NOAA Technical Memorandum OMPA-10

ALTERATIONS IN THE FREQUENCY OF SISTER CHROMATID EXCHANGES IN FLATFISH FROM PUGET SOUND, WASHINGTON, FOLLOWING EXPERIMENTAL AND NATURAL EXPOSURE TO MUTAGENIC CHEMICALS

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EXECUTIVE SUMMARY

Contamination of the aquatic environment by chemical pollutants has become a serious environmental problem which is heightened by the fact that a number of these contaminants have been demonstrated to be mutagenic or carcinogenic. This is of concern both from the standpoint of aquatic plant and animal health and of human health and it is, therefore, extremely important to be able to monitor the genetic effects of low level pollutants on marine organisms. One method that has been used recently to assess the effects of mutagenic chemicals on various organisms measures the rate of sister chromatid exchanges (SCE).

In the present study the technique of SCE was applied to English sole (Parophrys vetulus) collected from Puget Sound, Washington. SCE were observed 5.5 days after in vivo exposure to a thymidine analogue, bromodeoxyuridine (50 mg/g body wt) which allows for differentiation in the staining affinity of chromatids and visualization of exchanges between them. A baseline SCE rate of 2.16 exchanges per cell was determined. The sensitivity of this test was examined by subjecting the fish to the mutagen benzo(a)pyrene (B(a)P). The SCE rates of cells from fish exposed to three concentrations (0.05, 1.5, and 5 µg/g body wt) of intraperitoneally injected B(a)P were determined to be 2.99, 4.07, and 5.93 SCE/cell, respectively. The response was found to be dose-related and significantly higher than the baseline frequency at all concentrations of B(a)P. To test the efficacy of this technique as a useful tool in screening fish for evidence of exposure to mutagenic chemicals the chromosomes of English sole from a polluted site (Duwamish River, Seattle, Washington) were examined and found to have a significantly higher SCE rate (3.25 per cell) than the baseline.

This study showed that the technique of SCE could be successfully applied to a marine fish species and that the effects of low concentrations of mutagens could be detected using this method. Field data indicated that it was possible to sample fish from polluted sites, to examine them for SCE, and thus to use them as biological monitors of contamination of the marine environment.

1. INTRODUCTION

Epizootiological studies have revealed a higher incidence of neoplasms in fish living in polluted water than in those living in non-polluted water systems (Brown et al. 1973, Kligerman 1979, Stich and Acton 1976). Flatfish of the Puget Sound region, in particular members of the family Pleuronectidae, display a higher frequency of epidermal papillomas (Angell and Miller 1975, Cooper and Keller 1969, McArn et al. 1968) and also a high incidence of hepatomas (Pierce et al. 1978, Malins et al. 1980) at locations receiving industrial effluents.

Polluted waters often contain varying concentrations of mutagenic chemicals (Kraybill 1977, Kurelec et al. 1979, Brown et al. 1973). Evidence suggests that mutagens may be one factor involved in carcinogenesis (Ames et al. 1973, Loeb et al. 1974, McCann et al. 1975, Setlow and Hart 1974, Sirover and Loeb 1976, Stich and Acton 1976); and Ames et al. (1973), using a Salmonella test system, have shown that many carcinogens are in fact potent mutagens. Ames has proposed that those carcinogens that are also mutagens cause cancer by inducing somatic mutations.

Recently, a technique called sister chromatid exchange (SCE) has been employed to demonstrate chemically induced damage to the chromosomes of such species as mice (Allen and Latt 1976b), Chinese hamsters (Roszinsky-Kocher et al. 1979), humans (Solomon and Bobrow 1975, Rudiger et al. 1976, Latt 1974a), fish (Kligerman 1979, Alink et al. 1980, Barker and Rackham 1979), marine polychaete worms (Pesch and Pesch 1980) and the English broad bean (Kihlman 1975). The technique allows visualization of the exchange of genetic material between the two sister chromatids of a chromosome (Fig. 1) by exposing dividing cells (*in vivo* or *in vitro*) to the thymidine analogue 5'-bromodeoxyuridine (BrdU) for at least one round of DNA replication, followed by a second replication during which the presence of the analogue is optional. This method is generally used for *in vivo* exposures to BrdU, while *in vitro* methods normally apply BrdU to cultured cells for two replication cycles. Both techniques produce differentiation of chromatids upon staining.

During replication in the first cell cycle BrdU is incorporated into the newly synthesized strands of DNA in the place of normally incorporated thymidine so that each chromatid contains one BrdU-substituted DNA strand. Figure 2 shows a DNA strand that has replicated in the presence of BrdU. In Figure 3 the substituted strands are represented by the white bars. Chromosomes such as these will stain much lighter than unsubstituted chromosomes because the alteration of the DNA molecular structure on substitution with BrdU reduces their staining affinity (Goto et al. 1975).

During the second cell cycle, in the absence of BrdU, new thymidine-containing DNA strands are synthesized resulting in only one BrdU-substituted strand of the four DNA strands. The substituted chromatid will

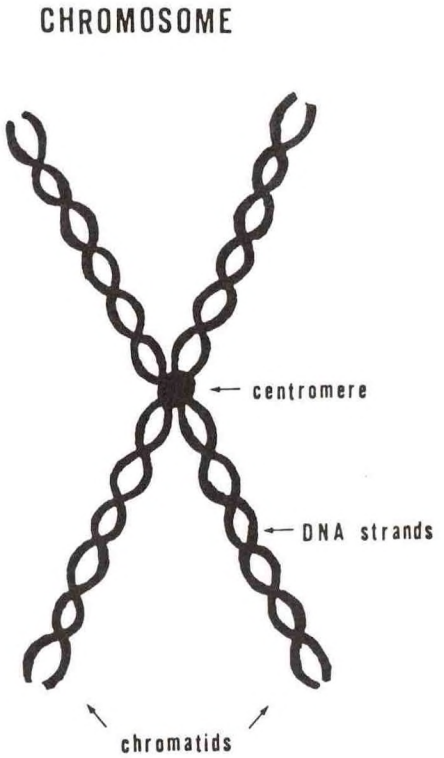

Figure 1. Diagram of a metaphase chromosome. Each chromatid is composed of two complementary DNA strands.

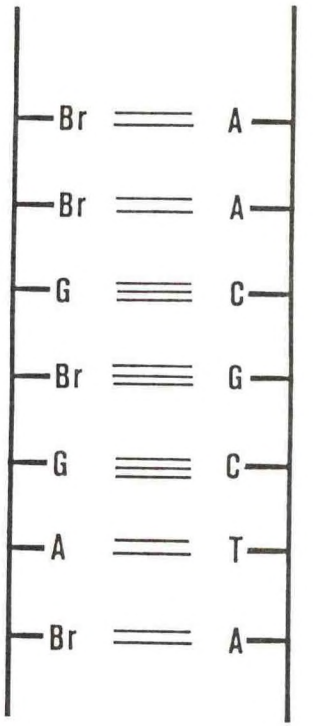

Figure 2. Diagrammatic representation of a DNA strand that has replicated in the presence of bromodeoxyuridine (Br). Br is substituted for thymidine and occasionally for cytosine in the new DNA strand.

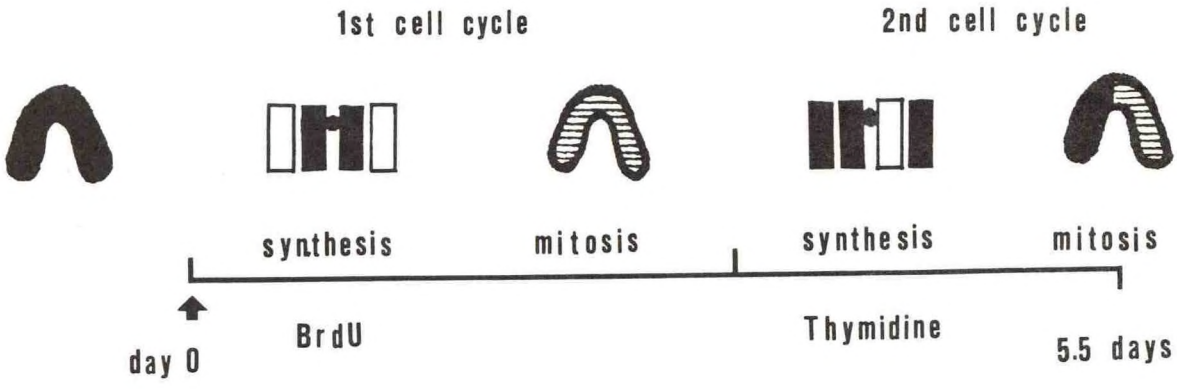

Figure 3. Diagrammatic representation of BrdU substitution for one round of DNA replication followed by one round of replication in the presence of native thymidine and resulting staining patterns. Black bars represent unsubstituted DNA strands and white bars represent substituted DNA strands. Black chromosomes represent chromatids in which no strands are substituted and which stain darkly with Giemsa. Hatched chromosomes represent chromatids in which one of the strands is substituted and which stain lightly with Giemsa.

stain lightly as before; however, the unsubstituted chromatid will stain darkly allowing differentiation between the two chromatids (sister chromatid differentiation-SCD) (Fig. 3). Exchanges between chromatids can be detected by the presence of a piece of darkly stained chromatid attached to a lightly stained chromatid and vice versa (Fig. 4).

Latt (1974a) was the first to apply this new technique to study the effects of mutagens on chromosomes. Using mitomycin C, an agent which is known to cross-link complementary DNA strands, he demonstrated the sensitivity of this technique by showing that significant increases in the number of SCE could be detected when as little as 0.003 µg mitomycin C/ml culture medium was applied to cells, while concentrations of 0.01 to 0.1 µg/ml were required to produce large numbers of gross chromosomal breaks.

