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CHANGES IN HEMATOCRIT AND THE
LEVEL OF THE SERUM ENZYMES GOT
AND ICD IN FUNDULUS HETEROCLITUS
SURVIVING SHORT AND LONG TERM
EXPOSURE TO THE SURFACTANT LAS

GARY F. HUNNISETT

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University of New Hampshire, Ph.D.,
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SERUM ENZYMES GOT AND ICD IN FUNDULUS HETEROCLITUS
SURVIVING SHORT AND LONG TERM EXPOSURE TO THE SURFACTANT LAS

by

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A THESIS

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To Sadie Stewart, biologist.

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LIST OF ABBREVIATIONS

- ABS - alkyl benzene sulfonate.
- AH₀ - accept the null hypothesis.
- C_{se} - standard error of control population values.
- C _{\bar{x}} - mean of control population values.
- (S)GOT - (serum levels of) glutamic-oxalacetic transaminase.
- (S)ICD - (serum levels of) isocitric-dehydrogenase.
- IR - infra-red.
- LAS - Linear alkylate sulfonate
- LD₅₀ - that concentration of toxicant lethal to 50% of the test population.
- mg/l. - milligrams per liter.
- ppm - parts per million.
- ppt - parts per thousand.
- RH₀ - reject the null hypothesis.
- SD - standard deviation.
- SE - standard error.
- (TLM) - that concentration lethal to 50% of the fish.
- T_{se} - standard error of treatment, population values.
- T _{\bar{x}} - mean value of the treatment.

ABSTRACT

CHANGES IN HEMATOCRIT AND THE LEVEL OF THE
SERUM ENZYMES GOT AND ICD IN FUNDULUS HETEROCLITUS
SURVIVING LONG AND SHORT TERM EXPOSURE TO THE SURFACTANT LAS

by

GARY HUNNISETT

F. heteroclitus was exposed to the anionic surfactant linear alkylate sulfuric acid (LAS) at 3, 2, and 1 ppm for 48 hours, and, in continuous flow fashion, to 0.8, 0.6, 0.5 and 0.4 ppm LAS for 60 days. Both tests were run in sea water diluted to 24 ppt with distilled water. Ambient temperature was $15^{\circ}\text{C} \pm 2^{\circ}$.

Changes in hematocrit, body weight, and the activity of the serum enzymes glutamic oxalacetic transaminase (GOT) and isocitric dehydrogenase (ICD) were monitored in both tests. Short term test results (N=180) were analyzed by using a Model III analysis of variance (hematocrit) or a Kruskal Wallace analysis of variance (SGOT, SICD). Long term test results (N=42) were expressed in terms of median and range.

Statistically significant graded increases in hematocrit, SGOT and SICD were evident at all concentrations used in the 48-hour tests. Weight loss was also proportional to concentration. Statistically significant rho correlation coefficients were found to exist between SGOT and SICD, but not between either of these and hematocrit. It was concluded that the observed changes were not due to hemoconcentration. The ratio of variances from control and test populations was statistically significant ($p > .01$) for the enzymes, but not for hematocrit.

Elevations in hematocrit, SGOT and SICD were also observed in most fish surviving the long term exposure. A low but statistically significant correlation existed between time of death and the presence of LAS.

Whether the liver was the source of the additional enzyme activity in the serum was not revealed in this study. No histologic changes in the livers of fish used in short term tests were noted. While such changes were observed in the livers of fish surviving long term tests, similar changes were obvious in the control fish. The evidence for alterations in the weights of the livers of the fish used in the long term tests was obscured by changes in the total body weight.

In light of similar elevated serum enzyme levels described in the literature resulting from disease and insecticides, it seems that these parameters are sensitive indicators of a variety of stresses.

SECTION I

INTRODUCTION

Modern household detergents contain from 8-12% surfactants (Duthie, 1966). These compounds serve to lower the surface tension of water, and to loosen, solubilize, and suspend oily soil. Linear Alkylate Sulfonic Acids (LAS) are compounds most common to such surfactants.

Because of the rapid biodegradability of LAS by a wide variety of naturally occurring bacteria (Heyman and Molof, 1967) the level in most surface waters is low. The distribution of surfactant components is widespread, however, since as Hahn (1966) has pointed out, the sewage from only 33% of the people in the United States is sufficiently treated to remove all detergents. The results of the investigation of Muzzi et al. (1968) indicate that, like the ubiquitous coliform bacteria, surfactant concentration can be used as an index of sewage treatment effectiveness.

The U.S. Public Health Service permissible standard for anionic surfactant in drinking water is 0.5 mg/l. as indicated by a standard methylene blue colorimetric assay (Lieber, 1969). Sullivan and Swisher (1968) have shown that this level is 10 times the concentration found in the Illinois River, which in previous years had been shown to be relatively contaminated. The level of surfactant in the wells, lakes, and streams of Wisconsin were even lower (Lawton, 1967).

Irrespective of this type of consideration, the Bavarian Biological Institute in Munich saw fit to recommend that a zero tolerance for LAS be established for outflows leading into the surface waters of the

German Federal Republic (Liebmann; 1966). The main reason for such a decision was the high toxicity of LAS to fishes. The four day LD₅₀ values for five species of fishes were found to range from 3.3 mg/l. to 6.4 mg/l. (Thatcher and Santer, 1967). These concentrations were two to four times higher than corresponding values for another alkyl benzene sulfonate type surfactant (ABS) used prior to 1965. The latter compound, however, is relatively non-biodegradable.

In a study by Swisher, O'Rourke, and Tomlinson (1964) the 24 hour LD₅₀ for bluegills to the 12 and 14 carbon homologs of LAS was found to be 3 mg/l. and 0.6 mg/l. respectively. Due to the rapid biodegradation of LAS, the fingerlings were able to survive in effluent from a continuous flow activated sludge system being fed 100 mg/l. Toxic intermediates did not accumulate during the bio-degradation process. The investigators concluded that LAS did not constitute any added threat to the nation's fish life.

Studies on the chronic effects of LAS on the well-being of fishes are more equivocal. At concentrations close to that found in the surface waters of Italy, both C₁₂ and C₁₄ LAS have been shown to affect the swimming ability of fishes (Marchetti, 1968). Failure of the lateral line system of Ictalurus natalis (Bardach, Fujiya, and Hall, 1965), and an increased sensitivity to endrin poisoning (Solon, Lincer, and Nair, 1969), have also been described as resulting from chronic exposure to LAS.

Alterations in the feeding behavior of the flagfish Jordaniella floridae (Foster, Scheier, and Cairns, 1966); loss of spermatocyte mobility, reduced growth rate, and increased susceptibility to epithelial invasion in trout (Mann, 1967); and increased sensitivity to DDT poisoning in goldfish (Dugan, 1967) have all been shown to result from chronic exposure to ABS.

A more direct response of fishes to both LAS and ABS type anionic surfactants is a degeneration of gill tissue. This is well documented, particularly at acute levels. Such changes are usually characterized by: altered levels of mucus secretion, thickening of the lamella and epithelial tissues, and the presence of blood clots in the circulatory vessels (Schmid and Mann, 1961; Mann, 1967; Dooley, 1968; Cairns and Scheier, 1966). The latter investigators demonstrated also that the gill damage resulting from the exposure of Lepomis gibbosus to the surfactant ABS at levels close to the physiologic limit did not impair the ability of this fish to regulate chloride ion. Extensive gill damage resulting from 120 days' exposure to ABS was shown also in Salmo gairdneri (Hassler, 1965). This investigator documented changes in plasma protein levels, numbers of leucocytes, and hematocrit values. When Fundulus heteroclitus was exposed to similar surfactant stress, there were no significant changes noted in hematocrit values, gonadosomatic index, growth rate, or liver condition (Eisler, 1965). The toxicity of ABS was shown to be partially a function of salinity.

It is thought that anionic surfactants like ABS and LAS act on fishes primarily via their surface tension reducing properties (Gloxhuber and Fischer, 1968). This view has not been completely accepted by Marchetti (1968). Alteration of pH is cited by the latter author as one important synergist causing mortality, in spite of the earlier observations of Dooley and Cavill (1964). These investigators exposed fishes to the same concentration of different anionic surfactants with pH ranges from 6.4 to 9.5. They found no relationship between mortality and pH at a given surfactant concentration, and concluded that surface tension reduction was primarily responsible for the observed mortalities. In either case, it seems to be generally agreed that mortality is due to

anoxia resulting from gill tissue damage.

The problem of determining the surfactant concentration which just causes gill alterations is compounded by the existence of susceptible and resistant individuals (Swisher, et al., 1964).

It is the purpose of this investigation to examine the effects of LAS on parameters which are distributed in a continuum fashion, and which may be related to the more discrete physiologic changes such as mortality or gross histology. Such parameters are commonly used in clinical situations to evaluate physical well-being and incipient damage, and to follow the course of treatment. The potential value in applying such an approach to the problems of fisheries workers has been alluded to by Waldichuk (1969) and Bouck and Ball (1965).

The parameters selected for the present investigation are hematocrit, and the blood fluid levels of the enzymes glutamic-oxalacetic transaminase (GOT) and isocitric dehydrogenase (ICD). Changes in the level of these parameters resulting from stresses similar to those resulting from LAS exposure have been described in many vertebrates. The following is a review of such studies.

In addition to the ABS surfactant induced hematocrit alterations already cited, changes in hematocrit level have been shown to result from sub-lethal exposure of carp to mercuric chloride (Tamura, Yasuda, and Fujiki, 1962); rainbow trout to chromium (Schiffman and Fromm, 1959); goldfish to low oxygen (Summerfelt, Lewis, and Ulrich, 1967); trout to low oxygen (Phillips, 1947); and various fishes to general disease (Young, 1949; Bell, 1968). A review of the use of hematocrit to fishery researchers is given by Snieszko (1960).

The use of the serum enzyme levels of GOT and ICD in clinical and veterinary situations is routine (Annino, 1964; Hoe and Sabara, 1967:

Cardinet, 1963). Such an approach is possible in homeotherms generally due to the constancy of the enzyme levels in the serum and of the demonstrated specificity of their response to the disease or trauma causing an elevation. Application of clinical procedures used with these enzymes to the problems of fisheries workers has been slow, and hindered by a lack of basic information relating to "normal" conditions.

In an effort to establish norms for SGOT in immature rainbow trout, Barnhart (1969) showed that the level of this enzyme was related to age, but not to sex or either of two commercial diets. Presumably both diets were sufficient in Vitamin B₆, as this strongly affects the level of SGOT in carp (Ogino, 1965).

No response to the fatigue of being caught on a hand line was evident in sharks with respect to SGOT, although the levels of a second serum enzyme (lactic dehydrogenase) were affected (Rasmussen, 1967).

Naturally occurring kidney disease, and experimentally induced liver necrosis each caused an increase in the level of SGOT in the Pacific Salmon (Bell, 1968). Such data confirm the possibility that enzyme levels reflect organ pathology as they do in homeotherms. Alterations in the level of five liver tissue enzymes of F. heteroclitus recently have been shown to reflect sublethal metal salt poisoning (Jackim, Hamlin, and Sonis, 1970). It would seem that such alterations, or the results of such alterations, may be reflected in the serum.

The classic study in the development of a bioassay based on altered tissue enzyme activity was that of Weiss (1959). The inhibition of brain cholinesterase activity by organophosphorous insecticides which he first demonstrated, became the basis of a bioassay for these compounds (Holland, Coppage, and Butler, 1962). Serum levels of cholinesterase were also shown to be affected by organophosphorous insecticides (Hayama

and Kuwabara, 1962).

Another study on the relation of serum enzymes of fishes to the chemical environment in which they live is that of Lue-Hing (1966). This investigator was able to show that time and concentration dependent changes in SGOT and ICD level of goldfish occurred in response to trace levels of the organic pesticides endrin and guthion. The specificity of such a change, or the statistical reliability, have not been yet determined.

In light of the preceding studies, the following investigation was undertaken to determine the chronic and acute effect of various concentrations of LAS on hematocrit, and the serum enzymes GOT and ICD. It may be that the serum enzyme response in fishes is not specific to insecticides, diet, and disease conditions described in this literature review. If it proves to be a sensitive non-specific response, it may provide the basis for the development of "application factors" to be used with mean tolerance limit (TLM) data to arrive at "safe" levels of contaminants in the environment. Conversely, it may prove to be useful in estimating the general well being of fishes exposed to waters whose facilities do not include equipment for removing anionic detergents, or in waters where the interaction of many substances, each in themselves below toxic limits, may combine to impair the normal functioning of physiologic processes.

SECTION II

MATERIALS AND METHODS

1. Care and Collection of Test Fishes

Fundulus heteroclitus is found locally in the shallow waters bordering the estuaries and bays. In two years of collecting with bread trap and seine it was found that these fishes frequent this area from the last days of May to the end of November. During these months, F. heteroclitus was routinely collected from two sites: the mouth of Crommet Creek at the level of the Durham Point Road; and at an inlet of Newcastle Island facing the north-east side of Pest Island.

After Fundulus had disappeared from the collecting sites, they were obtained from a bait dealer, who trapped in the deeper waters at the mouth of the Cocheco River. These fishes were stored in large continuous flow holding tanks, and were available for purchase throughout the period December to May. Fish from this population were used exclusively for the static 48-hour tests with LAS. Storage in the University cold room holding facilities was limited to a two day acclimation period.

Fish maintained for more than five days in the University holding facilities (i.e. those used in long term studies with LAS, or those used as a source of serum for working out assay techniques) were fed a commercial marine fish diet of Biorell* supplemented with chopped beef liver. The content of the commercial preparation is given in Table I.

* Sternco Industries, Harrison, N. J.

Table I. Content of commercial fish food preparation*
 fed to F. heteroclitus maintained in the cold room

Ingredients	Analysis
Fish Meal	Minimum Crude Protein 45%
Fish Roe Meal	Minimum Crude Fat 4%
Fish Liver	Maximum Crude Fibre 9%
Crayfish Meal	Maximum Ash 15%
Insect Larvae Meal	Maximum Moisture 8%
Dehydrated Kelp Meal	
Mussel Meal	
Brine Shrimp Meal	
Wheat Germ Meal	
Cod Liver Meal	

*Biorell, Sternco Industries, Harrison, N. J.

Vitamin B₆, a coenzyme of transamination reactions, has been shown to influence tissue levels of GOT in carp (Ogino, 1965). In addition, if dietary anemia results from eating a dry food diet in F. heteroclitus as it does in trout (Phillips and Brockway, 1957), this effect would also be circumvented by the addition of the fresh chopped liver supplement.

With the preceding diet, continuous aeration, and a salinity level of 24 parts per thousand, mortality was negligible over the course of the studies.

2. Hematology

Blood Sampling:

The fish were restrained by wrapping their anterior end with paper toweling. The caudal peduncle was then severed cleanly, and the exposed tissue quickly blotted to remove excess fluids. The end of a capillary tube (prepared as described later) was then placed directly over the open dorsal blood vessel.

No anesthetic was used in the study as it tended to reduce the rate of blood flow. This observation was also noted by Slicher (1961).

Blood collection was completed within 60 seconds. The capillary tube was sealed and color coded with modelling clay, and spun at 11,500 rpm for 2 1/2 minutes in an International Micro-capillary Centrifuge. Ice packs were used to keep the centrifuge head cool between runs.

Following centrifugation, the hematocrit was determined by use of a millimeter rule and the sample promptly refrigerated. If enzyme analysis was to be delayed more than six hours, the samples were frozen.

Prior to analysis, a file cut was made 3 mm above the erythrocyte level, the tube broken, and the serum drawn into a lambda pipette. The sample was discarded if clots were present, if blood flow was slow, or if hemolysis was visible by inspection.

Prevention of Clotting:

The blood of fishes clots rapidly (Vars, 1934) and the blood of F. heteroclitus is no exception.

Heparin is the recommended anticoagulant for fishes (Hesser, 1960). When standard capillary tubes* containing at least two USP units

*Scientific Products Division of American Hospital Supply Corp.

of heparin were used, clotting occurred. Usually this was within 90 seconds of the time of the incision. The clotting process was hastened by exposure to body fluids, an observation also noted by Wolf (1959) in fresh water fishes. Larsen and Snieszko (1961) have observed that when 10% heparin solutions are allowed to evaporate to dryness in standard capillary tubes, no clotting resulted when the tubes were used with trout blood. As this procedure was not effective with F. heteroclitus, the following modification was adopted.

