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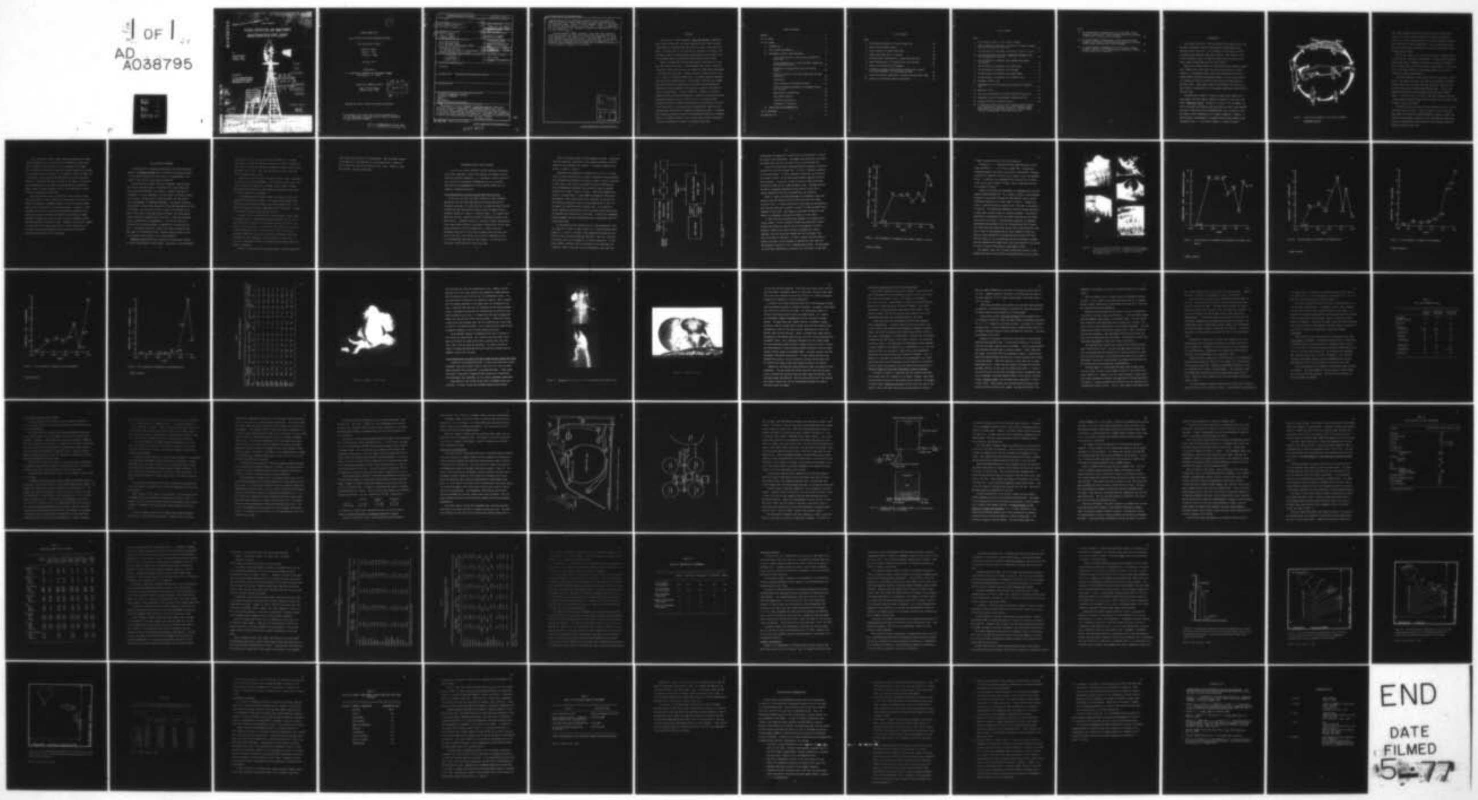

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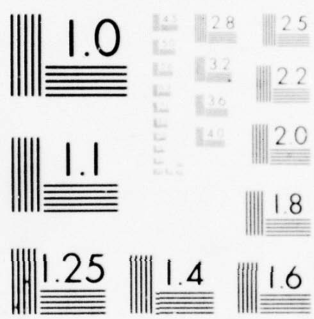

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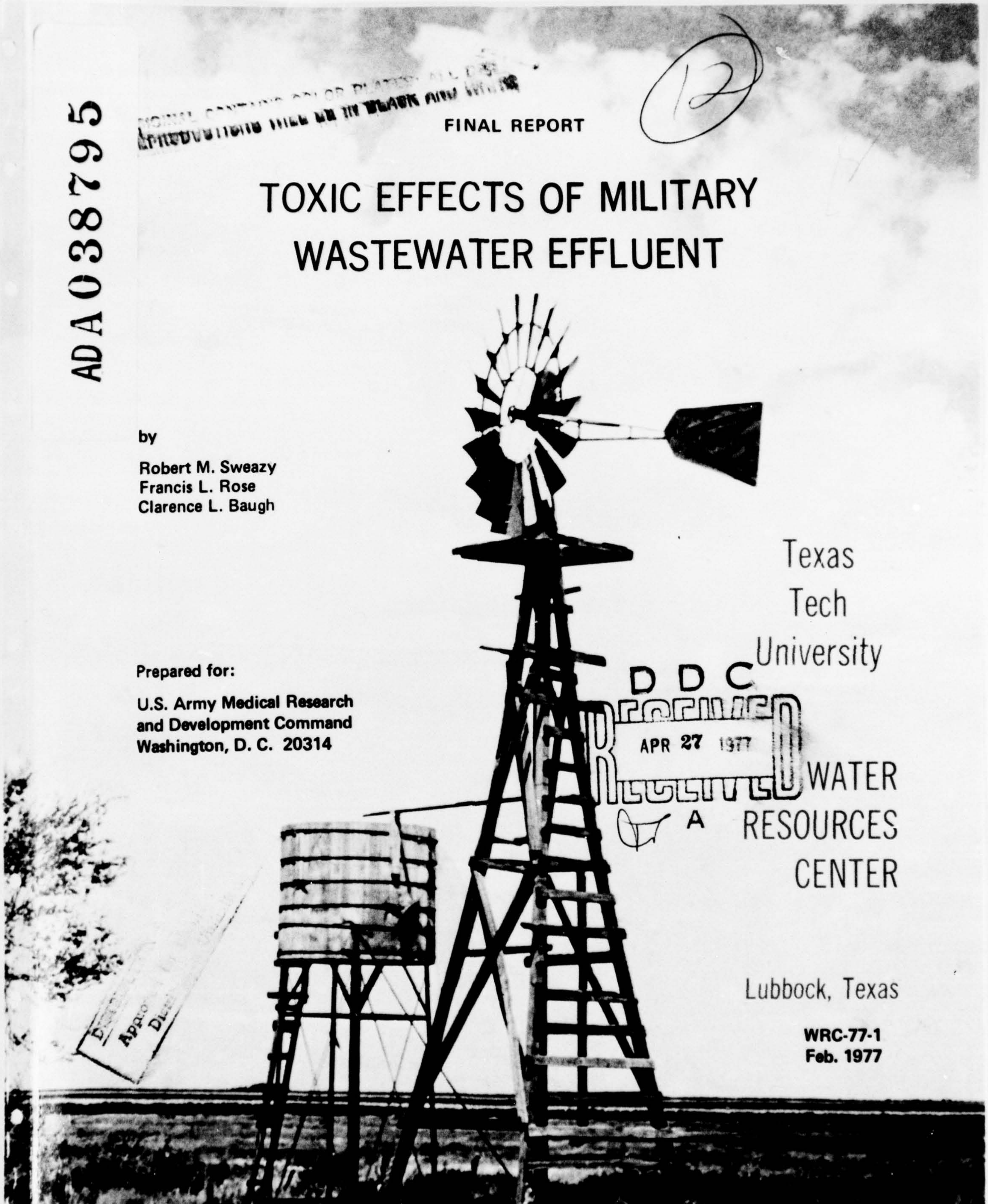

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TOXIC EFFECTS OF MILITARY WASTEWATER EFFLUENT

Final Comprehensive Report

Robert M. Sweazy

Francis L. Rose

Clarence L. Baugh

February, 1977

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Washington, D. C. 20314

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Texas Tech University
Lubbock, Texas 79409

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A population of tiger salamanders, Ambystoma tigrinum, inhabiting a playa lake receiving treated domestic sewage were found to have a high incidence of tumors. Research was aimed at determining the causative agent of the tumors, determining the transmittability of the tumors and to determine if conventional water treatment unit operations, coagulation and sedimentation, filtration and chlorination would reduce the tumor incidence.

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Although no specific tumorigenic agent was positively identified, there was evidence to suggest that polycyclic aromatic hydrocarbons were the tumorigenic agents. Sources of these compounds, diesel oil and asphalt, were known to have been applied to the playa lake. It was also determined that the conventional water treatment unit operations employed had no effect on tumor incidence.

From the enzymatic studies conducted, it was shown that salamanders inhabiting the playa lake were highly induced with the enzyme aryl hydrocarbon hydroxylase. This suggests that an inducing agent such as a polycyclic aromatic hydrocarbon was present in the playa lake. It was further found that the 4,5-epoxide of benzo-α-pyrene was not converted to the dihydrodiol form suggesting that carcinogenicity is related to the inability to the salamander to metabolize this epoxide.

ABSTRACT

A population of tiger salamanders, Abystoma tigrinum, inhabiting a playa lake near Reese Air Force Base, Hurlwood, Texas, which receives treated domestic sewage effluent was found to exhibit a high tumor incidence. The objectives of the study were to determine, if possible, the tumorigenic agent, to determine the transmittability of the tumors, and to investigate means of prohibiting the occurrence of such tumors.

Results indicated that neither heavy metals, pesticides, nor virus were the cause of the tumors. The salamanders were found to have been induced to produce high levels of the enzyme aryl hydrocarbon hydroxylase by the Reese AFB playa water. Animals induced by injection with a polycyclic aromatic hydrocarbon showed levels of enzyme induction comparable with those from the Reese playa. It was further found that the salamanders were incapable of converting the carciogenic epoxide matabolite resulting from the breakdown of the polycyclic aromatic hydrocarbon to harmless intermediates. The data, therefore, indicate a correlation between high aryl hydrocarbon hydroxylase induction, the apparent absence of epoxide hydrase activity, and the presence of an inducing agent (probably a polycyclic aromatic hydrocarbon) and the high rate of cancer in this population. Conventional water treatment unit operations and processes were ineffective in removing the tumorigenic agent. It appears that, because the tumors could not be transmitted to other test organisms, there is no immediate danger to man or other animals who may experience limited, nonconsumptive, exposure to the Reese playa water.

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INTRODUCTION

The southern extension of the Texas High Plains, known as the Llano Estacado, constitutes an area of approximately 35,000 square miles characterized by low relief. It is bounded on three sides by vertical escarpments which approach 1,000 feet in height. The surface is relatively featureless except for numerous depressions called playa lakes. Today, nearly 20,000 playas dot the Llano Estacado.

There are no extant streams or rivers on the Llano Estacado although their remnants are visible and carry water during periods of intense rainfall. In inhabitated areas, playas are often modified to serve as sewage effluent storage and evaporation ponds, as stock watering ponds, as irrigation tailwater pits and as collection and storage ponds for rainfall runoff. These playas, natural and modified, serve as the primary source of standing water for the numerous amphibians inhabiting this semi-arid area.

Although several species of frogs and toads reside temporarily in these playas, the most numerous amphibian inhabitant is the tiger salamander, Ambystoma tigrinum. Typically, the eggs of this salamander are laid in an aquatic environment where they hatch, and the larvae complete the aquatic stage and transform (metamorphose) into a terrestrial form which bears little resemblance to its aquatic progenitor. However, in some instances, transformation is uncommon and the larvae remain as the reproductive form. A life history schematic is shown in Figure 1.

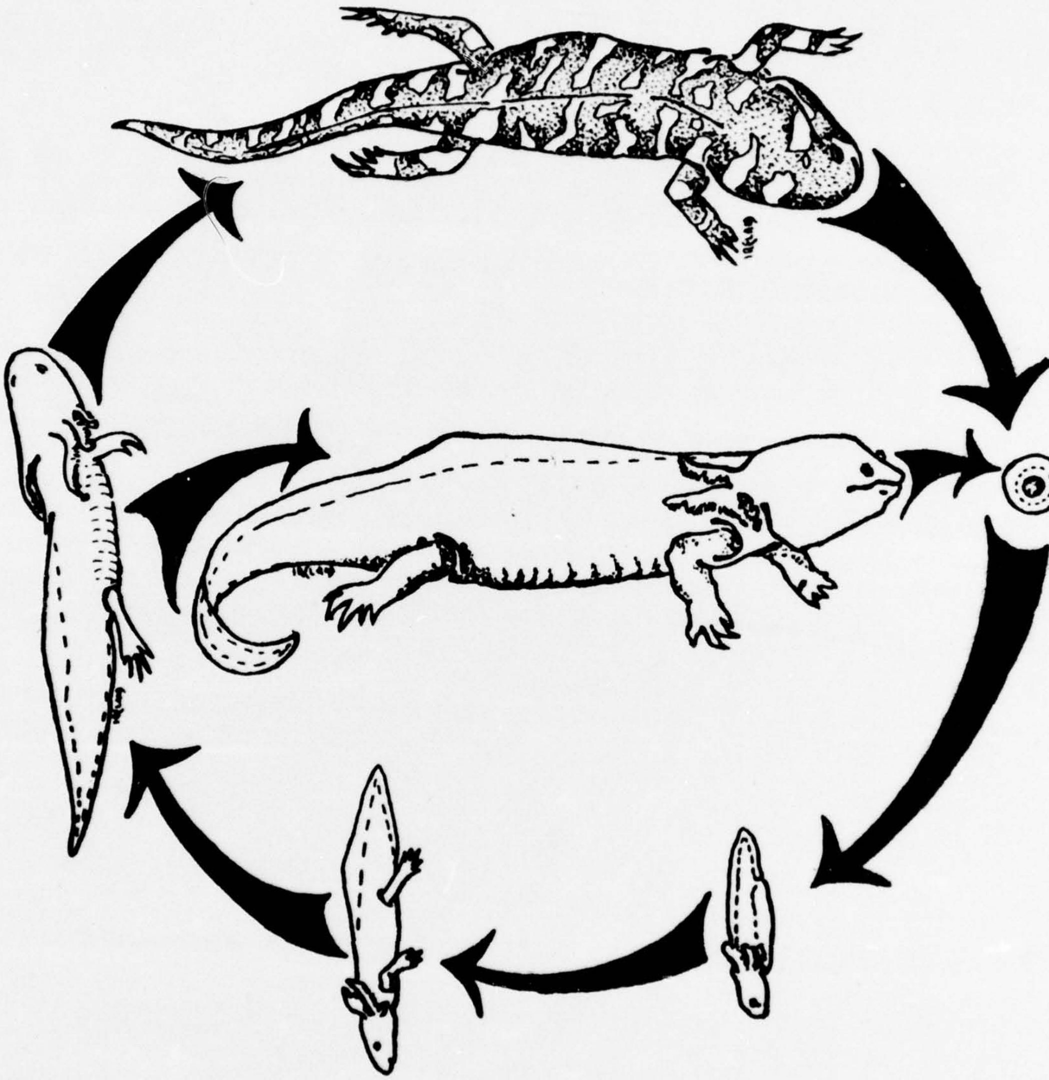

Figure 1. Life history schematic of the tiger salamander, *Ambystoma tigrinum*.

This large, reproductively mature larva is called a neotene and is, because of its very nature and metabolic reticence to transform, found only in bodies of water which are relatively permanent and which have high rates of primary productivity. On the Llano Estacado the ponds fulfilling these requirements are normally playas which receive treated sewage effluent.

Most of the playas in the area do not receive a secondary source of water and are, therefore, intermittent. In these playas, selection favors those tiger salamanders which have the capacity to transform quickly and at small sizes if the occasion necessitates. In fact, this form is extremely difficult to investigate because the very act of seining may trigger transformation.

The tiger salamander on the Llano Estacado has been under investigation since 1966 by the Texas Tech University Department of Biology. Primary interest was directed toward the ecological, behavioral and systematic associations between the different types. To this end, numerous populations from intermittent playa lakes were studied. However, it was not until the spring of 1969 that neotenes inhabiting a playa receiving treated sewage effluent at Reese Air Force Base, (AFB), Hurlwood, Texas, were studied.

The initial studies at Reese AFB were conducted from the spring of 1970 to the spring of 1972. A tumor incidence of five percent was found among the approximate 15,000 salamanders studied. Further examination during February and March of 1972 indicated that tumors were present on approximately thirty percent of the salamander population inhabiting the Reese AFB playa.