The chromosomal damage leading to the production of SCE's has been correlated with mutations. Carrano et al. (1978) found a linear relationship between induced SCE's and mutations occurring at a particular locus when Chinese hamster ovary cells were treated with alkylating agents (ethyl methanesulphonate and N-ethyl-N-nitroso-urea) and a cross-linking agent (mitomycin C). They also determined this relation to be dose-responsive; that is, a linear increase in both SCE rate and mutation rate was observed.

The technique of sister chromatid exchange can be a valuable tool for screening organisms for exposure to environmental pollutants. It can reveal damage caused by relatively recent exposure to chemical mutagens; it is dose-responsive; and it is sensitive to very low concentrations of a substance, at which no gross chromosomal aberrations are detected. An important application of this technique has been to the study of the effects of carcinogens on human chromosomes. Rudiger et al. (1976) determined a doubling of the SCE rate in cultured human lymphocytes after 70 hours incubation in benzpyrene. Significant increases in SCE's were observed by Latt (1974a) after exposure of cultured human lymphocytes to 3 µg/ml of mitomycin C, while few gross chromosomal aberrations were seen in cells that had as many as 100 SCE. SCE has also been used to screen humans for exposure to carcinogenic chemicals (Murthy 1979). For example, lymphocytes cultured from workers exposed to industrial chemicals show increased SCE rates as do those cultured from other members of their families (Funes-Cravioto et al. 1977). Patients undergoing treatment with cytostatic drugs such as andriamycin also exhibit increases in the SCE rates of their lymphocytes and marked decreases in the rate when taken off the drug (Perry and Evans 1975, Musilova et al. 1979).

The aim of this study was to evaluate the effectiveness of the SCE technique in screening marine fish for exposure to mutagenic chemicals. In this investigation the SCE procedures were adapted and applied to the English sole (Parophrys vetulus) and a baseline SCE rate was determined for these fish. To test the responsiveness of this technique laboratory exposures to several doses of a known mutagen were performed. To test its efficacy in screening fish for environmental exposure to mutagens the

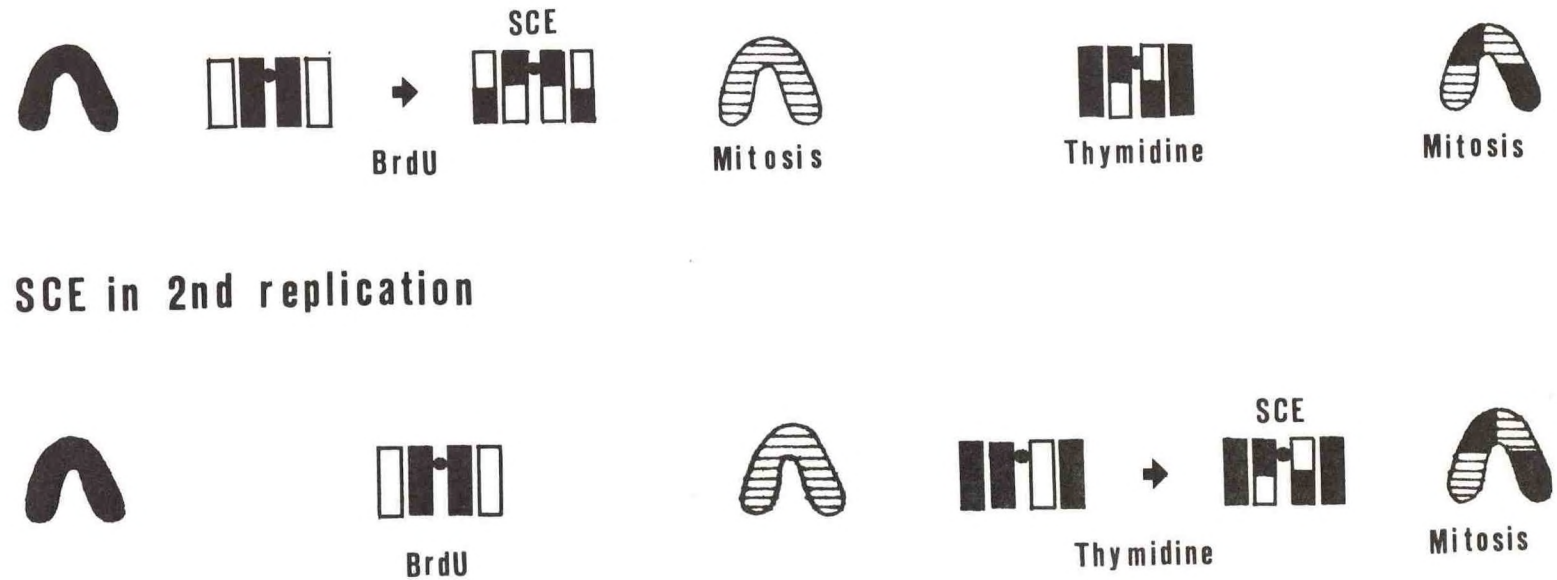

Figure 4. Diagrammatic representation of sister-chromatid exchanges and BrdU substitution and resulting staining patterns.

chromosomes of English sole from a polluted site (Duwamish River, Seattle, Washington) were examined for SCE to determine whether significant increases over the baseline frequency could be detected.

2. MATERIALS AND METHODS

2.1 Fish Capture and Maintenance

Juvenile English sole (Parophrys vetulus) were collected both by beach seine and by otter trawl from selected sites in Puget Sound and adjacent waters. The fish were housed in circular fiberglass tanks at the University of Washington College of Fisheries and held at 14°C. They were fed minced clams and oysters daily ad libitum.

2.2 In vitro Procedures

2.2.1 Leukocyte Culture

Leukocytes were cultured following procedures adapted from Thorgaard (1977). Three ml of blood was obtained from adult English sole by caudal vein puncture using a sterile heparinized syringe. The blood was transferred to a centrifuge tube and the leukocytes were separated by repeated centrifugation (three times) at 40 g for five minutes each. The leukocyte-rich plasma was then removed and pooled after each centrifugation. The plasma containing the leukocytes was added to 2 ml of Liebowitz-15 medium or Eagle's minimal essential medium in a culture bottle. The medium had final concentrations of 0.03 percent streptomycin and 0.03 percent penicillin and was supplemented with glutamine at a concentration of 0.29 mg/ml of medium. The initial concentration of cells was approximately 10^6 cells per 2 mls of medium. To adjust the osmolarity of the medium, 1.35 ml of 26 percent NaCl solution per 100 ml of medium was added.

To stimulate cell division mitogens and/or serum were used. Two mitogens, pokeweed mitogen (Gibco) and phytohemagglutinin (PHA-Burroughs Wellcome) were tried at final concentrations of 0.05, 0.1, and 1 percent. Fetal calf serum (Gibco), human serum (Gibco), and autologous fish serum were added to final concentrations of 5, 10, 15, and 20 percent. The cultures were incubated for five to six days at 18°C.

2.2.2 Chromosome Preparation

Five µg/ml of colchicine were added to the cultures six hours before harvesting the cells to suspend the chromosomes at metaphase. At the appropriate time the contents of the culture bottle were transferred to a centrifuge tube and spun for five minutes at 225 g, after which all but 0.25 ml of medium was removed from the tube. A hypotonic treatment (to

swell the cells) of 0.0375 M KCl at room temperature for 15 minutes was followed by another centrifugation at 225 g for five minutes. All but 0.25 ml of this liquid was removed. It was subsequently washed in 4 ml of freshly prepared absolute methanol-glacial acetic acid (3:1) and centrifuged again. After all but 0.25 ml was discarded 4 ml of methanol-acetic acid was added and the cells were fixed overnight at 4°C. Following fixation the cells were washed twice in fixative and then dropped on microscope slides, air dried, and stained with 5 percent Giemsa (Gurr's R66, Bio/Medical Specialties) in Sorenson's buffer pH 6.8.

2.3 In vivo Procedures

2.3.1 Bromodeoxyuridine Exposure

BrdU powder (Cal Biochem) was weighed to the desired amount (50 to 100 mg, giving a dosage of 0.5 mg/g body wt) and packed into tablet form using a pellet press. Cholesterol was added in equal amounts to some pellets prior to pressing to serve as a vehicle and to control the dissolution of the tablets.

Implantation of the pellets was accomplished by inserting the tablet through a 0.5 cm vertical incision into the lower right peritoneum on the blind side of an adult English sole which had been anesthetized with MS-222 (Crescent Research Chemicals, Paradise Valley, Arizona). After insertion of the tablet the incision was closed with suture and the fish were held for three to seven days.

BrdU was also administered by intraperitoneal (i.p.) injection. Unanesthetized juvenile English sole were injected with 0.5 mg/g body wt of BrdU in 0.1 ml of sterile saline-glycerol solution (40 percent glycerol) or 0.1 ml of 10 percent Dimethylsulfoxide (DMSO) solution. The fish were held five to seven days.

2.3.2 Processing Tissue

Six hours prior to sacrifice the fish were injected i.p. with colchicine (Eli Lilly 1 mg/2 ml) at a concentration of 0.01 ml/g body wt. Fish were sacrificed by overdose with MS-222. Kidney, spleen, intestine, gill, and fin samples were minced in 0.0375 M KCl solution. The tissues were fixed in absolute methanol-glacial acetic acid (3:1) for two hours or overnight at 4°C. Following fixation pieces of tissue were transferred to 60 percent acetic acid in absolute methanol for 15 minutes to dissociate the cells. This liquid was then dropped onto clean microscope slides and allowed to air dry.

Chromosome preparations to be examined by fluorescent microscopy were stained with 0.5 µg/ml of 33258 Hoechst in phosphate buffered saline pH 7.0 (Latt 1974a) for five minutes.

