

AUTHENTICATION REVIEW OF SELECTED MATERIALS SUBMITTED TO
THE FOOD AND DRUG ADMINISTRATION RELATIVE TO APPLICATION
OF SEARLE LABORATORIES TO MARKET ASPARTAME

Volume No. 1

Chapter I:	General Summary and Conclusions
Chapter II:	General Introduction
Chapter III:	106 Week Oral Toxicity Study in the Dog

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UNIVERSITIES ASSOCIATED
FOR RESEARCH AND EDUCATION IN PATHOLOGY, INC.

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SUBMITTED TO THE FOOD AND DRUG ADMINISTRATION
RELATIVE TO APPLICATION OF SEARLE LABORATORIES TO MARKET ASPARTAME

prepared by
Universities Associated for Research & Education in Pathology, Inc.

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TABLE OF CONTENTS

Chapter	FDA Entry Number	Title	Page
<u>Volume 1, pages 1-255</u>			
I	---	General Summary and Conclusions	1
II	---	General Introduction	28
III	E-28	106 Week Oral Toxicity Study in the Dog	117
<u>Volume 2, pages 256-710</u>			
IV	E-33,34	Two Year Toxicity Study in the Rat	256
V	E-70	Lifetime Toxicity Study in the Rat	458
VI	E-75	104 Week Toxicity Study in the Mouse	578
<u>Volume 3, pages 711-1062</u>			
VII	E-76	110 Week Toxicity Study in the Mouse	711
VIII	E-86	A Supplemental Study of Dog Brains from a 106 Week Oral Toxicity Study	825
IX	E-87	A Supplemental Study of Rat Brains from Two Tumorigenicity Studies	833
X	E-9	Toxicological Evaluation in the Neonatal Rat	846
XI	E-11	2 Generation Reproductive Study--Rats	864
XII	E-19	A Sweetening Agent: Endocrine Studies	885
XIII	E-88	Experiments in Mated and Pregnant Rhesus Monkeys	942
XIV	E-90	An Evaluation of Embryotoxic and Teratogenic Potential in the Rabbit	988

CHAPTER I

SUMMARY AND CONCLUSIONS

INTRODUCTION

It is the purpose of this initial chapter to present a brief overview of UAREP's validation studies of Searle projects with the principal conclusions. As studies may interrelate, an attempt will be made to draw correlative conclusions.

The general background and UAREP's approach to these authentication studies is presented in Chapter II. From nearly 100 studies which Searle has presented to the Food and Drug Administration to justify its application to market its artificial sweetener, aspartame, 12 were selected by the FDA and other parties for UAREP's review. There were five major studies of long-term toxicity effects involving the dog (Chapter III), rats (Chapters IV and V), and mice (Chapters VI and VII). Two special neuropathology studies (Chapters VIII and IX) reviewed the brain sections from the studies described in Chapters III, IV, and V. The balance of the UAREP report comprises smaller studies involving the effects of aspartame in newborn rats (Chapter X); a two-generation reproductive study in the rat (Chapter XI); a series of screening tests for endocrinological and physiological responses (Chapter XII); a minor report covering a few inconsequential observations on pregnant monkeys (Chapter XIII); and a major study of the effects of aspartame and its metabolites in embryogenesis and teratogenesis in rabbits (Chapter XIV).

UAREP did not participate in the selection of these studies. UAREP undertook this authentication without any prior bias regarding the studies, and adequate precautions were continued throughout its work to maintain independence from the views of any of the interested parties relative to the proposed use of aspartame. Both Searle and FDA requested that UAREP refrain from commenting on experiment design, or the safety of aspartame for human consumption. Any UAREP interpretation of results applies only to the experiments as designed. UAREP has addressed itself to the question of whether the experiments were carried out according to protocol plans and the accuracy and reliability with which the experiments were performed and reported to the FDA. We have, at times, commented on the interpretation of the significance of the data.

RESUME OF SEARLE STUDIES

In the following 12 sections, UAREP's conclusions regarding the various Searle studies will be summarized. Some attempt will be made to quantitate not only the number, but the magnitude and significance of discrepancies and problems noted. They will be presented according to the chapter numbering in this report, beginning with Chapter III.

E-28 (Chapter III) - 106 Week Oral Toxicity Study in the Dog

Forty Beagles, five animals of each sex per treatment group, were fed aspartame daily in three different dosages. Seven hematology, ten clinical chemistry, and eight urinalysis parameters were measured periodically throughout the experiment. An additional ten chemical constituents were determined at 78 and/or 106 weeks.

All of the dogs were killed at 106 weeks, and complete autopsies and histopathologic examinations performed. There was reasonably good agreement between the results of UAREP's and Searle's histopathologic examinations as well as the results of the clinical chemical, body weight, and food consumption determinations.

Protocols - The experiment was well documented in protocols and memos. The basic design was established before the study began. However, a number of important assays were added at later stages.

Body Weight and Food Consumption - The amount of compound consumed was somewhat variable, but never was more than 6% from the desired dose.

Food consumption in the treatment groups tended to be quite erratic in the early weeks of the study. There were statistically significant differences between some of the treatment groups and the controls when total food consumption for weeks 1-10 was compared.

Body weights were recorded periodically. The mean weights in the high dose groups were larger at the beginning of the study than those of the control groups. The high dose groups gained less weight during the first 26 weeks of the study. The high dose female group's weight gain was significantly less than that of the control females as well as the low and medium dose females.

Randomization was done rather haphazardly as evidenced by the occurrence of 2-3 littermates in several of the groups, as well as the significantly higher body weights in the high dose groups at the start of the study.

Clinical Laboratory Studies - UAREP's statistical evaluation showed fewer significant differences between treatment and control groups than were reported by Searle.

The raw data for the first four intervals showed that a correction had not been made in the leukocyte counts over 10,000 or in the erythrocyte counts. Although the correction does change the data to some extent it would not alter the results as reported to Searle.

Red cells were reported in significant numbers in urinary sediments at two intervals. They were numerous enough to cause bloody urine in these animals. Since there are no records of bloody urines or urinary tract disease, UAREP questions the validity of the data.

Necropsy and Histopathology - UAREP's review of the histopathology slides showed only two significant discrepancies in the diagnoses.

Chapters IV (E-33,34), V (E-70), VI (E-75), and VII (E-76)

For correlative purposes, these four experiments carried out for Searle by Hazleton will be discussed together. They share the objective of testing the effects of aspartame (E-33,34; E-70; E-75) or its conversion product, diketopiperazine, DKP (E-76) in life-time toxicity studies. E-33,34 involves 440 rats in five groups with the highest dosage being 8 gm/kg body weight per day. E-70 involved 280 rats in control and two treatment groups. E-75 and E-76 were very similar with the exception that E-76 provided three graded dosages of DKP whereas E-75 again evaluated aspartame. They each utilized 360 mice. The duration of the four experiments ranged from 103 to 108 weeks.

Protocols - A series of Searle protocols and amendments and Hazleton Project Sheets gave the specifications of each experiment. All four experiments involved numerous changes in parameters to be examined and intervals for testing. Although the results seem to indicate that the experiments were planned in detail and modified while in progress due to earlier findings or other information, UAREP found it difficult to reconstruct and document the progression of the changes of the experiment on the basis of a series of parallel amendments or written memoranda in the files of both Searle and Hazleton. This task was complicated by the fact that written instructions requested that all earlier versions of protocols be destroyed as they were updated. Apparently, some changes in protocol design were discussed over the telephone by Searle and Hazleton staff and, at times, there was a significant lapse of time after the change in the experiment was instituted before

written documentation appeared in the files of both Searle and Hazleton. Thus, the UAREP authentication shows a delay in documenting protocol change. For example, in E-33,34, it appeared that the experiment had been in progress a number of months before Hazleton's project sheets recognized the correct number of experimental animal groups. In addition, it seemed that the experiment was within a month of termination before Hazleton incorporated sheets from the Searle protocol to portray correctly the procedures to be carried out at necropsy. In E-70, the earliest Searle protocol available to UAREP was dated less than a week before the initiation of the termination of the experiments. Obviously, Searle and Hazleton did not carry out their protocol documentation with full recognition that the most minute changes in steps and procedures would be audited in detail eight years subsequently. Although E-75 and E-76, which were carried out by Hazleton several years later, also involved a considerable number of changes in the experiment plans as the work progressed, the protocol changes were somewhat easier to follow. The initial protocols for the clinical chemistry determinations on the mice in E-75 and E-76 listed numerous parameters to be determined serially during the experiment. The protocol suggested these tests be performed subject to the identification of suitable micromethods. Because reliable methods were not readily available, only a few clinical chemical determinations were done at termination of the experiment.

Statistical Procedures: Both Searle and Hazleton protocols tended to be vague as to the statistical methods to be employed. Whereas the protocols were more or less lacking in details, the Entry Books were documented

with impressive lists of names and references which, on occasion, included entire statistics books as the source for a particular method employed. UAREP particularly encountered problems in the application of a comparable life table analysis technique in which our results closely matched those of Hazleton at earlier intervals, but did not agree well at the final and important terminal periods. UAREP attempted to duplicate the methods employed by Hazleton and, on occasion, to add other especially suitable methods for comparative purposes. In general, inasmuch as UAREP comparisons produced less statistically significant differences than Hazleton, especially when using more critical methods, there was no evidence that Hazleton or Searle's biometricians were introducing bias to favor Searle.

Clinical Observations - There was generally close agreement in UAREP's validation of the clinical observations reported by Hazleton in these four experiments. This in part relates to the fact that halfway through experiment E-33,34, Hazleton switched to an INTEC computerized system for storage and retrieval of data on clinical observations, body weight, and food consumption and exceptions. Thus, for a number of parameters, a computer printout was the earliest raw data available to UAREP. The automation of handling data obviously reduces the opportunity for certain types of human error.

Body Weight, Food, and Compound Consumption - These four experiments appear to illustrate the tendency for higher consumption rates of aspartame and DKP to reduce the food consumption and body weight. It

was not always possible to demonstrate this tendency by statistical analysis of the figures. On the basis of the data available, there was little opportunity for, and no significant evidence of, UAREP's disagreement with the HLA handling of body weight and food consumption data. UAREP's analysis of the compound consumption agreed reasonably well with Hazleton's. At times our calculation of consumption for a group for one interval would differ from Hazleton's, but over the course of the total experiment, our calculations for total compound consumption were in very close agreement.

Survival Data - Although UAREP generally agreed with Hazleton regarding the absence of statistically significant differences in survival of the various groups of animals, UAREP did not derive precisely the same values for percentage survival at many of the terminal periods. There were other problems in the handling of the survival data. In E-33,34, HLA sacrificed the high dose group of female rats at 102 weeks, whereas all other groups were sacrificed at 104 weeks. Not only did the earlier sacrifice contribute to shorter survival, but HLA omitted the ten survivors at 102 weeks in their computation of mean survival time. Hence they reported 423 days mean survival instead of 602 days. The unusual situation also existed in this experiment, in which the females receiving large amounts of aspartame had significantly shorter survival, whereas the males receiving large amounts of aspartame had longer survival. This did not exist in the other aspartame experiments reviewed by UAREP. Some of the discrepancy in survival data in E-76 could

relate to the fact that HLA did not correct their data in their experimental groups for two animals whose sex was misinterpreted at the initiation of the experiments and not clarified until they were sacrificed. In E-75, the Entry Book reported six of the eight groups having mean survival times over 690 days. Since there was high mortality in many of the groups during the 720 day duration of the experiment, UAREP is unable to account for the HLA figures.

Clinical Laboratory Studies - E-33,34 and E-70 had sequential measurement of 5-6 hematologic, 15-17 clinical chemistry, and 7 urinalysis parameters with many of these being done at four to five intervals during the experiment. Both utilized groups of five rats. As animals were lost to the hematology studies, the next animal in sequence was added to the group of five. This meant that many of the rats sampled for chemistry at one period were subsequently sampled for hematology specimens. Although in theory it was planned to sample the same animal during the experiment, in actuality in some instances, it took 50 different rats over the course of the experiment to obtain the ten samples per interval measured; and in some groups, none of the rats initially sampled were resampled at the terminal interval.

E-75 and E-76 both measured five hematologic parameters at seven intervals on groups of six mice. In addition they both measured BUN, SGPT, Alkaline Phosphatase, and L-phenylalanine at the terminal interval with the additional measurement of serum insulin for E-75.

Hematology: Measurements of hematologic parameters in rodents frequently show not only biologic variation but further variability for technical

reasons which are not always easily ascertained. Thus, as one might expect, considerable variability within and between groups of mice or rats was noted. In these four experiments, transcriptional and computational discrepancies were very infrequent and altered individual figures by less than 1%. There was generally close agreement between Hazleton and UAREP when they applied the t-test to the same parameters. The largest number of discrepancies were encountered in E-75 when UAREP confirmed only 23 of HLA's 34 significant t-tests.

Clinical Chemistry: At the time these experiments were in progress, considerable variation in blood chemistries on the small volumes available from rats and mice occurred not infrequently. Such was the case in these experiments. In both clinical chemistries and hematologies there was a scattering of statistically significant differences in various parameters and among the various groups. UAREP agreed with Hazleton's interpretation that, under the conditions of these experiments, these differences were neither dose nor compound related. Not only were there problems in obtaining adequate amounts of blood to run all of the biochemistries, but this problem was so severe in some of the serum determinations on mice, that values were only obtained for one, two, or three animals per group, which would make significant comparisons impractical.

In the protocol, the measurement of serum L-phenylalanine was indicated as a monitor for the absorption of aspartame, since it is one of the main metabolites. L-phenylalanine was measured only at the

terminal interval so there was no opportunity for earlier comparative measurements. The fact that it was not reported to be increased in the high dose, aspartame fed rodents was not discussed in the Entry Books. In E-75, the number of samples in the groups was especially small, but UAREP was able to combine the results for males and females and show a statistically significant increase in the values for serum L-phenylalanine in the high dose mice.

Urinalysis: Seven parameters were measured for urinalysis at intervals during the experiment in rats. In mice, because of the scant amount of urine, it was obtained for study only terminally from the urinary bladder. Because of the type of urine measurements, data were not suitable for statistical analysis. In these experiments, there appeared to be no significant consistent changes in the urine of rats or mice eating aspartame or DKP. The only problem noted was an occasional transcriptional discrepancy from the earliest raw data to that reported in the Entry Book.

Ophthalmoscopic Observations - Although a number of the old rats and mice had a moderate incidence of lenticular opacities, there was no significant predilection for such changes in any of the treatment groups. There were no problems in validating these data except for an occasional transcriptional discrepancy. It was customary for Hazleton to replace mice or rats within the first four weeks of the experiment and assign them the number of the animal replaced. Such changes were not noted in the Entry reports. Two mice were replaced because of

cataracts in the fourth week of experiment E-76, although it was stated that all animals examined were normal.

Necropsy Studies - The plans for necropsies called for a complete examination with preservation of many tissues for potential histopathologic study. The necropsies were done by laboratory staff who were said to be experienced and to be under the supervision of a pathologist. Over the course of the experiments, it was necessary to use more than one person to perform necropsies being done throughout the week and on week-ends and holidays. Hazleton used a prosector and a recorder, both of whom initialed the necropsy sheets. It was evident that a considerable number of people participated in the necropsies which could contribute to variability. On the basis of the information available to UAREP, it appeared that the necropsy records were reasonably good. UAREP's validation of the transcription of organ weights and organ to body weight ratios, as well as the computation of means and statistical significance, generally showed good agreement with Hazleton's figures.

Histopathology - UAREP felt that the validation of the diagnoses on the 35,000 tissue sections for these four experiments, was an important, major undertaking. The initial diagnoses were made by pathologists experienced in the diseases of the species involved. They had copies of the necropsy sheets, but not of the diagnoses made by Experimental Pathology Laboratories (EPL) with whom Hazleton subcontracted. Lesions were graded in the same manner as done by EPL. An attempt was made

to use terminology which could be equated to that used by EPL and to de-emphasize certain minor or inconsequential lesions in which one might expect substantial variation from one pathologist to another, as to whether or not they chose to record their diagnosis. Special attention was devoted to the recording of the diagnoses of all malignant and benign tumors as well as proliferative lesions which may be suspected of progression to neoplasia. Considering the large number of tissue sections and the numerous diagnoses which may be applicable to some organs, UAREP felt that there was good correlation between their diagnoses and those of EPL. As expected, some discrepancies did exist. More than 99% of the slides diagnosed by EPL were available for UAREP review. The discrepancies in diagnosis and the occasional missing slides had no predilection for any of the animal groups, or organ systems.

In computations of tumor incidence, following the application of comparable methods of life table analysis, UAREP encountered difficulty similar to that found in the survival data in obtaining precisely the same results at the terminal intervals. Of the increased incidence of tumors observed, UAREP agreed with HLA that the control mice in E-76 had a higher incidence of benign tumors than in low dose DKP treated mice. Of the 12 increased incidences of tumors reported by Hazleton in these four experiments, this was the only one confirmed by UAREP. It is a remarkable coincidence that the higher tumor incidence was either in controls or in animals receiving less aspartame or DKP. Certainly there was no evidence that either aspartame or DKP enhanced the production of tumors in these experiments.

In E-33,34 and E-75, a summary comparison of diagnoses of non-neoplastic diseases was made. There was good agreement between EPL and UAREP.

On one segment of E-33,34, UAREP undertook a detailed time consuming analysis of serial correlation of clinical observations as compared with gross findings reported on the necropsy sheets and histopathologic diagnoses. This showed reasonably good correlation and UAREP recognized that some failure to correlate could be expected to occur without necessarily reflecting on the accuracy of observing and recording by employees of Hazleton or EPL. UAREP's review of the precision with which Hazleton and EPL staff followed the specifications of the protocol for necropsy procedures and sectioning of tissues indicated that this was generally well done.

The problem of animal tissues not being available for histopathologic examination seemed to be more evident in the mouse studies, than in the rat or dog histopathologic studies. Six of eight groups in E-75 had 10 to 27% of the mice without tissues available for histopathologic examination. In E-76, there were 24 animals whose tissues were not available, either because the animals were recorded as missing during the experiment, or autolysis was considered too advanced to warrant preparing sections for histopathologic examination. Such situations are at times, difficult to control although one prefers to avoid them whenever possible. UAREP has no reason to feel that the loss of these animals which were scattered through all the groups, would significantly change the results of histopathologic examination.

E-86 (Chapter VIII) - A Supplemental Study of Dog Brains from a 106-Week Oral Toxicity Study and E-87 (Chapter IX) - A Supplemental Study of Rat Brains from Two Tumorigenicity Studies

Because of the possibility that there might be an increased incidence of brain tumors in dogs in studies E-28 and in rats in E-33,34 and E-70, additional sections of brains were cut and reviewed by Searle's neuropathology consultant, Dr. J. R. M. Innes. UAREP convened a panel of neuropathology experts to review the neuropathology materials for these two studies.

On E-86, UAREP's expert panel agreed completely with Dr. Innes that there were no brain tumors present in the dog sections examined, nor other significant pathologic lesions relating to the treatment received. The presence of certain normal structures in the dog which might be confused with neoplasms were discussed by both Dr. Innes and the UAREP panel. UAREP's review of the neuropathology slides on the rat brains from E-33,34 and E-70, generally agreed well with that of Dr. Innes. The 20 brain tumors diagnosed showed no statistically significant increase in any group when the tumors for the two experiments were combined.

E-9 (Chapter X) Toxicological Evaluation of Aspartame in the Neonatal Rat

Groups of five male and five female newborn rats were sacrificed at 5, 15, and 21 days with analysis of five hematologic parameters and six chemical tests. Although data was variable, the white blood cell counts were statistically significantly depressed in the treatment groups. Although white blood counts were corrected for nucleated blood cells on

the laboratory sheets, the uncorrected figures were used in the Entry Report. This did not significantly affect the outcome of the statistical analysis.

UAREP lacks information to evaluate the exposure of these newborn rats to aspartame. We presume the mother's milk at most, could only contain the split peptides, L-phenylalanine and L-aspartic acid and that the baby rats might well not start to eat the aspartame diet until approximately two weeks of age. UAREP agreed with the EPL histopathologic diagnosis of nuclear changes in renal tubular cells in the 15 and 21 day old rats, which apparently related to aspartame exposure.

E-11 (Chapter XI) Two Generation Reproduction Study in Rats

This study was designed to characterize the effects of aspartame in the reproductive performance of the same strain of rats as used in other HLA-Searle experiments. UAREP's validation agreed with the findings in the E-11 report. UAREP noted that the consumption of aspartame was from 25 to 38% lower than planned at certain stages of the experiment. Otherwise, fewer discrepancies or problems were noted in this than in most of the other reports reviewed by UAREP.

E-19 (Chapter XII) - A Sweetening Agent: Endocrine Studies

This report covers a battery of screening tests (seven hormone related tests and six physiologic response tests) which were used routinely by Searle for compound clinical testing. Some of these routine tests appeared to be carried out with less precision or documentation than some might desire. Protocol design is not always clearly

indicated and some data were collected over a wide range of time with variable numbers of animals and substantial variation in data results. UAREP encountered some difficulty in reconstructing and interpreting the results of these routine tests.

E-88 (Chapter XIII) - Experiments in Mated and Pregnant Rhesus Monkeys;
A Compilation of Available Fragmentary Data

This fragmentary, incomplete material was gathered from the laboratory of Dr. Harry Waisman, University of Wisconsin, after his death and submitted to FDA by Searle in their "effort to make available all technical information regarding aspartame. . . whether scientifically meaningful or not." Searle informed the FDA that the E-88 report was based on fragmentary data developed by Dr. Waisman independently of Searle and without Searle's involvement in the design of the study.

UAREP felt that this report was without design, inconsequential, and based on woefully inadequate and confusing data from an inadequate number of animals.

E-90 (Chapter XIV) - An Evaluation of the Embryotoxic and Teratogenic
Potential of Aspartame in the Rabbit

These experiments, involving 300 female rabbits, studied the effects of administering by gavage, aspartame and its metabolites, L-phenylalanine and L-aspartic acid, during fetal organogenesis in pregnant rabbits. The dosage level of administration of the dipeptides was intended to be, but was not, equivalent to that recorded by the highest aspartame dose

group. Without prior knowledge of Searle findings, a UAREP teratologist authenticated the findings in the skeletal system of the cleared, whole fetuses. Seven of the 10 discrepancies UAREP found were minor malformations occurring in fetuses which were previously reported by Searle to have other malformations. Although it was not possible to examine the fetal body cross-sections because of their altered condition, UAREP felt that its validation of the skeletal specimens was adequate indication of the accuracy and significance of Searle findings.

A number of discrepancies in recording and/or reporting data on food consumption and some discrepancies in transcription of body weights were noted by UAREP. UAREP's analysis of statistical significance of food consumption agreed with Searle's in 76% of the comparisons. There was complete agreement by UAREP in the statistical evaluation of body weight data.

GENERAL DISCUSSION

Problems and Limitations in Validation Study

Although Searle and Hazleton turned over voluminous data, including the original raw data records, there were many instances in which the earliest recorded data were not available. For many types of data from Hazleton Laboratories, the earliest available information was a computer print-out. There were other instances in which data from the animal quarters or laboratory were presented in what appeared to be summary sheets in the same handwriting, although the data may have been collected over weeks or months. Much of our modern automatic chemical analyzing equipment prints out data on voluminous charts. It is customary to copy the pertinent parts of these data and discard the charts. All that UAREP could do was to check the earliest data made available to it with the data in the Entry Books.

Standards of Comparison

Laboratory methods for evaluation of the long-term effects of chemicals have been rapidly evolved over the past ten years, along with the development of standards for laboratory practice. At the time the Searle studies were performed there were few promulgated standards for this type of laboratory work. UAREP performed a meticulous validation of 12 Searle studies using methods of analysis and interpretation common to research laboratories circa 1969 to 1973. We have therefore

used this for a basis for all conclusions presented in this report. In the future, it is possible that standards for performing studies of the type that UAREP performed here will also be developed and promulgated.

Changes in Personnel, Facilities, and Methods with Elapsed Time

There have been many changes in personnel, facilities, and laboratory technology since these experiments were done. The great majority of the staff that carried out these studies is no longer employed by Searle or Hazleton. The scientific personnel with whom UAREP talked during visits to their laboratories, exhibited good knowledge of their work and responsibilities.

Animals and Animal Care

In biologic experiments such as those under review, the quality of the animals employed and the care which they receive is of critical importance to good research. The animals used in these experiments were obtained from standard commercial sources and are therefore presumed comparable to those generally in use at the time of the experiments. The animal care areas have been remodeled at both Searle and Hazleton in accordance with the recent trend toward progressive improvement. This makes it difficult to presently judge what the precise conditions were then. The American Association for Accreditation of Laboratory Animal Care carries out the most thorough and critical nation-wide evaluation of animal care facilities. The fact that both Searle and Hazleton had such accreditation while performing these experiments would indicate that their facilities were far above the average and would be considered

quite adequate for that time. Currently accepted quarantine procedures for laboratory animals were less frequently practiced with equivalent care ten years ago. The separation and holding of animals for only six or seven days to observe their general health before placing them on experiment, or the combining of rodents from more than one experiment in the same room, were not uncommon ten years ago. Neither of these might be considered desirable by today's standards.

