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Biochemical Genetic Studies of Fishes: Potentialities and Limitations

FRED M. UTTER, HAROLD O. HODGINS AND FRED W. ALLENDORF

*Northwest Fisheries Center,
National Marine Fisheries Service,
National Oceanic and Atmospheric Administration,
2725 Montlake Boulevard East,
Seattle, Washington 98112*

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I. Introduction

A new dimension in the understanding of protein variation had its basis in two events of the 1950's. The model of the structure of the DNA molecule proposed by Watson and Crick (1953) led to an understanding of the direct relationship between genes and proteins. Starch gel electrophoresis (Smithies, 1955) enhanced by the application of histochemical staining methods (Hunter and Markert, 1957) added simplicity and sensitivity to the study of protein variation. These events led to an explosion of information concerning protein variation and its significance which started in the 1960's and has continued in this decade. Studies of genetic variation

at the protein level have made major contributions to a diverse array of biologically oriented disciplines. Many fishes have been studied electrophoretically (reviewed by de Ligny, 1969, 1972) and our co-workers and we have been involved in some of these investigations.

This is not a review paper in a comprehensive sense, although considerable literature will be cited to illustrate various points. Rather, we attempt to evaluate the present status of biochemical genetic research of fishes from the particular perspective of our experience, giving a background for this perspective and an indication of what we regard as fruitful areas for future research. At the same time we urge readers of this review to also read a complementary review of a similar topic by Robertson (1972). For the reader who is not studying genetic variation at the protein level, we hope to clarify what can and cannot be accomplished using electrophoretic-histochemical methods. The less experienced reader who is involved to some degree in the type of research reviewed, will, we hope, through the sharing of our experiences, have enlarged his perspective. Finally, the equally or more experienced reader will find that some of our thoughts will at least complement his own experience, and we hope that some will be found provocative.

II. Background of Serological and Biochemical studies

Our present research program had its roots in the mid-1950's when George Ridgway of the U.S. Bureau of Commercial Fisheries (now the National Marine Fisheries Service) proposed that Pacific salmon, *Oncorhynchus* spp., caught on the high seas might be identified as to continent of origin by serologically detected differences reflecting, presumably, genetic differences. The problem was approached through studies of serum proteins and erythrocyte antigens.

Serum protein variants were detected in sockeye salmon, *O. nerka*, using immunodiffusion and immunoelectrophoresis and with antisera developed mainly in rabbits. One variant (called the A or SM antigen) was found in maturing females of all salmonid species tested and has proven useful for maturity rather than for racial studies. A pair of serum antigens (called antigens I and II), which appeared to vary markedly in their distribution between fish of Asian and American origin, initially seemed highly promising for assigning the continental origin of fish taken on the high seas. However, difficulties in producing adequate quantities of specific and potent antisera, coupled with strong indications that these variations reflected artifacts of differential preservation rather than valid genetically based differences, led to discontinuance of study of these antigens.

Erythrocyte antigens were examined with an array of agglutinating substances, including normal sera from a variety of animals, phytohemagglutinins, heteroimmune (xenogeneic) sera and isoimmune (allogeneic) sera; only the isoimmune sera gave any real promise for reliable identification of genetic differences in Pacific salmon species. These reagents, produced in rainbow trout, *Salmo gairdneri*, were used to identify differences between populations of sockeye; chum, *O. keta*; and pink salmon, *O. gorbuscha*; and to indicate inbreeding in a population of cut-throat trout, *S. clarki*. However, these antisera were difficult to produce and purify in adequate quantities; their patterns of reactivity were very complex and there was a loss of potency detectable after frozen storage from one year to the next.

In a detailed review of the above studies, Hodgins (1972) concluded: "Significant advances have been made in concepts of the nature of fish populations as a result of extensive serological and immunochemical studies. The magnitude of two types of problems militates against further extensive studies of this nature in our laboratory at present: (1) technical problems, such as difficulty in producing vast quantities of specific high-titered antisera and the critical nature of sample preservation; and (2) theoretical problems, such as relating blood groups to genes. These procedures can be used, however, to differentiate stocks under certain conditions."

An alternate approach was needed after a decade of limited success and considerable frustration through the use of serological methods in attempting to identify genetic differences in fish populations. Thus we turned to starch gel electrophoresis. It soon became apparent that this was the method of choice over any previous methods that we had used for the identification of intra-species genetic variations of fish species that we were studying.

The major advantage of biochemical genetic data obtained through gel electrophoresis compared to immunological data is that valid genetic interpretations can be made directly from raw data. Codominant expression of most variant alleles occurs on starch gels. That is to say that, in an individual containing different alleles of a given locus, each of the alleles is expressed as a single, distinct protein. Such a situation commonly permits designation of the genotypes of individual samples based on staining patterns on gels. The frequency with which a given gene occurs in a population of individuals can be directly determined and the distribution of phenotypes can be tested for deviations from expected values based on simple genetic models, an important one being the Hardy-Weinberg model. This states that in a randomly mating population in the absence of a variety of disturbing forces (e.g. selection, mutation, etc.) the

expected distribution of genotypes is determined by the random combination of alleles. In the case of two alleles (A — B) the expected genotypic frequency, therefore, is

$$q^2(AA) : 2q(1 - q)(AB) : (1 - q)^2(BB)$$

where q is the portion of A alleles in the population. Inferences can also be made directly from raw data regarding the subunit structure of proteins based on the number of bands observed for a particular system in heterozygous individuals. This is discussed in detail in a later section. Two other significant practical advantages of the starch gel method are that antisera are not required and that sample preparation and preservation have proven to be much simpler. The overall advantages of the starch gel method have resulted in an enormous differential in the amount of genetic information concerning intraspecies variation per unit of effort obtainable by comparing blood grouping and starch gel electrophoretic methods. As a hypothetical example (but based on actual experience), one worker examining a previously unstudied species could obtain more valid genetic information in a day using starch gel electrophoretic methods than a team of workers could obtain in 6 months using blood grouping methods.

By 1968, the major portion of our program's genetic efforts had shifted from blood grouping to electrophoretic and histochemical staining methods. The early studies in this phase were exhilarating because we were not yet accustomed to finding genetic variations with the ease made possible by our change in methods. A summary of our research through 1970 (Utter *et al.*, 1972) reported genetic variants at one or more loci in 16 species of marine and anadromous fish.

