The Passage of Las Across the Teleost Chorion and the Mouse Placenta

Annabella Juhasz

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THE PASSAGE OF LAS ACROSS THE TELEOST CHORION
AND THE MOUSE PLACENTA

by

Annabella Juhasz

A Thesis Submitted to the Faculty of the Graduate School
of Loyola University of Chicago in Partial Fulfillment
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VITA

The author, Annabella Juhasz, is the daughter of John Charles Juhasz, M.D. and Anna (Rizko) Juhasz. She was born July 22, 1950, in Budapest, Hungary.

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CHAPTER I

INTRODUCTION

Synthetic detergents are a diverse group of compounds and part of a larger group known as surface active agents or surfactants (Abel, 1974). Synthetic surfactants replaced soap in commercial cleaning formulations in the 1950's (Swisher, 1970) at which time interest arose in their toxicity (Abel, 1974).

Since 1965, United States detergent manufacturers have used biodegradable linear alkyl sulfonate (LAS) rather than "hard" tetrapropylene alkyl benzene sulfonate (ABS) surfactants in their detergent formulations (Arthur, 1970). Surfactants appear to be the primary toxic component of these formulations (Henderson et al., 1959) and LAS has been demonstrated to be one to four times more toxic than the old ABS compound (Pickering, 1966; Thatcher and Santner, 1967).

Studies in our laboratory have shown LAS to be the cause of neural anomalies in developing zebra fish embryos (Manner and Dewese, 1973), and to alter the membrane permeability of the fat head minnow chorion (Manner and Muehlman, 1975).

The interaction between detergents and proteins, and the influence of detergents on membrane permeability may be the basis of the biological action of detergents (Abel,
1974). The chorion is a protective membrane surrounding the fish embryo. In mammals, there exists a series of membranes, which surround the embryo, and together constitute the placenta. The purpose of this investigation is to determine the relative efficacy of these protective barriers in limiting the passage of LAS, and to determine where this compound is concentrated in the embryo.

Carbon-14, labeled LAS, will be used to study the permeability of the chorions of the fat head minnow, *Pimephales promelas*, and zebra fish, *Brachydanio rerio*, as well as the placental barrier of the mouse, *Mus musculus*. An autoradiographic study will be completed to examine the location of LAS in the fat head minnow embryo.
CHAPTER II

REVIEW OF THE LITERATURE

The literature related to this study will be surveyed from three areas: (1) the use of the zebra fish, fat head minnow, and mouse in toxicity studies; (2) studies of placental transport; (3) a description of LAS structure and function as well as a report on important studies on its toxicity and effects.

Use of the Zebra Fish

The zebra fish or zebra danio, Brachydanio rerio, is a member of the family Cyprinidae and is a native from Bengal to the Coromandel Coast of India (Axelrod and Schultz, 1955). This oviparous species can be bred with ease in captivity (Hisaoka and Battle, 1958). The eggs of the zebra fish lend themselves well to experimentation since abundant numbers can be obtained throughout the year; the chorion and embryos are relatively transparent; and development is rapid (Shirone and Gross, 1969). Only 96 hours are required from fertilization to hatching at 26°C (Hisaoka and Battle, 1958). The popularity of this fish is evidenced by the wide use it has received for many years in the laboratory. Earlier investigators studied embryological phenomenon such as bi-polar differentiation, cleavage, and
karyokinesis, using the zebra fish (Roosen-Runge, 1936, 1938, 1939; Goff, 1940). Experiments were conducted to study the structures developed in amphibians by implantation of living fish organizers from zebra fish (Oppenheimer, 1936). The clear chorion and rapid development of the zebra fish made it suitable for selection in a motion picture film on development of fish eggs (Lewis and Roosen-Runge, 1944). The effects of urethane on early development of the zebra fish were studied by Battle and Hisaoka (1952). The normal developmental stages of the zebra fish were studied by Hisaoka and Battle (1958) and Battle and Laale (1960) studied trypan blue induced anomalies in embryos of zebra fish.

Researchers have recently found the zebra fish to be particularly useful in toxicity studies. The zebra fish has been exposed to a variety of compounds to study the effects. The effects of ribonuclease on early development was studied by Hisaoka (1962). The effects of zinc sulfate at different stages of the zebra fish life cycle, after removal of the chorion, and oxygen uptake at different ages in relation to zinc sulfate resistance was studied by Skidmore (1965, 1967, 1968). A comparative study of the sensitivity of adult zebra fish and zebra fish eggs with that of adult bluegill was conducted by Cairns et al. (1965). Other studies showing the effects of chloramphenicol on cleavage (Anderson and Battle, 1967), the response of zebra fish larvae to folpet and defolatan (Abedi and Turton, 1970), and
ethanol induced eye defects and spinal cord duplications in embryos (Laale, 1971), indicate the wide use of the zebra fish in the laboratory.

Use of the Fat Head Minnow

The fat head minnow, *Pimephales promelas*, Rafinesque, is being used as a bioassay fish by the Environmental Protection Agency (Mount, 1971). Henderson et al. (1959) found ABS more toxic to *Pimephales promelas* in hard water than in soft, but sodium alkyl sulfate was more toxic in hard water. Pickering (1966) measured the 9-day LC$_{50}$'s (lethal threshold concentration) of LAS and ABS for *Pimephales promelas* eggs. The toxicity of ABS to fry and juvenile fish was also measured; one-day old fish were the most sensitive. Adult *Pimephales promelas* were less sensitive to ABS than their eggs. It was found that simultaneous exposure of *Pimephales promelas* to lethal concentrations of parathion and sublethal concentrations of LAS resulted in a drop of 50 percent in the 96h LC$_{50}$ for the pesticide: 1mg/l LAS caused a reduction from 1.4 to 0.7mg/l (Solon et al., 1969). LAS toxicity was found to differ in the life cycle of *Pimephales promelas*. LAS did not affect growth or hatching but did affect fry survival at 14 days after hatching (Pickering and Thatcher, 1970). Solon and Nair (1970) did a comparative study of the toxicity of eight phosphate pesticides in the presence and absence of 1mg/l LAS. The results indicated that detergents
alter the rate of uptake of poison by fish. Reducing the lethal threshold by 40 to 50 percent.

