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The Teratogenic Potential of Sodium Dodecylbenzene Sulfonate upon ICR CD-1 Strain of Mice

Raymond M. David
Loyola University Chicago

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THE TERATOGENIC POTENTIAL OF SODIUM DODECYLBENZENE
SULFONATE UPON THE ICR CD-1 STRAIN OF MICE

by

Raymond M. David

A Thesis Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirements for the Degree of Master of Sciences

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1975
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VITA

Raymond Michael David was born on February 1, 1949, in Wurzburg, Germany. He immigrated with his parents to the United States in May of 1951. He attended Calasanctius Preparatory School in Buffalo, New York, in 1960 and transferred to St. Ignatius High School, Chicago, Illinois, in 1963. He was graduated in June of 1965 and attended Marquette University the following September. He received a B.S. degree in Biology in September, 1969.

After four years of teaching at Calasanctius School, he enrolled at Loyola University of Chicago Graduate School in Biology.
ABSTRACT

The teratogenic potential of Sodium Dodecylbenzene Sulfonate, a biodegradable anionic surfactant, has been investigated in the ICR CD-1 strain of mice. Levels at which normal blastocyst implantation would occur were determined by the oral administration of a LAS-distilled, deionized water suspension to 3 to 5 month old pregnant females, from day 0 to 7 of gestation. Doses ranged from 0 to 300 mg/kg at 25 mg/kg increments. The highest dosage level at which the number of blastocyst implants equalled that of controls was 225 mg/kg.

In a subsequent 17 day study, doses of 225, 100, 5, and 0 mg/kg LAS were administered orally by intubation to 2½ month old pregnant females. No significant visceral defects or skeletal anomalies were observed at the gross level. The increased number of fetal deaths and resorptions observed suggests that embryotoxicity was evident after early exposure of the embryo to the surfactant. Results are compared with mammalian studies in which mode and timing of administration, as well as species tolerance, significantly affected the type of results which were observed.

Human teratogenic potential appears moot, since relatively high concentrations of the surfactant were required
to produce deleterious effects.
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INTRODUCTION

Linear alkylbenzene sulfonate (LAS) is a biodegradable, anionic surfactant commonly used in commercial detergents (Swisher, 1970; Harris et al., 1971). Because it is a synthetic surfactant, studies of its effects upon vertebrates, particularly mammals, have been undertaken to determine toxic and/or teratogenic levels. The question of its teratogenic potential is quite complex, and has led to conflicting conclusions.

Teratology, according to Webster's Dictionary, is "the scientific study of biological monstrosities and malformations." These malformations are normally produced during development. Thus, compounds that would ordinarily be relatively harmless to adults, may have deleterious effects on developing organisms because the fetus lacks the detoxifying capabilities of adults (DiPaolo, 1969). However, limitations arise in subsequent testing due to species tolerance. Past studies have shown that not all vertebrates, or even mammals, are equally susceptible to a teratogen. A good example is the thalidomide incident of the 1960's. Although initial testing showed that thalidomide did not harm fetal mice and rats, it was teratogenic to humans (Lenz, 1966). Thus, conclusions drawn from animal testing
are somewhat uncertain when compared with humans; no specific protocol for such tests can be outlined (Brent, 1972). A number of species must, therefore, be tested (Wilson, 1973).

Before considering the development of this environmental problem, a brief description of the surfactant and its properties is necessary. Surfactants, or surface active agents, are amphipathic molecules characterized by a polar, hydrophilic, and a non-polar, hydrophobic portion. Because of the dual nature of such a molecule, surfactants are able to lower surface energy along an interface. They are emulsifying agents and hence are found in most cleaning agents (Haney et al., 1954). Surfactants are divided into three types: anionic, nonionic, and cationic. The character of the polar group determines the class to which the specific molecule belongs. Linear alkylbenzene sulfonate is anionic due to the negative charge of its sulfonate group. The non-polar portion of the molecule consists of an alkyl chain attached to a benzene ring.

The nature of the alkyl group varies greatly in commercial surfactants, and significantly influences its chemical and biological action (Swisher, 1963; Amendt, 1967). The majority of alkylbenzene sulfonates (ABS) used before 1965 contained a tetrapropylene group as the alkyl chain (Swisher, 1970).
Due to the branching of the carbon skeleton, this compound was difficult for sewage bacteria to breakdown (Swisher, 1963). This unfavorable property of ABS prompted its replacement by a more biodegradable substance, LAS.

Conversion to LAS occurred in the middle Sixties, since LAS had been found to be more easily degraded by bacteria. One study performed by Halvorson and Isaque (1968) showed that 93% of a commercial preparation and 97.5% of an 11.3 preparation of LAS (an isomeric mixture containing an average carbon chain length of 11.3) were metabolized into non-detectable units by bacteria within 96 hours. In a similar study of the bacterial metabolism of dodecyl- and undecylbenzene-p-sulfonate, Willetts and Cain (1972) proposed a cleavage of the alkyl chain and the sulfonate group from the phenyl ring, with their subsequent metabolism into normal waste products. According to this scheme, the sulfite is oxidized to sulfate, the alkyl chain undergoes beta-oxidation, and the benzene ring is oxidized to succinate and acetyl CoA (Figure 1).