The randomization of animals being placed on an experiment can be an important part of experiment design. Hazleton used a randomization by weight method, which although not explained in the Entry Books, was recounted during personal visitation, and would appear to be quite acceptable. The randomization of dogs by Searle in E-28 was poor since there was some concentration of litter-mates and dogs of comparable weight in the same experimental group.

Protocols

The standards regarding protocols have varied not only with time, but with different segments of the scientific population, such as the regulated pharmaceutical industry. The earlier Searle protocols such as the one for E-28, would not serve as a model by today's standards. As mentioned earlier, possibly because all prior copies of documents were not preserved, on several occasions, UAREP was only able to identify a fully documented protocol just before the termination of the experiment. In other words, some of the protocols appeared to serve more as a record of what had been done than of what was to be done. Nevertheless, the

scientists seemed to know what they were doing, because the experiments carried out reflected the procedures subsequently revealed in the protocol. Presumably because such business was transacted by telephone, and written memoranda documenting such protocol changes were not available, in the larger, more complicated experiments in which there were numerous changes in experiment design, one was generally not able to document each and every change that was made in the files of both Searle and their subcontractor, Hazleton.

Data Production, Handling and Storage

UAREP's assessment of the accuracy of data handling produced a very small incidence of transcriptional discrepancies. UAREP failed to agree with Searle and Hazleton on less than 1% of the computations and in most instances the differences were small in magnitude.

As explained in Chapter II, UAREP fully recognizes the existence of a number of widely accepted methods of rounding numbers and makes no pretense that its choice is necessarily the correct one. Although in terms of interpreting the final results, it generally makes no difference whether one uses only one method or any combination of methods, UAREP feels that it is generally better to use one method throughout an experiment. This was usually, but not always, done in the experiments of Searle and Hazleton.

Among the infrequent examples of problems in Searle's data were the failure to apply the correction for the high hematologic readings made

with the Coulter Counter in some parts of E-28. Also, in experiment E-9 Hazleton personnel copied the uncorrected leukocyte counts from the data sheets rather than the figures corrected for nucleated red blood cells. In one instance, computing mean survival time, Hazleton overlooked including 10 animals in the group that lived to the time of sacrifice.

Statistical Analyses

The problems relating to statistical analysis have already been discussed as they pertain to E-33,34, E-70, E-75, and E-76. UAREP's study and use of not only the t-test as employed by Searle and Hazleton, but Analysis of Variance in conjunction with the Newman-Keuls and Least Significant Difference evaluations, showed that various statistical methods operating in somewhat different manners can give quite different interpretation of statistically significant differences. We repeatedly saw that the Newman-Keuls Q test was more critical than either the LSD or t-test and showed less frequent statistically significant differences. Although the write-up in the various Entry Books is generally quite clear as to whether the authors regarded results as being statistically significantly different, the specific details as to the methods employed to arrive at this conclusion, were, in UAREP's opinion, often vague.

Histopathologic Findings

UAREP felt that in some respects the review of the histopathologic slides provided a better basis for validation than for some numerical data, as for example, where the earliest available information was the

computer printout. For the histopathologic slides, the full information on which the original pathologist based his diagnosis was still available. Not only was the agreement in diagnoses generally good in the four larger studies in which EPL made the original diagnosis, but there was also good agreement in the smaller study in E-9. Inasmuch as EPL employed a system of grading the severity of lesions, there was a much better basis for comparing diagnosis than in the E-28 dog study with the initial microscopic diagnosis by Microscopy for Biologic Research. Here, the lesions were only indicated as present or absent. Although there were only two outright discrepancies in diagnosis of the slides in E-28, there were many instances in which MBR indicated a diagnosis in which the UAREP pathologist described the presence of the cells constituting the background for the MBR diagnoses, but did not personally feel that the degree of change was sufficient for the diagnoses to be made.

Autolysis of tissues was certainly present in both the rat and mouse slides from HLA. UAREP pathologists did not feel that it was sufficiently severe to materially interfere with making diagnoses on all but a few slides.

The two mouse experiments both had a higher incidence of animals whose tissues were not available for histopathologic examination than one might desire. The absences were caused by both animals that were missing during the course of the experiment and animals that were considered too autolyzed to cut sections, even though the carcass and organs were preserved. Although this involved more animals than one would

desire in these two experiments, it was UAREP's opinion that the absence of these relatively few animals did not produce a bias in their interpretation of results.

Some UAREP scientists expressed concern that the incidence of up to 50 to 85% tumors in some of the groups of old rodents sacrificed at termination of the experiment, might give such a high background of tumors that it would make it difficult to recognize a mild carcinogenic action. On the basis of a National Research Council Conference, the experiments in question were modified to extend the experiments until only 25% animals survived in one of the control groups. The life table method of analysis attempts to consider the actual time of production of a tumor, rather than only determining the number of tumors of a given type present at the time the animal died or was sacrificed. This correction is certainly better than none, although the fact is that the great majority of internal tumors in rats and mice are not recognized until the time of death.

Teratologic Observations

The teratologic studies are another example in which original data can be preserved for subsequent examination and evaluation. On those cleared specimens evaluated by UAREP, there was generally close agreement between the original Searle findings and those of UAREP.

Factors Producing Spurious Results

There was obviously considerable variation in the results within and between groups when measuring variability and statistical differences. In small rodents, some tests are difficult to carry out with the usual precision. When applying a test of statistical significance at the 5% level, to 100 comparisons, one would expect that an average of five comparisons would appear to be significantly different, when in actuality, they were not. In many parts of these experiments, there were spurious statistically significant differences that were not related to biologic response to treatment. Many of the old animals were obviously in poor health and had tumors or advanced renal or liver disease. When UAREP compared the mean values for hematology and clinical chemistry determinations in two groups of control mice (E-75, E-76), which were obtained from the same source, maintained under similar circumstances on experiments beginning within a few weeks of each other, there were substantially more statistically significant differences than one would expect at the 5% level. In all such situations, UAREP lacked information on which to determine the relative role of biologic variation in animals; variation in obtaining, transporting, storing, and analyzing for constituents; and sample size, experiment design, or other factors. From their own experience, some UAREP scientists feel that variability in technique and inherent problems of handling small samples from rodents contributed to getting less uniform results.

Magnitude and Consequence of Discrepancies Observed

In each of the following chapters, UAREP has assessed the accuracy and reliability of these Searle studies without comment on experiment design. Although a substantial number of minor and inconsequential discrepancies were noted in UAREP's validation studies, there were few, if any, discrepancies which would produce a change of greater than 5% in the final numerical data being compared. UAREP is unaware of other authentication reviews carried out in comparable scope and detail. Therefore, since established guidelines do not exist, UAREP leaves it to each reader to apply his own criteria as to the importance of the degree of precision and consistency in carrying out experiments.

Possible Intent to Mislead or Deceive

UAREP did not find evidence that, given the experiment design, there was any indication that animals in any one group had been treated deliberately to produce biased results. The discrepancies we observed appeared randomly distributed between treated and control groups of animals.

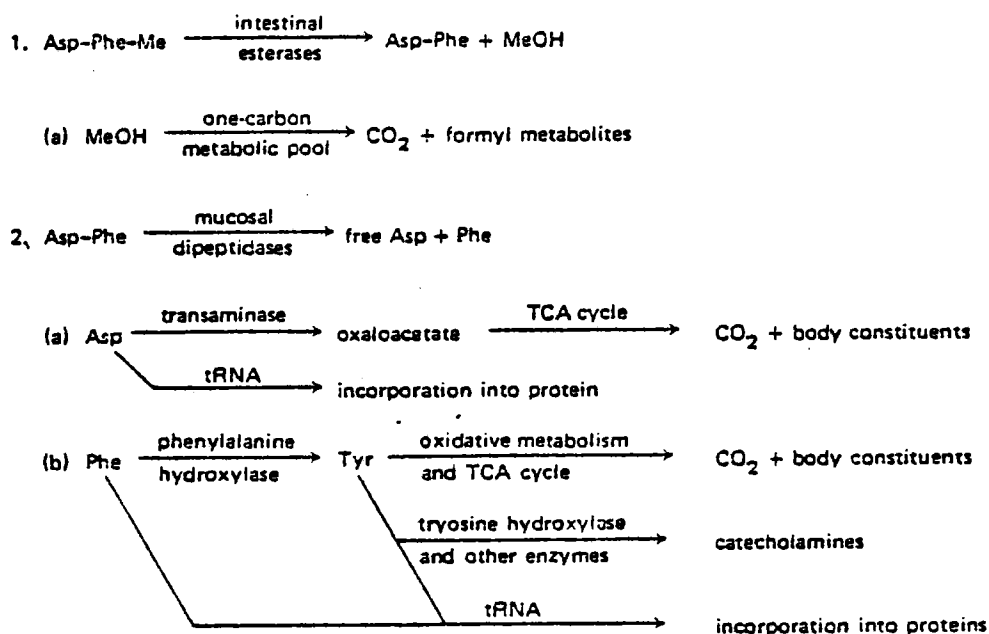
CHAPTER II

GENERAL INTRODUCTION

Background on aspartame and Searle

More than fifteen years ago, scientists at Searle Laboratories, a division of G. D. Searle and Co., accidentally discovered aspartame as a sweetening agent. Their petition to the Food and Drug Administration (FDA) to market aspartame as an artificial sweetening agent was provisionally approved, but approval was withdrawn by the FDA before it was marketed. This action was stimulated by statements from Dr. John W. Olney and Mr. James S. Turner and by questions raised by the FDA regarding some of the laboratory practices employed by Searle. The FDA carried out two on-site evaluations of the laboratory practices at Searle and some of its subcontractors, and it requested that Searle have a mutually satisfactory third party do a detailed, in-depth authentication of 12 selected aspartame studies which had been submitted by Searle to FDA. These 12 studies were selected because either the FDA or Olney and Turney felt that they had some inherent problems, and they are not necessarily representative of other Searle studies. Universities Associated for Research and Education in Pathology, Inc. (UAREP) was selected by the FDA and Searle for this task. Since Searle, rather than the FDA was responsible for paying the cost of the study, they contracted with UAREP under terms generally agreeable to FDA on August 19, 1977. (See Appendix II-1 and II-2.)

Aspartame is a nutritive artificial sweetening agent that has organoleptically about 180 times the sweetness of sugar. In the metabolism of this dipeptide, extensive degradation of the compound may occur after it enters the digestive tract. Although little change would be expected in the stomach, in the small intestine chymotrypsin would be expected to hydrolyze the methyl group to methanol and the peptide hydrolases of the microvillar membrane would cleave the dipeptide to its constituent amino acids L-aspartic acid (41%) and L-phenylalanine (55%). (5,6) Methanol is converted primarily to CO_2 . Aspartic acid is converted in the monkey intestine to CO_2 and is incorporated in protein and enters into purine and pyrimidine biosynthesis. Phenylalanine is metabolized differently so that the amino acid is incorporated directly into body protein to a great extent or metabolized to CO_2 and only 20 to 25% of the phenylalanine is excreted.

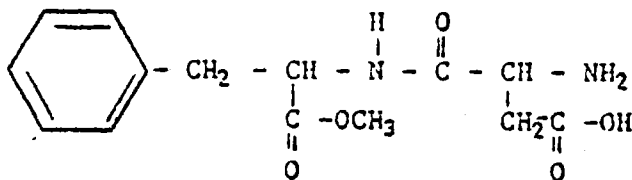


Aspartame Metabolic Pathways (6)

Searle compound 19192 is a diketopiperazine (DKP) which is formed as an in vitro conversion product of aspartame under certain conditions. The formulae and synonyms for these two compounds are:

A. aspartame

1. SC-18862



2. APM

3. Protid

4. aspartyl phenylalanine

5. L-aspartyl-L-phenylalanine
methyl ester

$C_{14}H_{19}N_2O_5$
MW 294.30

6. 3-amino-N-(α -carboxy phenethyl)
succinamic acid methyl ester

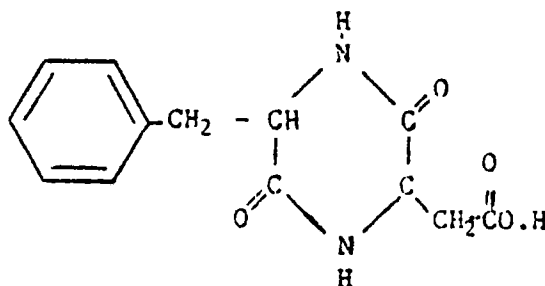
B. diketopiperazine of aspartame

1. SC-19192

2. DKP

3. diketopiperazine

4. 5 - benzyl - 3,6 - dioxo
- 2 - piperazineacetic acid



$C_{13}H_{14}N_2O_4$
MW 262.258

Aspartame was the compound being evaluated in 11 of the 12 Entry studies submitted by Searle to the FDA (Appendix II-3). DKP and aspartame were both evaluated in E-19, and DKP alone was tested in E-76. In E-90, in addition to aspartame, the dietary amino acids, L-phenylalanine and L-aspartic acid which are the principle constituents of aspartame, were also tested. All Searle preparations of aspartame evaluated contain small amounts of diketopiperazine, generally less than 2%.

Universities Associated for Research and Education in Pathology (UAREP) - was established in 1966 as a consortium of universities for the purpose of promoting research and education in pathology. It has served as a focus for projects between governmental and private agencies, as well as among institutions in the private sector. It has sponsored a variety of conferences on pertinent research and education projects, sponsored research projects, and published articles, monographs, books, and atlases. The nine universities which are members of the consortium, as well as a list of the directors and officers, are shown in Appendix II-4. UAREP has its offices on the campus of the Federation of American Societies for Experimental Biology, 9650 Rockville Pike, Bethesda, Maryland 20014.

The UAREP directors became interested in this data validation study because they felt it represented both an opportunity to serve the public and to gain insight into the problems of pharmaceutical firms and federal regulatory agencies, a situation which UAREP hopefully might help to improve.

UAREP-Searle-FDA Relationships - UAREP applied its knowledge and skills without bias toward any of the main interested parties, such as G. D. Searle and Company, the Food and Drug Administration, or any party to the proposed Board of Inquiry including James S. Turner and Dr. John W. Olney. UAREP staff maintained, as requested, a log of all contacts with these parties. UAREP named Dr. Robert E. Stowell as a Principal Investigator for its studies. The principal contacts for Searle Laboratories were Dr. Robert Bost and Dr. Fred McIlreath; and for the FDA the contact was Mr. Taylor Quinn. In order to avoid the potential for any unwarranted criticism that communication from Searle or FDA would in any way alter the nature or the scope of the final report, on August 21, 1978, Dr. Endicott was named to replace Dr. Stowell as UAREP's liaison contact person with Searle and the FDA during the terminal phases of report preparation.

The contract between Searle and UAREP further provided that "Persons employed or otherwise engaged for pay with UAREP to perform authentication and review functions contemplated by this contract shall be free of any financial interest in, or any other relationship, past, present, or anticipated with Searle, any manufacturer or distributor of a product directly competitive with aspartame, or any party to the proposed Board of Inquiry relating to aspartame, which would create, or appear to create, the potential for influencing UAREP's review of the submitted reports or the report of its findings." (See Appendix II-2.) All persons employed or otherwise engaged for pay by

UAREP relative to this project signed statements agreeing to maintain the confidentiality of any confidential information relative to this project. They also signed statements indicating they had no present or contemplated conflict of interest with any of the principal parties concerned with this contract. (See Appendix II-2.) The contract further indicates that UAREP shall make no statements relative to the safety of aspartame for human consumption nor concern itself with the design of any of the experiments that have been undertaken by Searle.

When UAREP met with Searle and FDA (Appendix II-1) only the state of progress toward completion of the study was discussed, not the nature of UAREP findings. All financial arrangements between Searle and UAREP were handled through the UAREP office in Bethesda, Maryland, and there were no direct discussions regarding finances with other UAREP employees.

Although independence from Searle was maintained during the study, it was necessary to obtain the materials and information from Searle and their subcontractors. Certain procedural questions regarding conduct of the study and preparation of the report were addressed jointly to Searle and FDA. As mentioned above, UAREP staff and employees maintained strict confidentiality and were free from any recognizable conflict of interest. Three members of the UAREP Board of Directors who had potential conflict of interest did not participate in this study or in any voting by the UAREP Board, relative to this study, or in the preparation of this final report.

UAREP Approaches and Methods of Authentication of Data

FDA Entries involved in the authentication - Of the original 15 entries listed in the contract (Appendix II-2, pg 2), E-77 and 78 were deleted from the study because an independent review was carried out by the FDA, and E-5, E-53, and E-89 were deleted because that work was said to be superseded by E-90. The remaining 12 entries, the authentication of which was undertaken by UAREP, are shown in Table 2-1. The total number of entries submitted by Searle to the FDA in support of its petition for marketing aspartame approximated 100.

Available information - UAREP was supplied with multiple copies of the Entry books which Searle had submitted to FDA and copies of all existing relevant background information on the experiments, results, statistical analyses, and preparation of reports, as well as the histopathologic slides which had been reviewed for pathologic diagnoses. (See Table 2-1). In addition, Searle supplied approximately 3000 sheets of general and miscellaneous background information. Available for inspection by UAREP, but not utilized systematically, were the paraffin blocks from which the tissue sections had been cut, and in many cases, fixed animal tissues comparable to those which had been imbedded in paraffin.

All histopathologic slides were transported personally by UAREP employees from Searle Laboratories in Skokie, Illinois, or from Hazleton Laboratories America in Vienna, Virginia, to the UAREP institution where the histopathologic review was accomplished. (Table 2-1)

Table 2-1
Scope Of Work

FDA Entry No.	Abbreviated Title	No. E Book Pages	Date Searle Submitted	Pages Background Material	Animals	Tissue Section(s)/ Specimens Reviewed by	Report Chapter
<i>Long Term Toxicity Studies</i>							
28	106 wk oral toxicity dog	217	8/72	5300	40 dogs	1430 sections UCD	III
33,34	2 yr toxicity study in rat	495	1/73	4900	420 rats	13,800 sections UM	IV
70	Lifetime toxicity study in rat	330	1/74	1600	280 rats	7450 sections UM	V
75	104 wk toxicity study in mouse	361	9/74	1830	360 mice	6950 sections NMU	VI
76	110 wk toxicity study in mouse (DKP)	352	9/74	1875	360 mice	7400 sections NMU	VII
86	Supplemental study dog brains, E-28	20	2/75	20	E-28	In E-28	VIII
87	Supplemental study rat brains, E-33,34	17	2/75	20	E-33,34 & E-70	In E-33,34 & E-70	IX
<i>Other Studies</i>							
9	Toxicological Evaluation of neonatal rat	106	1/72	440	160 rats	470 UCD	X
11	2-generation reproduction study in rats	33	10/72	590	108 rats	In E-9	XI
19	A sweetening agent: Endocrine studies	30	11/72	335	390 mice 79 rabbits 735 rats 55 hamsters	None	XII
88	Experiments in mated & pregnant monkeys	30	6/75	100	8 rhesus	UCD	XIII
90	Embryologic and teratologic potential in rabbits	213	10/75	1400	302 rabbits 1600 embryos	1600 specimens UC	XIV
TOTALS:		2204		Miscellaneous 3000 21,400	4897	39,100	

KEY: UC = University of Chicago
NMU = Northwestern University
UM = University of Maryland
UCD = University of California, Davis

Data and background materials were shipped from Searle Labs in Skokie, Illinois and from Hazleton Laboratories America in Vienna, Virginia to the University of California at Davis, California. As all materials were being copied before shipment, they were subject to FDA inspection in order to ensure that copies of all documents were provided to UAREP. This extensive transport of documents and slides was accomplished without any damage to the materials. Problems relative to a few missing slides will be discussed in the respective chapters. Some of the materials relative to E-28, which were from notebooks and laboratory manuals with faint lead pencil markings, were unsatisfactory for photocopying. Therefore, one of the UAREP staff visited the Searle Laboratories personally to copy the illegible material. On request, both Searle and Hazleton Laboratories supplied some additional materials to UAREP. At the time the FDA seals were removed from Searle materials at Skokie, Illinois or Vienna, Virginia, UAREP seals were immediately affixed.

Searle Installations and Subcontractors - As shown in Appendix II-3, the major part of the experimental research in these entries was subcontracted to Hazleton Laboratories America of Vienna, Virginia (HLA). They in turn subcontracted to Experimental Pathology Laboratories (EPL) in Herndon, Virginia, for the histopathologic examination of all the slides on E-9, E-33,34, E-70, E-75, and E-76. Searle subcontracted

through EPL for Dr. J. R. M. Innes to review the materials in E-86 and 87. Tissues for histopathologic slides on E-75 and 76 were processed and sections cut in the laboratories of EPL. Observations on E-88 were carried out at the University of Wisconsin but not under any specific contract by Searle. UAREP did not visit the University of Wisconsin relative to E-88 because the Principal Investigator was deceased and all materials had been turned over to Searle. The histopathology slides on E-28 were diagnosed at Microscopy for Biological Research (MBR). To obtain first hand information and additional background data, UAREP staff visited the facilities at Searle Laboratories in Skokie, Illinois, Hazleton Laboratories America in Vienna, Virginia, and EPL Laboratories in Herndon, Virginia on several occasions. UAREP did not visit MBR in Albany, New York because there was good agreement in diagnosis on slides examined by MBR and UAREP and there had been significant changes in staff at Albany. Because of their fragile nature, the 1600 embryo specimens on E-90 which were housed at the Searle Laboratories were reviewed in their laboratories by UAREP personnel. (Dr. Anthony Steffek)

When Searle subcontracted with Hazleton Laboratories to carry out experiments, Hazleton prepared the report in a form which was suitable for submission to the Food and Drug Administration. When this report was received by Searle, they forwarded such reports to the Food and Drug Administration. In connection with their histopathologic review and diagnosis of microscopic slides, Experimental Pathology Laboratories prepared a detailed report which they submitted to Hazleton Laboratories. The EPL report was submitted without page numbers and after suitable pagination, it was included by Hazleton as a part of their report to

Searle. UAREP was provided with copies of the EPL reports as well as the earliest and latest draft of reports prepared by Hazleton and with one copy of the report as it was received by the Food and Drug Administration. These reports were checked by UAREP and meaningful changes were not identified. In one or two instances, the sections of an EPL report were shifted in sequence. No instances were found in which Hazleton changed the meaning of any report submitted from Hazleton.

Definition of numerical discrepancies and categories - UAREP used the following procedures for determining the type of numerical discrepancies:

A discrepancy in "N" numbers (N) occurs when the UAREP determination of the number of animals used per treatment group or the number of data bits used in computing a mean does not agree with the corresponding number in the Entry Report.

A computational discrepancy (C) occurs when the UAREP calculation of a mean, standard deviation, etc., does not agree with that tabulated in the Entry Report and the disagreement is not due to rounding or to differences in number of significant digits.

A statistical discrepancy (ST) is a situation in which the results of a UAREP statistical analysis differs from the corresponding result tabulated in the Entry Book.

A rounding discrepancy (R) occurs when the entry in the Entry Report differs from the UAREP determination as a result of different rounding

procedures. For example, if UAREP determination of an entry is .666 and corresponding entry in the Entry Book is .66 instead of .67, then a rounding discrepancy results. In order to determine if such a discrepancy exists, it is necessary to calculate the entry to at least one more significant figure than is shown in the Entry Book.

A discrepancy in number of significant figures (SF) occurs when the entry in the Entry Book shows (after rounding) a different number of significant digits than that determined by UAREP validation. For example, for a series of body weights from necropsy sheets of 32.90, 32.00, 30.90, and 30.30, the mean calculated by UAREP is 31.53. This rounds to 31.5. If the corresponding figure in the Entry Book is 31.530, then there is a discrepancy in the use of significant figures.

A discrepancy in transcription (T) occurs when datum in one location (i.e., clinical lab sheets, necropsy sheets) is transcribed elsewhere as a different value. For example, if a datum on a clinical lab sheet is 15.13 and shows up on a summary sheet or in the Entry Book as 16.13, then a transcription discrepancy exists.

It is apparent that these discrepancies are not necessarily independent of each other. For example, if there is a substantial discrepancy in "N" numbers of treatment groups, this could result in discrepancies in means, standard deviation, and even in the statistical analysis for that group. In instances in which it is clear that one discrepancy has led to another, only the earliest discrepancy will be recorded. Since we are dealing with a very large volume of data it is important to keep in mind the ratio of discrepancies to agreements in validation.

Rounding discrepancies - There are several methods of rounding digits on numbers that end with a 5 in the place beyond the last significant digit. Three examples are given. Each of the methods is approved by those who use it and UAREP does not contend that one is correct and others incorrect.

- (a) One convention is to round up or down to the nearest even digit, so that 164.5 becomes 164, and 165.5 becomes 166.
- (b) Another method is to round down so that 164.5 becomes 164, and 165.5 goes to 165.
- (c) The third method, used by most calculators and computers is to round up, as for example 164.5 to 165, and 165.5 to 166.

