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DNA REASSOCIATION STUDIES IN FOUR SPECIES OF SALMONIDS

by

Ann Frances Hanham

B.Sc., University of Toronto, 1976

A THESIS SUBMITTED IN PARTIAL FULFILLMENT

OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in the Department

of

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DNA reassociation studies in four species of salmonids

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Abstract;

DNA reassociation kinetics were examined in three species of salmon and one of trout. Reassociation kinetic studies were carried out by hydroxyapatite chromatography with short (0.30-0.45 kb) DNA fragments from steelhead trout, chum, coho and sockeye salmon. At least 60% of the reassociated fragments had some portion of their length in repeated sequences. Computerized analyses of reassociation rates demonstrated that these repeated sequences range in reiteration frequency from 30 to 9×10^4 times per genome. A highly repeated or foldback component comprised about 10% of the DNA. Single copy component rates were found to be consistent with the estimated genome size of these species.

The interspersion of repetitive sequences with single copy sequences was examined in chum salmon. Long DNA fragments, 3.2 kb in length, were observed to have an accelerated rate of reaction and reduced hyperchromicity at low Cot values, typical of a "short period" or "Xenopus" interspersion pattern. Digestion with a single strand specific nuclease produced two characteristic fragment lengths of repeated sequences. The first of these was 0.35 kb in length with an average depression in T_m of 13°C, and the second was greater than 2.0 kb in length with a near native melting temperature.

Single copy sequence homology studies with ^{125}I-chum DNA demonstrated that approximately 100% of the reactive chum single copy sequences are also found in trout, 91% in coho, and 73% in sockeye salmon. Thermal elution of these heterologous reassociated fragments from hydroxyapatite exhibited a 2°C depression in T_m from that measured in a homologous reaction. This would indicate that approximately 1% single copy divergence per million years had occurred.

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I would like to thank my senior supervisor, Dr. M. J. Smith, for his help in this project. Appreciation is also extended to members of my committee, Dr. D. L. Baillie, and Dr. C. L. Kemp, for their interest and support. I would also like to thank Dr. R. J. Britten for providing S1 nuclease enzyme, Dr. S. Hayashi for preparation of the ^{125}I-labelled DNA, K. Gibson for the *E. coli* DNA reassociation curve, and V. Martin for translating pertinent journals from Russian. I would also like to thank Dr. B. Hartwick and the Queen Charlotte Fisheries for supplying fish gonad tissue. Special thanks to my husband Scott, for his support and aid.

Glossary of Special Abbreviations;

ATP	adenosine triphosphate
Cot	concentration (M) x time (s)
cpm	counts per minute
Ci	Curie (3.7×10^{10} dps)
dps	decays per second
g	gram
G	gravitational force
H	hyperchromicity
HAP	hydroxyapatite
^{125}I	isotope of iodine
K_{av}	partition coefficient
kb	kilobase, equal to 1000 nucleotide base pairs
M	molar
mCi	milliCurie
mg	milligram
µg	microgram
ml	millilitres
ul	microlitres
RMS	root mean square
s	second
T_m	thermal denaturation temperature

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Introduction:

Deoxyribonucleic acid (DNA) is the genetic material responsible for all structures and functions distinctive to a species. Characteristics so encoded are inherited from generation to generation. Recently it has become possible to perform comparative studies of taxa through the analysis of sequence homology in genomic DNA. Studies pioneering in this field include the Schildkraut et al., 1961, paper on bacterial homologies, McLaren and Walker's 1968 study of rodents, the Davidson et al., 1975a review of metazoa, and the Goldberg et al., 1975, study of marine invertebrates.

Surprisingly, no in depth study of DNA sequence organization or content has been undertaken in the Pacific species of salmonids; a recently evolved group which inhabit the north Pacific coast. DNA studies of the salmonids have been undertaken in a variety of ways, but none have examined sequence evolution in this manner. Simon, 1963, has made a detailed analysis of chromosome morphology in Pacific salmon, and Utter et al., 1973, examined several species of salmon at twelve loci for genetic heterozygosity. Gharrett et al., 1977, performed reassociation kinetic studies on several species of fish, but did not examine the sequence organization pattern or content in these species.

Vladychenskaya et al., 1977, examined the sequence interspersion pattern in another salmonid genus, Coregonus.

DNA sequence hybridization studies provide valuable insight into the evolution and phylogeny of the salmonids, since the genomic DNA should reflect the history of the organism. To date, emphasis on the classification of these organisms has been primarily restricted to their morphology and behavioural patterns. The various criteria developed to classify salmonids have resulted in diverse interpretations of the relationships within this group (see Hoar's 1976 review of salmonid evolution).

In molecular studies of DNA sequence homology, there is a logical progression of experiments which is necessary for meaningful interpretation of subsequent results. First of all, reassociation kinetics is determined to ascertain the number, frequency and total amount of repetitive and single copy DNA. Secondly, the arrangement or interspersion of repetitive sequences with single copy sequences is examined. Finally, with this information, one is able to isolate the single copy fraction and perform single copy sequence homology studies between species.

In 1968, Britten and Kohne demonstrated that a significant proportion of the eukaryotic genome contains sequences which are

present many more times than unique or single copy DNA. When randomly sheared fragments were denatured and allowed to reassociate, the reassociation was shown to follow approximate second order reaction kinetics, when monitored by hydroxyapatite chromatography. Hence the rate of reassociation was found to be dependent on the concentration of complementary strands in solution (Marmur et al., 1963; Wetmur and Davidson, 1968). Since rate of reaction is dependent on sequence concentration, repetitive sequences are observed to react at rates proportional to their reiteration frequency. Under standard conditions of reassociation (see Appendix 1) it becomes possible to make estimates of the number and fraction size of a series of frequency components contained within the genome (Britten and Kohne, 1968; Wetmur and Davidson, 1968). Furthermore, the rate of reassociation of non-repeating fragments may be expected to be inversely proportional to the genome size (Britten and Kohne, 1968). It is therefore possible to estimate genome size based on the kinetics of reassociating fragments, as well as the amount of unique sequence present. To this end, my work was designed to determine the reassociation kinetics of representative salmonids. The amount and repetition frequency of reiterated DNA, as well as amount of single copy DNA, was determined in Oncorhynchus keta (chum salmon), O. kisutch (coho salmon), O. nerka (sockeye salmon), and Salmo gairdneri (steelhead trout).

Recent information has demonstrated that short repetitive sequences (those present more than once per genome) and longer single copy sequences (those present only once per genome), are interspersed throughout the entire genome of most eukaryotes (Davidson et al., 1975b). If DNA is randomly sheared with respect to sequence, the resulting fragments may be considered to be in one of three categories:

a) total single copy sequence content

b) total repeated sequence content

c) repeat sequences adjacent to single copy sequences.

In longer fragment lengths, one would expect a greater proportion in category c), namely, repeat sequences adjacent to single copy sequences. Early in reassociation reactions these longer fragments may therefore be viewed as having some portion of their length as duplex structure (the reassociated repetitive sequences), and, some portion of their length as single strand regions or "tails" (the less repeated unreassociated sequences).

Analyses of this sort, have shown two characteristic interspersion patterns of repetitive and single copy sequences in eukaryotes. The first pattern known as the short period or "Xenopus" pattern, may be characterized by having a large fraction of the repetitive DNA in segments 0.3 kb long interspersed with single copy sequences approximately 1.0-2.5 kb long. In this pattern a substantial fraction of the DNA is also found in long repetitive segments of greater than 3.5 kb in

length (Davidson et al., 1973; Goldberg et al., 1975). Generally this pattern is associated with organisms having a large genome size, but has also been observed in the slime mold, Dictyostelium, which has a genome size of 0.1 pg (Firtel and Kindle, 1975).

A second pattern observed in such organisms as Drosophila melanogaster and Apis mellifera may be referred to as the long period or "Drosophila" interspersion pattern. This pattern is frequently observed in organisms with smaller genome sizes (Manning et al., 1975; Crain et al., 1976; Wells et al., 1976). An exception to this may occur in chicken DNA which has a 1.0 pg genome size (Arthur and Straus, 1978). This pattern is characterized by the presence of long repetitive sequences (5.6 kb) interspersed with even longer unique DNA sequences (13.0 kb in length). It is not yet known what the significance of this interspersion of repetitive and single copy DNA might be, but the predominance of such patterns suggests a possible regulative role (Britten and Davidson, 1969; Davidson et al., 1975). The second major object of this research is thus to determine the sequence interspersion pattern in a representative salmon. Chum salmon (O. keta) was chosen as the probe species.

Two independent techniques have been employed in this paper to determine the amount and interspersion pattern of such repetitive and single copy sequences present in renatured

fragments. The first involves hyperchromicity measurements of randomly sheared reassociated fragments. Since hyperchromicity is very nearly proportional to the fraction of nucleotides paired, thermal stability studies can provide information about the amount of reassociation which has occurred (Graham et al., 1974). The second technique employs an enzyme which selectively digests away the single strand regions of a reassociated fragment. Single strand specific nucleases such as S1, therefore provide information about the actual fraction of fragment which is in duplex (Ando, 1966; Vogt, 1973). These phenomena, together, enable one to ascertain both the amount and length of repetitive and single copy DNA.

Once careful analysis of sequence representation and organization is complete, it becomes possible to study molecular hybridization of various sequences, between species. In this study, single copy sequences of chum salmon were hybridized with trout, coho, and sockeye DNA. To accomplish this, chum salmon was thermally denatured and allowed to react to a Cot value at which only repetitive sequences had reassociated. Single copy sequences were then eluted from hydroxyapatite and radioactively labelled with ^{125}I using a modified Commerford technique (see below). This probe was allowed to reassociate with a large scale excess of DNA from the other species. Reassociation of fragments with members from another species was monitored by hydroxyapatite chromatography. In this way I was able to

compare the extent of single copy sequence conservation among four salmonid species. Previous studies of this nature have been performed on sea urchins (Angerer et al., 1976; Harpold and Craig, 1977; Harpold and Craig, 1978), plants (Stein and Thompson, 1977), primates (rev. in Kohne, 1970), rodents (Rice, 1974), and birds (Eden et al., 1978).

The purpose of this study was therefore to examine DNA reassociation kinetics and the sequence interspersion pattern in a representative species, the chum salmon. These findings provided information about the number, frequency and overall amount of repetitive sequences present in the chum genome. Reassociation kinetics could then be compared with those of steelhead trout, coho and sockeye salmon. Knowledge of the fraction and arrangement of chum single copy sequences allowed the preparation of a radioactively labelled probe which could then be used in determining single copy sequence homologies with trout, coho, and sockeye DNA.

Materials and Methods:

DNA isolation;

The DNA used in this study was extracted from the testes of Oncorhynchus keta (chum salmon), O. kisutch (coho salmon), O. nerka (sockeye salmon), Salmo gairdneri (steelhead trout), and Hexagrammos decagrammus (kelp greenling). Salmonid testes were obtained from and identified by Queen Charlotte Fisheries Ltd. and kelp greenling testes were the gift of Dr. B. Hartwick of the Bamfield Marine Station, Vancouver Island. All tissue was refrigerated for 24 hours post-dissection and stored at -70°C. Reagent grade chemicals were used in this study and all solutions were routinely filtered with 45μ Millipore Filters.

Frozen ripe testes were homogenized in a Waring blender in 5.0×10^{-2} M Tris(hydroxymethyl)aminomethane (Tris), 2.5×10^{-2} M KCl, 1.0×10^{-3} M $MgCl_2$, pH 7.4, for approximately 1 minute. The homogenate was passed through a double layer of gauze, and centrifuged at 4×10^3 G for 10 minutes at 4°C. The pellet was resuspended in one half volume of this buffer and again centrifuged at 4×10^3 G for 10 minutes (P. Candido, pers. comm.). The final pellet consisted of sperm heads and spermatocyte nuclei as determined by phase microscopy. The DNA was extracted by a modified Marmur method which has been

described (Britten et al., 1974). The pellet was suspended in 0.1 M NaCl, 0.05 M Tris, 0.025 M ethylenediamine tetraacetic acid (EDTA), pH 8.0. Pronase B (Cal Biochem) was added to 250 $\mu g\ ml^{-1}$ and sodium dodecyl sulfate (SDS) to 1% (w/v). The suspension became clear and viscous. After Pronase treatment at 37°C for 2 hours, sodium perchlorate was added to 1.0 M, and the solution was extracted by shaking for 20 minutes with a mixture of one part freshly distilled, buffer equilibrated, phenol and four parts chloroform which was 4% (v/v) isoamyl alcohol. The extraction mixture was centrifuged at 4×10^3 G for 10 minutes and the aqueous phase was twice resubmitted to extraction with an equal volume of chloroform:isoamyl alcohol (24:1, v/v). The resulting mixture was overlayed with 2 volumes of 95% ethanol and the DNA precipitate was spooled from the interface on glass rods. This procedure was repeated at least twice. The DNA was dissolved in 0.01 M EDTA at pH 7.0, and dialyzed into 0.12 M phosphate buffer (equimolar sodium mono- and dibasic phosphate, pH 6.8). This procedure typically yields greater than 2 mg of DNA per gram of gravid gonad tissue.

DNA characterization;

Ultraviolet absorption spectra of DNA preparations were measured from 220 to 320 nm, using a Gilford Model 250 UV Spectrophotometer. Only DNA with 260/230 ratios of > 2.0 and

260/280 ratios of > 1.8 (see Figure 1) was used for experimentation.

DNA which met this criteria was thermally denatured in a Gilford Spectrophotometer with an Analog Multiplexor, Thermalprogrammer, and Thermal Cuvette. The resulting optical measurements were corrected for thermal expansion of water prior to calculation of hyperchromicity (Mandel and Marmur, 1968). Hyperchromicity was calculated as defined by Britten et al., 1974, where:

$$H = \frac{A_{260}(100^{o}C) - A_{260}(55^{o}C)}{A_{260}(100^{o}C)} \qquad \text{(equation 1)}$$

where H stands for hyperchromicity and A_{260}, the absorbance at 260 nm.

The T_m or temperature (^{o}C) at one half the maximum hyperchromicity was also determined (Figure 2) in 0.12 M phosphate buffer (0.18 M sodium concentration). The guanine+cytosine (GC) content may be quantified from the T_m measurement by the relationship:

$$GC = 2.44(T_m - 81.5 - 16.6\log_e M) \qquad \text{(equation 2)}$$

where GC is the guanine+cytosine content expressed as a mole percentage, T_m is the temperature at half maximum

hyperchromicity and M, the solvent cation concentration. (Mandel and Marmur, 1968).

DNA shearing and fragment length determination;

DNA was sheared to one of two fragment lengths, 0.3 kb or 3.2 kb, in a Virtis Hi-Speed homogenizer as described (Britten, et al., 1974). The shorter fragment lengths were attained by shearing the DNA in 67% (v/v) gycerol, 33%(v/v) 0.06 M sodium acetate (NaAc) pH 7.2, for 30 minutes at 5 x 10^4 rpm in a dry-ice ethanol bath. Longer fragment lengths were produced by shearing in a 0.03 M NaAc (pH 7.2) solution for 20 minutes at 10^4 rpm in an ice water bath. The DNA was passed over a Chelex 1 (Bio-Rad, sodium form) column and the eluant was increased in salt concentration to 0.3 M. DNA was precipitated by the addition of two volumes of 95% ethanol and storage at -20°C for a minimum of 3 hours. The precipitate was centrifuged at 10^4 G, air dried, and taken up in the appropriate buffer.