Powdered sodium heparin* was allowed to drop through a microhematocrit tube (1.1 mm X 75 mm) so that minute grains adhered to the internal surface of the tube. Tubes so prepared were successful in preventing clotting, and were used throughout the study.

Both Wolf (1959) and Yuki (1960) have pointed out that the gelation of erythrocytes contributes to the coagulation process in fishes. Since the enzyme content of the erythrocyte is high, hemolysis could lead to spurious serum enzyme values. Some investigators have noted such values in fish blood assayed after clots had occurred (Barnhart, 1965; Bell, 1968). Others have allowed clotting to occur (Lue-Hing, 1966; Rasmussen, 1967).

Replicate enzyme assays were performed on the same F. heteroclitus blood sample treated to allow clotting in one portion, and no clotting in the other. The mean value of the GOT serum sample (N=10) was 37% higher than the plasma control, and 4% higher than the plasma sample control for ICD. The standard deviations of the serum samples were 31% and 27% higher than the plasma samples for GOT and ICD respectively. On the

*Sigma Chemical Company, St. Louis, Mo.

basis of these results, it was decided that control of clotting was essential both to insure that only the activity of the enzymes in the blood fluid was being measured, and to obtain homogeneous results.

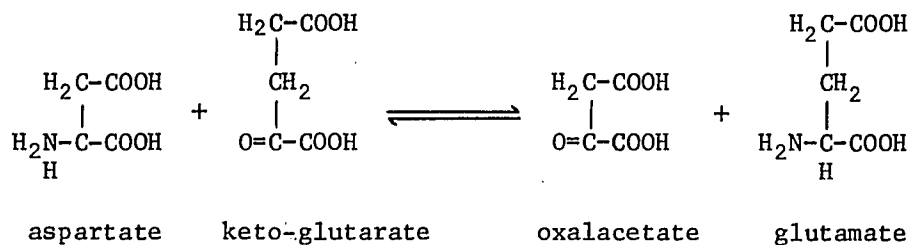
The more common term "serum enzymes" is used throughout referring to the blood fluid level of GOT and ICD, although the assays were performed on heparinized blood fluid containing serum with clotting elements or plasma.

Erratic hematocrit values have also been shown to result from the presence of even small clots in the serum of fishes (Snieszko, 1960).

3. The Serum Enzymes SGOT and SICD

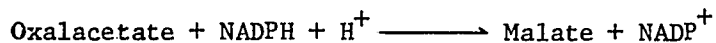
Theory of Analysis: GOT

GOT is involved with the catalysis of the following reaction:



The reaction can be followed by any of the following methods:

1. Paper chromatographic analysis of the reaction products after incubation. Quantification, however, is difficult.
2. Direct spectrophotometric measurement of oxalacetate formation at 280 m μ . Instability of the oxalacetate in solution makes this approach unreliable. (Karmen, Wroblewski, and LaDue, 1955).
3. Enzymatic decarboxylation of one of the products and subsequent manometric measurement of carbon dioxide. Karmen et al., (1955) have pointed out that the high bicarbonate content of the blood, along with a low level of transaminase, makes this procedure difficult to apply to serum analysis.
4. Measurement of the oxalacetate formed by use of:
 - a. A coupled reaction catalyzed by malic dehydrogenase..



The NADPH absorbs strongly at 340 m μ , and the progress of the reaction can be followed by recording loss of absorbance with time. Although this procedure is the basis for the definition of transaminase units, Annino (1965) states that it is "tedious and time consuming, employs unstable reagents, and requires the

use of an ultraviolet spectrophotometer."

- b. 2-4 dinitrophenylhydrazine as a ketone trapping reagent. This method (to be described in detail) was selected for use in the study because:
- (1) It seems to be the procedure most frequently used in clinical and experimental situations, as reported in the literature.
 - (2) The assay is relatively straightforward, and is available in kit form.
 - (3) It is reported (Sigma Technical Bulletin 505) to be the most reliable procedure for the assay of SGOT.
 - (4) Standard units of activity can be obtained. This is useful for comparisons with results of other studies.

The Sigma 505 procedure for SGOT analysis (Sigma Technical Bulletin 505) couples the reaction product oxalacetate formed in a unit time with 2-4 dinitrophenylhydrazine. Inasmuch as the alkaline stable hydrazones of pyruvate (formed from the spontaneous breakdown of oxalacetate) alpha ketoglutarate and oxalacetate are found in the reaction mixture (Bergmeyer, 1963), spectrophotometric measurements are made at 505 μ where the greatest differentiation between the optical densities of the three hydrazones occur.

Translation of optical density to units of enzyme activity is accomplished by use of a standard curve (optical density - ordinate, GOT activity - abscissa) recorded in Sigma Frankel Units.

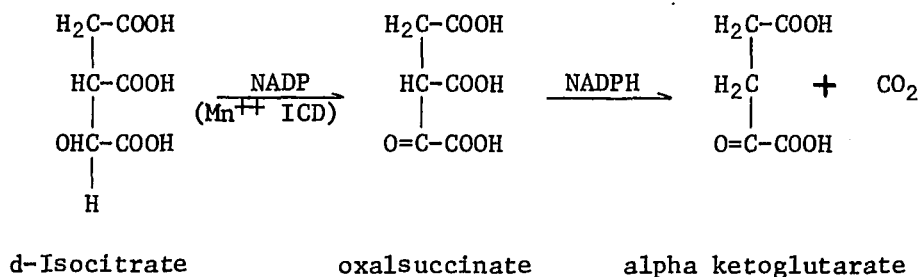
These units are defined as follows:

"One theoretical Sigma Frankel Unit of Glutamic Oxalacetic Transaminase will form 4.82×10^{-4} μ m of glutamate per minute, at pH 7.5 and 25° C."

(Sigma Technical Bulletin 505)

Theory of Analysis: ICD

Isocitric dehydrogenase is an enzyme which catalyzes the following reaction:



It is possible to monitor the course of the above reaction by:

1. Following NADPH activity by noting the increasing optical density at 340 m μ as the reaction proceeds.
2. Complexing the alpha ketoglutarate product with 2-4 dinitrophenylhydrazine. The resulting hydrazone is highly colored in alkaline solution, and absorbs strongly at 410 m μ .

A kit form (Sigma Technical Bulletin #175) of the colorimetric method was selected for use in this study because of its technical simplicity and reproducibility.

The optical activity of the colored reaction products is translated into enzyme units by the use of standard solutions whose optical density corresponds to known enzyme concentrations as determined by ultraviolet methods. Thus, one Sigma Unit of ICD activity will produce one millimicromole of NADPH per hour at pH 7.5 and 25° C.

Modification of Sigma Test Procedures:

The serum volumes which can be obtained from the test fishes is limited by the following facts:

1. As a class, the bony fishes have a total blood volume/body weight ratio 2-5 times lower than that of the other vertebrate classes

(Conte, Wagner, and Harris, 1963).

2. The maximum size of the fishes which can be obtained locally in sufficient numbers was found to be 6-10 grams. Only 40 lambdas of serum could routinely be obtained after centrifugation.

Thus, serum volume considerations necessitated the use of micro procedures.

The "normal" GOT and ICD levels in the plasma of F. heteroclitus were found, on preliminary inspection, to be in the order of ten times the accepted normal range for humans. These high values are in accord with the high normal values of GOT and ICD found in the blood fluid of carp by Zimmerman et al. (1965), and of GOT in the blood fluid of rainbow trout by Barnhart (1969). If enzyme tests designed for humans were to be used, it became apparent that an initial dilution of serum would also have to be made to obtain proportionate optical activity from the products of the test system.

Thiers and Vallee (1958) have pointed out the dangers in comparing enzyme values obtained when different substrate volume/serum volume ratios are used. More specifically, Annino (1964) in a chapter on SGOT analysis, states that a preliminary dilution of serum does not always result in values in exact proportion to the magnitude of the dilution.

Nonetheless, preliminary dilutions of serum in humans with elevated enzyme levels is a common lab practice. (Sigma Technical Bulletins 505 and 175.) Furthermore, Sterkel et al. (1958) report that the amount of serum added to the ICD test system was linear in every case studied, and Reitman and Frankel (1957), who developed the procedure on which the Sigma procedure is based, suggest that dilution of the serum by a factor of one to ten is convenient in cases where a high SGOT exists.

Assay procedures modified from standard tests most suitable for use with F. heteroclitus were developed in accordance with the following principles:

1. Serum dilution should be minimal, and directed towards obtaining average optical density readings in the more reliable portions of the spectrophotometric scale.
2. Scaling down of reagent volumes should be adjusted so that the total volume of reagents in the test should just be sufficient to obtain an optical density reading in the spectrophotometer cuvette, or cell.
3. Replicability of the test should be high.

Four dilution schedules based on these considerations were prepared by altering the Sigma Schedule for SGOT and SICD. Each schedule was used ten times with different subjects to determine the optical density which would be obtained with "normal subjects."

These data are presented in Tables 2 and 3.

Table 2. Test Schedules for SGOT

Schedule No.	Sigma	I	II	III	IV
Substrate* (ml)	1.0	0.5	0.4	0.1	0.2
Serum (ml)	0.2	0.02	0.01	0.02	0.02
Albumen (ml)	-	0.08	0.09	-	0.02
Incubation (min)	60	60	60	60	60
Color reagent** (ml)	1.0	0.5	0.4	0.1	0.2
0.4N NaOH (ml)	10.0	5.0	4.0	1.0	2.0
Total volume (ml)	12.2	6.1	4.9	1.22	2.44
OD at 505 m μ	-	0.48	0.40	0.68	0.60
Serum dilution	-	X5	X8	-	X2

* Aspartate alpha ketoglutarate (pH 7.5)

** 2-4 dinitro phenylhydrazine

Table 3. Test Schedules for SICD

Schedule	Sigma*	I	II	III	IV
Serum (ml)	0.2	0.01	0.01	0.01	0.02
0.9% Albumen (ml)	-	0.04	0.04	0.01	-
Isocitrate (ml)	0.4	0.10	0.20	0.04	0.04
MnCl ₂ (ml)	0.2	0.05	0.10	0.02	0.02
Water or TPN (ml)	0.2	0.05	0.10	0.02	0.02
Incubation (min)	60	60.00	120.00	60.00	60.00
2-4 DNPH (ml)	0.5	0.125	0.25	0.05	0.05
EDTA (ml)	0.4	0.10	0.20	0.04	0.04
NaOH (ml)	5.0	1.25	2.50	0.50	0.50
Total vol. (ml)	6.9	3.40	3.40	0.69	0.69
Dilution	-	X5	X5	X2	-
OD at 410 mμ	-	.17-.27	.19-.32	.18-.27	-

* Sigma Chemical Company Technical Bulletin #175

Legend:

TPN - Triphosphopyridine nucleotide

2-4 DNPH - 2-4-dinitrophenylhydrazine

EDTA - Ethelenediaminetetraacetate

NaOH - Sodium hydroxide

MnCl₂ - Manganese chloride

OD - Optical density

Reliability of Modified Assay Schedules:

GOT; Schedules I and II: From each of 10 large F. heteroclitus at least 0.06 ml of serum were obtained. SGOT tests were run in duplicate using both assay schedules I and II. No difference in sensitivity was apparent. The mean difference between replicate tests with the same assay schedule was approximately the same as the mean difference between methods. Schedule I was used throughout the experiment whenever sufficient serum was available.

ICD; Schedules I and II: When egg albumen was substituted for distilled water used as a serum diluent, there was no apparent difference between the sensitivity of the two schedules. When distilled water was used as the diluent, results tended to be erratic, particularly with Schedule III. This was thought to be related to the long incubation time. Absolute values obtained by both methods were approximately equal. The reaction was approximately linear over the two hour incubation in Schedule II.

Micro-cell procedures for Schedules III and IV: The possible advantage of not requiring extensive serum dilution did not compensate for the inherent slowness and lack of precision of the microcell procedures. Furthermore the mean OD values obtained with "normal" fish (Table 2, 3) were relatively high. Other specific objections noted were:

1. The microcell assembly had to be permanently installed in the spectrophotometer to obtain the best reliability.
2. The cell was extremely susceptible to scratching.
3. Proper filling, emptying, and cleaning of the cell was very time consuming.
4. The variance between readings (N=10) obtained with the microcell was greater than that between those obtained with standard cuvettes.

5. The standard curve was flatter, and consequently, there was a reduction in precision in translating optical density into enzyme units.
- For these reasons, the use of the micro-cell was abandoned.

4. Linear Alkylate Sulphonic Acid (LAS)

Physical Characteristics:

Reference samples of LAS were obtained from the Soap and Detergent Association.* These analytically standard samples are a "typical commercial linear alkylate in current use in the United States." The samples were identified as follows:

Lot # 2-5

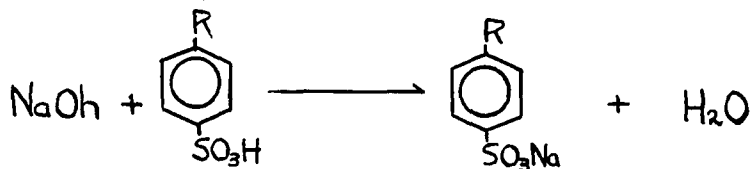
Active LAS 3.96%

Mean Molecular Weight 316

Biodegradability 90% (shake flask test)

95% (activated sludge test)

The average chain length of this sample was 11.8. (R. Sturm, Proctor and Gamble, personal communication) The free sulfonic acid form of the LAS in the ampoules is converted to the sodium salt according to the following equation:



Stock solutions, with a stable shelf life of 6 months, were prepared as follows:

1. 25.2 grams of the free acid solution was dissolved in 500 ml. of distilled water.
2. The pH of this solution was adjusted to 9.5 with 0.4 N NaOH. A Coleman Mettrion 111 pH meter was used.

*The Soap and Detergent Association, Technical and Materials Division:
485 Madison Ave., New York, N. Y. 10022.

3. The solution was brought to a total volume of 1 liter with distilled water to make a 1000 ppm solution, or to 0.75 liters to make a 1500 ppm solution.

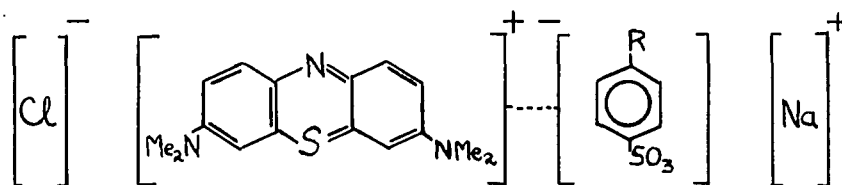
The rate of biodegradation of the test sample of LAS under the test conditions of salinity and temperature was tested by consecutive analyses of the same sample (Fig. 1.). On the basis of this breakdown rate, it was decided that there was little advantage to be gained in running the 48-hour tests with the continuous flow apparatus.