UAREP was unable to precisely ascertain any one method of rounding numbers that was consistently used by either Searle or HLA. We feel that consistency in the use of a method is more important than which method was used. UAREP chose method (a) for rounding numbers, and any one of the three methods would be expected to produce discrepancies with the other two. Further problems arise depending upon whether one is averaging rounded or unrounded numbers, and if one carries forward and considers more than one figure beyond the number of significant digits. For example, 164556 could be rounded to 164, if only 164.5 is considered, or to 165, if, contrary to usual convention, the third digit past the decimal raises the 5's progressively to 6's. Although all discrepancies which did not agree with method (a) are counted or illustrated in the Appendices of each chapter, UAREP regards most of these as prime examples of inconsequential discrepancies.

Protocol and Amendments - The contract (Appendix II-2) specifies that UAREP is to review the protocols and their amendments. Searle refers to its experiment design specifications as "protocols" and as its amendments are incorporated, the appropriate number is circled on the top line of the first page. On transmittal of revised protocols, recipients were requested to update their files by discarding earlier versions. At times, memoranda addressed to the members of the Searle protocol committee, with an information copy to the Hazleton Project Manager, specified changes in the experimental design. Hazleton used the term "Project Sheet," with the initial experiment specifications designated as Project Sheet no. 1, and amendments as sequential project sheets 2, 3, etc. When major revisions were made in a project, they also requested on the sheet that previous project sheets be destroyed. Although requested, UAREP was not able to obtain copies of the original protocols in those experiments in which the protocol had been amended and updated by being rewritten.

General Methods of Review of Numerical Data - Rather than checking each step in the handling of data, we chose to seek the earliest raw data available for comparison with the first typed data in the Entry report. These earliest data in the Entry Report were then used to check against the various summary tables. In many instances, the earliest raw data were no longer available, since it was many years after the experiments were performed. For example, the voluminous paper recordings of automated chemical analyses are generally discarded after the significant

figures have been transferred to other records. As the technology of data recording advanced with the progress of the various experiments, there was increasing use of computers with semi-automatic or automatic data input into an INTEC computer system, or other computer systems. Thus, in the case of the later experiments carried out at HLA, a computer print-out was frequently the earliest recorded data. At times, the computer output was actually reproduced in the Entry Report submitted to FDA. Although malfunction of computers does occur, it seldom goes unnoticed and UAREP did not consider it a profitable expenditure of time to spend large amounts of time checking the accuracy of the computers used by HLA. In general, UAREP chose to check in more detail the earliest and latest experiments carried out at Searle and at HLA. This comparison might reflect differences in methodology of handling data as well as inherent changes in accuracy and consistency resulting from differences in methodology and management.

UAREP checked all parameters which were included in the Entry Books submitted to FDA or were called for in the protocol and its amendments. This included the method of randomizing of animals included in the experiment, body weights, weight gain, food and compound consumption, clinical observations and ophthalmoscopic observations, clinical and special chemistries, hematology determinations, urinalysis, necropsy findings, organ weights, body to organ weight ratios, and histopathologic findings. Tables and graphs were reconstructed using the Searle or HLA data as well as any revised data developed by UAREP and any significant variations reported. The persons checking data did not know the answers for which they were making comparisons of accuracy. Since it has been

said that "to err is human" all discrepancies noted between Searle or HLA figures and UAREP figures were double, or at times, triple checked by different individuals.

In the interests of brevity, this report does not reflect all of the computations and analysis of results, or graphs, summary tables prepared by UAREP. Although UAREP checked all available data and reconstructed the handling of data, results and their analysis, we did not choose to reproduce all of the data in the Entry Report filed with the FDA with which we completely agreed. Drafts of graphs and other data not included in this report will be maintained by UAREP for a reasonable period of time.

Review of histopathologic diagnosis - UAREP pathologists reviewing slides were given the same information as the original slide reviewers, namely basic information regarding which animals belonged to which experimental groups, and the gross findings as indicated on the necropsy sheets. Pathologists working at the University of Maryland School of Medicine, Northwestern University School of Medicine, and University of California at Davis, School of Medicine and School of Veterinary Medicine, initially reviewed the histopathologic slides without any knowledge of any prior histopathologic diagnoses. Their diagnoses were then compared with those made by EPL or MBR, by third parties geographically removed from the site of the review of slide diagnoses. Discrepancies between EPL and UAREP diagnoses were reviewed by a panel of three or more UAREP pathologists who submitted a consensus diagnosis and indicated any minority opinions. In the final checking of the tabulation of their diagnoses, the UAREP pathologists were provided a copy of the EPL diagnoses and occasionally a UAREP diagnosis was changed at this time.

Histopathologic slides representing certain problem areas in the pathology of laboratory animals were reviewed by a special panel of expert pathologists of national stature. Their review included glial tumors of the central nervous system, (see Chapters VIII and IX) and hyperplasias and tumors of the pituitary, thyroid, and the adrenal glands. Fortunately, the perplexing problem of liver tumors and related non-neoplastic conditions in rats (10) and mice (12) have been the subject of recent national conferences.

Since it is well known that individual pathologists have their preferred terminology for certain diagnoses of the same or similar pathologic conditions, a preliminary conference was held by Dr. Robert E. Stowell of UAREP and Dr. John Ferrell, Chief Pathologist of EPL, for the purpose of discussing terminology and equivalence of diagnosis. This meeting was followed by a meeting of participating UAREP pathologists from University of Maryland School of Medicine, Northwestern University School of Medicine, and University of California at Davis, in which general agreement was reached regarding equivalence of diagnostic terminology. Minor differences in diagnostic terminology or in the degree of involvement of a lesion were not considered noteworthy. Agreement and discrepancies in histopathologic diagnoses are discussed with the review of each study. Errors produced by entering diagnoses in the incorrect column or transposing errors were counted as any other discrepancy. Other unpublished reviews have found 3-12% errors in rodent histopathology diagnoses by experienced pathologists.

Autolysis - While doing the histopathologic diagnoses, UAREP pathologists were requested to give an indication of the degree of autolysis of the tissues examined. When autolysis was present, the severity was denoted on a scale of 1 to 5 similar to that used for grading the severity of other tissue diagnoses:

1. slight autolysis
2. low degree of autolysis
3. moderate autolysis
4. moderate to high degree of autolysis
5. severe autolysis making histopathologic diagnosis difficult

UAREP pathologists noted that autolysis was present but rarely to a degree that interfered with appropriate tissue diagnoses. Only a few of the rats sacrificed at the termination of the experiment showed autolysis. Seventy-five to 90 percent of the rats that died prior to termination of the experiment had some degree of autolysis, which had an average reading of 3.4 on the scale of 5. Instances of more severe autolysis in mice dying during the course of the experiments are discussed in Chapters VI and VII.

Pituitary Tumors: UAREP defined criteria for differentiating between hyperplasia, adenoma, and carcinoma in pituitary lesions in rats. In doing this, they considered the criteria used by their own pathologists and by consultants as well as by the National Cancer Institute, and the criteria used in diagnosing human pituitary lesions. It is recognized that there may be some discrepancies in criteria applicable in different

species. In the rat, the amount of correlation of biologic behavior with the histopathologic criteria is inadequate. The same statement could be made for pituitary tumors in a number of other species.

Pituitaries in which there was a proliferation and hypercellularity of cells, which were not well circumscribed, were considered to be hyperplastic. No attempt was made to differentiate between hyperplasias or adenomas on the basis of cell type. Adenomas were considered to be lesions which were well circumscribed, composed of relatively normal appearing cells. Such adenomas were well demarcated and appeared as though they could be shelled out. Carcinomas, on the other hand, exhibited a substantial degree of cellular pleomorphism and at times, increased mitotic activity and invasive qualities. Some of these tumors consisted of cells with increased cytoplasm and in some instances, large droplets in the cytoplasm of the cells. The cells appeared to be somewhat larger and there was some tendency for the tumors to be larger. It was not considered, however, that size was a valid criteria for differentiating between the benign and malignant pituitary tumors.

In the human there is great conservatism in making a diagnosis of pituitary carcinoma. Very few such cases are diagnosed and they are essentially reportable in the literature when they occur (2, 8, 9). A close relationship between morphology and rarely observed metastases has not been noted in the human, where many of the adenomas will extend into the brain. Many pathologists would consider this more an extension by compression of the adjacent tissue rather than an active invasion.

UAREP therefore adopted criteria commonly used in judging pituitary tumors in rats, although such criteria are by no means universally accepted. It should be emphasized that these diagnoses are based on the histopathologic appearance and have not been adequately confirmed by biologic behavior of the tumors.

Tumors of the thyroid and parathyroid: Primary proliferative lesions of the thyroid involved only C-cells or parafollicular cells in E-33,34 and E-70. Because of the evidence that benign or malignant behavior is difficult or impossible to determine histologically in these neoplasms, and because such tumors often develop malignant potential, all those lesions judged to be neoplasms were termed C-cell carcinomas. This term is equivalent to medullary carcinoma of the thyroid.

C-cell hyperplasia was used for small collections of well differentiated parafollicular cells which were not well demarcated from surrounding follicular tissue and often interposed between follicles. There was no compression of the surrounding tissue and mitotic activity was not seen.

C-cell carcinomas were proliferations of C-cells which compressed or infiltrated the thyroid tissue. Some showed evidence of vascular or lymphatic invasion.

Other lesions seen included hyperplasia and adenomas of the parathyroid as well as one small lesion diagnosed as paraganglioma, which is a rare and unusual tumor.

Medullary carcinomas of the thyroid were first reported in man in 1959 (3), and large series of cases have been reviewed since then. In man, these tumors may be associated with bilateral pheochromocytomas (13), mucosal neuromas (14), Cushing's disease (11), and bilateral parathyroid hyperplasia and adenomas (7). Such lesions were not observed in the ten cases of C-cell hyperplasia or neoplasms in E-33,34 and E-70. C-cell tumors were recognized and began to appear in the literature 4-6 years ago. Medullary carcinomas in the rat thyroid were described in 1968 (1,4). Thus, at the time the slides were originally diagnosed, one might expect that EPL in 1972 might recognize medullary carcinoma of the thyroid but not call them C-cell tumors. However, discrepancies in diagnoses between UAREP and EPL are not all explainable by EPL's lack of use of C-cell hyperplasia or carcinoma. In one case (83-640) in E-33,34, and four cases (90-877, 91-037, 91-066, 91-930) in E-70, there was diagnostic discrepancy as to whether the cell of origin of the lesion was the thyroid or parathyroid. There were also significant discrepancies in diagnosis between normal hyperplasia, adenoma and carcinoma.

<u>Proliferations</u>	<u>E-33,34</u>	<u>E-70</u>
normal vs hyperplasia	83-723	90-965, 91-067
normal vs adenoma		91-937
hyperplasia vs adenoma	83-988, 83-779	
adenoma vs carcinoma	83-690	90-877, 91-037, 91-066, 91-930

UAREP regards the diagnosis of discrepancies in the thyroid and parathyroids as being based on distinct entities as described in the literature. The perception with which the pathologist recognizes such gradations of normal and abnormal proliferation of cells may be less precise in practice than in theory. In the rat and mouse, as compared with man, there may be relatively few cells to observe under the microscope, and less opportunity to observe and understand the biologic behavior of the neoplasms.

Adrenal gland diagnoses: In the adrenal cortex, hyperplasia is diagnosed on the basis of hypercellularity in areas which are circumscribed and not compressing the adjacent tissues. The cells tend to be somewhat larger, but are well differentiated. Adenomas on the other hand, apparently compress the surrounding tissue although they do not have a distinct capsule. They usually appear to be larger in size. The diagnosis of carcinoma was made primarily on the basis of the cellular pleomorphism and atypia and in some cases, increased size of the cells. It was recognized that angiectasis may at times constitute a problem in confusing the picture in the adrenal cortex.

The medullary lesions of the adrenal are somewhat more difficult to differentiate. The diagnosis of pheochromocytoma was employed for large medullary tumors composed of bizarre cells frequently with abundant cytoplasm. It was not possible to tell whether these tumors were malignant or benign on the basis of histologic appearance. The biologic behavior of malignancy in such tumors is very rarely observed. Medullary hyperplasia was used for lesions in which there were cells somewhat

larger than normal and apparently some hypercellularity. It could not be recognized whether this increased cellularity and cellular changes in any way relates to functional stimulation. The diagnosis of ganglioneuroma was based on the presence of large ganglion type cells.

Hyperplasia of the adrenal medullary or cortical cells was graded on a 1 to 5 scale as employed by EPL. At times the difference between categories of diagnoses was scarcely discernible, as for example, a hyperplasia of adrenal cortical cells grade 5 and a cortical adenoma or a medullary hyperplasia grade 5, and a pheochromocytoma. UAREP felt it was important to consider such a distinction as significant; however, because some government agencies have taken the position that the distinction between a non-neoplastic hyperplasia and a benign tumor is of critical importance, whereas they are less concerned about the distinction between a benign and a malignant tumor since they feel all benign tumors have the potential to become malignant.

In evaluating the pheochromocytomas, some showed substantial degrees of pleomorphism that one associates with malignancy in other tumors. Experience has shown, however, that this tumor, despite its histologic appearance, rarely behaves as a malignant tumor and that it is not possible to select the malignant pheochromocytoma on the basis of its histologic appearance. Therefore, we tabulated the pheochromocytomas as benign tumors.

Discrepancies in histopathologic diagnoses were classified in three groups, according to the nature of the discrepancy and its biologic significance.

1. Inconsequential discrepancies between UAREP and EPL diagnoses are those so minor that different pathologists might frequently use one or the other diagnosis. An example would be a minor difference in grading of the degree of a lesion as grade 1 vs grade 3 inflammation, on a scale of 5.
2. Minor discrepancies are those in which one would expect that most pathologists would recognize a degree of difference in a significant lesion. An example would be if one diagnosis of a lesion was slight (grade 1) acute inflammation of the lung, and the other was advanced (grade 5) inflammation of the lung.
3. Major discrepancies are those in which it is of substantial importance, in terms of a life-threatening process, that the correct diagnosis be made. An example would be if one pathologist diagnosed a malignant tumor of an organ and another diagnosed a non-neoplastic lesion.

Statistical and Biological Significance - In reviewing statistical computations, UAREP considered whether or not the methods employed by Searle were the most appropriate. If there were alternative methods which UAREP felt should have been considered, they were also used. Similarly, in evaluation of statistical significance, the method employed by Searle and their subcontractors was always used, but if other methods were considered possibly more appropriate, they were also employed and results compared. (See Appendix II-9). For example, in many instances, the Entry books compared each experimental group only with the control, whereas UAREP often compared each group with all other groups in the experiment. Not only was this considered more appropriate in seeking maximum information on intergroup and dose relationships, but it was generally in keeping with the Searle protocol instructions regarding statistics. In addition to seeking statistical significance, UAREP sometimes also independently commented on the biologic significance of data, but not the safety of aspartame for human consumption, which was contraindicated by our contract.

Analysis of significant interactions (analysis of variance, ANOVA) between test groups was performed by UAREP prior to pairing groups and analyzing statistically by the Newman-Keuls (Q) and the Least Significant Difference (LSD) methods. ANOVA indicates the exact probability that all group means are equal but based upon the F test for Analysis of Variance. UAREP applied ANOVA at a difference of $P < 0.05$. If values larger than 0.05 were obtained, the least significant difference and the Newman-Keuls test were not run. If the F statistic was greater than 0.05, we did not do the LSD or Q test because we accept the hypothesis

that (at the 5% level) all the means being compared were equal. As shown in the UAREP tables, the results of the ANOVA are indicated numerically, together with the significance of the Q and LSD tests run, and the significance of the UAREP and HLA or Searle t-test are given. The reader may then choose which of the statistical methods or combination of methods are regarded as most appropriate. In general, UAREP found less statistical significance of comparisons than HLA or Searle when the same groups were compared. This would favor one's interpretation of less likelihood of a significant biologic effect of the agent at test. It should be remembered, however, that there is no one statistical method of choice for all purposes, and that statistical significance at the 5% level can mean that if 1000 comparisons are made, that 50 would show up as significant when they were not really significant. Thus, an occasional statistical significance that is not dose-related should not be a cause for concern.

Degree of discrepancies - For purposes of this authentication, UAREP has defined degrees of discrepancies between UAREP and Searle observations as follows:

Minor and Inconsequential Discrepancies - Trivial differences which individually have no material effect upon the results and their interpretation. For numerical data, differences of 5% or less are minor and 1% or less are inconsequential. Examples of these would be differences in rounding of numbers or numbers of figures considered as significant digits.

Possibly Significant Discrepancies - This would include discrepancies of such magnitude that many scientists would disagree as to whether they made any material difference in results and especially in their interpretation. Numerical differences would be greater than 5%.

Probably Significant Discrepancies - These differences would be of an order of magnitude which the majority of scientists would feel produced a significant difference in terms of biologic interpretation of results.

Preparation, Organization, and Review of this Report. The various parts of this report were drafted and reviewed at the University of California at Davis by the UAREP Principal Investigator and his immediate staff of UAREP employees. Consultation was sought as necessary in preparing the final draft. The draft was always shown to the other UAREP investigators who had directly contributed information incorporated in the report.

The organization of the report is shown in the Table of Contents. UAREP has attempted to prepare a document which could be read and understood reasonably well by those who had some familiarity with the Entry Books submitted by Searle to FDA. Those with limited understanding of the contents of these books will need to obtain copies from Searle Laboratories or from FDA if they desire to explore the most minute details pertaining to this total project. Liberal use has been made of appendices to most of the 14 chapters, in order to provide a document

which can be easily read by those not interested in the details. Copies of drafts of various sections of this report were submitted to detailed study by an editorial group consisting of Drs. Brinkhous, Carter, Endicott, Scarpelli, Trump, and Stowell. The final total document was then reviewed by the officers of UAREP and submitted to Searle. These opinions expressed have not been altered by any statements made by any of the parties most concerned; Searle, FDA, or participants to the proposed hearing.

Total Scope of the Work Involved - UAREP and their employees have devoted 22,000 hours of work relative to this project, including the review of 2200 pages of Entry Books, 21,000 pages of background data, and more than a million bits of numerical data from experiments involving 4900 animals and the histopathologic diagnosis of 39,000 tissue sections and embryo specimens (Table 2-1). Extensive use was made of consultants, independently, and in conferences. (See Appendix II-5).

UAREP Participants - There were 60 participants in this study. The principal staff are shown in Appendix II-5.

General Discussion

Animal Care Facilities

The importance of good laboratory animal facilities is being increasingly recognized and the level of acceptable standards is rising. It is not easy to extrapolate backward five to 10 years in visiting animal care facilities to precisely ascertain conditions during the time these experiments were carried out. The animal care facilities of Searle Laboratories in Skokie were visited by consultant Dr. Dale L. Brooks and Dr. Robert E. Stowell on June 9, 1978. The present facilities for care of dogs, rabbits, rats, and hamsters were found to be satisfactory, and conversations with employees who were present during the time the experiments were carried out would lead one to believe that the facilities and care were also adequate at the time these experiments were in progress. The animal facilities at Searle Laboratories have been fully approved by the American Association for the Accreditation of Laboratory Animal Care (AAALAC) since 1968. The vivarium is also approved and inspected by the United States Department of Agriculture, Veterinary Sciences, and conforms to the Animal Welfare Act (Public Law 89-544 and Public Law 81-579). AAALAC accreditation is based on careful evaluation of overall animal care activities, and is probably the best evidence that can be obtained at this time as to the level of animal care at Searle during these experiments. In connection with AAALAC inspection and accreditation, constructive suggestions may be offered to the institution. Copies of the letters of accreditation for 1968 and 1973 are shown in Appendix II-7.

The Laboratory Animal Care Facilities at Hazleton Laboratories were visited by Dr. Frank M. Loew, consultant, and Dr. Robert E. Stowell on April 7, 1978. Their facilities were first examined by AAALAC in 1969, and approved in 1971. Accreditation has been renewed since that time. Copies of pertinent correspondence between AAALAC and Hazleton Laboratories were requested by UAREP and some information was provided four months later. Provisional approval by AAALAC was indicated in November 1970 (Appendix II-7) and full approval was given on June 4, 1971. The reasons for the provisional instead of full approval in November 1970 did not relate to species in experiments under review by UAREP. Earlier AAALAC correspondence which might have cited problems, if any, observed by AAALAC in 1969 or 1970 was not made available to UAREP by HLA. At the time these experiments were carried out, some of the animal rooms utilized were large, and animals from more than one experiment were kept in the same room (Appendix II-7). At the time of the UAREP visit it was said that new shipments of mice and rats were held in isolation for 14 days before they were started on experiments but later HLA said their correct time was one week. During this time the animals were observed daily, but, as not infrequently happened in those times, were not subjected to microbiologic or parasitic investigation.

Laboratory Facilities

There have been considerable changes in the laboratory facilities at both Searle and Hazleton since the time that these experiments were undertaken. UAREP feels that it is not feasible to precisely evaluate the laboratory facilities in view of such changes. The present laboratory facilities and laboratory staffing generally appear quite adequate.

Professional and Other Personnel

A substantial number of the people who were active in these projects at Searle and Hazleton are no longer employees at these firms. For example, Dr. Robert McConnell, Chief of the Pathology-Toxicology Department, is now at Massachusetts Institute of Technology. After weeks of negotiations between Searle and Dr. McConnell and his representatives, it was ascertained that, for personal reasons, Dr. McConnell would not be available for personal interview, but would only consider the submission of written questions. Dr. Rao, Dr. McConnell's associate, who participated in most of the projects under review, is now employed by Dow Chemical Company in Michigan. Similarly, Dr. Harris, the veterinarian in charge of animal facilities, Dr. Vondruska, the project advisor on E-90, and the two biostatiticians who were concerned with these problems are either employed elsewhere or retired and were not made available for interview by UAREP. The curriculum vitae for the professional personnel at Searle and Hazleton were reviewed by UAREP. Some of the laboratory personnel at both Searle and Hazleton were interviewed when those institutions were visited by the UAREP personnel, and such people evidenced good knowledge regarding their work. It is recognized that the Searle-UAREP contract encourages UAREP to interview former personnel. However, on the basis of the limited information available to UAREP, it does not appear feasible for UAREP to make any specific assessment relative to the qualities of Searle or Hazleton Laboratory employees.

Dosage Equivalence of Aspartame

In the protocol for E-33,34 and in a report written by Dr. Robert McConnell in January 1972, human consumption of aspartame was estimated at 1 g/day for a 50 kg adult, or 0.02 g/kg/day for the same 50 kg adult. The report mentioned that 1 g/day for a 27 kg child would be 0.037 g/kg/day. On this basis, an animal dose of 1 g/kg would be 27 times the human child dose and 50 times the adult human dose. A 4 g/kg/day consumption would be 108 times the child dose and 200 times the human adult dose. Multiples of this human equivalent adult dose were used in E-33,34 whereas in contrast in E-28, E-70, 75, and 76, 0.30 g/kg/day dose for a 27 kg child was assumed and 1 g/kg/day and 4 g/kg/day animal doses were reported respectively as 36 times and 133 times the human consumption

Stability and Mixing of Compounds in Diet

The production of aspartame and its conversion product, diketopiperazine, as well as their stability in pure state, may be considered outside the scope of the UAREP authentication study. (Appendix II-2) UAREP recognized that the stability of these compounds, under the conditions employed in these experiments, is obviously important to any interpretation of the results of these studies.

The Entry Books which are the subject of our review contain no documentary information relative to the stability of APM or DKP under these experimental conditions. Some of the earlier protocol sheets (E-34) indicate that the stability of APM was "uncertain" ("Drug vehicle

mixture stability; Rx weeks: uncertain") but later protocol sheets (E-70) say, "stable," in animal diets at room temperature. In the course of carrying out these 12 experiments, to UAREP's knowledge, no samples were analyzed for stability of APM or DKP, or uniformity of mixing. Hazleton states that Searle did not request such tests and that HLA only did things specified by their contractors. Other contractors have had uniformity of HLA mixing checked for their various products, but those results are the property of other firms than Searle and were not available to UAREP. Different products have different mixing characteristics with animal food. Searle declined an offer from UAREP to carry out an independent analysis on the mixing characteristics that would pertain to mixing equipment, aspartame, and animal food of the type used.

Another Entry (E-62), "An Evaluation of the Embryotoxic and Teratogenic Potential in the Rabbit," P-T No. 1048S73, submitted by Searle to FDA presents somewhat confusing evidence that over a six week interval, the aspartame content of APM pelleted with rabbit chow was relatively stable. In eleven individual samples, the aspartame content decreased up to 11% in one sample and increased up to 20% in another. The average of six samples of approximately 3% aspartame in pelleted chow, decreased only 1.5% whereas the average of five samples of 6% aspartame in pelleted chow increased 8.5%.

In response to a UAREP inquiry for information on stability of aspartame, Searle sent a brochure on aspartame, EQUA 200. On page 7 this states, "Dry EQUA 200 has been stored in a closed container at 40⁰ C for periods up to one year in order to determine the mode and extent of decomposition under storage conditions more severe than would be encountered in normal distribution. Only aspartylphenylalanine and

diketopiperazine were detected in the chromatograms of stored samples. After six months storage at 40⁰ C, some increases of as much as 1% in the level of diketopiperazine and of 0.5% in the level of aspartyl-phenylalanine were noted. The constituent amino acids were not seen in these chromatograms." UAREP interprets this to mean that neither phenylalanine nor aspartic acid were present.