In another study, in which the lethal toxicity of ABS was studied, *Pimephales promelas* was one of the 11 fish selected to study the effects (Thatcher, 1966). The fat head minnow was again used to study the acute toxicity of LAS (Thatcher and Santner, 1967). In 1974 a study of the early embryology of the fat head minnow was done by Manner and Dewese.

**Use of the Mouse**

The use of the mouse in the laboratory is well established. The small size, relatively short gestation period, large litter size, lack of seasonal breeding patterns, and ready availability of these animals are all responsible for their continued use as laboratory animals.

The small size can, however, be a disadvantage. The limited samples of tissues and body fluids hinders the examination of fetuses for defects as well as in studies on parental absorption, metabolism, and excretion of chemical agents (Wilson, 1973). Breeding performance is sometimes erratic and several inbred strains are known to have high and occasionally variable rates of background malformations and intrauterine death (Kalter, 1968; Namura, 1969), probably reflecting a greater degree of developmental instability than other rodents. Mice are known to respond readily to some substances that have limited teratogenicity
in other animals, for example, cortisone and the herbicide 2,4,5-T (Wilson, 1973), thereby earning a justified reputation for unusual sensitivity. In spite of these obvious disadvantages the low cost of maintaining large colonies of mice still makes this the preferred mammalian species for mutagenicity testing (Bateman and Epstein, 1971; Cottanach, 1971) and several inbred strains offer particular advantages in basic research and for teratogenicity testing (Smithberg, 1971). The genetics of the mouse is better known than that of any other mammalian species (Gruneberg, 1952, 1963).

**Transplacental Transport**

Transplacental passage of chemicals and drugs has been studied by many. The passage of DDT, dieldrin, and their metabolites into the placenta and fetus is well documented by autoradiographic studies and biochemical analysis of various tissues (Backstrom et al., 1965; Rapport and Hale, 1968; Zavon et al., 1969; Selby et al., 1969). The transplacental passage of organophosphorous insecticides was first suggested by the finding of parathion in the fetus of a mother who committed suicide with the agent (LeBreton et al., 1963). This compound has been widely used in Japan to control the rice stem borer, and has been suspected to cause fetal death and malformations (Ogi and Hamada, 1965). Rat fetuses from mothers injected with parathion, methyl parathion, and isofluorophate (DFP) showed diminished brain
cholinesterase activity when analyzed by the histochemical technique of Gomori (Fish, 1966). Biochemical assays have shown parathion, bromofos, and imidan to be present in placental tissue, fetal muscle, liver and brain (Ackerman and Engst, 1970).

Special interest in this area of research intensified as a result of the thalidomide induced extremity malformations in humans. Phocomelia was known to be rare. A few cases were observed in Germany in 1959. Cases of congenital malformations increased. In the latter part of 1961 the number of such cases in the United Kingdom, Australia, and Europe reached almost epidemic proportions. The common factor was thalidomide taken in early pregnancy (DiPaolo and Werner, 1964).

The transplacental passage of thalidomide with its accompanying congenital abnormalities were studied by Sommers (1962), Spencer (1962), and Giroud et al. (1962). Investigations of malformations of rat fetuses after thalidomide administration was conducted by Bignami et al. (1962). Thalidomide and the umbilical artery was studied by Kajii (1964). The effects of thalidomide were studied on a variety of animals including rabbits, hamsters, mice (Fratta et al., 1966), and monkeys (Drobeck, 1966). Researchers reported on the type of congenital anomalies associated with thalidomide. Symptoms ranged from mental deficiency to absence of proximal humerus and femur (Hagen, 1964). In the wake of the tragedies that occurred as a
result of thalidomide, research proliferated until the latter 1960's. Interest subsided as many of the questions about thalidomide had been resolved.

The passage of various chemical compounds across the placental barriers is still being investigated in several laboratories. Among the potentially dangerous drugs are procaine hydrochloride and paraamino benzoic acid (Usubiaga, 1969), ethanol (Waltman and Iniquez, 1972), carcinogenic agents such as 3-methyl cholanthracene (Guibert and Duperray, 1971), tetracycline (Kline, 1964), lidocaine (Shnider and Leongway, 1969), ampicillin (LeBlanc and Perry, 1969), phenobarbitone (Melchior et al., 1968), and fluoride (Gedalia, 1965). These drugs are being studied with reference to transplacental passage.

LAS

As a synthetic product it is inexpensive to produce and its special properties make it more efficient than soap as a laundering agent. Detergents contain 10 to 30 percent surfactant or the "active" part, larger portions of polyphosphate salts, the "builder," and a number of ingredients in small percentages (Swisher, 1970).

Synthetic detergents are a diverse group of compounds, and part of a larger group known as surface-active agents or surfactants. The otherwise diverse molecular forms of detergents have in common a hydrophilic-hydrophobic polarity whence stem three important
general properties: the tendency to concentrate at surfaces, the reduction of surface tension in solution, and the formation of aggregates of ions and micelles, when present in solution above a certain critical concentration. These properties are related aspects of the phenomenon of surface activity (Abel, 1974).

Linear alkylbenzene sulfonate (LAS) has served as the surfactant in commercial detergent formulations in several countries during the past (since 1965) years. LAS has replaced the poorly biodegradable tetrapropylene alkylbenzene sulfonate (ABS) which was formerly used. The change has resulted in substantial decreases in the foaming properties and methylene blue active substances (MBAS) of sewage treatment effluents and the receiving rivers because of the facile biodegradation of LAS by microorganisms (Swisher, 1967). In LAS the alkyl groups are straight chains, readily attacked by bacteria. In contrast ABS is a mixture characterized by highly branched alkyl groups, and approximately one-quarter to one-third of it is quite resistant to bacterial attack because of unfavorably compact alkyl structure. This has sometimes resulted in objectionable foaming in rivers and ground waters (Swisher, 1964).