Permanently stained chromosome preparations were made using a variety of published protocols but with variations in the concentration of reagents, staining times, and buffer pH (pH 6 to pH 8 at 0.2 increments). These included the hot phosphate technique of Korenberg and Freedlender (1974), the "reverse" staining method of Scheres et al. (1977), and the fluorescent plus Giemsa (FPG) method of Perry and Wolff (1974) and Wolff et al. (1975). According to the FPG technique the chromosome preparations were treated with Hoechst 33258 dye for five minutes, then immersed in phosphate buffered saline-distilled water (1:2) and exposed to a strong cool white light for up to 18 hours prior to staining with 5 percent Giemsa in Sorenson's buffer pH 6.8 for five to ten minutes. Direct staining of chromosomes in 0.3 molar Na_2HPO_4-Giemsa (5 percent) solution (unpublished method of Kocan) at various alkaline pH's accomplished the staining of chromosome preparations that did not stain using the FPG technique.

Photographs of permanently stained metaphases displaying SCD and SCE were taken on an Olympus photomicroscope through a 100X acromatic objective using a green substage filter and Kodak Panatomic-X film.

2.4 Radioactive Labelling

Radioactive labelling studies were performed to aid in determining the proper length of time required for exposure of the fish to BrdU.

Adult English sole, each weighing approximately 250 g, received single i.p. injections of 3 H-thymidine (Amersham, Arlington Heights, Ill., specific activity of 1 Ci/mmole) followed 60 minutes later by a single thymidine chase of 5 mg in 0.5 ml sterile saline.

Twenty fish were injected with a dose of 3 H-thymidine of 0.1 μCi/g body wt. Two fish were sacrificed by MS-222 overdose at twelve-hour intervals for up to 168 hours. One hundred milligrams of tissue from the anterior kidney was homogenized with 5 ml of distilled water. A 1 ml aliquot was transferred to a scintillation vial and treated with 1 ml of Protosol tissue stabilizer (New England Nuclear) for five minutes at room temperature. Finally 15 ml of scintillation fluor was added to this mixture and radioactivity of the samples was measured in a Packard Tri-Card liquid scintillation spectrometer.

Tissue samples were also taken from the anterior kidney for light autoradiography. They were fixed in half-strength Karnovsky's fixative (Karnovsky 1965) in phosphate buffer pH 7.4 for 24 hours, rinsed twice in buffer at 30 minutes each and dehydrated through graded alcohols (50, 70, 95, and 100 percent) and propylene oxide. Dehydrated samples were infiltrated and embedded in Epon (Luft 1961). One micron sections were cut on a Reichert OM-U2 ultramicrotome, dried onto microscope slides and coated with Kodak NTB-2 emulsion (1:1 with distilled water). Coated slides were stored at 4°C for two, six, and ten weeks and then developed in full-strength Kodak Microdol-X.

A second group of 10 fish was injected i.p. with 3 H-thymidine (0.4 μCi/g body wt) followed 60 minutes later by a thymidine chase (5 mg in 0.5 ml sterile saline) and sacrificed by MS-222 overdose every 24 hours for up to 216 hours post-tritiated thymidine injection. Radioactivity was measured by liquid scintillation. No tissues were taken for autoradiography.

2.5 Field Study

Juvenile English sole, weighing approximately 60 g each, were collected by otter trawl near the mouth of the Duwamish River, Seattle, Washington (Fig. 5). The fish were processed, as discussed in "*In vivo* Procedures," immediately upon return to the University of Washington.

2.6 Benzo(a)pyrene Exposures

Juvenile English sole, weighing approximately 30 g each, were collected by beach seine near Agate Pass in Puget Sound (Fig. 5) and held in glass aquaria in artificial seawater (14°C) at the University of Washington.

B(a)P was dissolved in sterile corn oil and injected intraperitoneally in doses of 0.5, 1.5, or 5 μg/g body wt. A single B(a)P injection was administered, followed by a single BrdU injection (0.5 mg/g body wt) two days later, and another B(a)P injection of the same dosage was administered on the following day. Table 1 outlines the protocol used. After sacrificing the fish the tissue was processed according to the methods discussed previously.

2.7 Additional Species Investigated

Metaphase chromosomes were prepared from the kidney tissue of three species of marine fish: saddleback gunnel (*Pholis ornata*), silver-spotted sculpin (*Blepsias cirrhosis*), and Pacific staghorn sculpin (*Leptocottus armatus*). The chromosomes were stained in 5 percent Giemsa in Sorenson's buffer (pH 6.8) and photographs were taken on a Leitz photomicroscope through a 100X acromatic objective using a green substage filter and Kodak Panatomic-X film.

3. RESULTS

3.1 Leukocyte Culture

No metaphase spreads were obtained from these cells using any of the differing culturing conditions; consequently, it was necessary to investigate preparations from other tissues.

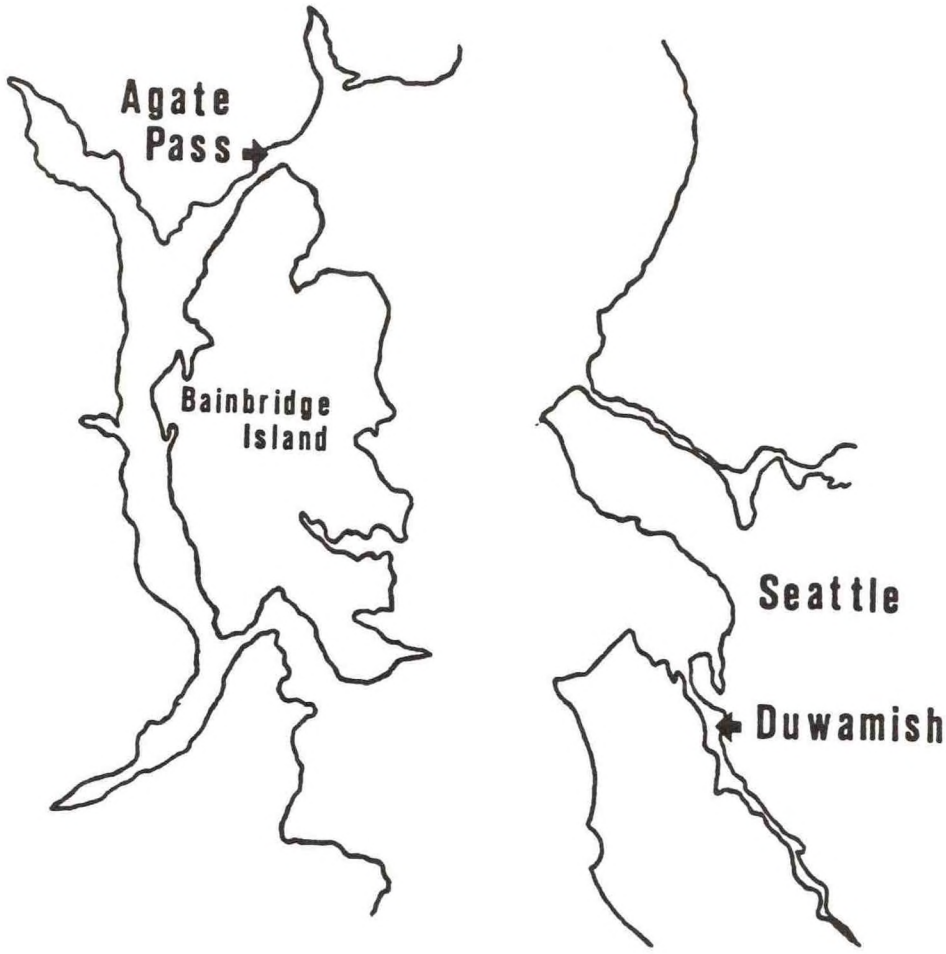

Figure 5. Map of central Puget Sound and sampling sites. Agate Pass was the control site and Duwamish the test site.

Table 1. Protocol for benzo(a)pyrene exposures.

Treatment	Time
1st B(a)P injection	day 0
BrdU injection	day 2
2nd B(a)P injection	day 3
Colchicine injection	day 7
Sacrifice	day 7

3.2 *In vivo* Procedures

3.2.1 Karyotype

Metaphase spreads were obtained from fish exposed to BrdU by both pellet implantation and i.p. injection methods. The chromosome complement of *P. vetulus* was determined to consist of 48 (2n) chromosomes of approximately 2-4 µm in length. Two of these possess a metacentric centromere while all of the others are acrocentric (Fig. 6).

3.2.2 Selection of Tissue for Study

The number of metaphases displaying SCD obtainable depended on several factors: the tissue used, the dose of BrdU, the length of exposure to BrdU and time of sacrifice, and the staining of the chromosome preparations. Kidney tissue was selected as the tissue for use in this study because it displayed the highest mitotic activity of all tissues sampled and routinely produced the greatest number of usable metaphase spreads per preparation in non-BrdU treated fish (Fig. 7).

3.2.3 Bromodeoxyuridine Exposure

A BrdU dose of 0.5 mg/g body wt appeared to have no adverse effect on the health of the fish and delivered a sufficient amount of BrdU for incorporation into the DNA. Higher doses (0.75 mg/g and 1.0 mg/g) produced adverse effects, including spotty livers and hemorrhagic enteritis. Many mortalities resulted at the highest dose. Electron microscopic examination of the livers of these fish revealed hepatocellular vacuolation, congestion, and swelling of the mitochondria and endoplasmic reticulum. The BrdU was dissolved for administration in a solution of glycerol (40 percent) in saline. This allowed dissolution of the drug and did not adversely affect the fish. Dimethylsulfoxide (DMSO) was also tried; however, these fish injected with DMSO exhibited inflamed vents, bloody anal discharge and in some cases prolapsed intestines.

3.2.4 Peak Time of Chromosomal Differentiation

Chromosome preparations made from fish sacrificed five to seven days post BrdU injection displayed some metaphases with BrdU incorporation; however, the peak rate of SCD occurred at 5.5 days.