The single strand length of the sheared DNA was determined by denaturing alkaline sucrose sedimentation (Noll, 1967). The sheared DNA was denatured for 3 minutes in 0.1 N NaOH at 60°C with DNA standards of known fragment length, and sedimented through exponential alkaline (0.1 N NaOH) sucrose gradients. These gradients were prepared using 14.2 ml Beckman polyallomer

tubes and a 9.6 ml mix volume of 7.5% (w/v) alkaline sucrose. This was mixed with a 32.6% (w/v) alkaline sucrose solution to produce gradients of 7.5% (w/v) to 25% (w/v) alkaline sucrose. The gradients were centrifuged at 5×10^5 G for approximately 24 hours at 20°C in a Beckman L2-65B ultracentrifuge. The position of the DNA fragments in the gradients was determined using an ISCO UA-5 absorbance monitor with a UV Type 6 optical unit attachment. The gradients were chased through this flow cell with a 60% sucrose solution and collected in 0.4 ml fractions. In the case of ^{125}iodine labelled DNA (see below), fractions were analyzed for gamma radiation with a Nuclear Chicago gamma counter. Estimates of the molecular weight were calculated by comparison of the sedimentation values and molecular weight of the internal standards according to the Studier (1965) relationship:

$$\left(\frac{S_u}{S_{std}}\right)^{0.4} = \left(\frac{M_u}{M_{std}}\right) \qquad \text{(equation 3)}$$

where S_{std} and S_u represent the $S_{20,w}$ coefficients of standard and unknown DNA, and M, the molecular weight of these fragments.

DNA reassociation;

The sheared DNA was dialyzed into either 0.12 M or 0.41 M phosphate buffer, thermally denatured at 100°C for 3 minutes, and reassociated at 60°C or 67°C respectively. The reassociation rate acceleration factor for the higher salt concentration was determined by the method of Britten et al., 1974, and all data are expressed in terms of equivalent Cot (product of moles per liter times seconds; M s). A summary of criteria for the reassociation of DNA may be found in Appendix 1. For convenience, 120-150 µg of DNA were used for each sample. At selected Cot values, the reassociation was terminated by quenching the reaction vials in a dry ice:acetone bath. The fraction of the DNA fragments which had reassociated at each Cot was determined by hydroxyapatite (Bio-Rad, DNA grade Bio-gel HTP) chromatography in 0.12 M phosphate buffer, 0.06% sodium dodecyl sulphate (SDS), at 60°C. Reassociated DNA fragments were diluted in 3 ml of 0.12 M phosphate buffer and loaded on the columns. This volume was routinely used because it diluted the DNA fragments to a concentration easily read in the spectrophotometer and was also convenient for the elution of fractions. Hydroxyapatite will bind DNA fragments which contain some portion of their length as duplex or double strand DNA. Double strand fragments were eluted from the hydroxyapatite columns with 0.5 M phosphate buffer or by elevation of the column temperature to 99°C. The amount of DNA in these

fractions was measured in the Gilford Spectrophotometer at 260 nm. Correction for light scattering caused by hydroxyapatite in solution was made by subtracting the net absorbance at 320 nm from the 260 nm value.

Analysis of reassociation kinetics;

When the degree of reassociation is plotted versus log equivalent Cot, ranges of sequence frequency can be observed in eukaryotic organisms. The reassociation of randomly sheared DNA fragments may therefore be analyzed as the sum of a number of apparent second order reactions (Britten and Kohne, 1968):

$$C/C_o = f_1(1+K_1Cot)^{-1} + f_2(1+K_2Cot)^{-1} \ldots + f_n(1+K_nCot)^{-1} \qquad \text{(equation 4)}$$

Where C represents the concentration of double strand DNA and C_o the initial concentration of single strand DNA, K the component rate constant, and f, the component fraction size.

The number, size as a fraction of the total DNA, and second order reassociation rate of these frequency components was determined using a computerized least squares fitting procedure which has been described in detail (Pearson et al., 1977). The computer programme for this procedure allows the operator the

choice of number and size of frequency components and permits these parameters to be varied or fixed in value. Data from the hydroxyapatite chromatographic separation of reassociated fragments could therefore be analyzed by allowing the fitting programme to float freely (hereafter referred to as a "free fit"), or by holding selected parameters constant. For example, the single copy rate constant in a three component fit could be fixed in value and its effect on the component fraction size, determined. The resulting solutions were compared on the basis of their root mean square (RMS) deviation. Component fits which did not differ more than 10% in their RMS were taken to be equally valid (Pearson et al., 1977, see also Appendix 2). For computerized fits of reassociation data, see Figures 3 through 6.

Interspersion pattern measurements;

Reassociated fragments were thermally denatured to determine their total hyperchromicity. Since hyperchromicity is directly related to the number of bases which are in duplex (Graham et al., 1974), these measurements allow one to compare the degree of reassociation in fragments of different lengths. Chum DNA fragments 0.3 kb in length were reacted to Cot 1, 10, and 100, and passed over hydroxyapatite to isolate duplex containing fractions. Double strand DNA was eluted from the columns with 0.5 M phosphate buffer and dialyzed into 0.12 M phosphate buffer

overnight. These fractions were thermally denatured in the Gilford spectrophotometer. This procedure could not be used for 3.2 kb fragments. Instead, long fragments were allowed to react to Cot 1, 10, and 100, and divided into two fractions. The first, was run over a hydroxyapatite column to determine the amount in duplex, while the remaining fraction was thermally denatured directly in the spectrophotometer. The correction of Goldberg et al., 1975, allowed me to make a rough estimate of the overall hyperchromicity of these fractions, by taking into consideration the fraction of fragments containing double strand material. Values for the calculated hyperchromicities of fragments reacted to Cot 1, 10, and 100 are listed in Table 2.

To determine the length of DNA duplex in a longer fragment length. The reassociation product was treated with the single strand specific nuclease, S1, from Aspergillus oryzae. This enzyme, under the proper reaction conditions, selectively digests single strand DNA (Ando, 1966; Vogt, 1973). The S1 nuclease preparation used in this work was the kind gift of Dr. R. J. Britten and has been extensively characterized (Britten et al., 1976).

The enzyme activity was found to be the same as that reported by Britten et al., 1976 (M.J. Smith, pers. comm.). An enzyme to substrate ratio of 4000 μL of enzyme preparation per mg of DNA substrate per min of digestion at 37°C (4000 μL min mg^{-1})

was used. Chum DNA, 3.2 kb in length, was denatured and reacted to Cot values of 1, 10, and 100, in 0.3 M NaCl, .01 M Piperazinè-N-N'-bis[2-ethane] Sulfonic Acid (Pipes) buffer (Sigma Co.) pH 6.8. The reaction rate and temperature were adjusted for this salt value (Britten et al., 1974). At the selected Cot values the reassociation reactions were stopped by freezing in a dry ice:acetone bath. This was made to pH 4.3 and 1 x 10^{-3} M $ZnSO_4$ with an equal volume of acetic acid and $ZnSO_4$. Mercaptoethanol was added to 5.0 x 10^{-3} M and the appropriate amount of enzyme preparation was added per mg of DNA. This mixture was incubated at 37°C for 45 minutes at which point the enzyme digestion was terminated by the addition of 1.0 M phosphate buffer to 0.1 M. Simultaneously the reaction mixture was chilled in an ice water bath. The enzyme digestion products were dialyzed into 0.12 M phosphate buffer and passed over a hydroxyapatite column at 60°C and the fraction of DNA in duplex was noted. The DNA was eluted from the hydroxyapatite in the duplex form with 0.5 M phosphate buffer. The thermal denaturation temperatures and hyperchromicity of this nuclease resistant DNA were measured. In all cases, enzymic digestion of single strand material was complete as judged by the near native hyperchromicity of enzyme resistant DNA products.

Aliquots of the nuclease resistant DNA were passed over a Sepharose CL-2B (Sigma) column to determine the length of DNA duplex, as illustrated in Figure 8. Sepharose CL-2B

fractionates DNA in the size range 2.0 to 0.2 kb. A linear relationship exists for the partition coefficient (K_{av}) of the DNA fragments between gel and eluant phases, and the log of the DNA fragment size in kb (M.J. Smith, pers. comm.). Each Sepharose column used in this study was calibrated with DNA standards whose fragment length was determined by alkaline sucrose sedimentation. The elution position of calf thymus, sea urchin, starfish, and salmon DNA of similar fragment length have been found to be reproducible within $\pm .05$ K_{av} units. The exclusion volume was noted for native DNA fragments 3.0 kb or greater in length and the column volume was marked with ATP or tritiated water.

Tracer preparation;

Single copy or unique sequence DNA was isolated by reacting chum DNA, 0.46 kb in length, to Cot 3000, and passing the reaction mixture over a hydroxyapatite column at 60°C. DNA fragments which had not reassociated were collected from this column by elution with 0.12 M phosphate buffer. This material was dialyzed into 0.3 M Na acetate, pH 6.8 at 4°C. The DNA was precipitated at -20°C by the addition of two volumes of ethanol. This was followed by centrifugation at 1.6×10^4 G for 30 minutes, and the pellet was air dried, dissolved in 0.05 ml of distilled water to give a final concentration of 1.0 mg/ml, and denatured by heating at 100°C for one minute. The DNA was iodinated using a modified Commerford technique (Commerford et al., 1971)

by Dr. S. Hayashi. Ten micrograms of denatured DNA were iodinated for 30 minutes at 70°C in 0.14 M Na acetate, pH 4.8, 0.018 M $ThCl_3$, 0.022 M NaI, and 0.5 mCi ^{125}I (Amersham Corp., carrier-free, 531 mCi ml^{-1}). The iodination reaction was terminated by the addition of sodium phosphate buffer, pH 7.0, to a concentration of 0.03 M and freshly prepared Na_2SO_3 to 2.0×10^{-3} M. The mixture was passed over a 0.3 ml hydroxyapatite column, and free ^{125}I was eluted from the mixture with 0.03 M phosphate buffer. DNA which had retained the ^{125}I was eluted from the column with 0.5 M phosphate buffer. Approximately 82% of the recovered ^{125}I was incorporated into the DNA, which had a specific activity of 1.26×10^7 cpm μg^{-1} when assayed in a Nuclear Chicago gamma counter, at 50% counting efficiency.

Hybridization measurements;

Unlabelled native chum, trout, coho, and sockeye DNA, were individually added to the ^{125}I Chum DNA in a greater than 10^4-fold single copy sequence excess. For example, in the case of chum salmon, in which the unique sequence component equals 30% of the 0.3 kb fragments, 30 ug of driver DNA were used for every ng of tracer. The iodinated or tracer DNA, had a mean length of 0.28 kb, as determined by alkaline sucrose gradients. Driver DNA consisted of chum, trout, coho, sockeye, and kelp greenling DNA with average fragment lengths of 0.50, 0.46, 0.46, 0.45, and 0.50 kb, respectively. The hybridization mixtures were

denatured and allowed to reassociate in 1 M NaCl, 0.01 M phosphate buffer, and 1 x 10^{-4} M EDTA, at 70°C (Angerer et al., 1976). This buffer causes a 25-fold reaction rate increase, allowing the reaction mixture to attain the required long Cot values. In a separate experiment, chum tracer DNA was reacted in the absence of any driver DNA to determine the amount of self reaction under these experimental conditions. The fraction of ^{125}I DNA in duplex was measured by hydroxyapitite chromatography and radioactive counting in a Nuclear Chicago gamma counter. These data were analyzed with the least squares fitting programme of Pearson et al., 1977, and are shown in Figure 9.

Thermal denaturation profiles were prepared to determine the degree of strand duplex formation, at completion of these reactions. This was accomplished by raising the temperature of the hydroxyapatite columns in 5°C increments and eluting the DNA with 0.12 M phosphate buffer (Figures 10, 11, and 12). The labelled DNA was prepared and experiments completed in a 30 day period. There was no significant deterioration of the iodinated product in this period. This was verified by the ^{125}I homologous Cot points reacted to Cot values greater than 100,000, which were repeated at the conclusion of the experimental period, and the alkaline sucrose sedimentation of both tracer and driver DNA.

Results:

Reassociation kinetics of salmonid DNA;

Computerized least squares analysis provides a number of equally valid solutions for the size and repetition frequency of sequence components within the salmonid genome. In Figure 3, the reassociation kinetics of chum salmon DNA at a fragment length of 0.3 kb is plotted. The fraction of the genome which is found in each frequency component and the reassociation rates are listed in Table 1. In a three component analysis, in which no constraints are placed on the fitting procedure, 65% of the 0.3 kb fragments react at a repetitive rate. In this solution, a middle repetitive fraction accounts for approximately 20% of the DNA and is reiterated on the order of 3×10^3 times. A slow repetitive component accounts for 40% and is reiterated on the order of 30 times. The remaining 10% reacts at a very fast rate which cannot be measured accurately with our existing data. The single copy or unique component comprises about 30% of the DNA and has a rate constant of 3.4×10^{-4} $M^{-1}s^{-1}$. When the single copy component rate is fixed at a value consistent with a 3.2 pg genome size, the rate and fraction size of these components does not change considerably (Table 1).

Reassociation curves plotted according to the same least squares fitting procedure are represented for trout DNA (Figure 4), coho DNA (Figure 5), and sockeye DNA (Figure 6). At a fragment length of 0.45 kb, steelhead trout DNA has approximately 55% of its fragments as repetitive DNA. Again, these repetitive components can be analyzed as two groups; the middle repeat component which accounts for 30% of the DNA and is reiterated on the order of 3×10^3 times, and the slow repetitive component which makes up 20%, and is observed to have a repetition frequency of about 100. The foldback or highly repeated component is found to comprise about 5% of the total DNA. The single copy component rate is analyzed to be 1.1×10^{-3} $M^{-1}s^{-1}$ which is consistent with the smaller genome size of 2.5 pg (Louie and Dixon, 1972).

Analogous results are obtained for coho and sockeye reassociated fragments. Coho DNA sheared to a fragment length of 0.45 kb, demonstrates 60% of its DNA to be repetitive, with a middle component repeated on the order of 9×10^4 times, and a slower component repeated about 800 times (see Figure 5 and Table 1). The foldback fraction accounts for about 10% of the total DNA. The single copy component comprised 40% of the DNA and its reassociation rate is measured to be 4.7×10^{-4} $M^{-1}s^{-1}$. These calculated rates are consistent with coho's genome size of 3.0 pg (Hinegardner and Rosen, 1972). Sockeye DNA, 0.46 kb in length, is found to be very similar with 60% of its DNA repetitive, and two repeated components; the middle component

which accounts for 15% of the DNA and is repeated approximately 2×10^4 times and the slow component, which is repeated 400 times and accounts for 38%. Again, the foldback fraction makes up about 10% of the total amount of DNA. The single copy rate constant is measured to be 1.1×10^{-3} $M^{-1}s^{-1}$ and the component accounts for 36% of the DNA (see Figure 6 and Table 1).

Chum DNA was further analyzed by reassociation kinetics to determine if repetitive and single copy sequences are interspersed. To accomplish this, longer DNA fragments were allowed to reanneal under the same experimental conditions. Reassociated chum DNA fragments sheared to a 3.2 kb length were assayed and plotted with the three component least squares fitting procedure. If short repeat sequences are interspersed with longer single copy sequences, one would expect a disproportionate increase in reassociation rate as compared to that measured for exclusively short fragment reassociation.

Free fit analysis of the reassociation of such fragment lengths demonstrates a single copy component rate of 4.35×10^{-2} $M^{-1}s^{-1}$ (Figure 3, Table 1). Using the Wetmur and Davidson (1968) correction for fragment length differences, one may calculate the expected single copy rate to be:

$$\sqrt{\frac{3.2}{0.3}} \times 3.4 \times 10^{-4} = 1.1 \times 10^{-3} \ M^{-1}s^{-1}$$

When the single copy rate is held constant at this rate, the RMS increases from 0.024 to 0.028. In Table 1, it is shown that only 5% of the total DNA reacts at such a rate. In fact, by Cot 0.004, more than 20% of the long DNA fragments are bound by hydroxyapatite. These accelerated values are consistent with the interspersion of repetitive and single copy sequences.