Surfactant biodegradation is defined as the activity by which microorganisms destroy surfactants. Biodegradation is considered in two phases. Primary biodegradation, in which the molecule is oxidized or otherwise altered by
bacterial action. The characteristic properties are no longer evident, it no longer responds to analytical procedure specific for detecting the original surfactant. Ultimate biodegradation is the complete conversion of the surfactant molecule to \( \text{CO}_2 \), water, inorganic salts, and products associated with the normal metabolic processes of the bacteria (Swisher, 1970).

In general, we can say that the biodegradation is easier the straighter the chain, the longer the chain, and the greater the distance between the sulfonate group and the far end of the chain (Swisher, 1963a). Apparent exceptions to this generalization for chain length beyond \( \text{C}_{12} \) arise from inhibitory properties and are brought into line by acclimation or by working with mixed systems (systems with a mixture of bacterial species) (Swisher, 1963b). With respect to the degradation of LAS benzene rings, Swisher (1967) showed that a \( \text{C}_{12} \) LAS mixture and two of its components in pure form, 3-phenyl and 6-phenyl dodecane-p-sulfonates, all showed approximately 90 percent ring degradation under a variety of conditions once acclimation had occurred.

The inhibition of degradation of LAS beyond \( \text{C}_{12} \) was studied by Ciattoni (1968). Throughout the range from \( \text{C}_{10} \) to \( \text{C}_{15} \) increasing the chain length by one carbon cut the limiting concentration by one-half. Ciattoni attributed the inhibition to interaction of the LAS with specific bacterial enzyme centers otherwise capable of attack on the alkyl
A surface active agent is a substance which, when present at low concentrations in a system, has the property of adsorbing onto the surfaces or interfaces of the system and of altering to a marked degree the surface or interfacial free energies of these surfaces (or interfaces) (Rosen, 1972). The characteristic structural feature of the surface active agent is a molecular structure containing a group which has a strong attraction for water, called the hydrophilic group, together with a group that has very little attraction for water, called the hydrophobic group. The hydrophobic group is usually a long chain hydrocarbon residue; the hydrophilic is an ionic or highly polar group (Rosen, 1972). Anionic detergents are the most studied, and most widely used group of detergents. These represent the major source of detergent pollution. The most common anionic detergents are the alkyl aryl sulfonates, such as LAS and ABS. Non-ionics, although used in significant quantities, have attracted less attention than anionics, and cationic detergents are mainly used as medical and laboratory disinfectant agents (Abel, 1974).

Another phenomenon characteristic of surfactants in aqueous solution is the aggregation of their molecules into larger, oriented groups called micelles. In very dilute solutions, e.g., 1 ppm, the individual single molecules are present, or their ions. Further increments of surfactants also dissolve to form separate molecules or ions up to a
certain point, known as the critical micelle concentration, abbreviated CMC. Beyond this point the concentration of single molecules remains relatively constant. Much more surfactant may still be dissolved to give clear solutions, but the added increments form micelles in the solution instead of appearing as individual molecules.

Many organic materials which are insoluble in water but soluble in organic solvents may be solubilized in aqueous solution to a certain extent by the presence of surfactant micelles. The organic material is found to be molecularly dispersed in the internal region of the micelle as a quasi-solution in the clustered hydrophobes, one or more (or less) molecules per micelle. This solubilizing action is no longer exhibited below the CMC of the surfactant, and is to be distinguished from the emulsification or dispersion of insoluble materials in water by the aid of surfactants. In the latter case the insoluble material is present in the form of a second phase of macroscopic droplets or particles, restrained from coalescing or precipitating by the presence of the oriented surface layer of surfactant (Swisher, 1970).

Much research has been carried out on detergent-protein interactions, early work along these lines being reviewed by Putnam (1948). It is established that proteins can be altered, reversibly or irreversibly, by detergents in low concentrations; the use of detergents as protein denaturants is a widely known technique in biochemical
research. At higher concentrations, when the detergent is present largely in the form of micelles, detergents have the property of solubilizing organic material. Most toxicological research is carried out on concentrations too low for the formation of micelles to occur, but even below the critical micelle concentration, detergents probably have more than one mode of action. At low concentrations, detergents alter membrane permeability. At higher concentrations, detergents undoubtedly exert a disruptive effect (Abel, 1974). Acute gill damage is a likely example of this. Swisher (1964) showed that LAS attacks the gill and mucous membranes of bluegill fingerlings and Goldacre (1968) demonstrated the effects of detergents on the cell membrane of the Amoeba providing further confirmation.

The role of surface tension reduction in detergent toxicity has excited controversy. Prat and Giroud (1964) accept uncritically the notion that detergent toxicity is due to surface tension reduction alone.

It is difficult to believe that substances of widely different chemical composition like lauryl sulphonate and dodecylbenzene sulphonate would be toxic in doses which are (s) ... close if they worked by chemical action. It may, therefore, be accepted that the action of surface active agents on fish is the purely physical one of reduced surface tension (Prat and Giroud, 1964, p. 34).

For the common anionic detergents, surface activity increases and solubility decreases with increasing chain length, resulting in a maximum in the $C_{12}$ to $C_{16}$ for both
detergency (Price, 1945) and toxicity. A toxicant must contact the fish in order to exert its effect, and a greater tendency to concentrate at interfaces must make the detergent more likely to do this. The toxicity of a detergent will, therefore, be a function of both its surface activity and its chemical toxicity (Abel, 1974).