3.2.5 Staining for Chromosomal Differentiation

Only two of the staining techniques investigated successfully stained the English sole preparations. Most exhibited SCD on staining with the FPG method (Wolff 1975); however, some did not. The disodium phosphate-Giemsa technique (Kocan, unpublished) at pH 10.7 revealed SCD in preparations that did not stain with FPG, and in fact produced adequate differentiation in preparations aged for up to two months. Figure 8 shows a control

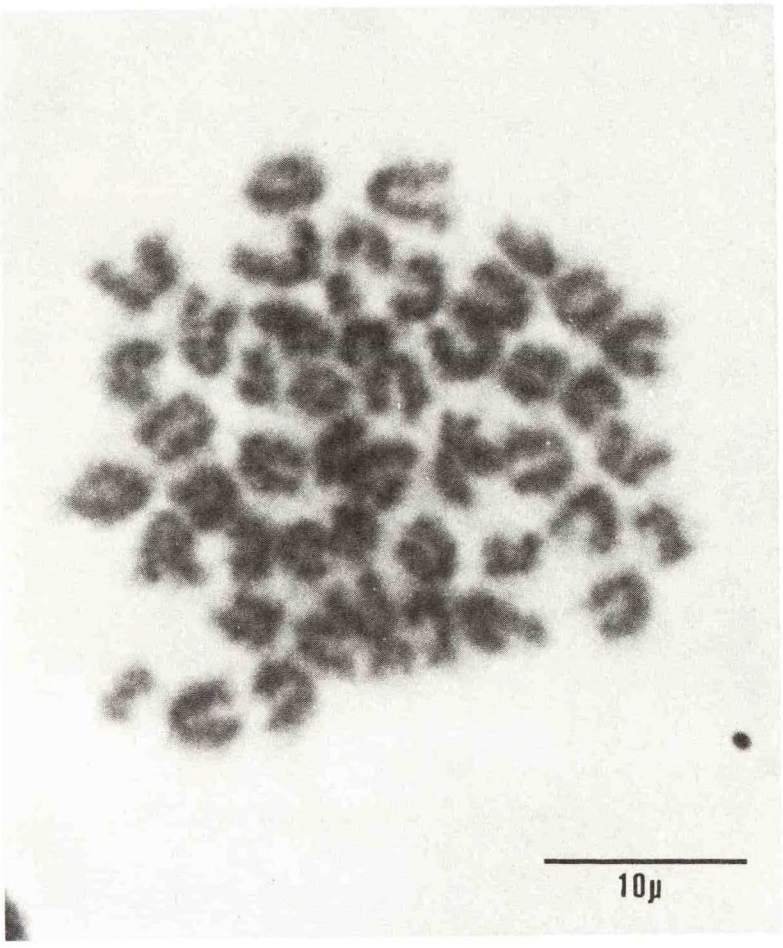

Figure 6. A normal metaphase chromosome spread from the kidney of an English sole (Parophrys vetulus).

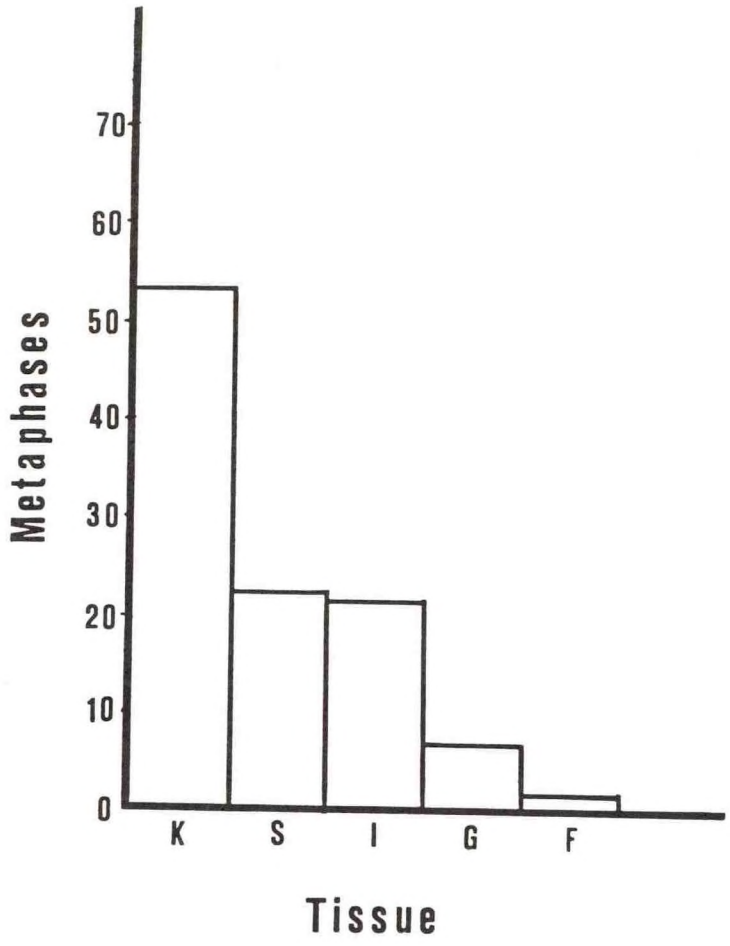

Figure 7. Average number of metaphases obtained per slide for different tissues of the English sole. K=kidney, S=spleen, I=intestine, G=gill, and F=fin epithelium.

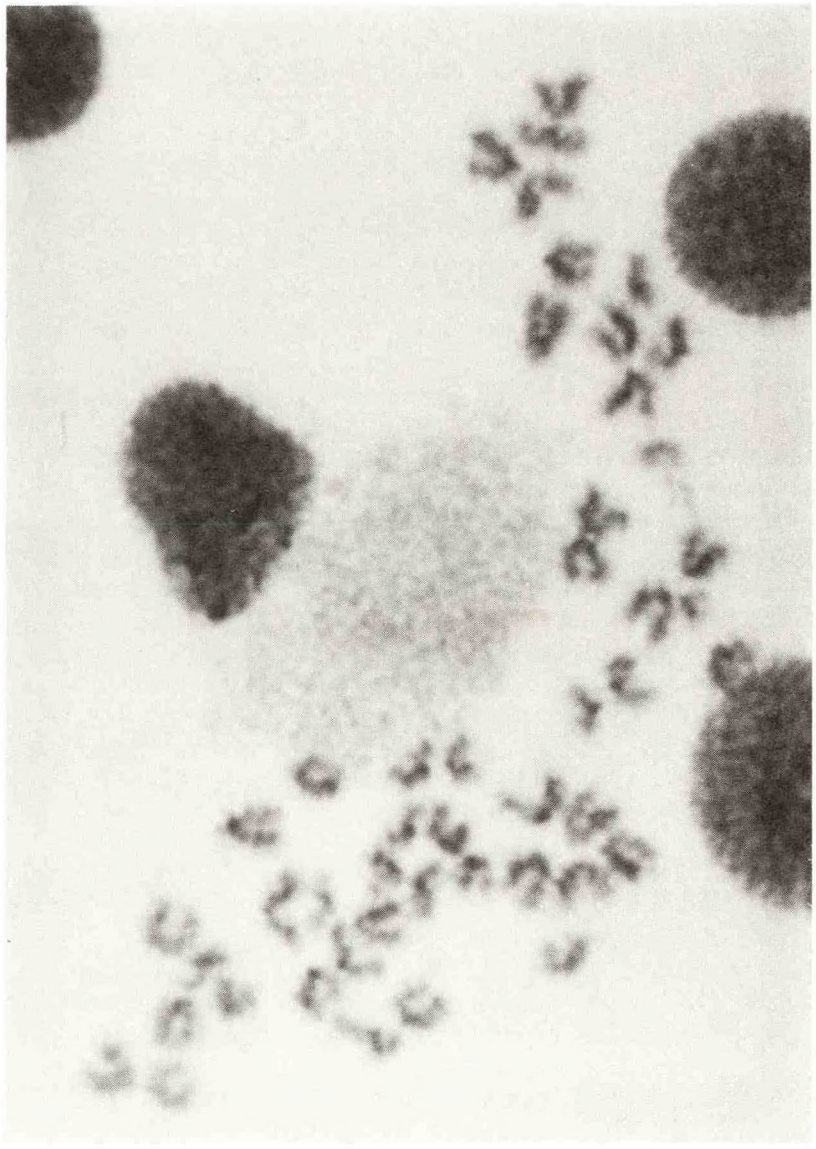

Figure 8. A control metaphase chromosome spread from the kidney tissue of an English sole. Stained with Giemsa.

metaphase stained with Giemsa, while in Figure 9 an FPG stained spread displaying SCD and two SCE is seen.

3.3 Radioactive Label

Radioactivity of the kidney tissue, measured in average counts per five minutes, for each time interval is shown in Figure 10.

Theoretically cells that are synthesizing DNA (in S-phase) at the time of the tritiated thymidine injection will become labelled as will cells that pass into S-phase during the labelling period. A peak in radioactivity occurred in the English sole between 12 and 24 hours after injection. This indicated an S-phase length of between 12 and 24 hours, after which time the cells entered premitosis (G_2-phase) and mitosis (M-phase).

A peak was also seen at approximately five days after tritiated thymidine injection; however, this may have been a sampling artifact and not actually a peak. Theoretically, a single peak of radioactivity should be evident, followed by a levelling-off, and finally an exponential decay as cells continue to replicate or leave the kidney as they mature. Confidence intervals for the sampling times overlapped, thereby implying no significant difference between the sample points except for times 0 and 12 hours. Since different fish were used for each time the large differences seen in amounts of radioactivity among fish may reflect a variety of conditions (fish health, metabolic differences, etc.) that could affect incorporation of the tritiated thymidine. Ideally, the sample size should be very large or if possible the same organism should be sampled over time.

3.4 Baseline Frequency of SCE

The baseline frequency of SCE in the kidney of English sole was determined to be 2.16 SCE per cell. The data are presented in Table 2. There were no significant differences in the number of SCE with respect to the sex of the fish. The mean SCE rate for females was 2.48 and for males was 2.10.