LAS Assay:

Considerations of proven reliability, simplicity of operation, low reagent costs, and availability of equipment suggested use of a methylene blue colorimetric method. Procedures using the methylene blue assay involve:

1. Formation of an ionically bonded, highly soluble, dye complex.

i.e.;



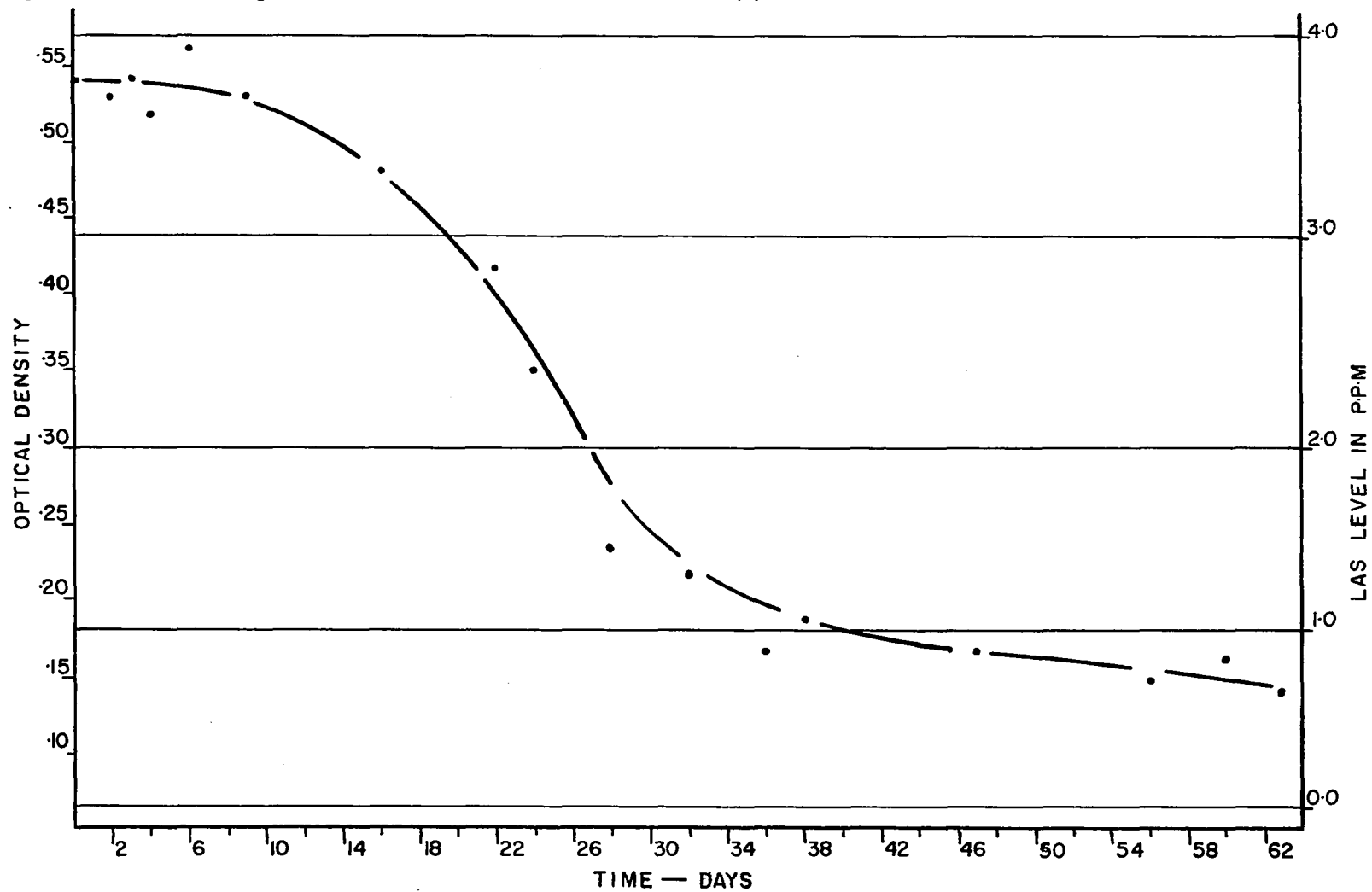
methylene blue

alkyl sulfonate

2. Extraction of complex from the aqueous phase into chloroform.
3. Separation of the aqueous and chloroform phases.
4. Determination of surfactant concentration by comparing the optical density of the methylene blue-surfactant-chloroform complex against a standardized curve of the methylene blue. (surfactant-dye concentration vs. optical density)

The procedure of Panowitz and Renn (1965) was modified for use

Figure 1. Biodegradation Rate of LAS in 24 ppt Sea Water



in these tests by omitting the use of a motor extractor, and using separatory funnels in its place. The volume of all reagents was also reduced by a factor of 5 to cut reagent costs.

The exact procedure followed in the laboratory was:

1. A 5 ml sample of test water was added to a 100 ml. separatory funnel.
2. 1 ml of methylene blue-alkaline borate solution was added and the resulting mixture shaken gently for 10 seconds.
3. 2 ml of chloroform were added, a teflon stopper was inserted, and the funnel shaken rhythmically for 3 minutes and 45 seconds. The funnel was allowed to stand upright for 15 seconds while the two phases separated, and the chloroform layer was collected from the bottom of the funnel.
4. 1 ml of chloroform was added to the aqueous phase, shaken for two minutes, and the chloroform layer was then added to the corresponding layer from step 3.
5. Same as step 4.
6. The total chloroform volume obtained in the last three steps was added to a second, clean, separatory funnel.
7. 5 ml of acid methylene blue wash solution was added, the mixture shaken for 3 minutes and 45 seconds, allowed to separate for 15 seconds, and the chloroform layer collected into a clean test tube.
8. The volume of the chloroform-methylene blue complex layer was brought to a total volume of 5 ml. in a 5 ml. pipette.
9. After standing 4 minutes, the optical density was read in a Bausch and Lomb Spectronic 20 Colorimeter at 650 m μ . This value was then changed to LAS concentration by use of a Standard Curve prepared under the same test conditions.

Reliability of the Modified Assay Used in Dilute Sea Water:

Seven consecutive assays were run on each of the same carefully standardized concentrations of LAS in sea water diluted to 24 ppt. The mean and standard deviations of such tests are reported in Figure 2. They serve to indicate the high sensitivity of the test.

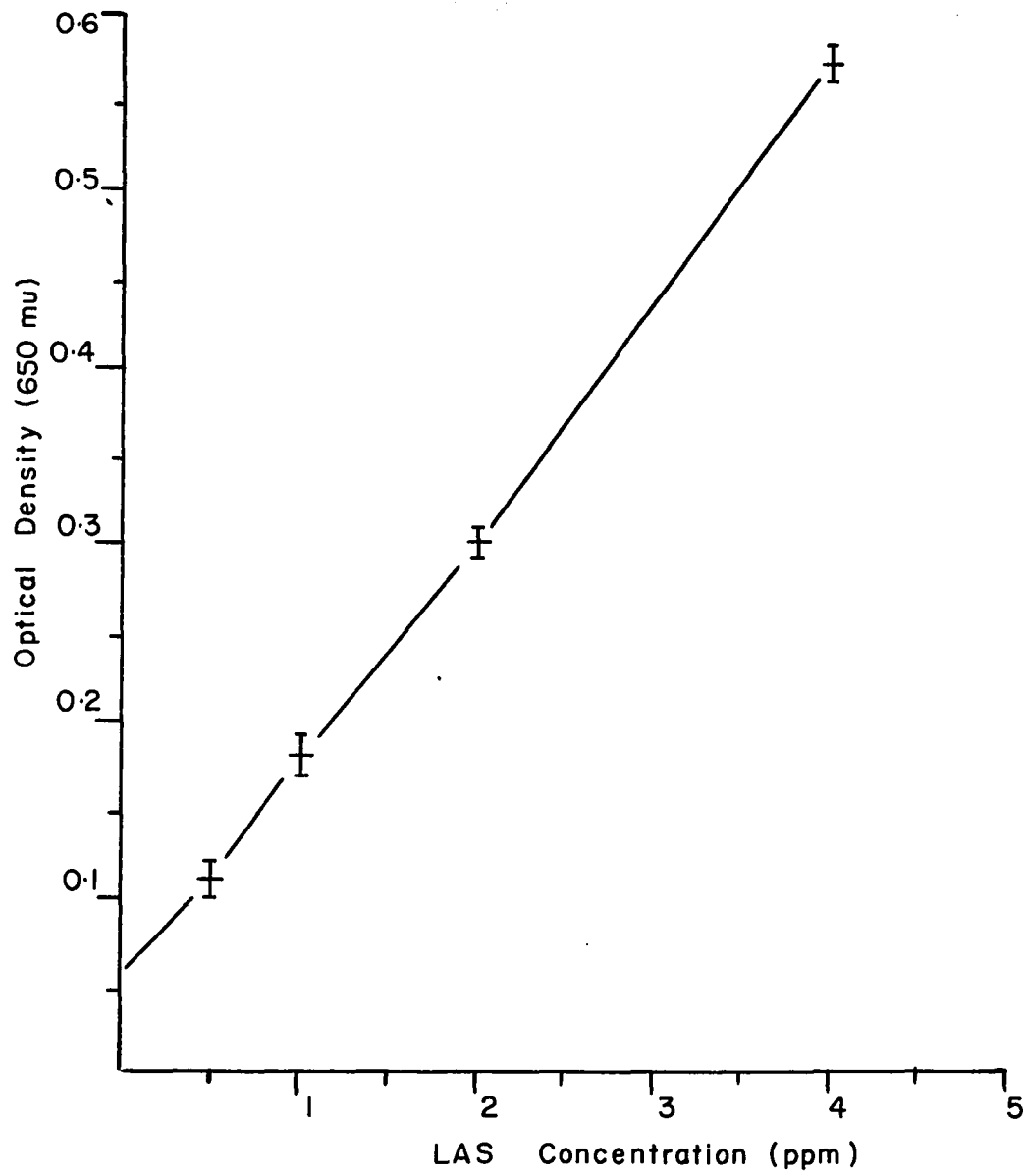
The fact that chlorides can interfere with the assay (Evans, 1950) probably accounts for the high optical density of the blank solution. It suggested also that the same LAS concentration in different salinities could result in different optical density readings. This had to be tested since chloride level could fluctuate (particularly in the long term tests) due to:

1. Errors stemming from the inherent limitations of the specific gravity method used throughout the study to measure chloride level.
2. The errors made possible in the mixing of the sea water with the distilled. In both short and long term tests, sea water of pre-determined salinity was introduced to a level line on the side of the reservoir barrel. Distilled water was added by hand to bring the total volume to a second line, precalibrated to result in the desired salinity. Fine adjustments were by trial and error.

Standard curves were run of LAS level (0, 1, 2, 4 ppm) vs. optical density in 18, 24, and 32 ppt chloride. Differences between optical densities of the same concentrations of LAS in these different salinities were not consistent, and did not fall outside of 2 standard deviation units of the 24 ppt values. It was concluded on this basis that the minor fluctuations to be expected in salinity over the long term testing period would not give rise to spurious LAS assay values.

The emulsification of aqueous and chloroform phases in the extraction process has been cited as a potential problem of the assay

Figure 2: Reliability of LAS Assay Procedures
Used in Sea Water Diluted to 24 ppt
(Mean OD value \pm 1 SD, N=10)



procedure (Standard Methods, 1965). No such difficulties were experienced with the dilute sea water, although the validity of the objections was verified when the procedures described were applied to the analysis of LAS in fresh water. The emulsification in this instance could be minimized by regulating the vigor of the mixing process.

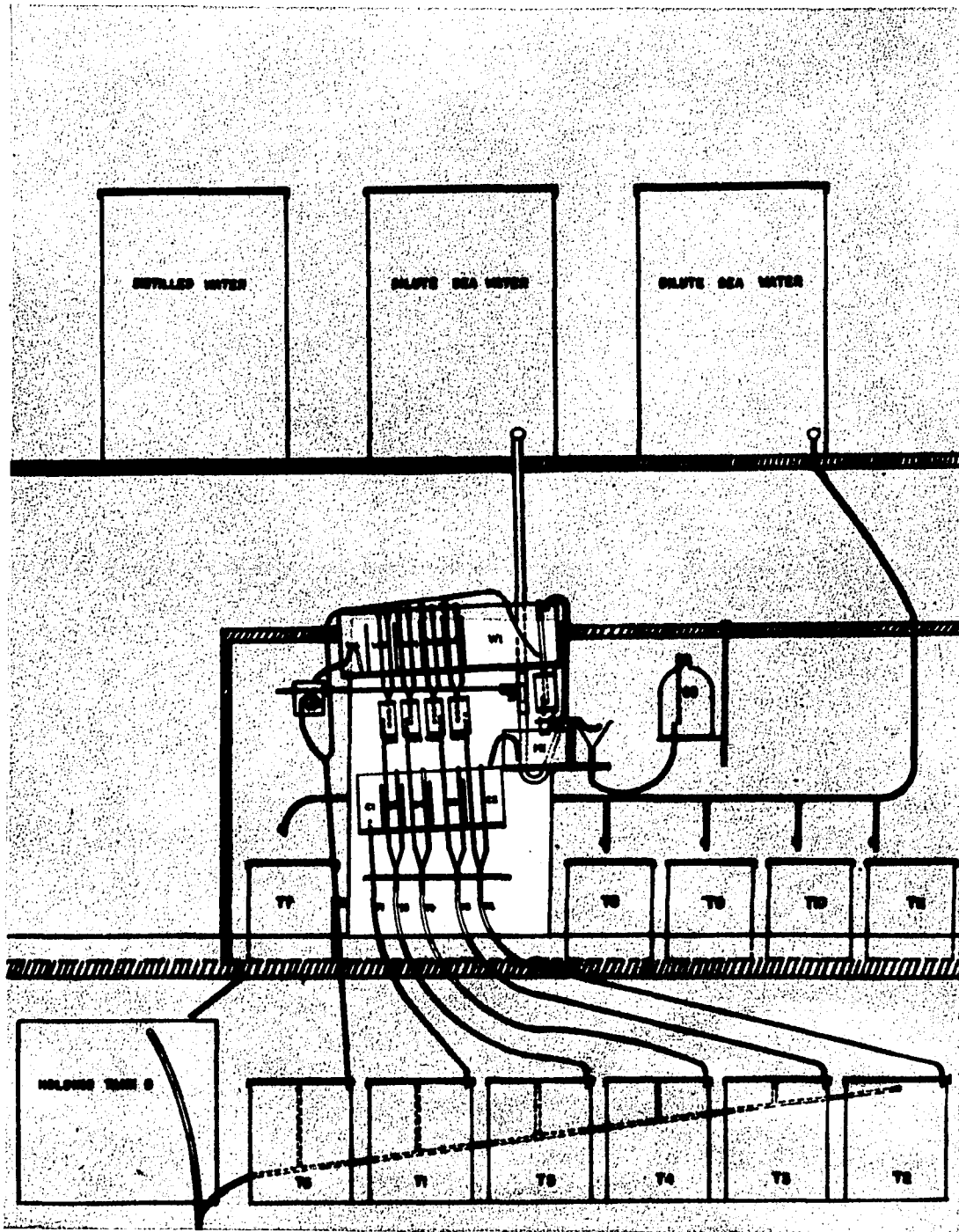
5. Continuous Flow Apparatus

Construction Modifications:

A Proportional Dilutor (Fig. 3), capable of delivering five toxicants and a control solution at flow rates up to 400 ml per minute was constructed from a design of Mount and Brungs (1967). The following modifications in this design were made:

1. Cells WB2-WB5 were made from solid plexiglass rods drilled to an internal diameter of 2".
2. The Intake Valve Plug (IV) in Fig. 3 was made by drilling a 3/8" hole in the base of a conical glass centrifuge tube. The seal, into which it is wedged to shut off the incoming water, was made by covering the apex of the cone with plastic film, and plunging it into a glass tube partially filled with Dow Corning Glass Sealant. After the sealant had set, the plug was removed, and the plastic film stripped off. The resulting sealant-conical glass plug valve proved to be superior to the rubber tubing-blown glass valve described by Mount and Brungs (1967) in preventing leakage at high water pressures. This was accomplished with a minimum of force on the glass rod actuating lever.
3. The tension on the glass actuating rod was found to be more easily controlled when the Valve Spring was replaced with a loop of surgical tubing which could be adjusted by lowering or raising a four inch eye bolt attached to the backstop by means of an L-bracket.
4. The dead water spaces between the ends of the siphons and the bottom of the cells was minimized by inserting glass plates approximately 1" below the level of the siphon. At slow cycling speeds, and maximal dilution between cells, this was a necessity to prevent

Figure 3. Continuous Flow Delivery System



excess biodegradation of LAS in the Dilutor itself.

Mechanics of Operation and Calibration:

Water from a 189 liter polyethelene storage barrel passed via the Intake Valve (IV) into Cell W1. (Fig. 3). It overflowed from Cell W1 to W2, to W3, etc. When it reached the top of the inverted U-Tube in Cell W6, it siphoned into Bucket V6. The weight of the water entering V6 depressed the lever actuating the intake valve shut off.