Hyperchromicity and thermal denaturation measurements;

As outlined in the Introduction, relative hyperchromicity can be used as an assay of the fraction of fragment length in duplex. Chum DNA 0.3 or 3.2 kb in length, was reacted to Cot 1, 10, and 100. 0.3 kb fragments at these Cot values were eluted over hydroxyapatite columns to isolate duplex containing fractions. These were thermally denatured and their hyperchromicity, measured. 3.2 kb fragments could not be separated into duplex containing fractions by elution with 0.5 M phosphate buffer on hydroxyapatite. It was therefore necessary to thermally denature the entire reassociation fraction and correct for any unreannealled fragments which may be present (Goldberg et al., 1975). Figure 7 illustrates the hyperchromicity of 0.3 kb fragments and estimated hyperchromicity of 3.2 kb fragments reacted to Cot 10 and 100. Both the 0.3 and 3.2 kb reassociated products had hyperchromicity measurements less than that of native DNA. In addition, it should be noted that 3.2 kb fragments demonstrated hyperchromicities significantly less than

0.3 kb fragments at the same Cot value. For example, Table 2 lists hyperchromicities of 23.0% and 11.9% for 0.3 and 3.2 kb fragments, respectively, reacted to a Cot value of 10.

The denaturation temperature, or T_m, of native chum DNA in 0.12 M phosphate buffer is 87.5°C. This corresponds to a 44% guanine+cytosine (GC) content (see Equation 2). At a fragment length of 0.3 kb the T_m was 85°C, or 2.5°C less than the T_m of longer fragment lengths. Duplex bearing 0.3 kb fragments at Cots of 10 and 100, have T_m's of 75.5°, and 77°C, respectively. The melting temperatures for long DNA fragments reacted to Cot values of 1, 10, and 100, are also depressed to values of 76°, 77°, and 78.5°C, respectively. Table 2 gives a complete list of these melting temperatures.

S1 nuclease digestion of repetitive fractions;

The Sepharose 2B elution profiles of enzyme resistant DNA duplex at Cots 1, 10, and 100 are illustrated in Figure 8. In each case there are clearly two size classes of enzyme resistant duplex (Table 3). At Cot 1, 12% of the 3.2 kb duplex bearing fragments are resistant to S1 nuclease. That is, an average 88% of the initial fragment length at this Cot value is free single strand DNA. With an increase in Cot value the fraction of fragment length in duplex increases to 21% at Cot 10 and to 44%

at Cot 100. Furthermore, at Cot 10, approximately 45% of the enzyme resistant duplex is found in fragments with an average length of 0.35 kb (Table 3, Figure 8). Although this material has a near native hyperchromicity of 30% (as opposed to 32.2%), showing completion of the enzymic digestion, the melting temperature is significantly decreased from 87.5°C to 73.5°C (Table 3). Similarly, at Cot 100, about 46% of the enzyme resistant DNA is approximately 0.35 kb in length with a subsequent depression in T_m from 87.5°C to 72.0°C (Table 3). The denaturation temperatures of Sepharose excluded, or long repetitive sequences, which result from nuclease digestion are identical to that of native DNA, in this case, 87.5°C (Table 3).

Hybridization of single copy sequences;

Single copy chum DNA was prepared by reassociating 0.50 kb fragments to a Cot value of 3000. DNA which had not reassociated with complementary strands, was eluted from hydroxyapatite and radioactively labelled with ^{125}I.

Alkaline sucrose gradients demonstrate the final length of the tracer DNA to be 0.28 kb. When reassociated with a 10^4-fold excess of homologous chum DNA, the total hybridization is observed to be 79±1.2% standard deviation (SD). Analysis,

using a least squares fitting procedure, demonstrates the presence of a single component with a rate constant of 2.6 x 10^{-4} $M^{-1}s^{-1}$ at an RMS value of 1.7 x 10^{-2}. To calculate the expected rate of hybridization of short tracer with a longer driver DNA, one must take into account the longer fragment length of the driver DNA (Chamberlain et al., 1978) according to the relationship:

$$K_T = K_S (L_T/L_D)^{1/2} \quad \text{(equation 5)}$$

where K_T = rate of tracer reaction when driven by excess DNA of fragment length, L_D

K_S = rate of tracer reaction when driven with DNA of an equal fragment length, i.e., L_T

L_T = tracer length

L_D = driver length

therefore, $K_T = .00034\ (280/500)^{1/2}$

$= 2.5 \times 10^{-4}\ M^{-1}s^{-1}$

When the same tracer was driven with a 10^4-fold sequence excess of DNA from other species (a heterologous reaction), the termination of reaction is found to differ. 80±1.0% SD of the iodinated single copy DNA from chum is observed to react to completion with trout driver, 73±1.5% SD with coho driver, and 57±1.6% SD with sockeye driver, DNA. These data are represented

in Figure 9, and also Table 4. The component rates are observed to be; 2.6×10^{-4}, 3.6×10^{-4} and 2.3×10^{-4} $M^{-1}s^{-1}$ for trout, coho and sockeye driven reactions respectively. To assess the self reaction of the tracer DNA, aliquots were incubated to maximum Cot values of 10. This would correspond to a Cot value of 10^5 in a driven reaction where a similar amount of tracer DNA was present. The degree of self reassociation is not observed to change and remains at approximately 6%. The amount of tracer DNA which is observed to react with kelp greenling DNA is no greater than that measured in the self reaction (6%). The fraction of reactive chum single copy DNA sequences which is homologous with trout, coho, and sockeye DNA, is 100%, 93%, and 73%, respectively.

To determine the number of bases which are mismatched in such a hybridization reaction, reassociated fragments were eluted over hydroxyapatite by increasing the temperature of the column in 5^oC increments. The thermal denaturation profiles for heterologous DNA reacted to completion are represented in Figures 10, 11, and 12. Iodinated single copy DNA reacted with trout, coho and sockeye DNA is observed to have a 2^oC depression in melting temperature from the T_m of the homologous ^{125}I chum reaction. All temperatures are considerably lower (by as much as 9^oC) than those measured for native double stranded DNA.

Discussion:

Reassociation Kinetics;

In 1968, Britten and Kohne introduced a procedure known as the "Cot method" for studying renaturation kinetics of thermally denatured DNA. Randomly sheared DNA was allowed to reassociate at constant temperature in standard salt solutions. The rate limiting step in renaturation was found to be the bimolecular reaction of complementary single stranded sequences of DNA (Wetmur and Davidson, 1968). This base pairing of complementary regions could therefore be described by second-order reaction kinetics and is given by the equation;

$$\frac{C}{C_o} = \frac{1}{1 + KCot} \qquad \text{(equation 6)}$$

where C is the concentration of single strand nucleotides at time, t, C_o is the concentration at initiation of reaction, and K, the second order rate constant for the reaction.

Graphic representation of Cot versus percent reassociation illustrates that a range of sequence frequencies occur in eukaryotic DNA (Britten and Kohne, 1968). Since rate of reassociation is dependent on sequence concentration, repetitive

sequences of a species, are observed to react at rates proportional to their reiteration frequency. Similarly, single copy sequences react at rates proportional to the total genome size (Britten and Kohne, 1968). The "Cot" curves so produced, are a composite of these repetitive and single copy sequence components. Statistically the entire curve can be analyzed as a series of components; the number and frequency of repetition being dependent on the criteria used (see Appendix 1). Generally, Cot curves are analyzed for the smallest number of frequency components which are consistent with the distribution of data (Pearson et al., 1977) at defined or standard criteria (see Appendix 1).

As in all observed eukaryotic DNA, chum reassociation curves are found to consist of these series of second order frequency components. The slowest component, i.e. the one in which each sequence is found to appear approximately once per haploid genome, is referred to as the single copy or unique sequence component. Those fractions which are observed to reassociate faster than the single copy sequence component are referred to as repetitive components and are found to be present more than once per haploid genome. Their repeat or reiteration frequency is expressed as the ratio of their second order rate constant to that of the single copy rate constant.

$$\frac{K \text{ (repetitive)}}{K \text{ (single copy)}} \qquad \text{(equation 7)}$$

where K is the inverse of Cot at one half the maximum value (Wetmur and Davidson, 1968).

It should be emphasized that the least squares fitting procedure as outlined above, is a statistical estimate for a minimum number of frequency components. These statistical frequency components only demonstrate average reiteration frequencies of a series of repetitive DNA sequences in the genome.

Two identifiable repetitive classes could be observed in chum DNA, 0.3 kb in length. The first is reiterated on the order of 3×10^3 times per genome (see equation 7) and makes up approximately 20% of the DNA fragments. The second repeated DNA component makes up approximately 40% of the genome and is reiterated on the order of 30 to 40 times per genome. These are referred to as the middle and slow repetitive components respectively. A third component reacts at a very fast rate. There is insufficient data at low Cot values to provide accurate measurement of its reiteration frequency, but it comprises about 10% of the 0.3 kb fragments and is reiterated at least an order of magnitude higher than the middle repetitive component. This may be referred to as the fast repetitive or foldback component.

Our least squares analysis for DNA components indicates that at least 30% of the 0.3 kb fragments are single copy sequence. From the free fit reassociation curve, the haploid genome size of chum DNA may be estimated to be 3.2 pg which is approximately 750 times that of E. coli. Estimation of the single copy reassociation rate constant for chum salmon is given below. The genome size for this organism has not been directly measured, and therefore values for O. tshawytscha (3.3 pg genome size) and O. kisutch (3.0 pg genome size) have been substituted (Hinegardner, 1972).

1 base pair = 618 Daltons

1 Mole of base pairs = 6.02×10^{23} molecules.

3.3×10^{-12} g is therefore equal to 3.2×10^{9} base pairs.

3.0×10^{-12} g therefore represents 2.9×10^{9} base pairs.

Since such prokaryotes as Escherichia coli, possess mostly single copy DNA, one can use the rate constant of their DNA reassociation as a standard in the calculation of expected rates for larger, eukaryotic genomes. Under our operating conditions, an E. coli 0.50 kb reassociation curve has a K value of 0.33 $M^{-1}s^{-1}$.

E. coli data; number of base pairs per genome = 4.2×10^6

(from Davidson, 1976)

Single copy rate constant = $0.33\ M^{-1}s^{-1}$

Length corrected rate constant = $0.33(300/500)^{.5}$

= $0.26\ M^{-1}s^{-1}$

Cot value at half maximum = 3.9 M s

Salmon data; (a) estmated number of base pairs per genome

(based on the *O. tshawytscha* genome)

= 3.2×10^9

Cot value at one half the maximum

$$= 3.9 \left(\frac{3.2 \times 10^9}{4.2 \times 10/6}\right)$$

$= 3.0 \times 10^3$ M s

single copy rate constant = $3.4 \times 10^{-4}\ M^{-1}s^{-1}$

(b) estimated number of base pairs per genome

(based on the *O. kisutch* genome)

= 2.9×10^9

Cot value at one half maximum

$$= 3.9 \left(\frac{2.9 \times 10^9}{4.2 \times 10^6} \right)$$

$$= 2.7 \times 10^3 \text{ M s}$$

single copy rate constant = 3.7×10^{-4} $M^{-1}s^{-1}$

Use of the computerized least squares fitting procedure (Pearson et al., 1977) allowed us to fix various parameters and detect any subsequent change in the root mean square (RMS) deviation of the fit. The range of possible fraction sizes for the single copy component are shown with their effective RMS in Appendix 2. According to this programme, all fits which differ less than 10% in their RMS value may be taken as equally valid. The second order reassociation rate for chum DNA's single copy component is found to be 3.4×10^{-4} $M^{-1}s^{-1}$ which is consistent with those calculated for other species of salmon DNA.

Similar kinetic analyses were generated for trout, coho and sockeye DNA (see Figures 4,5, and 6). In Table 1 it may be seen that for each species, the reassociation rate of the single copy component does not differ by more than a factor of two from predicted single copy rates. Variation of this magnitude can be attributed to the technique itself. As expected, the salmon reassociation curves were observed to be very similar. Coho and

sockeye DNA appear to have a slightly larger middle repetitive component size. This may be due to the larger fragment size used in the study. For example, if these fragments contained slower repetitive components (or single copy "tails"), their presence would not be detected, since hydroxyapatite only isolates fragments with some portion of their length in duplex. Trout 0.46 kb fragments, however, seemed to have a greater single copy component fraction size. Approximately 40% of the estimated 2.5 pg genome size (Louie and Dixon, 1972) or 1.0 pg of DNA, was observed to react at a single copy component rate. Salmon DNA generally had 30% of their approximate 3.0 pg genome size, or 1.0 pg of single copy DNA.

The results stated here, are in agreement with those of Britten and Kohne in their first report of repeated sequences in 1968. Salmon DNA of unspecified species was reacted to a maximum Cot value of 1000. However, they did not carry these reactions further, i.e. to the single copy region of the curve. Gharrett et al., (1977) published a report on reassociation in fish DNA. He employed an optical reassociation technique, however, and his reassociation values do not compare with ones given in this paper. He did report the occurrence of repetitive DNA in chinook salmon (O. tshawytscha) and steelhead trout (S. gairdneri). These sequences were observed to be reiterated on the order of 50 to 250 times per genome. Vladychenskaya et al., 1978,

reported the occurrence of repeated sequences which comprised 50% or more of DNA in selected species of the salmonid genus, Coregonus.

Interspersion Studied by Reassociation Kinetics;

The interspersion pattern of repeated and single copy sequences has been the subject of much interest in recent years. Reassociation kinetics of chum salmon at two fragment lengths, indicates that repetitive and single copy sequences are interspersed throughout the genome. About 30% of the short fragments reacted at rates consistent with that of the single copy component (Figure 3). At a fragment length of 3.2 kb, one can calculate the length corrected single copy rate to be (Wetmur and Davidson, 1968):

$$3.4 \times 10^{-4} \sqrt{\frac{3.2}{0.3}} = 1.1 \times 10^{-3} \text{ M}^{-1}\text{s}^{-1}$$

However, only 5% of these long fragments reacted at such a rate (Figure 3). This apparent decrease in amount of single copy DNA and increase in the amount of repetitive DNA suggests an interspersion of such sequences throughout the genome. Early in the reaction, repetitive DNA adjacent to single copy sequences would be bound by hydroxyapatite, carrying with them regions best described as single strand "tails". The entire fragment

would therefore be scored as duplex, leading to the measurement of apparent increase in amount of repetitive or early reacting DNA.

Interspersion Studied by Hyperchromicity Measurements;

Hyperchromicity can be used as a measure of the fraction of fragment in duplex (Graham et al., 1974) and can therefore provide information about the interspersion pattern of the organism. DNA reacted to a repetitive Cot (a Cot value at which only repetitive sequences have reacted), was therefore thermally denatured and analyzed for percent hyperchromicity. Figure 7 illustrates thermal denaturation curves for 0.3 and 3.2 kb fragments reacted to Cot 10 and 100. 0.3 kb fragments were reacted to these repetitive Cot values, collected on hydroxyapatite columns, and eluted with 0.5 M phosphate buffer. The isolated duplex fraction was dialyzed into 0.12 M phosphate buffer and thermally denatured in the Gilford spectrophotometer. Hyperchromicity of 0.3 kb fragments reacted to Cot 10, from 55° to 99°C is 23.0%. The hyperchromicity of totally single strand DNA was similarly measured to be 2.5% and of native DNA, 29.9%. Using the method of calculation of Graham et al., 1974, the fraction double strand in Cot 10, 0.3 kb DNA, is:

$$\frac{23.0 - 2.5}{29.9 - 2.5} = 0.77, \text{ or } 0.23 \text{ kb in total.}$$

A similar estimate for DNA fragments reacted to Cot 100 is 0.24 kb, as listed in Table 2.