Although conditions evaluating dry aspartame and those evaluating it in pelleted rabbit chow are not the same as the studies under UAREP review, they do at least suggest the probability that the aspartame was not greatly altered under the conditions of utilization in these experiments. In long-term experiments, minor variations in the concentration of aspartame or in uniformity of mixing of the compound in diet will probably average out to be of no significance.

Considerations of the relationships of APM or DKP to other compounds, including glutamates, is outside the scope of UAREP's authentication review.

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APPENDICES TO CHAPTER II

II-1.....	Calendar of Main Events
II-2.....	Excerpts from Contract Between UAREP and Searle
II-3.....	Study Numbers of FDA, Searle, and Subcontractors
• II-4.....	UAREP Membership
II-5.....	Principal Participants in Project
II-6.....	Glossary of Terms and Abbreviations
II-7.....	AAALAC Accreditation of Searle Animal Care Facilities
II-8.....	Background information on Hazleton Laboratories America
II-9.....	Comments by Consultant on Biostatistics

APPENDIX II-1

Calendar of Events

September 21, 1976.....FDA representatives first met with UAREP Board
of Directors suggesting authentication study
of aspartame

October 18-19, 1976.....Meeting of UAREP and FDA representatives

December 8, 1976.....Meeting with FDA to discuss contract terms

January, 1977.....FDA transmitted contract draft to Searle

June 30, 1977.....Searle first requested meeting with UAREP

July 5, 1977.....Meeting of Searle representatives with UAREP

August 19, 1977.....Contract between UAREP and Searle signed for
authentication studies

August 22, 1977.....First Searle shipment of documents arrived UCD

September 16, 1977.....First Hazleton shipment of documents arrived
UCD

October 7, 1977.....University of Maryland received slides from
Hazleton

October 14, 1977.....Slides received by Northwestern University

November 8, 1977.....FDA and Searle briefed on UAREP plan of study

November 11, 1977.....Visit to Searle Labs and histopathology slides
on E-28 transported to UCD

March 3, 1978.....Second briefing of FDA and Searle on progress
in authentication

June 9, 1978.....Meeting of UAREP and Searle representatives
to review progress

November 16-18, 1978.....UAREP Editorial Board reviewed total report

November 18, 1978.....UAREP Report submitted to Searle

APPENDIX II-2

Excerpts From

CONTRACT
FOR

AN INDEPENDENT AUTHENTICATION REVIEW OF THE DATA
FROM CERTAIN STUDIES SUBMITTED BY G. D. SEARLE & COMPANY
TO THE FOOD AND DRUG ADMINISTRATION
PERTAINING TO THE FOOD ADDITIVE PETITION FOR ASPARTAME

The Universities Associated for Research and Education in Pathology, Incorporated (UAREP), having its address at 9650 Rockville Pike, Bethesda, Maryland, and G. D. Searle & Company (Searle), having its address at P. O. Box 1045, Skokie, Illinois 60076, (the parties) agree to the following:

I. Purpose of Contract

The purpose of this contract is to provide for independent authentication review of the data contained in reports of certain studies conducted by or on behalf of Searle with respect to aspartame identified in Section II hereof. This review is designed to resolve questions relating to the factual accuracy and reliability of these reports.

II. Scope of Work

A. UAREP, as an independent contractor and not as an agent of either the Food and Drug Administration ("FDA") or Searle, will exert its best efforts and furnish the necessary personnel, materials, services, facilities, and otherwise do all things necessary for or incident to ascertaining and reporting on the accuracy and reliability of the reports of the following studies which were submitted to the FDA pertaining to Searle's food additive petition for aspartame:

- E-70 Lifetime Toxicity Study in the Rat of SC-18862 (aspartame)
- E-11 Two-Generation Reproduction Study in Rats of SC-18862 (aspartame)
- E-28 106-Week Oral Toxicity Dog Study of SC-18862 (aspartame)
- E-33,34 Two-Year Toxicity Study in the Rat of SC-18862 (aspartame)
- E-5 An Evaluation of Embryotoxic and Teratogenic Potential in the Rabbit of SC-18862 (aspartame)
- E-90 An Evaluation of Embryotoxic and Teratogenic Potential in the Rabbit of SC-18862 (aspartame)
- E-75 104-Week Toxicity Study in the Mouse of SC-18862 (aspartame)
- E-77,78 115-Week Oral Tumorigenicity Study in the Rat of SC-19192 (Diketopiperazine)
- E-76 110-Week Study in the Mouse of SC-19192 (Diketopiperazine)
- E-87 A Supplemental Study of Rat Brains from Two Tumorigenicity Studies of SC-18862 (aspartame) (Cross-reference E-34 and E-70)
- E-86 A Supplemental Study of Dog Brains from a 106-week Oral Toxicity Study of SC-18862 (aspartame) (Cross-reference E-28)
- E-89 An Evaluation of Embryotoxic and Teratogenic Potential in the Mouse of SC-18862 (aspartame)
- E-9 Toxicological Evaluation in the Neonatal Rat of SC-18862 (aspartame)
- E-19 A Sweetening Agent: Endocrine Studies of SC-18862 (aspartame)
- E-88 Experiments in Mated and Pregnant Monkeys of SC-18862 (aspartame)

B. As FDA has reviewed the data and reports of E-5, E-33,34, E-77,78, E-88 and E-89, and such review may render unnecessary any authentication of these studies by UAREP, the authentication review of these reports shall be held in abeyance pending notice from the FDA as to whether further review is required.

C. In order to perform the work specified in this contract, UAREP shall employ professional and other personnel, organize them in the manner UAREP determines appropriate, and provide the supervision for all persons who are employed.

D. Specifically, in order to determine the accuracy and reliability of the reports of the studies designated in paragraph II and any subsequent agreements, UAREP shall:

1. Review the protocols and any amendments thereto and reports as submitted to the FDA (hereinafter "submitted reports") in order to become familiar with the studies, objectives and the methodology employed.
2. Examine in detail data contained in the submitted reports, particularly the summary tabular data and the appended detailed data, identifying and documenting any discrepancies, inconsistencies, or errors.
3. Examine raw data, records, notebooks and other materials identified in accordance with paragraph VII(A) to determine whether they agree with the data as reported in the submitted reports. UAREP is encouraged to interview present and former Searle personnel for any purpose related to work under this contract.
4. Examine the tissue slides and blocks, to the extent deemed necessary by UAREP, in order to verify the condition of tissues in the diagnosis of lesions or the lack thereof as reported in the pathology records and data.

5. To the extent deemed necessary and feasible by UAREP, reconstruct the submitted reports from the raw data. Any inconsistencies between the raw data and the data contained in the submitted reports are to be noted in determining whether the reconstructed data support the submitted reports.

E. If requested by UAREP, prior to the review of each report of studies designated in paragraph II and any subsequent agreements, Searle shall provide UAREP with a written description of the manner in which the data for each study were compiled and reported, including a description of the way in which various forms and records were generated and utilized. Nothing in this paragraph shall be construed as precluding UAREP full access to and evaluation of all relevant records necessary for the authentication review other than or in addition to those described by Searle.

F. In the event that Searle determines that additional studies or data should be subjected to review, Searle and UAREP shall prepare a mutually satisfactory agreement for such additional work.

G. UAREP will use its best efforts to determine the accuracy and reliability of the data contained in the submitted reports. UAREP shall provide Searle with a written report setting forth its best judgment as to the accuracy and reliability of these data but shall not express an opinion with respect to the design of the studies or on the significance of studies as they relate to an evaluation of the safety of aspartame; nor shall UAREP make recommendations with respect to whether aspartame is safe for human consumption under any prescribed conditions of use. The report shall set forth UAREP's best judgment as to the accuracy and reliability of the data in the reports which are the subject of this contract.

H. Neither UAREP nor any subcontractor nor any persons employed or otherwise engaged by UAREP to perform work under this contract shall be liable to Searle for any damage to or injury suffered by Searle or its business as a consequence of any findings which may be made by any of them adverse to Searle's business interests as contained in UAREP's final written report except where such findings or findings are caused by or result from gross negligence or deliberate misrepresentation.

III. Period of Contract

UAREP shall exert its best efforts to complete the review of the reports of the studies designated in Section II and any subsequent agreements, including the delivery of the reports required by Section IX hereof, within six months after the execution of this contract. Three months after the execution of this contract, UAREP shall submit to Searle a progress report briefly describing the status of its review and providing an expected completion date. Such report is not intended to be for the purpose of expressing tentative conclusions that may have been reached with respect to any phase of the review but only to report on the time schedule. Should UAREP determine that its review and the delivery of its final report requires more than six months, UAREP may in the progress report so notify Searle and reschedule the completion date to a time mutually satisfactory to the parties.

IV. Personnel

A. In conducting its independent authentication review in accordance with this contract, UAREP will have the sole authority and responsibility for selecting persons to be employed or otherwise paid to discharge its obligations under this contract.

B. Upon execution of this contract, the following personnel designations shall be made:

UAREP shall designate a Principal Investigator.

Searle shall designate a Principal Contact. Each of these persons may, during the period of this contract, designate other persons to act on his behalf. Notice of such designation shall be in writing with a copy furnished at the time of designation to the parties. Such designees are hereinafter referred to as a "UAREP Investigator," and a "Searle Contact."

C. The role of the Searle Principal Contact and Searle Contacts with respect to any and all activities or functions performed by UAREP under the contract shall be to assist UAREP in any reasonable way to discharge its responsibilities for the review under this contract and to serve as points of contact between UAREP and Searle.

D. If UAREP decides to establish review teams for the performance of the work under this contract, a Searle contact shall be available to each team as described in Section IX throughout the lifetime of the team.

E. Persons employed or otherwise engaged for pay by UAREP to perform authentication or review functions contemplated by this contract shall be free of any financial interests in, or any other relationship, past, present, or anticipated, with Searle, any manufacturer or distributor of a product directly competitive with aspartame, or any party to the proposed Board of Inquiry relating to aspartame which could create, or appear to create, the potential for influencing UAREP's review of the submitted reports or the report of its findings. (The "parties" to the Board of Inquiry include James S. Turner, Washington, D.C., Dr. John W.

Olney, St. Louis, Missouri, and the Food and Drug Administration.) Any dispute between Searle and UAREP with respect to an alleged conflict of interest must be resolved between Searle and UAREP; where no agreed upon resolution is forthcoming, it is to be resolved in favor of the existence of the alleged conflict. Each person considered for employment or other engagement for pay by UAREP to perform authentication or review functions contemplated by this contract shall submit to UAREP a written signed statement fully disclosing any past, present or anticipated relationship with, or any financial interest in, Searle, any manufacturer or distributor of a product directly competitive with aspartame, or with any party to the proposed Board of Inquiry with respect to aspartame. Searle, upon request, shall have access to the written statement submitted to UAREP by any person considered for employment or other engagement by UAREP or employed or otherwise engaged by UAREP.

F. Any member of the UAREP Board of Directors who has any past, present or anticipated relationship with, or any financial interest in Searle, any manufacturer or distributor of a product directly competitive with aspartame, or with any party to the proposed Board of Inquiry with respect to aspartame shall submit to UAREP a written, signed statement disclosing such interest. Searle, upon request, shall have access to any such statement.

G. Each person employed or otherwise engaged for pay by UAREP who will be afforded access to confidential data under this contract shall submit a written, signed statement agreeing to maintain the confidentiality of any confidential information provided to him under this contract; the proposed findings to be incorporated into the final report; or to

refrain from making any statement to the public or the news media regarding the subject matter of this contract prior to the release of the final report by Searle or UAREP. Each person to be employed or otherwise engaged for pay by UAREP to perform authentication or review functions under this contract shall submit a written, signed statement agreeing to avoid any financial interest in or other relationship with Searle, any manufacturer or distributor of a product directly competitive with aspartame, or any other party to the proposed Board of Inquiry which could create, or appear to create, the potential for influencing UAREP's review of the select studies or the report of its findings; and not to accept any remuneration for work performed under this contract from Searle, any manufacturer or distributor of a product directly competitive with aspartame, or any party to the proposed Board of Inquiry, except the amount agreed to be paid under or pursuant to agreements under this contract. Searle, upon request, shall have access to these signed statements submitted to UAREP.

H. UAREP shall include all applicable provisions of this contract, including specifically the confidentiality and conflict of interest provisions, in any subcontract or other arrangements for the performance of work under this contract it may execute.

V. Termination of Contract

A. Either party may terminate this contract by serving 30 days' written notice of termination on the other party.

B. In the event of termination, UAREP shall be reimbursed for the following:

1. All costs actually incurred by UAREP under this contract until the date of termination;
2. Any reasonable financial obligations to employees, sub-contractors and others for purposes of performing under this contract.
3. The allowances in lieu of indirect costs due participating educational institutions under paragraph X(F) hereof, on a pro rata basis.

C. In the event of termination by Searle, in addition to the sums set forth in subparagraph B above, UAREP shall receive a fee in the amount of 15% of costs incurred to date of termination, but in no event less than \$25,000, less any amounts paid to UAREP prior to the date of termination under paragraph X(H) hereof allocable to its 15% fee.

D. In the event of termination by UAREP, UAREP shall provide with respect to the work performed up to the date of termination a written report as described in paragraph X hereof.

VI. Locations for Performance of Work

The work shall be performed in locations selected by UAREP. Searle shall cooperate in making personnel, documents, and other materials (or copies where appropriate) conveniently available for work at these locations. All documents or other material relating to the studies designated in paragraph II and any subsequent agreement shall be copied in a manner approved by or in the presence of, a UAREP Investigator and a Searle Contact. Nothing in this paragraph shall be construed as precluding UAREP from performing any of the work under this contract, where necessary, on the Searle premises or on the premises of any of Searle's contractors.

VII. Access to and Confidentiality of Data

A. UAREP shall be given full access by Searle to all records and materials, including those currently held under FDA seal (including but not limited to correspondence, formal or informal internal memoranda, memoranda of all communication, tissue slides, including any duplicates, tissue blocks, and similar materials) necessary to perform the work specified in this contract. The selection of the material to be provided to UAREP from the records and material in Searle's files shall be made by a Searle Contact and a UAREP Investigator. The final determination as to the availability or necessity of any particular record or document shall be made by the Principal Investigator. Prior to the copying of the written records and materials, the UAREP Investigator, and a Searle Contact shall each have an opportunity to document any features of the records which might not be noted from observation of copies of these records (e.g., notations made in different inks or different handwritings or which may be too faint to reproduce). UAREP is authorized to copy, or have copied, any or all of the records or materials necessary to perform the review of the studies designated under paragraph II and any subsequent agreement, and UAREP shall maintain a complete list of all documents. A copy of this list should be provided, upon request to Searle. UAREP is also authorized to take temporary possession of any or all of the materials not appropriate for copying (e.g., tissue slides and tissue blocks), in order to examine them at whatever locations UAREP shall select. UAREP shall maintain a complete record of all such materials in its possession and shall furnish a copy to Searle.

B. Searle shall authorize and obtain the agreement of Hazelton Laboratories ("Hazelton") and all other of its contractors to grant UAREP access to and copies of all records and materials in their possession, and over which Searle has control, that may be necessary to review the reports designated in paragraphs II and any subsequent agreement. The identification and copying of these records shall be done in the manner described in paragraph VII. Searle shall authorize and use its best efforts to obtain the agreement of Hazelton and all other of its contractors to grant UAREP full access to all present and former personnel that UAREP deems necessary to contact to perform the work specified in this contract.

C. Searle shall make all records and materials necessary to perform the work specified in this contract relating to the reports designated in the paragraph II and any subsequent agreement available to UAREP upon request. Searle shall immediately advise UAREP of the discovery of any additional information necessary for a complete review of the reports designated in paragraph II and any subsequent agreements as contemplated in this contract. UAREP shall maintain the confidentiality of all information provided by or obtained from Searle or its contractors. All such records, materials and information shall be returned to Searle as provided in subparagraph D below.

D. All working papers generated by UAREP in the course of the performance of this contract shall be retained by UAREP. All copies of data and information obtained by UAREP from Searle or any of its contractors, and all materials, e.g., tissue slides and tissue blocks, which UAREP obtained on a temporary basis shall be returned to Searle or

its contractors, as the case may be, upon the completion of work under this contract. Searle agrees to maintain and make available all such information to UAREP or any of its subcontractors, employees or personnel who were engaged in performing the authentication review under this contract in the event that any such person is ordered or agrees to testify before any administrative or judicial proceeding with respect to the food additive petition for aspartame.

VIII. Communications

A. As UAREP is to conduct an independent authentication review with the assistance of but free from any influence by Searle, all communications between Searle and UAREP relating to work under this contract, including interviews with laboratory personnel shall be appropriately documented by a UAREP Investigator.

B. In order for UAREP to conduct its independent investigation, the UAREP Investigators are authorized to communicate directly with Searle only in order to obtain such information as is necessary for their work. Such communications should ordinarily be made through a designated Searle Contact.

C. Except when providing responses to requests for information Searle shall not initiate communications with UAREP regarding the work to be done unless it is deemed essential to do so. As required by Paragraph A of this section, all such communications shall be appropriately documented by UAREP.

D. UAREP shall not discuss its conduct of the review or any of its findings with any member of the public. UAREP is free to acknowledge the existence of this contract and the general role of UAREP in its performance.

IX. Reports

A. UAREP shall prepare a final written report of its review of the studies designated in Paragraph II and any subsequent agreements. UAREP shall exert its best efforts to submit twelve copies of the report to the Searle Principal Contact no later than 30 days after completion of the review of the records and materials of each of the reports of the studies designated in Paragraph II and any subsequent agreement. UAREP shall provide Searle with an opportunity to comment on the report. No public comment concerning the report shall be made by Searle or UAREP prior to public release of the report.

B. For each of the reports of the studies designated in Paragraph II and any subsequent agreements, UAREP's final written report shall contain, when necessary for an appropriate evaluation of the Searle report, among other things, the following:

1. A description of the manner in which the study was conducted, including the source of test animals, quarantine and housing procedure, preparation of aspartame (or DKP) - diet mixtures, method(s) of daily observations and weighings, necropsy procedures, preparation of slides for microscopic examination, and histopathology procedures; and the method(s) of data analysis and reporting.

2. UAREP's results and conclusions regarding the accuracy and reliability of the report submitted by Searle, based on the records and materials provided by Searle and its contractors, including, where necessary, a summary of findings and tabular data on each animal or, in the case of group studies, group of animals involved in the study. The report shall also include a detailed rationale supporting UAREP's conclusions as to whether the data in each study report are accurate and reliable.

3. Specific documentation of all discrepancies between the submitted report and the data reviewed together with UAREP's analysis of the significance of any such discrepancy as it relates to the accuracy or reliability of the submitted report may be noted and listed as an appendix to the final report.

4. If differences of opinion exist within UAREP as to the accuracy and reliability of the report of a study, and such differences cannot be resolved, the report shall document the difference of opinion, explain its basis and significance, and set forth both the majority and minority views.

5. A summary of all interviews with the personnel involved in the conduct, analysis, and reporting of the studies set out in paragraph II which are deemed by UAREP to be relevant to its report.

C. If UAREP concludes that it will be unable to arrive at a final conclusion regarding the accuracy and reliability of any report of a study designated in Paragraph II and any subsequent agreements, UAREP shall immediately report such conclusions to a Searle Contact. As soon thereafter as possible, UAREP shall submit a written detailed explanation of its inability to render a final conclusion on that study.

X. Compensation

A. UAREP, or any person, institution or organization employed or otherwise engaged or paid by UAREP shall not receive from Searle or any other party to the proposed Board of Inquiry or any other source any sums in excess of and separate from, cost and the fees provided herein, or under any agreement entered into pursuant to this contract, for the work performed under this contract.

B. Searle shall be responsible for the payment to UAREP of all amounts due under this contract upon receipt of properly executed UAREP vouchers.

C. UAREP is authorized to claim reasonable and necessary expenses incurred in the performance of work related to this contract prior to the execution of this contract.

D. UAREP shall have its auditors audit this account during their regular annual audit and no other audit shall be made. Searle shall be given full access to that portion of the audit report and related documentation that deals with work under this contract.

E. UAREP shall be reimbursed for all direct and indirect costs incurred under this contract. The total indirect costs paid will be the ratio of salaries and wages incurred under this contract to the total salaries and wages incurred by UAREP for all of its operations, during each calendar year, multiplied by the total overhead cost actually incurred by UAREP each calendar year. For billing purposes under this contract, Searle will be billed for indirect costs at a provisional rate of

F. UAREP may select UAREP-affiliated educational institutions to perform work under this contract. Participating educational institutions shall be reimbursed by UAREP for reasonable direct costs incurred under this contract together with an allowance of 50% of such costs in lieu of indirect costs.

. . . .

I. Three months after the execution of this contract UAREP will provide Searle with an estimate of total costs to Searle for work performed under this contract.

The parties are authorized to exercise separately their discretion in giving public notice of this contract and making the contract available in whatever manner and to whomever they may designate.

In witness whereof, the parties have caused this contract to be duly executed by their duly authorized officers.

G. D. Searle & Company

Date 16 August 1977

By James R. Phelps (signature)
Vice President and General Counsel

Universities Associated for Research
and Education in Pathology, Inc.

Date 19 August 1977

By K. M. Endicott (signature)

APPENDIX II-3

STUDY NUMBERS OF FDA, SEARLE, AND SUBCONTRACTORS

FDA Entry Number	Searle Path-Tox Number	Compounds Evaluated	Subcontractors & Experiment Number	Histopath Contractor
28	P-T 855S70	APM	None	MBR
33,34	P-T 838H71	APM	HLA 700-233	EPL
70	P-T 892H72	APM	HLA 700-240	EPL
75	P-T 984H73	APM	HLA 700-259	EPL
76	P-T 985H73	DKP	HLA 700-260	EPL
86	P-T 1226	APM		Dr. Innes
87	P-T 1227	APM		Dr. Innes
9	P-T 893H71	APM	HLA 700-241	
11	P-T 867H71	APM	HLA 700-239	
19	None	APM & DKP	None	
88	None	APM		
90	P-T 1201	APM L-phenylalanine L-aspartic acids	None	

See Appendix II-7 for abbreviations

APPENDIX II-4

MEMBERSHIP

UNIVERSITIES ASSOCIATED FOR RESEARCH & EDUCATION IN PATHOLOGY, INC.

Member Institutions & Representatives

Case Western Reserve - John R. Carter, M. D.
Duke University - Robert B. Jennings, M. D.
University of California - Robert E. Stowell, M.D., Ph.D.
University of Chicago - Werner H. Kirsten, M. D.
University of Kansas - Harrison Clarke Anderson, M. D.
University of Maryland - Benjamin F. Trump, M. D.
University of North Carolina - Joe W. Grisham, M. D.
University of Rochester - Goetz W. Richter, M. D.
Yale University - Vincent T. Marchesi, M. D., Ph.D.

Officers

President: Dante G. Scarpelli, M. D., Ph.D.
Vice President: Benjamin F. Trump, M. D.
Secretary-Treasurer: Robert E. Stowell, M.D., Ph.D.
Executive Officer: Kenneth M. Endicott, M. D.

Directors

John R. Carter, M. D.
Joe W. Grisham, M. D.
Robert B. Jennings, M. D.
Werner H. Kirsten, M. D.
Vincent T. Marchesi, M. D., Ph.D.
Goetz W. Richter, M. D.
Harrison Clarke Anderson, M. D.

Directors-At-Large

Robert E. Anderson, M. D.
William H. Hartmann, M. D.
Fred V. Lucas, M. D.
Dante G. Scarpelli, M.D., Ph.D.
Peter A. Ward, M. D.

Honorary Directors-At-Large

Kenneth M. Brinkhous, M. D.
Robert W. Wissler, M. D., Ph.D.

