia (s)			*Petteria ramentacea* (s&b)		
Cercis griffithi (s)			*Phaseolus coccineus* (s)	x	
Cercis siliquastrum (s&b)			*Phaseolus angularis* (s)		
Cicer arietinum (s)			*Phaseolus limensis* (s)	x	x
Cladrastis lutea (b)			*Phaseolus vulgaris* (s)	x	
Colutea sp. (s&b)			*Pisum sativum* (s)		
Colutea persica (s)			*Pueraria thunbergiana* (s)		
Colutea gracilis (s)			*Rhynchosia precatoria* (s&b)		
Colutea orientalis (s)			*Robinia hispida* (s&b)	x	x
Cytisus albus (s&b)	x	x	*Robinia kelsii* (b)	x	x
Cytisus scoparius (s&b)	x	x	*Robinia luxurians* (s&b)	x	x
Cytisus sesilifolius (b)	x	x	*Robinia pseudoacacia* (s&b)	x	x
Cytisus batandieri (b)			*Robinia viscosa* (s&b)	x	x
Crotalaria spectabilis (s)			*Sophora japonica* (b)	x	x
Derris scandens (b)			*Spartium junceum* (s&b)	x	
Dolichos lab-lab (s)			*Trifolium repens* (s)		
Galega orientalis (s&b)			*Trigonella foenum-graecum* (s)		
Genista triacanthos (b)			*Ulex europus* (s&b)		
Gleditsia triacanthos (b)			*Vicia* sp. (s)		
Glycine max (s)	x		*Vicia faba* (s)		
Laburnum alpinum (s)			*Vicia gigantea* (s)		
Luburnum vulgare (s&b)	x		*Wistaria floribunda* (s&b)	x	x
Laburnum vossii (s)					

to be useful as species markers in certain instances at appropriate dilutions. The reciprocal relationship of the reaction of *Cytisus scoparius* bark and lima bean extracts with red and chinook salmon may be cited as an example. A factor complicating the interpretation of these results is discussed at the end of this section.

Table 5 shows reactions of Cyprinid species tested. Though titers were not determined, the qualitative aspects of this group are interesting. The only species failing to react with the *Maackia chinensis* bark extract and the only species reacting with the *Cytisus scoparius* seed extracts are also members of this group. A qualitative difference in the reactions of the Scotch-broom seed and bark extracts is apparent from this table. Studies of two interspecies hybrids in this group are included (these fish were supplied by Ben Patten). Regularity in the inheritance of certain reacting sites is suggested. The squawfish-chiselmouth hybrid erythrocytes reacted with *Cytisus scoparius* seed, *C. albus* bark, and *Maackia chinensis* bark extracts as did all squawfish,

Table 3.--Agglutination reactions of four primitive fish species with various plant extracts

Fish cells	Plant extracts																
	Number tested	*Arachis hypogae* peanut	*Caragana macrophylum* (seed)	*Caragana macrophylum* (bark)	*Cytisus scoparius* (seed) Scotch-broom	*Cytisus scoparius* (bark)	*Cytisus albus* (bark)	*Glycine max* soya bean	*Laburnum vulgare* (seed)	*Laburnum vulgare* (bark)	*Maackia amurensis* (seed)	*Maackia chinensis* (bark)	*Pahseolus limensis* lima bean	*Phaseolus vulgaris* golden wax bean	*Robinia pseudoacacia* (seed) black locust	*Robinia pseudoacacia* (bark)	*Sophora japonica* (bark)	*Wistaria floribunda* (bark)
Squalis suckleyi (dogfish shark)	3	0	0	0	0	5	0	0	0	0	3	6	0	0	0	0,2	0	0
Raja sp. (skate)	1	0	0	3	0	2	0	0	0	0	2	5	0	4	0	3	0	0
Hydrolagus colliei (ratfish)	1	0	2	3	0	5	3	0	0	3	2	7	W	0	0	7	6	0
Acipenser transmontanus (White sturgeon)	2	0	2	3	0	5	0	0	0	0	5	8	W	4	W	6	0	W