Ideally, LAS is dodecylbenzene sulfonate, but commer-
Bacterial degradation scheme of LAS according to Willetts and Cain (1972).

Figure 1
Figure 1
cially prepared LAS characteristically represents a mixture of straight chain carbon atoms ranging in number from 8 to 16. The majority of position isomers are secondary, with the benzene ring attached to the second carbon atom of the alkyl chain.

\[
\text{CH}_3\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_3
\]

(LAS)

The position of the phenyl ring and the chain length of the alkyl group can alter the detergency and biodegradability of the compound (Rubinfeld et al., 1964; McAteer and Kinnard, 1967). The mechanism of detergency is not well known. Certainly micellar formation and solubilization are key processes in detergency, but opinions differ with respect to the type of micellar organization and the energy requirements for that formation (Anacker, 1970).

Biodegradability in mammals was established by Michael (1968), who followed $^{35}$S-LAS in albino rats. His results showed that 92% of the label was excreted into the urine within 48 hours, while $^{35}$S-ABS accumulated in the liver and was excreted in the feces. Michael also established that both LAS and ABS were absorbed through the gastrointestinal tract and were probably transported by the venous blood. The extent to which LAS had been degraded was deter-
mined by Nuclear Magnetic Resonance (NMR) and showed that 60-65% of the LAS excreted in the urine after 72 hours was sulfophenyl butanoic acid and sulfophenyl pentanoic acid. These two compounds suggest omega- followed by beta-oxidation. Thus, the LAS molecule is relatively intact in the mammal, and if toxic or teratogenic effects are found, they can be attributed to either the intact molecule or one of its early degradation products.

The toxicity research with respect to LAS is most abundant for fish. Swisher (1964) determined the toxicity of dodecyl- and undecylbenzene-p-sulfonate to fish and showed that the 12 carbon chain had a 48 hour TLm (Median Tolerance Limit) of 3.0 ppm, while the 11 carbon chain was 75 ppm. A more refined test of toxicity and structure was reported by Hirsch (1963) who tested various structural isomers on the fish, Goldorfen, Idus idus. His results showed that greater toxicity as evidenced by lower TLm's was obtained as the chain length increased and the position of the phenyl ring came closer to the primary carbon. Similar results of the higher toxicity of a primary 12 carbon isomer of LAS have been reported in other species of fish (Borstlap, 1967). The work of Manner and Dewese (1974) has demonstrated that various mixtures of isomers differ in toxicity; a LAS preparation having an average carbon length of 11.8 was more toxic than one having a carbon length of 11.2. Although these results suggest an environmental hazard on the part of
these compounds, the hazard may not be relevant to mammals, since the manner of mammalian exposure to LAS is not the same as fish. Exposure to a developing mammal is only through the circulatory system of the mother. To achieve this, the mother must ingest the LAS or the material must be absorbed through the skin. The effects of LAS absorbed through the skin cannot be discounted, but the amount absorbed in relation to other modes of entry is minimal (Gale, 1974).

Two sources of oral exposure to the surfactant are possible: one from residual amounts in fresh water supplies, the other from inadequately rinsed dishes after washing. The United States Government has set allowable levels of LAS in waterways at 0.5 ppm (Swisher, 1970). Tests of the Calumet River conducted by the Federal Water Pollution Control Administration during 1966 showed an average Methylene Blue Active Substance (MBAS) of approximately 0.5 ppm. The reaction between Methylene Blue and the anionic surfactant involves a colorimetric assay of the salt formed from the two substances (Swisher, 1970). The value obtained from this test agrees with figures of MBAS obtained from the Illinois River (Sullivan and Evans, 1966; Sullivan and Swisher, 1969), the bulk of which represents LAS (Rickert and Hunter, 1973).

Residual amounts of LAS left on improperly rinsed dishes and glassware depend upon a number of factors. A
variety of reports have listed different figures for the average human intake, with a reliable estimate being from 0.3 to 3.0 mg/person/day (Swisher, 1968). For a 60 kg person, this means a maximal daily dose of 0.05 mg/kg. Nevertheless, individual amounts ingested may vary greatly, and mammalian studies to determine the oral toxicity of LAS have been performed. Experimentation on rats is extensive, perhaps because they are hardy animals and are large enough for easy biochemical assay. Bornmann and Loeser (1963) mixed LAS into the drinking water of rats and found no apparent ill effects. Both male and female rats were given 100 ppm LAS ad libitum for a period of 100 weeks; subsequent autopsies showed no significant difference from controls with respect to organ weights. Similar results have been obtained with dietary levels of LAS. In these cases, concentrations of 0.02, 0.1, and 0.5% LAS were mixed with commercial food and given ad libitum to rats (Paynter and Weir, 1960; Kay et al., 1965). Even the administration by intubation of commercial detergents containing 20% LAS have not shown significant toxicity (Snyder et al., 1964). An additional study of the effects of 0.02, 0.1, and 0.5% dietary LAS upon rat reproduction demonstrated no significant differences from controls through the F₁ and F₂ generations (Buehler et al., 1971).