3.5 Field Study

SCE data for fish collected in the Duwamish River, Seattle, Washington, are presented in Table 3. The mean SCE frequency was found to be 3.25 SCE per cell. The frequency of SCE was determined by the Student t-test to be significantly greater than the baseline ($p < 0.05$). There were no significant differences in the number of SCE with respect to the sex of the fish. The mean SCE rate for females was 3.46 and for males was 3.14.

3.6 Benzo(a)pyrene Exposures

The results of the three concentrations of B(a)P are presented in Tables 4, 5, and 6. Mean SCE were 2.99 per cell for 0.05 µg/g body wt,

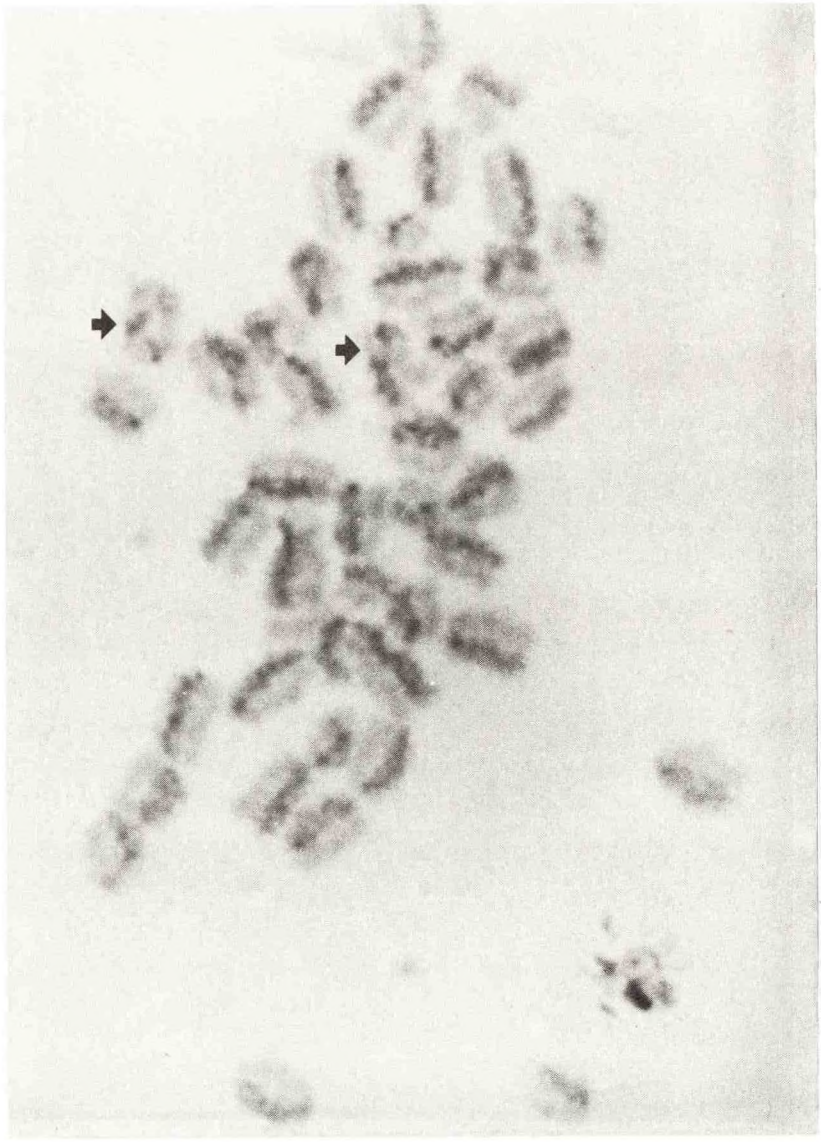

Figure 9. A metaphase chromosome spread from the kidney tissue of an English sole that had been treated with bromodeoxyuridine (BrdU). Sister chromatid differentiation (SCD) is evident and two sister chromatid exchanges (SCE) may be seen (arrows). Fluorescent plus Giemsa stain.

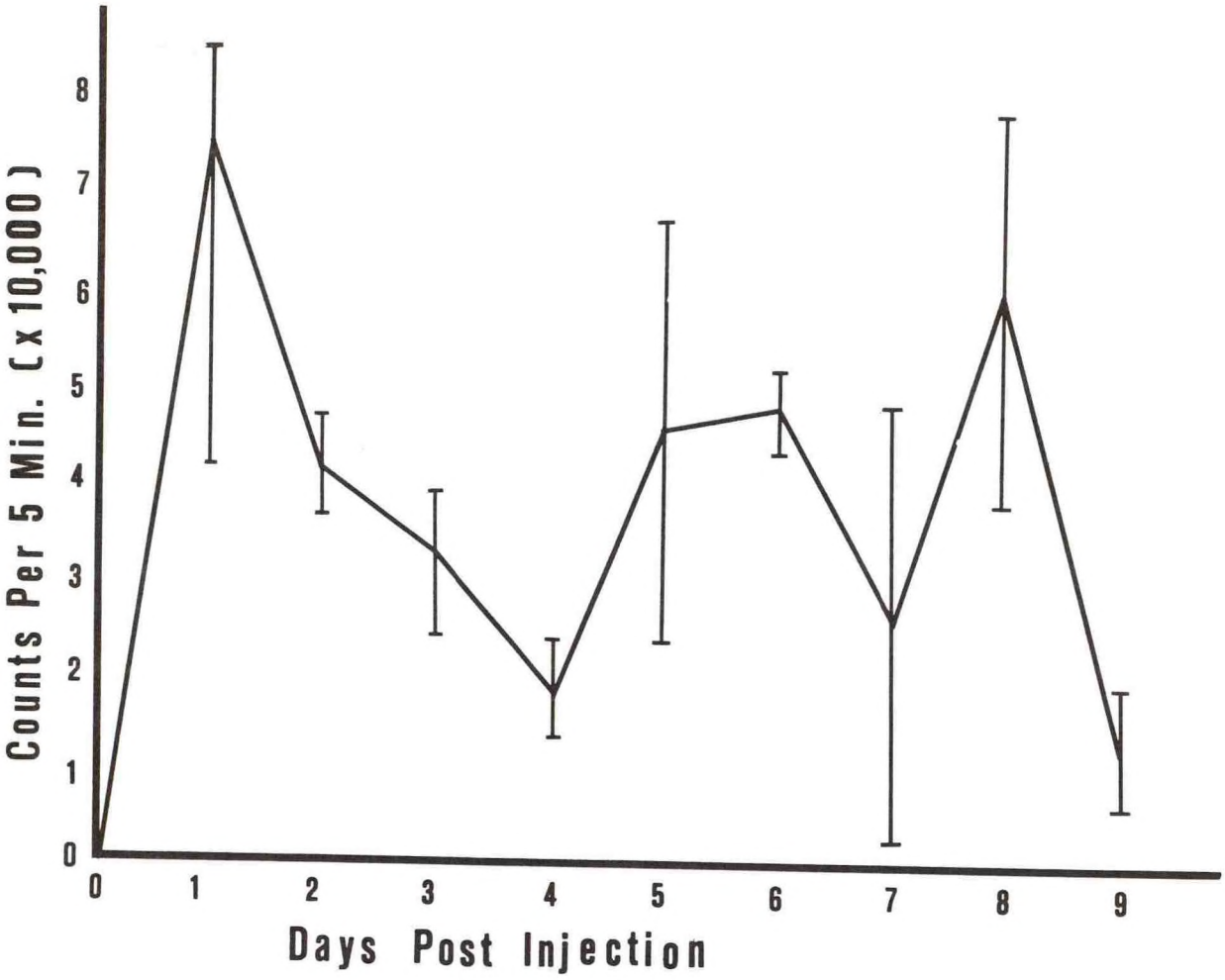

Figure 10. Radioactivity (measured in counts per 5 min) over time, of the anterior kidney tissue from a ^{3}H-thymidine-treated English sole. Bars represent 95% confidence intervals. Sample number (N) per point = 4.

Table 2. Summary of the SCE data of a sample of English sole from the control site near Agate Pass in Puget Sound, Washington.

Fish No.	Total No. Metaphases	No. Metaphases with SCD	No. Metaphases Displaying 0 to 6 SCE							Mean SCE/ Metaphase (X ± S.D.)
			0	1	2	3	4	5	6	
1	69	7	1	2	2	2				1.71 ± 0.49
2	54	12	2	1	5	3	1			2.00 ± 1.21
3	109	17		4	8	3	2			2.18 ± 0.95
4	43	2			2					2.00
5	7	1		1						1.00
6	39	1		1						1.00
7	70	16	-	1	8	5	1		1	2.63 ± 1.15
Total 7	391	56	3	10	25	13	4		1	2.16 ± 1.09

Table 3. Summary of the SCE data of a sample of English sole from the test site in the west waterway of the Duwamish River, Seattle, Washington.

Fish No.	Total No. Metaphases	No. Metaphases with SCD	No. Metaphases Displaying 0 to 7 SCE								Mean SCE/ Metaphase (X ± S.D.)
			0	1	2	3	4	5	6	7	
A	74	15	2	2	2	3	4	1	1		2.80 ± 1.78
B	6	1				1					3.0
C	20	5			1	1	1		1	1	4.40 ± 1.79
D	50	7	1			3	2		1		3.29 ± 1.80
E	71	16			2	5	4	2	3		3.94 ± 1.34
F	58	25	1	2	6	3	7	6			3.24 ± 1.43
G	56	13		2	3	3	3	1	1		3.08 ± 1.41
H	35	7		2	1	1	3				2.71 ± 1.20
I	23	3			1	1	1				2.66 ± 1.00
J	99	26	_	_	6	9	6	5	_	_	3.38 ± 1.00
Total 10	492	118	4	8	22	30	31	15	7	1	3.25 ± 0.55

Table 4. Summary of the SCE data of a sample of English sole (from the control site) that had been experimentally exposed to 0.5 µg benzo(a)pyrene/g body wt.