With the intake flow of water off, the water level in the W-series of cells stabilized. The water flowing into V6 eventually reached the top of the inverted U-Tube, and began to flow to the Control Tank T6. In so doing, it triggered the release of water from Cells W1-W5 by a Venturi Effect.

As the water from Cells W1-W5 flowed past the complementary C-Cells, a partial vacuum was set up which initiated the release of the solutions in these cells.

The glass scoop in the Funnel was made to deliver 4.0 cc (N=50, SD=0.2) of chemical from the Mariotte Bottle to the 998 cc (N=10, SD=2.2) of water in the Mixing Bowl (M1). The resulting mixture is therefore approximately 1/250 of the concentration in the Mariotte Bottle. Subsequent dilutions were made by the mixing of the water from the W Cells and the toxicant in the C Cells as described. The volumes of the cells were constructed so that a 50% dilution between tanks would result.

Reliability of Delivery System:

The ability of the apparatus to deliver a constant concentration each cycle was tested statically by catching the water as it left both the W and the C series of cells. These volumes were determined, and the dilutions calculated.

An optical procedure was used to test the concentrations being delivered while the dilutor was cycling at the speed to be used in the long term tests. A standard curve of methylene blue concentration vs. optical density at 650 m μ was prepared. The W Cells were filled normally, and the Intake Valve was shut off at the level of the reservoir barrel. The Mixing Bowl (M1) was filled with the methylene blue solution (optical density = 100%) and the apparatus allowed to cycle normally.

The optical density of each of the volumes delivered to the tanks T1-T6 was determined, and the delivery concentration determined from the standard curve. The average of ten such trials used with each of the two procedures is shown in Fig. 4. The reliability of the apparatus was thus confirmed by this testing procedure.

Use of the Dilutor with LAS:

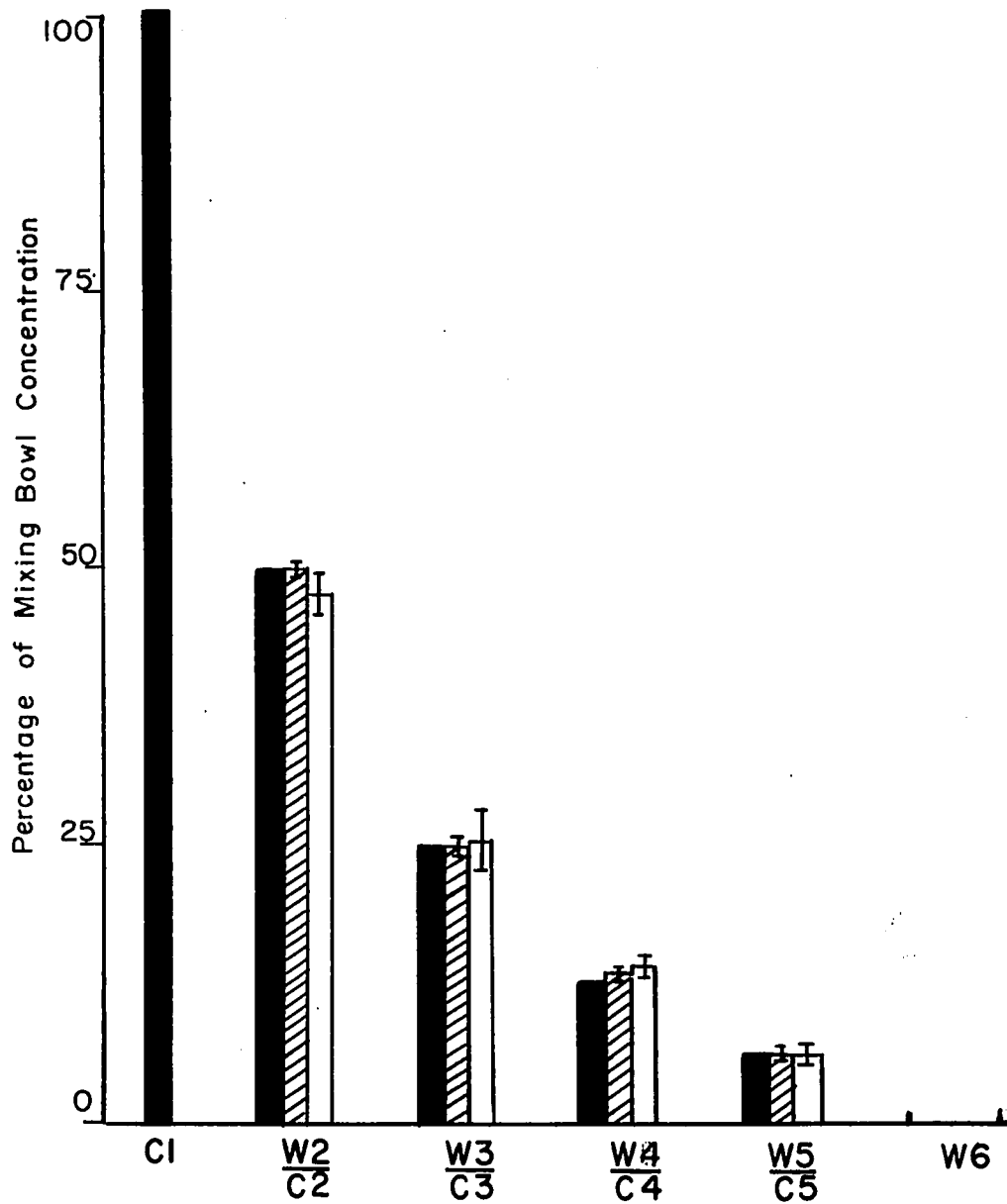
For the long term tests, a graded series of LAS concentrations less than 1 ppm in the tanks was desired. The following interrelated factors affect the level which will be obtained after a stabilization period:

1. Concentration of LAS entering the tanks.
2. Absorption and adsorption rate.
3. Microbial breakdown rate.
4. Flow rate through the tanks.

Variation of the concentration of the LAS entering the tanks was the only real control option. By trial and error it was found that a Mariotte Bottle concentration of 1500 ppm LAS resulted in the approximate desired equilibrium concentrations in the test tanks.

Occasionally, a microbial bloom... indicated by a clouding of the LAS solution, produced unusually low levels. In these instances,

Figure 4. Calibration and Reliability of Dilutor



Legend:

- Theoretical Delivery Concentration (% MI)
 - Static Concentration (% MI \pm ISD)
 - Dynamic Concentration (% MI \pm 1 SD)
- Letters refer to individual dilutor cells (Fig.)

the affected system was replaced with appropriate fresh preparations.

Using these procedures, it was possible to regulate LAS levels in the test tanks within reasonable limits. The advantages of using the continuous flow system were that constant aeration was possible without bubbling, fresh toxicant was added continually, and excretory products were prevented from accumulating.

6. Short Term Test Procedures

Twenty eight freshly caught F. heteroclitus were acclimated to the test conditions of salinity (24 ppt) and temperature (15°C) for 48 hours. Oxygen saturation was maintained during this period.

Seven fish were then placed in each of the 35 liter tanks T8 through T11 (Fig. 3). Both the total weight, and the range of weights (6-11 gm) of the fish in each tank was equal. The ratio of males to females was also made constant.

At the beginning of these 48 hour trials, the oxygen concentration of the test tanks was 100%. No further aeration was carried on during the exposure to LAS.

The approximate 48-hour LD₅₀ for large F. heteroclitus under the physical conditions just described, was found to be between 3 and 4 ppm. The tolerance level of the fish varied considerably from group to group. The concentration of LAS introduced into the tanks was 3, 2, 1 and 0 ppm respectively. If at least 5 of the 7 fish did not survive this exposure, the whole procedure was discontinued and repeated with a new set of fish, or a new high concentration point established.

After 48 hours, 20 fish were removed from the tanks and the following parameters examined: total body weight and length, hematocrit and liver weight.

Blood plasma was obtained by methods already described, and frozen along with the remains of the animal. Portions of the liver were placed in 10% calcium formalin for later histologic study. Representative liver tissue samples were processed in a Technicon. They were stained with heratoxylin and eosin, or Mallory's stain by procedures outlined by Gray (1958). A Periodic Acid Schiff (PAS) stain

was also used on some frozen sections, as well as those tissues fixed in formalin.

The order of enzyme assays was: (T8-T11) alternating with (T11-T8) to minimize the type of error which can result when multiple assays are run concurrently. No more than 12 assays were run at the same time. Equal numbers of subjects from each tank were in the same assay set. Assay procedures were completed within 48 hours.

The standard error per unit, and the mean differences between the means of the treatment and control data were then computed from the data obtained from the first 20 fishes. This information was then used in conjunction with Table 2-1 of Experimental Designs (Cochran and Cox, 1950) to arrive at an estimate of the number of replications which would be required to give statistically meaningful results (Table 4).

As a result of these calculations, the experiment as described so far was repeated 8 additional times between December and March. Between such repetitions the tanks were brush cleaned, rinsed in distilled water and dried with a sponge before reintroducing fresh sea water.

When the data from the last replication had been obtained, variance ratios of the combined data from the control and experimental populations were tested for statistical difference by means of an F-Test. If this ratio was significant at the .01 confidence level, distribution free statistics were used in the comparisons of central tendencies.

The Kruskal Wallace Analysis of Variance from Ranks was used extensively to evaluate the possibility at a given probability level that the sample values could have arisen by chance selection from the same population. It is, essentially, a Model I Analysis of Variance for use with ranked data. The procedure, as indicated in Statistics:

Table 4. Estimation of Replications Required to Detect a Difference
Between Means as Large as Sample

Parameter	LAS (ppm)	$\frac{T_{S.E}}{T_x}$	$\frac{T_x - C_x}{C_x}$	Replications Required
ICD	3	15.6	30.7	7**
	2	11.6	38.6	6***
	1	8.4	16.4	7**
GOT	3	20.3	28.4	9*
	2	10.4	95.6	4***
	1	8.6	23.0	8***
Hematocrit	3	4.0	9.4	7***
	2	3.3	4.7	9**
	1	3.7	6.2	9*

* 80% probability at the .05 level

** 90% probability at the .05 level

*** 95% probability at the .01 level

Methods and Applications (Griffin, 1962) is as follows:

1. Solve the following equation:

$$H_c = \left[\frac{\left(\frac{12}{(N)(N+1)} \right) \sum_{i=1}^K \left(\frac{R_i^2}{n_i} \right) - 3(N+1)}{\left(\frac{1 - \sum T}{(N^3 - N)} \right)} \right] \text{ with } (K-1)^\circ F$$

where:

N = total number of observations

R_i = sum of the column ranks

n_i = number of observations per column

K = number of columns

t = number of pairs of tied observations

T = $(t-1)(t)(t-1)$

$^\circ F$ = degrees of freedom.

2. Determine the confidence level of the value of H from Chi^2 Tables.

In some of the data, a statistically significant ratio between treatment and control variances did not exist. In such cases, a Model III Analysis of Variance was used to evaluate differences between means. The computer program used in this procedure was designed by the Health Science Computing Facility of UCLA, and identified by the number BMD02V. The program was run on the computer facilities of the University of New Hampshire.

The Spearman Rank Correlation method of evaluating the degree of correspondence between the variables tested was used. It can be applied to both parametric and non-parametric data. The procedure, as described by Griffin (1962) involves:

1. Solving the equation:

$$p = 1.00 - \left[\frac{6 \cdot \sum d^2}{n(n-1)(n+1)} \right]$$

where:

d = the difference between treatment ranks and control ranks.

n = total number of observations.

p = Spearman's correlation coefficient.

2. testing the value of the coefficient by using the criterion:

$$t = p \sqrt{\frac{n-2}{1-p^2}}$$

where:

t = value of the "t" distribution with (n-2) degrees of freedom.

In both the non-parametric (Kruskal-Wallace) and parametric (Model III analysis of variance) procedures, multiple comparisons were being made. If the results were statistically significant, procedures to evaluate which means, or set of means differed significantly from each other were implemented.

A Duncan Multiple Range Test was used in such cases with parametric data. Both the rationale and procedure were that of J.E. Freund, P.E. Livermore, and I. Miller (1960). A Kruskal-Wallace Analysis of Variance was used to compare each individual treatment and control means with the non-parametric data.

7. Long Term Test Procedures

A graded series of LAS concentrations (1:1/2:1/4:1/8:1/16:0 ppm) was introduced into the Test Tanks T1-T6. The Proportional Dilutor, set to deliver the same concentration series was started, and operated throughout the test, at a flow rate of 31.5 liters/68 liter tank/24 hour period. In 60 hours, a volume of test solution equal to the tank volume was added. In this period, the actual replacement time was approximately 50% (Sprague, 1969). While this was below the minimum recommended by the APHA (1965) for continuous flow bioassays it was thought to be more appropriate than static tests.

Ninety fishes were weighed individually and introduced into the six tanks such that the total weights of the fishes in each tank were within 1% of being equal.

Sea water from a 500 gallon basement reservoir was pumped to the polyethylene storage barrels shown in Fig. 3. It remained in the barrel 24 hours under vigorous aeration before being mixed with the LAS while passing through the Dilutor. Ambient temperature was 15°C.

Periodically, the pH of the water in the tanks was determined with a Coleman Metrion III pH Meter. Oxygen levels were checked with a Yellowstone Instrument Company Model YSI Probe.

LAS levels were monitored routinely both in the tanks and at the spouts entering the tanks. The assay used for LAS was the methylene blue procedure already described. It was not possible to obtain consistent LAS assay results in the tank receiving the lowest concentration of surfactant (T5 in Fig. 3). Results of fish from this tank were therefore not considered.

The feeding behavior and activity of the fishes throughout the

tests was noted in the course of daily observations which usually lasted about an hour. The diet and method of feeding have been described. Mortality checks were made 3-5 times between 8 a.m. and 12 p.m. The sex, weight, length, tank number, and time of death was recorded, and the fish discarded.

The experiment was run for 60 days. At that time, approximately 50% of the fish in the tank receiving the highest LAS concentration had died.

On the 60th day, the dilutor was stopped, and the fish were prepared for the same physical and analytical treatments received in the short term tests.

The order of removal from the tanks for serum sampling and analysis after freezing was: (T6-T1) alternating with (T1-T6). By the end of the 60th day, all fish had been removed from the tanks, and the serum samples obtained. Both the blood fluid sample and the carcass (individually wrapped in plastic film) were frozen. Three days later the enzyme analyses were completed and by the sixth day after removal from the tanks, the physical measurements had been taken.

The small sample size of the survivors (N=43) necessitated the use of non-parametric statistical procedures in the analyses of the data collected on serum enzyme levels, time of death, hematocrit and weight changes.

SECTION III

RESULTS

1. Short Term Tests

Due to absorption, and/or biodegradation, some loss of LAS methylene blue activity was experienced over the course of the experiment. The mean levels (based on assays of the first four trials) of LAS in the tanks at 24 and 48 hours are shown in Fig. 5.