Draft
9-22-78

APPENDIX II-5

PRINCIPAL PARTICIPANTS IN THE PROJECT

University of California, Davis

Richard O. Kellems, Ph.D., Coordinator
Joan A. Mayerle, Ph.D., Coordinator
Jack E. Moulton, D.V.M., Ph.D., Pathologist
Marrilyn J. Stein, M.D., Pathologist
Robert E. Stowell, M.D., Ph.D., Principal Investigator, Pathologist
Henry Tesluk, M.D., Pathologist

University of Chicago

Anthony J. Steffek, D.D.S., Ph.D., Teratologist

Northwestern University

Mauro C. Dal Canto, M. D., Pathologist
Jan E. Leestma, M. D., Pathologist; Coordinator
M. Sambasiva Rao, M. D., Pathologist
Janandan K. Reddy, M. D., Pathologist
Dante G. Scarpelli, M. D., Ph.D., Pathologist; Coordinator

University of Maryland

Daniel G. Branstetter, D.V.M.
Peter J. Goldblatt, M.D., Pathologist; Coordinator
Melvin Hamlin, D.V.M.
Gerald J. Kolaja, D.V.M., Ph.D.
Glen E. Marrs, Jr., D.V.M.
Ricardo Mandojana, M.D.
Benjamin F. Trump, M.D., Pathologist; Coordinator

UAREP Central Office

Kenneth M. Endicott, M. D., Executive Officer
Robert Learmouth, Business Officer

Appendix II-5
Principal Participants
page two

Consultants

Dale L. Brooks, DVM, Laboratory Animal Care
Dana D. Copeland, M.D., Ph.D.
Leon B. Ellwein, Ph.D., Systems Engineer
Julio H. Garcia, M.D., Pathologist
Andrew G. Hendrickx, Ph.D., Teratologist
Jiro J. Kaneko, DVM, Ph.D., Clinical Pathologist
Alvin Lewis, M.D., Pathologist
Franklin M. Loew, DVM, Laboratory Animal Care
Leon S. Rosenblatt, Ph.D., Biostatistician
Larry Ross, Ph.D., Teratologist
Elizabeth L. Scott, Ph.D., Biostatistician
John Strandberg, DVM, Pathologist
Stephen F. Vogel, M.D., Pathologist
Dorothy E. Woolley, Ph.D., Endocrinologist

Others

Robert G. Bost, Ph.D., Searle Liaison
John Ferrell, DVM, EPL Liaison
Frederick McIlreath, Ph.D., Searle Liaison
Taylor Quinn, FDA Liaison
Willard W. Weatherholtz, Ph.D., Liaison for Hazleton Laboratories

APPENDIX II-6

Glossary of Terms and Abbreviations

ANOVA.....	Analysis of Variance
AP.....	Alkaline phosphatase
APM.....	Aspartame = Searle Compound (SC) 18862
C.....	Computational discrepancy
DKP.....	Diketopiperazine = Searle Compound (SC) 19192
E.....	Entry material submitted to FDA
EPL.....	Experimental Pathology Laboratory
FDA.....	Food and Drug Administration
HLA.....	Hazleton Laboratories America
LSD.....	Least Significant Difference
MBR.....	Microscopy for Biologic Research
N or NS.....	Not significant at $P < 0.05$
ND.....	Not done
NMU.....	Northwestern University School of Medicine
PT.....	Pathology-Toxicology, Department of Searle Laboratories
Q.....	Newman-Keuls test value
QNS.....	Quantity not sufficient
R.....	Rounding discrepancy
S.....	Significant at $P < 0.05$
Searle.....	Searle Laboratories, Division of G. D. Searle and Company
SF.....	Significant figure discrepancy
SGOT.....	Serum glutamic oxalacetic transaminase
T.....	Transcriptional error
UAREP.....	Universities Associated for Research and Education in Pathology
UCD.....	University of California, Davis
UM.....	University of Maryland School of Medicine

Additional abbreviations for chemical terms are given in Table 3-18.

APPENDIX II-7

AAALAC Accreditation of Searle Animal Care Facilities

1. Letter from Consultant Dale Brooks
2. AAALAC letter of accreditation dated May 29, 1968
3. AAALAC letter regarding animal care program, 5/29/68
4. AAALAC letter of continuing accreditation of Searle Animal Care facilities, dated March 20, 1973

UNIVERSITY OF CALIFORNIA, DAVIS

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SANTA BARBARA • SANTA CRUZ

ANIMAL RESOURCES SERVICE
SCHOOL OF VETERINARY MEDICINE

DAVIS, CALIFORNIA 95616

June 21, 1978

Dr. Robert Stowell, M.D.
School of Medicine
University of California
Davis, California 95616

Dear Dr. Stowell:

This is the written report for Universities Associated for Research and Education in Pathology, Inc. concerning the care and use of laboratory animals by G.D.Searle Laboratories site visited June 9, 1978 with Dr. Stowell.

G.D.Searle Laboratories is a fully approved American Association for the Accreditation of Laboratory Animal Care *(AAALAC) facility since 1968. The vivarium is also approved and inspected by the United States Department of Agriculture, Veterinary Services, and conforms to the Animal Welfare Act*(Public Law 89-544 and Public Law 91-579). The vivarium is managed and environmentally maintained at acceptable standards. All animals are in good health and are obtained from reputable laboratory animal vendors. Each shipment of animals is housed in a separate room avoiding mixing of experiments and species.

The aspartaine rabbit experimental trials being questioned were performed at an acceptable level. Sexually mature rabbits, all over six months of age, were purchased. These were housed in a single room for a one month quarantine-acclimation period. Healthy rabbits were selected for artificial insemination. These inseminated rabbits were transferred to another room for 28 days of gestation prior to laparotomy for fetal examinations.

To the best of my knowledge, from the information available and the persons interviewed during this site visit, the animal care protocols and experimental procedures were performed at acceptable standards for the stage of practiced laboratory animal science of the time being investigated. Some of the indistinct results of the aspartaine experiments is due to the differing preliminary experimental trials. The earlier animal procedures and irregular record keeping of the first experiments were confusing. The later experimental trials essentially corrected the earlier deficiencies and uncertainties noted by other reports.

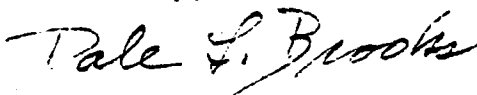
Dr. Robert Stowell

(2)

June 21, 1978

The overall end results concerning the care and use of the experimental rabbits and dogs for the G.D.Searle Laboratories for the aspartaine tests were conducted at acceptable standards for laboratory animals at the time these trials were conducted.

Sincerely,

A handwritten signature in cursive script that reads "Dale L. Brooks".

Dale L. Brooks, DVM
Director

DLB:pjr

*Enclosures

AMERICAN
ASSOCIATION
FOR
ACCREDITATION

OF
LABORATORY
ANIMAL
CARE

4 E. CLINTON STREET
P. O. BOX 13
JOLIET, ILLINOIS 60434
PHONE (815) 727-1955

May 29, 1968

Dr. Theodore W. Harris
Division of Biological Research
G. D. Searle & Company, Inc.
P. O. Box 5110
Chicago, Illinois 60680

Dear Dr. Harris:

The American Association for Accreditation of Laboratory Animal Care is pleased to accredit the animal care program at the Division of Biological Research of G. D. Searle and Company, Inc. and to send you the enclosed certificate of accreditation.

Sincerely yours,



Leslie R. Burrows, D.D.S., Ph.D.
Chairman
Board of Trustees

ek
enc.

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Y

AMERICAN ASSOCIATION FOR ACCREDITATION
OF LABORATORY ANIMAL CARE

4 E. CLINTON STREET • P. O. BOX 13 • JOLIET, ILLINOIS 60434 • PHONE (815) 727-1955

May 29, 1968

Dr. Theodore W. Harris
Division of Biological Research
G. D. Searle & Co., Inc.
P. O. Box 5110
Chicago, Illinois 60680

Dear Dr. Harris:

The Council on Accreditation would like to commend those responsible for the establishment and operation of the fine animal care program being carried on at your facility.

Here are two suggestions from the Council which are intended as constructive recommendations to improve your program:

1. Although the single elevator used to transport animals and clean and dirty equipment was in good sanitary condition, the use of two elevators to separately transport clean and dirty materials is preferred.
2. Animal identification seems adequate for the present research needs, however a more detailed identification using the investigators' names may be helpful to the animal care staff.

AAALAC requires an annual report detailing changes made during the year. We will be interested in hearing from you when we call for your report next spring.

Sincerely yours,

Bennett J. Cohen, D.V.M. Ph.D.
Chairman
Council on Accreditation

ek

cc: Dr. Edward C. Melby, Chairman-Elect

AMERICAN
ASSOCIATION
FOR
ACCREDITATION

2317 W. JEFFERSON STREET
SUITE 208
JOLIET, ILLINOIS 60435

OF
LABORATORY
ANIMAL
CARE

PHONE (815) 729-2020

March 20, 1973

Dr. Theodore Wm. Harris
Manager, Veterinary Care
G. D. Searle & Company, Inc.
Div. of Biological Research
P. O. Box 5110
Chicago, IL 60680

Dear Dr. Harris:

The Council on Accreditation of the American Association for Accreditation of Laboratory Animal Care (AAALAC) has reviewed the report of the November 30, 1972 site visit to the animal care facilities of G. D. Searle and Company, Inc., Chicago.

The Council is pleased to inform you that the animal care program is in accord with AAALAC standards as set forth by the Guide for the Care and Use of Laboratory Animals (DHEW Publication #NIH 73-23) and that full accreditation of the animal care facilities shall continue. Furthermore, the Council on Accreditation would like to commend the management and staff responsible, for an excellent animal care program.

While the Council found no problems which would jeopardize accreditation of the animal facilities of G. D. Searle and Company, Inc., it did note some areas in need of minor improvements. The Council, therefore, offers the following suggestions for improvement of the laboratory animal care program:

1. That the regulations established by the Manager of Veterinary Care regarding overcrowding of cages be rigidly observed. It was noted at the time of the site visit, for example, that in Room 248 housing mice, as many as 25 adult mice were housed in a $7\frac{1}{2} \times 13\frac{1}{2}$ " cage.
2. Closer attention should be given to utilizing cages of adequate size. For example, an occasional large rabbit was noted in a small cage even when larger cages were

empty and available in the same rack or room. In addition, one or two very large dogs were housed in cages which were borderline in terms of adequate size.

We will be interested to hear of any steps taken in response to these suggestions when we call for your next annual report.

Sincerely yours,

A handwritten signature in dark ink, appearing to read 'Alvin F. Moreland', with a stylized flourish extending from the end.

Alvin F. Moreland, D.V.M.
Chairman
Council on Accreditation

AFM:mh

APPENDIX II-8

Background Information on Hazleton Laboratories America

1. Notes of visit to Hazleton Laboratories, April 7, 1978
2. HLA response to some UAREP questions, June 30, 1978
3. AAALAC extension of provisional approval of HLA animal facilities, November 30, 1970
4. HLA current policy on replacement of animals following initiation of the study

NOTES OF VISIT TO HAZLETON LABORATORIES

Friday, April 7, 1978

Dr. Frank Loew and I arrived at Hazleton Laboratories at 10 a.m. and were met by Dr. Weatherholtz. He arranged for Clara Petrovics to talk with us. She has been working for Hazleton more than 20 years and seemed remarkably familiar with many aspects of the Searle Projects. She has worked in various capacities, including as a laboratory technician, doing necropsies, and in the animal room. She presently has some sort of supervisory status.

In response to a query regarding how necropsies were carried out, she stated that they were primarily done by six laboratory technicians and one or two recorders. There was always a recorder present to write down the findings as noted by the individual doing the necropsy. She felt that with the turnover that one might obtain in two years' time and with some changing around of recorders, there might be one or possibly two dozen people whose initials might be entered on these records. She seemed to think that a figure of 30 to 40, as we had noted in some counts, was unduly high. The animals were kept in wire mesh, galvanized cages, and an absorbent paper was kept in the tray underneath. A new mouse feeder was developed in 1967, and she felt that this was the feeder used during the studies. This was different that I had been told by Dr. Reno. The animals were given food for a week's time. At the end of this period, the unused food was discarded. She also gave information as to the receiving of Charles Rivers' Sprague-Dawley rats. A general excess of animals was usually ordered and any animals at the extremes of high and low weights were dropped out. The animals were then allocated to their experimental groups on a "random weight stratified basis." She felt that this was done first by picking animals that would have an average weight, and assigning them the earliest numbers in each group and then progressively moving to animals that would be further away from the mean. Mice and rats were kept in quarantine for a period of 14 days and checked daily, while they were on a control diet comparable to that which the control animals received during the experimentation. They were fed meal (Wayne or Purina) and not pellets. She stated that the method produced comparable groups of mean weights within ± 5 grams. It should be noted that the quarantine procedure outlined did not provide for any microbiologic or parasitic investigation of the animals so that this would not be considered the most effective quarantine method by today's standards. However, it was not an unusual practice at the time these experiments were undertaken.

She was quite definite that animals were sacrificed at periodic intervals during the course of the experiment in order to get clinical laboratory data. It is possible that she was thinking of one of the experiments with which we were not involved.

Hazleton Visit
4-7-78
page two

Ms. Petrovics stated that no records were kept in the animal rooms themselves, but that recorders always went into the animal rooms when clinical observations, weighings, and other procedures were being carried out. There was a weekly schedule for those working in the animal rooms that explicitly told them the procedures to be carried out. A group leader assigned about 6 people to various tasks to be done on an experiment. She stated that weights were taken on the third week of four week intervals, so that the diet could be changed to begin on the 4th week. Although the dosage on diet at the higher levels was calculated every 4th week, the actual food in the containers was changed every week. This would mean that there were intermediate weighings, or at least intermediate observations on which, as far as I know, we do not have data. Her initial reaction was that we should have such data, but Dr. Weatherholtz said that it was quite possible that we would not. An arrangement was made whereby two extra animals were started with each group of animals in an experiment. These two spare animals could be used to replace any animal in the first four weeks. Since these animals were on the same diet as the others in the experiment, this generally produced very little disruption in the experimental plan.

The paper in the animal trays under the animals was changed twice a week and the cages were changed and cleaned once a week. Blood for hematology studies was taken from the tail, but she admitted it was difficult to get more than 1 or 1.5 ml from the tail of a mouse. They were able generally to get 2 ml or slightly more from the tail of a rat. These procedures were used for hematology. For clinical chemistry, at least in the mouse, it was necessary to take blood from the aorta, which had to be done at sacrifice.

Metabolic cages were used for overnight collection of urine in rats. She felt that a preservative was used in connection with the urine collection. We saw the types of cages and feeders which were used in both the standard cages and the galvanized metabolic cages. We also visited the area in which the necropsy examinations were carried out, and noted the facilities for input to INTEC, the data computer system. We also saw the food mixing facilities, which are now outdated and have been replaced by more modern facilities. Typical animal rooms were visited, including ones such as were used for these Searle experiments, although they have now been subdivided into somewhat smaller rooms. We were told that it was probable that animals from more than one experiment were kept in the same room. There were program tapes for use in the animal rooms, which would print out an animal number on the teletype, and the weight from the balance would then be recorded also on the teletype, again with any clinical observations.

Hazleton Visit
4-7-78
page three

Clara Petrovics explained that after blood was drawn, it was placed on ice until a group of 5 or 10 specimens had been obtained, at which time they were taken immediately to chemistry, and she stated that the people in the laboratory were waiting to run the specimens. In a few instances where separation of blood was required, this was carried out in the animal room area. For determinations of the blood sugar, the animals were fasted overnight from 4 o'clock in the afternoon until 8 the next morning. This procedure was also used for collecting urines. She admitted that there had been trouble with the collection of urines in mice during this experiment and that they had only been able to get sufficient urine in a few instances. Terminally, some urine was collected from the bladders at the time of sacrifice. Attempts had also been made with some animals to collect the urine in plastic bags. This was presumably done in rats and was not very successful. She was emphatic that no determinations for glucose were done on animals which had not been fasted overnight. When animals that were to have blood drawn were fasted, all the other animals in the same group were fasted to avoid any possible differences produced from the fasting technique.

The animals were removed in a living state to the necropsy room. When asked whether there were instances in which swellings were noted to disappear, she stated that this did occur from time to time. The person making clinical observations had previous data on each animal available before them at the time, which they checked. All animals were kept on elevated wire bottom cages, except those in breeding studies, which were kept in pine shavings. In the early phases of the work, the animals were checked clinically once a day, but later they were checked twice a day. She was not certain when these changes were made. In general, animals were sacrificed when moribund, although if there was an outbreak of rat pneumonia they were treated with injections of penicillin/streptomycin. She made a parenthetical remark that one animal in group 4 did not get necropsied but this had been marked in the protocol book. She didn't explain the reason why the animal was not necropsied. At the termination of the experiments, since some animals were fasted for blood chemistries, all of the animals were fasted in order to obtain comparable organ weights.

In response to a query whether there were times when the people were too busy to do all the work assigned to them, she stated that there had only been trouble on one occasion because of a snow storm when she and another woman were the only ones who were able to get to work, and they had to stay at Hazleton for three days. The animals were subjected to controlled lighting conditions for 12 hours and she felt that the temperature in the animal rooms was maintained generally between 72-76 degrees.

Hazleton Visit
4-7-78
page four

It was mentioned that Dr. Kundzins did the eye examinations on the animals. He retired in 1971, but came back six months later to continue his work. He wrote down the results of the eye examinations on individual report forms, copies of which went to the protocol and to the project manager. Dr. Kundzins is a veterinarian, has been with Hazleton 25 years, and is still there. Ms. Petrovics claimed that all animals were examined at the start of the experiment, as well as at time of killing. There was a discussion regarding interim examinations and she was quite certain that they had been examined at intervals other than at the first and last, and stated that such information was available in a big book which she had. I asked her if she would be kind enough to get that information for us on E-76 and she demurred, saying that we should ask Dr. Weatherholtz for this information. When asked, he provided information that Experiment 700-260 had ophthalmologic data obtained at 9 (or as soon as practical), 20, 40, 60, and 80 weeks. When I told him that we were aware these examinations had been scheduled but that we presumed they had not been carried out in some cases because we did not have any data, he said first we should have such data and then said, "Well, perhaps it hadn't been utilized for some reason."

According to Clara Petrovics, all animals in one experimental group were kept on the same side of an animal rack, or at least in the same animal rack, and in weighing animals, they started with the controls. At one stage, they rotated the location of the cage racks to different parts of the room, to take into consideration any differences that might exist in air circulation within the room. The information which she gave us, however, was not substantiated by the head of the animal care facility as indicated earlier. Apparently, there could be animals from more than one experiment in the same room and there would be animals from more than one group on the same side of the cage rack. The cage racks for mice held 10 cages across and 10 down. Water bottles were cleaned and replaced with filled bottles twice weekly. The bottles were glass, equipped with rubber stoppers containing a metal drinking tube.

It was mentioned that there was an American Association for the Accreditation of Laboratory Animal Care (AAALAC) approval in 1971, following an examination by them in 1969. Later, I asked Dr. Weatherholtz to supply us with copies of these visitations, which he said he would do. They have also been reapproved by AAALAC since 1971. At the present time, some of the caretakers have American Association of Laboratory Animal Science (AALAS) certification, but probably few did at the time these Searle experiments were carried out. The technicians have, at times, gone for training in necropsy technique at courses given by NIH, but such courses have been discontinued.

Hazleton Visit
4-7-78
page five

When it came to the matter of inadequate materials to carry out all urine or blood tests, the decision was made by Dr. Reno and Dr. O'Connell as to which should be done and which eliminated. This apparently was a standing operating procedure.

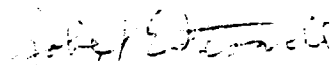
The material that went to Boeing Computer Services was submitted on punched cards. This gave information for individual values, mean values, and standard deviations which was printed out or available. Subsequent statistical analyses were done through the use of a dial commercial service. This included such things as F and "t" tests, and applied to lab data only. There were actually two systems used--the body weight, food consumption, clinical observations, and tumor incidence, all of which were recorded in INTEC. The paper tape which had been punched was converted to IBM cards, as input data. In the case of organ weights, the weight from the necropsy sheet was put on IBM cards which went to Boeing after which it was handled in the same way as body weight, food consumption, etc. The weight was put on punched paper tapes and printed out, after which it went to data processing and IBM cards.

Our discussions regarding the biostatistics unit were rather confusing, in that apparently such a unit was never activated to the degree the printing on the bottom of the necropsy form would suggest. At times, apparently, there was some biostatistical consultation available, but a good part of the time Hazleton was apparently doing whatever they were asked to do by Searle. The four part necropsy sheet actually apparently went to about three places, and one copy may have been discarded. The report writing group, the project director, and the pathology-histology were apparently the main sources for utilization.

Toxicological Resources Group is the name now given to what would have formerly been something like the Biostatistics Group. We met with Dale Strother, the head of that activity. At the present time, there are 13 people in the group, of whom 8 are writers of reports and 5 are typists and data coordinators. This is a considerably larger group than at the time the Searle experiments were being carried out. The report writer monitors the progress of data flow from the very initiation of the experiment, keeping track of when things are due and where the data goes. Original copies of materials such as the clinical laboratory data, eye examinations, and other data go to the report writer with other copies going to Dr. Reno. In response to queries regarding the frequency of communication between the report writers and those working in the laboratories and Dr. Reno, the answer seemed to be that it was done to the extent to which it was necessary. At one time this

Hazleton Visit
4-7-78
page six

group was called the World Processing Group, such as the time when this work was being carried out and a man by the name of a Ray Lennette (sp?) was in charge of the group then. It was mentioned that he had come up through working at various positions in the laboratory. The writer of the report always participated as one of the proofreaders. We inquired regarding the ways in which data was checked and were told that the input by IBM cards was checked by having two separate individuals punch the cards and then these were visually compared to see that there were no errors.


Robert E. Stowell

RES/ah



HAZLETON

LABORATORIES AMERICA, INC.

9200 LEESBURG TURNPIKE, VIENNA, VIRGINIA 22180, U S A

June 30, 1978

Dr. Robert Bost
G. D. Searle & Company
Searle Laboratories
P.O. Box 5110
Chicago, Illinois 60680

Dear Dr. Bost:

In response to our telephone conversation on June 6, 1978, the following information which you requested on Project 700-233, 700-240, 700-259, and 700-260 has been assembled. As indicated, in some instances there is no concise documentation for the information given. In such cases, the individual or reference source of the information is stated.

Copies of the following requested documents are enclosed:

1. The AAALAC visitation report of 1971 and the subsequent certificate of accreditation.
2. Signed inventories of slides checked out to the University of Maryland and Northwestern University.
3. A list of the corresponding animal numbers, histopathology numbers, and Experimental Pathology Laboratories (EPL) numbers for studies 700-259 and 700-260.
4. The current standard procedure for replacement of animals. At the time the studies in question were conducted there was no written policy. However, the attached procedure is based on the general practice followed at that time.

The following questions were answered by Dr. Reno based on standard operating procedures at the time these studies were performed as well as procedures peculiar to the conduct of these specific studies.

- Q. How often were feed and water containers changed? Was new feed added on top of old feed?
- A. Water bottles were changed twice weekly. Feed jars were changed once weekly with the following exception:

In project 700-233, dose levels were adjusted on an mg/kg/day basis three times weekly during weeks one through four, and twice weekly in weeks five through



Dr. Robert Bost
G. D. Searle & Co.

- 2 -

June 30, 1978

thirteen. In order to reduce the amount of test material wasted, old feed was removed and weighed, then additional feed or compound was added to the uneaten feed as necessary to achieve the new dietary level. This new diet was then presented to the animals. This procedure was followed only for the first thirteen weeks of the study. Only in this instance was new feed added to old feed.

- Q. If feed was changed more frequently than computer tapes were printed, how were feed consumption records maintained?
- A. Food consumption data is for a maximum of one week. (In case of 700-233 shorter intervals were measured during the first thirteen weeks of the study.) If, for example, feed consumption was determined monthly, the data represent the consumption for the one week reported. For the weeks when food consumption was not measured, old feed was discarded without weighing.
- Q. Dosages were changed by changing the concentration of test material in the feed based on food consumption data. What was the time lapse between determining feed consumption and preparation of the new diet levels?
- A. Feed consumption was measured in the morning; new diets were prepared and fed that afternoon. Maximum time lapse was about six hours.
- Q. What were animals fed during this time lapse?
- A. Nothing. Water was available ad libitum.
- Q. If a time lapse occurred, how were computations of food consumption and compound consumption adjusted to compensate?
- A. No adjustment was made. Computations were based on a seven day week.
- Q. How long were animals quarantined?
- A. 700-233, 700-259, and 700-260 were quarantined for one week. 700-240 animals were obtained as weanlings from offspring produced in project 700-239. Because they were born in-house no specific "quarantine" was observed prior to initiation.
- Q. Were these studies housed in separate rooms from other experiments?



Dr. Robert Bost
G. D. Searle & Co.

- 3 -

June 30, 1978

- A. There are no records of the exact room where each study was housed. Therefore, we cannot provide an accurate answer to this question.
- Q. How long were animals fasted before drawing blood for glucose determinations?
- A. Overnight - approximately sixteen to eighteen hours. Food was removed late in the afternoon and withheld until after animals were bled the following morning.
- Q. Were other animals on the study also fasted?
- A. No. Any weight loss resulting from the fasting period was normally regained within a few days. Whenever possible, blood sampling was scheduled on weeks when food consumption was not measured. This practice allowed additional recovery time so that food consumption values would not be affected.
- Q. Was a paired T-test used for hematology data?
- A. The answer for this question is not readily available. In the interest of time we have chosen to reply to your other questions, leaving this one unanswered. If this information is critical we will attempt to supply it.
- Q. Was a 1- or 2-tailed T-test used for clinical chemistry data? At what significant level?
- A. A 2-tailed T-test was used at the 5% level.

The following questions were answered based on data maintained at HLA and information from individuals who are familiar with the procedures used on these studies.

- Q. Regarding the use of a biostatistition in preparation of the study design (Projects 700-233 and 700-260): who approved the number of animals per group, the number of test levels, etc.?
- A. Searle provided the protocol for these studies.
- Q. Quarterly reports are required by the project sheets. Were they prepared? Are they available?
- A. Quarterly reports were issued. Searle provided a question/answer, fill-in type form which was completed at HLA and returned. No copies of these reports were kept at HLA.



Dr. Robert Bost
G. D. Searle & Co.

- 4 -

June 30, 1978

Q. What was the year and publication number of the U.S. Navy manual referenced for hematology parameters?

A. The referenced manual, Hematology, U.S. Naval Medical School, National Naval Medical Center, was published in 1943, 1945, and 1956. There is no publication number for any of these. The page numbers listed in the reports do not correspond exactly with the referenced methods in any of these three manuals.