Table 4.--Agglutination reactions of various plant extracts with the red cells of fish of order Clupeiformes

Fish cells	Plant extracts																
	Number tested	*Arachis hypogae* peanut	*Caragana macrophylum* (seed)	*Caragana macrophylum* (bark)	*Cytisus scoparius* (seed) scotch-broom	*Cytisus scoparius* (bark)	*Cytisus albus* (bark)	*Glycine max* soya bean	*Laburnum vulgare* (seed)	*Laburnum vulgare* (bark)	*Maackia amurensis* (seed)	*Maackia chinensis* (bark)	*Phaseolus limensis* lima bean	*Phaseolus vulgaris* golden wax bean	*Robinia pseudoacacia* (seed) black locust	*Robinia pseudoacacia* (bark)	*Sophora japonica* (bark)	*Wisteria floribunda* (bark)
Clupea harengus (herring)	10	0	4	5	0	0	0	0	0	0	6	9	5	10	0	7	0	0
Oncorhynchus keta (chum salmon)	20	0	-	9	0	3,6	0	0	0	0	+	7	6	11	+	7	2	4
O. gorbuscha (pink salmon)	40	0	+	+	0	4	-	0	0	-	+	6	2,4	7	+	+	-	-
O. nerka (red salmon)	50 +	0	+	12	0	0,4	0	0	W	0	6	11	5,8	7	4	7	0	4,7
O. kisutch (silver salmon)	6	0	5	7	0	6	0	0	0	0	+	10	W,2	+	-	+	0	W,3
O. tschawytscha (chinook salmon)	280	0	0	7	0	5	0	0	0	0	+	10	0,5	5	0	3,6	0	1
Salmo gairdneri (steelhead trout)	1	0	3	7	0	4	0	0	0	0	5	10	7	1	2	8	0	0
Prosopium williamsoni (mountain whitefish)	2	0	4	7	0	0	0	0	0	0	5	10	2	10	2	9	W	W

Table 5.--Agglutination reactions of plant extracts with red cells of fish within the family *Cyprinidae* (including two interspecies hybrids)

Fish cells	Plant extracts																
	Number tested	*Arachis hypogae* peanut	*Caragana macrophylum* (seed)	*Caragana macrophylum* (bark)	*Cytisus scoparius* (seed) Scotch-broom	*Cytisus scoparius* (bark)	*Cytisus albus* (bark)	*Glycine max* soya bean	*Laburnum vulgare* (seed)	*Laburnum vulgare* (bark)	*Maackia amurensis* (seed)	*Maackia chinensis* (bark)	*Phaseolus limensis* lima bean	*Phaseolus vulgaris* gold wax bean	*Robinia pseudoacacia* (seed) black locust	*Robinia pseudoacacia* (bark)	*Sophora japonica* (bark)	*Wistaria floribunda* (bark)
Richardsonius balteatus (shiner)	12	0	+	+	0	+	0	0	0	0	±	0	+	+	+	+	+	+
Rhinichthys cataractae (dace)	9	0	+	+	0	+	0	±	0	0	+	0	0	W	0	+	±	+
Acrocheilus alutaceus (chiselmouth)	9	0	+	+	0	+	0	0	0	0	+	0	0	W	0	+	+	+
Ptychocheilus oregonensis (squawfish)	7	0	+	+	9	0	+	0	0	0	+	+	0	−	−	+	±	+
Shiner X dace hybrid	2	0	+	+	0	+	0	0	0	0	0	0	0	−	−	+	±	+
Chiselmouth X squaw hybrid	3	0	+	+	+	±	+	0	0	0	+	+	0	+	0	+	0	+

Table 6.--Agglutination reactions of plant extracts with the red blood cells of two species of the family Gadidae.