Toxicological and teratological studies of LAS on mice have produced conflicting results. In 1949, Hopper
et al., using an early preparation of LAS designated Santomerse No. 3, found that the oral LD$_{50}$ was 2000 mg/kg in mice, as opposed to the 650 mg/kg established by Oser and Morgareidge (1965) in rats. Recently, Mikami et al. (1973) administered 60 mg/kg of LAS in the drinking water of pregnant ICR mice, and reported that litters exhibited one-third to one-half cleft palate, a small percentage of encephaly and microphthalmia, and one-third to one half fetal death. Another report, by Sakai (1973) of the Mikami group, stated that concentrations of as low as 0.015 mg/kg LAS produced cleft palate, spina bifida, and anencephaly in a percutaneous study on ICR/JCL mice. However, Palmer et al. (1973) also applied LAS to the skin of ICR CD-1 mice in concentrations of 0.03, 0.3, and 3.0%. He found that at 3.0%, an 80% implantation loss occurred along with a decrease in normal weight gains during pregnancy. Minor skeletal variations were the most common abnormality, but they were within "laboratory limits". A more recent work by Palmer et al. (1975) reported that no teratogenesis was evident in CD-1 mice that had been given 0.2, 2.0, 300, and 600 mg/kg of LAS by intubation from days 6 to 15 of gestation. However, maternal toxicity was evident at the 600 and 300 mg/kg doses.

Although these results appear conclusive with respect to the teratogenic potential of LAS, the basis for the re-testing of LAS in mammalian systems is based upon several
recent findings. Manner and Dewese (1974) demonstrated that an 11.8 LAS preparation produced neural anomalies in Zebra fish, Brachydanio rerio, at concentrations of less than 5 ppm. Curvature of the spine, anophthalmia, microphthalmia, and even deaths were observed when the embryo had been exposed at the late cleavage stage of development. These results were consistent with those of Mikami et al. (1973), who had reported that 60 mg/kg of orally administered LAS produced exencephaly, microphthalmia, cleft palate, and even fetal death in ICR/JCL mice. The previously described results had not been reported in fish, mice, or rats. Palmer et al. (1975), in reassessing the teratogenic potential of LAS upon mice, rats, and rabbits, found the rat to be more tolerant of the surfactant than other species. Since generalizations regarding lack of teratogenesis of LAS toward rats had been projected to include all mammals, there is now doubt regarding the validity of such a conclusion. Further, the procedure employed by Palmer had been to dose the animals after blastocyst implantation. The teratogenic potential of LAS administered during early mammalian embryonic development had been overlooked. Manner and Dewese (1974) had shown that this period was most susceptible to LAS teratogenicity in fish embryos. Thus, their observations, suggesting early stages of embryogenesis are most susceptible to LAS teratogenicity, form a working hypothesis to be tested in mammals.
MATERIALS AND METHODS

Two basic differences from the majority of toxicological work done with LAS were employed here. First, the mode of administration, intubation, is used infrequently except for the determination of LD$_{50}$ values; however, it provides a more accurate record of the amount of material which the animal has received. Second, chronic or prolonged doses were used instead of dividing the gestation period into sections and administering the dose only at specific times (King et al., 1972). The amount of time available to study a given number of animals was the limiting factor of this method.

One ambiguity of teratology is the distinction between physical and functional anomalies. In the examination of fetuses, only physical defects were considered, since functional deviations often take time to verify, a process which can take place only if the fetuses are living.

General Procedures

Forty-two day old virgin ICR CD-1 mice were obtained from the Charles River Breeding Laboratory, Wilmington, Massachusetts, and allowed to mature to 2½ months of age before experimentation. Throughout the study, two to four animals were housed in plastic shoe-box type cages (6½ x 11
in.) containing an inert bedding material called Sanicel®. Purina Mouse Chow for Breeding Mice® and tap water were given ad libitum, with distilled, deionized water being substituted for tap water during experimentation. Fresh water was provided every other day; water bottles and cages were cleaned weekly. A mild Alconox® solution was employed during cleaning, followed by 6-10 rinses under running tap water. Temperature was regulated at 22 ± 1°C under a 12 hour cycle and a relative humidity of 50 ± 10%.

Females were exposed to males overnight in a ratio of 3-4 to 1. Presence of vaginal plugs or sperm in vaginal smears were taken as indications of fertilization and this day was designated day 0 of gestation. Pregnant mice used for experimentation were separated from stock females and grouped according to dose.

Sodium dodecylbenzene sulfonate having an average carbon length of 11.8 was provided by the Procter and Gamble Co., Cincinnati, Ohio. The test material was an off-white paste whose composition was assayed as 57.5% active sodium dodecylbenzene sulfonate, 2-3% unreacted alkylbenzene, 0.91% sodium sulfate, and a remaining fraction of water. A correction factor of 1.74 was used in calculating doses to adjust to 100% activity. The test material was refrigerated to maintain consistency, but was allowed to come to room temperature before weighing. Since small amounts were needed, a factor of 10 was employed to insure accuracy to
within 0.5 mg; the volume of distilled, deionized water was also adjusted by a factor of 10. Concentrations of LAS administered were in relation to body weight to allow comparison with other species; all amounts varied according to the daily weight changes of the animal. Vials used for weighing were thoroughly rinsed and dried beforehand; suspensions were mixed with a teflon stirring bar. A gavage needle was formed from a 17 gauge needle by making a small bend at the open end and attaching a ball of lead solder. The needle was thoroughly rinsed before and after each intubation, and a fresh plastic 1 cc syringe was used each day. Single, daily doses were administered at 12:00 P.M. ± 2 hours.