Body Weight:

The process of confinement under the test conditions was sufficient to reduce the mean weight of fishes in the control tank. A similar weight loss was experienced by subjects in the 1 ppm LAS. At concentrations of 2 and 3 ppm LAS respectively, weight loss was considerably more. (Fig. 6)

Hematocrit, SGOT, and SICD:

The values obtained for individual fish for hematocrit, SGOT, and SICD are presented in Table 5, Table 6, and Table 7 respectively. Data are arranged so that the levels of any of the three variables in an individual can be identified by reference to the replication number (abscissa) and the letter (ordinate). The results of statistical treatment of this data follows:

1. Hematocrit:

LAS concentrations of 3, 2, and 1 ppm respectively cause an increase in the mean hematocrit level of F. heteroclitus. This response is proportional to concentration over this range (Fig. 7) and the mean increase of the experimental populations over the control is statistically

Figure 5. LAS Levels During Static Tests

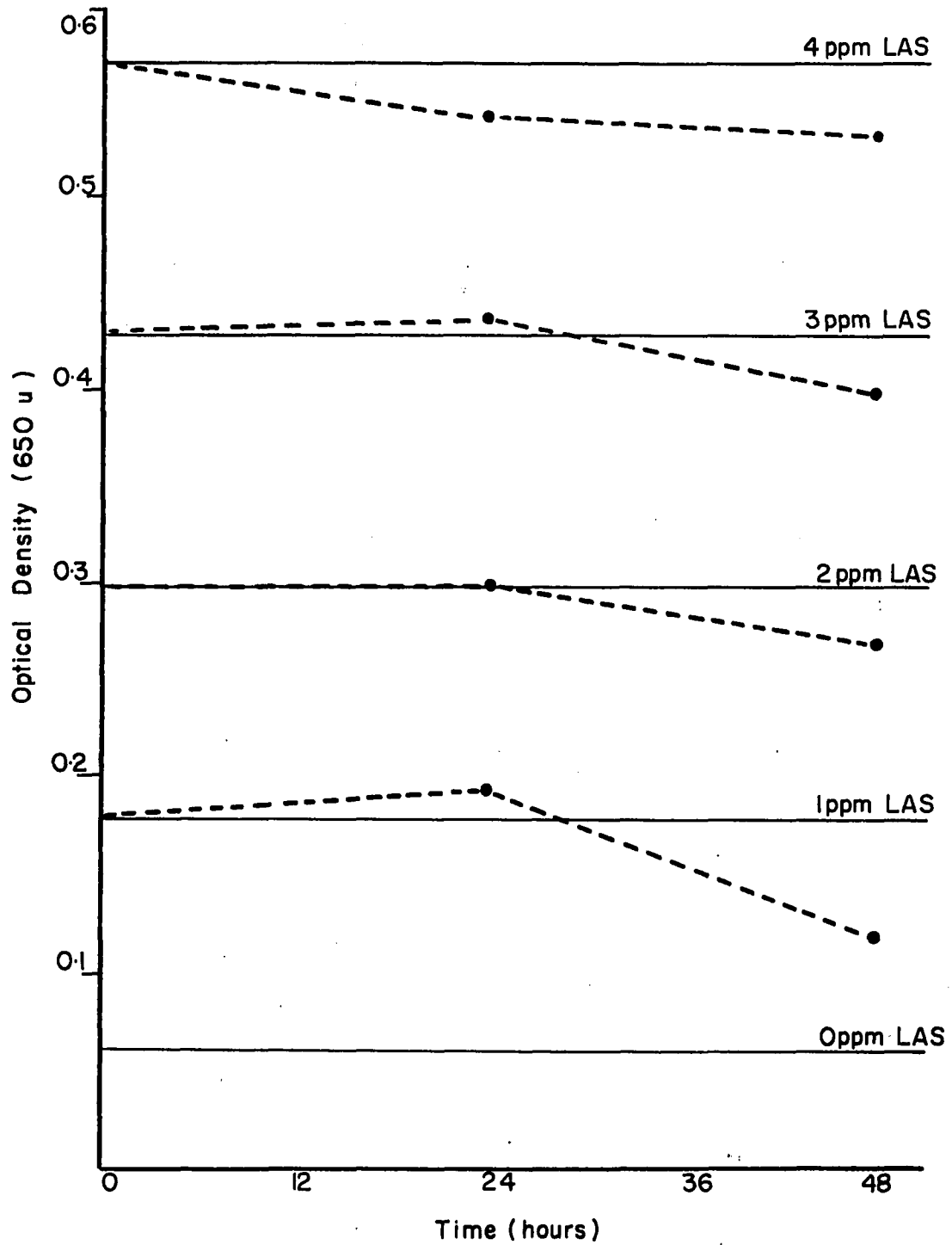


Figure 6. Mean Body Weight Loss in F. heteroclitus After 48-Hour Test Procedures With LAS

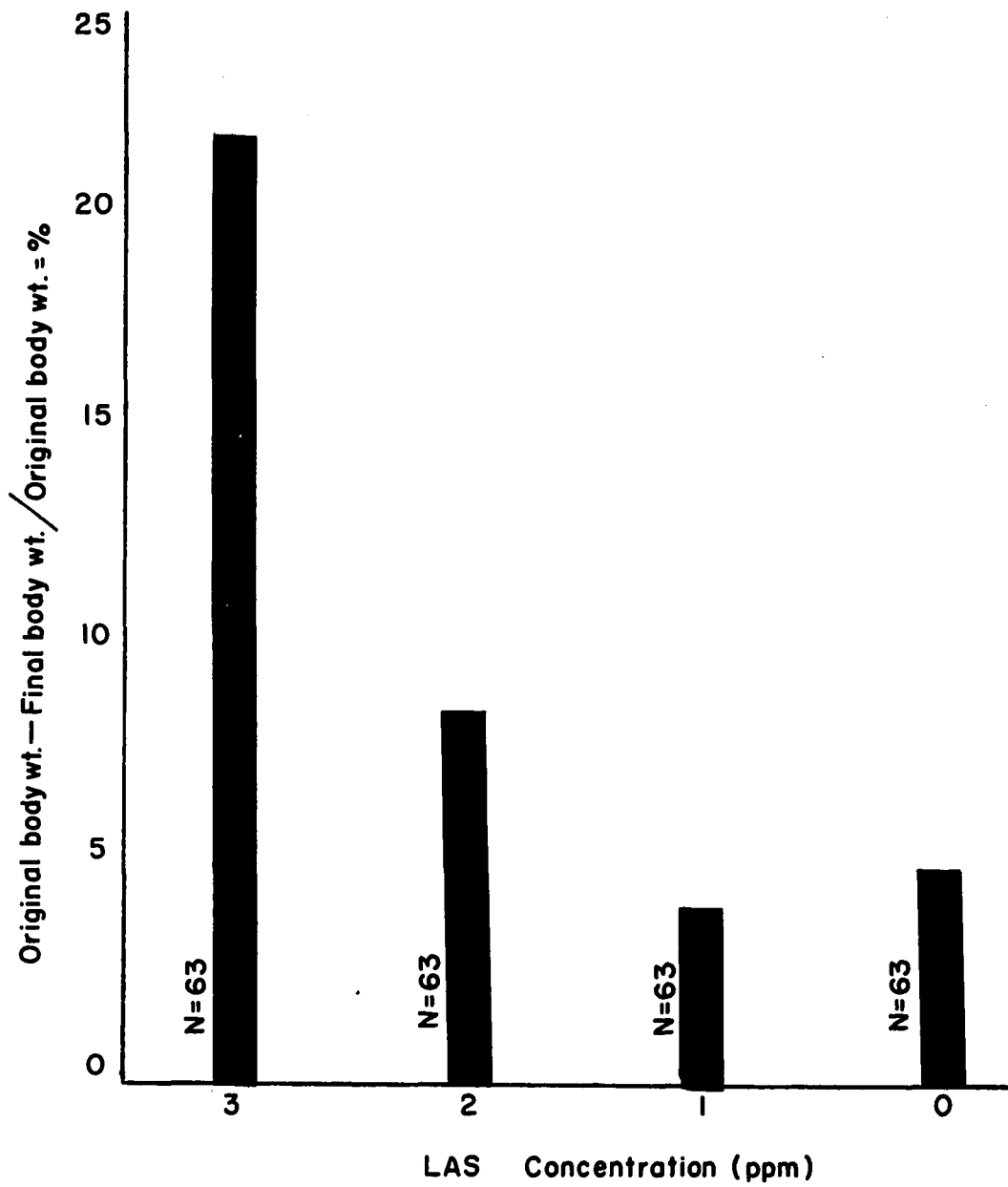


Table 5. Hematocrit Levels in F. heteroclitus After 48-Hour

		Test Procedures								
		Replications								
	I	II	III	IV	V	VI	VII	VIII	IX	
<u>3 ppm LAS</u>										
A)	346	500	545	508	402	470	330	340	475	
B)	360	354	492	420	384	396	325	520	385	
C)	346	310	362	344	340	400	380	320	405	
D)	420	423	418	410	400	520	450	400	400	
E)	<u>348</u>	<u>465</u>	<u>450</u>	<u>435</u>	<u>402</u>	<u>340</u>	<u>385</u>	<u>485</u>	<u>450</u>	
	364	410	453	423	385	425	374	413	423	
<u>2 ppm LAS</u>										
F)	374	374	344	320	370	378	376	500	320	
G)	326	429	413	388	440	380	418	395	500	
H)	375	402	451	320	384	376	394	328	420	
I)	330	358	412	348	334	448	372	515	340	
J)	<u>328</u>	<u>310</u>	<u>360</u>	<u>321</u>	<u>402</u>	<u>425</u>	<u>304</u>	<u>328</u>	<u>348</u>	
	347	375	396	339	386	401	373	413	386	
<u>1 ppm LAS</u>										
K)	386	386	384	316	402	326	306	314	315	
L)	330	383	413	370	320	445	242	354	355	
M)	329	364	448	326	326	390	304	378	265	
N)	380	372	378	368	330	375	455	460	380	
O)	<u>328</u>	<u>362</u>	<u>318</u>	<u>313</u>	<u>348</u>	<u>404</u>	<u>339</u>	<u>378</u>	<u>385</u>	
	351	372	388	339	345	388	329	377	340	
<u>0 ppm LAS</u>										
P)	364	334	450	364	304	379	280	318	320	
Q)	298	298	372	353	342	280	273	395	380	
R)	283	380	326	351	370	346	329	384	372	
S)	388	350	308	323	308	358	306	380	325	
T)	<u>321</u>	<u>340</u>	<u>388</u>	<u>370</u>	<u>330</u>	<u>216</u>	<u>338</u>	<u>384</u>	<u>355</u>	
	331	340	369	352	331	316	305	372	350	

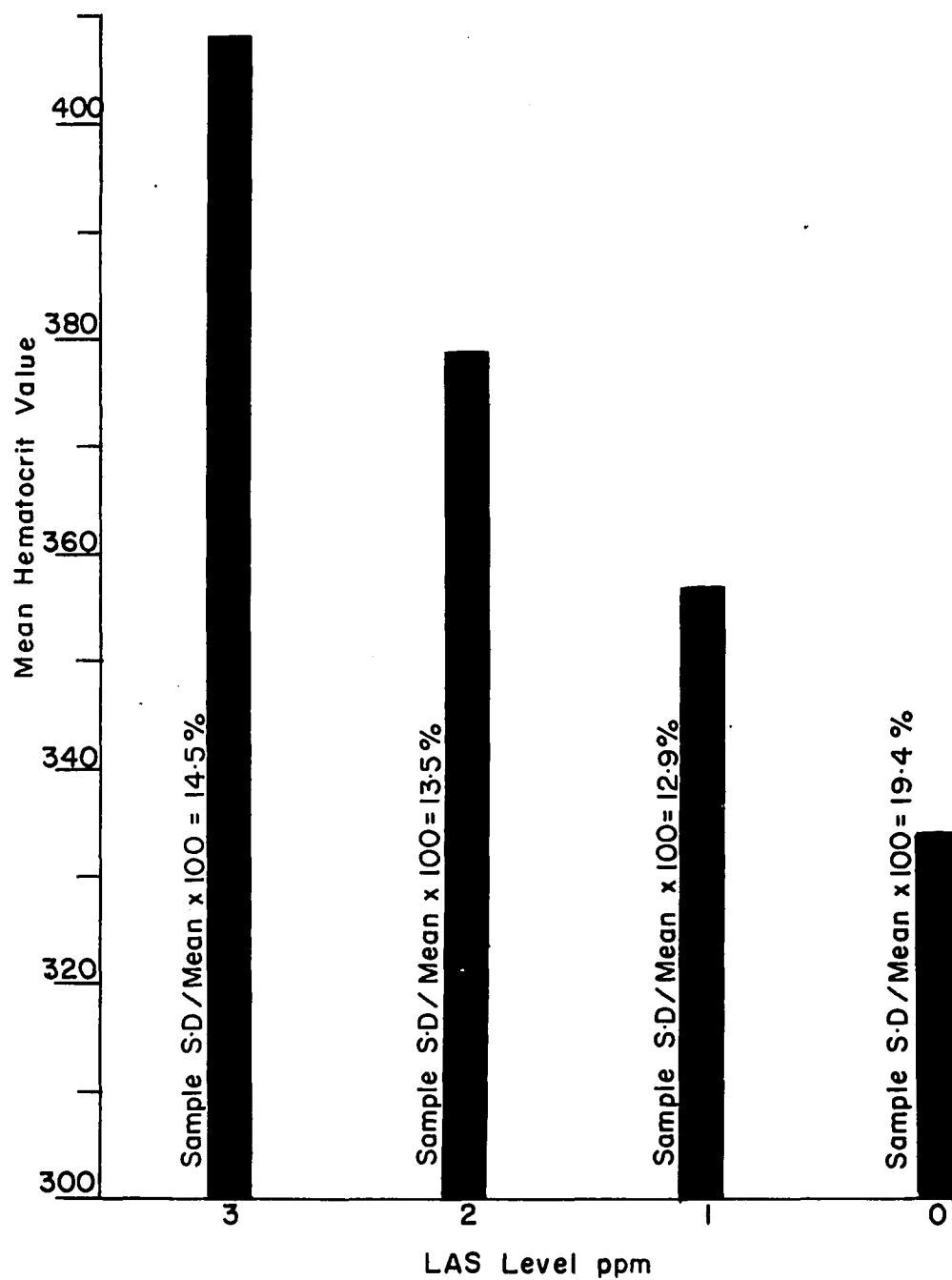
Table 6. Serum Levels of GOT (Sigma Frankel Units) in
F. heteroclitus After 48 Hour Test Procedures

	Replications								
	I	II	III	IV	V	VI	VII	VIII	IX
<u>3 ppm LAS</u>									
A)	460	875	1072	465	740	740	950	800	980
B)	350	600	750	735	900	345	1010	850	560
C)	880	345	1310	610	550	1010	550	1200	430
D)	295	400	345	670	434	850	745	385	385
E)	<u>485</u>	<u>1100</u>	<u>475</u>	<u>1150</u>	<u>340</u>	<u>365</u>	<u>550</u>	<u>1072</u>	<u>740</u>
	494	664	790	726	593	662	761	861	619
<u>2 ppm LAS</u>									
F)	545	440	339	1100	430	720	315	1100	305
G)	650	460	450	465	945	465	770	390	785
H)	812	505	275	610	660	420	650	1100	750
I)	1010	550	1010	810	710	365	1010	800	705
J)	<u>770</u>	<u>1600</u>	<u>1010</u>	<u>275</u>	<u>290</u>	<u>365</u>	<u>275</u>	<u>700</u>	<u>740</u>
	757	711	617	652	607	467	604	818	657
<u>1 ppm LAS</u>									
K)	495	440	315	240	500	275	495	900	875
L)	405	420	600	425	465	700	600	740	300
M)	405	380	450	810	425	315	315	520	600
N)	450	450	500	270	520	315	495	295	720
O)	<u>625</u>	<u>720</u>	<u>700</u>	<u>275</u>	<u>250</u>	<u>650</u>	<u>740</u>	<u>570</u>	<u>315</u>
	476	482	513	404	432	451	529	605	562
<u>0 ppm LAS (Control)</u>									
P)	360	545	360	205	395	225	600	495	485
Q)	250	450	450	250	420	700	680	340	700
R)	225	360	405	660	275	420	450	428	500
S)	450	495	770	485	520	450	225	800	425
T)	<u>650</u>	<u>295</u>	<u>382</u>	<u>870</u>	<u>290</u>	<u>265</u>	<u>650</u>	<u>500</u>	<u>325</u>
	387	429	473	494	380	412	521	513	487

Table 7. Serum Levels of ICD (Sigma ICD Units) in
F. heteroclitus After 48 Hour Test Procedures

	Replications								
	I	II	III	IV	V	VI	VII	VIII	IX
<u>3 ppm LAS</u>									
A)	340	410	675	415	520	785	1300	900	700
B)	455	565	485	610	480	345	715	695	480
C)	785	610	475	480	535	475	485	550	440
D)	398	580	1065	875	740	1065	800	500	500
E)	<u>494</u>	<u>1080</u>	<u>990</u>	<u>660</u>	<u>345</u>	<u>640</u>	<u>420</u>	<u>800</u>	<u>860</u>
	495	649	738	608	524	662	744	689	596
<u>2 ppm LAS</u>									
F)	415	370	440	515	890	640	710	760	500
G)	400	385	315	453	555	445	1150	460	620
H)	620	380	475	380	520	635	790	500	900
I)	710	540	1265	695	385	635	1100	630	800
J)	<u>475</u>	<u>660</u>	<u>985</u>	<u>515</u>	<u>345</u>	<u>480</u>	<u>330</u>	<u>800</u>	<u>630</u>
	524	467	696	511	539	567	816	630	690
<u>1 ppm LAS</u>									
K)	453	560	600	290	585	470	710	605	645
L)	415	640	400	495	430	613	830	1100	400
M)	455	240	475	455	525	1010	400	345	820
N)	553	990	685	430	430	618	430	600	360
O)	<u>323</u>	<u>660</u>	<u>1100</u>	<u>380</u>	<u>438</u>	<u>645</u>	<u>1080</u>	<u>360</u>	<u>600</u>
	440	618	652	410	482	671	690	602	565
<u>0 ppm LAS (Control)</u>									
P)	255	460	435	305	420	400	455	765	360
Q)	305	460	420	245	620	575	760	360	515
R)	360	240	560	438	300	400	650	610	700
S)	455	580	845	515	475	515	330	800	605
T)	<u>515</u>	<u>380</u>	<u>515</u>	<u>710</u>	<u>420</u>	<u>640</u>	<u>275</u>	<u>475</u>	<u>575</u>
	378	424	555	442	447	506	494	602	551

Figure 7. Mean and Standard Deviation of Hematocrit Values From F. heteroclitus Exposed to LAS for 48 Hours



significant at the .05 confidence level (Table 8).