Fish cells	Plant extracts																
	Number tested	*Arachis hypogae* peanut	*Caragana macrophylum* (seed)	*Caragana macrophylum* (bark)	*Cytisus scoparius* (seed) Scotch-broom	*Cytisus scoparius* (bark)	*Cytisus albus* (bark)	*Glycine max* soya bean	*Laburnum vulgare* (seed)	*Laburnum vulgare* (bark)	*Maackia amurensis* (seed)	*Maackia chinensis* (bark)	*Phaseolus limensis* lima bean	*Phaseolus vulgaris* golden wax bean	*Robinia pseudoacacia* (seed) black locust	*Robinia pseudoacacia* (bark)	*Sophora japonica* (bark)	*Wistaria floribunda* (bark)
Gadus macrocephalus (cod)	3	0	1,3	1,5	0	5	0	0	0	0	2,5	5,8	W	3,5	0,3	7,9	0	0
Merluccius productus (hake)	3	0	2,4	2,4	0	0	0	0	0	0	2,4	2,5	W,5	W,4	0	5,7	0	0

Table 7.--Agglutination reactions of plant extracts with the red cells of various species within the order Perciformes.

Fish cells	Plant extracts																	
	Number tested	*Arachis hypogae* peanut	*Caragana macrophylum* (seed)	*Caragana macrophylum* (bark)	*Cytisus scoparius* (Seed) Scotch-broom	*Cytisus scoparius* (bark)	*Cytisus albus* (bark)	*Glycine max* soya bean	*Laburnum vulgare* (seed)	*Laburnum vulgare* (bark)	*Maackia amurensis* (seed)	*Maackia chinensis* (bark)	*Phaseolus limensis* lima bean	*Phaseolus vulgaris* golden wax bean	*Robinia pseudoacacia* (seed) black locust	*Robinia pseudoacacia* (bark)	*Sophora japonica* (bark)	*Wisteria floribunda* (bark)
Perca flavescens (perch)	2	-	+	-	0	-	-	+	0	-	+	-	0	W	+	-	-	-
Lepomis gibbosus (pumpkinseed)	1	-	+	-	0	-	-	0	0	-	-	-	0	+	+	-	-	-
Pomoxis nigromaculatus (Black crappie)	1	-	0	-	0	-	-	0	0	-	-	-	0	+	+	-	-	-
Micropterus salmoides (largemouth bass)	1	0	+	-	0	-	-	+	0	-	+	-	+	+	+	-	-	-
Rhacochilus vacca (sea perch)	3	0	2	5	0	0	0	0	0	0	4	8	0	7	1	6	-	-
Sebastodes auriculatus (brown rockfish)	6	0	1	3	0	3	2	3	0	0	+	6	0	5	0	7	0	0
Sebastodes caurinus (copper rockfish)	6	0,2	2	4	0	2	0	6	0	0	+	6	0	5	0	8	0	0
Scorpaenichthys marmoratus (giant sculpin)	1	2	2	+	0	W	3	6	0	0	W	5	3	5	0	7	0	W
Leptocottus armatus (sculpin)	1	0	4	7	W	8	5	6	0	0	4	7	4	8	3	8	0	5
Anoplopoma fimbria (sablefish)	1	0	4	3	0	0	0	0	0	0	3	7	0	5	0	7	0	0

Table 8.--Agglutination reactions of plant extracts with the red blood cells of three species of the family Pleuronectidae and the midshipman, *Porichthys notatus*.