At the time of the initial study, concurrent examination of about 15,000 salamanders from playa lakes not associated with sewage treatment discharges failed to reveal a single salamander with a tumor. Thus, an apparent correlation between the tumorous growths and the water in the Reese Playa was indicated. In an attempt to confirm or deny such a correlation, this study was undertaken with the primary objectives being to (1) determine the causative agent of the tumors, (2) determine the transmittability of the tumors and thereby define the potential danger to man and other organisms, and (3) investigate means of prohibiting the occurrence of such tumors.

This project bears special significance to the military for two particular reasons. First, because the military makes extensive use of lagoons for wastewater treatment purposes, the possibility that these waters are health hazards is of extreme importance. Secondly, as more stringent water quality restrictions (approaching elimination of discharge) are placed on military installations, the storage of sewage effluents in natural or artificial lakes or ponds is perhaps the most feasible alternative available to them. Therefore, such practice appears to figure heavily in future sewage disposal methods employed by the military and any potential health threat associated with effluent receiving ponds should be fully researched.

LIFE HISTORY AND BEHAVIOR

Although specific information pertaining to the life history and behavior of *Ambystoma tigrinum* will be reported in following sections, a brief, general discussion of these topics is presented here in an attempt to add clarity to the project's dimension.

As was previously stated, the tiger salamanders inhabiting West Texas are of two basic types, a large morph and a small morph, and have drastically different life histories. However, both types of salamanders, like most amphibians, lay their eggs in the water, usually in the spring. The eggs hatch in about two weeks, but the precise rate of development is temperature dependent. The aquatic larvae are characterized by having large external gills and well-developed tail fins for swimming. The legs are too weak to support the body mass out of water. After about a nine month period of growth, the larvae become sexually mature and normally mate at this time. Later the larvae may undergo transformation or metamorphosis. The gills are lost as is the broad tail fin and the animal is prepared for a terrestrial existence. Transformed animals may return to the water to breed and lay their eggs at the appropriate time. However, most egg laying is done by the sexually mature larvae prior to transformation.

Ambystoma tigrinum which are classified as large morphs transform at snout to vent lengths of about 160 mm. They occur in large permanent lakes usually with an extra input of organic matter such as sewage. They remain as larvae for two years or more and a large percentage lack the ability to transform, remaining as permanent sexually mature larvae throughout their lives. Those that transform do so very slowly and with great physiological stress.

Apparently, selection favors the aquatic mode for these salamanders in the arid to semi-arid southwest. This particular mode of their life cycle is adaptive if the pond is permanent because there is greater than 99 percent mortality at transformation associated with the rigors of the dry terrestrial environment.

The secondary input of organic matter is essential to the maintenance of large morph populations. The deeper, more permanent playa lakes are very turbid with extremely low primary productivity. Thus, they cannot support a constant large population of vertebrates. Females of this morph lay about 6,200 eggs each per year.

The small morphs are associated with non-permanent, usually undisturbed playa basins. They normally transform at about 100 mm snout to vent length and go from egg to transformation within one year or less. Because some of the lakes are intermittent, the larvae must be able to transform completely within a short period of time. This morph is extremely susceptible to any kind of stress and transforms under such conditions as simple handling. They are difficult to work with in the larval stage unless they are collected at a size at which they are unable to transform.

If water is available for long enough periods, the small morphs will reach sexual maturity prior to transformation. When the female returns to the pond she is not dependent on a male because she is capable of storing sperm from the larval mating for over a year. Females of this morph normally lay about 3,000 eggs.

METHODOLOGY RESULTS AND DISCUSSION

To satisfy the project objectives several separate simultaneous studies were undertaken. Rather than separate the studies into conventionally reported components, i.e., procedures, results and discussion, each will be considered independently and in its entirety. Conclusions and recommendations from the combined studies will be reported in subsequent sections.

Tissue Abnormalities on Larvae from the Reese AFB Playa Lake

As has been the case since 1969, sampling of tiger salamander populations from the Reese AFB playa lake continued periodically throughout the study. As stated earlier, the Reese AFB playa receives effluent from the installation's domestic waste treatment facility. Industrial wastes are routed to a separate lagoon. This sewage treatment plant utilizes a modified activated sludge process known as the Hayes Process. Treatment consists of screening, primary sedimentation, first stage contact aeration, intermediate sedimentation, second stage contact aeration, and final sedimentation. Contact aeration is achieved by passing air across asbestos-cement sheets arranged vertically in the tank. The effluent from the final sedimentation tank is chlorinated and flows into two small lagoons. The effluent from the lagoons flows directly into the playa lake.

Once in the playa, water has three avenues of escape: evaporation into the atmosphere, percolation to the underlying aquifer, and irrigation of the surrounding golf courses. A schematic diagram of the process is shown in Figure 2.

Salamanders were generally collected with a 12.2 m x 1.8 m bag seine, and each catch was kept in plastic containers or wire enclosures until subsequent seinings were initiated in order to prevent recaptures. In some cases, animals were toe-clipped for future identification. Every salamander was examined at the collection site for external abnormalities (tumors, lesions or cysts). Those with abnormalities, except in instances where a large number of abnormality duplication was apparent, were either taken to the laboratory for observation, sacrificed and autopsied, used for tissue preparation, or sent live to experts for identification or further study. In instances of obvious abnormality duplication, only a few animals were retained and the remainder were placed back in the playa. A few of the salamanders without abnormalities were retained and used in experiments which will be described later.

All abnormalities were identified by Dr. John Harshbarger, Director, Registry of Tumors in Lower Animals, U. S. National Museum, Washington, D. C. and Dr. Clyde Dawe, Head, Comparative Oncology Section, National Cancer Institute, Bethesda, Maryland. Some live, afflicted larvae were sent to Dr. Harshbarger for tissue preparation. In most cases, however, abnormal tissue was prepared in the laboratory and forwarded. Before tissue was sent for examination, each animal was

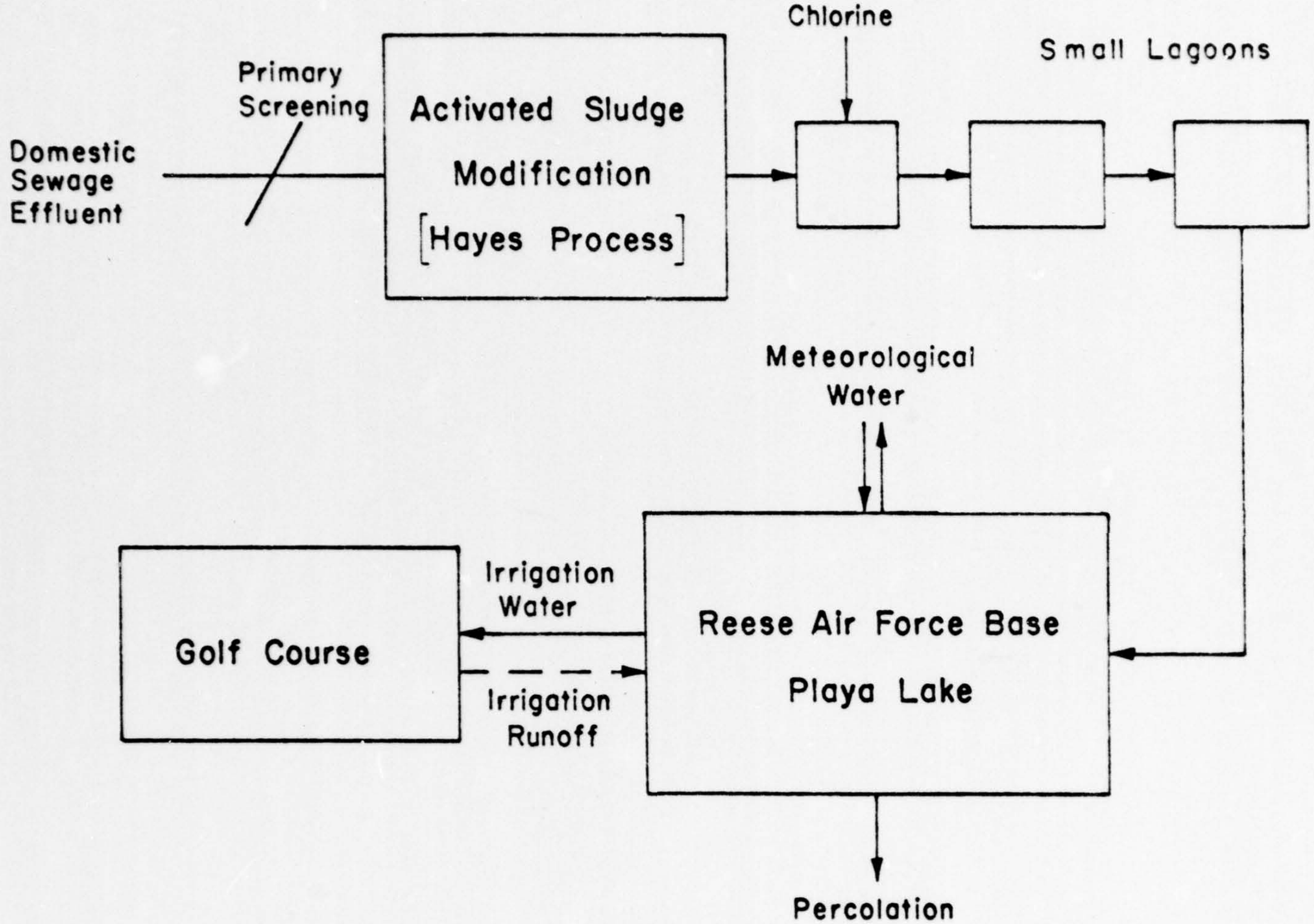

Fig. 2. Flow, treatment, interchange, and disposal of domestic sewage effluent from Reese Air Force Base.

photographed and numbered for identification and the animal's external and internal state determined. The numbers and pictures of the animals and growths were then sent with the tissue to avoid mislabeling.

A total of 23,559 larvae from Reese AFB were examined for external abnormalities from 1970 through 1974. Of these, 2,100 were autopsied for internal growths. Of those salamander populations not associated with sewage effluent, 12,100 larvae were examined externally and 475 were autopsied. In addition, in May 1973 nearly 11,000 larvae were collected at Reese AFB for a mark-recapture study. Although time did not permit a thorough examination of each animal at that time, a cursory examination for bizarre abnormalities was conducted.

Shown in Figure 3 are the percentages of salamanders seined and examined from 1970 through 1974 which were afflicted with tumors, lesions or cysts. To ensure statistical significance to the population, only seinings resulting in catches in excess of 1,000 salamanders were reported in this study. The figures on the graph indicate the total numbers of salamanders collected and examined in each sample.

In 1970, only one of 2,430 larvae examined in one sample had an obvious growth. It was a roundish fibroma associated with the tail. Because this growth was noted on one of the salamanders initially examined, interest was spurred and each salamander was closely examined; the point being that growths were not overlooked, they were not present at that time. In 1971, a drastic increase (approximately 25 percent) in the incidence of abnormalities was noted and many growths in excess of 2 cm in diameter were evident. The percentage of afflictions remained fairly constant until the middle of 1974 when

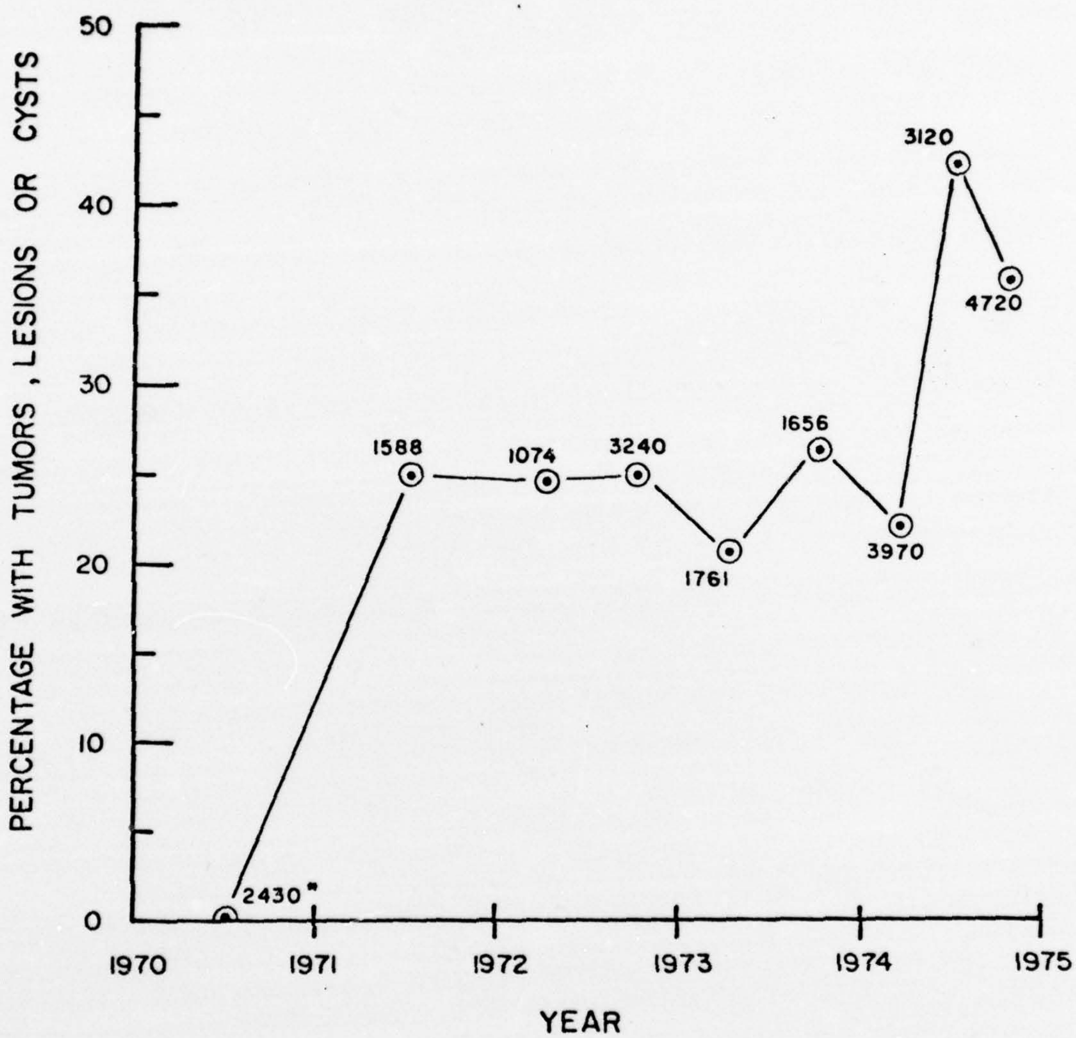

Figure 3. The percentage of salamanders with tumors, lesions, or cysts.

* Number examined

it again increased drastically to over 40 percent.

Eight basic tissue abnormalities were identified by Drs. Harshbarger and Dawe from animals seined at Reese AFB. Included were melanomas, epidermal papillomas (lace tumors), myxofibromas, fibromas, hyperplasias, epidermal inclusion cysts, cephalic cysts, and abdominal acites. In addition, two salamanders were collected with cirrhotic livers and one with a hepatic fibroma. Some of these abnormalities are pictured in Figure 4.

Figures 5-9 indicate the percentages of salamanders with the more common tissues abnormalities. As is shown in Figure 5, the percentage frequency of lace tumors followed the same general trend as did the percentage frequency of all tumors shown in Figure 3. Hyperplasia, Figure 6, remained relatively constant until 1973 when it increased to 4.2 percent. The hyperplasia appeared as a whitish raised area which, in some cases, covered as much as a square centimeter. Histological examinations of hyperplasia and lace tumors revealed that the former gives rise to the latter. The percentage frequencies of melanomas, myxofibromas and epidermal cysts all increased during 1974 (Figures 7,8, and 9) with the latter being the only one to decrease during 1975. Table I shows the percentages and the actual numbers of total and specific tissue abnormalities observed during the period 1971-1974. From this table, it can be determined that the percentage of salamanders with abnormalities ranged from a low of 20.9 percent in the spring of 1973 to a high of 42.9 percent in the summer of 1974.