The relationship between detergents and other pollutants has been studied. Natural and synthetic surfactants have been shown to increase membrane permeability to a variety of substances including barbiturates, ethanol, insulin, and antibodies (Gibaldi and Feldman, 1970). Goldfish with a history of ABS exposure were shown to be more susceptible to the toxic affects of dieldrin and DDT (Dugan, 1967).
CHAPTER III

MATERIALS AND METHODS

Procedure for Zebra Fish

Zebra fish were conditioned for breeding by daily feedings of brine shrimp, tubifex worms, and Tetra Min Growth Food or Tetra Min Staple Food, a product of the Tetra Min Werke Company, Western Germany. The fish were acclimated to a 16-hour photoperiod. The temperature of the tank was maintained at a range of 28°-31°C (Shirone and Gross, 1969). The water used was dechlorinated tap water.

Adult males and females were kept in separate tanks until breeding. For breeding a single mature (plump) female was selected and placed in a breeding tank with three or four males. The breeding tank was equipped with a nylon net, which was two inches below the surface of the water, and was attached to the tank at all sides. The mesh was large enough to allow eggs to fall quickly through -- preventing them from being eaten by adult fish. Eggs were available for year 'round study and each breeding yielded from 50-100 eggs.

Thirty eggs were selected each time. Eggs with cloudy chorions, apparent fungus infection, or retarded development were rejected. Preliminary work in our laboratory showed that concentrations of 2ppm LAS or below
had no affect on the embryos and concentrations of 10ppm were lethal. Concentrations of 4, 6, and 8ppm LAS were chosen for this work.

LAS, Linear Alkyl Benzene Sulfonate, was obtained from the Proctor and Gamble Company of Cincinnati. The length of the carbon chain was fixed at 12 carbons with the benzene ring located on the first carbon and the sulfonate group on the para position on the benzene ring.

The eggs, at the preneurula stage, were placed in finger bowls containing the above concentrations of tagged LAS. There were 10 eggs per finger bowl. The eggs remained in the LAS for four hours. They were then removed and rinsed with four washings of tank water to remove residual LAS which may have adsorbed to the surface of the chorion. The rinsed eggs, with chorions still intact, were placed in glass scintillation phials containing a fluor or scintillation fluid. The following is Bray's solution for the liquid scintillation counter:

Naphthalene 60gm
ppo 4gm
POPOP 0.02gm
Absolute MeOH 100ml
Ethylene Glycol 20ml
p-Dioxane to make one liter
Stable at 25°C for one month

A Packard Tri-Carb liquid scintillation spectrometer was used to determine radioactivity. Samples were run for 100
minutes. Results were reported as counts per minute per embryo.

**Procedure for Fat Head Minnow**

Adult fat head minnows were conditioned for breeding by a diet of brine shrimp, bug larvae, Tetra Min Conditioning Food, and tubifex worms. They were acclimated to a 16 hour photoperiod and kept at the temperature found most suitable for breeding, 23°C (Manner and Dewese, 1974). The water used was dechlorinated tap water.

Two males and eight females were placed in a breeding tank. Inverted halves of asbestos pipes (three inch in diameter) were placed on the bottom of the tank. The eggs were deposited on the inside of the inverted pipes. Eggs were laid for a period of six hours beginning with the onset of light in the morning. The eggs were removed from the inverted tiles by pipette.

Eggs, past stage 12 of development (Manner and Dewese, 1974), were placed in finger bowls containing labeled LAS. Concentrations of 4, 6, and 8ppm LAS were contained in each finger bowl. These concentrations were selected so a comparison could be made between the same concentrations and their affects on the zebra fish and fat head minnow. There were 10 eggs per finger bowl. The eggs remained in the LAS for four hours. They were then removed and rinsed with four washings of tank water to remove residual LAS which may have adsorbed to the surface of the
chorion. The rinsed eggs, with chorions still intact, were placed in glass scintillation phials containing a fluor or scintillation fluid. A Packard Tri-Carb liquid scintillation spectrometer was used to determine radioactivity. Samples were run for 100 minutes. Results were reported as counts per minute per embryo.

Procedure for Mouse

The mouse, *Mus musculus*, was chosen to study the effects of LAS on mammals. The Charles River ICR CD-1 strain was used. The mice were kept in an isolated and restricted room to prevent disturbance from unnecessary noise. They were maintained on a 12-hour photoperiod. The humidity in the room was maintained at approximately 50 percent. The ears of the mice were punched and a numerical system was used for identification of individual animals. Males and females were kept separate, with four animals per cage, until mating.

For mating, one male was placed in a cage with four females over night. In the morning the males were returned to their cages. Prior to mating the females were weighed. After mating they were weighed daily. An increase in weight of over five grams by the twelfth day indicated pregnancy. Vaginal plugs, observed on the morning of mating, were also noted and those females were watched for the expected correlative weight gain.

Females whose weight gain indicated pregnancy were
selected on the twelfth and fifteenth day of gestation. The females were placed in a glass container with ether soaked cotton on the bottom. When they were sufficiently stunned they were removed and given an intra peritoneal injection of the LAS solution.

From earlier work in our laboratory the following three dosages were selected so as not to be lethal to the mother nor be too small to have any effect on the embryo:

1. 100mg LAS/Kg body weight;
2. 200mg LAS/Kg body weight; and
3. 300mg LAS/Kg body weight.

Four hours after injection the females were killed and the embryos were removed. The embryos were then left to dry on filter paper for four days. The paper was trimmed from around the embryo and the remaining paper and dried tissue was placed in a scintillation counter vial containing Bray's solution. The embryos remained in this solution for one week and then were placed in the Tri-Carb Liquid Scintillation Counter. Samples were run for 10 minutes each and reported as counts per minut per embryo. The number of embryos per female varied, all retrieveable embryos were used and counted. The LAS solutions were made up from LAS with Carbon-14 randomly labeled on the benzene ring and 12 carbon chains in length. This provided the radioactive label. To bring the solution up to the appropriate concentration unlabeled LAS, averaging 11.8 carbons in chain length, was used. Both types of LAS were obtained from the
Proctor and Gamble Company.