Fish species	Plant extracts																
	Number tested	*Arachis hypogae* peanut	*Caragana macrophylum* (seed)	*Caragana macrophylum* (bark)	*Cytisus scoparius* (seed) Scotch-broom	*Cytisus scoparius* (bark)	*Cytisus albus* (bark)	*Glycine max* soya bean	*Laburnum vulgare* (seed)	*Laburnum vulgare* (bark)	*Maackia amurensis* (seed)	*Maackia chinensis* (bark)	*Phaseolus limensis* lima bean	*Phaseolus vulgaris* golden wax bean	*Robinia pseudoacacia* (seed) black locust	*Robinia pseudoacacia* (bark)	*Sophora japonica* (bark)	*Wistaria floribunda* (bark)
Platichthys stellatus (starry flounder)	1	0	0	–	0	–	–	+	–	–	+	–	0	W	+	–	–	–
Parophrys vetulus (English sole)	3	0	0	0	0	0	0	0	0	0	3	7	0	W	0	6	0	0
Psettichthys melanostictus (sand sole)	3	0	W	W	0	0	0	0	0	0	4	7	6	8	2	7	0	1
Porichthys notatus (midshipman)	3	0	3	5	0	4	0	0	W	0	4	9	0	3	W	6	0	W

tested, while all chiselmouths tested were negative for the three extracts. Particularly significant is the *Cytisus scoparius* seed extract reaction due to the apparently high species specificity of this reagent for squawfish cells. On the other hand, the negative reaction of the shiner-dace hybrid with lima bean extract compared to the positive reaction of all shiners tested suggests that the gene controlling the inheritance of this reacting site may be segregating in the shiner population or that dosage may be involved. The soy bean extract reacts for the first time in this group demonstrating qualitative individual variations in dace. This indicates a possible absence of reacting sites specific for this extract in more primitive species and is consistent with the recent work of Sprague and Nakashima (1962) which shows that certain individual tuna react with soy bean extract.

The reactions of two species of the cod family are shown in table 6. Since eight of the extracts give evidence of individual variation in one or both species, it appears likely that further research can produce a number of phytoagglutinin reagents useful for blood typing studies in this group.

Table 7 includes species of the order *Perciformes* tested. The only reactions to the peanut extract, including evidence of individual variation in *Sebastodes caurinus*, are found in this group.

Reactions of three species of the family *Pleuronectidae* are given in table 8. The midshipman is included in this table although taxonomically distinct from the Pleuronectidae. Species separation of the three pleuronectid species appears possible on the basis of these limited samples. The starry flounder is the only flatfish reacting with the soy bean extract. The sand sole is the only flatfish reacting with the lima bean extract. The English sole can be tentatively separated from the other two species by its lack of reaction with either extract.

Results of this study indicate a relationship between seed extracts and bark extracts that varies according to species. The *Cytisus scoparius* seed extract was specific for the squawfish and its hybrids throughout the range of fish species tested, whereas the *C. scoparius* bark extract agglutinated red blood cells of a broad range of fish species but failed to agglutinate squawfish erythrocytes. Other direct comparisons that can be made are the reactions of seed and bark extracts of *Caragana macrophylum*, *Laburnum vulgare*, and *Robinia pseudoacacia*. In none of these is there strong evidence of a qualitative difference between the seed and bark agglutinins as is found in *C. scoparius*.

Variations in the strengths of reactions when the cells age is a problem that has complicated the interpretation of many of the apparently promising reactions of the salmonid group. Though the degree of variation changed from one fish species-plant extract system to another, cells kept over 1 week have shown detectable alteration in specificity in every variable system studied. The alteration of specificity proceeded at approximately the same rate in untreated samples as in those in which an antibiotic was used to inhibit bacterial growth (chlortetracycline at 80 parts/million). Perhaps the most striking example of this alteration is seen in the reaction of chinook salmon cells against lima bean extract: no fresh cells reacted, but week-old cells reacted strongly. A reverse situation was found in the reaction of chinook cells with *Cytisus scoparius* bark extract, for fresh cells reacted strongly and 10-day old cells reacted considerably less. Table 9 and figure 1 illustrate the aging phenomenon when the same cells were tested at various time intervals with a given reagent. The immune antiserum reaction with chinook cells is included to demonstrate the relative reactive stability of fish erythrocytes with immune reagents when compared with phytoagglutinins. The score used is the sum of the agglutination strengths of three consecutive serial dilutions. Each individual test is scored ranging from 4 to 0. A maximum score for the three dilutions is therefore 12.