The cephalic cysts (Fig. 10) were striking and initially it was thought that these fluid filled sacs connected directly to the cranial

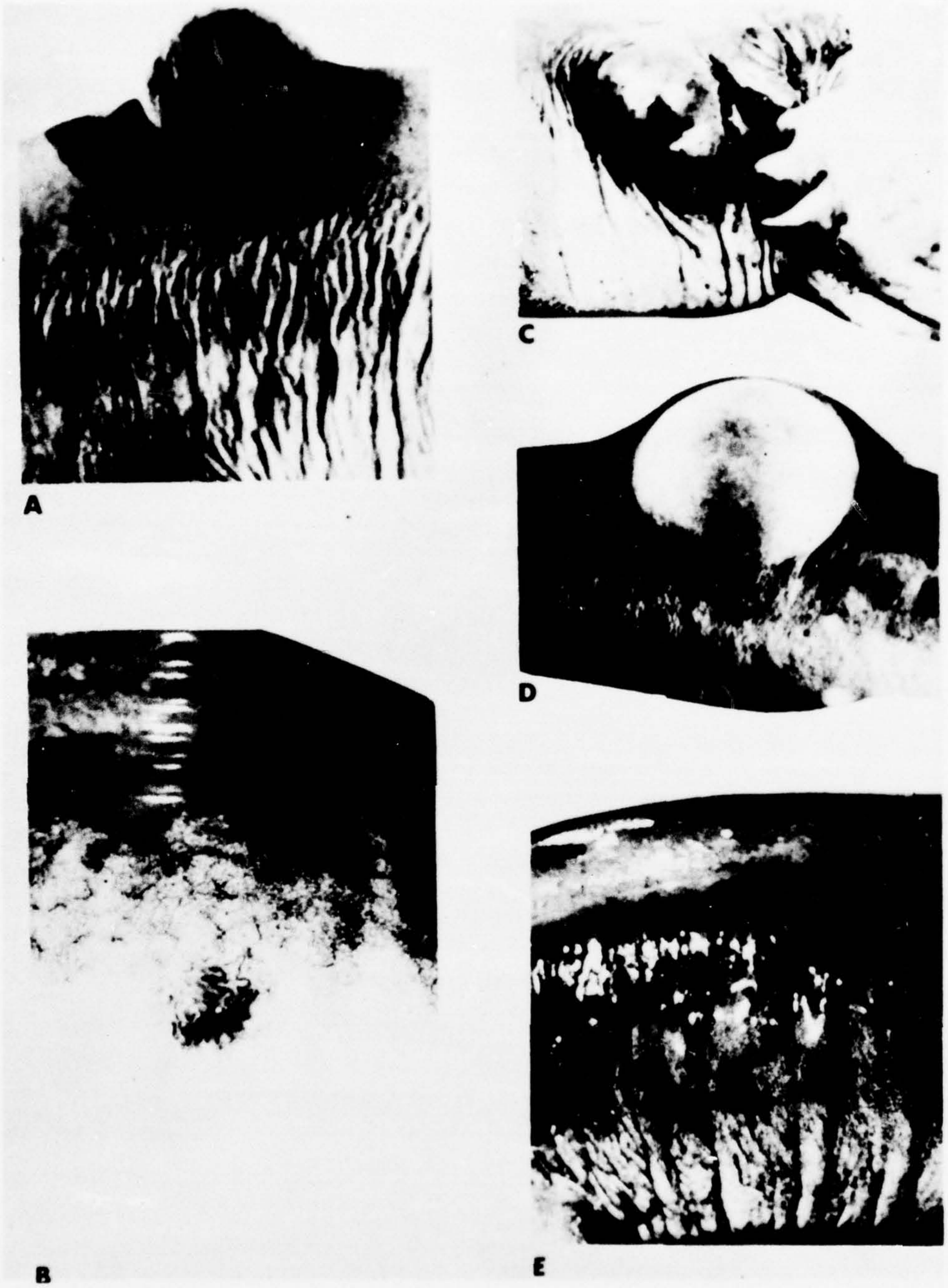

Figure 4. Tissue abnormalities observed on Ambystoma tigrinum seined from the Reese AFB Playa Lake. (A) Melonoma; (B) Epidermal papilloma (lace tumor); (C) Myxofibroma; (D) Fibroma; (E) Epidermal cyst.

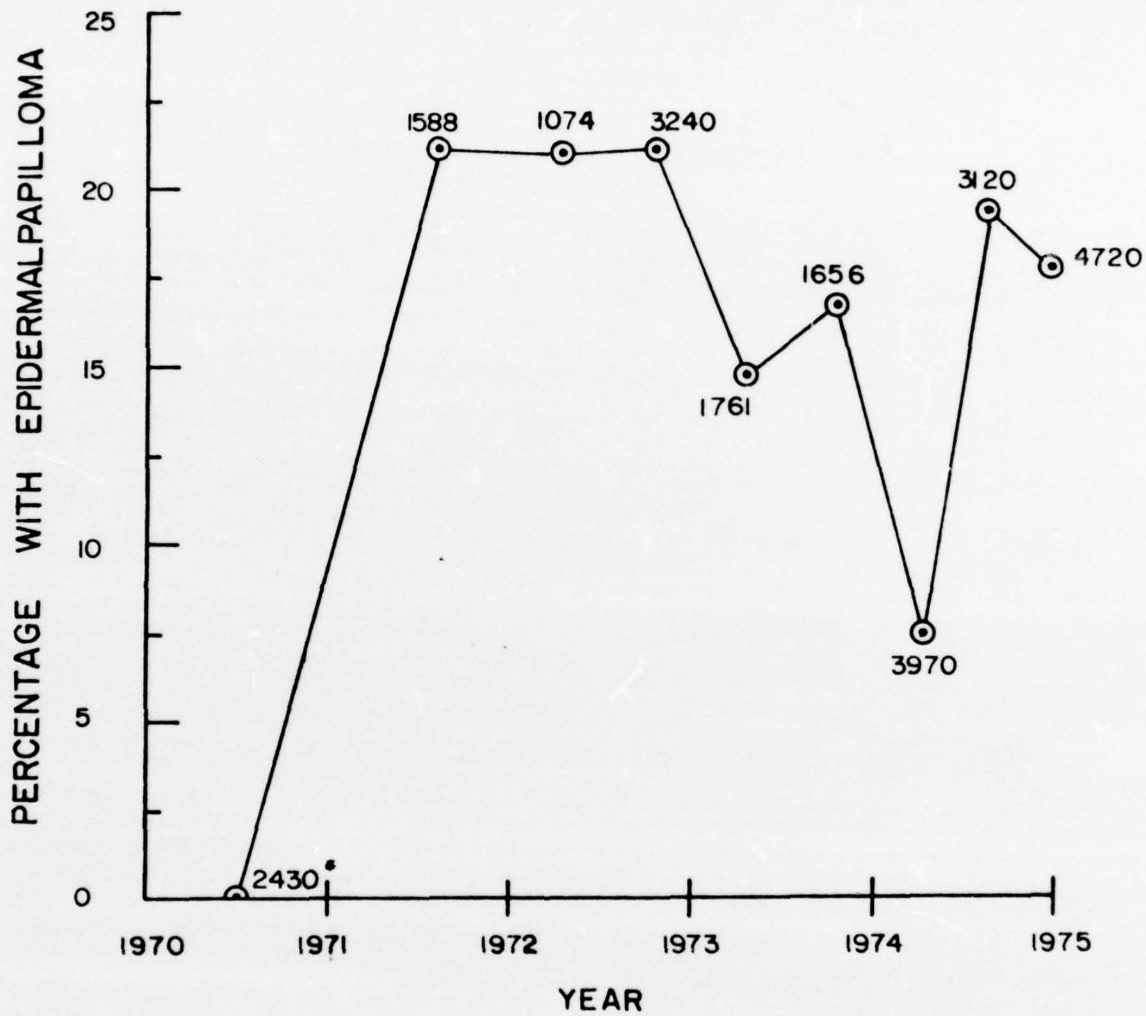

Figure 5. The percentage of salamanders with epidermal papillomas (Lace tumors)

* Number examined

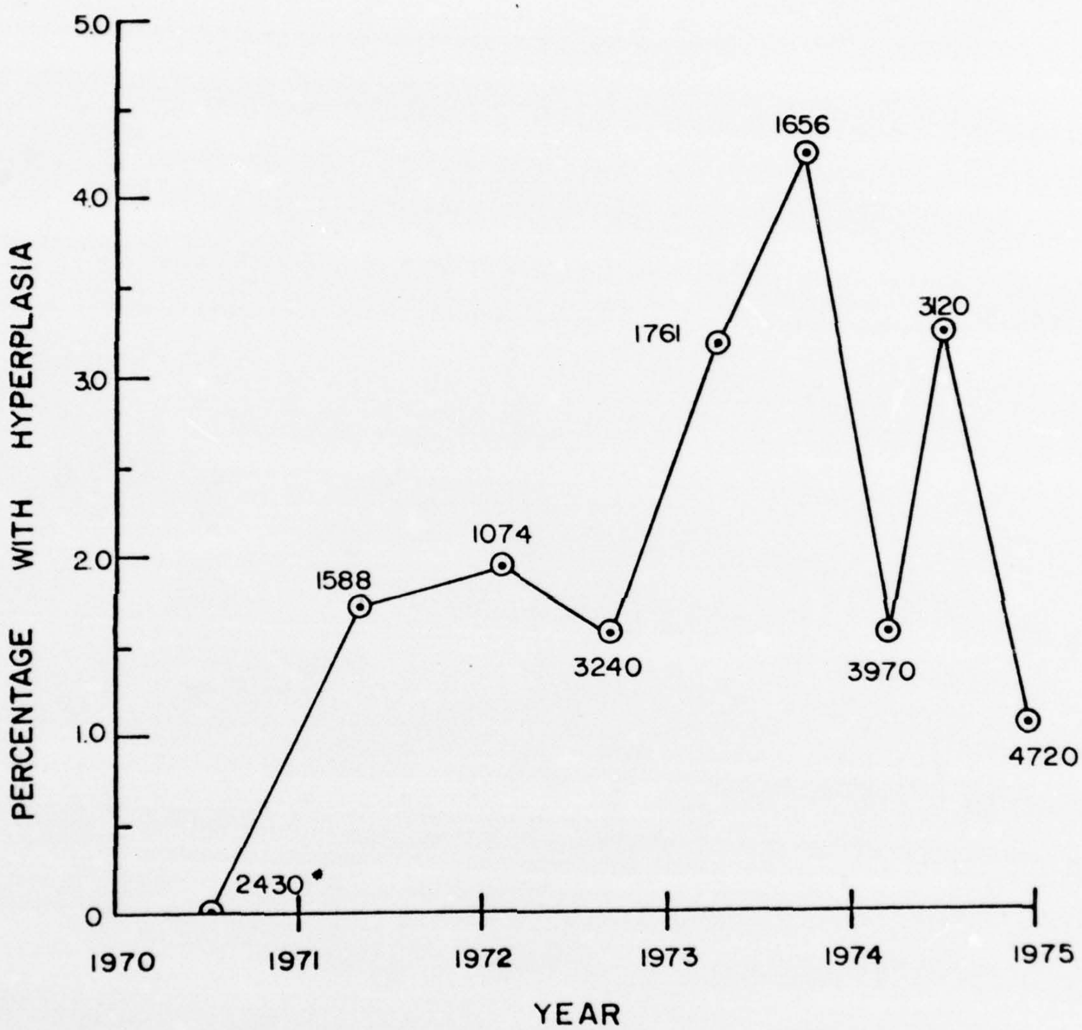

Figure 6. The percentage of salamanders with hyperplasias.

* Number examined

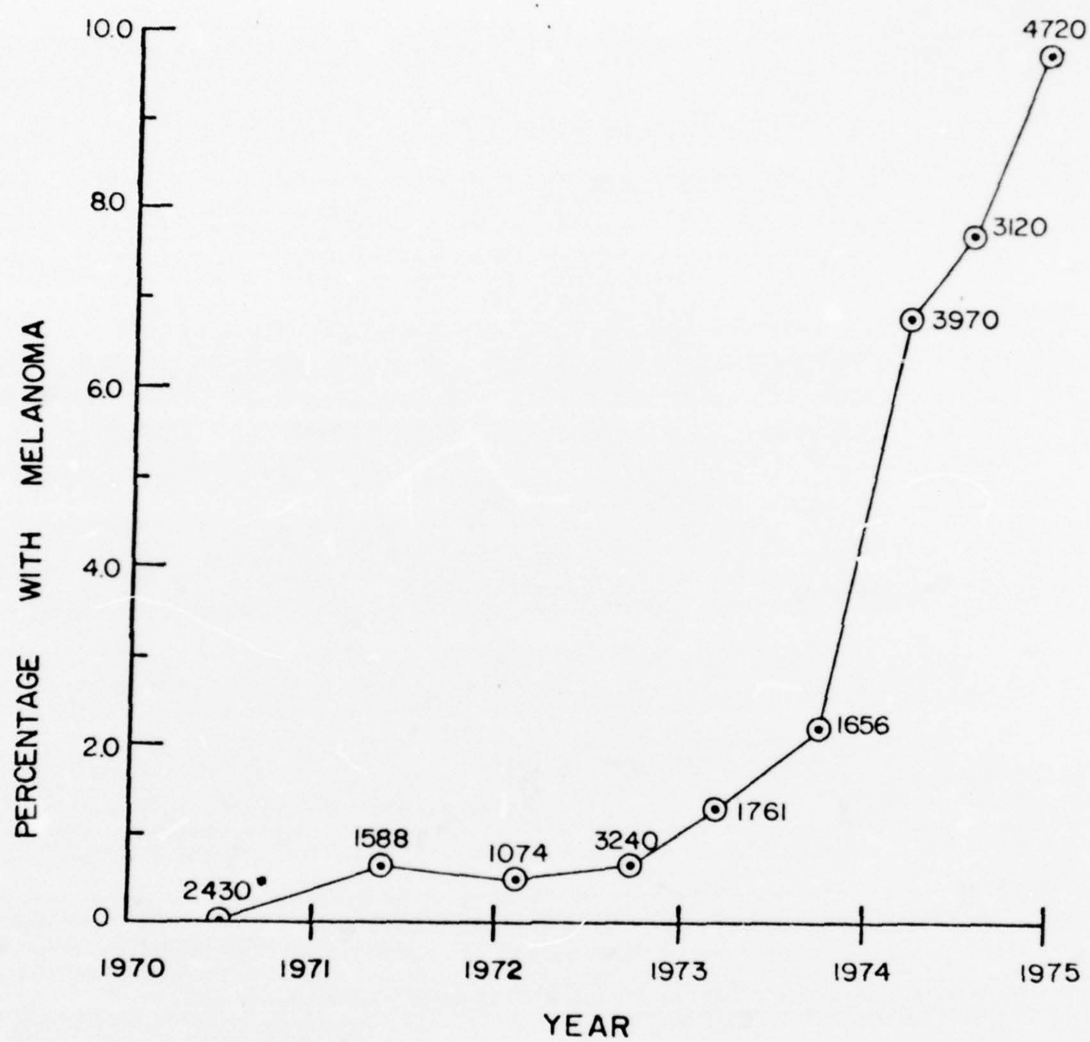

Figure 7. The percentage of salamanders with melanomas.

* Number examined

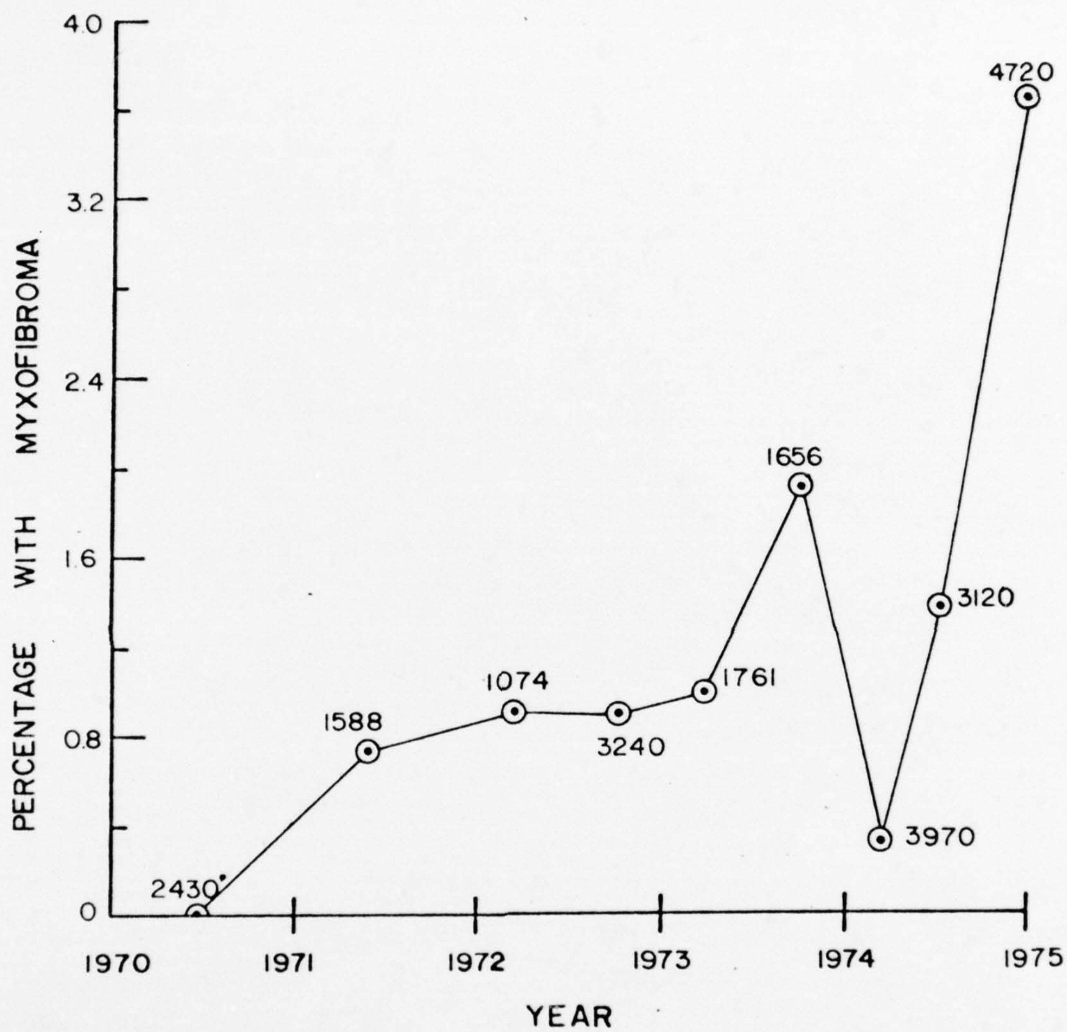

Figure 8. The percentage of salamanders with myxofibromas.