All animals were killed by excess etherization, the uteri excised, fetuses checked for movement, and uteri plus contents weighed; individual fetal weights were not taken. Two-thirds of the fetuses from each litter were placed into vials containing Bouin's solution and allowed to fix for a period of at least 10 days. One-third of the fetuses were placed into 95% ethanol, cleared, and stained with Alizarin Red S according to Knudsen (1966).

Seventeen day old fetuses which had been preserved in Bouin's fluid were sectioned according to Wilson (1965). This method is used for gross examination; histologic variations were not considered. Fetuses were examined for syndactyly, polydactyly, adactyly, micromelia, phocomelia, spina bifida, anophthalmia, microphthalmia, hydrocephaly,
anencephaly, exencephaly, cleft palate, incomplete closure of the ventricular cardiac septum, diaphragmatic hernia, umbilical hernia, position of the aortic arch, number and position of the kidneys and adrenal glands, and the presence of gonads. In addition, the volume of each fetus without umbilicus was measured by water displacement as an indication of relative size. Fetuses stained with Alizarin Red S were examined for number of ribs, cervical vertebrae, sternal vertebrae, digits, ossification of vertebrae centrae, and general skeletal anomalies.

Range Study

Early in the research it had become apparent that the high dose of 300 mg LAS/kg maternal body weight was interfering with normal gestation. A range of doses was therefore tested to determine at what level implantation would occur. Pregnant females were randomly divided into groups and administered 0.25 ml of a LAS-distilled, deionized water suspension by intubation on a daily basis from day 0 to 7 of gestation. Doses from 300 to 100 mg/kg at 25 mg/kg increments were used with the addition of a 50 and 0 mg/kg dose to complete the spectrum of doses. Controls received 0.25 ml of the vehicle without LAS.

Females were killed on day 8 of gestation and the number of implants recorded. A minimum of three mice either bearing or lacking implants was taken as sufficient indication of toxicity for each dose. Sectioning of embryos was
not possible due to small embryonic sizes at this early stage of development.

Term Study

Pregnant females were randomly distributed into groups receiving 225, 100, and 5 mg LAS/kg body weight and controls. The teratogenic activity of 300 mg/kg of sodium sulfate was also tested in a preliminary study upon 5 dams; the results showed no significant abnormalities when compared with controls (Table I). This dose was chosen to compare the effects of sodium sulfate with an equal concentration of LAS. Also, since only 0.91% of the LAS preparation was sodium sulfate, the level administered in the dose is well below the lowest normal sulfate concentration in the blood (Guyton, 1971). Sodium sulfate was then minimized as a teratogenic possibility.

All mice were placed into cages with animals receiving the same dose level. Experimentals received 0.20 ml LAS-distilled, deionized water suspension, while controls received 0.20 ml distilled, deionized water. Animals were gavaged for 17 days beginning on day 0, and killed on day 17 of gestation. Fetuses were scored, placed in appropriate solutions, and examined in the manner outlined previously. Records were kept of the anomalies and resorptions found in each litter. The Kruskal-Wallis test, Wilcoxon Rank test, and the Chi square test were used to ascertain statistical
significance of the results (Sokal and Rohlf, 1969). Non-parametric values such as litter data are particularly suited to the Kruskal-Wallis and Wilcoxon tests, while Chi square is a well established test of frequencies.
RESULTS

Table I shows the results of oral administration of 300 mg sodium sulfate/kg body weight. The observation of one fetus with cleft palate does not appear to be teratogenically significant, since cleft palate is the most common sporadic visceral defect with a high incidence in this strain of mice (Palmer, 1972).

Table II presents the results of the range study. It can be seen that of the concentrations of LAS used, 225 mg/kg was the upper limit of surfactant which allowed the number of implantations of blastocysts of experimentals to approximate that of the controls at almost every dose level below 225 mg/kg. Because of borderline maternal toxicity at 200 and 225 mg/kg, more than 3 experimental animals were required to ascertain toxicity. Dams receiving concentrations of 250, 275, and 300 mg/kg failed to exhibit implantation of blastocysts. Maternal toxicity rather than embryotoxicity is believed to have caused interference with the normal processes involved in fertilization and development leading to implantation, since the usual signs of maternal toxicity such as weight loss and diarrhea were evident. Since 225 mg/kg was the highest tolerable dose, it was used as the
### TABLE I

RESULTS OF ADMINISTRATION OF 300 mg Na$_2$SO$_4$/kg BODY WEIGHT

<table>
<thead>
<tr>
<th>NUMBER OF LITTERS</th>
<th>LITTER SIZE ± S.D.</th>
<th>PERCENTAGE RESORBED AND DEAD FETUSES ± S.D.</th>
<th>LITTER WEIGHT (gm) ± S.D.</th>
<th>NUMBER OF FETUSES EXAMINED</th>
<th>NUMBER OF FETUSES AFFECTED</th>
</tr>
</thead>
<tbody>
<tr>
<td>3*</td>
<td>13.0±0.7</td>
<td>2.8±4.8</td>
<td>21.63±2.7</td>
<td>39</td>
<td>1**</td>
</tr>
<tr>
<td>2***</td>
<td>11.0±1.0</td>
<td>0</td>
<td>5.57±1.1</td>
<td>22</td>
<td>0</td>
</tr>
</tbody>
</table>

* Pregnancies terminated on day 18 of gestation. Animals dosed from day 0 to 17 of gestation.