It is difficult to say if this method of calculation is indeed valid. Theoretically, no free single strand DNA should be present in native DNA. It is therefore difficult to assess why a value for single strand hyperchromicity should be subtracted from the native measurement. Without this correction for native single strand hyperchromicity, the duplex length of Cot 10 and Cot 100 reassociated fragments can be estimated to be 206 and 0.22 kb, respectively. In either case, approximately two-thirds of the fragments have reassociated. Electron micrographs have, however, verified the observation of sequence interspersion in reassociated fragments of Xenopus DNA (Chamberlain et al., 1975). Calculations obtained from these micrographs were not significantly different from those obtained by the above method (Davidson et al., 1973). Perhaps it is sufficient to say that a variety of factors play a role in hyperchromicity measurements and that these calculations are, therefore, only estimates of the fraction of fragment in duplex.

The melting temperatures or T_m of 0.3 kb reassociated fragments are shown in Table 2. In each case, I observed a significant depression in T_m, beyond that expected from fragment length considerations. This decrease may be attributed to mismatch of repetitive sequences. At Cot 10, for example, the

melting temperature of repetitive duplex was 75.5°C, or 9.5°C below that measured for native 0.3 kb fragments. According to Kohne, 1970, a 1°C drop in T_m corresponds to 1% mismatch of sequences in hybrid prokaryotic DNA fragments. If one applies this relationship to reassociated repetitive fragments, the 9.5°C depression in T_m would correspond to 9.5% mismatch at Cot 10, and 8% mismatch at Cot 100. Bautz and Bautz, 1964, increase this estimate to 1.5% base mismatch per 1°C depression in T_m. Cot 10 and Cot 100 reassociated fragments may therefore contain as much as 14 and 12% respectively, mismatch of sequences.

It is difficult to say whether these differences in T_m are significant. According to Britten and Kohne, 1968, precise matching of repetitive sequences should occur in sequences most recently evolved, since he proposes that they arise by saltatory replication. Members of a related set of sequences would subsequently diverge from each other, with time. Our measurements show the slower repeating component to have less sequence mismatch indicating that these sequences are either relatively new, or more highly conserved. However, it is not known what role selection might play in divergence of repetitive sequences or at what state these sequences may be in the evolution of the organism.

Experimentally I was unable to isolate duplex 3.2 kb fragments by eluting from hydroxyapatite with 0.5 M phosphate buffer. This was most likely due to extensive networks of reassociated fragments, that form during renaturation. I was therefore unable to thermally denature exclusively duplex fractions. Approximate estimates for the amount of duplex in long fragments were obtained by thermally denaturing the entire reassociation mixture, and correcting our measurements for the free single strands as reported by Goldberg et al., 1975. The resulting hyperchromicity curves are found in Figure 7. Although I was unable to determine an exact measurement for hyperchromicity in 3.2 kb fragments it is significant that the estimates are greatly reduced relative to those of 0.3 kb fragments at the same Cot values. For example, 3.2 kb fragments at Cot 10 had an estimated hyperchromicity of 11.9% as opposed to 0.3 kb fragments reacted to the same Cot value with a hyperchromicity of 23.0%. This illustrates that the fraction of the 3.2 kb fragment length which is in duplex, is less than that of the shorter fragment, again demonstrating the interspersion of reassociated and unreassociated DNA sequences.

The lower hyperchromicity in reassociated long fragments is consistent with the theory that repetitive sequences are interspersed with unique sequences. By Cot 100, few of the single copy or unique sequences would have reassociated. Fragments containing both repetitive and single copy sequences

would therefore show a significant fraction of their length as unreassociated or single strand regions. These findings are consistent with a short period or "Xenopus" interspersion pattern of repetitive and single copy sequences.

Interspersion Studied by S1 Nuclease Resistance;

The reassociation kinetics of 3.2 kb fragments and hyperchromicity of such fragments at repetitive Cot values indicate the interspersion of repetitive sequences with single copy DNA. However, these measures do not reveal the length or amount of repetitive sequence in the reassociated products. In order to determine the length of the repetitive sequence in duplex containing long fragments, reassociated fragments were digested with the single strand specific nuclease, S1 at Cot 1, 10, and 100. This enzyme under the proper reaction conditions, will digest single strand DNA, but leave duplex intact (Ando, 1966; Vogt, 1973). DNA reacted to these repetitive Cot values and treated with enzyme, was passed over hydroxyapatite to determine the amount which was resistant to S1 enzyme. Estimates of the amount of duplex material are listed in Table 2.

When enzyme resistant fractions were passed over Sepharose CL-2B columns, distinct size classes were observed (Figure 8). Sepharose CL-2B fractionates double strand eukaryotic DNA in the

size range 2.0 to 0.2 kb. A linear relationship exists for the partition coefficient (K_{av}) of the DNA fragments between gel and eluant phases, and the log of the DNA fragment size in kb (M.J. Smith pers. comm.).

At Cots 1, 10, and 100 approximately 25% of the enzyme resistant fragments were excluded from the Sepharose column. These fragments would typically have a length of greater than 2.0 kb The peak of DNA fragments included by Sepharose may be estimated in the size range of 0.35 kb $\pm$ 0.1 kb. Distinctive to this separation, is the bimodal distribution of fragment sizes. This distibution of fragment sizes is characteristic of the short period interspersion pattern (Davidson et al., 1973; Graham et al., 1974).

To ensure that the S1 nuclease reaction was complete, Sepharose included and excluded fractions were thermally denatured in a Gilford spectrophotometer. Completion of reaction was indicated by the near native hyperchromicities of both fractions. In addition, the melting temperature, T_m, of the excluded fraction was observed to be very near that of native DNA. This would suggest almost complete base pairing of the reassociated strands. It is not yet known why these strands are so highly conserved, but this pattern has also been observed in sea urchins and star fish (Eden et al., 1977; Smith and Boal, 1978). The included fractions, however, showed a 11.5°C depression in

T_m. According to Kohne, 1970, and Britten et al., 1974, a 11.5°C decrease in T_m would correspond to approximately 11.5%, or 0.03 kb, which are mismatched. This estimate is in agreement with the 9.0-11.5°C depression in T_m measured using hyperchromicity of 3.2 kb fragments.

Hybridization of Single Copy Sequences;

There has been much speculation on the role of single copy DNA sequences in the development and maintenance of organisms. Recent findings have shown that most structural genes are coded in single copy sequences (Galau et al., 1974; Klein et al., 1974; Campo and Bishop, 1974; and Angerer et al., 1976). However the large quantity of single copy DNA (approximately 1.0 pg in salmonids) and the variation in quantity from species to species, suggests that much of this DNA is not made up of structural genes. Evidence for limited transcription of single copy genes has been presented in papers by Hough et al., 1975, and Galau et al., 1976.

Measurement of the degree of single copy sequence homology can, however, provide valuable information about the relationship of salmonid genomes. Isolated single copy sequences of chum DNA were radioactively labelled and allowed to reassociate with a large excess of DNA from the other species. These homologous and heterologous reactions were assayed by hydroxyapatite

chromatography to determine the extent to which they would hybridize. This technique has been employed for such groups as sea urchins (Harpold and Craig, 1978; Angerer et al., 1976), primates (Kohne, 1970), rodents (Rice, 1974), and birds (Eden et al., 1978). In these reports, a positive correlation is usually found between morphologic data, and single copy relatedness in evolution. If this is the case, then DNA hybridization studies may provide additional and much needed information on evolution in salmonids.

Chum DNA, 0.46 kb in length, was reacted to a Cot value of 3000 and passed over hydroxyapatite to isolate single copy DNA from the repetitive sequences. Reassociation kinetic measurements demonstrate that the repetitive DNA will have reacted by this value. The longer fragment length was used to ensure that a sufficiently long, labelled DNA preparation would be obtained. The isolated single copy DNA was iodinated using a modified Commerford (1971) technique. This method covalently bonds an ^{125}I molecule to cytosine to make a 5-iodo cytosine derivative (Prensky, 1976). Approximately 0.1% of the cytosine bases were iodinated in this preparation (see Appendix 3 for a detailed calculation of this value). Iodination was the method of choice because of its high specific activity and ease of handling. One drawback to this isotope is its short half-life, and all experiments had to be completed in as short a period of time as possible (in our case, one month) under strictly controlled conditions (see below).

^{125}I labelled chum single copy DNA was allowed to react with a 10^4-fold single copy sequence excess of heterologous DNA. By reacting with such a vast amount of driver DNA, the probability of tracer DNA reacting with other tracer molecules becomes very small. Hence the rate of reaction was dictated, or "driven", by the excess heterologous or "driver", DNA. We have verified this phenomenon by our self-reaction controls (see below). Disparity in the fragment length of tracer and driver DNA has been shown to affect the rate of reaction (Wetmur, 1971; Hutton and Wetmur, 1973; Bishop, 1975; and Chamberlain et al., 1978). However one can correct for length effects with knowledge of tracer and driver fragment size (Chamberlain et al., 1978). In each case, hybridization rates obtained in this study, are extremely close to expected values (Table 4). These values verify that only single copy sequences are reacting.

In order to determine the maximum amount of reassociation possible, chum tracer DNA was allowed to react with an excess of chum driver DNA. Figure 9 illustrates this homologous reaction. The expected rate of reassociation for tracer DNA with a longer driver DNA is 2.5×10^{-4} $M^{-1}s^{-1}$ (Chamberlain et al., 1978, see equation 5). The rate observed was 2.6×10^{-4} $M^{-1}s^{-1}$, with a root mean square value of 1.9×10^{-2}. The least squares kinetic analysis of this reaction demonstrate that there is no detectable contamination of the ^{125}I-DNA with repetitive sequences. Approximately 79% of the tracer DNA was observed to

react to completion with excess chum DNA. A measurement of 6% self reaction would reduce this estimate to 73% of the total.

We can only speculate as to why a greater proportion did not hybridize in this reaction. One possible reason may be that I have enhanced for unreactable DNA in the isolation of single copy sequences. From Figure 3, it can be observed that 5% of the total DNA was unable to reassociate. Since 24.8% of the DNA was isolated as single copy, then that 5% unreactable material would constitute almost 20% of the final preparation. This is in numerical agreement with our findings.

Since our rate estimates indicate that only single copy DNA is reacting, any disparity in fragment length between the tracer and driver DNA cannot be expected to affect which sequences are assayed as double stranded. We can therefore calculate the amount of tracer DNA present in hybrid molecules at termination of reaction, in terms of mass units. Furthermore, the amount of driver DNA which hybridizes with the single copy tracer DNA must be at least equal to it in mass.

The hybridization of chum ^{125}I-DNA with an excess of trout, coho, and sockeye DNA is shown in Figure 9. 80% of the chum single copy DNA was observed to react with trout DNA, a value indistinguishable from the homologous reaction.

Based on free fit analysis of the reassociation kinetics of 0.3 kb chum DNA, one can predict that a 0.28 kb tracer would have 1.01 pg (31.7% of 3.2 pg) present as single copy DNA. Trout driver, on the other hand, would have 43.5% of 2.5 pg which is equal to 1.09 pg in single copy sequence. That is to say that virtually all of the chum single copy or unique sequences are contained in trout (1.09 - 1.01 = 0.08 pg difference). If time had permitted, it would have been interesting to examine the reciprocal experiment using trout single copy DNA as the probe.

In the case of coho DNA, 92% (67%/73% = 92%) of the chum sequences reacted. This would account for 0.93 pg of the total 1.2 pg present as coho single copy DNA. The difference of 0.27 pg is enough DNA to code for 50 times the information content of the *E. coli* genome. Sockeye DNA was found to hybridize with 73% of the chum single copy sequences. Therefore 0.74 pg is homologous with an estimated 0.9 pg sockeye single copy component. A difference of 0.16 pg can be calculated from these figures. It is important to note that these data do not indicate the sequence homology between coho and sockeye, for it cannot be determined if the same single copy sequences have reacted.

None of the chum single copy sequences were observed to react with *Hexagrammos decagrammus* (kelp greenling) DNA. This may be explained if the criteria of my conditions are taken into account. Since elution from hydroxyapatite took place at 60°C

(16.0°C below the T_m of the homologous reaction), mismatch of 16.0% of the sequences would lead to elution from the column.

This lack of sequence hybridization may be explained if one examines evolutionary divergence between the two groups. McAllister, 1968, illustrates in a chart of ordinal phylogeny of the Teleostomi, last common ancestors occurring in the late Jurassic period, approximately 160-180 million years ago. If as much as 1% sequence divergence per million years has occurred, than this factor alone could account for lack of hybridization under my experimental conditions. Thus this measurement served as a control of specifity for the ^{125}I chum probe.

Reassociated tracer-driver mixtures were thermally denatured in 5°C increments on hydroxyapatite columns. Since the T_m of a reassociated fragment is directly related to the fraction of bases paired, this technique allowed us to measure any mismatch that might have occurred in the heterologous reactions (Laird et al., 1969; Britten et al., 1974).

The T_m for the chum tracer-chum driver DNA reacted to completion (Cot = 225,000) is 76.0°C. This represents a 9°C depression in temperature from that measured for native 0.3 kb fractions. It is not known why this depression occurred; it may be related to thermal instability caused by the iodination reaction. However, since only 0.1% of a 0.3 kb fragment was

iodinated, this would only account for a 0.1°C depression in T_m, at maximum. This phenomenum has been reported by other workers using other radioactive labels. For example, Harpold et al., 1978, reported a 78.5°C melting temperature for ^3H-labelled DNA reacted to long Cot values.

More likely, the low melting temperature of tracer fragments may be attributed to a combination of factors. In a paper by Smith et al., 1975, experiments were performed to measure the nucleation rate of single strands on partially reassociated structures. Since the average fraction of the tracer fragment that is in duplex with driver DNA, is about 55%, one may expect a 4°C depression in T_m from that of native DNA (Britten et al., 1974). In addition, contributions from polymorphism and heterozygosity have accounted for as much as 4°C depression in T_m in sea urchins (Britten et al., 1978).

Heterologous reactions using trout, coho, and sockeye driver DNA, were observed to have a 2°C depression in T_m relative to the chum reaction (Figures 10, 11, and 12). According to the Laird et al., 1969, and Bautz and Bautz, 1964, relationships, this would correspond to approximately 2-3% mismatch of reassociated sequences. This represents significant divergence in the evolution of such sequences. Neave, 1958, dates the last common ancestor in Oncorhynchus at 1.0 million years ago. The

evolutionary time separating these species would therefore be at maximum, 2 million years. If, as in the case of reassociated chum-trout hybrids, there is 2% mismatch in single copy sequences, one can calculate that 1% divergence per million years, has occurred.

This value is higher than those reported for sea urchins (Angerer et al., 1976; Harpold and Craig, 1978). Based on a minimum divergence time of seven million years, their findings suggest 0.06-0.35% single copy change per million years. Kohne, 1970, measured a single copy divergence rate of 0.7% per million years in primates, and Galau et al., 1976, using similar experimental procedures observed a rate of 0.2% single copy divergence per million years, in two Xenopus species. However, values published by Rice, 1974, suggest as much as 1.6% divergence per million years in rat-mice single copy hybrids.

In terms of single copy sequence homology, this study demonstrates that chum has more single copy sequences in common with coho, than it does with sockeye. Furthermore, it demonstrates a close relationship between the single copy sequences of trout and chum salmon. It does not however, demonstrate that the same sequences have been conserved, nor does it demonstrate the proportion of sequences present in the reciprocal case. Our findings do not seem to support

morphological data, but are more consistent with studies based on biochemical, genetic, and cytological evidence.