**Procedure for LAS Solutions**

Several shipments of labeled LAS were received for this study. The LAS was in powder form, was 12 carbons in chain length, and had a Carbon-14 label located randomly on the benzene ring. Each shipment differed in the weight and radioactivity of the sample received. For the study of the zebra fish and fat head minnow eggs the concentration of LAS required was 4, 6, and 8 ppm. The radioactivity was standardized at approximately 0.5 microCurries (mCi). The following are the calculations required to make up the solutions with the desired concentration and radioactivity of LAS. Deionized and distilled water was used for the stock solution. Tank water was used to make up the final concentrations into which the eggs were placed.

**Dilution Method of LAS -- October Shipment.** To the stock supply of LAS (5.1 mg and 14.28 mCi), 1 cc of deionized and distilled water was added.

1. 0.1 cc was removed. This contained 0.51 mg LAS with a radioactivity of 1.428 mCi.

Using the formula: \[ V_1 C_1 = V_2 C_2 \]

\[ V_1 = 1000 \text{cc} \]
\[ C_1 = 0.51 \text{mg} \]
\[ C_2 = 8 \text{ppm} \]
\[ V_2 = x \]


\[(1000) \times (0.51) = (x) \times (8 \text{ppm})\]

\[x = 63.75 \text{cc}\]

To 0.1cc of LAS addition of 63.6cc tank water yields 8ppm LAS at 1.428mCi. Removing 21.3cc of the solution contains 8ppm LAS at 0.476mCi.

2. 42.88cc remain. Addition of 14.27cc of tank water increases the volume to 55.65cc. Removing 27.81 of the solution contains a concentration of 6ppm LAS at 0.476mCi.

3. 27.84cc remain. Adding 13.92cc of tank water the solution contains 4ppm LAS at 0.476mCi.

This method allowed for complete use of 0.1cc LAS through serial dilution.

**Dilution Method of LAS -- January Shipment.**

**Weight:** 29mg

**Radioactivity:** 80mCi 2.75mCi/mg

16cc of de-ionized, distilled water was added:

- 5mCi/cc 1.8225mg/cc
- 0.5mCi/0.1cc 0.18125mg/0.1cc

1cc of labeled LAS was added to 99cc de-ionized, distilled water:

- 0.05mCi/cc 0.018225mg/cc

10cc aliquots were used:

- 0.5mCi/10cc 0.18125mg/10cc

Contents of fingerbowls:

- 4ppm -- 30.2ml H₂O
6ppm -- 16.8ml H₂O
8ppm -- 10.1ml H₂O

plus the addition of 10cc labeled LAS solution to each dish.

**Dilution Method of LAS -- April Shipment.**

Weight: 56mg
Radioactivity: 150mCi

\[
\frac{150\text{mCi}}{X} = 5\text{mCi}
\]

\[X = 30\text{cc de-ionized, distilled H₂O}.

30cc of de-ionized, distilled water was added:

- 5mCi/cc = 1.8666mg/cc
- 0.5mCi/0.1cc = 0.1866mg/0.1cc

1cc of labeled LAS was added to 99cc de-ionized, distilled water:

- 0.05mCi/cc = 0.018666mg/cc

10cc aliquots were used:

- 0.5mCi/10cc = 0.1866mg/10cc

**Contents of fingerbowls:**

- 4ppm -- 31.47cc H₂O
- 6ppm -- 16.90cc H₂O
- 8ppm -- 10.73cc H₂O

plus the addition of 10cc labeled LAS solution to each dish.

**Formula for Determining Dosage of LAS to Mice**

The following method was used to determine how much unlabeled LAS was required to make up a solution with the appropriate dosage. The dosages (100, 200, 300mg/kg body
wt.) were calculated from this formula which took into account the variability in the weights of individual mice. To the calculated amount of unlabeled LAS was added labeled LAS, from the stock solution, which contained 0.5mCi.

Let \( Z \) = actual body weight of mouse

Let \( Y \) = the dosage \( \left( \frac{mg}{Kg \text{ wt.}} \right) \) of LAS

Let \( X \) = unknown weight of LAS needed to make the appropriate dosage.

\[
\frac{Y \times Z}{1000} = X
\]

\[
X - 0.1866 = M
\]

\[
\left( \frac{X}{10} \right) \times \left( \% \text{ activity of LAS} \right) = P
\]

\[
\left( \frac{X}{P} \right) \times \left( \frac{M}{1} \right) = R
\]

\[
\left( \frac{0.9}{10} \right) \times \left( \frac{R}{1} \right) = \text{mg of unlabeled LAS required for dosage.}
\]

Make up a 10cc solution with unlabeled LAS. Take 0.9cc of this solution. Add 0.1cc of labeled LAS.

Calculations: Day-12 Mouse, Dosage 100mg LAS/Kg

Body Weight.

Animal #12

\[
\frac{100}{1000} \times \frac{X}{36} = 3.6
\]

3.6 - 0.1866 = 3.41

\[
\frac{3.6}{X} \times \frac{62}{100} = 5.80
\]

3.6 \( \times \frac{5.80}{X} \) = 5.49

0.9 \( \times \frac{5.49}{X} \) = 61.00mg
Animal #14

\[
\frac{100}{1000} \times \frac{X}{38} = 3.8
\]

3.8 - 0.1866 = 3.61

\[
\frac{3.8}{X} \times \frac{62}{100} = 6.12
\]

\[
\frac{3.8}{6.12} \times \frac{3.61}{X} = 5.81
\]

\[
\frac{0.9}{10} \times \frac{5.81}{X} = 64.55 \text{ mg}
\]

Animal #16

Weight = 36g

". calculations same as Animal #12.