III. Methodology

The methodology we use is similar to that employed in other laboratories using horizontal starch gel electrophoresis to study a wide variety of organisms. Some modifications which we routinely employ, which may result in saving time and money, may not be apparent from the literature. Although we continually look for ways to simplify our methods while maintaining or improving the quality of the protein resolution, the basic electrophoretic methodology described below is that taught to us by C. N. Stormont and Y. Suzuki of the University of California at Davis.

Starch gel preparation generally follows that described by Kristjansson (1963). The gel is poured into a frame composed of a 7 x 10-1/2 inch glass plate bounded by removable 1/4-inch thick Plexiglass strips which are held in place with paper clamps. We have observed a strong preference among our colleagues for this arrangement rather than complete preformed

electrophoretic units. After pouring, the gel is cooled to room temperature, and covered with plastic wrap. The cooling process may be accelerated in the refrigerator and the gel made ready for electrophoresis within 15 min after pouring. Freshly poured gels are preferred, although we have obtained satisfactory results from gels that have been poured as long as 48 h previously. We have found that placing a glass plate on top of the gel immediately after pouring is not desirable.

Prior to application of samples, the gel is cut 3 cm from the end along the long side and the smaller section of the gel is pulled back about 1 cm. Samples are drawn onto filter paper inserts as small as 4×6 mm and placed side by side along the gel separated by 1 mm; in this manner, more than 40 samples can be tested on a single gel. For electrophoresis, the smaller gel section is placed firmly against the larger section and inserts. Plastic wrap is folded back to expose about 1 cm at each end of the gel.

Buffer trays used are plastic dishes containing 150-200 ml of buffer. The tray buffer is transmitted to the gel with disposable utility cloths which are re-used indefinitely. Initial current for electrophoresis varies according to the buffer systems; we use as much as 300V but never exceed 100 ma. Sample inserts are removed and discarded following 10 min preliminary electrophoresis. Gel sections are again placed firmly together. Ice packs composed of gelled refrigerant repackaged to fit on a gel-sized glass plate are placed on top of the gel, and electrophoresis is continued until dye marker or boundary has migrated the appropriate distance. Following electrophoresis, the 1/4-inch plastic strips are removed from the gel frame. The gel is sliced into four layers, with nylon thread guided by 1/16th-inch plastic strips placed sequentially on top of one another; each layer is placed in a separate staining tray and stained differentially as desired.

It is desirable to use a variety of buffer systems in the initial phases of an electrophoretic investigation. No single buffer system can resolve all of the proteins that can be detected by starch gel electrophoresis. We have generally found, however, that a protein that is clearly resolved by a particular buffer system in one species is usually resolved in other species by the same system. We routinely use four different buffer systems. The components of these systems and the proteins that they best resolve are given in Table I. Additional buffer systems have been described elsewhere which may provide better resolution for certain proteins (Shaw and Prasad, 1970).

Staining methods we routinely use have followed directly or were modified from procedures of Shaw and Prasad (1970) in most instances. We have saved a considerable amount of money and time by reusing many solutions requiring tetrazolium salts. These salts plus substrates, cofactors

TABLE I: Different buffer systems for starch gel electrophoresis.

Buffer System	Comments
<p>1. After Ridgway <i>et al.</i>, 1970.</p> <p><i>Electrode Buffer</i>—LiOH 0.06M, H₃BO₄ 0.3M, pH 8.3</p> <p><i>Gel Buffer</i>—Tris 0.03M, citric acid acid. 0.005M, pH 8.0</p> <p>Use 99% gel buffer and 1% tray buffer for making gels.</p> <p>Use electrode buffer undiluted in electrode compartments.</p>	<p>Best system for resolving LDH,* PGM, Tfn, SDH, CrK.</p> <p>Gives only fair to poor resolution for some proteins that are clearly resolved by other systems.</p>
<p>2. After Markert and Faulhaber, 1965.</p> <p><i>Stock Solution</i>—Tris 0.9M, H₃BO₄ 0.5M, NaEDTA 0.02M, pH 8.6</p> <p>Dilute stock solution 1:20 for making gel, 1:4 for electrode compartments</p>	<p>Best system for AGPD. Adequate resolution for most proteins.</p>
<p>3. After Wolf <i>et al.</i>, 1970.</p> <p><i>Stock Solution</i>—Na₂HPO₄ 0.1M with pH adjusted to 6.5 with 0.1M NaH₂PO₄.</p> <p>Dilute stock solution 1:10 for gel, use undiluted in electrode compart- ments.</p>	<p>The only system of those listed to adequately resolve IDH and all MDH isozymes.</p>
<p>4. After Utter and Hodgins, 1972.</p> <p><i>Stock Solution</i>—one part 0.3M H₃BO₄ to four parts 0.3M tris, pH 9.0</p> <p>Dilute stock solution 1:10 for making gel, use undiluted for electrode compartments.</p>	<p>Gives adequate resolution for most proteins. Particularly useful for resolution of non-specific muscle proteins migrating towards the anode.</p>

*See Table II for full names of abbreviated proteins.

and coupling enzymes can be quite expensive. We have found that some solutions are stable for periods exceeding 1 week when stored refrigerated in the dark. Appropriate controls are of course required to ensure that negative results are caused by no enzyme activity rather than by deterioration of a component of the staining solution.

We have found that many samples can be prepared for electrophoresis with a minimum of effort. Skeletal muscle that has been frozen more than a few days is more readily extractable and can be placed in a suitable container with an equal amount of extracting fluid and tested without further treatment. Liver samples can often be similarly extracted.

Stability of proteins under frozen storage varies according to the particular protein itself and the tissue in which it is expressed. Muscle proteins have been quite stable with some enzymes, e.g., PGM and LDH (see Table II for full names of abbreviated enzymes), retaining good activity well in excess of a year. Proteins expressed only in the liver have proven considerably more labile, some enzymes—e.g., ADH—losing activity after only a few weeks.

Reliable blood samples for transferrin testing are obtained up to 48 hours after the fish was killed, provided the fish was promptly placed on ice but not frozen. The flesh is pierced with a sharp scalpel and the blood removed from the pericardial cavity with a Pasteur pipette; the blood is then expressed into a suitable container holding an anticoagulant solution. Dilution of the blood as much as 5:1 still results in reliable transferrin phenotypes. This method has proven useful in small fish where only minimal amounts of blood can be collected. Although freshly collected serum is preferable, transferrin phenotypes can also be found in eye fluids (Utter, 1969). The investigator should always run tests initially with both serum and eye fluids to ensure that parallel phenotypes are being expressed. We found that transferrins in eye fluids are sometimes detectable only after concentration.