Fish No.	Total No. Metaphases	No. Metaphases with SCD	No. Metaphases Displaying 0 to 6 SCE							Mean SCE/ Metaphase (X ± S.D.)
			0	1	2	3	4	5	6	
1	136	12	1	1	4	3	3			2.50 ± 1.20
2	228	25		1	6	13	4	1		2.92 ± 0.86
3	138	20			6	9	3	1	1	3.10 ± 1.07
4	220	37	_	2	8	9	9	8	1	3.43 ± 1.60
Total 4	722	94	1	4	24	34	19	10	2	2.99 ± 0.39

Table 5. Summary of the SCE data of a sample of English sole (from the control site) that had been experimentally exposed to 1.5 µg benzo(a)pyrene/g body wt.

Fish No.	Total No. Metaphases	No. Metaphases with SCD	No. Metaphases Displaying 0 to 7 SCE								Mean SCE/ Metaphase (X ± S.D.)
			0	1	2	3	4	5	6	7	
1	152	17				2	6	5	2	2	4.76 ± 1.20
2	146	39	1	6	8	12	9	2	1		2.82 ± 0.84
3	230	44				9	18	11	4	2	4.36 ± 1.06
4	190	33				10	16	5	2	1	4.18 ± 0.68
5	198	27	–	–	1	8	8	5	3	2	4.26 ± 1.32
Total 5	916	160	1	6	9	41	57	28	12	7	4.07 ± 0.74

Table 6. Summary of the SCE data of a sample of English sole (from the control site) that had been experimentally exposed to 5.0 µg benzo(a)pyrene/g body wt.

Fish No.	Total No. Metaphases	No. Metaphases with SCD	No. Metaphases Displaying 3 to 14 SCE									Mean SCE/ Metaphase (X ± S.D.)
			3	4	5	6	7	8	9	10	14	
1	55	15	1	6	5	1	1	1				4.86 ± 1.30
2	151	33		1	11	9	5	3	2	2		6.36 ± 2.30
3	145	26	_	1	6	9	6	1	1	1	1	6.58 ± 2.78
Total 3	351	74	1	8	22	19	12	5	3	3	1	5.93 ± 0.94

4.07 for 1.5 µg/g, and 5.93 for 5 µg/g. Statistical analyses were performed using the Student t-test. Significant increases in SCE were found for each concentration and SCE number was determined to be highly correlated with the dose of B(a)P ($r = 0.8644$). The dose-response curve to B(a)P is shown in Figure 11.

B(a)P had no apparent effect on the total number of metaphases in most fish at all doses. One fish exposed to 5.0 µg/g showed a significant reduction in the total number of metaphases (Table 6). This fish appeared moribund at the time of sacrificing.

The percentage of metaphases displaying SCD was not affected by B(a)P treatment (Table 7). Health of the fish was adversely affected by B(a)P exposure and could be correlated with dose (Table 7).

3.7 Additional Species Investigated

Chromosomes of three additional species of marine fish (Pacific staghorn sculpin, silver spotted sculpin, and saddleback gunnel) were examined. All had a diploid (2n) number of approximately 40 and the chromosomes were about 3-5 µm in length (Fig. 12).

4. DISCUSSION

4.1 SCE Analysis

The initial objective of this study was to induce peripheral leukocytes to divide in culture and to pass through two rounds of replication in the presence of BrdU so that they might be used in SCE analysis. This was not possible using the English sole. Chromosome spreads have been obtained from the cultured cells of marine fish but these have been from established cell lines (Regan et al. 1968) or from cultured kidney cells (Park and Park 1979). Kang and Park (1975) obtained large numbers of metaphase spreads from PHA-stimulated eel leukocytes only when the cells were cultured without autologous serum. This method and many other methods were attempted using leukocytes from the English sole but with no success.

Since the *in vitro* culture of leukocytes did not produce dividing cells, *in vivo* methods of BrdU incorporation were attempted. Two methods of exposure were used: implantation of pellets and i.p. injection. Although both treatments effected BrdU incorporation, implantation of pellets was abandoned and only i.p. injection was used since this procedure proved to be more rapid and less stressful to the fish.

The anterior kidney was selected as the best tissue to use because of its high mitotic activity and its ability to yield large numbers of useable

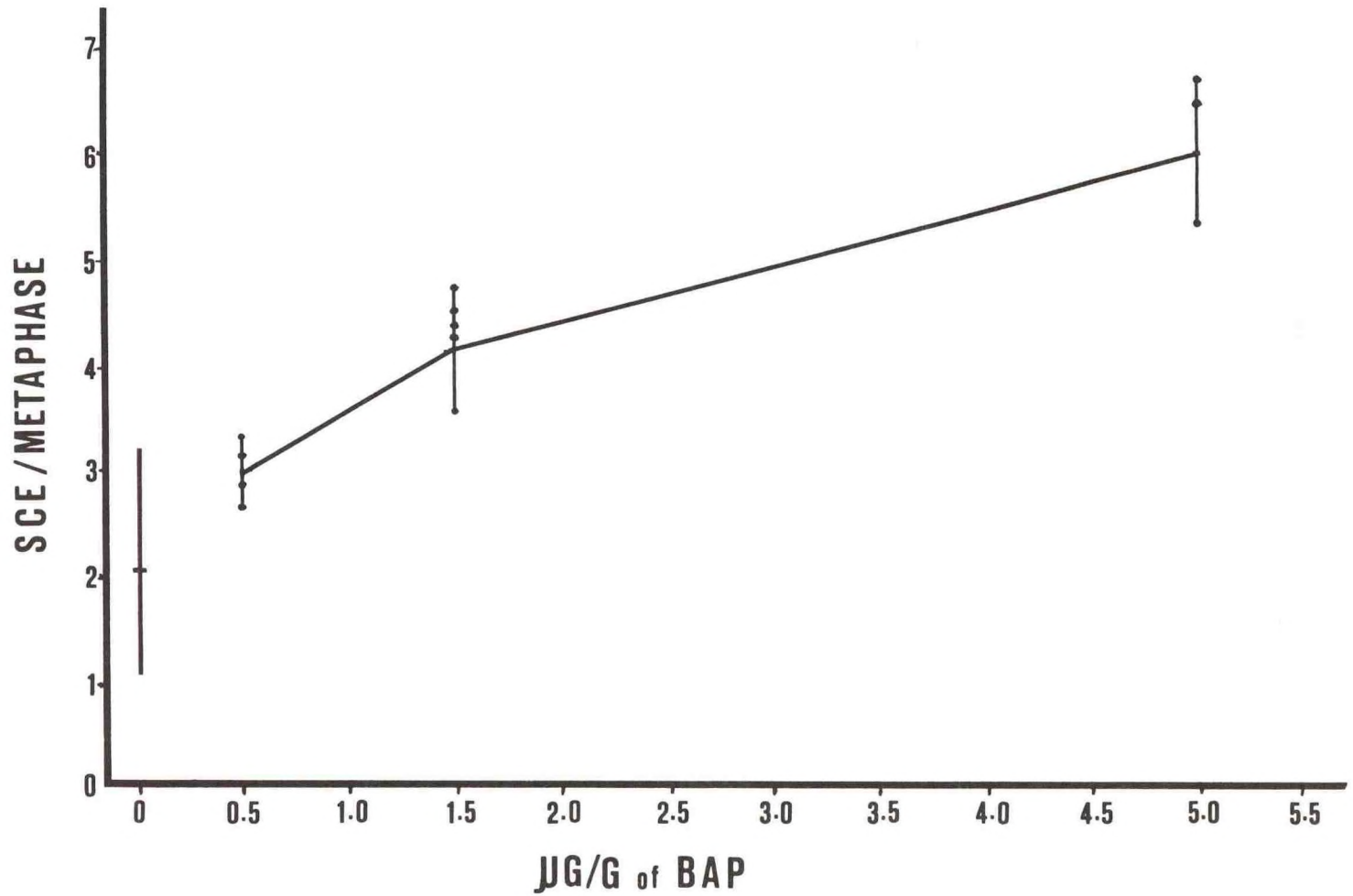

Figure 11. Dose-response curve for SCE frequencies in English sole exposed to three concentrations of benzo(a)pyrene (X ± S.D.).

Table 7. Summary of the effects of the benzo(a)pyrene treatments.

Dose	Percent SCD	Mean No. SCE	Exposed ÷ Control	General Physical Characteristics
0.0	14	2.16	--	normal
0.5	13	2.99	1.38	normal, actively feeding
1.5	17	4.07	1.88	tan liver, intestinal vessels prominent, enlarged gall bladder, reduced feeding
5.0	21	5.93	2.75	tan, congested liver, gastro-intestinal tract empty, enlarged gall bladder containing clear fluid, lethargy