** Fetus exhibited cleft palate.

*** Pregnancies terminated on day 13 of gestation. Animals dosed from day 0 to 12 of gestation.
TABLE II
RANGE STUDY: LEVELS OF LAS AFFECTING NUMBER OF IMPLANTS

<table>
<thead>
<tr>
<th>DOSE (mg/kg)</th>
<th>0</th>
<th>50</th>
<th>100</th>
<th>125</th>
<th>150</th>
<th>175</th>
<th>200</th>
<th>225</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAMPLE SIZE</td>
<td>9</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>AVERAGE IMPLANTS OF VIABLE LITTERS</td>
<td>12.4</td>
<td>13.3</td>
<td>12.3</td>
<td>13</td>
<td>15</td>
<td>14.6</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>± S.D.</td>
<td>±3.3</td>
<td>±0.6</td>
<td>±2.8</td>
<td>±2.0</td>
<td>±1.0</td>
<td>±1.2</td>
<td>±1.0</td>
<td>±2.6</td>
</tr>
<tr>
<td>NUMBER NOT PREGNANT</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

Presented are the results of the range study in which dams received 0.25 ml LAS-vehicle from day 0 to 7 of gestation. These results show the average number of implants observed on day 8. The "number not pregnant" is the number of confirmed matings which on examination did not bear blastocyst implants. The "sample size" represents all confirmed matings.
highest dose in the 17 day study; 100 mg/kg represented
the mid-region of the range; and 5 mg/kg was a close ap-
proximation of the amount of normal intake (Swisher, 1968).
Experimental parameters are summarized in Table III, and
include values for litter size, in utero litter weight,
fetal volume, and the percentage of dead and resorbed
fetuses of viable litters. As can be seen, the litter
sizes and weights, as well as fetal volumes, are all over­
lapping values and therefore not statistically different
from the controls. Although the percentages of dead and
resorbed fetuses overlap and were not significantly differ­
et from controls, a trend toward increasing embryotoxicity
with increasing concentrations of LAS administered is seen.
Also, 5 of the fetal deaths at 225 mg/kg represent retarded
growths, whereas no retarded growths were observed in the
controls or any other group. Possible embryotoxicity at
225 mg/kg is further evidenced by the number of females not
bearing litters. None of the aforementioned signs of mater­
nal toxicity were present.

Although a similarly high number of non-pregnancies
is seen in the control group, they may reflect a non-random
selection of females by the experimenter. In these cases,
although copulation had occurred, the likelihood of preg­
nancy may have been diminished due to a decreased sperm con­
centration in the semen.

It should be noted that although the percentage of
TABLE III
FETAL AND LITTER PARAMETERS

<table>
<thead>
<tr>
<th>DOSE mgLAS/kg BODY WEIGHT</th>
<th>NUMBER OF DAMS BEARING VIABLE YOUNG/NUMBER OF CONFIRMED PREGNANCIES</th>
<th>AVERAGE LITTER SIZE ± S.D.</th>
<th>PERCENTAGE RESORBED AND DEAD FETUSES ± S.D.</th>
<th>AVERAGE LITTER WEIGHT (gm) ± S.D.</th>
<th>AVERAGE FETAL VOLUME (ml) ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15/21</td>
<td>12.00±2.9</td>
<td>4.17±5.8</td>
<td>18.5±3.7</td>
<td>1.03±0.14</td>
</tr>
<tr>
<td>5</td>
<td>13/15</td>
<td>12.54±1.9</td>
<td>4.37±5.4</td>
<td>18.1±2.8</td>
<td>0.99±0.09</td>
</tr>
<tr>
<td>100</td>
<td>12/15</td>
<td>13.00±1.9</td>
<td>5.66±5.6</td>
<td>17.8±2.7</td>
<td>0.95±0.12</td>
</tr>
<tr>
<td>225</td>
<td>13/21</td>
<td>12.69±2.9</td>
<td>10.77±9.7</td>
<td>18.0±3.5</td>
<td>0.97±0.12</td>
</tr>
</tbody>
</table>

Presented are the fetal and litter parameters of dams in the 17 day study which received either 0.20 ml LAS-vehicle or 0.20 ml vehicle alone beginning on day 0 of gestation.
dead and resorbed fetuses differ between control and experimental groups, all other parameters are comparable. Thus the production of anomalies in the experimental groups does not appear to have been influenced by maternal factors or diet.