It may be noted here that we have not included polyacrylamide gel electrophoresis in this discussion largely because of our more limited experience with it. We can use a somewhat smaller volume of sample with acrylamide but starch is faster and more flexible, results are more repeatable and we see little significant decreased resolution of fish protein bands with starch rather than acrylamide.

We may also mention that electrophoresis can be combined with immunodiffusion to give immunoelectrophoresis (IEP, see Williams and Chase, 1971). This test has great resolving power for mixtures of proteins. In human serum, for example, about 6 components are detected by electrophoresis in agar alone, while 30 or more components are detected by IEP. IEP in agar gels has been used in this laboratory to characterize serum proteins in sockeye salmon (Krauel and Ridgway, 1963) and in other species for population studies. Methods routinely used are described in the paper by Krauel and Ridgway in which they reported that as many as 25 components were detected in sockeye salmon serum. We have also extensively applied this procedure to identifying and characterizing immunoglobulin in rainbow trout serum (Hodgins *et al.*, 1965; Hodgins *et al.*, 1967).

Other matrices than agar (such as cellulose acetate, agarose, and polyacrylamide) are used in IEP. We have used a combination of an acrylamide disc gel imbedded in agar in several experiments. This entails prior electrophoresis of the sample in a disc gel followed by immediate

embedding of the entire unfixed gel in agar on a 2 × 3 inch glass slide and then proceeding with usual IEP procedures of cutting troughs and applying antiserum. With specific antisera and this technique, we have characterized female-specific antigens in salmonid fish sera and eggs (Gronlund *et al.*, 1973).

IV. Criteria for Mendelian variation

In spite of the many advantages that we have found for using biochemical data for studying genetic variation within species, it is necessary to impose some rather stringent restrictions on all biochemical variation observed on starch gels before assuming that a particular pattern of variation is actually a reflection of genetic variation. The strongest data are those obtained from progeny of parents having known biochemical differences; a genetic basis is regarded as confirmed if these data conform to models of simple Mendelian inheritance. A genetic basis is assumed for variants that are similar, and presumably homologous, to variants observed in closely related species for which breeding data have been obtained.

In the absence of breeding data, several requirements are imposed. The bands resolved in starch must fit interpretable patterns based on simple genetic hypotheses. These patterns must be repeatable with sub-sampling of the same tissue of an individual. It is useful, though not essential, if a pattern of variation is expressed over a broad range of development of individuals in a species. Any significant deviations of phenotypic distribution from predictions of a genetic model must be explained.

We have observed four basic types of variation that may be interpreted as reflecting simple codominant expression of allelic genes (Fig. 1). Patterns

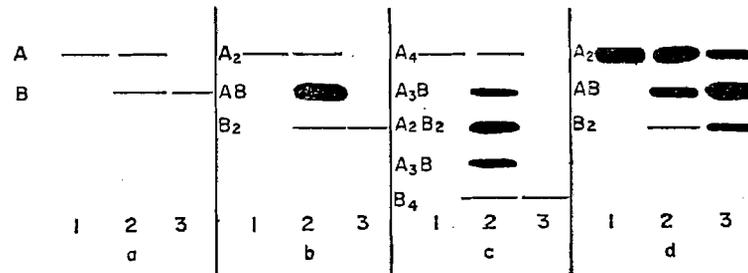


FIG. 1. Patterns of biochemical genetic variation observed in fishes reflecting codominant expression of two alleles. Subunit compositions are given to left of each band. 1. fast homozygote, 2. heterozygote, 3. slow homozygote. a—monomeric pattern, b—simple dimeric pattern, c—tetrameric pattern, d—dimeric pattern with two loci—one fixed and one polymorphic. Similar patterns with more phenotypes are observed when three or more alleles are found.

a, b, and c are typical of allelic expression for their respective subunit combinations in a wide variety of organisms. The enzymes yielding patterns a, b, and c are proteins consisting of one, two, and four subunits respectively. In each case two genetically distinct enzymes are involved and both are present in the heterozygote. That is, we are dealing in the examples shown with two codominant alleles. The patterns assume that the individual subunits of the enzymes in question combine in a random manner. For the interested reader, Shaw (1964) has discussed in detail the patterns expected for allelic proteins having from one to four polypeptide chains in the active molecule. In Fig. 1d we show a more complex pattern for a dimeric protein with two genetic loci—one fixed and one polymorphic. Pattern d has been reported for MDH and IDH in salmonid fishes (Bailey *et al.*, 1970; Wolf *et al.*, 1970; Allendorf and Utter, 1973). An hypothesis of two loci, one fixed and one polymorphic, has been verified for both loci through actual breeding data (Bailey *et al.*, 1970; Allendorf and Utter, 1973). The need for breeding data must be emphasized for proper interpretation of this kind of variation; it had been previously proposed only on the basis of observation that IDH variation reflected tetrasomic inheritance at a single locus (Wolf *et al.*, 1970).

Initial observations of patterns a, b, and c in Fig. 1 have generally, but not invariably, proven to be valid reflections of simple codominant allelic variation. Exceptions have been with some non-specific muscle proteins and esterases in various tissues where pattern a-type variations have not been repeatable on subsampling of the same individual. We have also encountered difficulty in obtaining liver esterase patterns in progeny of rainbow trout based on phenotypes of parents, although subsampling data from individual fish are consistent. The nature of this variation remains questionable and may reflect ontogenetic changes in gene expression superimposed on typical Mendelian variation.

With a few clearly noted exceptions, the remaining sections of this review are devoted to a consideration of the applications of codominantly expressed genetic variants that have met our requirements for allelism. Such variations are an intrinsic property of the individual fish, and their expression is not directly affected by the environment (although there may be an indirect effect through processes of natural selection).