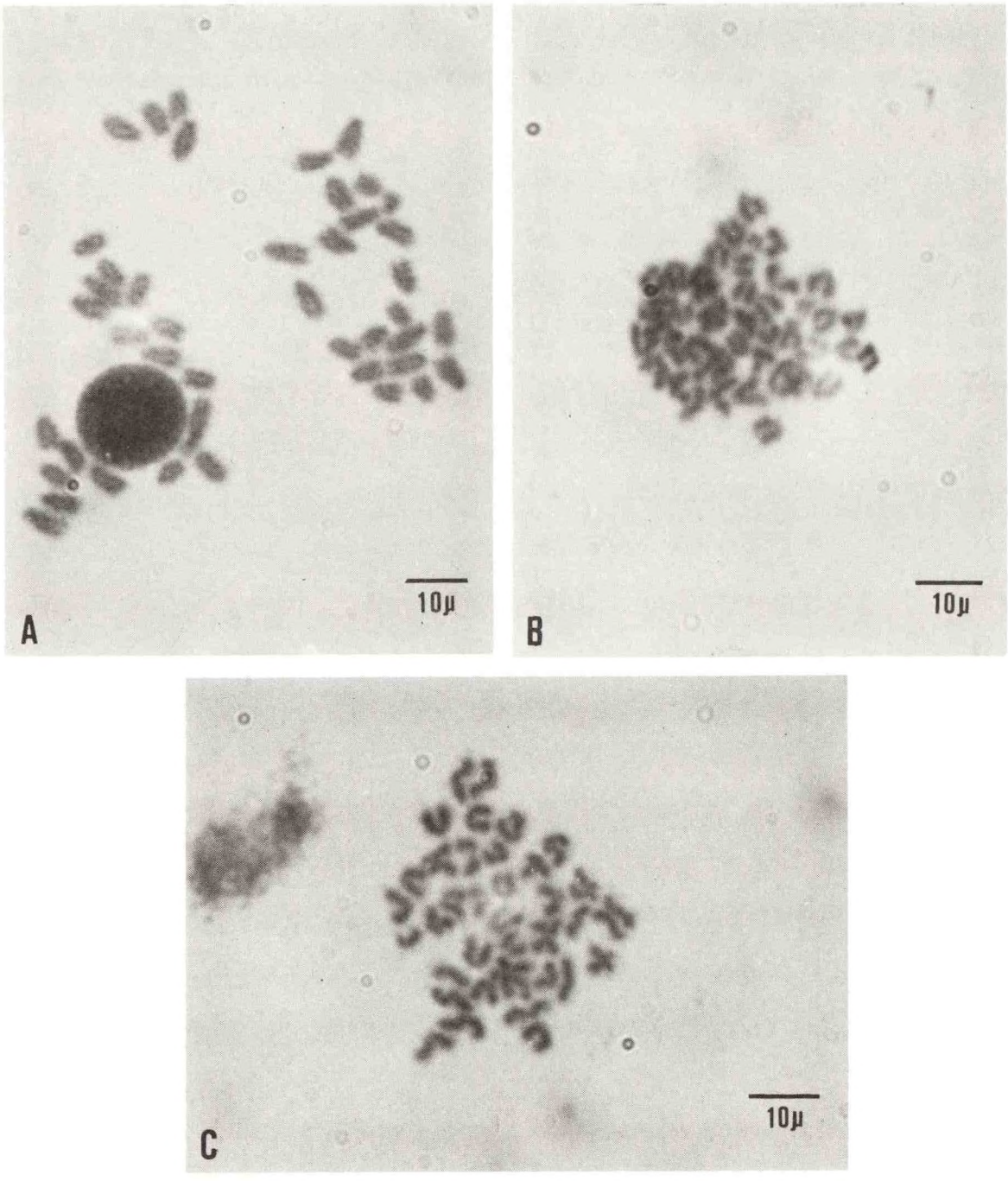

Figure 12. Metaphase chromosome spreads from three species of marine fish: (A) silver-spotted sculpin (Blepsias cirrhosis), (B) saddleback gunnel (Pholis ornata), and (C) Pacific staghorn sculpin (Leptocottus armatus).

metaphases. It is important to select a tissue such as this because less than optimum general health and poor feeding behavior of the test organism and exposure to BrdU can depress mitotic activity and the incorporation of BrdU considerably. Kligerman and Bloom (1976) also found this to be true in the central mudminnow (Umbra limi), but they determined that the intestines produced the highest number of metaphases per preparation of all tissues sampled and the kidney the next highest.

Although large numbers of metaphase figures may be obtained from English sole kidney tissue, the fish's moderately high chromosome number (2n = 48) and most importantly the extremely small size (2-4 μ) of its chromosomes makes analyses involving them very difficult. Kligerman, Bloom, and Howell (1975) chose to use the central mudminnow for SCE analysis because it has a relatively low diploid number of 22 and because it has larger chromosomes (2.5-7.5 μ (estimated)) than other fish they investigated.

Similarities between the time required for exposure to BrdU and SCE rates of the English sole and the central mudminnow are apparent. Kligerman and Bloom (1976) found the peak SCD in the kidney to occur five days after exposure and the SCE rate in the intestines to be 2.64, while an exposure time of 5.5 days gave a peak number of SCD and an SCE rate of approximately 2.16 in the English sole.

Baseline frequencies for a variety of in vivo systems are compared in Table 8. With the exception of the broad bean, Vicia faba, the SCE frequencies are quite similar. Different BrdU concentrations, organs examined and species differences may contribute to the slight discrepancies observed in the SCE rates among the species. It has been suggested that the number of SCE's for a species is proportional to the length of its chromosomes (Kihlman 1975, Wolff 1977). Vicia faba has the largest chromosomes of all the species studied in vivo and also the highest SCE rate, while the English sole has very small chromosomes and exhibits a relatively low SCE rate.

Kligerman and Bloom (1976) used a single i.p. injection of BrdU in the central mudminnow. Other in vivo studies using mammals revealed that a single injection gave insufficient incorporation of BrdU into DNA because of rapid debromination and inactivation of the compound and it was therefore found necessary to use the tablet implantation method, continuous intravenous infusion of BrdU solution, or hourly injections (Allen et al. 1977, Allen et al. 1978, Allen and Latt 1976a, Nakanishi and Schneider 1979). Based on the findings of this study and those of Kligerman and Bloom (1976) this is apparently not the case with fish, at least with P. vetulus and Umbra limi. In a comparative study of the effects of BrdU on cultured leukocytes of various species (humans, cows, pigs, sheep) McFee and Sherrill (1979) observed a species-related difference in the minimum BrdU concentration required for chromatid differentiation. Species differences in the amount of BrdU required for SCD may explain why a single i.p. injection of 0.5 mg/g body wt is adequate in these fish and not in mammals.

Table 8. Summary of some *in vivo* SCE investigations.

System	BrdU Dose*	Chromosome Size (μM)*	SCE/ Metaphase*	SCE/ Chromosome*	Reference
English broad bean (*Vicia faba*)	100 μM/l	8-12	20.6	1.72	Kihlman and Kronborg (1975)
Chick embryo	25 μg/ embryo	micros/ macros	0.75	0.07	Bloom and Hsu (1975)
Mouse	8.6 mg/ mouse	2-5	1.18	0.045	Allen and Latt (1976a)
Mouse	40 μg/g	2-5	4.0	0.10	Vogel and Bauknecht (1976)
Central mudminnow (*Umbra limi*)	500 μg/g	2.5-7.5	2.59	0.12	Kligerman and Bloom (1976)
Eastern mudminnow (*Umbra pygmaea*)	500 μg/g	2.5-7.5	1.33	0.06	Alink et al. (1980)
Polychaete worm (*Neanthes arenaceodentata*)	30 μM/l	3-5	2.5	0.14	Pesch and Pesch (1980)
English sole (*Parophrys vetulus*)	500 μg/g	2-4	2.16	0.045	Present study

* Calculated from original paper where required.

Staining is also a very important factor in the visualization of SCD. Unless the staining is effective SCD will not be seen even if optimum incorporation of BrdU and exact timing of cell harvesting is achieved. Many different staining techniques may be used to visualize SCD in mammals (Korenberg and Freedlender 1974, Perry and Wolff 1974, Wolff et al. 1975, Scheres et al. 1977). The technique which usually produced SCD upon staining was the FPG method which requires treatment with the fluorescent dye Hoechst 33258 and exposure to light prior to Giemsa staining (Wolff et al. 1975). Kligerman (1979) used a similar technique in his work with the mudminnow.

The FPG method did not always produce SCD. Chromosome preparations from approximately 20 percent of the fish did not stain with FPG even though these fish had been treated exactly the same as fish whose chromosomes stained readily for SCD with FPG. These chromosomes did stain, however, with the disodium phosphate-Giemsa method of Kocan (unpublished). Kocan used 0.3 M Na_2HPO_4 at pH 10.4 to stain human leukocyte metaphases, while SCD were seen in English sole metaphases at pH 10.7. It is interesting to note that the English sole chromosome preparations that stained with FPG also stained with Kocan's stain at pH 10.4. Apparently pH is a critical factor for a difference of 0.2 in pH can affect staining considerably (Kocan, personal communication). Takayama and Sakanishi (1979) employed a similar direct Na_2HPO_4-Giemsa technique in staining Chinese hamster chromosomes for SCD using a 0.1 M Na_2HPO_4 solution at pH 9.0 and a Giemsa concentration of 1.5 percent.

Dissimilar binding of proteins to DNA in BrdU-substituted and non-substituted chromatids has been cited as a major factor responsible for differential staining (Ikushima and Wolff 1974, Wolff 1977, Takayama and Sakanishi 1979). Differences in the DNA proteins, however slight, between species and even between individuals of the same species may account for the modifications in staining conditions required for chromosomal differentiation of some metaphase preparations. The mechanisms involved in these dye methodologies have been investigated but the evidence, at present, is inconclusive.

4.2 Radioactive Label

The radioactive labelling study was initiated because exposure of the fish to various doses of BrdU failed to produce SCD in the metaphase spreads. It was assumed that BrdU incorporation was the problem and that knowledge of the cell cycle times and the lengths of the various phases that make up the cycle (G, S, G_2, M) would give a clear indication of the time required for BrdU exposure and also the amount of time necessary for the completion of two cell cycles. The S-phase length is especially important because BrdU must be available for incorporation into newly synthesized DNA during this period. If tablet implantation is used as the method of exposure the tablet must be formed in such a fashion that it will dissolve over the length of the S-phase giving continuous exposure to the compound.

The cell cycle times are not known for any tissues of P. vetulus. In this study kidney tissue was used in an attempt to establish cell cycle times; however, the level of radioactivity was insufficient for the excitation of enough silver grains in the emulsion coat of the autoradiographs even though radioactivity could be detected by scintillation techniques. Since labelled cells could not be counted the labelling index could not be determined for the calculation of the cell cycle times.