* Number examined

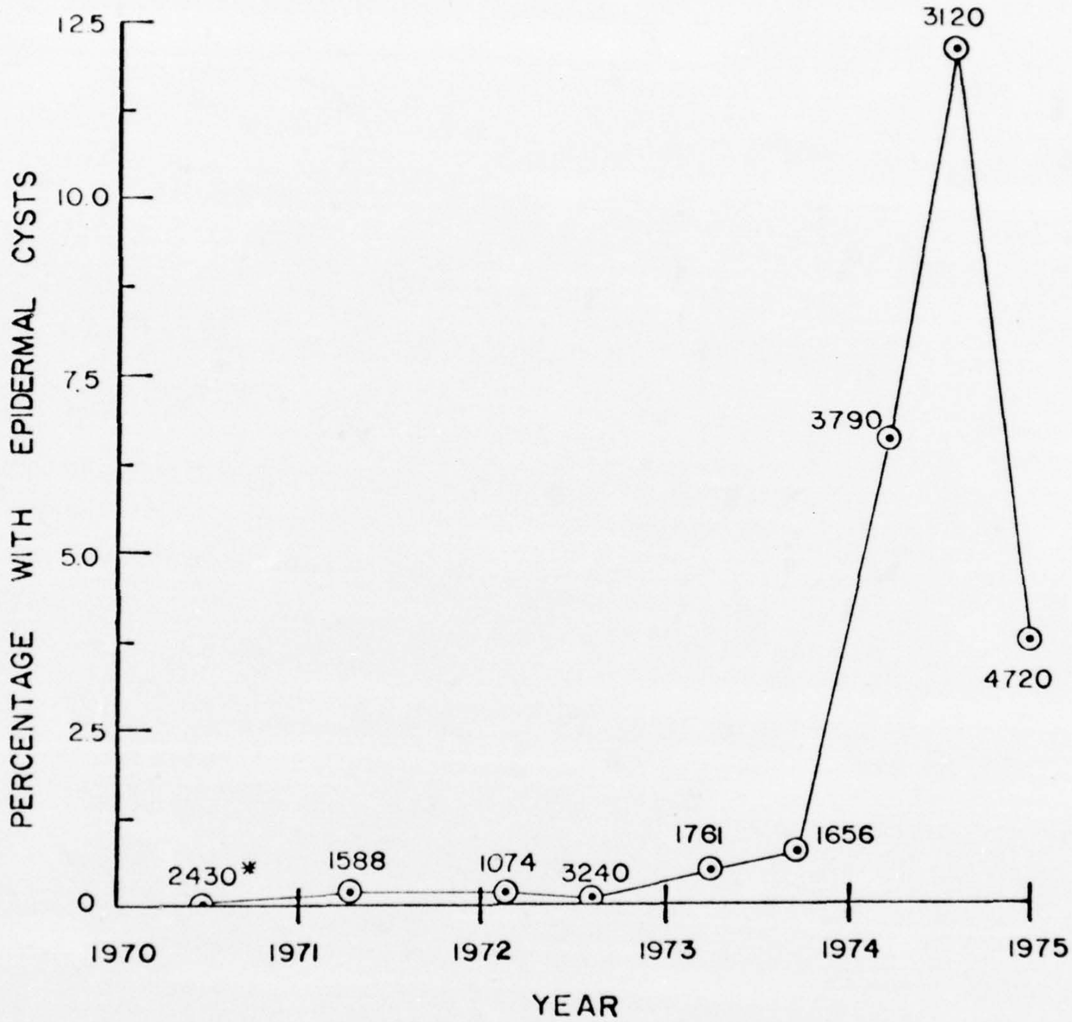

Figure 9. The percentage of salamanders with epidermal cysts.

* Number examined

TABLE I

PERCENTAGE AND NUMBER OF TISSUE ABNORMALITIES

Tissue Abnormality	Epidermal Papilloma (lace)		Hyperplasia		Melonoma		Myxofibromas		Epidermal Cysts		Unspecified		Total With Abnormalities		Total Without Abnormalities	
Date	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Summer 1971	331	20.8	25	1.6	9	0.6	12	0.7	1	0.1	19	1.2	397	25.0	1191	75.0
Spring 1972	220	20.5	19	1.8	5	0.5	10	0.9	1	0.1	13	1.2	268	25.0	806	75.0
Summer 1972	671	20.7	49	1.5	19	0.6	29	0.9	0	0.0	39	1.2	807	24.9	2433	75.1
Spring 1973	261	14.8	55	3.1	21	1.2	18	1.0	3	0.2	10	0.6	368	20.9	1393	79.1
Summer 1973	275	16.6	70	4.2	35	2.1	31	1.9	8	0.5	20	1.2	439	26.5	1217	73.5
Spring 1974	290	7.3	60	1.5	270	6.8	12	0.3	250	6.3	0	0.0	881	22.2	3089	77.8
Summer 1974	586	18.8	100	3.2	240	7.7	40	1.3	371	11.9	0	0.0	1338	42.9	1782	57.1
Fall 1974	812	17.2	47	1.0	458	9.7	170	3.6	170	3.6	38	0.8	1694	35.9	3026	64.1

Figure 10. Epidermal cephalic cyst.

vault and that the fluid was cerebrospinal fluid. However, precise analyses of the fluid were conducted and compared to plasma analyses which indicated the cyst fluid was not of cerebrospinal origin. The sacs were either unilaterally or bilaterally situated. When bilateral, pressure changes genreated in one chamber were not transduced to the other, indicating that they were not connected via the central nervous system. Subsequent dissections and investigations using barium injections followed by X-ray (Fig. 11) showed that there was no connection between the cyst and the central nervous system. In May 1973, nearly 11,000 larvae were examined solely for this condition and it was found in only six (.06 percent) animals. At no time during the study did the percentage frequency of this condition exceed 0.8 percent.

The percentage frequency of abdominal acites was 0.2 percent or less during the study period. This condition was also either unilateral or bilateral and some animals were so swollen with fluid that their limbs could not reach the substrate. The animal pictured in Figure 12 weighed 342 gm before 244 gm of fluid were removed from its abdominal cavity with a syringe.

Tissue Abnormalities on Larvae from Other Sewage and Non-Sewage Playa Lakes

In addition to the Reese AFB playa, 17 other playa lakes were seined. Salamander larvae were found in most of them, but in no case did their density approach that encountered in the Reese AFB playa. Those playas that weren't recipients of sewage effluent tended to be intermittent and oligotrophic and, therefore, did not sustain salamander populations.

Conservatively, over 21,000 larvae from 13 nonsewage playas were collected. Of these, 12,100 were examined externally with careful

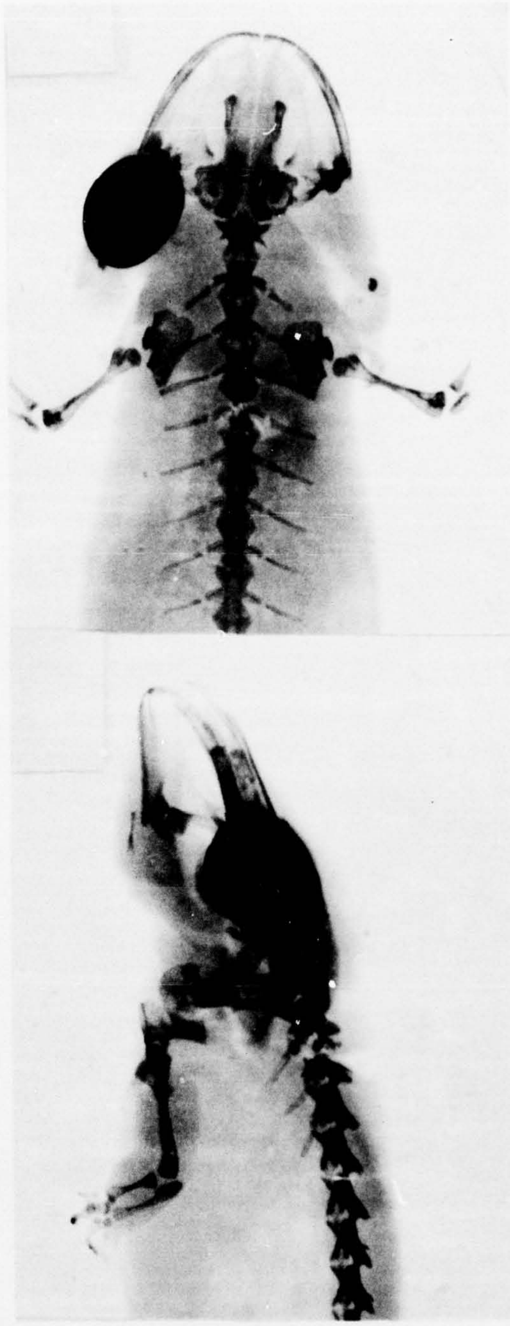

Figure 11. Photograph of X-ray following barium injection of cephalic cyst.

Figure 12. Abdominal acites.

scrutiny and 475 were autopsied. Aside from tiny internal cysts (100 percent occurrence), apparently caused by round worms, the only tissue abnormality noted was abdominal acites which occurred in a similar percentage frequency (0.2 percent) as it did at Reese AFB.

Included in the playas seined were four which received sewage effluent that had undergone primary or secondary treatment. In general, these playas were either very shallow or very deep. The shallow ones tended to overheat and become anaerobic during the summer months. As a result, the salamanders tended to metamorphose prior to the onset of warmer weather. The deep playas were normally smaller in diameter, thereby providing a smaller surface area to volume ratio than the shallow playas. Consequently, winds did not induce mixing, reaeration was minimal and thermal stratification resulted. Layers of unstabilized sludge rapidly accumulated on the bottoms impairing the activities of the bottom dwelling salamander larvae. Also, subsequent degradation of the sludge blanket left the hypolimnetic waters devoid of oxygen forcing the larvae to spend inordinate amounts of time and energy swimming through the vertical water column to obtain oxygen via pulmonary means. For these reasons, very few salamanders inhabitated these playas. Of those seined none had tissue abnormalities except in one case extensive lesions, which proved to be of bacterial and fungal origin, were present on many of the animals.

Apparently, the Reese AFB lagoon offered an ideal environment for the salamanders. The water depth and surface area were such that the water rarely stratified and the wind mixed the water enough to maintain a high dissolved oxygen concentration. When the playa did stratify, the shallowness (about 2 meters) was not an insurmountable distance for them to continually swim for oxygen.

Association of Abnormalities with Life History Stages

From careful observation during the seinings, it was noticed that larvae from Reese AFB rarely developed neoplasms or cysts until they were from 14 to 17 months of age. For example, in one sample, only 4.5% of the larvae approximately 15 months old had abnormalities, whereas 42% of those two years or older had growths. Since those individuals in nonsewage playas experienced larval periods of only about six months, they may not have had ample exposure time to any tumorogenic agent which may have been present in the water.

It was also noted that individuals which had large neoplasms rarely metamorphosed. It appears unlikely that the neoplastic conditions block the physiological and biochemical pathways for metamorphosis, but rather the animals have developed the neoplasia because they have a low propensity to metamorphose. Specifically, the neotenes at Reese AFB lay their eggs in late winter or spring. The young grow quickly and reach a size compatible with metamorphosis around November. A certain segment of the population will metamorphose at this time and others will do so in the spring. Those which do not metamorphose during the first year are destined to become the neotenes. Thus, it appears it is not that the neoplasms block metamorphosis, but rather that larvae which do not metamorphose may be exposed for a sufficient period of time to any agent(s) which may cause tissue abnormalities.

Effects of Reese Air Force Base Playa Water on Other Vertebrates

If length of exposure is important then the transient organisms, such as water fowl, will probably not be harmed. However, those organisms utilizing the water for long periods may be affected. For example, of eight kildeer (Charadrius vociferus) hatched along the shore of the lagoon in 1974, seven were found dead within one month of hatching.

When the eighth fledgling was last seen it was weak and had lesions on its feet. Whether exposure to the water via drinking was the cause of the poor condition of this kildeer and the deaths of the other seven is still not known.

Controlled experiments using catfish, mice and quail were conducted to determine if prolonged exposure to the Reese Air Force Base playa water would induce tissue abnormalities in these animals.

Young channel catfish (Ictalurus punctatus) were purchased from a fish hatchery in Wolforth, Texas, and were placed in fiberglass containers (4' x 4' x 3') located in an outside enclosure. Two groups of 20 animals each were used. A control group was placed in aged tap water and an experimental group was placed in water from Reese Air Force Base. They were fed standard catfish chow.

Because of an unusually cold winter, both groups of catfish became emaciated and eventually died. A second group, obtained in the spring, died from low oxygen tensions during the summer. Later ten young catfish were purchased and maintained in 15-gallon aquaria. Again, there were two groups; five control and five experimental animals. Sewage and tap water were replaced weekly and both were filtered through sand to remove particulate matter. These fish grew well in both tanks for six weeks then began to die. A check with the supplier indicated that they were extremely difficult to raise and such deaths were typical. In view of the sensitivity of the fish and the comments from Dr. Harshbarger that, to his knowledge, no one had reported tumors on this species, bullhead catfish (Ictalurus melas), were obtained from a State Park, 7 miles S. of Post, Texas. These animals were selected because they were young and had not been associated with sewage. They were maintained in the laboratory in two groups of ten such as those described for the channel catfish.

After six months, only six controls and five experimental animals remained. Fish in neither group exhibited external lesions or growths. Neither internal examination of dead animals nor autopsies of living animals revealed any type of abnormality.

White mice were obtained from the general stock at the department of biological sciences, Texas Tech University. In one experiment, paired males and females, were placed in 10 different cages. Five pair had access to Reese Air Force Base water which had been sand filtered. The other five pair were allowed access to sand filtered tap-water. For some unknown reason neither of the pairs produced young. After five months the mice were autopsied. One experimental and one control animal had small fibroids associated with the kidneys. Since white mice often show spontaneous tumorogenesis these results were not startling.

The experiment was repeated using immature mice obtained from the Texas Tech University stock. They were assigned cages as before, and this time reproduction was successful but a definite decrease in reproductive success was noted in those females which had access to Reese's playa water. Because of space problems the progeny were destroyed, but the parents were used for additional experimentation.

The mean number of second generation young from the Reese group was four (range 2-6) and the mean number of young for the controls was eight (range 6-12). Because it was not readily apparent if fewer young were actually produced or whether some young were killed and devoured by the parents, a second experiment with different mice was conducted which confirmed the previous results. That is, those females given Reese water had a lower reproductive rate than those given aged tap water. However, there was no evidence of tumorogenesis in any of the animals.

Chuka quail were purchased locally and placed into two groups of ten each. One group was allowed access to Reese water and the other group was provided tap water. The quail were maintained for approximately nine months and at the end of this time they were autopsied. At the time of autopsy, two experimental and one control had died of unknown causes. A strong peck-order was established by the birds and they fought frequently, which probably contributed to the deaths. No growths were noted in either group of quail; however, the experimental quail were decidedly lighter in weight than the controls. In addition, the amount of visible adipose tissue was far less in the experimental group.

Hematological examination of the quail was conducted, but interpretation was difficult because there was no literature from which to base a comparison. Therefore, we could compare only the experimental to the control group. Comparisons of cell types were facilitated by the Atlas of Avian Hematology (Lucas and Jamroz, 1961).

Hematocrits were determined by collecting blood in a microhematocrit tube and then spinning the sample for five minutes in an International micro-capillary centrifuge. Hemoglobin concentration (gm/100 ml blood) was determined with a Spencer hemoglobinometer. The hemoglobin values were undoubtedly high because of the nucleated erythrocytes found in birds. Blood cells were counted directly from thin smears stained with Wright's stain.