There is no statistically significant relationship between hematocrit level and the interaction of time and LAS level (Table 8).

2. Serum Enzymes:

The response of GOT and ICD in the blood fluid of F. heteroclitus to 3, 2, and 1 ppm LAS was highly individual. That the response of the population was not uniform is indicated by the fact that the variability of the data from the test populations was greater than that of the control. This was significant at the .01 level of confidence for SICD levels at all concentrations, and of SGOT levels at 3 and 2 ppm LAS (Fig. 8).

When the values from Table 6 and Table 7 were arranged in rank order, and a Kruskal Wallace Analysis of Variance from Ranks used to evaluate differences less affected by the extreme variance ranges, significant results were noted at all concentrations tested for both SGOT and SICD (Table 8).

These changes in the level of central tendency and dispersion (as a deviation from the control values) can be seen in the frequency histograms for SGOT (Fig. 9) and SICD (Fig. 10) respectively.

3. Histology:

The livers of fishes exposed to 3, 2, and 1 ppm LAS showed no evidence of having been altered histologically.

4. Interaction of Test Parameters:

Statistically significant rho-correlations existed between the enzymes GOT and ICD, but not between either of them and hematocrit (Table 9).

Table 8. Statistical Comparison of Average Results of 48-Hour LAS Tests

Analysis of Variance	Parameter	Comparison	Required Value	Obtained	Statistical Conclusion
Model III	Hematocrit	vs. LAS level	$.05F(3,144) = 2.67$	F=15.2	RHo
		vs. Replications	$.05F(8,144) = 2.00$	F= 2.96	RHo
		vs. Interaction	$.05F(24,144) = 1.59$	F= 0.64	AHo
Duncan Multiple Range		3 ppm LAS vs. 0 ppm	Rp = 20.7	$T_{\bar{x}} - C_{\bar{x}} = 73.5$	RHo
		2 ppm LAS vs. 0 ppm	Rp = 21.4	$T_{\bar{x}} - C_{\bar{x}} = 45$	RHo
		1 ppm LAS vs. 0 ppm	Rp = 22.2	$T_{\bar{x}} - C_{\bar{x}} = 24.3$	RHo
Kruskal Wallace	SICD (ranks)	vs. LAS levels	$.05X^2(4) = 9.49$	Hc=573.1	RHo
		3 ppm LAS vs. 0 ppm	$.05X^2(1) = 3.84$	Hc=281.1	RHo
		2 ppm LAS vs. 0 ppm	$.05X^2(1) = 3.84$	Hc=278.1	RHo
		1 ppm LAS vs. 0 ppm	$.05X^2(1) = 3.84$	Hc=261.3	RHo
Kruskal Wallace	SGOT (ranks)	vs. LAS levels	$.05X^2(4) = 9.49$	Hc=572.4	RHo
		3 ppm LAS vs. 0 ppm	$.05X^2(1) = 3.84$	Hc=287.2	RHo
		2 ppm LAS vs. 0 ppm	$.05X^2(1) = 3.84$	Hc=284.7	RHo
		1 ppm LAS vs. 0 ppm	$.05X^2(1) = 3.84$	Hc=274.5	RHo

Figure 8. Sample Variance Ratios of Serum Enzyme Values from F.heteroclitus after 48-Hour Exposure to LAS

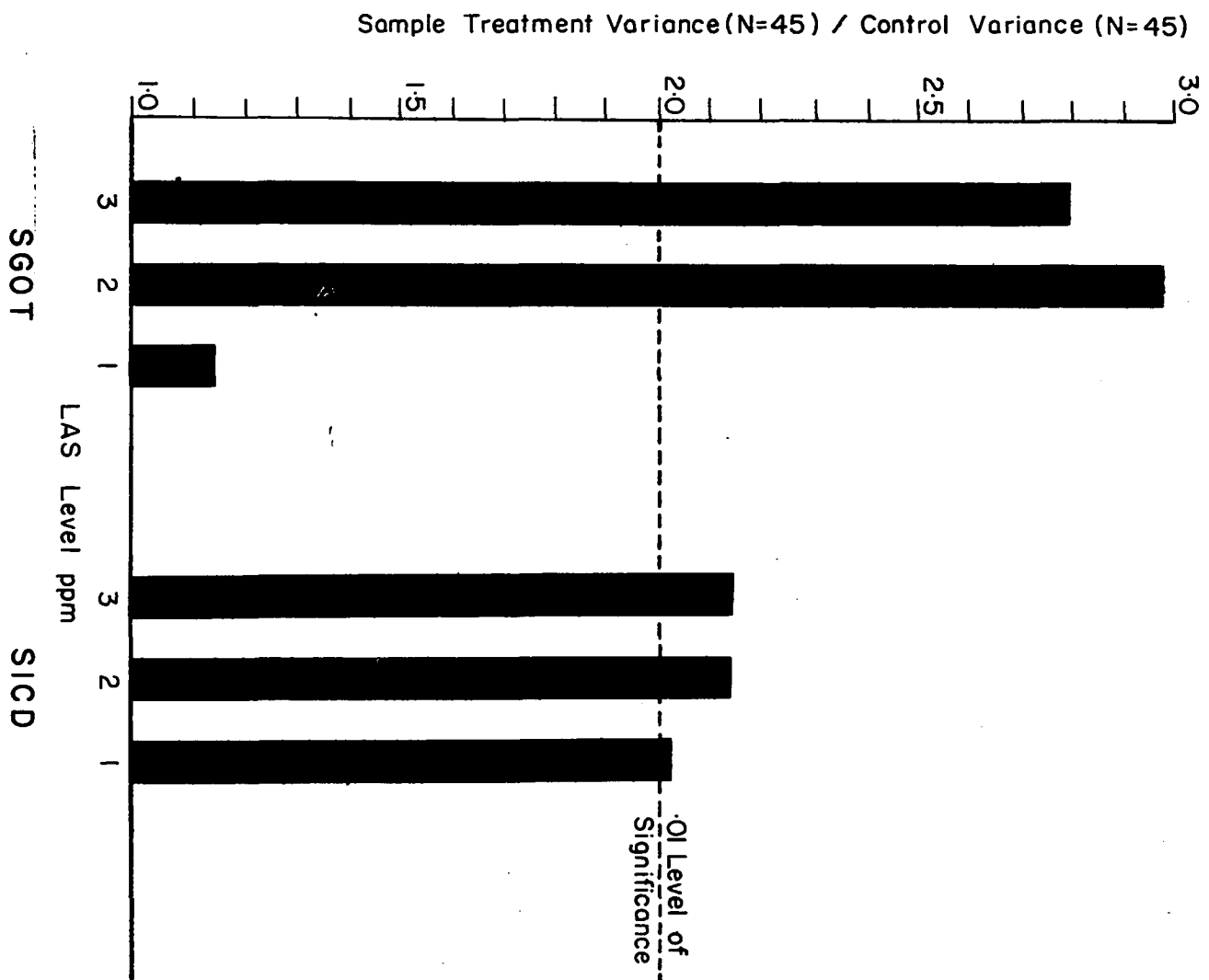


Figure 9. Frequency Histogram of SGOT Values From F. heteroclitus Exposed to 48 Hours LAS

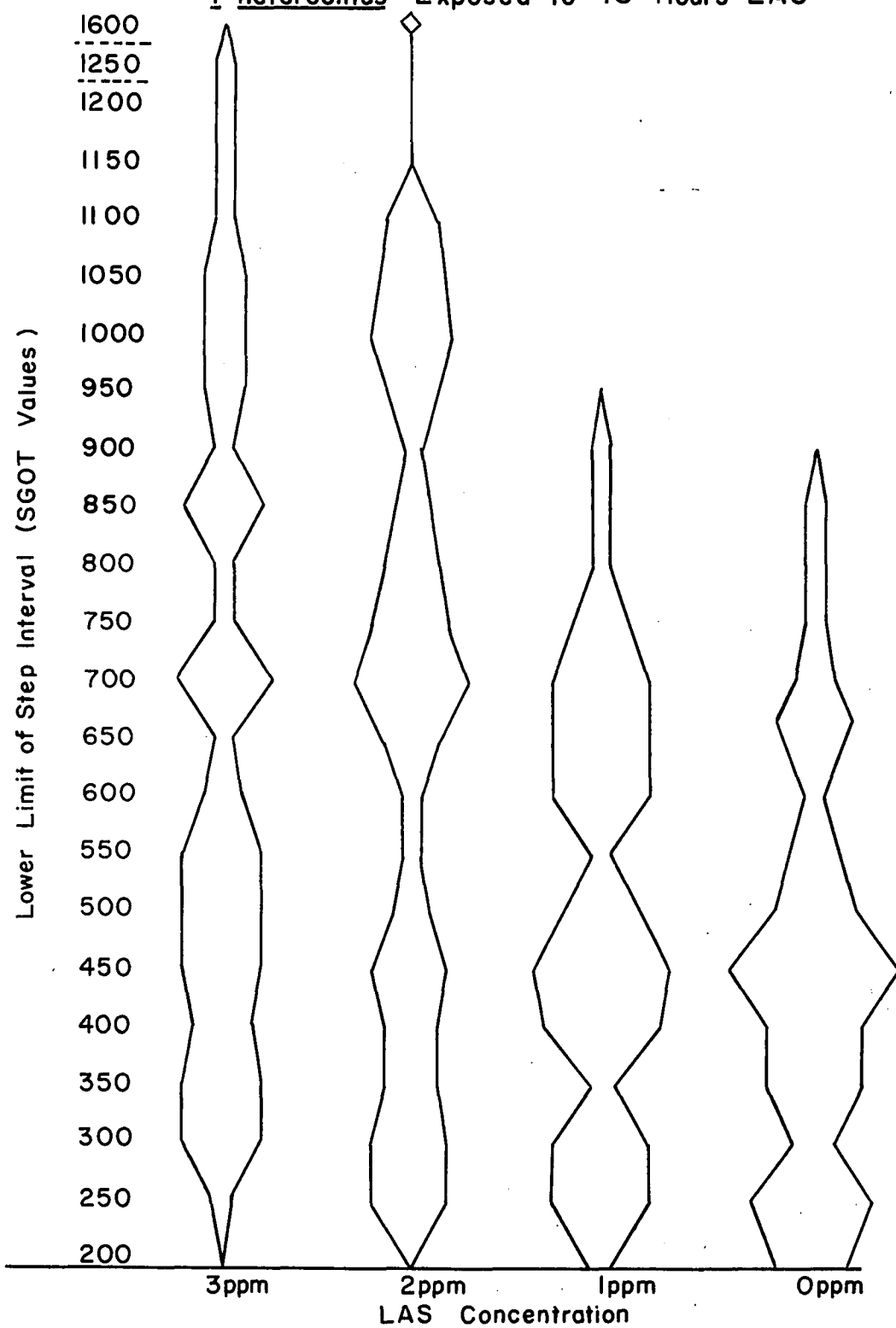


Figure 10. Frequency Histogram of SICD Values From F. heteroclitus Exposed to 48-Hours LAS

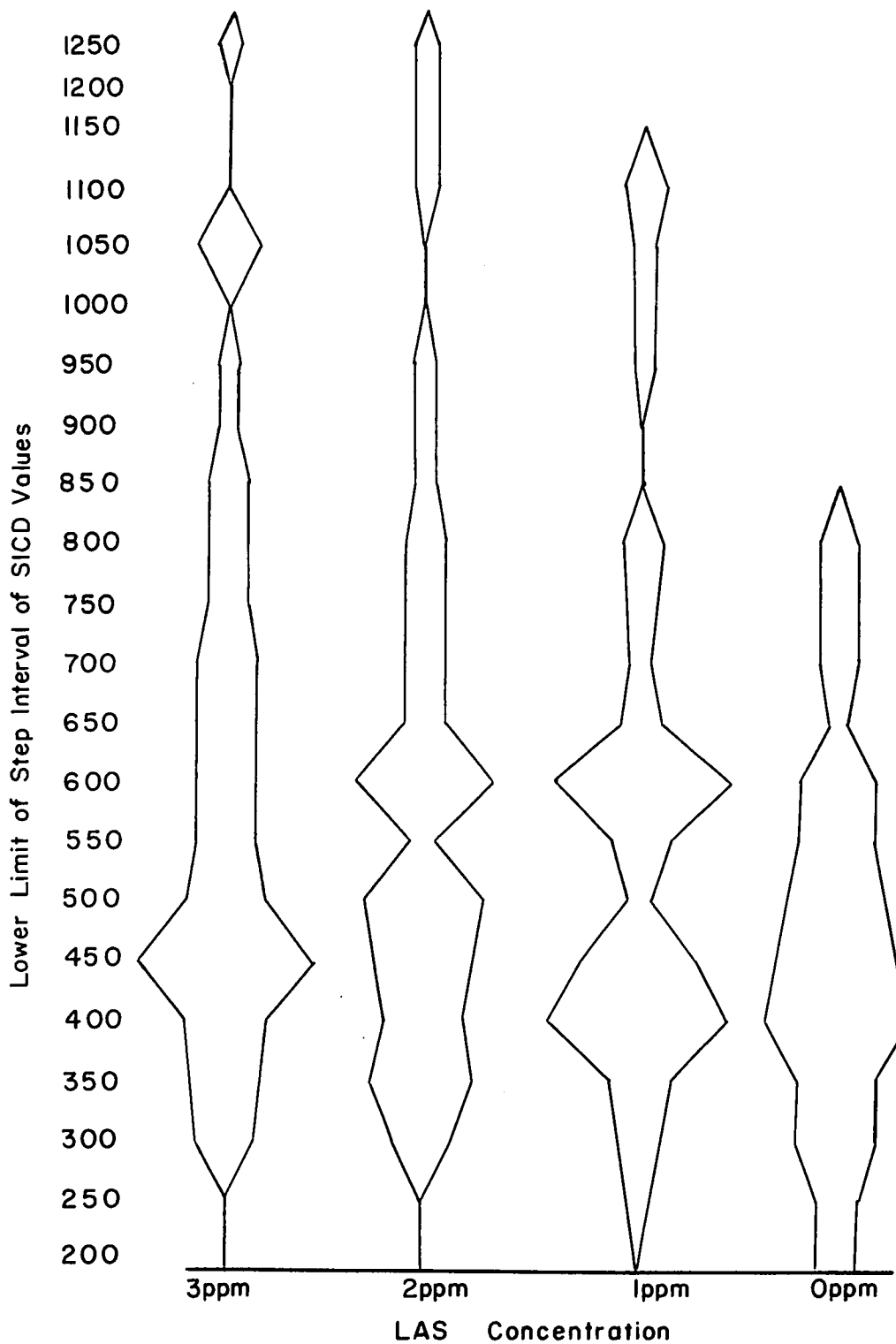


Table 9. Rho Correlation Coefficients Between
Results Obtained in 48-Hour Test Procedures

Correlation Tested	LAS Level (ppm)	Rho Value
SICD vs SGOT	3	+.365*
	2	+.520*
	1	+.365*
	0	+.581*
SICD vs Hematocrit	3	+.163
	2	+.113
	1	-.074
	0	-.038
SGOT vs Hematocrit	3	+.168
	2	-.014
	1	-.016
	0	-.075

* Correlation significant at the .05 level of confidence.