Scintillation data revealed a peak in radioactivity between 12 and 24 hours after tritiated thymidine injection indicating an S-phase occurring somewhere during this time period. Hightower (1978) reported an average S-phase length of 18 hours in the newt, Notopthalmus viridescens. It is conceivable that the S-phase is at least this long in the English sole and probably even longer given the fact that the sole were held at 14°C while the newts were maintained at 23°C.

4.3 Benzo(a)pyrene Exposures

To test the sensitivity of the SCE technique in the English sole, a known mutagen was administered in three different doses. These doses represent only the amount of the mutagen administered to each fish and not the total tissue levels or body burden levels of the chemical. B(a)P was chosen because it has been demonstrated to be a water contaminant in municipal and industrial areas (World Health Organization 1973, Dunn and Stich 1975, Malins et al. 1980) and it is also mutagenic (World Health Organization 1973, Ames et al. 1973, Roszinsky-Kocher et al. 1979, Haseltine et al. 1980). Haseltine et al. (1980) and Gamper et al. (1977) observed binding of the 9, 10-oxide of the B(a)P metabolite trans-7 β, 8 α-dihydro-7, 8-dihydroxybenzo(a)pyrene (BP 7,8-dihydrodiol) to DNA and subsequent DNA strand scission. The chemical structure of B(a)P and this epoxide are shown in Figure 13. Pal et al. (1980) determined this epoxide to be an extremely potent inducer of SCE in Chinese hamster ovary cells. Varanasi and Gmur (1980) identified a significant portion of the liver extracts of starry flounder (Platichthys stellatus) which had been injected intraperitoneally with B(a)P to be this metabolite. Incubation of DNA with these extracts produced a high degree of covalent binding of the dihydrodiol epoxide to DNA.

The major product of B(a)P metabolism in three species of marine fish (sand goby, Gillichthys mirabilis; sculpin, Oligocottus maculosus; sand dab, Citharichthys stigmaeus) was identified as the 7,8-dihydrodiol (Lee et al. 1972). It is conceivable that this is also the major B(a)P metabolite produced by the English sole and that it, too, binds to DNA causing strand scission and SCE.

In the present study two intraperitoneal injections of B(a)P, three days apart, were given to each fish. The purpose of the first injection was to stimulate the mixed function oxygenase system so that B(a)P would be readily converted to its mutagenic epoxide derivatives. Significant

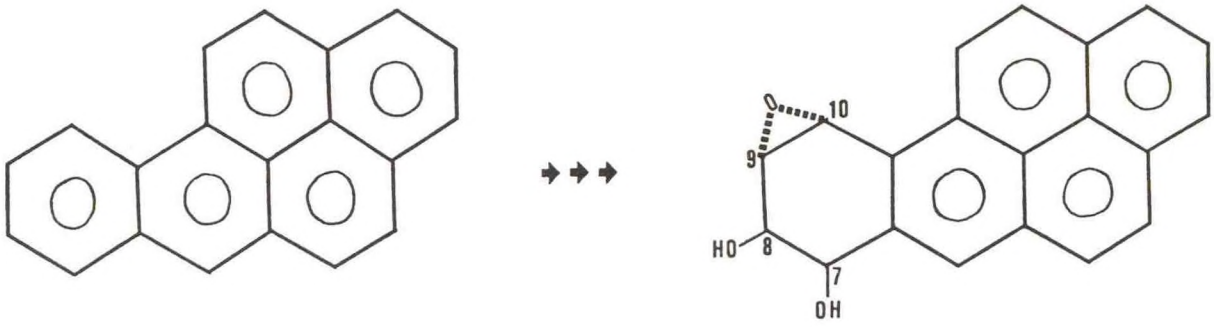

Figure 13. The chemical structure of benzo(a)pyrene and one of its mutagenic metabolites.

increases in SCE rates were obtained for each dose of B(a)P studied. SCE tests using cultured mammalian cells have revealed large increases in SCE rates with increasing doses of B(a)P (Pal et al. 1980); however, it is difficult to compare the results of *in vivo* with *in vitro* exposures. SCE rates obtained from *in vivo* exposures to a mutagen are usually lower than *in vitro* exposures to the same agent (Allen et al. 1977). Metabolic activation, dilution, and excretion are mechanisms the body uses to deal with xenobiotics. These mechanisms will be active during a whole body exposure and will influence the effect of the mutagen on the cells and therefore the SCE rate. An *in vivo* SCE analysis may more closely approximate the actual SCE rate.

The results obtained in this exposure study indicate that the SCE test, when applied to the English sole, can reveal exposure to extremely low doses of a mutagen. SCE analysis using the English sole may be a useful tool for screening the aquatic environment for mutagenic contaminants.

4.4 Field Study

The Duwamish River, which empties into Puget Sound at Seattle, Washington, received industrial, agricultural and municipal wastes. Several species of fish inhabiting this river exhibit high incidences of fin erosion, epidermal papillomas (Pierce et al. 1978) and many other histopathological conditions, especially hepatic lesions, similar to those seen in animals exposed to toxic chemicals under experimental conditions (Malins et al. 1980). The SCE rate of a sample of English sole collected in the west waterway of the Duwamish River (MESA station 10038, Malins et al. 1980) was determined to be 3.25 SCE per metaphase. It was demonstrated to be significantly higher than the baseline SCE rate ($p < 0.05$). This suggests that exposure to an as yet unidentified mutagen or mutagens is occurring in the Duwamish River. Chemical analyses of sediment samples at this site revealed high concentrations of several mutagenic chemicals (Malins et al. 1980). Benzo(a)anthracene (2700 µg/g dry wt. (ppb)) and B(a)P (1500 µg/g dry wt. (ppb)) levels were particularly high. The SCE response is not specific for any mutagenic chemical. The SCE rate observed in fish from this site reflects the combined effects of several mutagens which are present. It is not known whether these effects are synergistic or additive.

The Duwamish SCE rate was not significantly different from the SCE rate of the lowest concentration of B(a)P in the exposure experiments. This dose gave a total body exposure of 0.05 µg/g or 50 ppb B(a)P.

4.5 Additional Species Investigated

Chromosome numbers of the other three species investigated are not sufficiently lower than that of the English sole to warrant them better candidates for SCE studies. As in the English sole nearly all of the chromosomes are acrocentric (the Pacific staghorn sculpin appears to have

two more metacentric chromosomes than the English sole) and are approximately the same size as the chromosomes of the English sole.

Of the three species, the Pacific staghorn sculpin was the easiest to handle, it adapted best to aquarium conditions, and it produced the largest number of well-spread metaphases. Use of the Pacific staghorn sculpin in SCE tests deserves further investigation. Its diploid chromosome number is approximately 40, slightly less than that of the English sole (48).

5. CONCLUSIONS

1. Many staining techniques which have been shown to differentiate sister chromatids in mammalian tissues were applied to the English sole; however, of these only the fluorescent plus Giemsa (FPG) method of Wolff et al. (1975) and the 0.3 M Na_2HPO_4-Giemsa method of Kocan provided differential staining of these chromosomes.

2. The baseline frequency of SCE in the kidney of the English sole is approximately 2.16 SCE per metaphase.

3. The SCE technique can be applied to the English sole and can reveal exposure to low doses of mutagens. In experimental exposures using B(a)P significant increases in SCE rates over the baseline frequency were obtained. It was also shown to be dose-responsive.

4. The SCE rate of a sample of fish from the Duwamish River, Seattle, Washington was found to be significantly higher than the baseline SCE frequency. It was similar to the lowest dose of B(a)P studied. This may indicate that there are some low-level mutagenic contaminants present in the Duwamish River system.

5. Although the English sole appears to be a sensitive test organism for SCE analysis quantitation of SCE proved very difficult owing to the extremely small size of their chromosomes (2-4 μ). The fact that its chromosomes are all approximately the same size and nearly all acrocentric would likely make certain other types of studies difficult as well (karyotyping, etc.).

6. Chromosomes were prepared from three other species of marine fish: Pacific staghorn sculpin, silver-spotted sculpin, and saddleback gunnel. The chromosome complements of all three proved to be very similar to that of the English sole with respect to number, size, and centromere placement. Of the three the Pacific staghorn sculpin deserves further investigation.

6. RECOMMENDATIONS

The present study has demonstrated that it is possible to sample English sole in the field and to detect increases in the SCE rates of these fish over their baseline SCE rates, which presumably reflects exposure to mutagenic chemicals. A field survey utilizing this technique has yet to be undertaken; however, the results of this study and those of others using the SCE technique in fish suggest that it is entirely feasible.

Although there is no way, as yet, to relate a specific level of an environmental contaminant to a corresponding SCE rate, a linear relationship between the dose of a mutagen and SCE rates has been experimentally demonstrated. It can be assumed then, that a high SCE rate indicates the presence of a significant level of a mutagenic contaminant or contaminants. A field survey in which the SCE rates of English sole from several sites (polluted and non-polluted) are compared with the level of mutagenic contaminants found at these sites is a logical extension of the present study. Such an investigation would confirm, in the field, the relationship between SCE rates and level of mutagens; would verify the usefulness of the SCE technique as a screening tool; and would provide a profile of the genetic effects of the contamination of a geographical region with mutagenic chemicals.

A study which samples whole, live fish has many drawbacks, however, since it requires collecting fish in the field and transporting them back to the laboratory where they are maintained in salt water aquaria for the duration of their exposure to BrdU (at least 5.5 days). This method limits the number of fish that can be sampled at one time and can be complicated and costly.

If it were possible to perform the SCE test using cultured leukocytes from English sole, blood could be drawn, in the field, from a large number of fish and easily transported back to the laboratory. Many cultures, requiring little space and small amounts of reagents, could be set up at one time. To date, attempts at culturing leukocytes from English sole have been unsuccessful; however, investigations into the culturing of leukocytes from the Pacific staghorn sculpin (Leptocottus armatus) are presently being conducted at our laboratory. The present study has indicated that this fish may be a suitable alternative to the English sole. If the SCE test can be applied to leukocyte cultures from these fish, a survey of a large number of fish from several different sites could be readily accomplished.

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