The blood parameters studied revealed only one significant difference between control and the experimental groups. The eosinophil polymorphonuclear cells were greater in number in the experimental group ($\overline{X}$=31) than in the controls ($\overline{X}$=10). This difference was significant at the .05 level. Results of these experiments are shown in Table II.

While data obtained from the mice and quail experimentation do not indicate that the Reese water is tumorogenic, they do imply, however, that the species used in this study were affected by the water. The reduction in reproductive potential of the mice coupled with the obvious weight differences and high eosinophil counts of the quail indicate metabolic dishormony. Since high eosinophil counts are often associated with chemical intrusion into organisms, one might logically suspect the water quality to which the experimental animals were exposed.

Inoculations

These experiments were conducted on locally captured grass frogs (Rana pipiens), salamanders from Reese AFB and white mice. Myxofibroma and Melanoma tumor tissue were minced in a phosphate buffer solution (1:1 ratio) and 0.01 ml of the mixture was injected subcutaneously into ten mice; five with each preparation. Ten frogs and ten salamanders were likewise injected with 0.1 ml of the mixtures. The frogs were injected in the dorsal lymph sac and the salamanders were injected subcutaneously on the tail just posterior to the left rear limb.

Only one melanoma on one salamander initiated what might be termed an induction. The frogs showed no reactions except a slight inflamation in the area of the injections. The mice reacted violently and tissue rejection was intense.

TABLE II

QUAIL BLOOD PARAMETER STUDIES

	Control (Average)	Experimental (Average)	Significance @ 5% Level
Hemoglobin (gm/100 ml-blood	13.6	14.2	N.S.
Hematocrit	52	52	N.S.
Cell Type			
Erythrocytes	11,489	11,262	N.S.
Lymphocytes	115	120	N.S.
Monocytes	110	117	N.S.
Thrombocytes	39	41	N.S.
Heterophilis	38	40	N.S.
Eosinophiles	10	31	$p < 0.05$
Basophiles	21	19	N.S.

Bacteriological and Virological Studies

The major emphasis of this portion of the study was to determine if any transmissable tumorigenic agents were present in the Reese Playa water or in the salamanders.

The primary difficulty encountered in the experimentation was that a tissue culture method utilizing salamander cells had not been previously described. Therefore, a major effort was required to develope a method of culturing salamander cells in vitro. In addition to the problem encountered with cell culture, the rate of bacterial contamination of adult tissue is usually extremely high compared to embryonic tissue. Inspite of adding double strength antibiotics to our cell cultures, the contaminate ratio remained high throughout the entire study.

Many different variations of tissue culture growth media, temperature of incubation, growth flasks, methods of preparing the cells, and amount of supplement serum were tested throughout the course of the study.

The method which gave the best results on the salamander tissue culture is as follows:

A salamander was killed by heat shock (several rapid extreme changes in temperature) and the torso pinned to a board and rinsed with 70% ethanol. The kidneys were removed aseptically and placed in a sterile Petri dish. The kidneys were minced into fragments (1-2 mm) with sterile surgical scissors and transferred to a trypsininzing flask containing 25 ml of 0.25% trypsin (Difco) in normal saline. The minced tissue suspension was stirred magnetically for 30 minutes at 5°C. The suspension was then centrifuged and the trypsin solution decanted from the cell pellet. The cells and small fragments were washed once with normal saline, centrifuged, and suspended in Amphibian Tissue Culture Medium containing 20% calf serum and double strength antibiotics (penicillin and streptomycin). The cell suspension was then transferred into four glass perscription bottles (8 oz) containing 15 ml of growth medium and incubated at 26° C. A confluent monolayer of cells was attained in 5-7 days. These cells were transferred to new bottles or tubes after being trypsinized or being scraped off the glass surface with a rubber policeman. A new monolayer usually would be formed in 7 days if 3 new bottles were planted with the cells obtained from one bottle.

The critical points in the growth conditions appeared to be a glass substrate (instead of plastic), 20% calf serum instead of 5-10%, and the extra wash of the cells in saline. Such treatment seemed to remove toxic products of the kidneys.

Following the establishment of a suitable growth procedure, an endogenous viral survey with salamander tissue culture cells was conducted. Primary salamander kidney cells were planted into 8 oz glass perscription bottles and incubated for periods up to 2 months without transfer to determine if any virus in the tissue would express itself over a long time period. The growth medium was changed at intervals when the pH of the medium became too acidic.

The cells finally stripped off the glass surface as the cultures aged but a viral type of cytopathic effect (CPE) never developed during the incubation period.

Other cultures of this type were transferred at 7-10 day intervals for 10 passages (1-2 splits) without any apparent viral activity. After 10 passages, the cell cultures no longer attached to the glass surface and were discarded. No apparent viral activity was noted throughout the culture period.

A survey of tumor tissue for viral agents using primary salamander kidney cells in culture was next performed. Epithelial tumor tissue was removed from salamanders sacrificed for Tissue Culture studies and frozen at -20° C until the cell culture system was developed. Two of these tumors were later thawed and finely minced with sterile surgical scissors into 1-2 mm fragments. The fragments and the tissue extract were suspended in 5 ml of tissue culture medium, passed through a 0.2 μ filter and 1.0 ml was inoculated into each of 4 prescription bottle cultures of primary salamander kidney cells. The cells were incubated at 26° C and observed at weekly intervals until the cultures finally stripped off the glass (4-6 weeks). During this period of culture no evidence of viral-like cytopathic effect (CPE) was noted. In addition, no evidence of cell transformation or change in cell morphology, increased growth rate, colonies or foci of cells with different characteristics was found.

The effluent from the Reese Air Force Base sewage treatment plant was then screened using salamander kidney cell cultures. Prescription bottle cultures of primary salamander kidney cells were prepared as previously described. The cells were used as they reached confluency.

The effluent was filtered with 0.2 μ millipore filters and 0.1 ml of the filtrate was added to the culture medium (25 ml) and incubated at 25° C. At 24 hours, a toxic effect was noted with the cells becoming rounded and detached from the glass surface. One ml of this culture fluid was added to each of 3 new confluent kidney cell cultures and incubated at 26° C. No toxic effect was observed at 24 hours indicating that the toxicity noted previously was probably due to chemical toxicity and not to a filterable infectious agent although some viruses may have passed the filter. The cultures were observed microscopically until they stripped off the glass surface. No evidence of later toxicity or cell transformation by the toxic chemical(s) was found.

The effluent was then subjected to virus screening with human tissue culture cells. HeLa cells, a human cell line established from a cervical carcinoma, were planted in plastic Falcon flasks containing 25 ml of Eagle Minimum Essential Medium supplemented with 10% calf serum and penicillin and streptomycin.

One tenth ml of the filtered sewage effluent was added to three confluent cell cultures and the culture incubated at 35° C. A toxic effect was noted in all of the cultures at 48 hours but the causative agent did not transfer to another culture. The toxicity was not as marked as that found in the salamander tissue culture. The toxic agent or material caused the cells to become rounded and granular with some cells becoming detached. It did not appear to be viral in nature since no definite foci of infection were found and, as mentioned previously, transfer of the culture medium to a new culture did not result in toxicity. Long term culture of other HeLa cells in the medium from the toxic culture did not result in expression of a virus.

The bacteriological condition of the water was determined at three sites; from the plant effluent, from the small holding lagoons and from the Reese Playa. Samples from these sources were tested for total viable count and also for a coliform count. A sample of the effluent was placed in selenite enrichment broth for 12 hours. The culture was then streaked onto Salmonella-Shigella agar plates. The cell counts were as follows (organisms/ml):

	Effluent	Holding Pond	Reese Playa Lake
Coliform	2.5×10^4	1.2×10^4	4.8×10^3
Total count	2.6×10^5	2.4×10^5	1.4×10^4

No Salmonella or Shigella were recovered from the Selinite enrichment.

Effect of Diethylnitrosoamine on Salamander Kidney Cell Cultures

Because of the possibility of nitrosoamines being the tumorogenic agent existed, their effects on salamander kidney cells were investigated.

Salamander kidney cells were planted into prescription bottles until a monolayer of cells had formed. The culture medium was replaced with fresh medium containing 0.01% diphenylnitrosoamine. The cultures were fed with medium containing the test compound weekly.

Some cells showed a limited toxicity by forming large clumps, but the majority of the cells appeared normal. No evidence of cellular transformation such as a change in morphology or increase in growth rate was noted after prolonged culturing.

Water Quality Investigations

The purpose of this experimental work was to investigate means of prohibiting the occurrence of tumorous growths on the salamanders by treating the water with water treatment unit operations and processes; namely filtration, flocculation and chlorination. The site selected to conduct the study was the northwestern corner of the Reese Playa Lake as shown in Figure 13. The vessels used to culture the salamanders, and the treatment operations were arrnaged as shown in Figure 14. The animals were introduced into vessels which had previously been filled with experimentally treated Reese playa water, or in the case of the control, with untreated Reese playa water. Water was treated and added to the culture vessels three times a week to replace water lost by evaporation. The salamanders inhabiting the vessels were seined and examined for external growths about every two months. More frequent observation, which involved handling, probably would have encouraged metamorphisis.

The vessels used to culture the salamanders were circular galvanized sheet metal stock tanks nine feet in diameter and two feet high. The tanks were extended one foot vertically by riveting and soldering sheet metal to

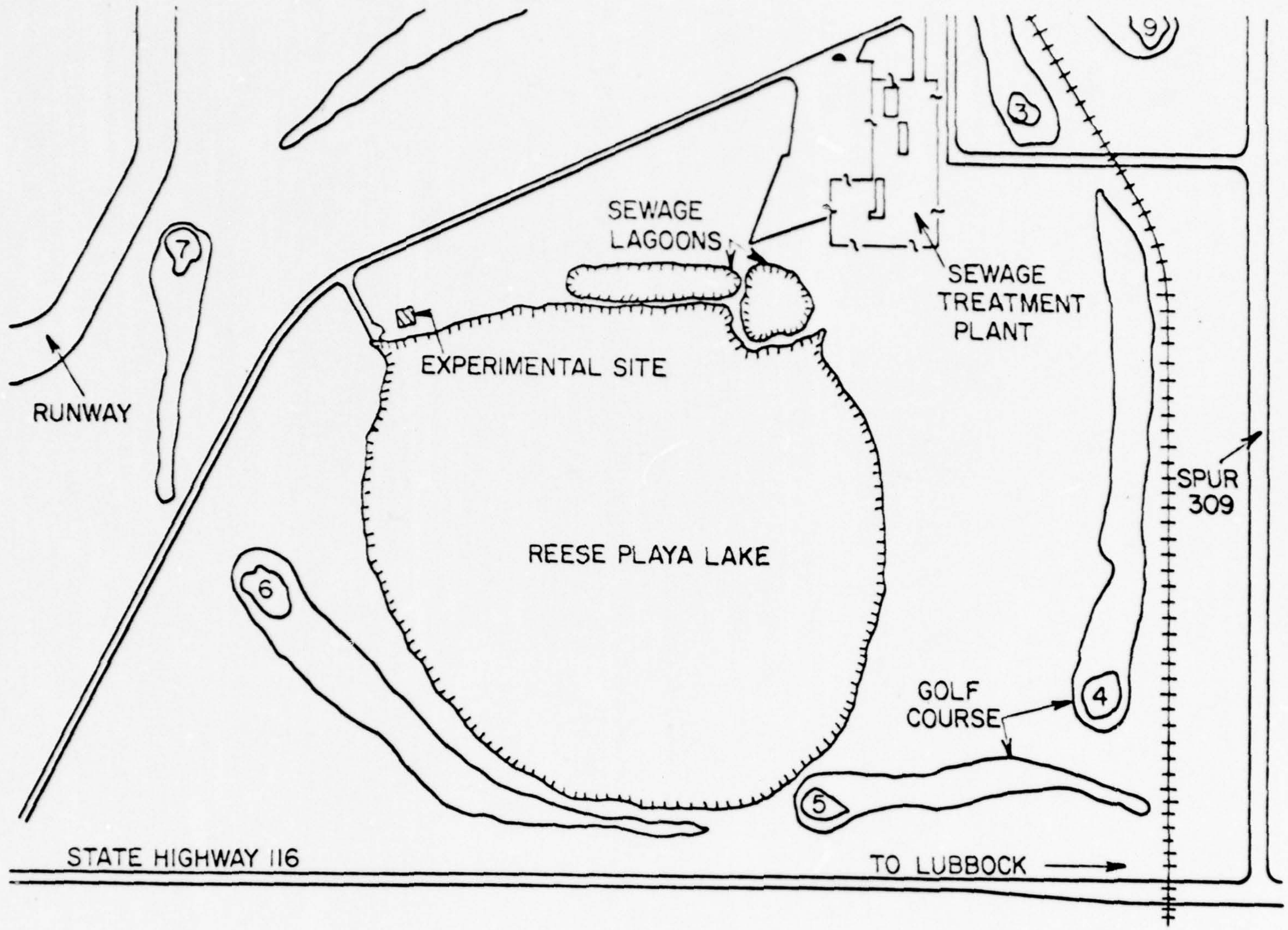

Fig. 13. Location of experimental site witnin Reese Air Force Base.

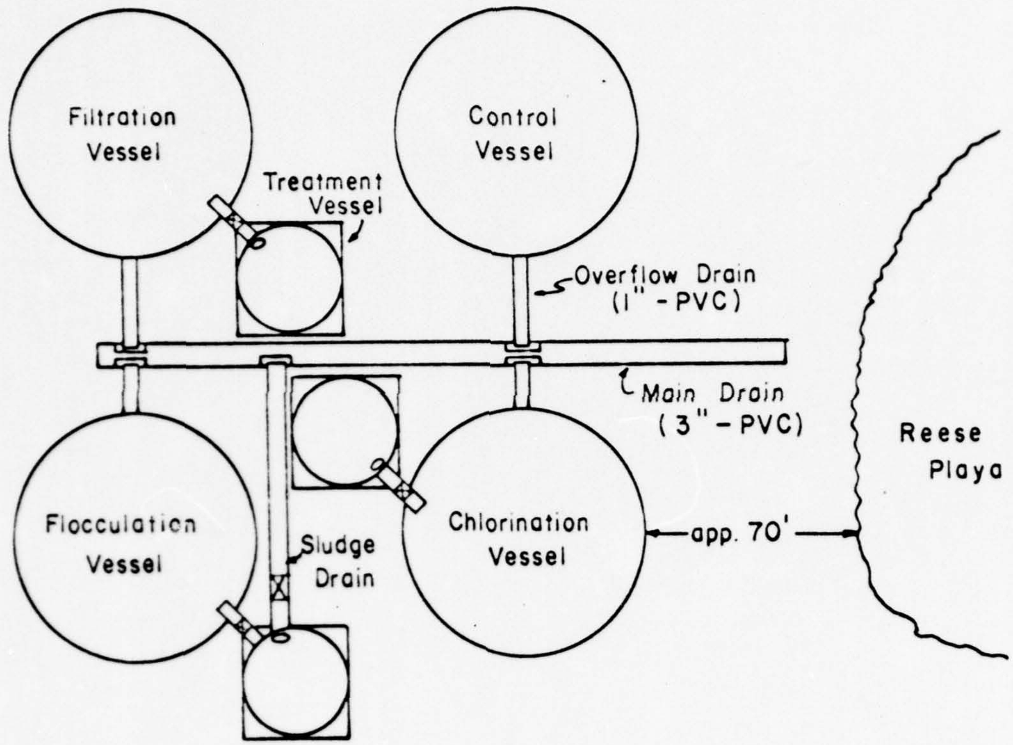

Fig. 14. Diagramatic sketch of experimental treatment facility.

the top edge. With the extension attached, each tank had a volume of 1430 gallons. The inner surfaces of the vessels were painted with zinc chromate primer (Technical Coatings Inc., P501 Zinc Chromate Red Oxide Primer) to prepare the metal for an epoxy base paint (Technical Coatings, Inc., Tec-Tile-Epoxy, 50:50 mixture: E 4000 White and E 0535 catalyst). The culture vessels were placed in a site adjacent to the Reese playa which was excavated to a depth of two and one-half feet. Dirt was backfilled against the tanks so that three inches of the side of the tank were left exposed. Overflow drains were made by cutting a hole in the side of each tank one inch below the top. One-inch-diameter polyvinyl chloride (PVC) pipe was fabricated to connect the overflow drain from each tank to a three-inch PVC pipe main drain. Hail screen (3 inch high, 1/4 inch mesh) was attached to the lip of the culture vessels to prevent salamanders from escaping and small animals from falling and jumping into the tanks.

Floating water heaters (Pyroil Heatmaster Stock Tank Water Warmer, 1000 watt, Model ST 1000 Master Speciality Company) were used to prevent the culture vessels from completely freezing over. A transformer and circuit breaker were installed by Reese personnel to provide the electricity.