Answer = 61mg

Animal #22

\[
\frac{100}{1000} \times \frac{X}{37} = 3.7
\]

3.7 - 0.1866 = 3.51

\[
\frac{3.7}{X} \times \frac{62}{100} = 5.96
\]

\[
\frac{3.7}{5.96} \times \frac{3.51}{X} = 5.65
\]

\[
\frac{0.9}{10} \times \frac{5.65}{X} = 62.77 \text{ mg}
\]

Animal #23

\[
\frac{100}{1000} \times \frac{X}{37} = 3.7
\]

3.7 - 0.1866 = 3.51

\[
\frac{3.7}{X} \times \frac{57.5}{100} = 6.43
\]

\[
\frac{3.7}{6.43} \times \frac{3.51}{X} = 6.09
\]
\[
\frac{.09}{10} \times \frac{6.09}{X} = 67.66\text{mg}
\]

**Calculations: Day-12 Mouse, Dosage 200mg LAS/Kg**

**Body Weight.**

**Animal #7**

\[
\frac{200}{1000} \times \frac{X}{36} = 7.2
\]

\[
7.2 - 0.1866 = 7.01
\]

\[
\frac{7.2}{X} = \frac{62}{100} = 11.61
\]

\[
\frac{7.2}{11.61} \times \frac{7.01}{X} = 11.30
\]

\[
\frac{.09}{10} \times \frac{11.30}{X} = 125.55
\]

**Animal #15**

\[
\frac{200}{1000} \times \frac{X}{34} = 6.8
\]

\[
6.8 - 0.1866 = 6.61
\]

\[
\frac{6.8}{X} = \frac{62}{100} = 10.96
\]

\[
\frac{6.8}{10.96} \times \frac{6.61}{X} = 10.65
\]

\[
\frac{.09}{10} \times \frac{10.65}{X} = 118.33
\]

**Animal #26**

\[
\frac{200}{1000} \times \frac{X}{35} = 7
\]

\[
7.0 - 0.1866 = 6.81
\]

\[
\frac{7}{X} = \frac{62}{100} = 11.29
\]

\[
\frac{7}{11.29} \times \frac{6.81}{X} = 10.98
\]
Animal #29

\[
\frac{200}{1000} \times \frac{X}{31} = 6.2
\]

\[
6.2 - 0.1866 = 6.01
\]

\[
\frac{6.2}{X} = \frac{62}{100} = 10
\]

\[
\frac{6.2}{10} \times \frac{6.01}{X} = 9.69
\]

\[
0.9 \times \frac{9.69}{X} = 107.66
\]

Animal #31

\[
\frac{200}{1000} \times \frac{X}{37} = 7.4
\]

\[
7.4 - 0.1866 = 7.21
\]

\[
\frac{7.4}{X} \times \frac{62}{100} = 11.93
\]

\[
\frac{7.4}{11.93} \times \frac{7.21}{X} = 11.62
\]

\[
0.9 \times \frac{11.62}{X} = 129.11
\]

Calculations: Day-15 Mouse, Dosage 100mg LAS/Kg

Body Weight.

Animal #1

\[
\frac{100}{1000} \times \frac{X}{44} = 4.4
\]

\[
4.4 - 0.1866 = 4.2
\]

\[
\frac{4.4}{X} \times \frac{57.5}{100} = 7.7
\]

\[
\frac{4.4}{7.7} \times \frac{4.2}{X} = 7.4
\]
\[
\frac{0.9}{10} \times \frac{7.4}{x} = 82.2
\]

**Animal #9**

\[
\frac{100}{1000} \times \frac{x}{48} = 4.8
\]

\[
4.8 - 0.1866 = 4.61
\]

\[
\frac{4.8}{x} \times \frac{57.5}{100} = 8.35
\]

\[
\frac{4.8}{8.35} \times \frac{4.61}{x} = 8.01
\]

\[
\frac{0.9}{10} \times \frac{8.01}{x} = 89.00
\]

**Animal #13**

Weight = 48g

'. calculations same as Animal #9.

Answer = 89.00

**Animal #27**

\[
\frac{100}{1000} \times \frac{x}{49} = 4.9
\]

\[
4.9 - 0.1866 = 4.71
\]

\[
\frac{4.9}{x} \times \frac{57.5}{100} = 8.52
\]

\[
\frac{4.9}{8.52} \times \frac{4.71}{x} = 8.18
\]

\[
\frac{0.9}{10} \times \frac{8.18}{x} = 90.88
\]

**Calculations**: Day-15 Mouse, Dosage 200mg LAS/Kg

**Body Weight**.

**Animal #5**

\[
\frac{200}{1000} \times \frac{x}{44} = 8.8
\]
\[
8.8 - 0.1866 = 8.6
\]
\[
\frac{8.8}{15.30} \times \frac{57.5}{100} = 15.30
\]
\[
\frac{8.8}{15.30} \times \frac{8.6}{X} = 14.95
\]
\[
\frac{0.9}{10} \times \frac{14.95}{X} = 166.11
\]

**Animal #10**

\[
\frac{200}{1000} \times \frac{X}{38} = 7.6
\]
\[
7.6 - 0.1866 = 7.41
\]
\[
\frac{7.6}{X} \times \frac{62}{100} = 12.25
\]
\[
\frac{7.6}{12.25} \times \frac{7.41}{X} = 11.94
\]
\[
\frac{0.9}{10} \times \frac{11.94}{X} = 132.66
\]

**Animal #20**

Weight = 38g

\[\therefore\] calculations same as Animal #10.

Answer = 132.66mg

**Animal #30**

\[
\frac{200}{1000} \times \frac{X}{41} = 8.2
\]
\[
8.2 - 0.1866 = 8.01
\]
\[
\frac{8.2}{X} \times \frac{62}{100} = 13.22
\]
\[
\frac{8.2}{13.22} \times \frac{8.01}{X} = 12.91
\]
\[
\frac{0.9}{10} \times \frac{12.91}{X} = 143.9
\]
Animal #33

\[
\frac{200}{1000} \times \frac{x}{39.5} = 7.9
\]

\[
7.9 - 0.1866 = 7.71
\]

\[
\frac{7.9}{x} \times \frac{62}{100} = 12.74
\]

\[
\frac{7.9}{12.79} \times \frac{7.71}{x} = 12.43
\]

\[
\frac{0.9}{10} \times \frac{12.43}{x} = 138.11
\]

**Autoradiographic Procedure**

Fat head minnow eggs, at approximately stage 12, were exposed to 8ppm LAS for four hours. They were then removed and rinsed with four washings of tank water. They were prepared for histological sectioning. The eggs were fixed in Tellesnicky Solution for 24 hours and then rinsed in water for 24 hours. The eggs were then dehydrated in 70 percent, 80 percent, 95 percent, and 100 percent alcohol for one hour periods. They were cleared in Toluene for 30 minutes. The eggs were infiltrated with wax, imbedded in wax, and sectioned at 10 microns, and mounted on slides.