The only visceral anomalies detected using Wilson's sectioning techniques (1965) were 1 hydrocephalus (Figure 2), 2 cleft palates (Figure 4), and one case of mispositioning of the aortic arch. The 4 affected fetuses were of 110 examined in the 225 mg/kg group. No visceral defects were found in any of the 144 fetuses in control, 115 fetuses in the 5 mg/kg, or 102 fetuses in the 100 mg/kg groups. The placenta of one fetus with cleft palate was fused with the placenta of the neighboring fetus; how this may have influenced the production of the cleft is analyzed in the discussion. Five fetuses in the 225 and 100 mg/kg groups exhibited underdeveloped hearts, but it was not clear whether these represented miscounted fetal deaths or abnormalities; these data were not included.

Results of skeletal examinations are summarized in Table IV, which tabulated the incidence of extra ribs, unossified 5th sternebrae (Figure 6), and incomplete fusion of sternebrae. The latter abnormality was designated as "dumbbell shaped" (Figure 7). The column marked "Other" tabulated unfused sternebrae other than the 5th. In assessing the skeletal anomalies, some attention must be
Figure shows a picture of a longitudinal section through a brain suspected as having hydrocephalus. Enlarged lateral ventricles are characteristic of hydrocephalus.

Figure 2

Figure shows a picture of a longitudinal section through a normal brain.

Figure 3
Figure shows a picture of a fetus with cleft palate.

Figure 4

Figure shows a picture of a normal palate.

Figure 5
### TABLE IV

**SKELETAL VARIATIONS**

<table>
<thead>
<tr>
<th>DOSE (mg/kg)</th>
<th>NUMBER OF FETUSES EXAMINED</th>
<th>NUMBER OF FETUSES AFFECTED</th>
<th>PERCENTAGE FETUSES AFFECTED</th>
<th>EXTRA RIBS</th>
<th>UNOSSIFIED 5th STERNEBRAE</th>
<th>DUMBELL SHAPED</th>
<th>OTHER*</th>
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<td>17</td>
<td>54.84</td>
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<td>2</td>
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<td>13</td>
<td>37.14</td>
<td>6</td>
<td>4</td>
<td>3</td>
<td>2</td>
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</tbody>
</table>

* Dumbbell formations at the second and fourth sternebrae.

The data represents the incidences of observed skeletal variations of fetuses stained with Alizarin Red S.
Figure shows a picture of a fetus with no ossification of the 5th sternebra.
Figure shows a picture of a fetus with incomplete fusion of the 2nd and 5th sternebrae; these were designated "dumbbell shaped".

Figure 7
Fig. 7
paid to normal variations which occur within a population. For example, Palmer (1972) describes extra ribs as such a variant with no particular significance to teratogenic activity. For this reason, the skeletal variations observed do not appear to be in any way related to LAS administration.

Table V lists multiple defects and the distribution of anomalies within groups and litters. The purpose of this table is to assess the occurrence of related anomalies and litter effects. No instances of litter effects or multiple visceral anomalies in one fetus were found. The occurrence of multiple skeletal defects is due to the high incidence of extra ribs. Omission of these values of extra ribs decreases the number of fetuses with multiple defects to 0. A random distribution is seen in the number of affected litters.
TABLE V
DISTRIBUTION OF ANOMALIES

VISCERAL ANOMALIES

<table>
<thead>
<tr>
<th>DOSE (mg/kg)</th>
<th>NUMBER OF FETUSES WITH MULTIPLE DEFECTS</th>
<th>NUMBER OF AFFECTED LITTERS/TOTAL NUMBER OF LITTERS</th>
<th>NUMBER OF LITTERS WITH 2–3 FETUSES WITH SAME DEFECT</th>
<th>NUMBER OF LITTERS WITH 4 OR MORE FETUSES WITH SAME DEFECT</th>
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<td>0</td>
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<tr>
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<tr>
<td>225</td>
<td>0</td>
<td>4/13</td>
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</table>

SKELETAL ANOMALIES

<table>
<thead>
<tr>
<th>DOSE (mg/kg)</th>
<th>NUMBER OF FETUSES WITH MULTIPLE DEFECTS</th>
<th>NUMBER OF AFFECTED LITTERS/TOTAL NUMBER OF LITTERS</th>
<th>NUMBER OF LITTERS WITH 2–3 FETUSES WITH SAME DEFECT</th>
<th>NUMBER OF LITTERS WITH 4 OR MORE FETUSES WITH SAME DEFECT</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>3</td>
<td>7/8</td>
<td>4</td>
<td>1* (extra ribs)</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>8/11</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>1</td>
<td>9/11</td>
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<td>225</td>
<td>2</td>
<td>5/10</td>
<td>1</td>
<td>1* (extra ribs)</td>
</tr>
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</table>
DISCUSSION

In evaluating the data for teratogenicity, the firm establishment of normal incidences of malformations is required. Data from this experiment have not provided enough background data of frequencies of sporadic anomalies to allow acceptable statistical analysis. However, when compared to 2.23 hydrocephalic mice per 10,000 fetuses and 45.56 mice with cleft palate per 10,000 fetuses for sporadic incidences of anomalies (Palmer, 1972), differences to the 225 mg/kg group are observed. Although these values may be statistically significant, they are not teratogenically significant, since the values are low enough to be within normal limits of a population (Wilson, 1975, personal communication) and they do not stress one single defect or related defects. The administration of LAS may have only slightly exceeded the threshold for these common anomalies (Fraser, 1971). However, conclusions will be drawn later.