Fifty-five gallon drums were adapted for use as treatment or reaction vessels. One end of each of the barrels was cut off and the inside surface of each of the barrels was prepared using the same technique applied to the culture vessels. Strap iron reinforcement (1/8 inch thick by 2 inches wide and 5 inches long) was welded to the side of the barrels used for flocculation and chlorination to facilitate mounting of a variable speed electric mixer. Figure 15 shows a sketch of the reaction vessels.

The flocculation treatment vessel was fitted with a drain (2-inch PVC pipe) on the bottom to remove the sludge after treatment. The outlet to

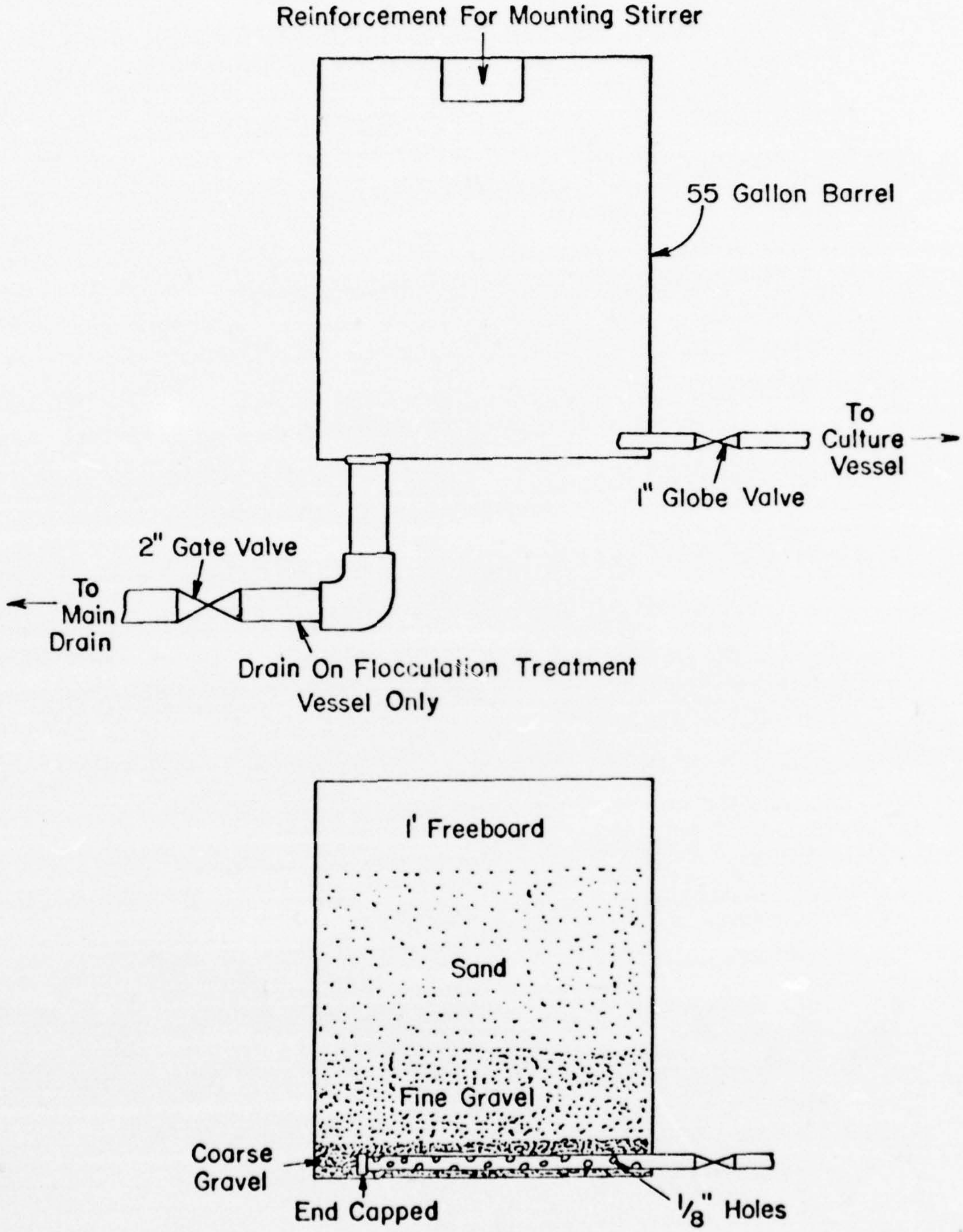

Fig. 15. Cutaway sideview of treatment vessels (a.) chlorination, flocculation (b.) filtration.

the tank was planned to be a drain along the lower side wall. Originally a float arrangement attached to this drain by plastic tubing was used to decant the treated water. However, the plastic tubing stirred up settled floc particles. A simple solution to this problem was siphoning off the treated water. With the siphon technique, water was removed to within three inches of the settled floc.

The chlorination treatment vessel was essentially the same but without the two inch drain on the bottom. A one inch drain was fitted to the filtration treatment vessel and was connected to the underdrain on the inside. The underdrain was fabricated from one-inch PVC pipe by plugging the end and drilling randomly spaced 1/8 inch holes in the side wall.

Once preliminary fabrication of the treatment vessels was completed, the vessels were placed on concrete blocks adjacent to the culture vessels to make use of gravity flow. With the flocculation treatment tank in place, the two-inch drain pipe was connected to the main drain with a gate valve in the line. Coarse gravel was placed in the bottom of the filtration tank until it just covered the underdrain (about three inches). Then a five-inch layer of fine gravel was shoveled into the tank. Two feet of sand were applied to complete the filter bed.

Following construction of the vessels, dosages for the aluminum sulfate coagulant and chlorine disinfectant were determined. The dosage of calcium hypochlorite was calculated by constructing a chlorine demand curve following the procedure outlined in <u>Standard Methods for the Examination of Water and Wastewater</u> (1). A slight alteration in the procedure outlined was necessary due to the interference of turbidity. Standard chlorine solution was added to a sample of Reese water. The system was allowed to react 28 minutes. The chlorinated sample was filtered (Whatman No. 4 filter paper) to remove the suspended solids causing turbidity. The clear filtrate was analyzed for residual chlorine by the orthotolidine arsenite (OTA) method after exactly 30 minutes contact time. The dosage of calcium hypochlorite (0.000852 lbm/gal) needed to give 6 mg/l free chlorine after 30 minutes was determined from the chlorine demand curve previously constructed.

The standard Jar Test was employed to find the optimum dosage of coagulant. Various amounts of alum solution were added to 1000 ml samples arranged on a stirring machine. The samples were rapidly mixed (80 RPM) for one minute and then slowly mixed (30 RPM) for thirty minutes. Size of floc, filtrate turbidity and rapidity of settling were the criteria used to decide on the optimum dosage of alum (0.00286 lbm/gal).

Water was pumped from the playa into the treatment tanks. The flocculation treatment method consisted of adding a 48 percent by weight aqueous solution of alum to 45 gallons of water in the reaction vessels. The alum was added while rapidly stirring the water to disperse the coagulant uniformly. After about one minute of rapid stirring, the mixture was gently agitated for thirty minutes to allow the floc to form. The water thus flocculated was allowed to settle until the next treatment period (2-3 days). Once settled, the treated water was siphoned off the top into the culture tanks. The floc that had settled to the bottom of the treatment tank was drawn off through the drain.

Water was chlorinated in 45 gallon aliquots by treatment with calcium hypochlorite (Wel Chlor Granules, Cotey Chemical Corporation, Lubbock, Texas, 49 percent by weight available chlorine). The water was stirred for twenty minutes to insure uniform dispersion of the chlorine throughout the water. Free chlorine was dissipated by leaving the water in the tank exposed to the atmosphere until the next treatment period.

Filtration only required that water be introduced to the top of the filter tank. More water was added to the top of the filter as the level went down. A head of approximately one foot was maintained in order to obtain a reasonable flow rate through the filter.

After the culture vessels had been filled with treated water and the control tank filled with Reese playa water, salamanders were seined from the Reese playa and thirty were placed in each vessel. Animals were selected that showed no evidence of tumors. The salamanders chosen were approximately a year old at the beginning of the study. One year old salamanders inhabiting the Reese playa exhibit a low incidence of tumors, shortening an otherwise lengthy screening operation, and are less apt to transform from aquatic larvae to amphibous adults.

Upon initiation of the project, several analytical tests were made on Reese playa water including determination of manganese, iron, Kjeldahl nitrogen, orthophosphate, nitrate, nitrite, and dissolved oxygen concentration; turbidity; alkalinity (phenopthalein and total); hardness (calcium and total); chemical oxygen demand; total residue; fixed residue; total suspended solids; and volatile suspended solids. These preliminary analyses were used as a basis for the selection of analytical methods to be employed throughout the study. After reviewing the results, the water quality analyses were limited to orthophosphate, (stannous chloride method), nitrogen as nitrate (ultraviolet spectrophotometric method), chemical oxygen demand (dichromate reflux method), dissolved oxygen (membrane electrode method), total residue, volatile residue, total suspended solids, volatile suspended solids, and pH.

The dissolved oxygen measurements were originally taken using the

Winkler titration method. Interference of particulate matter necessitated adopting the membrane electrode method. A Beckman Fieldlab Oxygen Analyzer (Model No. 100801) was used to take the dissolved oxygen readings which were taken in the field at the surface of each of the culture vessels. A Beckman Century SS pH Meter (model 76006) was employed to obtain pH values.

The water samples analyzed for nitrate and phosphate were filtered through a 0.45 micron millipore filter which had been soaked 48 hours in deionized water to remove any phosphate present in the filter. The tests mentioned previously were performed using the procedures outlined in the 13th Edition of Standard Methods for the Examination of Water and Wastewater (1).

Samples were taken for analysis from each of the culture vessels, from the treatment tanks effluent and from the Reese playa. The sample was taken from the flocculation tank after the floc had settled to be sure floc particles were not present in the sample. This represented water that was treated during the previous treatment period. The sample to be analyzed from the chlorination treatment vessel was taken thirty minutes after the addition of the calcium hypochlorite which corresponds to the typical detention time for chlorination in waste treatment practice.

The results of the initial chemical analysis performed on the Reese playa water are shown in Table III and the range and average values of the subsequent monthly analyses made during the course of the study on the Reese playa water and the various treatment, culture and control vessels are shown in Table IV.

There was significant monthly and seasonal variation in the quality of the Reese water, and, therefore, in the quality of the treated water and that in the culture vessels. However the various unit operations

TABLE III

INITIAL ANALYSIS OF REESE PLAYA WATER

Parameter	Concentration in mg/l except as noted
Manganese	< 1.0
Dissolved Oxygen	4.0
Turbidity	88*
Nitrite	0.627 (as NO_2)
Nitrate	15.4 (as NO_3)
Orthophosphate	3.0
Alkalinity	
(Phenothalein)	26.0
(Total)	174.0
Hardness	
(Calcium)	70.0
(Total)	150.0
Iron	0.08
COD	303
Kjeldahl Nitrogen	
(Free Ammonia)	1.12
(Organic Nitrogen)	3.64
Suspended Solids	193
Volatile Suspended Solids	86
Total Residue	900
Volatile Residue	220

* Jackson Turbidity Units

TABLE IV

RANGE AND AVERAGE VALUE OF ANALYSES

	Control Vessel	Filtration Treat. Vessel	Filtration Culture Vessel	Chlorination Treat. Vessel	Chlorination Control Vessel	Flocculation Treat. Vessel	Flocculation Control Vessel	Reese Playa Lake
Suspended Solids								
High	172	16	393	189	135	25	107	170
Low	33	0	22	18	3	0	5	53
Average	109	7	132	77	75	14	44	93
Volatile S. S.								
High	120	7	127	148	65	17	97	97
Low	20	0	3	3	3	0	0	20
Average	61	3	52	41	37	6	19	50
Total Residue								
High	2063	1317	2677	1203	1620	1127	1737	1133
Low	900	517	1000	897	1153	850	1217	753
Average	1335	1003	1429	1055	1385	1018	1489	997
Volatile TR								
High	733	468	633	393	320	353	520	377
Low	107	17	113	60	120	53	87	73
Average	321	239	272	186	217	173	245	223
COD								
High	255	107	232	219	222	116	364	232
Low	98	11	70	80	70	38	37	91
Average	178	76	149	131	155	78	191	141
Phosphate								
High	1.10	1.65	0.87	1.20	0.92	0.05	0.06	1.15
Low	0.42	0.92	0.62	0.25	0.22	<0.01	<0.01	0.41
Average	0.60	1.25	0.77	0.60	0.52	<0.02	<0.02	0.67
Nitrate								
High	1.60	3.50	1.50	2.30	3.10	1.70	0.77	1.90
Low	0.95	3.00	0.76	2.00	1.30	0.91	0.64	1.20
Average	1.25	3.22	0.96	2.20	1.58	1.31	0.72	1.49
Dissolved Oxygen								
High	14.8		16.0		14.2		12.5	13.0
Low	9.2		9.2		8.4		8.6	5.8
Average	11.1		11.4		10.5		10.3	8.9

and/or processes performed in anticipated fashion. For example, suspended solids were removed effectively by filtration and flocculation and phosphate concentrations were significantly decreased by flocculation and subsequent sedimentation. Concentrations of many of the measured constituents increased with time in the culture vessels mainly because of atmospheric additions. While not shown in tabular form, infrequent microbiological examination showed the effluent from the chlorination treatment vessel to be free of bacteria. Substantial regrowth occurred later but their numbers never reached the level reported previously for the Reese Playa (1.4×10^4 bact/ml). The highest count observed was 3.6×10^3 bact/ml.

Periodically, water from the Reese playa and each of the treatment and control vessels was analyzed for copper, chromium, iron, lead, mercury, nickle and zinc by atomic absorption spectroscopy at Texas Tech University. None of these elements, except iron, were detectable. Iron concentrations ranged from .5 mg/l to 1.8 mg/l in the playa water and in the control vessel but was removed to below detectable limits by each of the treatment operations.

In addition to the water quality studies conducted at Texas Tech University, testing was also done by the Texas State Department of Health and by the Environmental Health Laboratory at Kelly Air Force Base, Texas.

On two different occasions during the study, samples were collected from the Reese Air Force Base sewage plant, the playa lake and from each of the treatment and culture vessels and sent to the Texas State Department of Health for selected heavy metal analysis, parathion analysis, malathion analysis and polychlorinated biphenyls (PCB) analysis. One analysis was also made on the effluent from the sewage plant for asbestos by the health department. In no case was any parathion, malathion or PCB detected and heavy metals were below detectable limits in each instance.

Specifically, the following heavy metal results were obtained:

mercury < 0.0005 mg/l; copper < 0.1 mg/l; lead < 0.05 mg/l;

chromium < 0.05 mg/l.

In the sample analyzed for asbestos, none was detected.

The results of the metal analyses on water and sediment taken from the Reese playa and conducted at Kelly Air Force Base Environmental Health Laboratory are shown in Tables V and VI. Samples of the Reese playa water were collected at depths ranging from 8 to 12 inches from south, east, west, and north stations on the lake near the shoreline (10-15 feet from shore) and from the center of the lake. Sediment samples were taken from each of the points of the lake near shore (10-15 feet from shore) and also out nearer the center of the lake (30 feet from shore).

No detectable concentrations of heavy metals other than iron were found in the water, but typical concentrations of calcium, magnesium, sodium, and potassium were present. However, as seen in Table VII, relatively high concentrations of chromium, cobalt, copper, iron, lead, magnesium, nickle, silver, and zinc were detected. As will be discussed later, the metals present in the sediments had no apparent effect on the cultured salamanders.

During the second year of the study, an additional culture vessel was utilized for copper studies. These were initiated because it had been reported by Reese Air Force Base personnel that occassionally, in past years, copper sulfate ($CuSO_4$) had been used to control algae growth in the Reese playa.