Q. What were the weights of the feed and the food containers from which feed consumption was determined?

A. For rats, the feed jars weigh approximately 236-260 grams, average around 250 grams. Sufficient feed was added to total 500 grams. Feeders used on mouse studies weigh approximately 120 grams and feed was added to give a total weight of 175 grams. It is the total weight that is significant. Feed consumption is the difference between the total weight at the beginning of the measured interval (500 or 175 grams) and the total weight of the container and remaining feed at the end of the interval. Thus, the weight of the individual feeder and/or feed does not enter into the computation and need not be the same for each animal.

On some studies (for example, the initial phase of 700-233) a different type of feed container or different amount of food may have been used, resulting in a different total feed weight. However, the procedure of computing the difference between the gross initial weight and the gross remaining weight remained the same.

Q. What is the meaning of the entry 99.9 as recorded under body weights for various intervals on Project 700-260?

A. 99.9 was the code used to indicate a dead or missing animal for which a final body weight was not available. 99.9 was not included in body weight calculations.

Q. Were Searle ophthalmologic examination worksheets used for recording data on experiments 700-260 and 700-233?

A. No. Abnormalities were recorded in notebooks then summarized as reported in the study data. Searle forms were not used.



HAZLETON
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Dr. Robert Bost
G. D. Searle & Co.

- 5 -

June 30, 1978

I hope this information will be satisfactory. If you have further questions, please contact me.

Sincerely,

A handwritten signature in cursive script that reads "Susan Hinkle". The signature is written in dark ink and is positioned below the word "Sincerely,".

SUSAN HINKLE
Office of Quality Assurance

SH:kb

AMERICAN
ASSOCIATION
FOR
ACCREDITATION

OF
LABORATORY
ANIMAL
CARE

4 E. CLINTON STREET
P. O. BOX 13
JOLIET, ILLINOIS 60434

PHONE (815) 727-1755

November 30, 1970

Dr. William S. Broughton, Jr.
Technical Administrator
Toxicology-Biosciences Laboratory
Hasleton Laboratories, Inc.
P. O. Box 30
Falls Church, VA 22046

1829
REF. NO. _____
ACCT. Broughton
DEPOSIT DATE 1-31-71
DATE REC'D 12-3-70
FROM H. L. G. G. G.
DET. D. L. G. G. G. O.E.

Dear Dr. Broughton:

The Council on Accreditation of the American Association for Accreditation of Laboratory Animal Care had in earlier action extended the provisional accreditation of Hasleton Research Laboratories to January 31, 1971. On or before that date we would appreciate your notification of correction of the following provisions:

1. Animal care and caging in the Pharmacology area of building 19, particularly room 1137, should be improved to meet the standards outlined in the Guide for Laboratory Animal Facilities and Care.
2. In the canine toxicology area the old dog cages with rusty metal rods for doors and poorly designed expanded metal floors should be completely replaced with suitable caging. The site visitors indicated that this had already begun at the time of their visit.
3. Cage sizes for non-human primates throughout the facilities should be reviewed with reference to the space recommendations for housing in the Guide. Where necessary, larger cages or smaller animals should be utilized to meet the Guide recommendations.

Please notify us as soon as these provisions have been corrected. Where the purchase of cages is required, copies of purchase orders will temporarily suffice as evidence of correction.

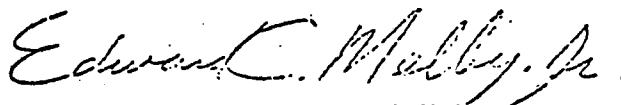
Dr. William S. Broughton, Jr.

2

November 30, 1970

In extending provisional accreditation to January 31, 1971 we have granted the maximum total period of provisional accreditation permitted by the AAALAC Bylaws; no further extensions can be made. We realize that this does not allow much time for completion of the required improvements. However, in view of the considerable improvements instituted since provisional accreditation was first granted and in recognition of the highly competent personnel we are confident that the provisions can be met within two months.

Sincerely yours,



Edward C. Melby, Jr., D.V.M.
Chairman
Council on Accreditation

ek

cc: Dr. Dan Dalgard

E. Replacement of Animals Following Initiation of the Study

Following initiation of the study, animals which die or appear unthrifty may be replaced only if the project coordinator has specified in writing before initiation that replacements may be used. Unless otherwise specified, animals may be replaced only within the first four weeks of the study.

Two animals or the number of animals housed in one cage are routinely selected per group to act as potential replacements from the extra animals remaining after the selection of the study animals. The procedures used to select the study animals and place them in groups are again used to select and place the potential replacements. Each animal is identified as Replacement Animal No. 1, No. 2, etc. in each group and individually housed in a clearly labelled cage. These animals may be housed in the same rack but there should be sufficient spacing between housing so that group identification is easily discernible. All remaining extra animals are then discarded.

The replacements receive the same experimental treatments (e.g. diets, injections, etc.) as the corresponding groups of animals on study. Observations, body weights, and food consumption are recorded as for the assigned animals. However, these body weight and food consumption values are not included in calculations of group mean values and are to be used only for reference purposes during the four weeks of maintenance.

If any assigned animal is replaced, the replacement is recorded on the record of the permanent identification number and on the Daily Observations form (see Appendix A, Form 6-12) along with the number of the animal replaced and the number of the replacement animal. The replacement then will assume the permanent number of the animal it replaces and for all purposes is considered thereafter as one of the original population assigned to that group. Those animals which are replaced are ordinarily discarded without necropsy. At the end of the first four weeks of study, all remaining potential replacements are likewise discarded without necropsy; date of discard is recorded.

APPENDIX II-9

Comments By Biostatistical Consultant, Dr. Leon S. Rosenblatt

I have gone over the statistical methodology section of several of the studies. In general, there was little there with which I would take issue. I do not know, however, whether the methods cited were the tests actually performed in all cases.

A major area requiring discussion is that of the analysis of clinical chemistry and hematology data. Such data were obtained from a small group of animals sequentially sampled. Analysis of such data is complicated by the fact that using the same animals over a period of time introduces serial correlations, making a single analysis difficult. In the original analysis, the problem was avoided by analyzing each sampling period individually. For UAREP to have attempted a more sophisticated approach would have been costly and difficult, so that my advice was to continue the analysis of individual sampling periods but with some significant modification.

Comparisons made in the original analysis were based on standard t-tests and the use of least significant differences (LSD). The LSD analyses were based on results of analysis of variance (ANOVA). If the ANOVA indicates a significant difference among the means tested, one wishes to know which of the several differences are responsible for the rejection of the hypothesis that means are all equal. The least significance difference statistic is one method which has been utilized. The problem is that the LSD is not a very conservative statistic.

If we say a difference is significant at the 5% level, we are going to be wrong 5% of the time. That is by chance alone, five percent of the differences are not real differences. The more tests we make, the greater the number of times we are wrong. Recently, Tukey (Science, 198:679, November 18, 1977), commenting on clinical trials of long duration, warned against taking too many "peeks" at the data--the more peeks the more "significant" differences we are likely to observe. In the present studies, there were numerous samples taken during the two-year course of the studies. Thus, we are likely to observe a fairly significant number of differences which are not, in fact, significant.

The appended four pages from Snedecor and Cochran Statistical Methods, 6th. ed, Iowa State Univ. Press, 1967, are relevant to the above remarks. In the paragraph marked A on page 272, the authors object to LSD from the point of view that the true probability of observing at least one significant difference among a set of means, is not, say, $p < 0.05$ in all cases, but depends upon the number of means in the test. Where there are five means the probability is not 5% but 29%.

Paragraph B discusses this matter further. As more tests are made, one becomes more certain of finding significant differences. A somewhat more conservative procedure is that of the Newman-Keul's test. Snedecor and Cochran on page 273 (paragraph A) point out that using the Q-test (Newman-Keuls) we can obtain the probability at the 5% level that no erroneous claim of significance will be made, but that the price paid is that fewer differences that are real will be detected. It was my opinion that in the analysis of Searle data, because of the large number of tests being performed and the possible consequences of erroneous significant results, the Q-test was more appropriate, although these

authors (page 275, paragraph A), point out that no method is uniformly best.

In a sense, the preceding discussion is almost academic, in that the original analyses, while citing the LSD in the methods section in some experiments, rarely utilized it in the general series of experiments. Instead, the most commonly utilized method was the simple t-test, with comparisons made only between the individual dose groups and the controls. In so doing, the implication is that the difference between those groups themselves is unimportant, or less important, in comparison with the controls. This procedure throws away half or more of the available information. In the Student t-test, there are only two independent estimates of the error. In the ANOVA, with four dose groups, there are four six such estimates. Clearly the ANOVA is preferable, if one wishes to obtain substantially more information from the data.

For the above reasons, we decided on the following procedures:

- (1) Repeat the t-test of the original analyses.
- (2) Perform the analysis of variance and if the F statistic was significant at the $p < 0.05$ level, utilize the results of the ANOVA to do the LSD and Q-tests. (See paragraph C, page 272).

Finding of discrepancies between the results of the t-test and the LSD and Q-tests would not be particularly surprising. It is my opinion that on balance, the Q-test results should be given preference to the LSD or the t-test and the LSD preference over the t-test. As an aside, I feel if a desire was to compare the treated group with the controls, only, the method of Dunnett would have been more appropriate.

In concluding this part of the discussion, I would stress that the interpretation of observed significant differences, however many, needs to be tempered by judgment; as many of them may not in fact be real. Further discrepancies between the t-test and Q-test which are set out in a table may give the impression of lack of agreement. The table, in fact, will not show the many instances where there was agreement between these tests.

The other major area to be discussed relates to survival data and tumor incidences. In the methodology section there are references to a paper by Sacks (1959). This paper utilizes a life table method to handle concomitant risks in estimation of cumulative incidence rates for tumors. Her method, with a single trivial exception--measurement from the end of the end of a period rather than from the beginning of the period--is statistically identical with my paper Health Physics (21:869, 1971). Thus, utilizing my method for which my computer is programmed, is the same as using the Sacks method. I understand, however, that the original analyses did not, in fact, employ the Sacks technique. In addition to estimating cumulative survival rates by the method of Cutler and Ederer (J. Chron. Dis. 8:699, 1958) and cumulative tumor incidence rates by my method, we followed these up by application of the Mantel-Haenszel test of significance between two survival or incidence curves. The methodology section refers to a t-test which was used to compare survival and tumor incidence. I do not feel that a t-test is appropriate here, and in any case compares only the final survival or incidence rates. The Mantel-Haenszel test developed at the National Cancer Institute compares entire survival or incidence curves, taking times of death into account. In addition we tested all possible comparisons among dose groups.

272 Chapter 10: One-Way Classifications. Analysis of Variance

differences among them appear to be real. The most frequent example is when the treatments are qualitatively similar, as in tests on working gloves made by different manufacturers.

Taking the doughnut data from table 10.2.1 as an illustration, the means for the four fats (arranged in increasing order) are as follows:

TABLE 10.8.1

Fat	4	1	3	2	LSD	D
Mean grams absorbed	62	72	76	85	12.1	16.2

The standard error of the difference between two means, $\sqrt{(2s^2/n)}$, is ± 5.80 , with 20 *d.f.* (table 10.2.1). The 5% value of *t* with 20 *d.f.* is 2.086. Hence, the difference between a specific pair of means is significant at the 5% level if it exceeds $(2.086)(5.8) = 12.1$.

The highest mean, 85 for fat 2, is significantly greater than the means 72 for fat 1 and 62 for fat 4. The mean 76 for fat 3 is significantly greater than the mean 62 for fat 4. None of the other three differences between pairs reaches 12.1. The quantity 12.1 which serves as a criterion is called the *Least Significant Difference (LSD)*. Similarly, 95% confidence limits for the population difference between any pair of means are given by adding ± 12.1 to the observed difference.

A Objections to indiscriminate use of the *LSD* in significance tests have been raised for many years. Suppose that all the population means μ_i are equal, so that there are no real differences. With five types of gloves, for instance, there are ten possible comparisons between pairs of means. The probability that at least one of the ten exceeds the *LSD* is bound to be greater than 0.05: it can be shown to be about 0.29. With ten means (45 comparisons among pairs) the probability of finding at least one significant difference is about 0.63 and with 15 means it is around 0.83.

B When the μ_i are all equal, the *LSD* method still has the basic property of a test of significance, namely that about 5% of the tested differences will erroneously be declared significant. The trouble is that when many differences are tested, some that appear significant are almost certain to be found. If these are the ones that are reported and attract attention, the test procedure loses its valuable property of protecting the investigator against making erroneous claims.

C Commenting on this issue, Fisher (8) wrote: "When the *z* test (i.e., the *F*-test) does not demonstrate significance, much caution should be used before claiming significance for special comparisons." In line with this remark, investigators are sometimes advised to use the *LSD* method only if *F* is significant.

Among other proposed methods, perhaps the best known is one which replaces the *LSD* by a criterion based on the tables of the Studentized Range, $Q = (\bar{X}_{\max} - \bar{X}_{\min})/s_{\bar{X}}$. Table A 15 gives the upper 5% levels

of Q , i.e., the value exceeded in 5% of experiments. This value depends on the number of means, a , and the number f of $d.f.$ in s_X . Having read $Q_{0.05}$ from table A 15, we compute the difference D between two means that is required for 5% significance as $Q_{0.05}s_X$.

For the doughnuts, $a = 4$, $f = 20$, we find $Q_{0.05} = 3.96$. Hence $D = Q_{0.05}s_X = (3.96)(4.1) = 16.2$. Looking back at table 10.8.1, only the difference between fats 2 and 4 is significant with this criterion. When there are only two means, the Q method becomes identical with the LSD method. Otherwise Q requires a larger difference for significance than the LSD .

The Q method has the property that if we test some or all of the differences between pairs of means, the probability that no erroneous claim of significance will be made is ≥ 0.95 . Similarly, the probability that all the confidence intervals $(\bar{X}_i - \bar{X}_j) \pm D$ will correctly include the difference $\mu_i - \mu_j$ is 0.95. The price paid for this increased protection is, of course, that fewer differences $\mu_i - \mu_j$ that are real will be detected and that confidence intervals are wider.

EXAMPLE 10.8.1—In Case III of the constructed example in table 10.4.1, with $\mu_1 = 3$, $\mu_2 = 5$, $\mu_3 = 9$, the observed means are $\bar{X}_1 = 2.9$, $\bar{X}_2 = 4.0$, $\bar{X}_3 = 9.25$, with $s.e. = \sqrt{(s^2/n)} = 0.75$ (3 $d.f.$). Test the three differences by (i) the LSD test, (ii) the Q test. Construct a confidence interval for each difference by each method. (iii) Do all the confidence intervals include $(\mu_i - \mu_j)$? *Ans.* (i) $LSD = 3.37$. \bar{X}_3 significantly greater than \bar{X}_2 and \bar{X}_1 . (ii) Required difference = 4.43. Same significant differences. (iii) Yes.

EXAMPLE 10.8.2—In example 10.5.1, the mean gains in weight of baby chicks under four feeding treatments were $\bar{X}_1 = 43.3$, $\bar{X}_2 = 71.0$, $\bar{X}_3 = 81.4$, $\bar{X}_4 = 142.8$ while $\sqrt{(s^2/n)} = 12.0$ with 16 $d.f.$ Compare the means by the LSD and the Q methods. *Ans.* Both methods show that \bar{X}_4 differs significantly from any other mean. The LSD method gives \bar{X}_3 significantly greater than \bar{X}_1 .

Hartley (30) showed that a sequential variant of the Q method, originally due to Newman (10) and Keuls (31), gives the same type of protection and is more powerful: that is, the variant will detect real differences more frequently than the original Q method.

Arrange the means in ascending order. For the doughnut fats, these means are as follows:

Fat	4	1	3	2	S.D.
	62	72	76	85	± 4.10 (20 $d.f.$)

As before, first test the extreme difference, fat 2 — fat 4 = 23, against $D = 16.2$. Since the difference exceeds D , proceed to test fat 2 — fat 1 = 13 and fat 3 — fat 4 = 14 against the D value for $a = 3$, because these comparisons are differences between the highest and lowest of a group of three means. For $a = 3$, $f = 20$, Q is 3.58, giving $D = (3.58)(4.10) = 14.7$. Both the differences, 13 and 14, fall short of D . Consequently we stop; the difference between fats 2 and 4 is the only significant difference in the

274 Chapter 10: One-Way Classifications. Analysis of Variance

experiment. If fat 3 - fat 4 had been, say, 17, we would have declared this difference significant and next tested fat 3 - fat 1 and fat 1 - fat 4 against the D value for $\alpha = 2$.

Whenever the highest and lowest of a group of means are found not significantly different in this method, we declare that none of the members of this group is distinguishable. This rule avoids logical contradictions in the conclusions. The method is called *sequential* because the testing follows a prescribed order or sequence.

Since protection against false claims of significance is obtained by decreasing the ability to detect real differences, a realistic choice among these methods requires a judgment about the relative seriousness of the two kinds of mistake. Duncan (32) has examined the type of policy that emerges if the investigator assigns relative costs to (i) declaring a significant result when the true difference is zero, (ii) declaring non-significance when there is a true difference, (iii) declaring a significant result in the wrong direction. His policy is designed to minimize the average cost of mistakes in such verdicts of significance or non-significance. These costs are not necessarily monetary but might be in terms of utility or equity. His optimum policy resembles an *LSD* rule with two notable differences. In its simplest form, which applies when the number of treatments exceeds 15 and $d.f.$ in s exceed 30, a difference between two means is declared significant if it exceeds $s\delta t_{\alpha} \sqrt{F/(F-1)}$. The quantity t_{α} (not Student's t) depends on the relative costs assigned to wrong verdicts of significance or non-significance. If F is large, indicating that there are substantial differences among the population means of the treatments, $\sqrt{F/(F-1)}$ is nearly 1. The rule then resembles a simple *LSD* rule, but with the size of the *LSD* determined by the relative costs. As F approaches 1, suggesting that differences among treatment means are in general small, the difference required for significance becomes steadily larger, leading to greater caution in declaring differences significant. The F -value given by the experiment enters into the rule because F provides information as to whether real differences among treatment means are likely to be large or small. In Duncan's method, the investigator may also build into the rule his *a priori* judgment on this point.

In a large sampling experiment with four treatments, Balaam (33) compared (i) the *LSD* method, (ii) the revised *LSD* method in which no significant differences are declared unless F is significant, (iii) the Newman-Keuls method (as well as other methods). Various sets of values were assigned to the population means μ_i , including a set in which all μ_i were equal. For each pair of means, a test procedure received a score of +1 if it ranked them correctly, a score 0 if it declared a significant difference when $\mu_i = \mu_j$ or found no difference when $\mu_i \neq \mu_j$, and a score -1 if it ranked the means in the wrong order. These scores were added over the six pairs of means.

When all μ_i were equal, the average scores were: *LSD*, 5.76; Revised *LSD*, 5.91; *NK*, 5.94. With three means equal, so that three of the six differences between pairs were equal and three unequal, average scores

were: *LSD*, 3.80; Revised *LSD*, 3.57; *NK*, 3.51. With more than three inequalities between pairs, average scores were: *LSD*, 1.92; Revised *LSD*, 1.73; *NK*, 1.63. To sum up for this section, no method is uniformly best. In critical situations, try to judge the relative costs of the two kinds of mistakes and be guided by these costs. For routine purposes, thoughtful use of either the *LSD* or the Newman-Keuls method should be satisfactory. Remember also Scheffé's test (p. 271) for a comparison that is picked out just because it looks large.

10.9—Shortcut computation using ranges. An easy method of testing all comparisons among means is based on the ranges of the samples (13). In the doughnut experiment, table 10.2.1, the four ranges are 39, 20, 30, 21; the sum is 110. This sum of ranges is multiplied by a factor taken from table 10.9.1. In the column for $a = 4$ and the row for $n = 6$, take the factor 0.95. Then

$$D' = \frac{(\text{Factor})(\text{Sum of Ranges})}{n} = \frac{(0.95)(110)}{6} = 17.4$$

D' is used like the D in the Q -test of the foregoing section. Comparing it with the six differences among treatments, we conclude, as before, that only the largest difference, 23, is significant.

TABLE 10.9.1
CRITICAL FACTORS FOR ALLOWANCES, 5% RISK*

Sample Size, n	Number of Samples, a								
	2	3	4	5	6	7	8	9	10
2	3.43	2.35	1.74	1.39	1.15	0.99	0.87	0.77	0.70
3	1.90	1.44	1.14	.94	.80	.70	.62	.56	.51
4	1.62	1.25	1.01	.84	.72	.63	.57	.51	.47
5	1.53	1.19	.96	.81	.70	.61	.55	.50	.45
6	1.50	1.17	.95	.80	.69	.61	.55	.49	.45
7	1.49	1.17	.95	.80	.69	.61	.55	.50	.45
8	1.49	1.18	.96	.81	.70	.62	.55	.50	.46
9	1.50	1.19	.97	.82	.71	.62	.56	.51	.47
10	1.52	1.20	.98	.83	.72	.63	.57	.52	.47

* Extracted from a more extensive table by Kurtz, Link, Tukey, and Wallace (13).

EXAMPLE 10.9.1—Using the shortcut method, examine all differences in the chick experiment of example 10.5.1 (p. 267). Ans. $D' = 49$. Same conclusions as for the Q method in example 10.8.2.

10.10—Model I. Fixed treatment effects. It is time to make a more formal statement about the assumptions underlying the analysis of variance for single classifications. A notation common in statistical papers is to use the subscript i to denote the class, where i takes on the values 1, 2, ..., a . The subscript j designates the members of a class, j going from 1 to n .

CHAPTER III

E-28: 106 WEEK ORAL TOXICITY STUDY IN THE DOG

INTRODUCTION

Searle Laboratories conducted this 106 week oral toxicity study (P-T No. 855S70) using aspartame in pure-bred Beagles. A screening test was run in early March of 1970 (Appendix III-1) to determine if the dogs would eat Purina dog meal with aspartame added. Four dogs were fed once daily for eight days, with a mixture of feed and compound (4 g/kg). Maximum anticipated human consumption is 30 mg/kg per day. The dogs ate the above ration without any problems. The initial plan was to feed once daily, the amount of compound to be based on group means of body weights. Purina meal plus compound was to be given once a day in the later afternoon, and a meat supplement given once a day in the morning.

The first protocol sheet was dated March 26, 1970 (Appendix III-2). The proposed feeding schedule was 200 g of Purina meal plus compound with Dermo supplement and water added, given to each dog around 9 a.m. It was presumed that each dog consumed the entire amount within two hours. After two hours, each dog received 250 grams of Wayne Lab-blox. After four and a half additional hours, the food dish was removed from the cage and weighed. The amount of food consumed was calculated and recorded. Uneaten food was then discarded. The concentration

of compound in the diet was calculated for each group based on the group mean body weight and each dog was fed accordingly. Individual aspartame consumption in grams per kilogram was calculated retrospectively.

An amendment dated March 30, 1970 (Appendix III-3) stated that this was a chronic toxicity study and would include careful attention to food consumption and body weight gains. Animals were divided into four groups; control, low dose (1 g/kg), medium dose (2 g/kg) and high dose (4 g/kg), each consisting of five males and five females. Three of the male dogs were 70 days older than the others and therefore heavier (Table 3-1). They were distributed, one each, in the low, medium, and high dose groups. They received compound-diet mix plus the basal diet of Wayne Lab-blox and tap water ad libitum. Each dog, irrespective of size, age, or sex, received 250 g daily of dry Wayne Lab-blox in pellet or powder form. This amount was to be increased by approximately 56 calories for control dogs, an amount equivalent to the caloric value of the medium dose level of aspartame. According to the amendment, dogs were to be fed the compound one to three times daily as required, under supervision of the Pathology-Toxicology Department.

The clinical laboratory procedures, including hematology, clinical chemistry, and urinalysis were performed at the Department of Biological Research at Searle Laboratories. Hematology studies included the packed cell volume, hemoglobin, red blood cell count, white blood cell count, differential, prothrombin time and activated prothrombin time. These were done at minus 2 weeks (some at minus 1), 0, 4, 14, 26, 52, 78, and 106 weeks. The beginning of compound administration was considered week zero.

Clinical chemistries assayed in plasma included urea nitrogen, glutamic pyruvic transaminase, alkaline phosphatase, bromsulfophthalein retention (BSP), bilirubin, glucose, sodium, potassium, calcium, and chloride. These were also done by Searle at the time intervals indicated previously. Urine samples were assayed for specific gravity, pH, bilirubin, protein, sugar, ketones, and the sediment was examined for microscopic components; red blood cells, white blood cells, crystals, bacteria, and casts. Urine was also assayed for phenylketones, urobilinogen, and occult blood.

A change in feeding schedule was made on April 16, 1970, the 17th day of study (Appendix III-4). Beginning then, the first feeding was to be at 9 a.m. Purina meal (200 g) was mixed with compound (except in controls), Dermo supplement, and 180 ml of water. After two hours, each feed dish was checked. A second feeding of 200 g of Wayne Lab-blox was given each dog that had consumed the initial feeding. After 3½ to 4 additional hours, the feed dish was removed and weighed. Food consumed (in grams) was calculated and recorded. Uneaten food was discarded. Dogs not consuming the first feeding after two hours, but finishing the entire amount by 4:30 p.m., were likewise given 200 grams of Wayne Lab-blox. In the morning before the following day's feeding, the dish of dog pellets was removed, weighed and the food consumed was calculated and recorded. Uneaten food was discarded. Dogs not consuming the initial feeding of diet containing compound by 4:30 p.m. were left with food overnight. Food uneaten overnight was calculated and recorded. Effective April 6, 1970 (Appendix III-4), one week after the start of the test, the dosage calculation was based on individual body weight.