Making a distinction between normal and hydrocephalic fetuses was quite difficult, since the development of the lateral ventricles was not completed at this developmental stage (Figures 2 and 3). Examination of a representative section of a fetus with hydrocephalus did not confirm this
interpretation (Wilson, 1975, personal communication). The scoring of cleft palate did not have this ambiguity; the observed clefts were clear (Figures 4 and 5). In one case of observed cleft palate, the placentas of a malformed fetus and its neighbor were fused. This fusion of placentas could have contributed to the formation of the cleft; a fetus with decreased placental supply may lack the necessary metabolites for proper palatal closure (Fraser, 1971). A similar occurrence of placental fusion in the same litter produced one normal and one underdeveloped fetus.

The relationship of anomalies to sex and uterine position have been explored. Of a total of 207 male and 204 female fetuses examined, males show a higher incidence of anomaly than females (4 to 0). This observation suggests that males exhibit a higher susceptibility toward such anomalies than females, but lack of sufficient numbers of affected fetuses warrants caution not to overstate this observation. The uterine positions of abnormal fetuses in the examined litters appeared to be random.

The mechanism of LAS action on the fetus is difficult to ascertain. The development of cleft palate may be influenced by a decrease of amniotic fluid which produces clefts by overconstriction of the fetus, or a decrease in availability of mucopolysaccharide necessary for palatal closure (Fraser, 1968). Overconstriction of the fetus presses the tongue against the palatal shelves, thereby
preventing their union. Mucopolysaccharides are necessary for shelf extension and growth. The mechanism by which LAS produces cleft palates depends largely on placental transfer of the compound which has not yet been proven. Assuming gastro-intestinal absorption, placental transfer might result in solubilization of mucopolysaccharide. It should be noted that for this mechanism to operate the concentration of the surfactant must be sufficient to overcome excretion by the kidney and oxidative degradation into products which lack surfactant properties (Michael, 1968).

Hydrocephalus may be produced by one of three mechanisms: overproduction of cerebrospinal fluid (CSF), defective absorption of CSF, or obstruction of CSF drainage (Fishman, 1971). Little is known about the overproduction of CSF, but defective absorption can be caused by high CSF protein concentration which decreases the absorption through the arachnoid villi. Obstruction of CSF pathways can be at the level of the ventricular aqueduct (non-communicating hydrocephalus) or at the level of the spinal subarachnoid space (communicating hydrocephalus) (Foley, 1974). Excess amounts of amniotic fluid may also play a significant role in the formation of hydrocephaly (Posner, 1972). However, since only one case of mild hydrocephalus was observed, discussion of the mode of action by which LAS produces hydrocephaly appears moot.

There appear to be two periods of gestation during
which the embryo or fetus is most susceptible to the action of LAS. One period of embryo susceptibility is seen in the term study, in which a high number of early resorptions was observed at 225 mg/kg. This period is essentially the first trimester of gestation. Susceptibility during this time may be due to the presence of a yolk sac placenta (Bellairs, 1971) and fetal deaths might be a result of the denaturation of membrane or nutrient protein by LAS (Yang and Foster, 1953). It should be noted that large concentrations of the surfactant are not necessary for protein-surfactant complex formation (10.5 x 10^{-5} M). The occurrence of fetal deaths after early exposure of the embryo to LAS corresponds to the observation of Manner and Dewese (1974) in fish. Such observed deaths and anomalies after early exposure to the surfactant may be related to yolk protein-surfactant complexing.

The second period of susceptibility to LAS may affect early fetal development as illustrated by the cleft palates, hydrocephalus, and retarded growths. The critical period for the production of cleft palate and hydrocephaly encompasses the early portion of the last trimester (Dagg, 1966; Rugh, 1968), a time which would correspond to the developmental age of many of the retarded growths observed at the 225 mg/kg dose. Although the formation of the hemato-endothelial placenta begins at this time (Rugh, 1968), susceptibility to LAS may again be influenced by a maternal factor.
sac placenta.

One question that can be posed at this time is why such varying results have been reported in many mammalian studies. It is known that the results of any toxic or teratogenic study are always dependent, to some extent, on mechanical or procedural factors such as time and method of administration of teratogen (Halberg, 1974; Haus et al., 1974). An important factor is the particular type of LAS preparation tested, which can vary with respect to chain length and positional isomeric composition from manufacturer to manufacturer. Variable composition may account in part for varying levels of toxicity in mammals; such variances have been documented in fish (Hirsch, 1963; Borstlap, 1964; Manner and Dewese, 1974).

An equally important factor is the timing of administration, not only with respect to gestation, but also with respect to circadian rhythm (Halberg, 1974). It has been reported that varying degrees of drug susceptibility are present at different times of the circadian rhythm (Haus et al., 1974). Thus, the time in the animals natural rhythm at which the dose is administered may play a significant role in obtaining varying results.