Thirty salamander larvae, sans tumors, were placed in the vessel which contained Reese playa water to which enough copper sulfate was added to bring the dissolved copper concentration to 0.7 mg/l. The relatively high alkalinity of the Reese water caused much of the copper to precipitate in the carbonate

TABLE V

DISSOLVED METAL CONCENTRATIONS IN REESE AFB PLAYA LAKE
(mg/l)

	South Shore (near shore)	East Shore (near shore)	West Shore (near shore)	North Shore (near shore)	Center
Arsenic	<0.01	<0.01	<0.01	<0.01	<0.01
Beryllium	<0.001	<0.001	<0.001	<0.001	<0.001
Cadmium	<0.01	<0.01	<0.01	<0.01	<0.01
Calcium	27.5	47.5	35.0	42.5	35.0
Chromium	<0.05	<0.05	<0.05	<0.05	<0.05
Cobalt	<0.05	<0.05	<0.05	<0.05	<0.05
Copper	<0.02	0.05	<0.02	0.54	<0.02
Iron	0.54	0.26	0.70	0.69	0.60
Lead	<0.05	<0.05	<0.05	<0.05	<0.05
Magnesium	14.6	13.2	15.0	15.3	14.3
Mercury	----	----	----	----	----
Molybdenum	<0.01	<0.01	<0.01	<0.01	<0.01
Nickel	<0.05	<0.05	<0.05	<0.05	<0.05
Potassium	14.5	14.3	16.6	15.0	14.5
Silver	<0.01	<0.01	<0.01	<0.01	<0.01
Sodium	37.5	42.5	40.0	35.0	37.5
Zinc	<0.05	<0.05	<0.05	<0.05	<0.05

(Source: Environmental Health Lab; Kelly AFB)

TABLE VI

METAL CONCENTRATIONS IN REESE AFB PLAYA LAKE SEDIMENTS
(micrograms/gram of sediment*)

	South Shore		East Shore		West Shore		North Shore	
	Near Shore	Center	Near Shore	Center	Near Shore	Center	Near Shore	Center
Arsenic	0.91	0.42	< 0.22	< 0.22	< 0.15	< 0.62	0.18	0.85
Beryllium	0.30	0.18	0.03	0.02	0.29	0.22	0.11	0.31
Cadmium	0.233	0.299	1.241	.0.445	0.553	0.003	0.001	0.238
Chromium	11.65	9.92	75.21	1.62	3.72	7.32	6.81	1.95
Cobalt	4.2	3.4	2.3	0.7	4.8	3.7	2.7	2.7
Copper	16.52	5.65	6.57	1.39	5.90	12.73	3.48	9.66
Iron	8,842.7	3,960.5	1,531.9	1,358.5	5,531.9	4,854.4	3,145.6	10,971.1
Lead	9.74	13.41	18.18	4.85	11.34	19.85	11.10	12.26
Magnesium	9076	2841	1516	941	6195	5264	3160	4883
Mercury	0.017	0.010	0.010	0.006	0.008	0.020	0.005	0.006
Molybdenum			1.38		1.66			
Nickel	8.6	5.2	4.1	2.3	6.9	7.7	3.2	4.2
Silver	2.116	0.679	3.885	0.302	0.402	1.406	0.574	0.217
Zinc	44.39	18.43	19.56	3.70	14.41	21.38	11.77	3.69
% H_2O	61.71	38.58	29.45	17.31	17.69	70.21	25.57	24.22

*Dry wt. basis

(Source: Environmental Health Lab; Kelly AFB) form. Periodic additions of $CuSO_4$ were made to maintain the dissolved copper concentration at about 0.7 mg/l. After eight months, the copper concentration in the sediment had reached 15 mg/gm.

The results after seventeen months of containment for the salamanders in the control, flocculation, filtration, and chlorination culture vessels and eight months in the copper vessel are reported in Table VII.

Since no tumors were observed on animals inhabiting the control vessel, any results from this expermentation are inconclusive. Some speculative explanation of these results is needed however. By comparing the live salamanders seined and the dead salamanders discovered it is noted that some salamanders were unaccounted for; the greatest number, 8, from the flocculation vessel which also was the only vessel containing salamanders which had tumors. It is probable that the unaccounted for salamanders were almost completely decomposed between observation periods. This belief is partially substantiated by the fact that the highest bacterial counts and COD concentrations were found in the flocculation vessel.

The tumors found on the four salamanders were all epidermal papillomas (lace tumors), the most frequently occurring tumor on Reese playa salamanders. No reason for their occurrence on the test animals is known, but three explanations are plausible. The tumors may have been present initially but were overlooked; something in the flocculation vessel, such as aluminum, may have induced the tumors; or because of the low primary productivity in this vessel (note the low suspended solids concentrations from Table IV), which probably was a result of the existing low nitrate and phosphate concentrations (Table IV), sufficient food for the salamanders wasn't provided which resulted in undue physiological stress on the organisms, thus inducing tumor development.

TABLE VII

RESULTS OF EXAMINATION OF SALAMANDERS

	Control	Filtration	Chlorination	Flocculation	Copper
Initial Number of Salamanders	30	30	30	30	30
Live Salamanders actually seined	22	23	20	8	29
Dead Salamanders discovered	6	3	8	14	1
Number of Salamanders transformed	4	2	6	0	0
Number of Salamanders with tumors	0	0	0	4	0

Nitrosoamine Analysis

The possibility that nitrosoamines were the cause of the tumors was investigated because they are known to be carcinogenic and because their precursors, secondary amines and nitrites are almost certain to be present in the Reese Playa. Secondary amines are found in urine, feces, detergents, higher plants and algae and nitrites are intermediate in the nitrification and denitrification processes.

Unfortunately, however, detection of nitrosoamines is very difficult. The most accepted technique for their analysis is gas chromotography, the method employed in this study.

Solutions of diethylnitrosoamine, di-n-proylitrosoamine and N-nitrosodiphenylamine were purchased and used as standards to calibrate the gas chromatographs. Two chromatographs were used; the first was a Perkin-Elmer 990 equipped with a 7.62 m. by 4.67 mm. steel column packed with Porapak Q-S (100-120 mesh) and the second was a Varian Aerograph (1200 series) with the same sized steel column but packed with 5% chromosorb W, S-F 30. Both instruments had flame ionization detectors and employed N_2 at a rate of 40 ml/min as the carrier gas. Respective operating temperatures used with the P-E unit were 250° C, 180°C, and 250°C for the injector, column and detector. Those used with the Varian were 230°C, 130°C, and 230°C.

Although difficulty was encountered in obtaining and isolating peaks with the standard solutions below concentrations of 1 mg/l, repeated injection of playa water samples yielded no peaks whatsoever in the range of the nitrosoamine intervals.

Enzymatic Investigation

Members of the Departments of Biology and Basic Health Sciences from North Texas State University in Denton, Texas, in cooperation with the Texas

Tech University Biology Department and Water Resources Center conducted independent enzymatic studies on salamanders seined from the Reese Air Force Base playa lake. The following paragraphs summarize their findings. More precise information regarding the materials and methods utilized can be found in a thesis by Colvin (2).

The enzymatic studies centered around Aryl hydrocarbon hydroxylase (AHH), an inducible enzyme of the microsomal mixed-function oxygenase group also known as benzo(α)pyrene-3-hydroxylase. Previous work (3) with this enzyme by Dr. David Busbee (the principal investigator of this facet of this study) had suggested possible correlation between malignant tumors and AHH induction. Because known carcinogens such as polycyclic aromatic hydrocarbons (PAH), PCB and DDT are metabolized by this enzyme, it was decided to investigate AHH induction in salamanders in an attempt to provide insight into identifying the tumorigenic agent at Reese Air Force Base. Specifically the study objectives were to determine: (a) if the salamanders were competent for AHH induction, (b) the ratio of PAH metabolites that were produced in salamanders and (c) if the enzyme system could be compared with that of mammals including man.

Both water and sediment were analyzed for halogenated hydrocarbons and organophosphate pesticides at North Texas State, and none were found. Even though the presence of PAH has not been determined, research activity focused on these compounds.

The polycyclic aromatic hydrocarbons are metabolized by AHH to various mono- and dihydroxy derivatives and quinones and highly reactive expoxides such as the 4,5-epoxide of benzo(α)pyrene may be produced as intermediates in the metabolism of PAH (4). These epoxides are generally considered to be the proximate carcinogens of polycyclic hydrocarbons.

To meet the study objectives, salamander AHH activity was characterized, a comparative investigation of matabolites between a representative mammal (C57Bl/6J mice) and the salamander, Ambystoma tigrinum was conducted and a test of naturally occurring animals for environmentally induced AHH activity was made.

3-Methylcholanthrene (3MC) was used to induce the AHH enzyme with benzo-α-pyrene (BP) serving as the enzymatic substrate. AHH activity was indicated by monitoring the metabolite 3-hydroxybenzo α-pyrene by utilizing fluorescence measured spectrophotoflurometrically at indicated wavelengths, and the metabolite study was performed using thin layer chromotography methods.

Collected salamanders from the Reese playa lake were held in clean, aerated water until testing. Their diet consisted of earthworms obtained from Sargents Worm Garden, Lake Dallas, Texas. C57Bl/6J mice were obtained from the Jackson Laboratory, Bar Harbor, Maine.

To determine if AHH was being environmentally induced in animals from the experimental population of the Reese playa lake, animals taken directly from the lake were tested for AHH activity.

In vivo induction of AHH was accomplished using two methods. 3-Methylcholanthrene (3MC) (10 mg/Kg body weight) in corn oil was given by intraperitoneal injection. Secondly, 3MC (75 ug/ml final 3MC concentration) was dissolved in acetone and added to the water contained in the experimental holding tanks.

Microsomal preparations from mouse and salamander livers, tissue preparations used to indicate the presence of AHH activity and lymphocyte isolation and culture were done according to procedures outlined in the previously referenced paper by Colvin *et al*.

Animals taken directly from the Reese playa were found to have hepatic microsomal AHH levels approximating those animals injected with 3-methylcholanthrene as seen in Figure 16. After being held several months in clean water, the AHH levels of salamanders from the Reese playa could not be distinguished from those of control animals. These data suggest that an inducing agent is present in the Reese playa.

^{3}H-benzo α-pyrene metabolites produced by salamander liver microsomes and separated by thin-layer chromatography (Fig. 17) exhibited a thin-layer chromatographic distribution of metabolites similar in mobility to those produced by mouse liver microsomes (Fig. 18) and cultured human lymphocytes (Fig. 19). A number of the metabolites were tentatively identified by comparison of chromatographic mobilities with those of known standards. Percentages of total metabolite formed by mouse and salamander liver microsomes were calculated (Table VIII) and were seen to vary considerably between the two animals. In mice, 3-hydroxy benzo α-pyrene made up 69.9% of the total metabolites while in the salamander preparations the 3-hydroxy metabolite constituted only 1.7% of the total. The 4, 5-epoxide of benzo α-pyrene was converted to 4, 5-dihydrodiol by a similar, and perhaps genetically related, inducible cytochrome P-450-associated enzyme, epoxide hydrase.

Mouse liver microsomes produced relatively little 4, 5-epoxide and apparently converted it reasonably well to the dihydrodiol form, the two being present at 1.6% and 1.29% respectively. Salamander microsomes produced the 4, 5-epoxide as about 9% of the total metabolites, and, since the 4, 5-dihydrodiol comprised only about 1.7% of the total metabolites, they apparently converted it poorly to the transdiol form. Treatment of salamander hepatic microsome preparations with cyclohexene oxide, an epoxide hydrase inhibitor, did not effect a change in the ratio of 4, 5-epoxide to 4, 5-dihydrodiol. The lack of ability to convert the 4, 5-epoxide of BP to 4, 5-dihydrodiol upon treatment with cyclohexene oxide suggests that these salamanders are deficient

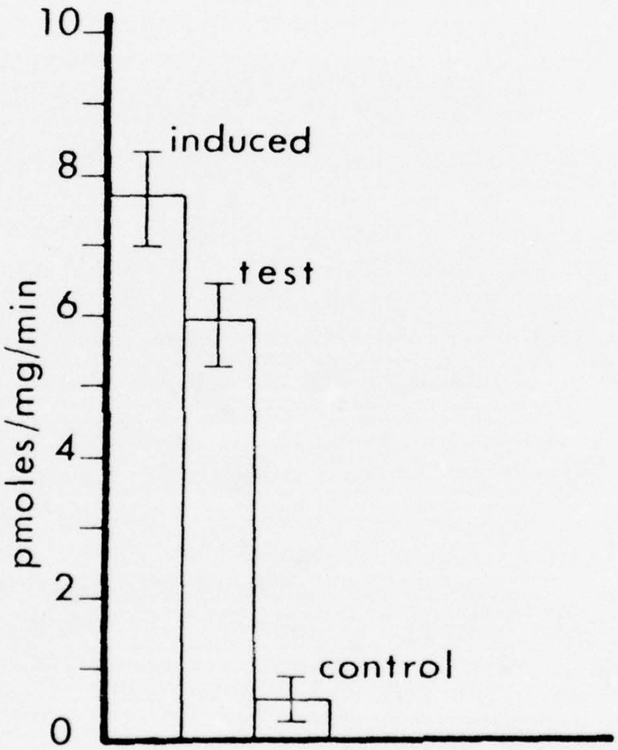

Figure 16. A comparison of AHH activity levels between animals induced by intraperitoneal injections in corn oil and test animals taken directly from the Reese Playa Lake. Control animals received intraperitoneal injections of corn oil.

Source: Colvin, *et al.* 1974.

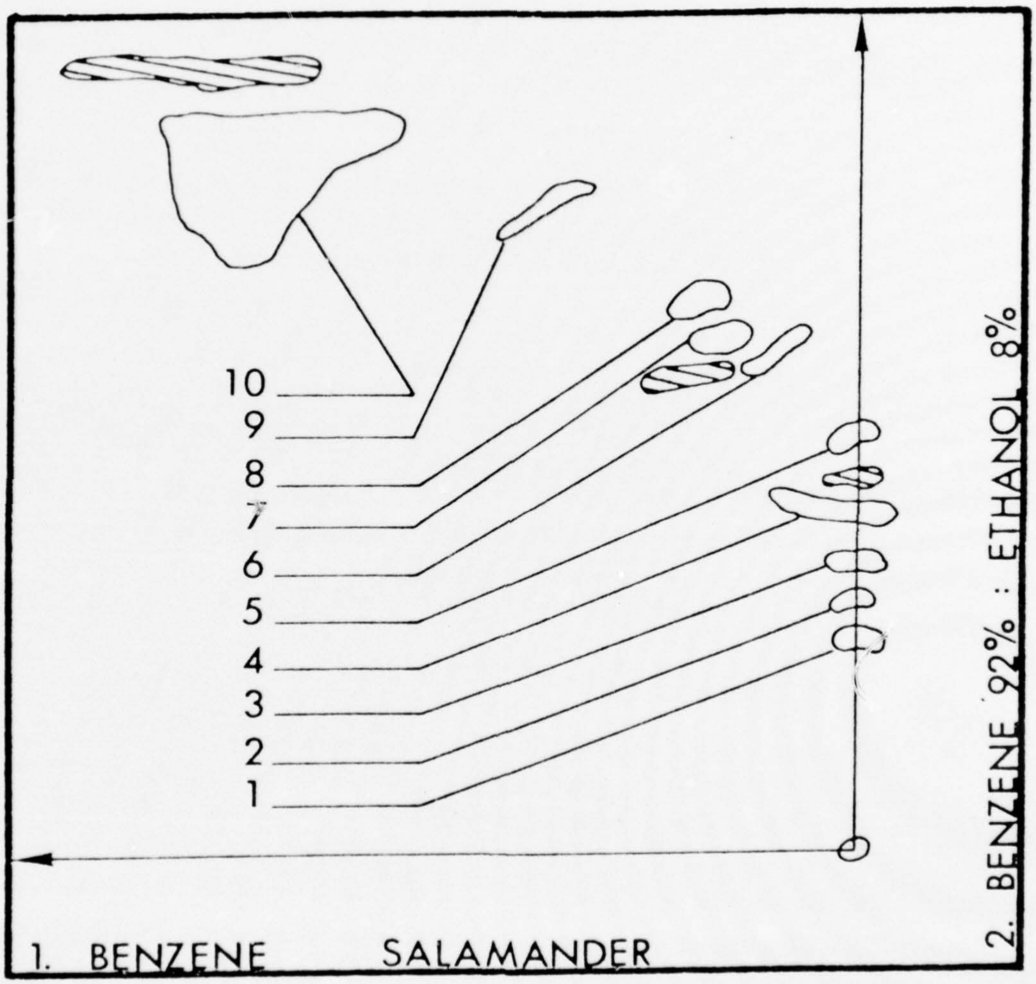

Figure 17. An autoradiographic determination of the thin-layer chromatographic separation of ^{3}H-benzo(a)pyrene metabolites produced by 3MC-induced salamander liver microsomes.

Source: Colvin, et al. 1974.