Table 9.--Variability of agglutination strength of various reagents with respect to storage age of red cells

Fish cells and reagents	Interval between collection of sample and test	Mean agglutination score	Range of agglutination scores	Size of sample
	Days			*Number*
Soos Creek chinook salmon against rabbit antichinook erythrocyte serum	1	7.7	5-10	4
	8	6.7	6-8	
	15	6.2	5-7	
Soos Creek chinook salmon against *Robinia luxurians* bark extract	1	0.1	0-0.5	4
	8	5.5	2.5-7.5	
	15	12.0	all 12	
Soos Creek chinook salmon against *Cytisus scoparius* bark extract	1	12.0	all 12	4
	8	4.8	0.3-6	
	15	0.8	0.5-1.5	
Skagit River chinook salmon against lima bean extract	0	0		12
	2	0		
	4	0		
	7	3.5	0-12	
	10	8.7	5-12	3
Horsefly River red salmon (British Columbia) against *Cytisus scoparius* bark extract	1	1.1	0-3	12
	5	0.6	0-3.5	
	7	3.2	0-12	

Sugars have been tested for their ability to inhibit a portion of the reactions of certain extracts in attempts to increase the specificities (Morgan and Watkins, 1953). A saline solution of L-arabinose (10 percent) has shown marked inhibition of *Robinia luxurians* bark extract at higher titers when tested against chinook salmon erythrocytes. There is no conclusive evidence of an increase in specificity associated with the use of this sugar in the above reaction.

PRELIMINARY STUDIES OF PHYTOPRECIPITINS

The general necessity for fresh material in the study of erythrocyte antigens has led to a search for variation in serum antigens. Using the double diffusion technique of Ouchterlony and immune antisera we have been studying the variation of serum antigens within and between the five species of Pacific salmon indigenous to North America (Ridgway, Klontz, and Matsumoto, 1962).