Timing with respect to the gestation period may also influence results. For example, Palmer et al. (1975), using methods approximating the ones employed here, gavaged ICR CD-1 mice with an LAS preparation on days 6 to 15 of gesta-
tion. Deaths occurred in 19 of 20 mice at 600 mg/kg and 7 of 20 mice at 300 mg/kg. In the latter group, only 9 of the 13 surviving dams bore young. At dosages of 2.0 and 0.2 mg/kg, no toxicity was evident in dams or their offspring. Results of the present experiment indicate that LAS administered during the first 6 days of gestation was sufficiently toxic at levels above 225 mg/kg to interfere with the processes leading to normal blastocyst implantation.

The mode of administration employed in any teratogenic study often reflects actual means of exposure and greatly influences the results. Percutaneous studies which assume primary exposure to the compound have shown both positive and negative results (Sakai, 1973; Palmer, 1973). However, absorption through the skin may be minimal in comparison with other modes of administration (Gale, 1974). One striking example of the effects of different modes of administration can be seen in a comparison of oral versus intraperitoneal administration levels; a 5 fold difference exists in the dose of surfactant that can be tolerated for normal implantation (David et al., 1975, manuscript submitted for review). The cause of this difference is not known, but several possibilities exist: metabolic inactivation of the compound by omega- followed by beta-oxidation (Michael, 1968), a slow rate of absorption through the gastro-intestinal tract (Michael, 1968), or a combination of factors including rate of placental transfer (Villee, 1965; Wilson,
1973). Whatever the mechanism, it is evident that extremely large amounts of oral LAS were necessary to produce effects similar to those obtained with other methods.

**Conclusions**

The primary question of the present research has been to ascertain the teratogenic potential of LAS in ICR CD-1 mice. The hypothesis that LAS is teratogenic in mice is suggested only by the data of embryotoxicity at the 225 mg/kg level, since fetal deaths and resorptions may represent embryotoxicity produced by teratogenicity (Beck and Lloyd, 1963). However, this embryotoxic activity may correspond only to the presence of the yolk sac placenta. The later formation of the hemo-endothelial placenta characteristic of rodents apparently acts as a sufficient barrier to prevent any teratogenic activity by the surfactant. The observation of a few common visceral defects at the 225 mg/kg dose may reflect 1) the lower tolerance of a few dams, or 2) a normal incidence of defects in a population. In either case, the defects were not considered to be teratogenically significant.

An underlying question, however, concerns the potential hazard to humans. As mentioned in the introduction of this paper, species tolerance to various compounds plays a significant role in evaluating teratogenic potential. Such a problem can be illustrated by the results obtained by Palmer et al. (1975) in which CD rats and CD-1 mice were
intubated with comparable concentrations of LAS. It was found that 600 mg/kg was lethal to 19 of 20 mice, while not lethal to any rats. Similarly, while 300 mg/kg had no effect on rats, 7 of 20 mice died, with 4 of the survivors failing to bear young. Explanations which cite the size of the animal as the sensitivity factor were untenable, since NZW rabbits also used in the study showed responses to LAS which were comparable to those obtained from mice. Results of a comparable nature have been reported for other compounds (Schumacher et al., 1972). The effects of such species tolerance, particularly of the rat, are secondary to this study, but in assessing LAS teratogenicity to humans, such resistance of a widely used test animal complicates any conclusions to be drawn from the available data. However, the environmental hazard of LAS to humans appears minimal, since 1) levels of LAS in the environmental watershed are low, due to rapid degradation by sewage bacteria (Swisher, 1963; Halvorson and Isaque, 1968); 2) fairly low levels of LAS are consumed in comparison to body weight (Wedell, 1966; Swisher, 1968); and 3) any LAS which is ingested is metabolized and excreted into the urine (Michael, 1968). Further, the adverse effects found in mice were within the theoretical teratogenic range of the compound (Wilson, 1973), with the dose closest to physiological levels demonstrating no effect. It can only be concluded that at levels currently found in water supplies, 11.8 LAS
poses no teratogenic hazard to humans.
LITERATURE CITED


APPENDIX
Kruskal-Wallis test

Determination of significance of percentage of dead and resorbed fetuses.

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Kruskal-Wallis test (cont.)

Sum of ranks within groups.

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<tr>
<td>45</td>
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Calculation of "H".

\[
H = \frac{12}{(53)(54)} \left( \frac{341^2}{15} + \frac{312.5^2}{13} + \frac{330^2}{12} + \frac{447.5^2}{13} \right) - 3(54)
\]

\[
H_{\text{adjusted}} = \frac{H}{D}
\]

\[
D = 1 - \frac{14202}{(52)(53)(54)}
\]

\[
H_{\text{calc}} = 5.1287
\]

Critical \( H \) (\( p = 0.05 \)) = 7.815
Chi square test

Determination of significance of skeletal defects.

<table>
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</table>

Expected Anomalous = \(\frac{63}{143}\) (Total of group)

Expected Normal = Total of Group - Expected Anomalous

Calculated Chi square = 2.52

Critical Chi square (p = 0.05) = 7.815
APPROVAL SHEET

The thesis submitted by Raymond M. David has been read and approved by the following Committee:

Dr. Albert J. Rotermund, Jr., Chairman
Assistant Professor, Biology, Loyola

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

Dr. Kirt Vener
Assistant 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 Sciences.

May 12, 1975
Date

Director's Signature