There may be considerable variation expressed on starch gel other than that reflecting (or appearing to reflect) codominant allelic variation. Although this variation may be partially or totally under genetic control, it cannot be analyzed with the precision that is possible for data reflecting codominant gene expression. Such variation includes the presence or absence of single or multiple bands, or variations in the intensity of such bands. This can often reflect artifacts arising from storage and preser-

vation of samples (see Eppenberger *et al.*, 1971). It may also arise from different environmental or pathological conditions (see Hochachka, 1971) or may reflect gene modifications that do not alter the electrophoretic mobility of the protein in question (see Yoshida, 1973). Null (i.e., inactive) alleles may also account for some of this kind of variation; such variation can be analyzed similarly to codominant variants (i.e., a genotype can be assigned to each phenotype) if the active alleles of this locus interact with gene products of another locus, as for a null allele described for an LDH locus in rainbow trout (see Wright and Atherton, 1970). Some variation may have a codominant basis but cannot be clearly resolved electrophoretically; this observation emphasizes the need for using a variety of buffer systems in the early phases of an electrophoretic study. Regardless of cause, non-codominant variation is generally excluded from detailed analysis in our investigations.

V. Genetic variations of proteins within species

A. Relative Frequency of Variations

We have observed that some protein systems tend to vary more than others. A survey of protein variation observed in as many as 20 species that we have studied (Table II) indicates that over half of the species tested were polymorphic for PGM while none were polymorphic for 6PGD, ADH, or CrK. This observation is not unusual. It is well documented from amino acid sequencing data comparing similar proteins of diverse organisms (Dickerson, 1972) that proteins evolve at different rates; the less variable proteins observed here may represent more conservative evolutionary lines. It is interesting that G. B. Johnson (1971) predicted that ADH should be among the more variable proteins generally, on the basis of observed variations in *Drosophila* spp. and the equilibrium constants of ADH. It is possible of course that ADH of teleost fishes and *Drosophila* have different evolutionary dynamics. In a more practical vein, the data summarized in Table II offer a guide to other workers studying polymorphisms in fishes, whose methods do not yet include staining techniques for some of the proteins listed, by establishing priorities on the basis of the more variant proteins.

B. Different Patterns of Variations

The diversity of biochemical genetic variation within species that has been observed in our studies is typical not only of that observed by other workers in fishes (reviewed by de Ligny, 1969, 1972), but also of a continually broadening sampling of animal and plant species in general (see

TABLE II: Average frequencies of polymorphic proteins in fish species.

Protein	Number of species tested	Number of species polymorphic	Proportion of species polymorphic
Phosphoglucomutase (PGM)	19	11	0.58
Alpha-glycerophosphate dehydrogenase (AGPD)	19	9	0.47
Transferrin (Tfn)	7	3	0.43
Lactate dehydrogenase (LDH)	20	8	0.40
Malate dehydrogenase (MDH)	20	7	0.35
Esterase (Est)*	20	5	0.20
Sorbitol dehydrogenase (SDH)	6	1	0.17
Aspartate aminotransferase (AAT)	20	3	0.15
Isocitrate dehydrogenase (IDH)	20	3	0.15
Non-specific muscle protein	20	3	0.15
Diaphorase (Dia)	10	1	0.10
Tetrazolium oxidase (TO)	20	2	0.10
Peptidase (Pep)	20	1	0.05
6-phosphogluconate dehydrogenase (6PGD)	20	0	0
Alcohol dehydrogenase (ADH)	10	0	0
Creatine kinase (CrK)	10	0	0

*As noted earlier (p. 221), we found artifacts in analyses of esterases and urge investigators to be particularly stringent in their criteria for allelism in this class of enzymes.

Gottlieb, 1971). Intraspecies patterns of geographic variation that we have observed have been quite different, even among closely related species. It is clear from these observed patterns of genetic variation that the potential of biochemical genetic variation for use in our original purpose of stock identification differs considerably among species. We have interpreted significant differences at one or more loci between two groups of fish of a particular species as evidence for different breeding groups. Conversely, however, non-significant differences between two groups of fishes are regarded only as indicating that these groups are not necessarily different, but not as evidence that they are the same. An analogous situation at the individual level is a paternity test where the putative parent is exonerated when his genotype conflicts at one or more loci with that of the offspring in question, but is not incriminated in the absence of conflicting genetic data. This interpretation has generally proven to be accurate when other kinds of evidence, such as data from tagging experiments and growth studies, are also available for different groups of a species that we are investigating. It is fallible, however, in instances where subgroups of a single breeding population reflect differential selective pressures through different allelic frequencies, and that topic is discussed separately. In

Table II are presented some of these different patterns of polymorphism that have been interpreted as described above.

The simplest pattern of variation was found in the Pacific hake, *Merluccius productus* (Utter, 1969; Utter and Hodgins, 1969, 1971; Utter *et al.*, 1970). Two major population units were identified one in Puget Sound, Washington, and the other in the northeastern Pacific Ocean extending from British Columbia southward to Mexico. Highly significant differences of allelic distribution were found in each of four polymorphic systems; LDH, muscle protein, transferrin and an esterase system expressed in vitreous fluids. Very large fish occasionally caught in Puget Sound were originally suspected of being immigrants from the Pacific Ocean but were determined to be indigenous to Puget Sound by their gene frequencies.

A clinal distribution, where the frequency of polymorphism gradually changes with the geographical area, has been observed for protein variation in some species. The distribution of variant forms of two proteins—PGM and LDH—are clinal in sockeye salmon; much higher frequencies of variant forms are found in the more westward range of the species and gradual decreases occur as sampling progresses eastward (Hodgins *et al.*, 1969; Utter and Hodgins, 1970; Hodgins and Utter, 1971). It can therefore be assumed that samples of sockeye salmon taken on the high seas, lacking LDH variants and having low frequencies of PGM variants, originated from the southeastern range of the species. A clinal distribution has also been observed for a muscle protein variation in the greenstriped rockfish, *Sebastes elongatus*, in samples collected between Queen Charlotte Sound, B. C. and Puget Sound (Johnson, 1972).

An abrupt reversal of gene frequencies has been found for a three allele system of the serum protein transferrin in samples of coho salmon, *O. kisutch*, in different areas of Washington State (Utter *et al.*, 1970; Utter *et al.*, 1973). The A allele has a frequency greater than 90% in samples taken from streams entering the Columbia River. The C allele is most frequent in samples taken from streams entering Puget Sound and the Pacific Ocean, including those entering the ocean adjacent to the Columbia River. A third allele (B) is found at fairly high frequencies in fish from coastal and Puget Sound streams, but has not been detected in Columbia River fish. The pattern of transferrin variation in coho salmon from streams south of the Columbia River and north of Washington State is presently unknown, and knowledge of whether or not similar discontinuities occur in other areas must await further sampling. This system appears to have considerable potential for determining whether fish caught in the ocean in the vicinity of the Columbia River are destined to spawn in Columbia River tributaries or other areas.