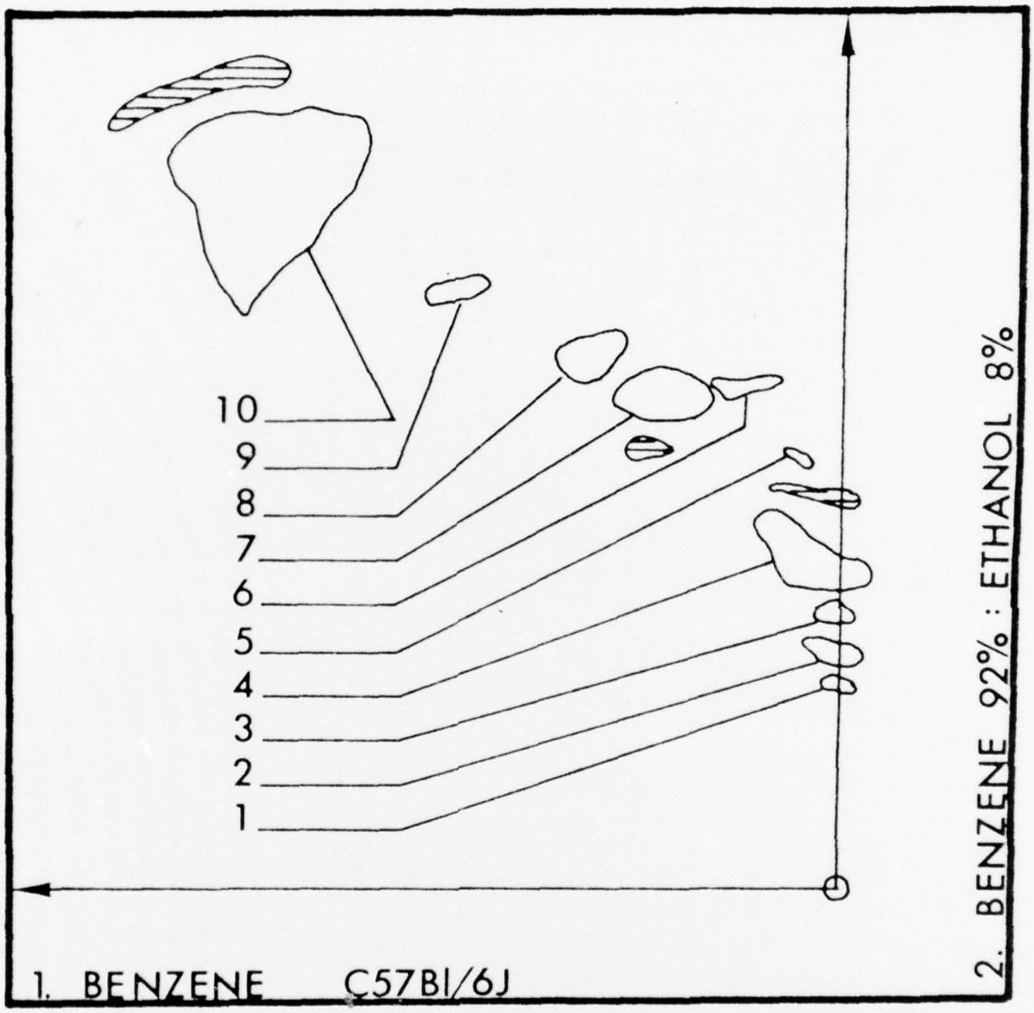

Figure 18. An autoradiographic determination of the thin-layer chromatographic separation of ^{3}H-benzo(a)pyrene metabolites produced by 3MC-induced C57Bl/6J mouse liver microsomes.

Source: Colvin, et al. 1974.

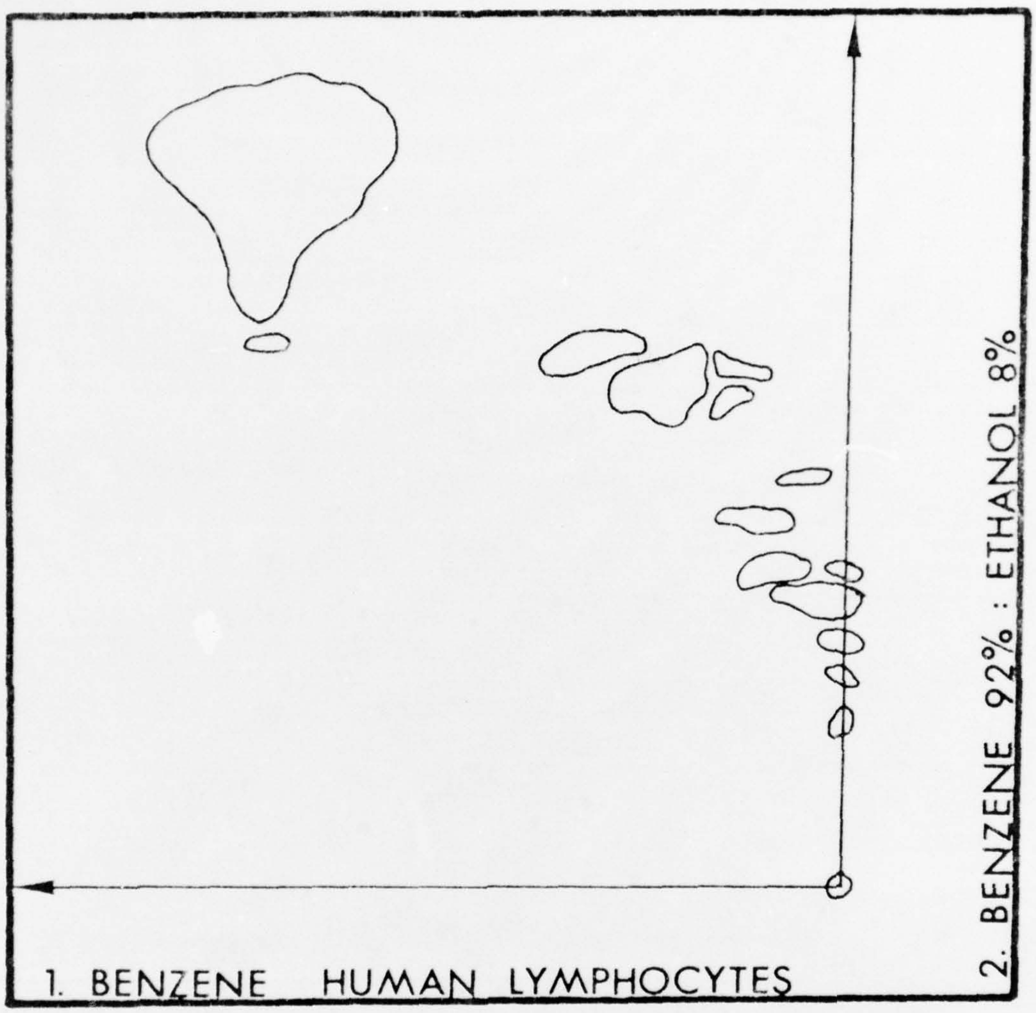

Figure 19. An autoradiographic determination of the thin-layer chromatographic separation of ^{3}H-benzo(a)pyrene metabolites produced by 3MC-induced human lymphocytes.

Source: Colvin, et al. 1974.

TABLE VIII

A COMPARISON BETWEEN ^{3}H-BENZO(a)PYRENE METABOLITES OF MOUSE LIVER AND SALAMANDER LIVER MICROSOMES IN VITRO

	Metabolites	C57Bl/6J cpm	% of Total Metabolites	Salamander cpm	% of Total Metabolites
	Origin	2140	0.0068	725	0.0213
1	9,10 di	1469	0.0046	486	0.0142
2	7,8 di	1269	0.0041	368	0.0110
3	4,5 di	4052	0.0129	580	0.0169
4	3-OHBP	218682	0.6992	4384	0.1350
5	unknown	943	0.0030	1170	0.0348
6	unknown	2676	0.856	3289	0.0957
7	3,6 qui	32020	0.1014	10083	0.2938
8	1.6 qui	46765	0.1495	10098	0.2550
9	4,5 epox	3651	0.0116	3094	0.0893

Source: Colvin, et al. 1974.

in epoxide hydrase activity. Burki and Bresnick (5) reported an increase in cancer production when animals were treated with 3MC in concert with an epoxide hydrase inhibitor, suggesting the carcinogenicity increase to be related to inability of the animal to metabolize the K region (4, 5-), epoxide of 3MC.

Supplemental Information

As alluded to throughout the report, no specific tumorigenic agent was identified. The data generated by Busbee and his coworkers at North Texas State University suggest, because of the high tumor index, high AHH levels in the salamanders inhabitating the Reese playa, and the apparent recessive condition for epoxide hydrase, that correlation exists between the microsomal enzymes, an unknown water pollutant which is probably a polycyclic aromatic hydrocarbon (PAH) and the development of cancer in this population of animals.

Samples of water and sediment were sent to the Eppley Institute in Omaha, Nebraska for analysis of polycyclic aromatic hydrocarbons, also referred to as polynuclear aromatic hydrocarbons. None were found in the Reese playa water, but significant concentrations of perylene (300 ppb) were found in the Reese playa bottom sediments. Trace amounts of other polycyclic aromatic hydrocarbons were found in the sediment and are reported in Table 9. While perylene is not generally thought to be a carcinogen as are many of the other polycyclic aromatic hydrocarbons, such determinations were made using dated bioassay procedures. From the results of this study, it appears that additional research aimed at determining the carcinogenicity of perylene should be undertaken.

No specific sources of the perylene or other polycyclic aromatic hydrocarbons were identified, but they are known to be contained in petroleum

TABLE IX

POLYCYCLIC AROMATIC HYDROCARBONS ISOLATED FROM REESE AFB SLUDGE
ppb=mg/kg

POLYCYCLIC AROMATIC HYDROCARBON	CONCENTRATION (ppb)
Perylene	300.0
Pyrene	5.8
Fluoranthene	5.7
Alkyl pyrenes	4.9
Benz (a) anthracene	1.4
Chrysene	1.3
Triphenylene	0.5
Benzo (a) pyrene	0.5
Benzo (e) pyrene	0.2
Anthanthrene	0.2

products and from pyrolytic sources such as automobile, and presumably, aircraft exhausts.

Table X, taken from a paper authored by Koons, et al. (6) lists several sources of PAH. As shown, petroleum and petroleum products are significant sources. At least two petroleum products, asphalt and diesel oil, have been applied to the Reese playa lake. Diesel oil was, in past years, sprayed on the surface of the lake near the shore for mosquito control purposes. Infrequent applications were confirmed by the Reese Air Force Base entomologist (7).

Sometime between 1962-1964, an old asphaltic runway at Reese Air Force Base was scarified, removed and used for rip-rap along the northwestern corner of the Reese playa to prevent bank erosion. This action was confirmed by a conversation with Mr. W. C. Teal of the Reese Air Force Base grounds maintenance department, and remnants of the rip-rap still remain. In addition to the asphalt itself being a source of PAH, it is likely that from time to time, airplane fuel, another potential source of PAH, was spilled on the runway and was absorbed by the asphalt, thus increasing the amount of PAH applied to the water. Also small amounts of jet fuel may be contributed even now to the lake via runoff following a rain; although most runoff from the runways and hangar areas drains to a different locale.

Although none was detected by analysis, the chlorinated hydrocarbon, Toxaphene, was used, in 1959 or 1960 to eradicate the salamander population so that fish stockings might be successful. It was determined by conversation with a Texas Parks and Wildlife Department official that an overabundance of the pesticide was used. Apparently the salamander population was destroyed or at least its numbers were drastically decreased because reports, confirmed by Mr. Teal, indicated that several 2-1/2 yard dump truck loads of dead salamanders were hauled from the playa to a landfill.

TABLE X

SOURCES OF POLYNUCLEAR AROMATIC HYDROCARBONS

Source	Amount per Year
Forest and prairie fires	A high percentage of the total
Other pyrolytic sources - automobiles, coking, power plants, refuse burning	1,400 tons BAP*
Total petroleum input, 42,000,000 bbls	5 tons BAP*
Oil in discharged produced water, 65,000 bbls.	0.008 tons BAP*

*BAP is benzo(a)pyrene, the polynuclear aromatic most often measured.

Source: Koons, *et al.* 1976

Analysis of tissue from fish used to stock the Reese playa in 1963 was made for chlorinated hydrocarbons in 1964. No Toxaphene was detected, but significant amounts of DDT were found. Again, as previously stated, no DDT was detected in the water or sediments during the course of this study. A check with Reese AFB personnel substantiates that no chlorinated hydrocarbons have been used on the base for "several years" (8).

As a result of a thorough study conducted by the Texas Tech Water Resources Center in 1970 (9) to detect the presence or absence of chlorinated hydrocarbons in playa lake waters and sediments, further credence is given to analyses that fail to detect any measurable quantities of these pesticides. No chlorinated hydrocarbons were found in any of the playa waters, and only on rare occasions were trace amounts detected in the sediments. These results were not surprising when the very low application rates of these compounds to Texas High Plains crops was considered.

CONCLUSIONS AND RECOMMENDATIONS

The conclusions and recommendations drawn from this research work are peculiar to the particular circumstances found to exist at the Reese Air Force Base playa lake. They are based solely on the data generated during the course of the study and on documented history. While the conclusions reached may not be firm in every detail, they do reflect the best judgement of the authors. In order to further substantiate these findings, much additional time, expertise and work would be required. Because of the nature of the findings, the study objectives, and the authors' perceived opinion regarding the mission of the U.S. Army Medical Research and Development Command, it would be difficult to justify the additional required investment. Therefore, the following conclusions and recommendations, although perhaps partially indeterminant, are offered.

1. Eight basic tissues abnormalities were identified throughout the course of the study. Included were melanomas, epidermal papillomas (lace tumors), myxofibromas, fibromas, hyperplasias, epidermal inclusion cysts, cephalic cysts, and abdominal acites.
2. There was a substantial increase in the total numbers of salamanders with abnormalities during the course of the study, with epidermal papillomas occurring in the highest frequency.
3. Salamander seinings from playa lakes other than the Reese playa, either associated or non-associated with sewage effluent, revealed no tissue abnormalities.

4. Salamander larvae from the Reese AFB playa lake rarely, if ever, developed neoplasms or cysts until they were from 14 to 17 months of age, which suggests that lengthy exposure to the Reese playa water is necessary to stimulate tumor development.
5. No evidence of tumor development was observed on catfish, mice, or quail that were maintained exclusively with Reese playa lake water. However, experimental mice raised on the water had a lower reproductive rate than their control counterparts and quail raised on Reese playa water experienced weight reduction and high eosinophil counts.
6. Inoculations of frogs, mice and healthy salamanders with minced tumor tissue resulted in only one possible tumor induction in salamanders and none in the frogs or mice. This result, coupled with those from Conclusion 5, suggests that the tumorigenic agent is not vertically transmissible along the phylogenetic scale or that the agent simply is not tumorgenic to the other organisms tested.
7. Results of bacteriological and virological studies indicate that the tumors were not induced by either bacteria or viruses.
8. Conventional water treatment unit operations, namely filtration, flocculation and sedimentation and chlorination had no discernable effect on the presence or absence of tumors on the salamanders.
9. Standard chemical analyses, heavy metal analyses and pesticide analyses of Reese playa water showed concentrations of these constituents to be present either in normal, expected concentrations or below detectable limits. Therefore, it was concluded that none of the constituents analyzed for were responsible for tissue abnormalities.

10. None of the nitrosoamine forms analyzed for were found to be present in the Reese playa water, thereby, disavowing any relationship between nitrosoamines and tumor development.
11. From the enzymatic studies conducted, it was shown that salamanders inhabiting the Reese playa lake were highly induced for the enzyme aryl hydrocarbon hydroxylase. This suggests that an inducing agent was present in the Reese playa lake. It was further found from these studies that the 4,5-epoxide of benzo-α-pyrene was not converted to the dihydrodiol form suggesting that carcinogenicity is related to the inability of the salamander to metabolize this epoxide.
12. Although no specific tumorigenic agent was positively identified, there is evidence to suggest that polycyclic aromatic hydrocarbons served to induce the high levels of aryl hydrocarbon hydroxylase activity and may well be the tumorigenic agent. Sources of these compounds, diesel oil and asphalt, are known to have been applied to the Reese playa lake.
13. From the results of these studies, it cannot be concluded that salamanders are ultrasensitive to chemical carcinogens and therefore should be widely used in bioassay procedures developed to determine carcinogenicity. Because the salamanders were continuously in contact with the carcinogen(s) involved in this study and therefore surely ingested large quantities of the carcinogen without revealing any internal tumors, they may in fact be insensitive organisms. However, it appears, because all the tumors were of an external nature, that their skins may be sensitive to carcinogens of the nature found in the Reese playa. Further research aimed at determining the usefulness of salamanders as indicator organisms should be undertaken.

14. In summary, all the data collected during the course of the study lead the authors to conclude that the salamanders are induced to a high level of aryl hydrocarbon hydroxylase activity by polycyclic aromatic hydrocarbons and the inability of the salamanders to convert the carcinogenic epoxide intermediate to nonreactive metabolites causes tumor development in the salamanders. Based upon this finding, it appears that no immediate danger exists to man or other animals who may experience limited exposure to the Reese playa water.

In view of these conclusions, it is recommended that the practice of storing treated sewage effluent in the playa and subsequently utilizing it for irrigation of the Reese AFB golf course should not be discontinued.

It is further recommended that before widespread utilization of treated sewage effluents for purposes other than irrigation of nonforage crops is contemplated, such effluents be thoroughly analyzed for compounds such as polycyclic aromatic hydrocarbons and that bioassays be conducted as a matter of routine.

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