The twelve-week interim progress review on June 24, 1970 (Appendix III-5) summarizes percent body weight change (means for each group of dogs), food consumption, nutritional status, mortality rate, appearance, behavior, and elimination. Body weight gain was 22% lower in the high dose males and 12% lower in the high dose females when compared to the corresponding controls. Food consumption was listed only as greater than 200 g/day indicating that all animals consumed the desired amount of aspartame, but giving no indication of whether their total food consumption was affected by treatment. The other parameters were simply noted as unremarkable. Under hematology and clinical chemistry, a note states, "See separate sheets." These may be the intermediate data sheets supplied to UAREP.

The seventeen week progress review on July 28, 1970 (Appendix III-5) was similar to the twelve week review, except there was 29.8% less body weight gain in the high dose males compared to control males and 29.9% less in the high dose females compared to control females. It was also noted that phenylketones in the urine were all negative after fourteen weeks of treatment.

The twenty week progress review on August 19, 1970 (Appendix III-5) was similar to the previous ones, except that there was 38.4% less body weight gain in the high dose male group and 32.4% less in the high dose female group when compared with the controls.

On September 28, 1970 (Appendix III-6), a memo was sent from Dr. McConnell extending the study indefinitely. Another memo was sent from Dr. McConnell on October 29, 1970 (Appendix III-7), stating that the study was tentatively extended to 104 weeks duration with a termination

date of April 1, 1972. The profile of clinical lab work including clinical chemistry, hematology, and urinalysis, as indicated in the original protocol, was repeated at 52, 78, and 104 week intervals. Ophthalmoscopic examinations were to be performed at the same intervals specified for the laboratory observations.

The twenty-six week progress review on September 30, 1970 (Appendix III-5) was basically similar to the other progress reviews. At this point there was 41.76% less body weight gain in the high dose male group compared to controls, and 39.0% less in the high dose female group compared to controls. Dr. McConnell noted that there was a methodological problem with the GPT assay. He also noted that the body weight gain decrease was definitely compound-related, but did not require lowering the dosage.

The forty week progress review on January 26, 1971 (Appendix III-5) noted that there was 48.02% less weight gained in the high dose male group compared to the controls, and 37.39% less gained in the high dose females compared to controls.

A memo dated May 28, 1971 (Appendix III-8) from Dr. McConnell to Dr. Hemm requested review of the 52 week differential blood smear data to evaluate the presence of treatment related changes.

A memo dated June 11, 1971 (Appendix III-10) from Dr. Hemm to Dr. McConnell discusses review of the 52 week differential smear data. Dr. Hemm says the data shows no evidence of treatment related changes.

A memo dated September 10, 1971 (Appendix III-11) was sent from Dr. Polk to Dr. McConnell stating that the toxicology protocol design committee had decided to assess the glucogenic potential of the compound, and measure insulin secretion in addition to glucose. To assess lipo-

genic potential they decided to assay free cholesterol, cholesterol esters, triglycerides, free fatty acids, and, if possible, lipoprotein electrophoresis.

A memo dated October 1, 1971 (Appendix III-12) stated that data on insulin secretion would be obtained by the Pharmacology Department. Total cholesterol, free fatty acids, and triglycerides were added as routine measurements for weeks 78 and 106 of the dog studies. If total cholesterol was affected by the compound, free and esterified cholesterol fractions would be measured. Lipoprotein electrophoresis would be performed once in the study and repeated if indicated.

The protocol design committee was composed of the following members:

- 1) Dr. Sammeta, Biostatistician
- 2) Dr. F. Saunders, Biological Research Director
- 3) Dr. Ranney, Drug Metabolism Representative
- 4) Dr. Polk, Clinical Representative
- 5) Dr. Rao, Pathology-Toxicology Department Monitor
- 6) Dr. McConnell, Pathology-Toxicology Department Advisor

The technical staff included:

- 1) Mrs. Sprenger, Pathology Laboratory
- 2) Mr. Martinez, Autopsy Laboratory
- 3) Dr. Hutsell, Bioanalytical Laboratory
- 4) Dr. Rao, General Toxicology Laboratory
- 5) Mrs. Perkins, Hematology Laboratory
- 6) Dr. McConnell, Pathologist

A protocol amendment dated April 7, 1972 (Appendix III-16) requested assay of insulin, L-phenylalanine, ornithine carbamyl transferase, protein electrophoresis, and total protein for assay in terminal blood samples. The tissue slides of up to 37 organs from 40 dogs were prepared in Searle labs and sent to Microscopy for Biological Research in Albany, New York, where they were examined by Dr. Jacqueline Mauro. Slides were received at Microscopy for Biological Research on July 6, 1972 and the finished report was sent back to Searle on August 15, 1972. This report is included in the Entry Book on pages 108i to 217. Additional brain material submitted for pathological examination was received at Microscopy for Biological Research on May 10, 1973, and the report was sent back to Searle on May 21, 1973, signed by Dr. Ellen Dugan, the pathologist who examined the brain slides.

Dogs were acclimated for five weeks before the study began. They were housed in separate cages in air-conditioned quarters that were maintained at 72 degrees F with 14 hour photoperiod. They were fed as indicated previously, had water ad libitum, and were observed daily for survival and illnesses. The report did not indicate that the dogs were held in quarantine after their arrival.

In a letter dated June 21, 1978 (Appendix II-8) Dr. Dale Brooks discussed his site visit to evaluate the animal facilities at Searle. He concluded that these facilities were adequate, according to AAALAC standards, at the time this study was conducted.

Since the UAREP contract contraindicates our being concerned with protocol design, we have not considered whether the addition of testing for parameters such as L-phenylalanine and glucogenic and lipogenic factors only at later stages of the experiment might have resulted in important early changes being overlooked.

In a memo dated August 19, 1974 (Appendix III-17), Dr. Rao noted that the graph of mean body weights of the female dogs (E-28, Figure 2) had been labeled incorrectly. The uncorrected and corrected versions are included in Appendix III-18. Dr. Rao felt that this error would not affect the outcome of the report.

RESULTS AND DISCUSSION

Food Consumption and Body Weight

On page 2 in the Entry Book in the Experimental Design section, it is stated, "Animals were separated according to sex, and assigned individual cages by a standard randomization procedure." Tables 3-1 and 3-2 show groupings of animals as littermates. There are four groups of five littermates, two groups of four littermates, and some smaller groups of two or three littermates in the study (Appendix III-21). In the control male group, three out of the five dogs are littermates, as are three out of five in the high dose male group. In the high dose female group, two of the five are littermates. In the medium dose female group, there are two sets of two littermates each out of the five dogs. From this it appears that the animals may not have been randomized in terms of parentage.

Furthermore, the mean starting body weight of the dogs in the high dose group was significantly higher ($p < 0.05$) than the mean starting weight of the animals in the control groups when compared by t-test (Table 3-3). This result also raises questions regarding the randomization. In addition, it should be noted that animal E10 in the low dose male group and E20 in the high dose male group, besides being littermates, were about two months older than any of the other animals. Table 3-1 contains birthdates, ages, and relationships of all the dogs. Searle has not responded to a UAREP query as to the precise method of randomization employed in E-28.

Table 3-1

Ages of Dogs at Start of Experiment in E-28

(based on starting date of 3/30/70)

<u>Date Whelped</u>	<u>Age (days)</u>	<u>N</u>	<u>Number of Littermates</u>
8/2/69	240	1	
8/14/69	228	2	2
10/17/69	164	3	3
10/18/69	163	5	5
10/21/69	160	10	5/5*
10/26/69	155	6	4/2*
10/28/69	153	7	5/2*
10/29/69	152	4	4
11/2/69	148	2	2

* group composed of two different litters

Table 3-2

Distribution of Littermates in E-28

	<u>Animal Number</u>	<u>Dam</u>	<u>Sire</u>	<u>Date Whelped</u>
Control Males	A1CM	HI06	Randall	10-21-69
	B2CM*	MKH4	CII6	10-28-69
	C3CM*	MKH4	CII6	10-28-69
	D4CM*	MKH4	CII6	10-28-69
	E5CM	FL06	ACQ4	10-29-69
Low Dose Males	A6LM*	FL06	ACQ4	10-29-69
	B7LM*	FL06	ACQ4	10-29-69
	C8LM	DHR6	ANI6	10-21-69
	D9LM	DYL8	CII6	10-17-69
	E10LM	BAH7	PBI4	8-14-69
Medium Dose Males	A11MM	HA-574289	HA-979986	8-02-69
	B12MM	AJ-FX4	J	11-02-69
	C13MM	EBN7	HYI5	10-26-69
	D14MM*	DWP6	ACQ4	10-18-69
	E15MM*	DWP6	ACQ4	10-18-69
High Dose Males	A16HM*	ECJ8	FXM9	10-26-69
	B17HM*	ECJ8	FXM9	10-26-69
	C18HM*	ECJ8	FXM9	10-26-69
	D19HM	HI06	Randall	10-21-69
	E20HM	BAH7	PBI4	8-14-69
Control Females	A21CF	HI06	Randall	10-21-69
	B22CF	ECJ8	FXM9	10-26-69
	C23CF	EBN7	HYI5	10-26-69
	D24CF*	MKH4	CII6	10-28-69
	E25CF*	MKH4	CII6	10-28-69
Low Dose Females	A26LF	FL06	ACQ4	10-29-69
	B27LF*	HI06	Randall	10-21-69
	C28LF	DHR6	ANI6	10-21-69
	D29LF*	HI06	Randall	10-21-69
	E30LF	DWP6	ACQ4	10-18-69
Medium Dose Females	A31MF*	DWP6	ACQ4	10-18-69
	B32MF*	DWP6	ACQ4	10-18-69
	C33MF*	Randall-2	Randall-1	10-28-69
	D34MF*	Randall-2	Randall-1	10-28-69
	E35MF	DYL8	CII6	10-17-69
High Dose Females	A36HF	DYL8	CII6	10-17-69
	B37HF*	DHR6	ANI6	10-21-69
	C38HF*	DHR6	ANI6	10-21-69
	D39HF*	DHR6	ANI6	10-21-69
	E40HF	AJ-FX4	J	11-02-69

* littermates included in the same group

Table 3-3

Body Weights (kg) at Start (Week 0) of Study

Control Males

A1 6.1
B2 6.0
C3 5.9
D4 5.7
E5 5.6
 \bar{x} 5.9 \pm 0.21

t = 3.25

High Dose Males

A16 6.2
B17 7.0
C18 7.4
D19 6.1
E20 6.9
 \bar{x} 6.7 \pm 0.55

Control Females

A21 5.5
B22 5.0
C23 4.9
D24 5.7
E25 5.1
 \bar{x} 5.2 \pm 0.34

t = 2.97

High Dose Females

A36 6.6
B37 6.3
C38 5.8
D39 5.3
E40 6.3
 \bar{x} 6.1 \pm 0.51

The high dose groups were both significantly higher than the control groups ($P < 0.05$) when compared by the two-tailed t-test. $t_{.05}$ with eight degrees of freedom = 2.31.

The various weeks for which there is difficulty correlating data on body weights are listed in Table 3-4. It shows that data for week 36 are recorded in the Entry Book (p 62) under week 34. Data for week 40 are those recorded for week 36. The original weights for week 44 are recorded in E-28 (p 62) for week 40. This four week discrepancy persists throughout the remainder of the study. Such discrepancies in a 106 week study would not be expected to have a significant effect on the biological interpretation of results.

Apparent discrepancies in recording individual body weights are pointed out in Table 3-5. The underlined values are those which appear to show substantial deviation for the sequence in which they occur. UAREP believes that dogs don't normally gain or lose 4 kg actual weight over a two week interval. The raw data are difficult to evaluate since weights are recorded in groups of five according to dosage and sex with no indication of animal number. UAREP proceeded on the assumption that weights were always recorded in the same order.

Table 3-6 shows weight gain in kilograms in all groups for the period from 0 to 26 weeks. Mean weight gain for the male groups shows a downward trend. Least weight gain occurred in the medium dose group. One extremely low value contributed to making this mean lower than the high dose group. The high dose group had the lowest weight gain of the females (Figure 3-1). In plots of food consumption, the high dose groups showed more variation in the early part of the experiment than the control groups. Table 3-7 shows the percent weight gain for weeks 0-26. The medium and high dose male groups were significantly lower than the controls using the Least Significant Difference test, while the high dose female group was significantly lower than all the other groups by both the LSD and Q tests.

Table 3-4
Discrepancies In Reporting Body Weights When Drug Dosage Records,
"Raw" Data And Entry Book Are Compared

1 Date	2 Week	3 E-29	4 Drug Dosage Record	5 "Raw" Data for Mean Body Weight
3/20/70	-1	-1	-1 3/7 (3/20/70)	
3/30/70	0	0	0 - 1 (3/30/70)	
4/6/70	1	1	1 - 2 (4/6/70)	1 (4/6/70)
4/13/70	2	2	2 - 3 (4/13/70)	
4/20/70	3	3	3 - 4 (4/20/70)	3 (4/20/70)
4/27/70	4	4	4 - 5 (4/27/70)	4 (4/27/70)
5/4/70	5	5	5 - 6 (5/4/70)	5 (5/4/70)
5/12/70	6	6	6 - 7 (5/12/70)	6 (5/12/70)
5/18/70	7	7	7 - 8 (5/18/70)	7 (5/18/70)
5/25/70	8	8	8 - 9 (5/25/70)	8 (5/25/70)
6/8/70	10	10	10-11 (6/8/70)	10 (6/8/70)
6/22/70	12	12	12-13 (6/22/70)	12 (6/22/70)
7/6/70	14	14	14-15 (7/6/70)	
7/20/70	16	16	16-17 (7/20/70)	16 (7/20/70)
8/3/70	18	18	18-19 (8/3/70)	18 (8/3/70)
8/17/70	20	20	20-21 (8/17/70)	20 (8/17/70)
8/31/70	22	22	22-23 (8/31/70)	22 (8/31/70)
9/14/70	24	24	24-25 (9/14/70)	24 (9/14/70)
9/28/70	26	26	26-27 (9/28/70)	26 (9/28/70)
10/12/70	28	28	28-29 (10/12/70)	
10/26/70	30	30	30-31 (10/26/70)	30 (10/26/70)
11/10/70	32	32	32-33 (11/10/70)	32 (11/10/70)
11/24/70	34			
12/8/70	36	34	33-36 (12/8/70)	36 (12/8/70)
1/4/71	40	36	36-40 (1/5/71)	
2/1/71	44	40	40-44 (2/2/71)	44 (2/1/71)
3/2/71	48	44	44-48 (3/2/71)	48 (3/2/71)
3/30/71	52	48	48-52 (3/30/71)	52 (3/30/71)
4/28/71	56	52	52-56 (4/27/71)	
5/25/71	60	56	56-60 (5/25/71)	60 (5/25/71)
6/21/71	64	60	60-64 (6/21/71)	64 (6/21/71)
7/19/71	68	64	64-68 (7/19/71)	
8/16/71	72	68	68-72 (8/16/71)	72 (8/16/71)
9/13/71	76	72	72-76 (9/13/71)	76 (9/13/71)
10/11/71	80	76	76-80 (10/12/71)	
11/8/71	84	80	80-84 (11/8/71)	
12/7/71	88	84	84-88 (12/7/71)	88 (12/7/71)
1/4/72	92	88	88-92 (1/4/72)	92 (1/4/72)
2/1/72	96	92	92-96 (2/1/72)	
3/1/72	100	96	96-100 (3/1/72)	100 (3/1/72)
3/29/72	104	100	100-104 (3/29/72)	104 (3/29/72)
4/10-14/72	106	106	106 (4/10/72)	

Dates and weeks listed in the first two columns were calculated with the Hewlett-Packard-97 Calendar Function Program.

Column 3 lists the weeks reported in the Entry Book.

Column 4 lists dates and weeks recorded on the Drug Dosage Records sent to UAREP from Searle.

Column 5 lists dates and weeks recorded on worksheets for calculating group means also sent to UAREP from Searle.

Table 3-6

Body Weight Gain (kg) Weeks 0 to 26 of Study E-28

Males

<u>Control</u>	<u>Low Dose</u>	<u>Medium Dose</u>	<u>High Dose</u>
3.9	4.0	1.0	3.8
5.5	4.7	5.0	5.2
5.4	3.8	2.0	3.7
4.9	3.4	3.8	3.4
<u>3.2</u>	<u>5.1</u>	<u>5.1</u>	<u>1.4</u>
\bar{x} 4.58	4.20	3.38	3.5
SD 1.0 (CV=22%)	0.69 (CV=16%)	1.83 (CV=54%)	1.36 (CV=39%)

No significant difference by analysis of variance because of such large coefficient variation (CV)

Females

<u>Control</u>	<u>Low Dose</u>	<u>Medium Dose</u>	<u>High Dose</u>
3.5	3.9	3.6	1.9
4.5	2.7	2.8	2.7
2.5	2.9	4.4	1.6
4.9	3.7	4.8	1.1
<u>3.5</u>	<u>3.2</u>	<u>3.1</u>	<u>3.5</u>
\bar{x} 3.8*	3.28*	3.74*	2.16
SD 0.94	0.51	0.85	0.95

* Significantly higher than the high dose ($P < 0.05$) when compared by analysis of variance followed by Newman-Keuls (Q) test.

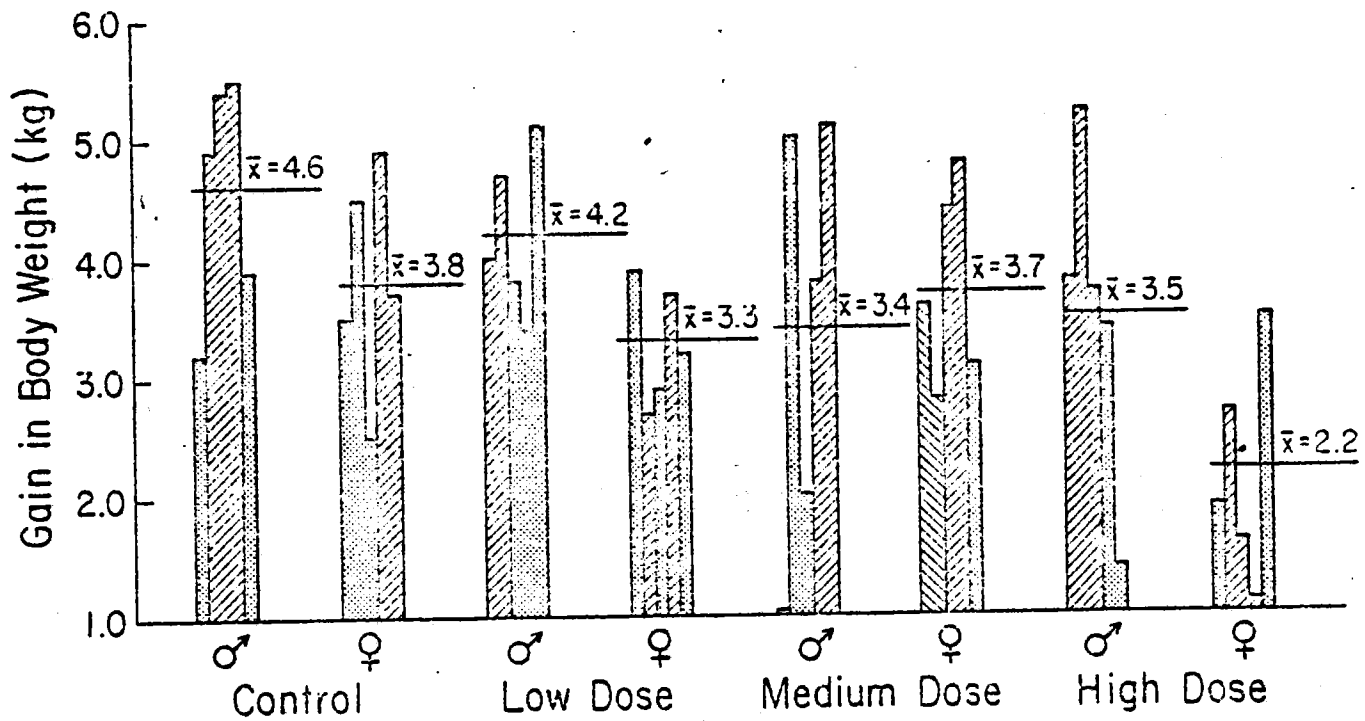


Figure 3-1: Body Weight Gain (kg) in Individual Animals from Week 0 to Week 26.

Table 3-7

Percent Body Weight Gain Weeks 0-26

MALES

	<u>Control</u>	<u>Low Dose</u>	<u>Medium Dose</u>	<u>High Dose</u>
	64	73	18	61
	92	71	77	74
	92	61	38	50
	86	56	63	56
	57	85	77	20
	<hr/>	<hr/>	<hr/>	<hr/>
\bar{x}	78.2	69.2	54.6	52.2
SD	16.5	11.3	25.9	20.0

The high dose group was significantly lower ($P < 0.05$) than the control group by the LSD but not the Q test.

FEMALES

	<u>Control</u>	<u>Low Dose</u>	<u>Medium Dose</u>	<u>High Dose</u>
	64	76	50	29
	90	53	47	43
	51	51	65	28
	86	63	80	21
	71	70	63	56
	<hr/>	<hr/>	<hr/>	<hr/>
\bar{x}	72.4	62.6	61.0	35.4
SD	16.0	10.7	13.2	14.0

The high dose group was significantly lower ($P < 0.05$) when compared with each of the other groups by both the LSD and Q tests for significance.

That the high dose groups were heavier to start with (Table 3-3), may be an important aspect of the study. Erratic food consumption in the high dose groups may be related simply to the altered taste of the food the animals were getting rather than anything chemically inherent in the compound. Conversely, diminished weight gain and erratic food consumption in the high dose groups could be compound related. The variance is so great in both food consumption and body weight gain data that statistical significance may be obscured.

From the age of 6 months on (throughout the study), the dogs were presented with a total of 400 grams of feed per day equal to 1410 kilocalories/day. The caloric requirement for puppies under twelve months of age (4.5 kg/body weight) is 900 kilocalories/day, while the requirement for dogs one year of age and over (13.5 kg body weight) is 1000 kilocalories/day.

It should also be noted that the dogs were divided into five groups (A, B, C, D, E) of eight, with four males and four females per group. There was one dog from Group A in each treatment group for each sex. Compound feeding in Group A was begun on Monday, March 30, 1970. Group B was begun on Tuesday, and continuing until Friday, when Group E was started. The dogs were sacrificed in the same sequence over a five day period at the termination of the experiment.

Table 3-8 lists food consumption in all groups for weeks 1-26. There is depressed food intake during this period in the treated groups which is not significantly different by Analysis of Variance. However, this effect of aspartame might have biological significance if it also occurred in humans ingesting the compound.

Table 3-8

Total Food Consumption (kg) Weeks 1 to 26

Males

	<u>Control</u>	<u>Low Dose</u>	<u>Medium Dose</u>	<u>High Dose</u>
	72.46	67.45	64.33	62.63
	70.99	65.29	66.11	63.90
	70.02	59.80	68.98	71.87
	69.93	64.16	63.79	68.82
	<u>65.57</u>	<u>68.28</u>	<u>64.86</u>	<u>67.87</u>
\bar{x}	69.79	65.00	65.61	67.02
SD	2.57	3.34	2.07	3.76

Females

	<u>Control</u>	<u>Low Dose</u>	<u>Medium Dose</u>	<u>High Dose</u>
	67.85	62.81	58.54	54.52
	66.56	66.78	57.50	64.74
	69.05	53.16	66.12	64.66
	68.15	67.35	63.83	60.96
	<u>60.25</u>	<u>55.85</u>	<u>57.29</u>	<u>65.10</u>
\bar{x}	66.37	61.19	60.66	62.00
SD	3.54	6.42	4.05	4.51

Table 3-9 lists total food consumption in all groups for weeks 1-10 when the most erratic consumption was occurring in the treated groups. In the male dogs, all the treated groups ate less than the controls, but the only statistically significant difference was between the medium dose group and the controls. In the females, there was essentially no difference in food consumption in the low and medium dose groups versus the controls; however, the high dose group was significantly lower than the controls as well as the other two treated groups.

UAREP confirmed the decrease in total mean food consumption which occurred at weeks 52 and 53 (E-28, Figure 3, p 16). Food consumption records show zero consumption for most animals at the time of the second feeding on several days during these two weeks. UAREP is unable to ascertain whether the animals did not eat at these times or were not given the second feeding for some reason.

Compound Consumption

Amount of aspartame used in the study, lot numbers, percent of DKP, and number of days each lot was used were recorded (Appendix III-22). Consumption of aspartame (Table 3-10) was checked at selected intervals by recalculation, using correct body weights (Table 3-4), for the intervals being checked. Raw data for body weights were missing for weeks 14, 68, and 84.