Genetic variation of TO in chinook salmon, *O. tshawytscha*, may be correlated to the time of their return to freshwater (i.e., their spawning migration). A low frequency of a variant TO allele (F) was observed in four populations of fish returning to rivers of origin in the spring in populations sampled from southeastern Alaska and the Columbia River. However, the average frequency of the variant TO allele in five populations of fish from the Columbia River and Puget Sound that returned to the rivers in the fall was much higher (Utter *et al.*, 1973).

The rainbow trout is of particular interest with regard to the use of biochemical polymorphisms for racial studies because of the relatively large number of variant loci that have been reported in this species. Fourteen loci identified by electrophoretic variants are listed in Table III.

TABLE III: Polymorphic loci of rainbow trout.

Locus	Number of alleles	Reference
LDH E	2	Wright and Atherton, 1970.
LDH B ¹	2	Wright and Atherton, 1970.
LDH B ²	2	Willisroft and Tsuyuki, 1970; Utter and Hodgins, 1972.
MDH A	2	Allendorf, unpublished data.
MDH B	4	Bailey <i>et al.</i> , 1970; Allendorf, unpublished data.
Tfn	3	Utter and Hodgins, 1972; Utter <i>et al.</i> , 1973.
PGM	2	Roberts <i>et al.</i> , 1969.
AGPD	2	Engel <i>et al.</i> , 1971; Utter and Hodgins, 1972.
TO	3	Utter, 1971; Utter and Hodgins, 1972.
Est I	2	Kingsbury and Masters, 1972.
Est II*	2	Allendorf, unpublished data.
IDH	4	Wolf <i>et al.</i> , 1970; Allendorf and Utter, 1973.
SDH	2	Engel <i>et al.</i> , 1970.
Hemoglobin	2	Tsuyuki and Ronald, 1970.

*Some question exists concerning the genetic nature of the variation. See discussion under "Criteria for Mendelian Variation".

Some of this variation has proven useful for distinguishing groups of rainbow trout, particularly when data from more than one locus are included in comparisons (Utter and Hodgins, 1972; Utter *et al.*, 1973). A comparison of gene frequencies in anadromous rainbow trout (steelhead) taken from hatcheries of tributary streams of the Columbia River (Table IV) indicates considerable heterogeneity in a group of fish that is managed in some instances as a single population. Of particular interest is a comparison of allelic frequencies of LDH and TO in

TABLE IV: Gene frequencies of biochemical genetic variants at seven loci in Columbia River steelhead trout populations.

Population	Frequency of most commonly observed allele						
	AGPD	Est II	IDH	LDH	MDH-B	PGM	TO
<i>Upstream</i>							
Clearwater	1.00	—	—	0.29	0.97	0.99	1.00
Pahsimeroi	0.99	0.72	0.45	0.40	0.99	1.00	0.94
Hell's Canyon	1.00	0.45	0.53	0.46	1.00	1.00	0.94
Deschutes	1.00	0.50	0.28	0.40	0.98	1.00	0.96
<i>Downstream</i>							
Big Creek	1.00	0.74	0.40	0.99	0.79	1.00	0.55
Skamania	0.92	0.81	0.10	0.80	0.88	0.99	0.60
Cowlitz	0.98	0.60	0.22	0.83	0.83	1.00	0.70

—no data available.

upstream and downstream populations. Data from these loci alone may be useful in predicting the extent of migration in steelhead entering the Columbia River.

Two species that have considerable biochemical polymorphism but which have failed to reveal any significant variations among populations—in spite of fairly extensive sampling—are the Pacific herring, *Glupea harengus pallasi*, and the Pacific saury, *Cololabis saira*, (see Utter *et al.*, 1972, and Utter, 1972, for preliminary reports of these variants). The variations of these species are summarized in Table V. Herring have been

TABLE V: Variant loci of Pacific herring and Pacific saury.

Species	Average frequency of most common allele							
	LDH-A	LDH-B	Est	AAT	MDH	PGM	AGPD	IDH
Pacific herring	>0.99	>0.99	0.85	0.98	0.96	0.74	1.00	1.00
Pacific saury	1.00	1.00	0.68	1.00	0.63	1.00	0.70	0.52

sampled from off the coast of Oregon northward to Kodiak Island and Saury were sampled off the coast of Japan and from southern California northward to British Columbia. The only differences in gene frequencies were for the LDH variants of herring; variants at two loci occurred at low frequencies in samples collected in Puget Sound and in the North Pacific Ocean near Vancouver Island, but so far these variants have not been observed in samples collected from Alaskan waters. It is premature to speculate in any detail concerning possible reasons for the stability of

allelic frequencies over broad geographic areas in these two species. It is interesting, however, that both species serve as forage fish for larger carnivorous species (the herring in inshore areas and the saury on the high seas) and thus occur in large numbers throughout their respective ranges. It may be that their gene frequencies are less subjected to random factors because of large breeding groups and that gene frequencies are stabilized among these groups, in the absence of strong selection, by some degree of genetic interchange.

To summarize the potential of biochemical genetic data in racial studies of fish, we believe that data based on reliable Mendelian variants should be collected in the initial phases of any study examining the population structure of a fish species. If the geographic range of sample collection is sufficiently broad, a situation similar to that observed in the Pacific hake should soon become apparent and biochemical genetic data, alone, may be sufficient to define adequately the populations in question. In instances such as the one exemplified by the sockeye salmon, biochemical genetic data may be used for broad definition of stocks taken at sea, and alternate methods, such as scale characteristics, may then be employed for more precise identification. Conversely, in the case of Columbia River fish vs. populations of coho salmon from coastal streams, biochemical genetic data may be used for more precise definition, but may be less useful alone for identification over a broader geographic range. Similarly, differences in gene frequencies of LDH and PGM variants between populations of sockeye salmon within major river systems have been observed in Alaska and may be useful for characterization of these stocks (Hodgins and Utter, 1971; Utter, unpublished observations). Finally, in instances such as that of Pacific saury, biochemical genetic data are of value in supporting hypotheses of homogeneity established by other criteria, but cannot be used alone for either broad or restricted definitions of populations.