Kodak NTB3 liquid emulsion, for highest sensitivity recording of all charged particles, was used following the procedures for routine preparation for autoradiographs (Kopriwa and Leblond, 1962). The package of emulsion was removed from the refrigerator and allowed to come to room temperature. In the darkroom, equipped with a Wratten safe light #2, 25W, the emulsion was placed in a water bath to
warm for 30 minutes at 43°C. The contents were slowly rotated, to mix well, before pouring into a Coplin jar used for dipping. The contents were allowed to stand for a few minutes to allow any bubbles that may have formed to dissolve. The emulsion was diluted 1:1 with distilled water.

The slides which had been deparaffinized (four changes of xylene and alcohol for three minutes each and 15 minutes under running water) and warmed on the cover of the water bath were then dipped into the emulsion for approximately 15 seconds. The slides were removed from the emulsion, allowed to drain on a paper towel, and placed in a drying rack dip side down. The slides were allowed to dry and harden for approximately 15 minutes. The dried slides were then placed in a light proof box containing Drierite at one end, sealed with black plastic tape, and placed in a refrigerator.

The slides remained in storage for two weeks then removed, brought to room temperature and developed. The developed slides were then stained with Eosin and Hematoxylin.

Procedure for Developing Autoradiographic Slides.
1. The slides were allowed to come to room temperature.
2. The slides were placed in Kodak, D-19, developer for two minutes.
3. The slides were placed in Kodak fixed for two
4. The slides were washed in running water for 15 minutes.

5. The slides were stained.

**Staining Procedure for Autoradiographic Study.**

1. While still wet, slides were placed in hematoxylin for one minute.

2. Differentiated in running tap water.

3. Emulsion removed from back of slides with razor blade.

4. Dipped in 80 percent alcohol for 30 seconds - one minute.

5. Quick dip in Eosin (80 percent ETOH).

6. Quick rinse in 95 percent ETOH (three times in different jars).

7. Then absolute ETOH (four times in different jars).

8. The four rinses of xylene, for three minutes each, in four jars.

9. Dip 80 percent ETOH.

10. Dip Eosin.

11. Ninety-five percent ETOH.

12. Four rinses of absolute alcohol.

13. Three rinses of xylene.

14. Cover slip for permanent slide.
CHAPTER IV

DISCUSSION

An attempt has been made to answer two questions important to all aquatic pollutants: (1) Can the substance reach the embryo? and (2) Is there a site of concentration within the embryo?

Figure 1 represents the increase in counts per minute per embryo, as the concentration of LAS is increased, for the zebra fish. There is an overlap in data when considering the standard deviations (see Table 1). This wide range of data is explainable on the basis of recent information which was not available when this work was done. Embryos from the preneurula stage to the second day of development were used for this study. Recent work (Manner and Meuhlmann, 1975) has indicated that the amount of uridine which passes an LAS treated chorion increases, over 800 percent, during that two day period when the concentration is kept the same on the outside. Also, because of the large number of animals used the mean becomes more significant than the range or standard deviation. When examining the means alone (see Fig. 1) there is an increase from 4 to 6 to 8ppm. However, this is of secondary importance. Of primary importance, is the fact that the material is reaching the embryo. From this data it is clear...
Figure 1. Counts per Minute per Embryo for Zebra Fish at 4, 6, and 8 ppm LAS
Table 1. Counts per Minute per Zebra Fish Embryo*

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<th>6ppm</th>
<th>8ppm</th>
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<td>Mean</td>
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Table 1. (continued)

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<th>Concentration of LAS</th>
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<td>Standard Deviation</td>
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<tr>
<td>Standard Error</td>
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</table>

*The counts per minute reflect the amount of Carbon-14 diffusing across the chorion after a four hour pulse dose. The counts are based on the average count for 10 embryos.
that the LAS reaches all of the embryoes.

Figure 2 represents the increase in counts per minute per embryo, as the concentration of LAS is increased, for the fat head minnow. Again the overlap in the standard deviations (see Table 2) and the occasional high count for individual animals is explained by the variability in the age of the eggs used.

In comparing Figure 1 and 2, it is apparent that the total counts were lower for the fat head minnow, for each of the three concentrations, than for the zebra fish. Also, there is a decrease in counts from 6 to 8ppm for the fat head minnow, where there is an increase in the corresponding range for the zebra fish. The difference in total counts may be due to a difference in metabolism in the embryoes of the two species. The fat head minnow has a longer development time, its metabolism may be slower, and uptake of pollutants may be smaller. The decrease in counts from 6 to 8ppm may indicate that the fat head minnow is more sensitive to the toxic effect of LAS (even though the net amount reaching it is less) and that the embryoes are dying at the 6ppm level. Again, there are many variables that can affect how much LAS reaches the embryo, but it is clear from this data (see Fig. 2) that LAS does reach the embryo of the fat head minnow.

In the work with zebra fish and fat head minnow, although care was taken to rinse the eggs to wash off any LAS that might have adhered to the chorion, no rigorous proof was available to show that the LAS was really removed.
Figure 2. Counts per Minute per Embryo for Fat Head Minnow at 4, 6, and 8ppm LAS
Table 2. Counts per Minute per Fat Head Minnow Embryo*

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</table>

Mean              | 43.26| 58.84| 53.83|

Standard Deviation | 31.32| 24.16| 27.72|

Standard Error     | 7.00 | 5.27 | 6.05 |

*The counts per minute reflect the amount of Carbon-14 diffusing across the chorion after a four hour pulse dose. The counts are based on the average count for 10 embryos.
An autoradiographic study was done on the fat head minnow egg to see if the LAS did reach the embryo.