For example, Simon, 1963, has correlated chromosome morphology with species evolution in five species of Pacific Salmon. He concluded that chum salmon, with 2n=74, was the most primitive form, with the remaining species occupying positions of increasing specialization characterized by decreases in chromosome numbers. If this is true, then coho, with 2n=60, would occupy an intermediate position, and sockeye with 2n=56, would represent the most advanced form. This is in agreement with our results.

This hypothesis was further supported in a paper on genetic variation in Pacific salmon by Utter et al., 1973. This group examined 12 loci for average heterozygosity, using horizontal starch gel electrophoresis. In a dendogram plot of relative genetic similarity, chum, coho, and chinook (O. tshawytscha) salmon separate out as a distinct group from a sockeye-pink stem group. Interestingly, chum salmon is the first to branch from these linkage points. These results are in agreement with our findings.

Papers relating evolutionary trends on the basis of morphology and behaviour of salmonids, show less agreement. For example, in 1962, Hikita (as reviewed by Hoar, 1976), linked chum and

sockeye as a distinct group from coho salmon. Clemens, 1953, using similar criteria linked spring and coho salmon on one hand, pink and chum on the other hand, and sockeye at a position intermediate to the two pairs. Milne, as reviewed by Hoar, 1976, poses another point of view. Based on morphologic characteristics, he felt that coho salmon were most like steelhead trout, with sockeye next, and chum the most advanced.

Problems seem to arise from judging which features contribute or result from the development of a species. For example, it can be argued that behavioural and morphological characters result from environmental modulation of genetic information. How much environmental influence may obscure these characters, is difficult to determine. It is our belief that DNA sequence homology studies, will provide valuable insight with this respect.

In conclusion, one may state that chum DNA exhibits a "short period" interspersion pattern of repetitive and single copy sequences. This form of sequence organization is typical of many metazoa so far examined (Davidson et al., 1975a). Reassociation kinetics demonstrated that repeated sequences made up a significant fraction (as much as 70% of reannealled fragments) of the genome in all four species. Variation in the amount and reiteration frequency of these sequences were measured using the

techniques mentioned above. Single copy sequence homology studies demonstrated a close relationship between chum and trout, and to a lesser degree, chum and coho and chum and sockeye DNA.

Table 1 Footnotes.

a; The fraction of the genome which reassociates at the indicated rate. It is expressed as a fraction of the total genomic DNA used in the reaction.

b; The second order reassociation rate constant ($M^{-1}s^{-1}$) for the indicated components. The reciprocal of the second order rate is $Cot_{1/2}$, which is the Cot at which 50% of a component has reannealed.

c; RMS is the root mean square deviation of the data from the least squares solution.

d; The reassociation rate was fixed in the least squares analysis at a value appropriate to the fragment length and estimated genome size of the organism.

e; The value of the second order rate constant was calculated on the basis of the relationship established by Wetmur and Davidson (1968) for the effect of fragment length on reassociation rate.

Table 1. The reassociation kinetics of salmonid DNA.

SPECIES	FRAGMENT LENGTH (BP)	FINAL UNREACTED	INTERMEDIATE REPETITIVE		SLOW REPETITIVE		SINGLE COPY		RMS[c]
			F_1[a]	K_1[b]	F2	K2	F3	K3	
Chum	300	.03	.216	1.07	.371	.012	.317	.00034	.025
		.03	.212	1.11	.346	.015	.346	.00042[d]	.025
Chum	3200	.03	.199	16.28	.343	1.07	.240	.04350	.024
		.03	.360	6.31	.358	.175	.050	.00110[e]	.028
Trout	460	.02	.305	4.08	.211	.148	.435	.00123	.017
		.02	.425	1.74	.357	.004	.146	.00037[d]	.026
Coho	450	.03	.155	40.1	.372	.391	.400	.00047	.015
		.03	.150	48.1	.373	.429	.407	.00051[d]	.014
Sockeye	460	.05	.155	25.8	.376	.481	.357	.00113	.008
		.05	.199	13.5	.388	.238	.294	.00051[d]	.018

Table 2 footnotes.

a; T_m is the temperature at half maximum hyperchromicity.

b; $-dT_m$ refers to the difference between measured T_m and native T_m, for the indicated fragment lengths.

c; H is the hyperchromicity as defined by Britten et al., 1974, see Equation 1.

d; % bases in duplex represents the ratio of measured hyperchromicity to native hyperchromicity.

e; Number of bases in duplex is calculated from the estimated fraction of the total length that is base paired.

Table 2. Thermal denaturation characteristics of chum salmon repetitive DNA.

FRAGMENT LENGTH (bp)	CoT (M s)	FRACTION HYDROXYAPATITE BOUND	T_m[a] (°C)	$-dT_m$[b] (°C)	% H[c]	PERCENT IN DUPLEX[d]	NUMBER OF BASES IN DUPLEX[e]
300	10	0.31	75.5	9.5	23.0	77.0	230
	100	0.50	77.0	8.0	24.8	81.0	243
	Native		85.0		29.2		
3200	1	0.58	76.0	11.5			
	10	0.77	77.0	10.5	11.9	31.6	1012
	100	0.93	78.5	9.0	15.7	44.4	1422
	Native		87.5		32.2		

Table 3 Footnotes.

a; Chum DNA was sheared to a fragment length of 3.2 kb, denatured, and reassociated to Cot 1, 10, or 100. At the desired Cot value an aliquot of the reassociation mixture was passed over hydroxyapatite (HAP) and the fraction of fragments bearing duplex was scored as bound to HAP before S1 nuclease digestion. The remainder of the reassociation mixture was submitted to S1 nuclease digestion. At the completion of the enzymatic digestion, the reaction mixture was passed over HAP and the DNA which bound was scored. The Sepharose partition is expressed in terms of the fraction of post-nuclease DNA duplex.

b; The melting temperature of the excluded and included fractions was determined directly. Fractions which contained these portions of the Sepharose eluant were pooled, dialyzed into 0.12 M phosphate buffer, and thermally denatured in the Gilford Spectrophotometer (see Materials and Methods).

Table 3. The S1 nuclease digestion of 3200 bp chum DNA reassociated to Cot 1, 10, and 100,[a]

CoT	FRACTION BOUND TO HYDROXYAPATITE		SEPHAROSE PARTITION	
	BEFORE S1 DIGESTION	AFTER S1 DIGESTION	EXCLUDED FRACTION $K_{av} \leq 0.1$ (T_m °C)[b]	INCLUDED FRACTION $K_{av}=0.5-0.8$ (T_m °C)
1	0.52	0.12	0.27	0.35
10	0.77	0.21	0.22 (87.5 °C)	0.45 (73.5 °C)
100	0.93	0.44	0.25 (87.5 °C)	0.46 (72.0 °C)

Table 4 footnotes.

a; The extent of hybridization was corrected for ^{125}I-DNA self reaction, see Figure 9A. Error is expressed as standard deviation.

Table 4. Hybridization characteristics of chum single copy ^{125}I-DNA

DRIVER DNA	HYBRIDIZATION EXTENT (%)[a]	HYBRIDIZATION RATE $M^{-1}s^{-1}$	FRACTION OF CHUM UNIQUE SEQUENCE (%)	$-dT_m$ (°C)
Chum	73.5±1.2	.00027	100	0
Trout	74.0±1.0	.00026	100	2
Coho	68.5±1.5	.00036	93	2
Sockeye	53.5±1.6	.00023	73	2

Figure 1. A representative absorbance spectrum of chum native DNA. Purified chum native DNA was diluted in 0.12 M phosphate buffer and placed in a Gilford Spectrophotometer. The absorbance spectrum from 220 nm to 320 nm was recorded. DNA with 260/230 and 260/280 ratios of greater than 2.0 and 1.8, respectively, were used for experimentation.

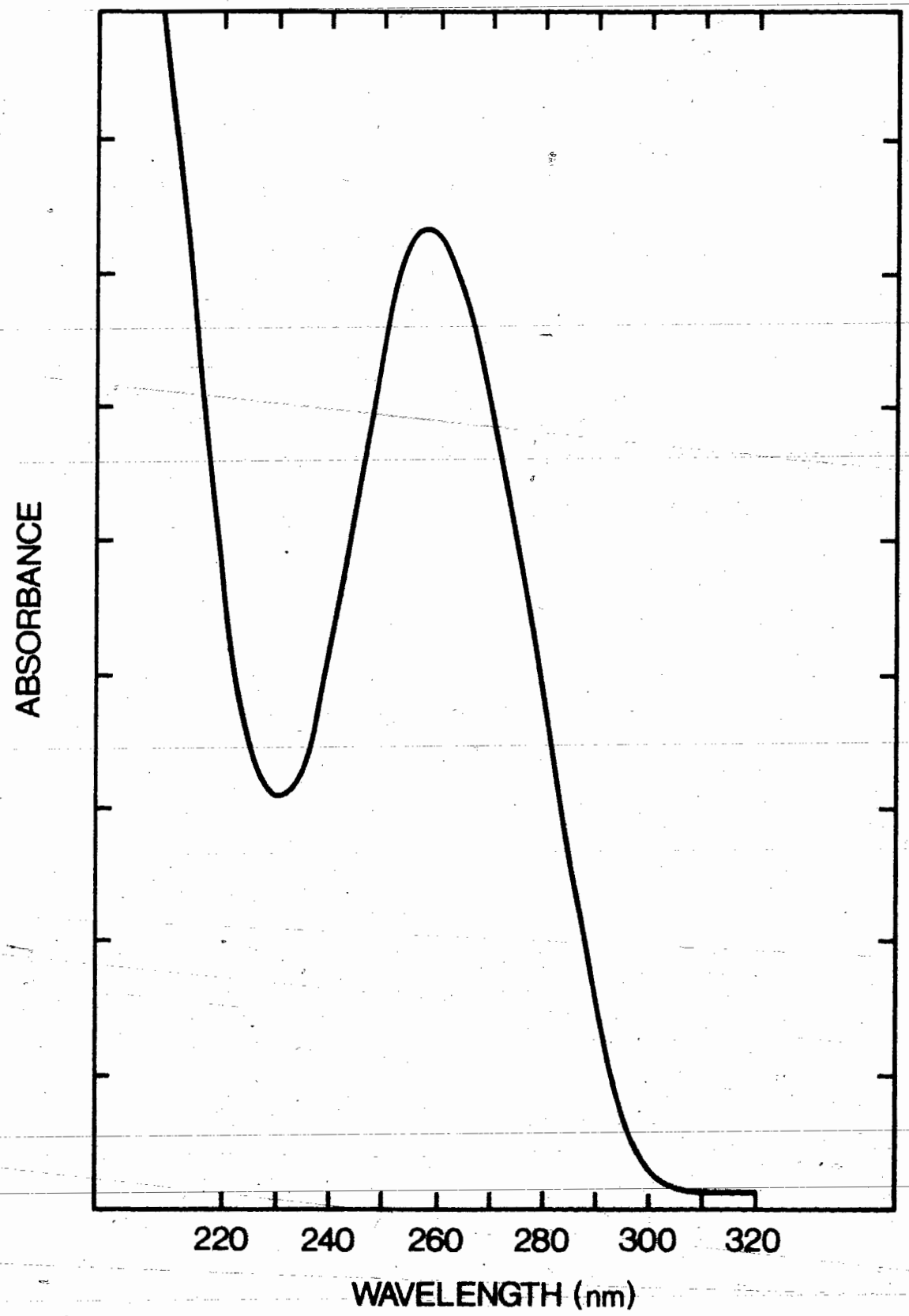

ABSORBANCE
220
240
260
280
300
320
WAVELENGTH (nm)

Figure 2. Thermal denaturation of chum DNA. Chum DNA, 0.3 and 3.2 kb in length was diluted to approximately 40 ug/ml in 0.12 M phosphate buffer and loaded in a Gilford Thermal Cuvette. Phosphate buffer was used as a reference baseline (dotted line). The cuvette was placed in a Gilford Spectrophotometer with an Analog Multiplexor and Thermalprogrammer, and heated at a rate of 0.5°C/min. The absorbance of the sample was measured relative to the buffer reference. T_m is shown as the temperature at half the maximum absorbance, $A_{1/2}$.

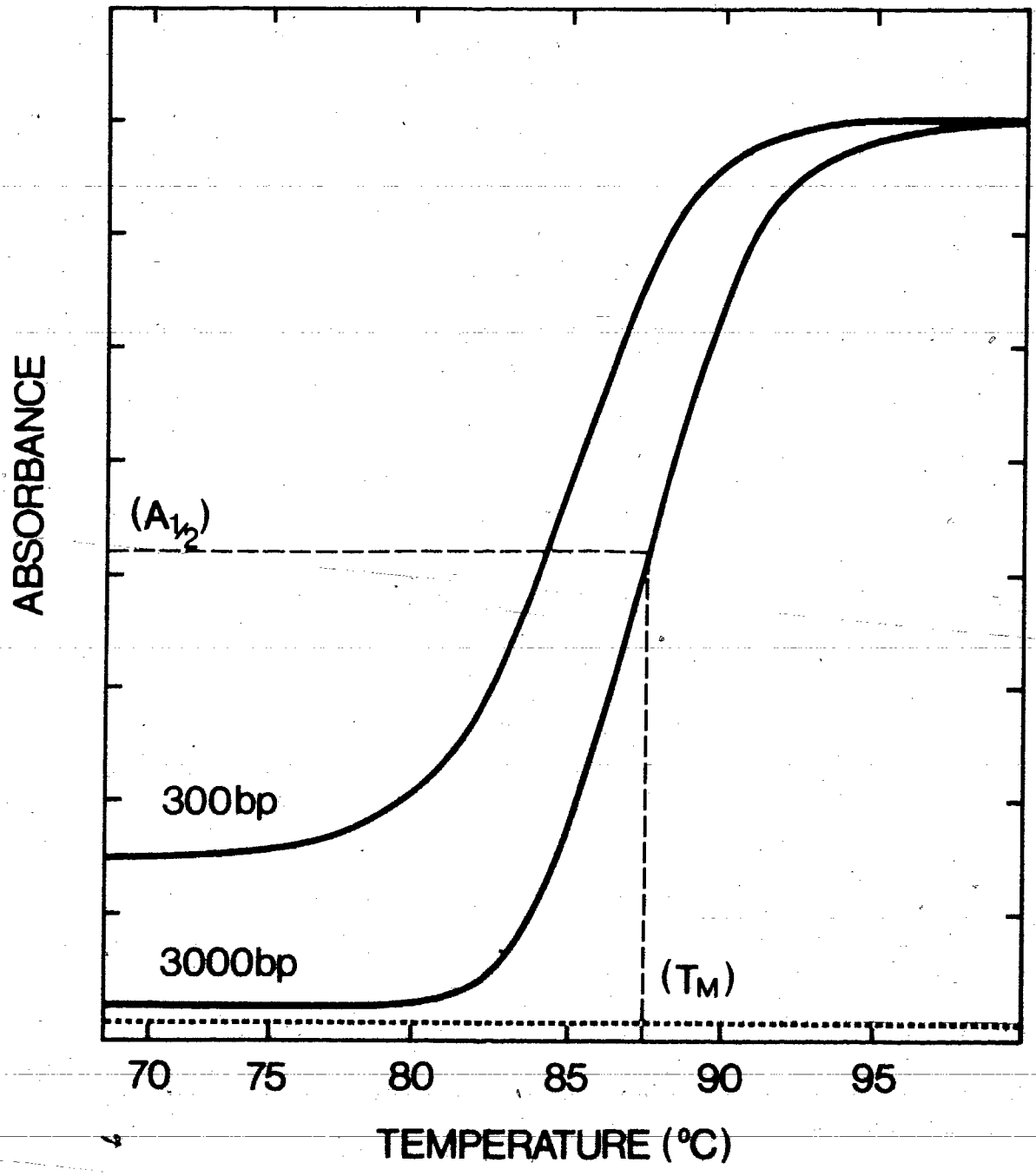

ABSORBANCE
(A1/2)
300bp
3000bp
(TM)
70
75
80
85
90
95
TEMPERATURE (°C)

Figure 3. The reassociation kinetics of chum salmon DNA. Chum salmon DNA, sheared to fragment lengths of 0.3 and 3.2 kb was denatured and reassociated as specified in Materials and Methods. Duplex formation was determined on hydroxyapaptite columns at 60°C in 0.12 M phosphate buffer, 0.06% SDS. Solid circles represent a 0.3 kb reassociation curve while open circles designate a 3.2 kb curve. The solid lines indicate computer fitted least squares solutions for multiple second-order reassociation components. The dashed lines are the best fit lines for three such components at a fragment length of 0.3 kb (see Table 1).