The unique value of biochemical genetic studies is their direct reflection of genetic differences coupled with their ease of application. As indicated above, we believe that biochemical genetic methods should generally complement rather than replace other methods of examining population differences.

C. Gene-Environment Interactions

Considerable uncertainty presently exists concerning the role of natural selection in the maintenance of biochemical polymorphisms in populations. The large amount of biochemical polymorphism observed in most organisms has been explained, both on the basis of selection (Prakash *et al.*, 1969) and random factors (King and Jukes, 1969). The potential usefulness

of biochemical polymorphisms could be extended considerably with a better understanding of the degree to which allelic forms of different proteins interact with components of the environment. Variants could then have greater value in examining patterns of dispersal and questions of systematics.

In fishes, as in other groups of organisms that have been studied, very little is presently known concerning the extent to which genotype-environment interactions of biochemical genetic variants can account for the amount of variation that has been described. Koehn and his associates have related allelic esterases of certain fresh water teleosts to temperature on the basis of geographic distribution and biochemical activities (Koehn, 1969; Koehn *et al.*, 1971). M. S. Johnson (1971) observationally and experimentally related allelic forms of LDH in the high cockscomb, *Anoplarchus purpureus*, to environmental temperature differences.

In our own studies (Johnson *et al.*, 1971; Johnson, 1972), significant excesses of heterozygous individuals were observed for PGM and AGPD polymorphisms in deep water collections of Pacific ocean perch, *Sebastes alutus*, that were not observed in collections taken from shallower waters. There were also significant non-random associations of phenotypes observed for these two polymorphic systems at greater depths which were not found in shallower collections. These observations suggested that selective forces were acting upon these two loci at greater depths and pointed to the need for including other gene products of the individual, as well as external influences, when considering components of natural selection at a particular locus. This is one instance where selective forces may be acting differently on different segments of the same breeding population (see also Williams *et al.*, 1973).

Our current studies include a detailed examination of the kinetics of the allelic forms of LDH in sockeye salmon described above. These studies were carried out by L. J. Guilbert during a 1-year post-doctoral appointment with our research program. The data—to be published in detail elsewhere—have indicated firstly, that differences in Michaelis constants (K_m 's) exist in different phenotypes of liver LDH in sockeye salmon at physiological temperatures*; and secondly, that forms of the enzyme appearing identical in electrophoresis have different K_m 's. The latter results suggest that "silent" alleles (see Boyer, 1972) reflected in substitutions of similarly charged amino acids may be present, altering functional properties of the enzymes without changing their electrophoretic mobilities. These investigations have proven to us the value of a

*A similar phenomenon has been described by Merritt (1972) for allelic LDH variants of the fat head minnow and related to a north-south cline observed in this species.

team approach to studies of complex biological problems, where complementary training and skills are emphasized in different members of the team.

Gaining a better understanding of the relationships between biochemical genetic variation and environmental factors is a major, although difficult, direction for intensive investigation during the present decade. The vast amount of descriptive data collected during the 1960's and early 1970's, that illustrate considerable biochemical genetic variation in diverse organisms, have provided an empirical basis for a change in thinking regarding the amount of genetic variation that can be tolerated in natural populations and the role of this variation in evolutionary processes. The far more complex task of demonstrating the biological significance of this variation lies ahead.

D. Fish Culture

The relating of biochemical genetic variants at single loci to production characteristics such as growth rate has not been notably successful to date (Robertson, 1972). This may be explained, in part, by the highly multi-genic nature of most production characteristics of economic importance and very likely by the complexities of gene-gene interactions where the product of a single gene may interact with a variety of mechanisms relating to production characteristics. The limited success of the use of biochemical genetic variation in breed improvement appears to be related to the limited present knowledge concerning the degree of interaction of this variation with components of the external or internal environment of the organism. As more knowledge accumulates concerning the nature of gene-environment and gene-gene interactions, it seems likely that this knowledge can be usefully applied to animal breeding programs, perhaps from the simultaneous consideration of data from many loci.

Biochemical genetic variation may find useful application in many areas of fish culture other than direct relationships to production characteristics, however. For example, one problem of concern to fish culturists is inbreeding. Relative degrees of inbreeding could be directly estimated by comparing average heterozygosities observed in different brood stocks of a given species. A routine registry of gene frequencies in artificially maintained stocks of widely cultured fish species, such as rainbow trout, is a feasible means of facilitating such extension of biochemical genetic methods to fish culture.

Degrees of genetic differences can be directly estimated from biochemical genetic data. Methods for comparing genetic similarity at the species level (discussed in a later section) can be applied to intraspecies variation as well. Knowledge of the nature of genetic differences among

stocks can be very useful when it is desirable to introduce additional genetic variation into a particular stock. As a rational basis for maximum extension of the gene pool, introductions could be made from stocks showing the greatest biochemical-genetic differences. Similarly, two stocks showing large biochemical-genetic differences could be reared separately and the F_1 progeny between them used as production fish in attempts to maximize heterotic effects.

Biochemical identification of the hatchery of origin, based on a limited number of samples, is a possibility that presently exists for rainbow trout (Utter and Hodgins, 1972). It is possible to breed towards unique homozygous combinations involving multiple loci where no strong environmental interactions have been detected. A unique genetic brand on all fish of a given hatchery could thereby be achieved in a few generations, and could be used in many situations where it is desirable to identify fish of that hatchery.

E. Hybrids

Some of the advantages offered by biochemical genetic methods for studies of intraspecific variation can be extended through studies of species hybrids because of the greater amount of genetic variation that exists between any two species than that which exists within either of them. More loci are available for studies of linkages if sufficient fertility exists between the F_1 generation and either of the parent species (Wheat *et al.*, 1973) (see also the discussion in the following section). Evidence can be obtained for identification of parent species in natural and artificial hybrids (Abramoff *et al.*, 1968; Aspinwall and Tsuyuki, 1968). If hybridization coupled with subsequent backcrossing and intercrossing has been part of a breeding program, the exotic portion of the genome can be estimated by biochemical-genetic data. Intraspecific differences in activation and control of allelic genes can be observed in hybrid individuals (Hitzeroth *et al.*, 1968; Whitt *et al.*, 1972). Evidence for heterosis has been described in hybrid sunfish (Whitt *et al.*, 1973). On several occasions it has been possible to determine whether or not certain individual salmonids suspected of being hybrids are indeed hybrids. We have also used hybrid data to examine questions of allelism and sub-unit composition of proteins that are monomorphic in individual species but which differ among them (Utter *et al.*, 1973).