The results indicate that the highest density of Carbon-14 granules are clustered in three regions: the neural tube; the yolk; and the eye. Figure 3 shows a section through the somites and notochord. The regular dispersion of granules is in contrast to the clusters of dark granules in the yolk cells (bottom right). Figure 4 shows the large yolk cells with the cluster of Carbon-14 granules at a higher magnification. Figure 5 is a cross section through the eye, the dark outer layer is the pigmented layer. At a higher power the Carbon-14 granules are visibly clustered around the lens (see Fig. 6).

The results of this work indicate that the LAS did reach the embryo. Once in the embryo the LAS reached the yolk then passed through the blood stream to the nervous system, especially to the brain. This may explain the findings of other researchers who have shown that the developing zebra fish embryo, at the neaurula stage is extremely sensitive to the lethal effects of LAS (Manner and Thompson, 1974).

The question of whether or not LAS can reach the embryo was extended to a terrestrial animal, the mouse. The oral administration of LAS was not considered acceptable as the liver and digestive system had to be considered as potential sites for the breakdown of the LAS molecule. The intraperitoneal route was chosen.
Figure 3. Autoradiograph of Stage 12 Fat Head Minnow Embryo After Four Hour Exposure to 8ppm LAS. Section Through Somites and Notochord.
Figure 4. Autoradiograph of Stage 12 Fat Head Minnow Embryo After Four Hour Exposure to 8ppm LAS. Section Through Large Yolk Cells.
Figure 5. Autoradiograph of Stage 12 Fat Head Minnow Embryo After Four Hour Exposure to 8ppm LAS. Section Through the Eye.
The highest dosage, 100mg/kg was the only one that the embryos and only the 100mg/kg and 200mg/kg doses were used.

Figure 7. A graph of the data. There is an increase in counts per minute per embryo between the 60mg/kg and 200mg/kg doses for both days. For both days, 12 and 18, in the 100mg/kg dose the standard deviations (see Table 3 and 4) are small. In contrast at the 200mg/kg dose for these two days, the standard deviations are large (see Tables 5

Figure 6. Autoradiograph of Stage 12 Fat Head Minnow Embryo After Four Hour Exposure to 8ppm LAS. Section Through the Eye.
The highest dosage, 300mg/kg was lethal to the mother and only the 100mg/kg and 200mg/kg dosages were used. Figure 7 is a graph of the data. There is an increase in counts per minute per embryo between the 100mg/kg and 200mg/kg doses for both days. For both days, 12 and 15, at the 100mg/kg dose the standard deviations (see Tables 3 and 4) are small. In contrast at the 200mg/kg dose for these two days the standard deviations are large (see Tables 5 and 6). Apparently at the 100mg/kg level the amount of LAS reaching the embryos is greater on day 15 than on day 12 but this distinction cannot be made so clearly for the 200mg/kg dose. There is an overlap of data when the standard deviations are considered. The LAS is reaching the embryos on both days and at both concentrations, but at the higher dosage the correlation between the age of the embryos and counts cannot be made. This is understandable, if at 300mg/kg the LAS was lethal to the mother, at 200mg/kg the toxicity of the LAS to the mother may have affected her circulation and consequently fetal circulation.

The intraperitoneal method used in this work prohibits conjecture about placental transfer. There is no way of knowing whether the injected LAS entered the maternal blood stream and crossed the placental barrier. It is possible that the LAS passed through the ostium of the oviduct. In this case the LAS still had to cross the chorio-allantoic membrane to reach the fetus. This data indicates that the LAS did reach the embryo. Further work
Figure 7. Counts per Minute per Embryo for 12 and 15 Day Mouse at 100 and 200mg/Kg LAS
Table 3. Counts per Minute per Mouse Embryo*

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*The counts per minute reflect the amount of Carbon-14 diffusing across the chorio-allantoic membrane of the 12 day old mouse embryo after a four hour pulse dose of 100mg/kg LAS.
Table 4. Counts per Minute per Mouse Embryo*

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*The counts per minute reflect the amount of Carbon-14 diffusing across the chorio-allantoic membrane of the 15 day old mouse embryo after a four hour pulse dose of 100mg/kg LAS.
Table 5. Counts per Minute per Mouse Embryo*

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*The counts per minute reflect the amount of Carbon-14 diffusing across the chorio-allantoic membrane of the 12 day old mouse embryo after a four hour pulse dose of 200mg/kg LAS.
Table 6. Counts per Minute per Mouse Embryo*

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*The counts per minute reflect the amount of Carbon-14 diffusing across the chorio-allantoic membrane of the 15 day old mouse embryo after a four hour pulse dose of 200mg/kg LAS.
in this area could be carried out to study the affects of LAS at ages other than the one chosen here, at concentrations between 100mg/kg and below 200mg/kg (which may be less toxic), and an autoradiographic study would be of interest to see where the LAS was localized in the mouse embryo.

Summary

It was shown that LAS did reach the embryo of the zebra fish and also of the fat head minnow. Autoradiographic study revealed that, for stage 12 fat head minnow, after a four hour exposure to Carbon-14 labeled LAS, the LAS was concentrated in the yolk, the neural tube and the eye.

LAS was also shown to reach the embryo of the mouse after intraperitoneal injection.
BIBLIOGRAPHY


APPROVAL SHEET

The thesis submitted by Annabella Juhasz has been read and approved by the following committee:

Dr. Harold W. Manner, Director
Professor, Biology, Loyola
Chairman, Biology, Loyola

Dr. Mark Goldie
Assistant Professor, Biology, Loyola

Dr. Edward Palincsar
Professor, Biology, Loyola

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

Date: Jan. 13, 1976

Director's Signature: [Signature]