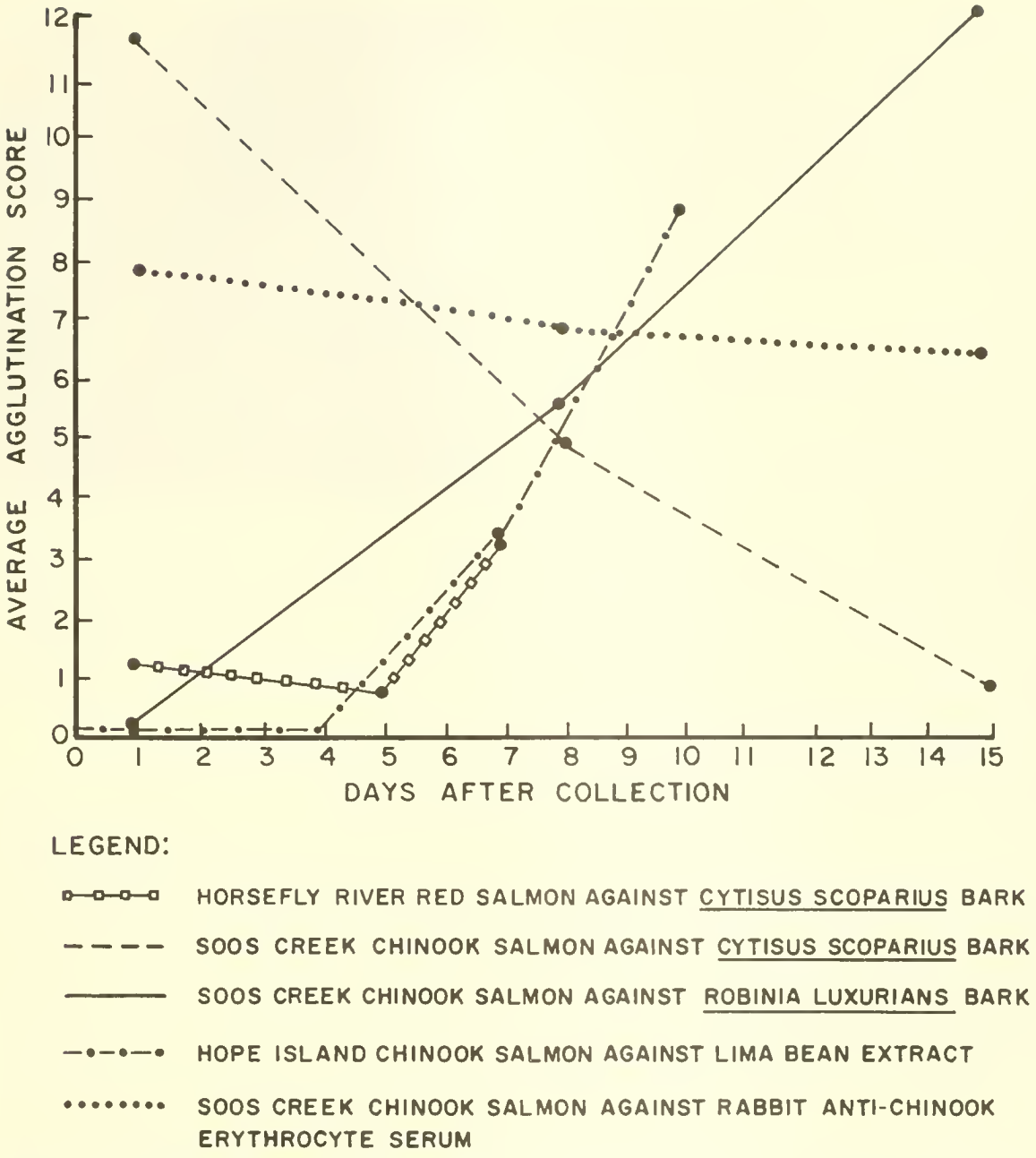

Figure 1.--Variability of agglutination strength of various reagents with respect to storage age of red cells.

Mäkëla (1957) reports plant precipitins were first demonstrated over 70 years ago against mammalian sera. Boyd and Shapleigh (1954) have shown that human soluble blood group substances are specifically precipitated by certain blood group specific plant extracts. Bird (1961) through double diffusion precipitin tests has demonstrated a specificity of *Ricinus communis* and *Abrus precatorius* seed extracts for type XIV pneumococcus polysaccharides.

Using a microslide adaptation of the double diffusion technique, we have tested a number of plant extracts with a variety of fish sera. Some of these extracts have precipitated components of certain fish sera. So far, the most promising of these extracts is from *Robinia pseudoacacia* bark. With this extract, a distinct though quantitative variation has been found in the reaction of chinook salmon sera (fig. 2). With this reagent, significant variations, independent of sex, were found in sera of geographically separate populations of chinook salmon (table 10). Since all samples were taken at the start of upstream migration after the fish have ceased to feed, the variation also appears to be independent of stage of maturity and diet.

Figure 2.--Individual variation in chinook salmon sera as demonstrated by *Robinia pseudoacacia* bark extract. Positions 1 and 4--Spring Creek (Columbia River) sera; position 2--Anchor River; positions 3, 5, and 6--Stikine River. Observe strong reactions in positions 1, 3, and 4, a weaker reaction in position 6, a questionable reaction in position 2 and a negative reaction in position 5.

DISCUSSION

The above results indicate that phytoagglutinins have a considerable potential value as useful supplements to immune and normal sera now employed in fish serology. From the point of view of economy the use of phytoagglutinins is desirable, for they eliminate the disadvantages of maintaining animals. If a useful plant extract is found, the investigator has some assurance of an almost limitless supply of uniformly specific reagent, providing proper identification has been made and plant components are available locally or by request. In the field, where refrigeration is impractical, seeds and presumably dried barks are ideal because extracts can be made whenever needed.