F. Cytogenetic and Linkage Studies

Biochemical genetic data, in conjunction with classical cytogenetic techniques, have played a major role in the determination of both the existence and mechanisms of gene duplication. The role of gene duplication in

providing the material for vertebrate evolution has been discussed in detail by Ohno (1970b) who has postulated that gene duplication by means of autotetraploidy occurred in an ancestral salmonid species on the basis of DNA content, chromosomal characteristics, and the duplication of LDH loci (Ohno *et al.*, 1969; Ohno, 1970a). Subsequent reports of numerous duplicated biochemical genetic loci in salmonids have supported this hypothesis (Bailey *et al.*, 1970; Wilkins, 1972). A recent paper, however, using karyotypes and an LDH locus has reported the presence of non-deleterious trisomy in brook trout, *Salvelinus fontinalis*, and has suggested recurrent trisomy as a possible alternate means of gene duplication in salmonids (Davisson *et al.*, 1972).

Our own studies do not indicate as much gene duplication in salmonids as is generally accepted (Ohno, 1970a, Bailey *et al.*, 1970). We recently reported polymorphisms of six loci in rainbow trout of which only two demonstrated evidence of gene duplication (Utter and Hodgins, 1972).

Single locus variants represent a basic tool of the geneticist. The genome of an organism such as *Drosophila* has been extensively mapped through the use of external morphological traits controlled by single loci, and has provided information which is extremely useful when utilizing this species as an object of genetic research. This type of variation is largely absent in fish species and because of this there have been very few reports of linkage relationships in fish. Biochemical genetics, however, provides a number of single locus markers which makes the study of linkages possible. We have examined the linkage relationships of five biochemical loci—LDH, MDH, TO, AGPD, and IDH (Allendorf, unpublished data)—as a part of our investigations of rainbow trout. Each two-way segregation was examined with the exception of LDH-AGPD where a double-heterozygote parental fish was not available. In no instance did the observed two-way segregation differ significantly from that expected with independent assortment. Only in a single case (AGPD-IDH) did recombination approach a non-random rate. However, Wheat *et al.* (1973) examined possible linkage relationships among six loci which varied in hybrid sunfish and found linkage between 6PGD and AGPD loci (recombination frequencies 15%–22%).

Because of the large number of potential linkage groups in rainbow trout— $2N = 60$ (Wright, 1955)—the chances of finding linkage between any two randomly chosen loci is small. Potentially more promising areas for detecting unusual two-way segregation are duplicated loci. Linkage between duplicated loci is more likely than between two randomly chosen loci because of the nature of gene duplication mechanisms. If duplication occurs through some form of tandem duplication these loci will lie on the

same chromosome. If duplication occurs through tetraploidy or tetrasomy, the expected fusion of homologous chromosomes (Ohno *et al.*, 1969) would result in linkage between duplicated genes. Wheat *et al.* (1973) found no significant deviation from random segregation between duplicated MDH loci in sunfish hybrids. Morrison (1970) however, did discover non-random segregation of two LDH loci in brook trout x lake trout, *Salvelinus namaycush*, hybrids. There are additional instances of gene duplication in salmonids where linkages between duplicated loci could be determined (e.g., MDH-A and MDH-B loci). One of our future objectives is to discover variants at these loci so that the linkage relationships can be explored.

VI. Variation at the species level

An extension of studies of allelic protein variation within a species is a comparison of the total amount of such variation that exists in a species relative to the total number of loci that can be reliably detected. If it is assumed that data from a limited number of loci coding for proteins can be extrapolated to the entire genome (Lewontin and Hubby, 1966), such data can be used to estimate the proportion of polymorphic loci and average individual heterozygosity in a species. These estimates can provide a useful basis for comparison among species.

We have estimated the proportion of polymorphic loci and average individual heterozygosity in three scorpaenid and six salmonid species (Johnson *et al.*, 1973; Utter *et al.*, 1973). In all species but rainbow trout, our estimates have been considerably lower than the average values reported in a wide variety of plant and animal species (reviewed by Gottlieb, 1971). The higher values observed in rainbow trout may reflect the greater habitat diversity of this species relative to any of the closely related species of Pacific salmon. If extrapolation to the remainder of the genome is valid, the greater genetic variation of rainbow trout suggests that heritabilities for a given characteristic should be higher in rainbow trout than in Pacific salmon, a possibility of potential interest to fish culturalists.

Biochemical genetic methods have proven useful in examining relationships among related animal species ranging from *Drosophila* (Ayala *et al.*, 1970) to mammals (Johnson and Selander, 1971). This is not surprising because of the direct relationship between the protein and the gene. We have used criteria for allelism that are similar to those imposed for intra-species variations for comparing protein differences between closely related species, although it is more difficult to obtain direct evidence for allelism by breeding tests. Homology for a single locus has been assumed

for proteins differing among closely related species that are expressed as single bands. Differences of multiple banded protein patterns can be analyzed on the basis of two fixed loci if the banding patterns conform to those typically observed for intra-species variants (e.g., invariant 5-banded LDH patterns or 3-banded MDH patterns). There are occasional instances where multiple-banded protein patterns do not conform to known models of molecular interaction but the repeatability of such patterns strongly suggests that the variation observed is genetically based. In these instances two fixed loci have also been assumed and comparisons between species have been made by ratios obtained by dividing common bands by total bands (Utter *et al.*, 1973).

We have examined biochemical genetic variation among related species for two somewhat different objectives, firstly, to identify species from biochemical characteristics of unknown samples and secondly, to examine systematic relationships based on biochemical data. Rockfishes of the northeastern Pacific Ocean have been examined primarily for the first objective, although a considerably more detailed study of this group towards its second objective is in progress.

In a study of muscle proteins from 27 Pacific Ocean species of *Sebastes* (Johnson *et al.*, 1972), 14 different biochemical groups were identified, based on non-specific muscle protein patterns and five enzyme systems. No group had more than 4 species, and 10 species had unique biochemical profiles. These biochemical criteria have considerable practical potential in these species because of the economic importance of rockfishes and the fact that some species in different groups are morphologically indistinguishable to the non-expert. Accidental or intentional mislabeling can be minimized through routine testing of muscle samples obtained from commercial sources.