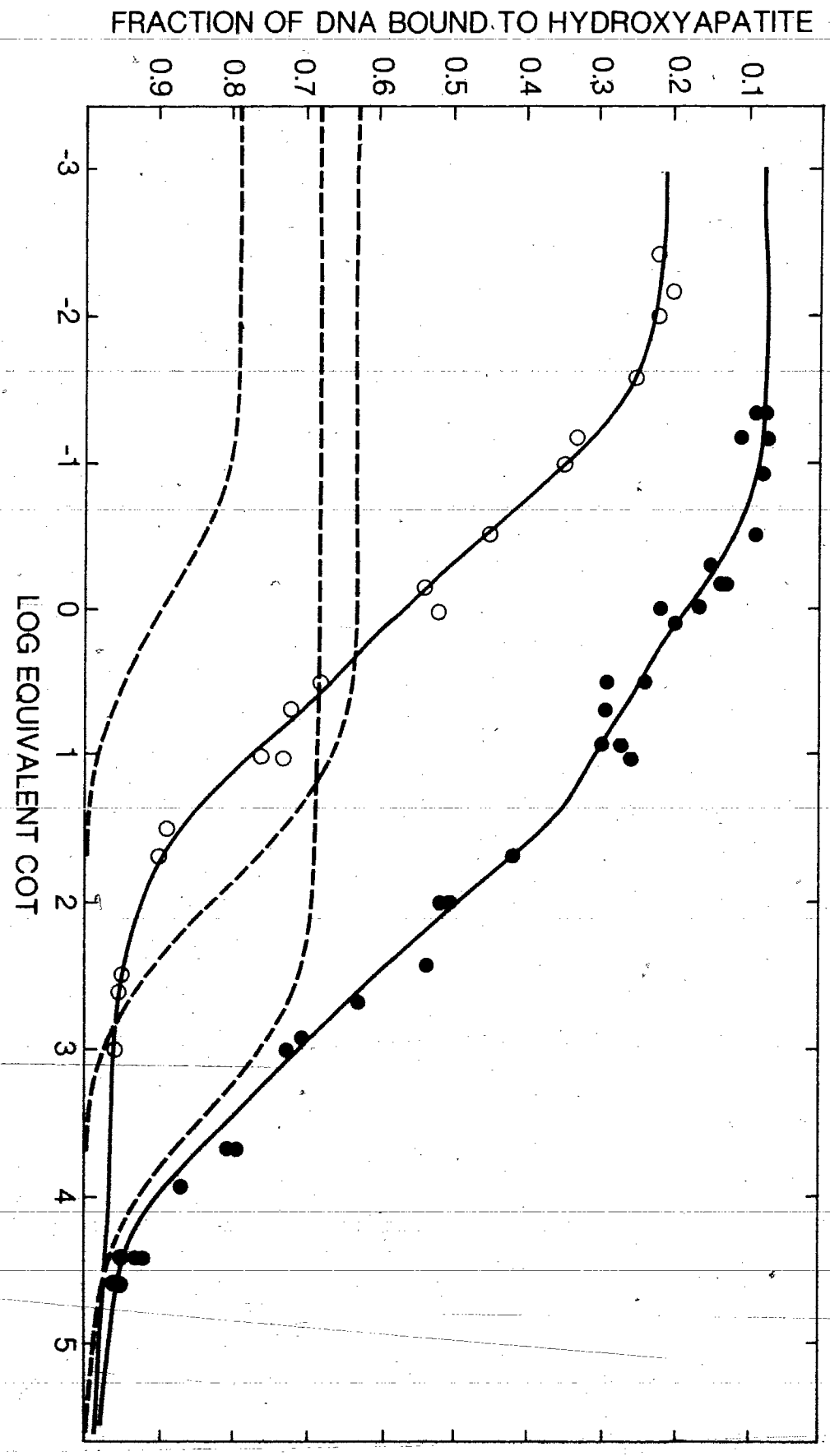

FRACTION OF DNA BOUND TO HYDROXYAPATITE
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
LOG EQUIVALENT COT
-3
-2
-1
0
1
2
3
4
5

Figure 4. The reassociation kinetics of steelhead trout DNA. Trout DNA, sheared to 0.46 kb fragment lengths, was denatured and reassociated as specified in Materials and Methods. Duplex formation was determined on hydroxyapatite columns at 60°C in 0.12 M phosphate buffer, 0.06% SDS. The solid line is a computer-fitted least squares solution for multiple second-order reassociation components. The dashed lines represent fit lines for three such components The second order rate constant for the single copy component was held constant throughout (see Table 1).

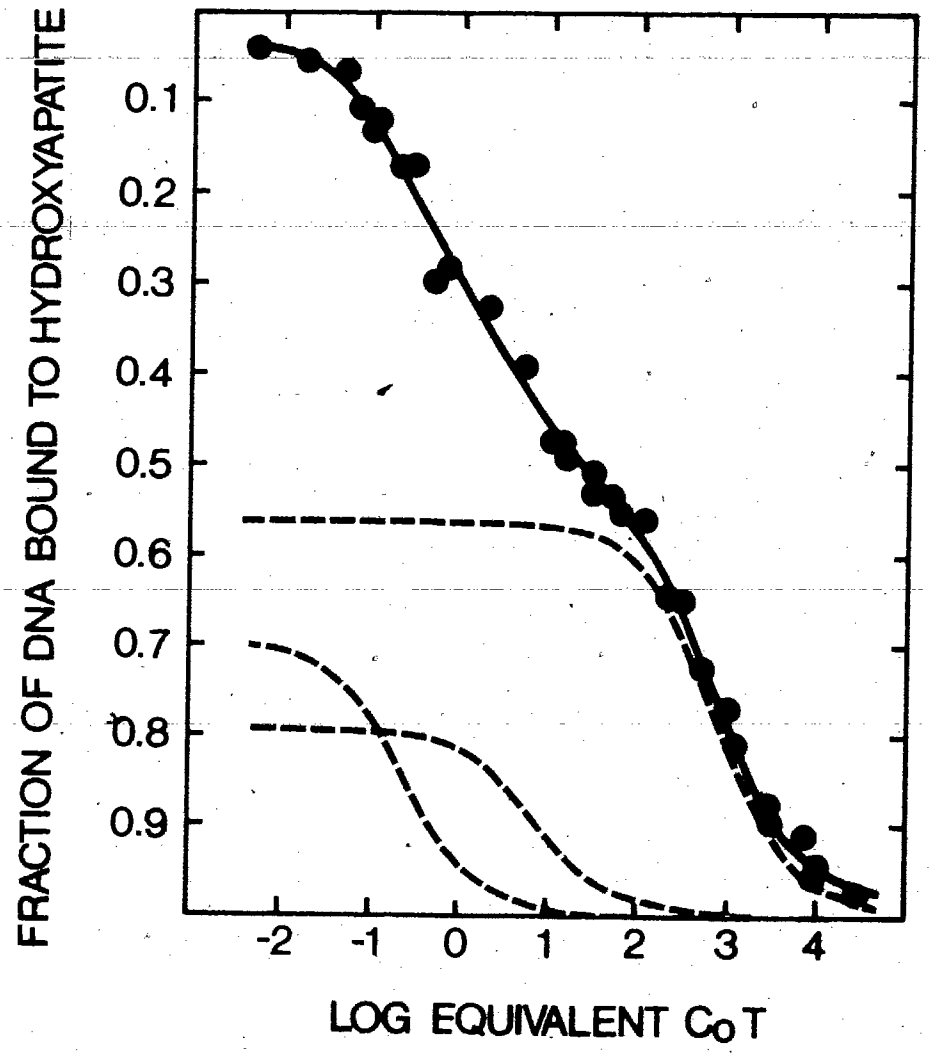

FRACTION OF DNA BOUND TO HYDROXYAPATITE
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
-2
-1
0
1
2
3
4
LOG EQUIVALENT C_0T

Figure 5. The reassociation kinetics of coho salmon DNA. Coho DNA, sheared to 0.46 kb fragment lengths, was denatured and reassociated as specified in Materials and Methods. Duplex formation was determined on hydroxyapatite columns at 60°C in 0.12 M phosphate buffer, 0.06% SDS. The solid line is a computer-fitted least squares solution for multiple second order reassociation components. The dashed lines represent fit lines for three such components (see Table 1).

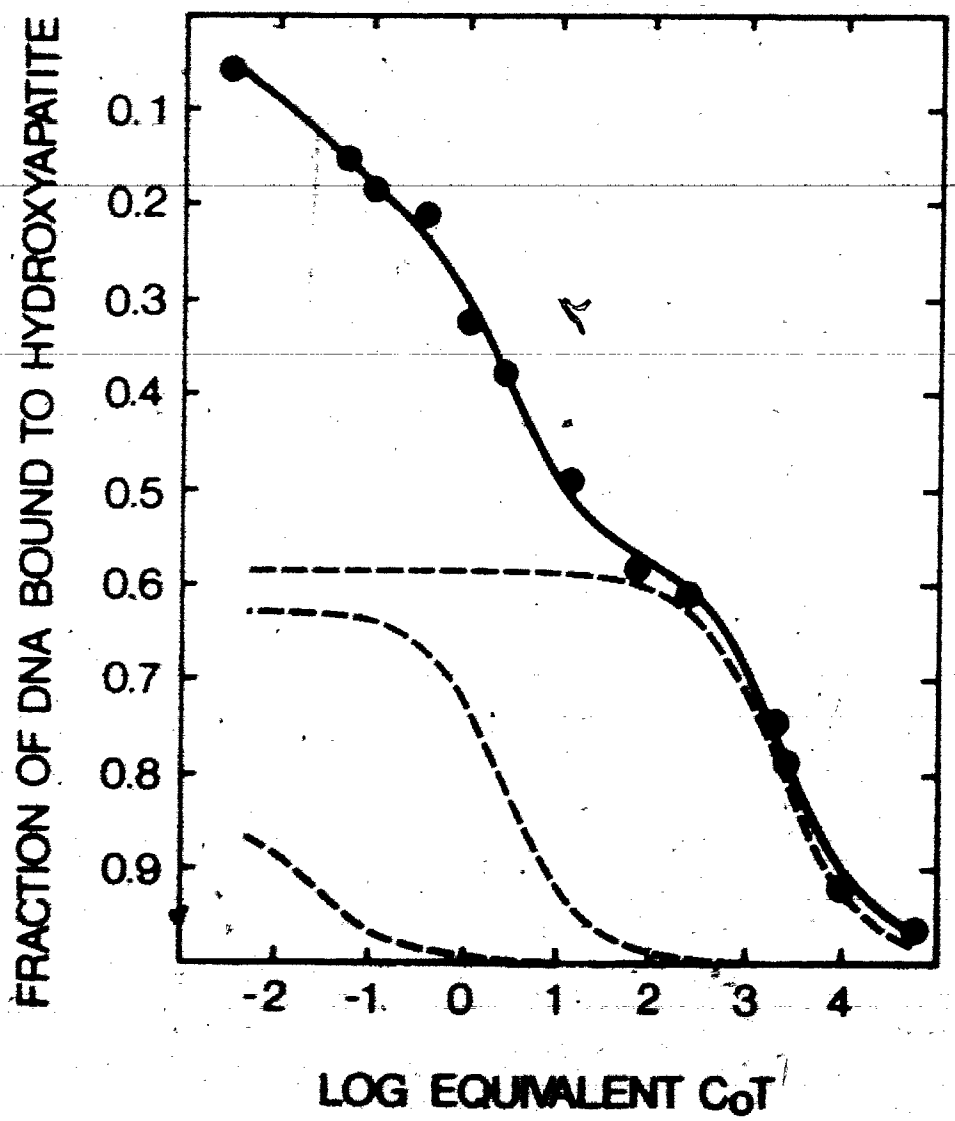

FRACTION OF DNA BOUND TO HYDROXYAPATITE
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
-2
-1
0
1
2
3
4
LOG EQUIVALENT C_0T

Figure 6. The reassociation kinetics of sockeye salmon DNA. Sockeye salmon, sheared to 0.45 kb fragment lengths, was denatured and reassociated as specified in Materials and Methods. Duplex formation was determined on hydroxyapatite columns at 60°C in 0.12 M phosphate buffer, 0.06% SDS. The solid line is a computer-fitted least squares solution for multiple second order reassociation components. The dashed lines represent fit lines for three such components (see Table 1).

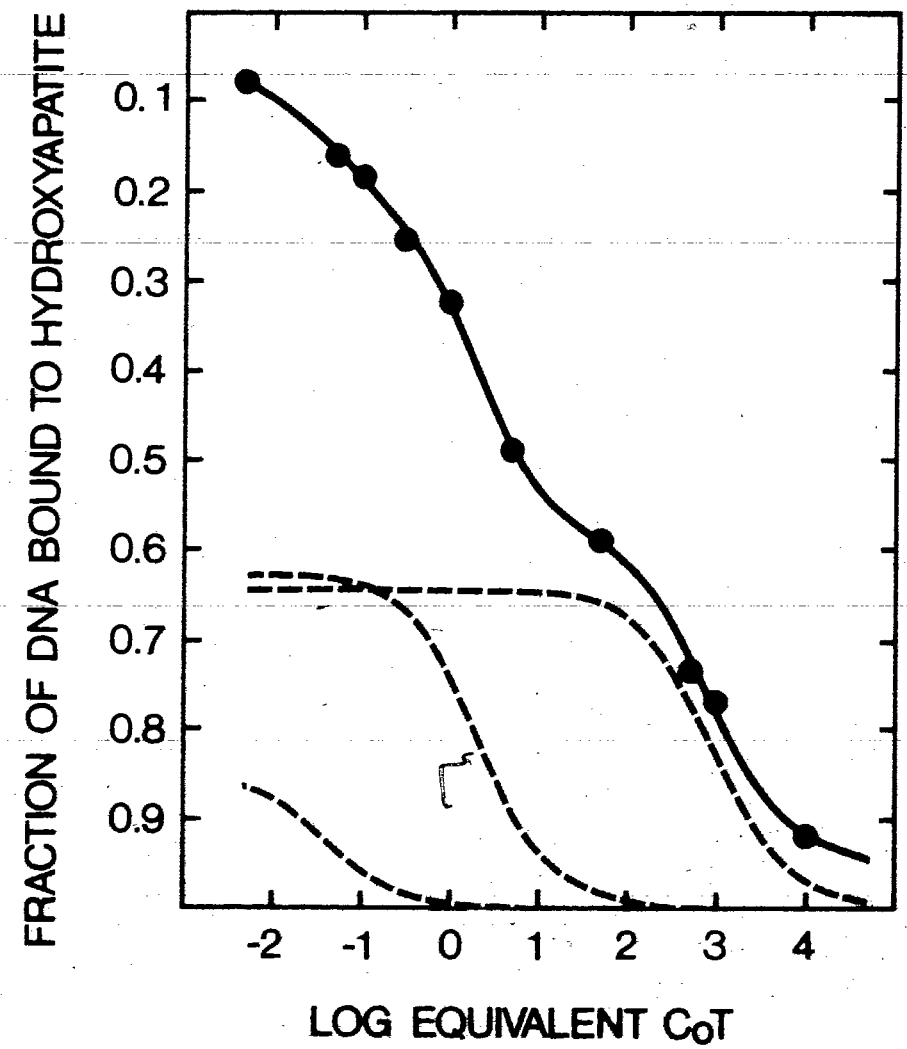

FRACTION OF DNA BOUND TO HYDROXYAPATITE
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
-2
-1
0
1
2
3
4
LOG EQUIVALENT CoT

Figure 7. The thermal denaturation of chum DNA. Chum DNA sheared to fragment lengths of 0.3 and 3.2 kb was denatured and reassociated to Cot 10, solid circles and triangles, or Cot 100, open circles and triangles. At each Cot, reassociated fragments were thermally denatured in the Gilford Spectrophotometer as outlined in Materials and Methods. The thermal denaturation profile of native chum DNA, closed squares, is shown for reference.