2. Long Term Tests

The mean delivery concentration of LAS into the test tanks was close to the desired 50% dilution. The mean levels of LAS in the tanks were constant, but dilution between the tanks was closer to 25%. These data, along with mean values of LAS for the 60 day experiment are presented in Fig. 11.

There were no significant differences in the mean values for oxygen concentration and pH between the test tanks and the controls. The pH varied from 7.0 to 10.1 and the oxygen tension from 1.5 to 2.5 (N=8). There was no correlation between individual tanks and pH or oxygen.

The changes noted in the test parameters were as follows:

Body weight and liver weight.

Based on the fact that the median weight of all of the post-treatment populations was less than the pre-treatment weight, and that this was not true of the control population, it was concluded that one result of exposing F. heteroclitus to LAS at concentrations of 0.3 to 0.8 ppm is a loss of weight. (Fig. 12) When liver weight was plotted against body weight of surviving fishes, the variability of the treatment data was different from the control data (Fig. 13).

Hematocrit:

The distribution of hematocrit values of the treatment populations was such that in tanks T4, T3 and T6 the median values were higher than that of the controls. Hematocrit levels from the lowest range of the distribution of treatment values were higher than corresponding control values. In all the treatment tanks, the upper limit of the hematocrit range of values was also higher than corresponding control tank values.

Figure II.

Mean and range values of LAS during long term tests

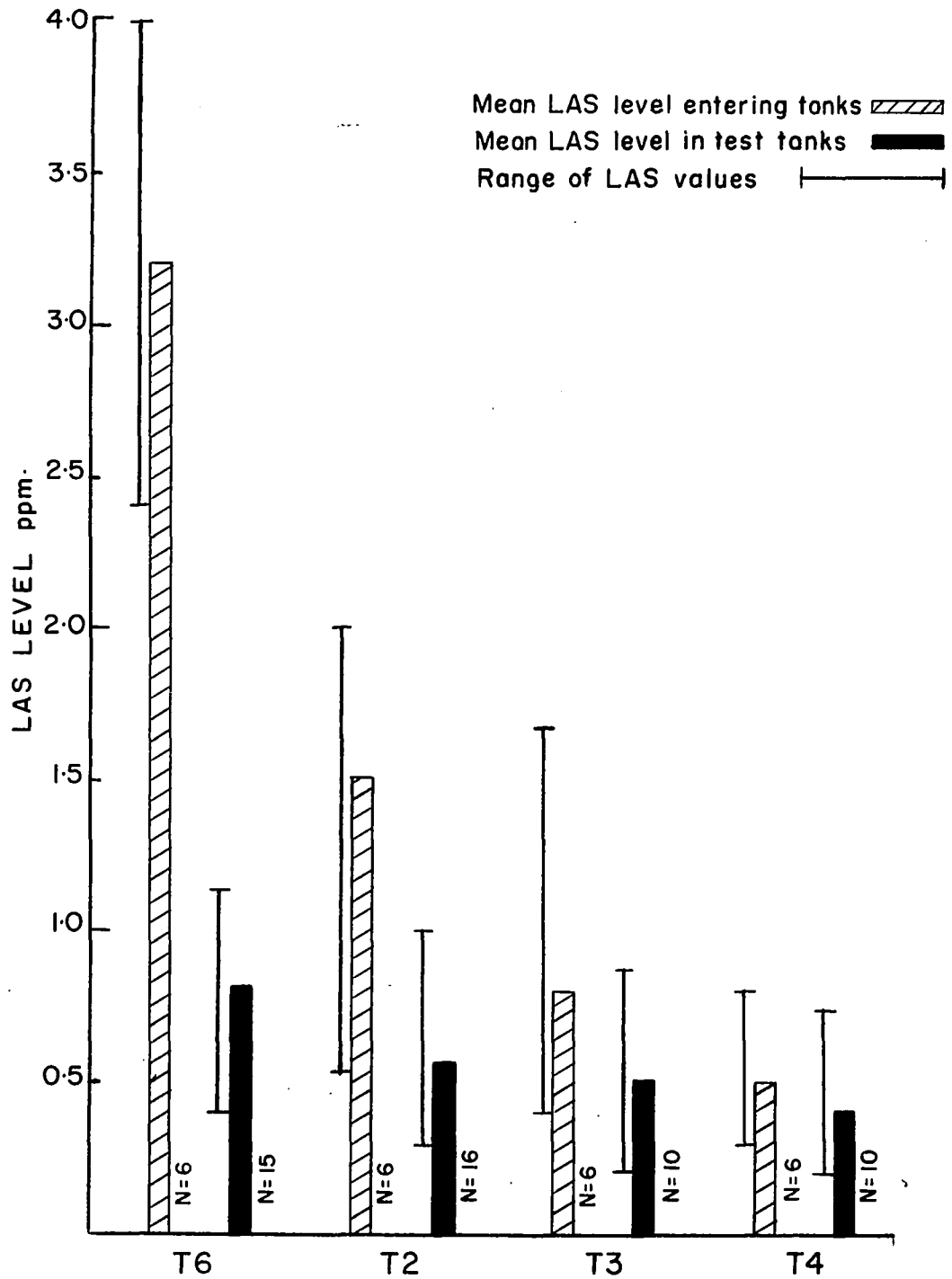
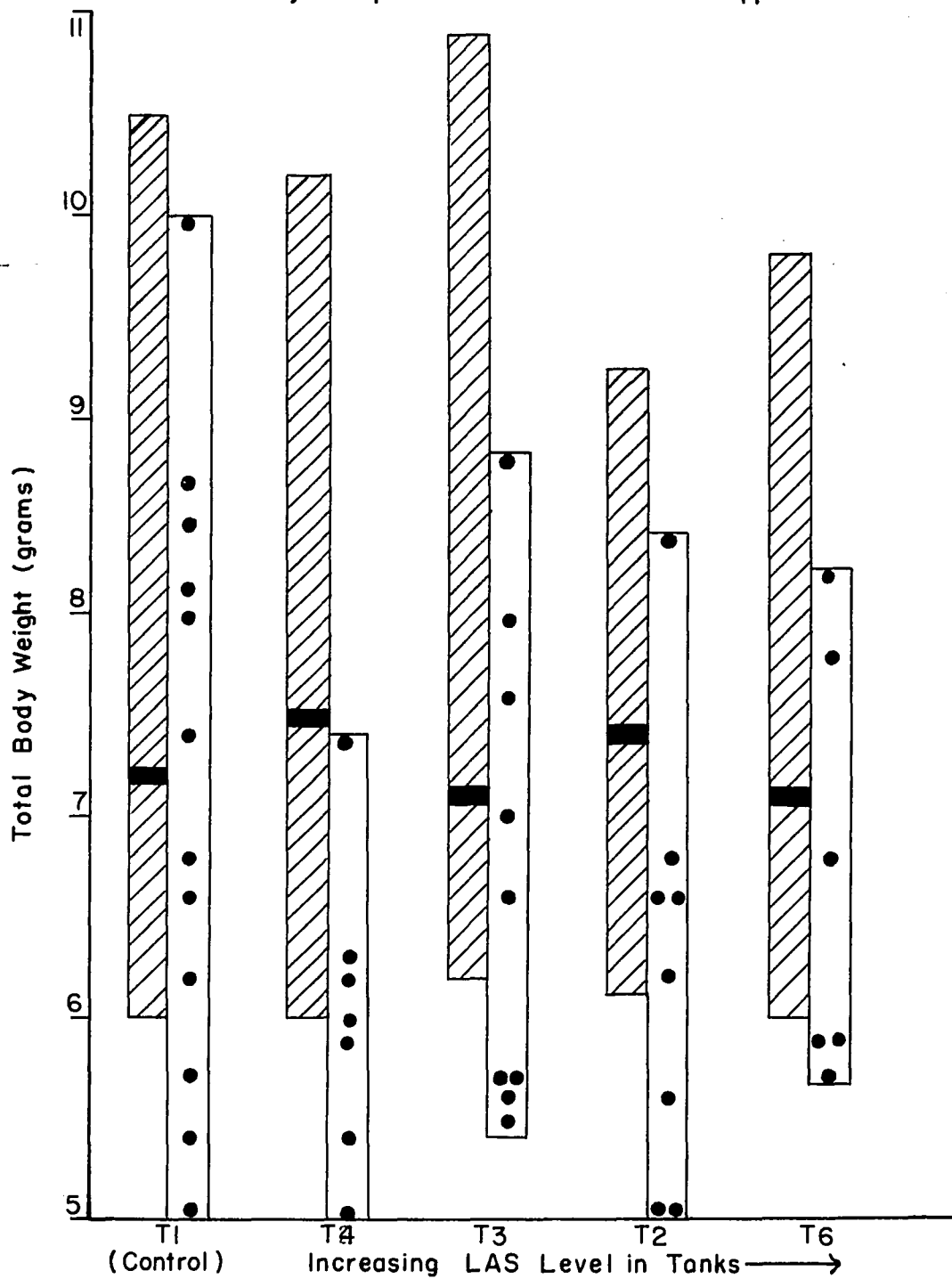


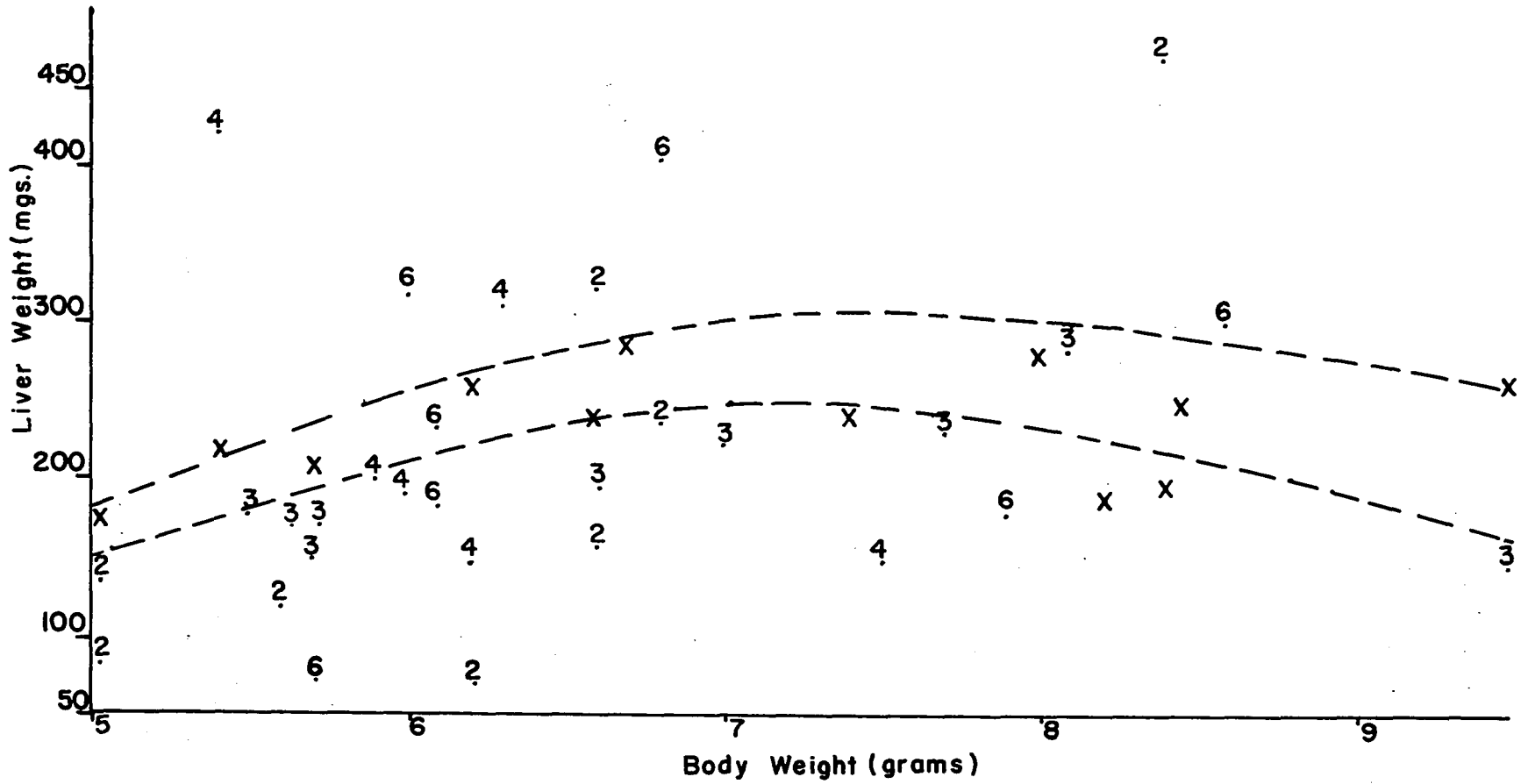
Figure 12. Weight Changes in F. heteroclitus After 60 Days Exposure to LAS Levels <1ppm



Legend:



Figure 13. Liver Weight—Body Weight of Individual F. heteroclitus Surviving 60-Day Exposure to LAS. (Numbers represent tank concentrations in Fig. II. Control=X).



These results are shown in Fig. 14.

Serum Enzymes:

The distribution of values representing serum levels of the enzymes GOT and ICD of all of the four populations exposed to LAS from 0.8 - 0.3 ppm was such that:

1. The median enzyme value of fish in the 4 experimental tanks was higher than the control median in 2 cases for GOT and 3 for ICD.
2. Both the upper and lower limits of the range of treatment values of SGOT and SICD were generally higher than corresponding control values.

These data are presented in Fig. 15.

Toxicity:

The pattern of death in the tanks throughout the tests was not random, as indicated by the results of a Kruskal Wallace analysis of variance of time to death vs. treatments. (Table 10).

Behavior:

No clear cut alterations in feeding behavior were noted. There seemed to be a tendency for fish in the control tank to spend more time actively swimming, particularly at the surface of the water. This was impossible to quantify, and may represent bias on the part of the observer. During the course of the experiment, 6 fish jumped from the tanks. None of these were from the control tank.

Histology:

The livers of experimental and control groups of fish used in the long term tests showed dramatic increases in the amount of fat present when compared with fish livers from the 48-hour tests. No other changes were apparent.