We have examined the phylogenetic relationships of the six species of Pacific salmon and two related trout species, based on biochemical genetic data. Interspecies comparison of muscle protein patterns (including enzymes) were made, based on related proteins of eight loci and more complex protein patterns assumed to reflect four additional loci (Utter *et al.*, 1973). A dendrogram (Fig. 2), constructed from indices of similarity which reflected pairwise interspecies protein differences, separated the species into two major groups; one group contained the two trout species which were paired closely and, more distantly, the masu salmon, *O. masu*, while the other group contained the remaining five species of salmon. The data generally support classifications of this group of fishes, based on other criteria (reviewed by Behnke, 1965 and 1968), and indicate that the masu salmon is phylogenetically closer to the two trout species than to the other species of Pacific salmon.

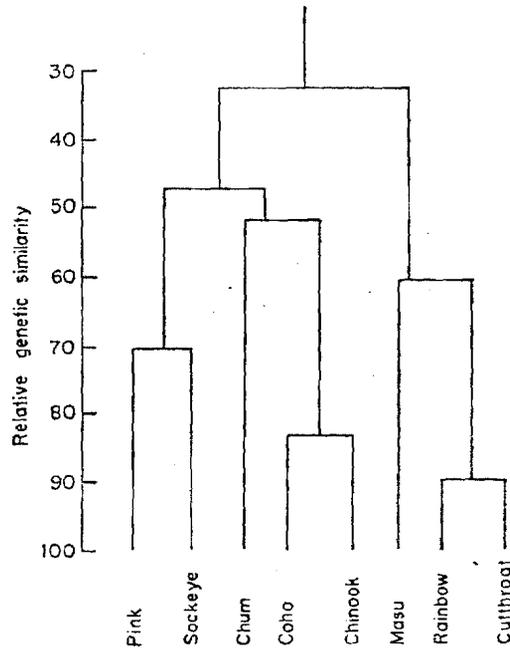


FIG. 2. Genetic relationships of eight salmonid species based on biochemical genetic data from 12 loci (from Utter *et al.*, 1973).

It is of interest to examine the data from which Fig. 2 was drawn from the point of view of species identification as a practical exercise because of the considerable potential for commercial mislabeling of salmonid flesh. Figure 3 is a biochemical key for identifying these species from samples of muscle tissue.

The genetic potential of electrophoretic criteria becomes less and less applicable for systematic comparisons between organisms as the taxonomic differences rise beyond the species level. Even electrophoretic identity within a species does not necessarily mean genetic identity, but merely that there is no detectable difference. (The K_m data for sockeye salmon LDH certainly suggests a genetic difference for electrophoretically identical isozymes). It has been estimated that only 40% of the amino acid substitutions can be electrophoretically detected on the basis of charge differences (Nei, 1971). It seems, because of the time elapsed between common ancestry, that many electrophoretic identities of analogous proteins beyond the family level may be merely fortuitous. On the other hand, immunological methods—which have been of very limited use to us

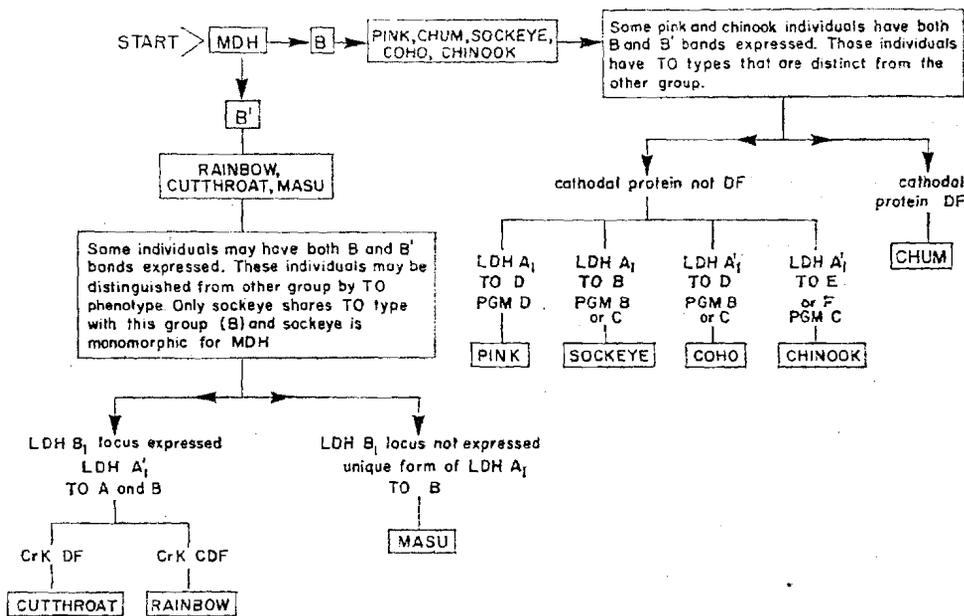


FIG. 3. A key for identifying six species of Pacific salmon and two related trout species based on genetic variations of skeletal muscle proteins. See Utter *et al.*, 1973 for allelic designations.

for studies of genetic variations within species—appear to have greater potential for comparative studies as the taxonomic distance increases (Gorman *et al.*, 1971).

VII. Summary

We have reviewed potentialities and limitations of biochemical genetic studies of fishes using electrophoretic methods. These methods can be usefully applied in numerous areas of fisheries research by virtue of their direct reflection of genetic differences and their ease of application.

The principle conclusions presented were:

(1) The potential of biochemical genetic data for use in identifying fish populations varied considerably among species. This ranged from clear separation of populations based on significant differences at many loci to no detectable differences over a broad geographic range in spite of the existence of numerous polymorphic loci.

(2) Studies of gene-environment interactions appear to be a difficult but rewarding field for the application of biochemical genetic methods.

Variations in catalytic activities of genetic variants of certain enzymes have been observed. These have occurred in some forms of enzymes that are electrophoretically identical as well as in electrophoretically distinct forms.

(3) Biochemical genetic data may presently be applicable to aspects of fish culture that include detection of inbreeding, brood stock identification and registration, and estimations of genetic differences among stocks. However, useful associations between genetically related proteins and production characteristics have not yet been found.

(4) Hybrids between species are readily detected by biochemical genetic methods.

(5) Proteins that vary either among or within species are useful for studying genetic linkages and ancestral gene duplication.

(6) Protein differences among species can be used for estimating relationships and for identification of species of tissues of questionable identity.

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