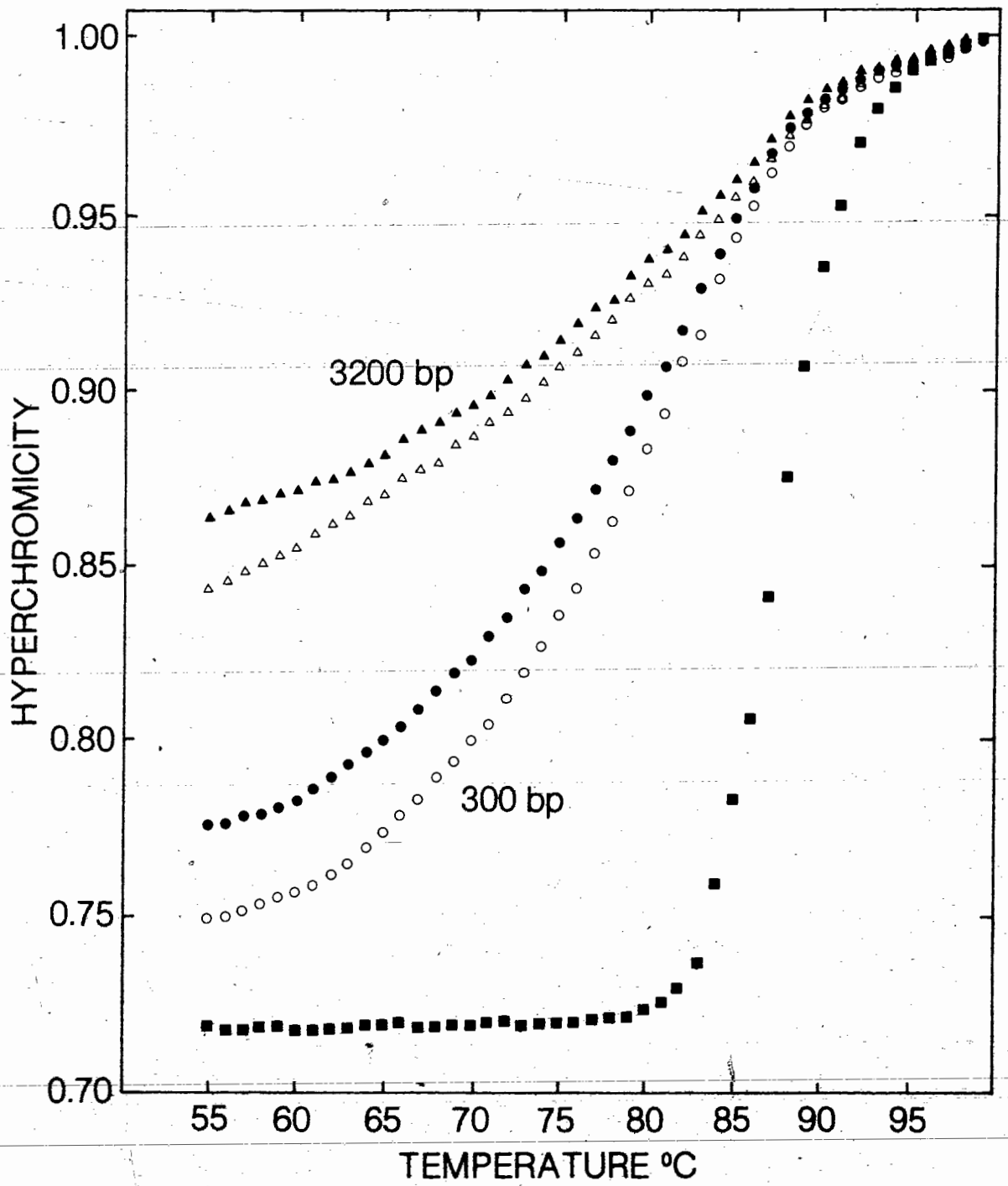

1.00
0.95
0.90
0.85
0.80
0.75
0.70
HYPERCHROMICITY
3200 bp
300 bp
55
60
65
70
75
80
85
90
95
TEMPERATURE °C

Figure 8. The Sepharose CL-2B elution of S1 nuclease resistant reassociated chum DNA. Chum DNA, 3.2 kb in length, was reacted to Cot 1, 10, or 100 and digested with S1 nuclease as specified in Materials and Methods. The S1 nuclease resistant duplex was collected from hydroxyapaptite columns and chromatographed on Sepharose CL-2B columns. The amount of S1 resistant DNA was measured in each eluted fraction and plotted as a function of K_{av}, the partition coefficient between gel and solvent phases. V_o is the exclusion volume; V_t is the column volume. The fraction in which a 0.33 kb double strand DNA marker elutes is indicated. Panel a is the enzyme resistant duplex at Cot 1, Panel b at Cot 10, and Panel c at Cot 100.

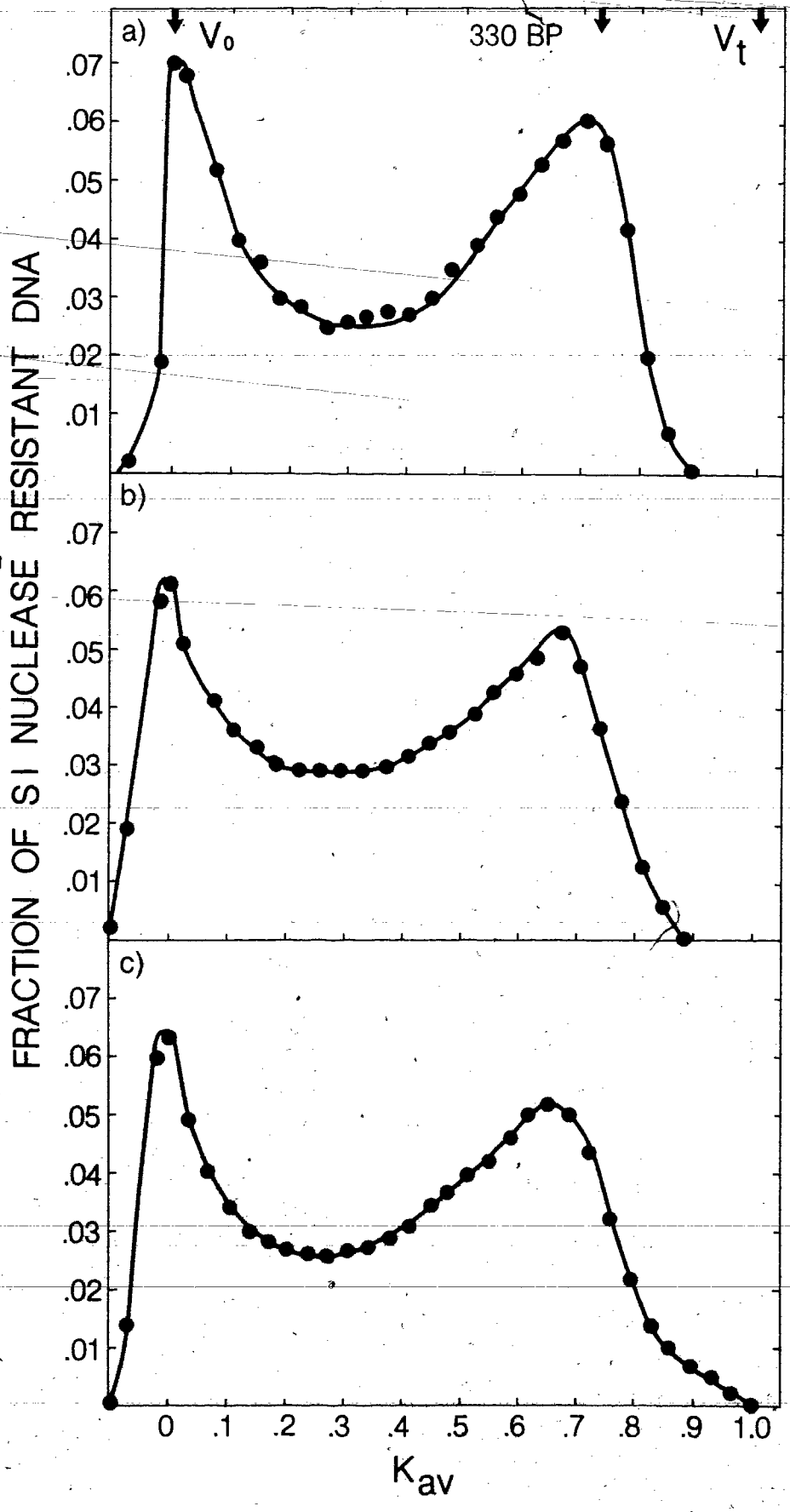

a)
b)
c)
V_0
330 BP
V_t
FRACTION OF S1 NUCLEASE RESISTANT DNA
.07
.06
.05
.04
.03
.02
.01
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
K_{av}

Figure 9. The reassociation kinetics of chum ^{125}I-DNA driven with a 10^4-fold sequence excess of chum (Panel A), trout (Panel B), coho (Panel C), and sockeye (Panel D) DNA. Mixtures of tracer and driver DNA were thermally denatured and allowed to reassociate as specified in Materials and Methods. Duplex formation was assayed on hydroxyapatite columns at 60°C in 0.12 M phosphate buffer, 0.06% SDS. The solid lines represent computer-fitted least squares solutions for single component second order reactions. The dashed lines in Panels A through D indicate the single copy component region of the reassociation curve of the excess DNA driving the hybridization reaction. The open circles in Panel A indicate a chum tracer self reaction curve. Here, chum tracer was allowed to react under identical reaction conditions, with no driver DNA present. Chum tracer driven with kelp greenling DNA is illustrated by closed triangles in Panel A.

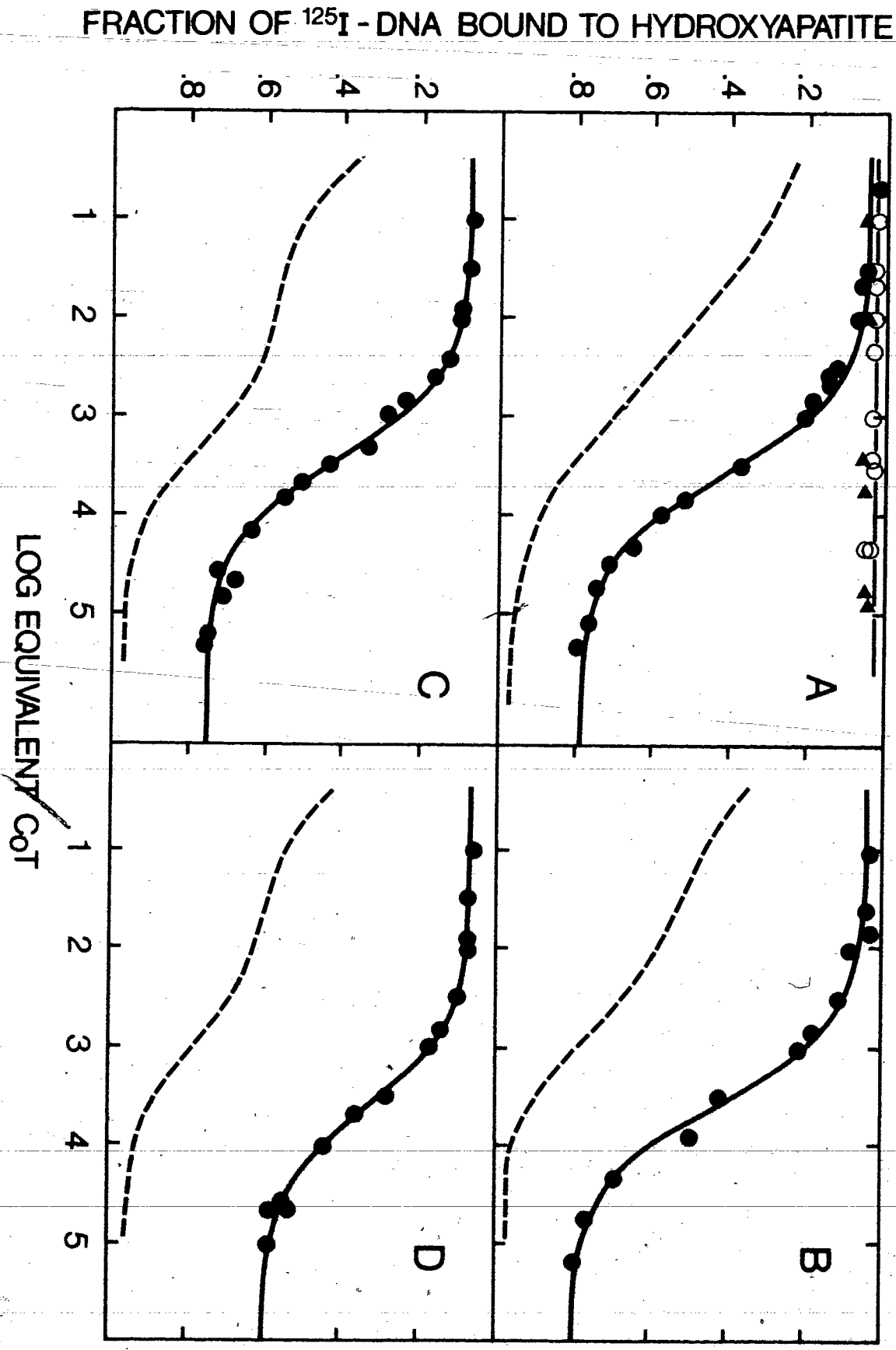

FRACTION OF ^{125}I - DNA BOUND TO HYDROXYAPATITE
.8
.6
.4
.2
1
2
3
4
5
LOG EQUIVALENT C_0T
A
B
C
D

Figure 10. Thermal denaturation profile of ^{125}I-chum DNA and trout driver DNA hybrids. Chum tracer DNA was reacted with a 10^4-fold sequence excess of trout DNA to a Cot of 169,074, and loaded on hydroxyapatite columns as outlined in Materials and Methods. Reassociated fragments were thermally eluted by raising the temperature of the column in 5°C increments. The graph in the left panel is a plot of the cumulative percent eluted from the column, while the bar graphs on the right have been plotted from the percent of total counts eluted at each temperature. Data from a chum driven reaction is represented by closed circles in the left panel, and open boxes of the bar graph on the right.