Figure 14. Hematocrit Levels of Individual F. heteroclitus Surviving 60 Days Exposure to LAS <1ppm

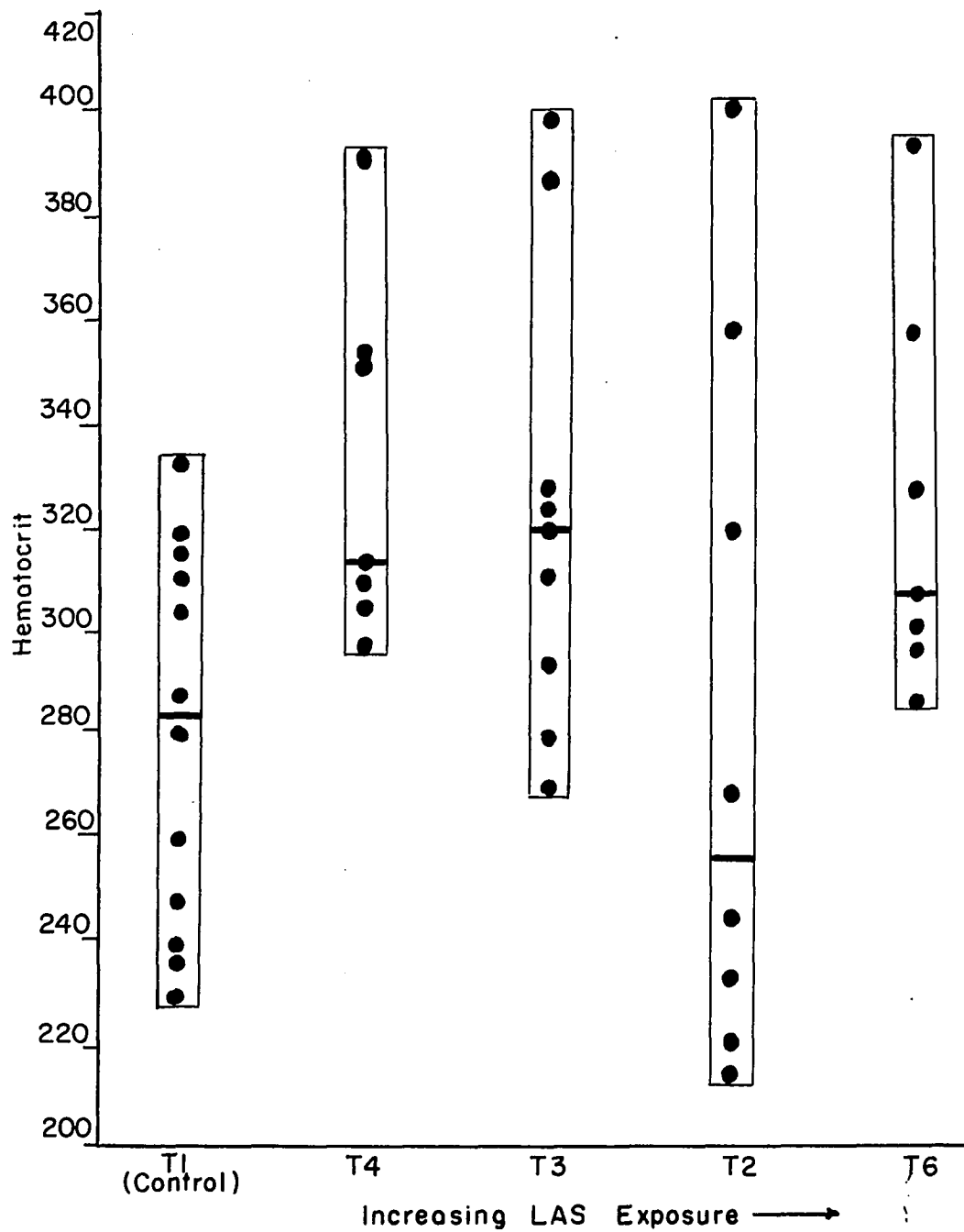


Figure 15. SGOT and SICD Levels of Individual F. heteroclitus Surviving 60 Day Exposure to LAS <1ppm

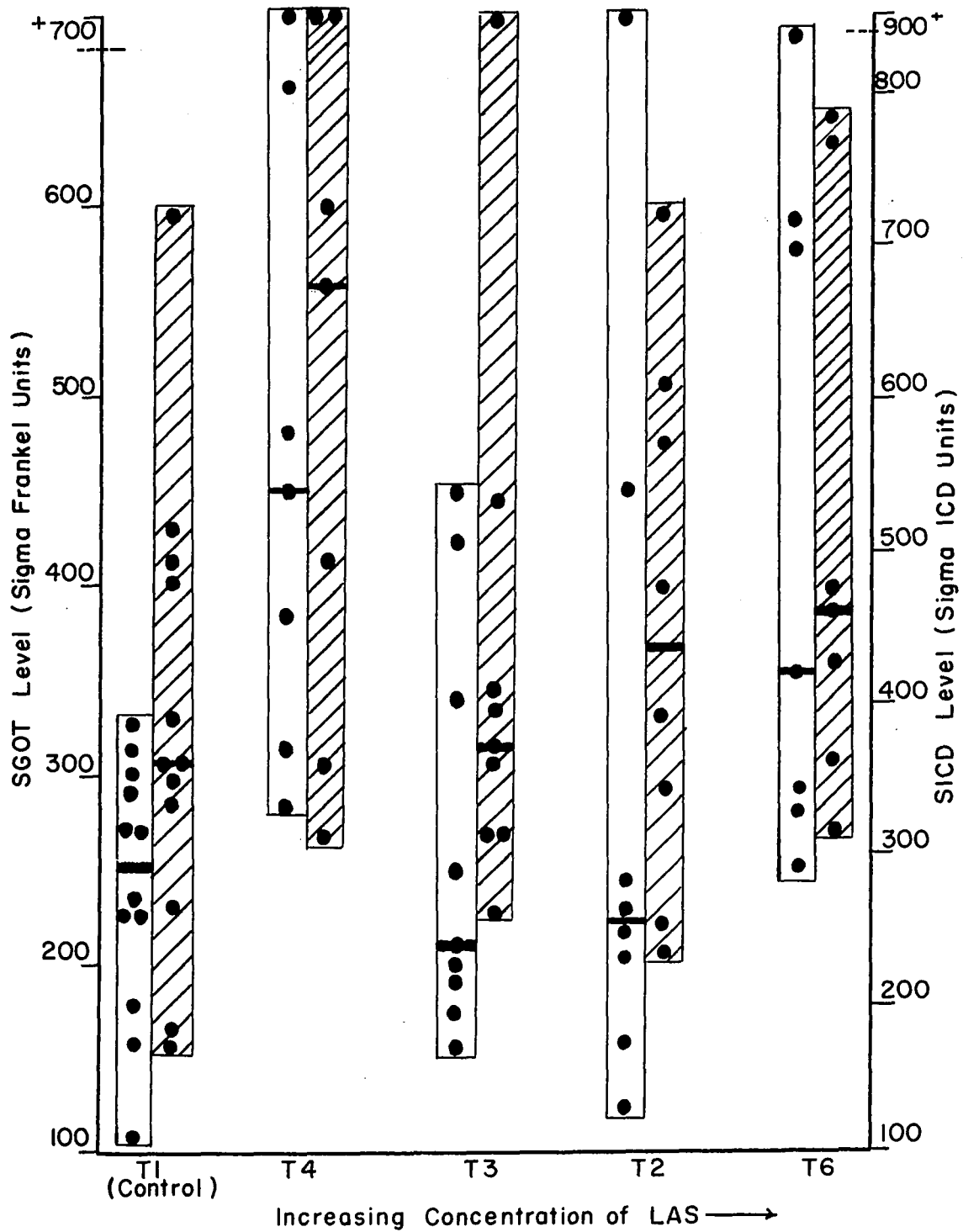


Table 10. Time to Death vs. Exposure Level of LAS in F. heteroclitus:

Kruskal Wallace Analysis of Variance of Long Term Test Results

T6	days to death	3	17	22	24	25	43	55	55	-	-	-	-	-	-	-
	rank order	68	67	66	65	63	53	48.5	48.5	22	22	22	22	22	22	22
T2	days to death	25	26	42	51	-	-	-	-	-	-	-	-	-	-	-
	rank order	63	61	54	51	22	22	22	22	22	22	22	22	22	22	22
T3	days to death	44	56	58	-	-	-	-	-	-	-	-	-	-	-	-
	rank order	52	46.5	44.5	22	22	22	22	22	22	22	22	22	22	22	22
T4	days to death	27	31	31	38	41	52	56	58	-	-	-	-	-	-	-
	rank order	60	58.5	58.5	57	55.5	50	46.5	44.5	22	22	22	22	22	22	22
T1	days to death	25	41	-	-	-	-	-	-	-	-	-	-	-	-	-
	rank order	63	55.5	22	22	22	22	22	22	22	22	22	22	22	22	22

$$H_c = \left[\frac{\left(\frac{12}{N(N+1)} \right) \sum \left(\frac{R_i^2}{n_i} \right) - 3(N+1)}{\left(\frac{1 - \sum T}{(n-1)(n)(n-1)} \right) * } \right] = \frac{8.0}{0.747} = 10.7$$

Table value of χ^2 at the .05 level with 4°F = 9.49

Legend: N = number of subjects
 n_i = number of subjects per tank
 R_i^2 = sum of squares of rank orders per tank
t = number of pairs of tied observations
T = (t-1) (t) (t-1)
* = correction factors for tie scores

SECTION IV

DISCUSSION

It was the purpose of this study to determine if the levels of the serum enzymes GOT, ICD and of the red blood cell packed volume were affected by short and long term exposure to LAS. A potential use for this kind of information was anticipated for use with bioassays. The determination of levels which would have no effect on these parameters was considered to be relevant in light of current interest in determining levels of foreign materials to be tolerated in the environment. Whether the changes that have been documented represent initial disorders which finally terminate in death, or whether they represent a degree of metabolic alteration stemming from adaptation is not known. An appreciation of the significance of the increased levels of GOT and ICD in the serum and of hematocrit in F. heteroclitus is intimately connected with studies concerned with the origins of the enzymes in the serum.

The practical value of using the serum levels of GOT and ICD as a bioassay for sub-lethal LAS detection is limited by the following considerations:

1. There is no gross difference in degree or kind of the elevations of SGOT and SICD experienced in the stress of sublethal exposure to various insecticides (Lue-Hing, 1966). A bioassay based on SGOT and SICD levels then would not be capable of distinguishing between stress resulting from either of these or possibly other compounds.
2. The SGOT and SICD response in F. heteroclitus was not at all uniform with respect to individual members of the population.
(Fig. 8.)
3. The variability of so called "normal values" of the enzymes is

broader than in homeotherms generally. This is indicated in this study by the range of "control" values in Figs. 9 and 10.

4. The bioassay procedures required for statistically significant results are more difficult to standardize and conduct than more direct and sensitive chemical assays. This is not to say, however, that the enzyme levels do not reflect a more generalized stress which could result from mixtures of materials for which no suitable chemical assay existed.

An increase in hematocrit, and in the mean levels of SGOT and SICD were shown to have been caused by 3, 2 and 1 ppm LAS in F. heteroclitus. Similar changes occurred at graded concentrations less than 1 ppm in tests running 60 days. These increases could be accounted for by hemoconcentration, in which case no pathology may be involved at all. The low rho correlations obtained between the subjects with extreme enzyme levels and those with correspondingly altered hematocrits, however are not consistent with such a possibility (Table 9).

A hypoxia-induced response (related to the well documented effects of surfactants on gills) is a reasonable explanation which is particularly applicable in accounting for hematocrit level increases resulting from graded exposure to LAS. Blood clot formation in the gill capillaries with the possibility of clotting hemolysis would account for increased serum enzyme activity. No attempt was made in this study to test this possibility by correlation of results from individual fishes with degrees of alteration in gill histology, or by testing for hemolysis by means other than visual inspection.

It is difficult to isolate the factors which cause a rise in the activity of the serum enzymes GOT and ICD in response to LAS. In homeotherms, comparable elevations in SGOT would normally indicate cardiac or

liver damage (Agress, 1959). An elevation of the level of SICD is even more specific and is usually elevated to extremes only in cases of widespread liver damage (Sterkel et al., 1958).

These relationships are dependent upon the fact that the source of the supra-normal quantity of enzymes in the serum is cells affected by necrosis, inflammation, or submicroscopic alterations of permeability (White, 1959). The levels of specific enzymes in the serum should therefore reflect the enzyme makeup of the tissue or organ cells from which it came. There are numerous objections to such a "leaky cell membrane" theory, and other investigators favor a broader explanation wherein the enzyme elevation is part of a general "acute syndrome" which may not involve a specific tissue (Hauss and Leppelman, 1959; Garbus, Highman and Altland, 1964). Thus, even in the well studied homeotherms, the origin of the serum enzymes is only vaguely understood, although their empirical value is not in question.

Histologically verifiable gill damage resulting from exposure of fishes to LAS at levels close to the TLM have been documented (Swisher et al., 1964; Dooley, 1968). It may be that the increase in SGOT and SICD activity reflects direct gill tissue destruction at these and lower levels. It remains to be shown that the GOT and ICD content of gill tissue is sufficiently high to account for the increases in serum levels which were shown. Bell (1968) demonstrated that the SGOT activity of Pacific Salmon tissues increased in the following order: muscle, kidney, liver, and heart. Comparable gill tissue values would be difficult to arrive at due to the extensive capillary system with related interference by the high enzyme content of the erythrocytes. Whether LAS is able to enter the general circulation by cell lysis of the gills has not been resolved (Dooley and Cavill, 1962; Gloxhuber and Fisher, 1968). In

light of the in vitro and in vivo inhibition by other surfactants on enzyme activity (Manwell and Baker, 1967; Wills, 1954) an inhibition of SGOT and SICD activity might reasonably be expected. At all levels of LAS studied, no such inhibition occurred when the population is considered (Table 8). Perhaps the lowest values experienced within a treatment population (Tables 6 and 7) represent individuals in which LAS has penetrated the general circulation. Such a possibility is consistent with the increased variance ratios of both enzymes at the highest levels (Fig. 8) and the high rho correlation observed between the levels of the two enzymes (Table 9). An analysis of the blood of F. heteroclitus for LAS using IR spectrophotometry or gas chromatography would be of related value in any future study.

LAS may alternatively or concurrently gain access to the liver by a direct circulatory route from the intestinal mucosa, there to cause a release of GOT and ICD into the general circulation. Such a possibility is consistent with the increased toxicity of the anionic surfactant ABS with salinity above 24 ppt observed by Eisler (1965). In this view, more osmoregulatory sea water would be taken in at the higher salinities and with the water, more LAS. Bock (1966) found, however, no evidence of intestinal damage in carp exposed to LAS at concentrations even above those required to destroy the epithelial gill cells. It is not necessary to postulate any direct effect of LAS on the liver. Anoxia may induce the release of catecholamines from the adrenal gland. These in turn may cause the cells of the liver to release the enzymes into the serum. Such a situation has been described in rats (Highman and Altland, 1960).

If detoxification of LAS does take place in the liver, and if the mechanism for this activity is comparable to the same processes in

homeotherms, then the following changes could also be noted: an increase in the level of the enzyme systems engaged in detoxification, a rise in the amount of microsomal protein, and an increase in liver weight (Kupfer, 1970). When the weights of the livers of fishes exposed to graded doses of LAS for 60 days was plotted against their own body weight there was no consistent difference with comparable control data (Fig. 13). Individual differences were noted however. It is interesting to note that all of the values outside of the broad band of most frequently occurring values were in one of two of the experimental tanks, and that none of these values outside the norm was that of a control. Like the rise in serum enzyme values, the liver weight response appears to be highly specific. The effects may have been masked by the obvious histologic alteration of livers from both control and experimental populations.

A study of the distribution of enzymes and their isozymes will shed more light on the origin of the enzymes appearing in the serum. The possible role of the liver in detoxification could be evaluated by a study of the microsomal fraction. The probability of there being changes in the weight of the liver of fish exposed to LAS in long term tests (Fig. 13) would seem to warrant the implementation of such a study.

SUMMARY

When F. heteroclitus was exposed to the anionic surfactant LAS at 3, 2 and 1 ppm for 48 hours, a statistically significant graded increase in the levels of the serum enzymes GOT, ICD and in hematocrit was noted. Significant rho correlations existed between enzyme values, but not between either of the enzymes and hematocrit. Statistically significant ratios of treatment to control variances existed for SICD at all concentrations tested, for SGOT at 3 and 2 ppm, and for hematocrit at 3 ppm. No histological changes in the liver were noted.

When a graded series of LAS concentrations less than 1 ppm were delivered in continuous flow fashion to F. heteroclitus for 60 days, similar alterations in both hematocrit and the levels of the serum enzymes GOT and ICD occurred. A low, but statistically significant relation existed between time to death and LAS treatment. Fatty alterations observed in liver histology were similar in control and experimental populations. They were attributed to confinement.

Both short and long term tests were carried out in sea water diluted to 24 ppt. Ambient temperature was $15^{\circ}\pm 2^{\circ}$.

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