Certain extracts appeared to possess either species specificity or reactivity within a limited taxonomic range. The *Cytisus scoparius* seed extract reacted exclusively with all squawfish and squawfish hybrid cells tested. The only reaction of the *Laburnum vulgare* bark extract was with the single ratfish tested. The peanut extract appeared to be specific for some individuals of the somewhat closely related corpaenids and cottids. The lack of reaction of the soybean extract with more primitive species may be indicative of a broad group specificity of this reagent. Conversely, the *Sophora japonica* bark extract reacted only with certain more primitive species. The remainder of the extracts tested exhibited no strong group or species specific qualities. There are, however, indications that testing a battery of reagents at appropriate dilutions can give species identification within groups. This conclusion, however, should be considered as tentative because the samples were small in most instances.

The specificities detected by phytoagglutinins appear to be much more sensitive to changes caused by red blood cell aging than those specificities detected by normal or im-

Table 10.--Reactive frequency of chinook salmon sera reacting with *Robinia pseudoacacia* bark extract

Area	Year	Tested number	Positive number	Negative number	Positive percent
Nushagak (Bristol Bay)	1959	13	1	12	8
Anchor River (Cook Inlet)	1959	12	5	7	42
Stikine River (S.E. Alaska)	1959	17	12	5	71
Skagit River (Puget Sound)	1961	9	8	1	89
Spring Creek (Columbia River)	1959-1960	50	49	1	98
Columbia River Spring run	1961	18	18	0	100

X^2 = 75 (1 percent significance level with 5 d.f. = 30)

mune antibodies. Using strictly fresh material precluded this phenomenon from interfering with results obtained on the nonsalmonid species tested. The presence of the phenomenon in salmonids, however, suggests that the aging of cells is a limiting factor in the usefulness of phytoagglutinins in fish blood typing work. Therefore, one should be cautious in interpreting results of tests with these extracts unless fresh cells are being used or previous work has shown that storage of cells does not alter the reaction. Sprague and Holloway (1962) have also commented on the variability of reactions obtained when tuna cells are agglutinated with various plant extracts.

Further research is necessary before the potentialities of phytoprecipitins in fishery research can be properly assessed. Relationships to immune precipitins and genetic implications of their variability are unknown. It is of interest, however, that such variabilities do exist and that they can be studied in the same manner as those detected by immune antisera. The apparent geographic variation of the reaction of chinook salmon sera with *Robinia pseudoacacia* bark extract is certainly of practical interest. It seems probable that similar plant extract-fish serum relationships exist which are capable of analysis by the double diffusion technique. This variation is probably an expression of a basic genetic difference in these populations since it is independent of sex and all individuals were collected at the same stage of maturity. Effects of diet are also excluded because these fish had ceased feeding.

SUMMARY

1. Phytoagglutinins extracted from the seeds and bark of various legumes were shown to possess a variety of specificities against fish erythrocytes, some of which detected intra- and interspecies differences.

2. Significance of individual variations found in salmonids is complicated as a result of alteration of reactive strengths of cells on aging.

3. Some extracts contained specific phyto-precipitins which reacted with components in fish sera when tested by the double diffusion technique of Ouchterlony.

4. The results of this research indicate that plant extracts may be usefully applied as haemagglutinins or serum precipitins in studying fish populations.

ACKNOWLEDGMENTS

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ADDENDUM:

Since submitting this manuscript for publication, two additional publications have come to light which are pertinent to this subject and warrant mentioning. Suzuki and Higasa (1962) found variable agglutinins for certain tuna species in seed extracts of *Glycine max*, *Ginkgo biloba*, and *Virgilia divaricata*. They found certain sugars to be inhibitory to some of these agglutinins and noted that agglutinability was destroyed in each extract by heating at 80° C. for 30 minutes. Sindermann (1963) found seed extracts of certain lima bean strains, and large lentils to give differential reactions in three clupeoid species indigenous to coastal areas of the northwestern Atlantic. Evidence was obtained for heterogeneity in four spawning populations of alewives *(Alosa pseudoharengus)*.

The above entries are in reference to the following:

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Suzuki, Akimi, and Shichiro Higasa.
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