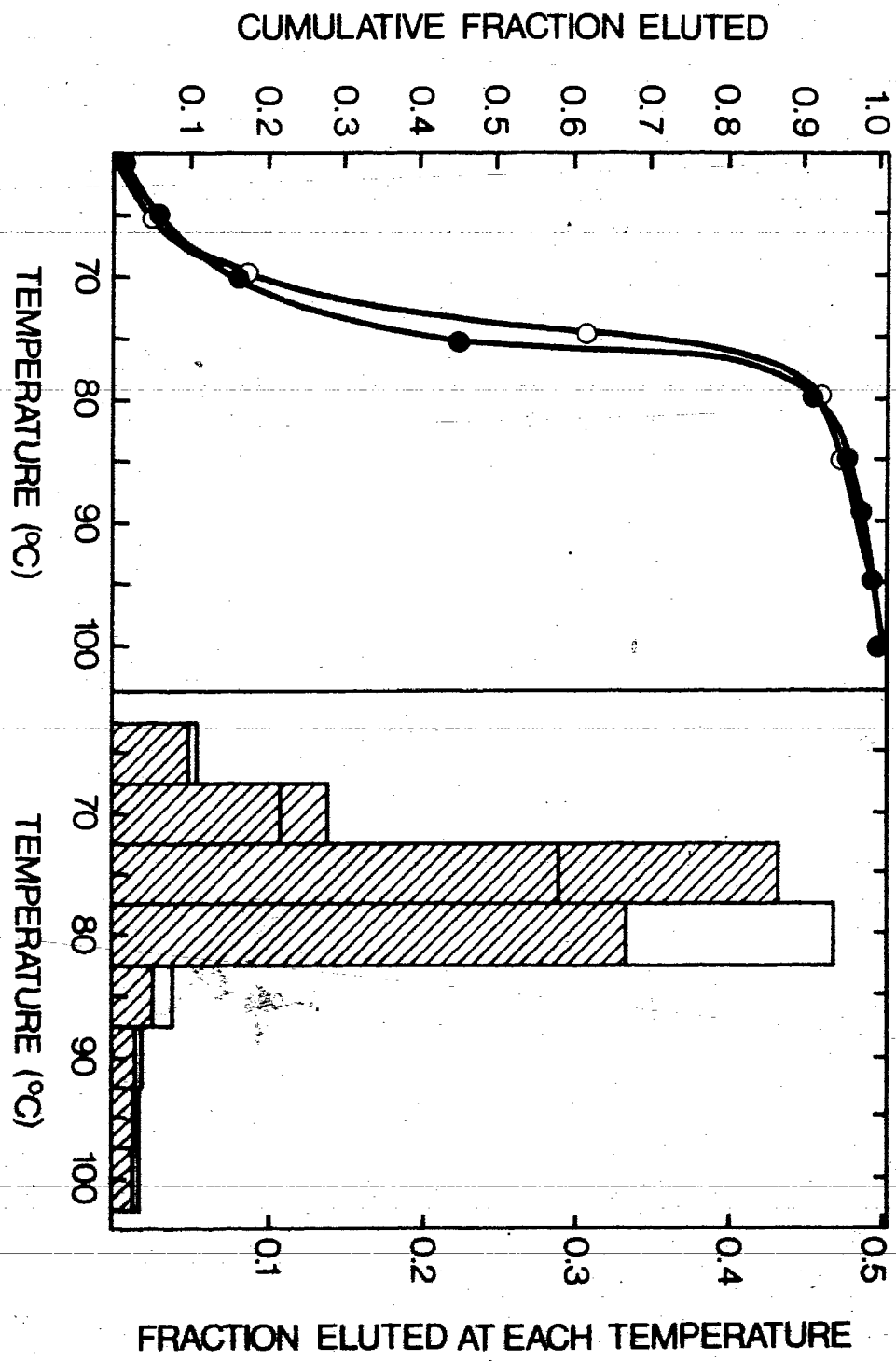

CUMULATIVE FRACTION ELUTED
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
TEMPERATURE (°C)
70
80
90
100
TEMPERATURE (°C)
70
80
90
100
0.1
0.2
0.3
0.4
0.5
FRACTION ELUTED AT EACH TEMPERATURE

Figure 11. Thermal denaturation profile of ^{125}I-chum DNA and coho driver DNA hybrids. Chum tracer DNA was reacted with a 10^4-fold sequence excess of coho DNA to a Cot of 129,082, and loaded on hydroxyapatite columns as outlined in Materials and Methods. Reassociated fragments were thermally eluted by raising the temperature of the column in 5°C increments. The graph in the left panel is a plot of the cumulative percent eluted from the column, while the bar graphs on the right have been plotted from the percent of total counts eluted at each temperature. Data from a chum driven reaction is represented by closed circles in the left panel, and open boxes of the bar graph on the right.

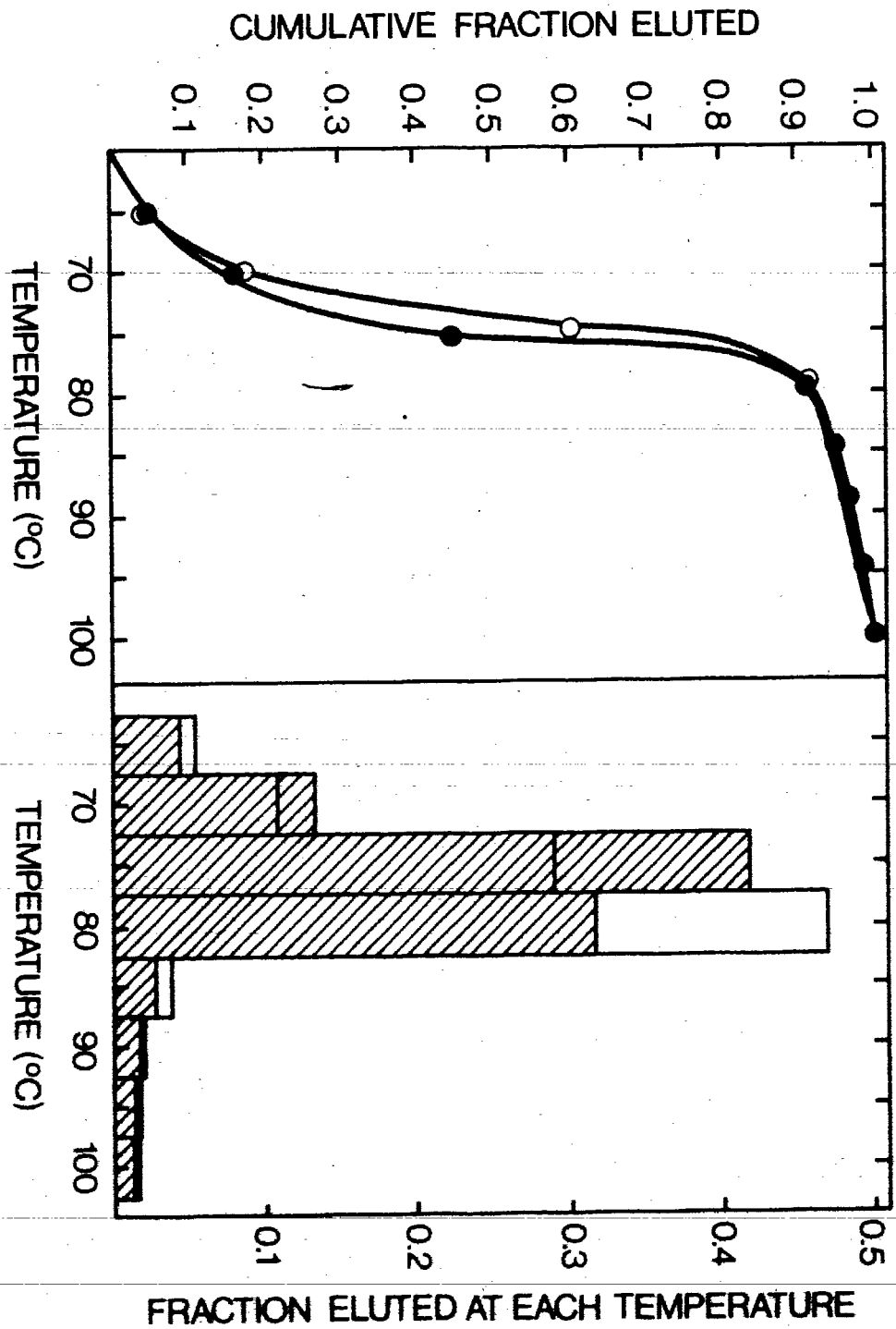
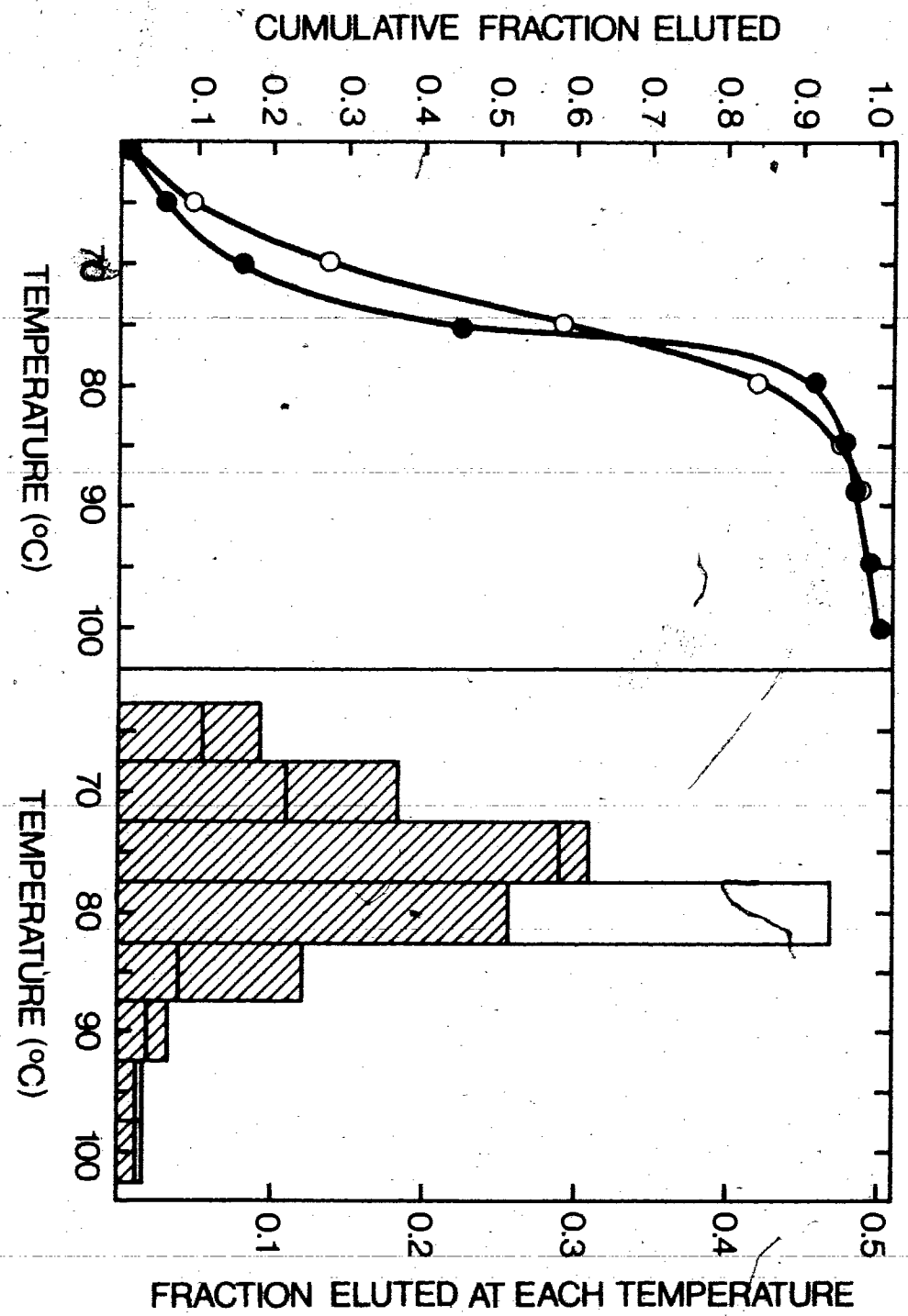

CUMULATIVE FRACTION ELUTED
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
TEMPERATURE (°C)
70
80
90
100
TEMPERATURE (°C)
70
80
90
100
0.1
0.2
0.3
0.4
0.5
FRACTION ELUTED AT EACH TEMPERATURE

Figure 12. Thermal denaturation profile of ^{125}I-chum DNA and sockeye driver DNA hybrids. Chum tracer DNA was reacted with a 10^4-fold sequence excess of sockeye DNA to a Cot of 150,000, and loaded on hydroxyapatite columns as outlined in Materials and Methods. Reassociated fragments were thermally eluted by raising the temperature of the column in 5°C increments. The graph in the left panel is a plot of the cumulative percent eluted from the column, while the bar graphs on the right have been plotted from the percent of total counts eluted at each temperature. Data from a chum driven reaction is represented by closed circles in the left panel, and open boxes of the bar graph on the right.


CUMULATIVE FRACTION ELUTED
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
TEMPERATURE (°C)
70
80
90
100
TEMPERATURE (°C)
70
80
90
100
0.1
0.2
0.3
0.4
0.5
FRACTION ELUTED AT EACH TEMPERATURE

Appendix 1. DNA reassociation criteria.

The rate at which complementary sequences reassociate depends basically, on four parameters:

1) the concentration of monovalent cations, which act to decrease the intermolecular repulsion of negatively charged DNA strands,

2) the incubation temperature which is optimally 25°C below the melting temperature, or T_m,

3) the DNA concentration which affects the frequency of strand collisions, and

4) the length of the DNA fragment which determines how many characteristic sequences will be present at any collision.

By keeping all four parameters constant, a reassociation curve can be generated which will be characteristic of the sequences to be examined. Standard criteria of reassociation are; 0.18 M cation concentration (0.12 M phosphate buffer) at 60°C. Incubation with 0.41 M phosphate buffer requires a reassociation temperature of 67°C and causes a five-fold increase in reaction rate. Reactions of this nature are therefore expressed in equivalent Cot units (Britten et al., 1974).

Appendix 2. Table illustrating computer-generated RMS values.

This table illustrates the relationship between fixed fraction size and subsequent RMS, of chum 0.3 kb fragments. Data was generated using a computerized least squares fitting procedure as outlined in Materials and Methods. The second rate constant for the single copy component was held constant at 3.4×10^{-4} $M^{-1}s^{-1}$.

Fraction Size	RMS Fragment Length (kb) 3.0	3.2
.04		0.027
.05		0.028
.1		0.034
.15		0.045
.20	0.028	
.25	0.026	
.30	0.025	
.35	0.026	
.40	0.031	

Appendix 3. Sample calculation of the percent cytosine bases iodinated.

specific activity = 1.26×10^7 dpm / ug DNA.

(assuming 50% counting efficiency)

^{125}I half-life = 60 days = 8.64×10^4 min.

therefore $K = \frac{\ln 2}{8.64 \times 10^4}$

$= 8.02 \times 10^{-6}$

$$N(t) = N_o e^{-KT}$$

$$\frac{-dN}{dT} = KN_o$$

at $t = 0$, $N_o = \frac{1.26 \times 10^7}{8.02 \times 10^{-6}}$

$= 1.57 \times 10^{12}$

1 µg DNA = 1.79×10^{15} nucleotides

GC content of chum salmon = 44.0%

therefore cytosine content = 22.0%

1 µg DNA = 3.95×10^{14} cytosine molecules

$$\frac{1.57 \times 10^{12}}{3.95 \times 10^{14}} = .004$$

Therefore 0.4% of the cytosine molecules or 0.1% of the total number of bases are iodinated.

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