Since the data at the intervals checked were within 6% of the desired dose (except as indicated below, in three groups in which body weight data was unclear), UAREP felt that further checking was not necessary to verify test dosages.

Table 3-9

Total Food Consumption (Kg) Weeks 1-10

Males

	<u>Control</u>	<u>Low Dose</u>	<u>Medium Dose</u>	<u>High Dose</u>
	27.97	25.07	20.83	22.79
	26.85	25.36	25.71	21.85
	26.20	24.09	24.71	27.62
	25.85	23.02	22.64	25.07
	24.49	25.40	23.74	23.19
	<hr/>	<hr/>	<hr/>	<hr/>
\bar{x}	26.27	24.59	23.52*	24.10
SD	1.23	1.02	1.88	2.29

* significantly lower than control group by two-tailed "t" test (P<0.05).

Females

	<u>Control</u>	<u>Low Dose</u>	<u>Medium Dose</u>	<u>High Dose</u>
	25.31	24.19	23.79	21.63
	22.71	24.74	23.31	20.74
	25.36	20.33	25.64	20.63
	24.67	24.30	25.78	20.96
	20.43	22.43	22.16	21.07
	<hr/>	<hr/>	<hr/>	<hr/>
\bar{x}	23.69	23.29	23.94	21.00†
SD	2.12	1.92	1.55	0.39

† significantly lower than all other groups by two-tailed "t" test (P<0.05)

Table 3-10 lists compound consumption in grams per kilogram at various intervals. All the doses were close to the intended amount. Minor fluctuations in dosage would tend to average out over the long term of the experiment.

Actual mean dosages ranged as follows:

Test Group	Actual Amount Received	Percent Desired Dose
low dose males (1g/kg)	0.94 to 1.03 g/kg	94 to 103%
med. dose males (2g/kg)	1.89 to 2.08 g/kg	94 to 104%
high dose males (4g/kg)	3.77 to 4.07 g/kg	94 to 102%
low dose females (1g/kg)	0.96 to 1.03 g/kg	96 to 103%
med. dose females (2g/kg)	1.90 to 2.12 g/kg	95 to 106%
high dose females (4g/kg)	3.79 to 4.08 g/kg	95 to 102%

In the high dose male group at week four the animals received 4.01 - 5.18 g/kg. At week 36 animal C13 (medium dose) received 3.08 g/kg, animal B12 (medium dose) received 1.62 g/kg, and animal A16 (high dose) received 2.91 g/kg. At week 106 animal B17 (high dose) received 1.01 g/kg. These calculations were done with the body weights as recorded. It is possible they could have been recorded out of sequence. Because of the variations mentioned above, the means for these groups were not calculated.

The Entry Book (E-28, p 4) states: "The compound was administered orally each day by incorporating appropriate amounts of SC-18862 into 200 g of powdered basal diet individually. . . Usually this compound diet mixture would be consumed by the dog in about two hours. At the end of two hours (about 11 a.m.) another 200 g basal diet (without the

compound) was presented to each dog." The Entry Book does not clearly indicate if dishes were checked to be sure the initial feeding (containing the compound) had been consumed. The second feeding might simply have been added to remaining food from the first. In this case if the total amount of food was not consumed, the animals would not receive the full daily dose of compound. Searle staff, however, assured UAREP that the first feeding containing the aspartame was virtually always eaten before the second feeding was given.

Table 2A, E-28, pp 21-22 shows an interesting, unusual variation in intervals used for demonstrating compound consumption. For weeks 33 to 72, the periods run 33-34-36-37-40-42-44-48-49-52-55-56-60-62-64-68-69-72 and the net weeks in each interval is 1, 2, 1, 3, 2, 2, 4, 1, 3, 3, 1, 4, 2, 2, 4, 1, 3. UAREP was interested to see that the 3-3 point at 52 weeks coincided with the maximum dip in food consumption in Figure 3 (E-28, p 16). UAREP demonstrated that if the food consumption had been plotted using a similar floating time interval instead of weekly intervals which were used, the dip would not have been nearly as pronounced. Searle, when queried, could not explain the dip in food consumption at 52 weeks. UAREP can not explain it or the coincidence with the varying intervals in reporting compound consumption.

Clinical Observations

Observations were made at the time of each body weight measurement. Weights were measured weekly for the first eight weeks, and at longer intervals following that. To quote the Entry Book: "An evaluation of general motor and behavioral activity, locomotion, external appearance of teeth, nose, eyes, ears, perineum, hair coat, and digital palpation for tissue masses was conducted immediately prior to the initiation of treatment, and subsequently concurrent with each body weight measurement. Unusual signs including indications of systemic pharmacologic or toxicologic effects were routinely recorded at this time and whenever warranted."

UAREP was not supplied with any records of clinical observation data. A book labeled "Canine Registration and Health Records" was received. The health records consist of data on five animals giving dates of immunizations and weaning.

There was a note that animal B17 of the high dose male group had some kind of mishap requiring amputation of the last two vertebrae of the tail.

Due to the lack of records, UAREP is unable to evaluate the adequacy of clinical observation procedures in this study.

Ophthalmoscopic Examinations

These examinations were performed by Drs. W. Magrane and G. Youkilis on all dogs at 24, 30, 37, 52, 80 and 105 weeks of compound administration (Table 3-11).

As pointed out in the Entry Book (E-28, p 17) no pretreatment examination of the eyes was performed. The initial examination was done at 24 weeks, at which time B12 (medium dose male) and E40 (high dose female) were found to have bilateral anterior lens subcapsular and nuclear cataracts. Reference to Table 3-2 will show that these animals were littermates. These lesions did not progress during the course of the study.

Since the type and location of the cataracts mentioned above were consistent with the characteristics of congenital cataracts according to E-28, page 19, it was decided to remate the parental dam and sire of B12 and E40. Two male full siblings were obtained (Appendix III-13), one of which showed (at about six months) a unilateral cataract involving the temporal aspect of the lens cortex. Follow-up examinations (Appendix III-14) at intervals of 2-4 weeks showed little progression of this cataract. Searle therefore concluded that the cataracts in the two dogs in the study were not treatment related.

A total of 34 lesions were recorded. Percent distribution of the lesions was as follows:

<u>Control</u>	<u>Low Dose</u>	<u>Medium Dose</u>	<u>High Dose</u>
9	8	3	14
(26.5%)	(23.5%)	(8.8%)	(41.2%)

Table 3-11

Dates of Necropsies and Ophthalmoscopic Exams as Verified

<u>Path #</u>	<u>Animal #</u>	<u>Necropsy Date</u>	<u>Initials</u>	<u>Dates of Eye Exams</u>
91939	A1CM	4/10/72	RGM	10/31/71, 4/6/72
91990	B2CM	4/11/72	RDH	No record
91991	C3CM	4/12/72	RGM	9/7/70, 10/28/70, 12/15/70, 4/1/71, 10/13/71, 4/10/72
91992	D4CM	4/13/72	RDH	12/15/70, 3/31/71, 10/13/71, 10/21/71, 4/11/72
91993	E5CM	4/14/72	RDH	10/3/71, 4/12/72
92019	A6LM	4/10/72	RDH	10/13/71, 10/21/71, 4/6/72
92020	B7LM	4/11/72	RGM	No record
92021	C8LM	4/12/72	RDH	4/10/72
92022	D9LM	4/13/72	RGM	3/31/71, 10/13/71, 4/11/72
92023	E10LM	4/14/72	RGM	10/13/70, 4/12/72
92009	A11MM	4/10/72	RGM	No record
92010	B12MM	4/11/72	RDH	9/17/70, 10/28/70, 12/15/70, 4/2/71, 10/13/71, 10/21/71, 4/7/72
92011	C13MM	4/12/72	RGM	4/10/72
92012	D14MM	4/13/72	RDH	4/11/72
92013	E15MM	4/14/72	RDH	No record
91999	A16HM	4/10/72	RDH	10/13/71, 10/21/71, 4/6/72
92000	B17HM	4/11/72	RGM	9/17/70, 10/28/70, 12/15/70, 4/2/71, 10/13/71, 4/7/72
92001	C18HM	4/12/72	RDH	No record
92002	D19HM	4/13/72	---	9/17/70, 10/28/70, 12/15/70, 3/31/71, 10/13/71, 10/21/71, 4/11/72
92003	E20HM	4/14/72	RGM	3/30/71, 10/13/71, 4/12/72
91994	A21CF	4/10/72	RDH	No record
91995	B22CF	4/11/72	RGM	No record
91996	C23CF	4/12/72	RDH	9/17/70, 10/28/70, 12/15/70, 4/1/71, 10/13/71, 4/10/72
91997	D24CF	4/13/72	RGM	No record
91998	E25CF	4/14/72	RGM	4/12/72
92024	A26LF	4/10/72	RGM	No record
92025	B27LF	4/11/72	RDH	No record
92026	C28LF	4/12/72	RGM	4/1/71, 10/13/71, 4/10/72
92027	D29LF	4/13/72 - 4/12/72 ?	RDH	No record
92028	E30LF	4/14/72	RDH	No record
92014	A31MF	4/10/72	RDH	No record
92015	B32MF	4/11/72	RGM	No record
92016	C33MF	4/12/72	RDH	No record
92017	D34MF	4/13/72	RDH	No record
92018	E35MF	4/14/72	RGM	No record
92004	A36HF	4/10/72	RGM	10/17/70, 10/28/70, 12/15/70, 3/28/71, 10/13/71, 4/6/72
92005	B37HF	4/11/72	RDH / RGM	No record
92006	C38HF	4/12/72	RGM	4/13/70, 10/21/71, 4/10/72
92007	D39HF	4/13/72	RDH	10/13/70, 4/11/72
92008	E40HF	4/14/72	RDH	10/23/70, 12/15/70, 3/30/71, 10/13/71, 10/21/71, 4/12/72

Time of onset of lesions varied from week 24 to week 105. Eight dogs were first noted as having lesions at week 24. Thirteen were first observed at week 105, then ten were first noted at week 80.

No eye examination records were available for the following dogs:

B2 Control male

B7 Low dose male

A11
E15 Medium dose male

C18 High dose male

A21
B22 Control female
D24

A26
B27 Low dose female
D29
E30

5 Medium dose female

B37 High dose female

Total 18

UAREP is unable to evaluate the significance of the missing records. Possibly these animals had negative findings and, therefore, were not included.

Hematology

General Comments: Tables of individual values for hematology parameters are found in E-28 on pages 65 to 74, and summary tables for the hematology data are found on pages 23 to 31 (E-28). These tables give means for each group and show significant differences found in the statistical analyses. Hematology findings are discussed on page 20. "No biologically meaningful changes in hematologic parameters were observed although transient differences reaching statistical significance were frequently present. Statistically significant changes in red cell parameters (hemoglobin, hematocrit, and/or total red cell values) were variably present in the treated groups and were consistently significantly lower in the high dose males, perhaps reflecting both a slight hypovolemia and a slight reduction in total RBC's." Hypervolemia would more likely cause an apparent reduction in erythrocyte parameters than hypovolemia. The report also states that these alterations in the high dose males were not considered to have any biological significance.

Erythrocyte Parameters: There is an upward trend in erythrocyte parameters (hemoglobin, hematocrit, and total red blood cell count) during the two year period. This upward trend appears to be normal in dogs as they mature. However, the high dose male group had significantly lower values for hemoglobin and hematocrit at most intervals in the study. These calculations were checked by us with corrected data, using Analysis of Variance, Least Significant Difference (LSD), and the Newman-Keuls (Q) test for significance (Table 3-12), in addition to the t-test.

Table 3-12
continued

Parameters	Week	Sex	ANOVA	Group	LSD	Q	UAREP t test	t Value	Searle t test
Hemoglobin (cont'd)	26	M	0.00	2>4	S	S	S	4.152	ND
		M		3>4	S	S	S	3.775	ND
		M		1>4	S	S	S	4.230	S
	52	M	0.00	2>4	S	S	S	7.409	ND
		M		3>4	S	S	S	3.699	ND
		M		1>4	S	S	S	6.600	S
		F		1<2	S	S	S	3.020	S
		F		2>4	S	S	S	6.684	ND
	78	F	0.00	2>3	S	S	S	3.524	ND
		M		2>4	S	S	S	4.313	ND
		M		3>4	S	S	S	3.312	ND
		M		1>4	S	S	S	2.710	S
		F		1>3	ND	ND	N	---	S
	106	M	0.00	2>4	S	S	S	2.542	ND
		M		1>3	S	N	S	3.420	S
		M		3>4	S	S	S	2.520	ND
		M		1>4	S	S	S	4.530	S
PMI	-1	F	0.23	1<3	ND	ND	S	2.390	N
	4	F	0.04	2<4	S	S	S	3.471	ND
	14	M	0.20	1<4	ND	ND	N	(2.290)	S
	52	M	0.17	1<4	ND	ND	S	2.330	S
Prothrombin Time	-1	F	0.01	1<4	S	N	S	2.360	S
		F		2<3	S	N	N	---	ND
		F		2<4	N	N	N	---	ND
	52	M	0.11	1<2	ND	ND	S	2.700	S
	106	M	0.38	1<2	ND	ND	N	(2.280)	S
		F	0.00	1<2	S	S	S	4.780	S
		F		1<3	S	S	S	3.620	S
PTTA	-1	F	0.01	1<4	S	S	S	4.110	S
		M		1>2	S	S	S	3.080	S
		M		1>3	S	S	S	3.920	S
		F		1<3	S	N	N	---	S
		F		2<3	S	S	S	2.579	ND
	4	F	0.05	2<4	S	N	N	---	ND
		M		1>2	S	N	N	(2.200)	S
		M		1>3	S	S	S	4.550	S
	14	F	0.23	1<4	ND	ND	N	---	S
	52	F	0.38	1<3	ND	ND	N	---	S
		F		1<3	ND	ND	N	---	S

ND = Not Done

ANOVA indicates the exact probability that all group means are equal based upon the F test for Analysis of Variance (ANOVA).

LSD (Least Significant Difference) S = significant at $P < 0.05$; N = $P > 0.05$.

Q (Newman-Keuls test) S = significant at $P < 0.05$; N = $P > 0.05$.

All ANOVA values of 0.00 in this report indicate less than 1% chance that the means being compared are equal.

Verification of the tables of individual values for hematology parameters was done as follows. Raw data books were Xeroxed. We received copies of these for most of the hematology assays. We were able to cross-check from raw data to the table of individual values in the Appendix of E-28 and verify that the data recorded were in fact those recorded in the laboratory.

Red and white blood cells were counted in a Coulter counter. Results from this counter over 10,000 require correction because a certain percentage of the time two cells will be seen as one (E-28, p 58, ref. 3) and hence a coincidence correction factor should be applied to any count over 10,000. The raw data books do not show this correction as being made for the erythrocyte counts (Table 3-13), nor for the white blood cell counts at weeks -2, (-1), 0, 4, and 8, although it was made at other intervals. Table 3-14 gives samples of red and white cell counts as they appear in the raw data and as they should appear if the correction had been made. In cases where this correction was not made, we have checked the calculations with the data as recorded. In addition, we have recalculated with corrected data to obtain the means and check significance.

After receiving the data in Tables 3-13 and 3-14, Dr. Bost checked with the Searle laboratory personnel. He advised UAREP by phone on 8/14/78 that the laboratory people said that the Coulter Coincidence correction was made before the data were recorded in the laboratory notebooks for weeks -2 to 8 and that, therefore, the data as presented in the Entry Book were correct.

However, UAREP notes that five of the white cell count figures reported by Searle for these times fall within the range of 10.0 to 10.2. Since 10.0 would be corrected to 10.3 if the coincidence factor is

Table 3-13
Examples of Comparison of Means for RBC ($\times 10^6/\text{cmm}$)
and WBC ($\times 10^3/\text{cmm}$) using Corrected* and Uncorrected Data

Week	-2	-1	0	4	8	14	26	52	78	106
<u>Control Males</u>										
RBC*	4.75		5.49	5.48	5.76	5.85	5.93	5.95	6.61	7.05
RBC‡	4.27		4.89	4.88	5.10					
WBC*		10.40	15.34	12.86	12.24	14.32	13.06	11.84	13.40	9.58
WBC‡		10.26	14.74	12.42	11.86					
<u>Control Females</u>										
RBC*	4.68		5.01	5.13	5.25	5.39	5.92	5.87	6.44	6.90
WBC‡	4.21		4.51	4.58	4.68					
WBC*		9.94	16.26	14.00	11.72	12.44	11.80	11.74	10.40	9.72
WBC‡		9.81	15.62	13.44	11.44					
<u>High Dose Males</u>										
RBC*	4.78		5.06	4.27	4.88	5.03	5.79	5.55	6.63	6.61
RBC‡	4.29		4.52	3.87	4.38					
WBC*		9.84	14.84	10.64	9.56	14.84	15.56	11.40	10.88	10.34
WBC‡		9.78	14.28	10.42	9.42					
<u>High Dose Females</u>										
RBC*	4.95		4.89	4.36	5.36	5.41	6.10	5.93	6.22	6.53
RBC‡	4.44		4.39	3.95	4.77					
WBC*		12.40	11.78	10.14	13.12	14.72	14.32	11.32	12.08	9.70
WBC‡		12.04	11.5	9.94	12.72					

* Individual values corrected with Coulter Coincidence Table

‡ Individual values through week 8 appear not to have been corrected for Coulter Coincidence

Table 3-14

Examples of Discrepancies in Hematology Data

From Appendix Table 2, E-28

Code #	RBC (x10 ⁶ /cmm)	Corrected*	WBC (x10 ³ /cmm)	Corrected*
	(week -2)		(week -1)	
<u>Control</u>				
A1CM	4.34	4.83	6.8	--
B2CM	4.34	4.83	9.7	--
C3CM	4.02	4.44	11.3	11.6
D4CM	4.42	4.93	14.0	14.6
E5CM	4.25	4.72	9.5	--
A21CF	4.31	4.80	12.1	12.5
B22CF	4.06	4.49	9.0	--
C23CF	4.00	4.41	7.7	--
D24CF	4.50	5.03	10.5	10.8
E25CF	4.20	4.66	9.7	--
<u>Low Dose</u>				
A6LM	5.31	6.01	11.1	11.4
B7LM	4.34	4.83	8.8	--
C8LM	4.23	4.70	6.0	--
D9LM	4.57	5.12	16.3	17.0
E10LM	4.37	4.87	15.9	16.3
A26LF	4.61	5.16	10.7	11.0
B27LF	3.94	4.34	11.6	11.9
C28LF	4.12	4.56	7.5	--
D29LF	4.56	5.10	10.2	10.5
E30LF	4.05	4.48	13.6	14.1
<u>Medium Dose</u>				
A11MM	5.48	6.21	9.3	--
B12MM	4.81	5.41	11.6	11.9
C13MM	4.68	5.25	11.0	11.3
D14MM	4.63	5.19	10.2	10.5
E15MM	4.43	4.94	10.4	10.7
A31MF	4.25	4.72	11.8	12.2
B32MF	4.33	4.82	7.9	--
C33MF	4.48	5.01	14.9	15.5
D34MF	4.41	4.92	13.3	13.8
E35MF	4.68	5.25	11.8	12.2
<u>High Dose</u>				
A16HM	4.55	5.09	8.7	--
B17HM	4.20	4.66	9.9	--
C18HM	4.44	4.96	10.8	11.1
D19HM	4.19	4.65	9.9	--
E20HM	4.10	4.54	9.6	--
A36HF	4.39	4.89	14.7	15.3
B37HF	4.42	4.93	9.4	--
C38HF	4.55	5.09	8.0	--
D39HF	4.31	4.80	14.0	14.6
E40HF	4.52	5.05	14.1	14.7

*Values over 10,000/cmm should be corrected by a coincidence factor

used, the only counts possible are 9.9 and 10.3. A count of 9.9 would not be corrected, while a count of 10.0 would be corrected to 10.3.

The values reported after eight weeks do not contain any counts of 10.0 to 10.2 indicating that the correction table was used. In addition, the laboratory notebooks for weeks 14 on show the original and the corrected figures.

UAREP recognizes the difficulty of recalling precisely how things were done after a lapse of more than eight years, and has no better explanation to offer to account for the data which it feels were not appropriately corrected.

The coefficient of variation ($\frac{\text{standard deviation}}{\text{arithmetic mean}} \times 100$) was so great in most of the white cell counts that the statistics were recalculated by UAREP using logarithms to obtain geometric means. Analysis of Variance using these data revealed no significant differences in the white cell data whether calculated with logarithms or actual values. This contradicts the findings reported in the summary tables (E-28, pp 26-29) in which Searle reported the following values as significantly different from controls: week 78, low dose males and medium dose females; week 106, medium dose females.

UAREP's t-test showed that only the low dose males were significantly different from controls at week 78.

Wintrobe Indices of erythrocyte parameters were calculated according to the standard formulae:

$$\text{Mean Corpuscular Volume} = \frac{\text{Hematocrit} \times 10}{\text{RBC} \times 10^6/\text{cmm}}$$

$$\text{Mean Corpuscular Hemoglobin (MCH)} = \frac{\text{Hemoglobin (g\%)} \times 10}{\text{RBC} \times 10^6/\text{cmm}}$$

$$\text{Mean Corpuscular Hemoglobin Concentration (MCHC)} = \frac{\text{Hemoglobin (g\%)} \times 100}{\text{Hematocrit}}$$

Normal values for dogs (1) are:

MCV 59-58 μ^3

MCH 21-25 g $\times 10^{-12}$

MCHC 30-35%

Table 3-15 shows the Indices calculated for the dog study using arithmetic means of all data. Erythrocyte counts were corrected for Coulter Coincidence. MCV in the high dose males was in the low normal range except at weeks 26 and 52 when it was below the normal range. MCH in the high dose males was lower than the normal range in weeks 8 to 52 and 106. MCHC in the high dose males was lower than the other groups, but within the normal range. These results could indicate a trend to simple microcytic anemia in the high dose males. The females did not show such a trend, however, so the biological significance of the low values in the males is obscure.

Table 3-15
Wintrobe Indices
SC-18862: 106 Week Oral Toxicity Study In The Dog

Treatment Group	Weeks of Treatment								
	- 2			0				4	
MCV	MCH	MCHC	MCV	MCH	MCHC	MCV	MCH	MCHC	
MALES									
Control	72.4	24.9	34.4	69.2	23.5	33.9	68.4	23.7	34.6
Low Dose	73.6	25.0	34.0	68.7	23.5	34.2	69.0	23.8	34.5
Medium Dose	71.5	24.6	34.4	69.6	24.0	34.4	67.4	23.0	34.1
High Dose	72.4	25.3	35.0	70.4	24.2	34.3	62.8	21.6	34.5
FEMALES									
Control	72.6	25.5	35.1	70.3	24.5	34.8	66.1	22.7	34.3
Low Dose	75.3	26.1	34.7	71.4	24.4	34.2	69.1	23.8	34.4
Medium Dose	71.7	25.1	35.1	70.9	24.3	34.3	67.2	22.9	34.1
High Dose	72.3	25.0	34.8	71.2	24.2	34.0	68.4	22.6	32.5
WEEKS OF TREATMENT									
	8			14			26		
MALES									
Control	68.4	23.4	34.2	72.3	25.1	34.8	70.8	25.9	36.6
Low Dose	69.8	24.0	34.4	74.1	25.4	34.3	68.6	23.9	34.8
Medium Dose	65.6	22.2	33.8	68.5	23.7	34.5	66.6	23.1	34.7
High Dose	62.9	19.7	31.3	61.4	19.5	31.8	55.3	19.3	35.0
FEMALES									
Control	70.3	23.7	33.7	74.1	25.4	34.3	73.0	24.4	33.4
Low Dose	72.1	24.3	33.7	76.1	26.6	35.0	72.6	25.1	34.6
Medium Dose	69.8	23.5	33.7	68.2	23.6	34.6	68.4	22.9	33.4
High Dose	72.0	24.1	33.4	69.9	23.1	33.1	66.6	22.9	34.4
WEEKS OF TREATMENT									
	52			78			106		
MALES									
Control	73.3	26.4	36.1	69.3	24.8	35.8	68.4	24.0	35.1
Low Dose	72.7	26.4	36.3	71.1	25.6	36.0	69.3	24.3	35.0
Medium Dose	65.4	23.5	35.9	65.1	23.5	36.1	62.5	22.1	35.3
High Dose	58.8	19.8	33.7	61.9	21.2	34.3	61.5	20.7	33.6
FEMALES									
Control	71.2	25.6	35.9	68.3	25.9	38.0	66.1	23.6	35.7
Low Dose	71.8	26.1	36.3	71.0	24.6	34.7	69.0	24.5	35.5
Medium Dose	65.9	23.2	35.2	64.9	22.2	34.3	61.9	22.2	35.8
High Dose	66.8	23.6	35.4	66.2	24.6	37.1	65.5	23.1	35.3

Microcytic anemias may result from inadequate intake or absorption of iron, copper, and pyridoxine (19). A common cause of iron deficiency is chronic bleeding. There were, however, no observations in any of the dogs at autopsy that would indicate or explain chronic blood loss during the study. A transient infection with intestinal parasites could cause a low grade anemia. UAREP has no data to show whether the animals were routinely screened for ova and parasites or not.

The means (\pm SEM) of hemoglobin values for the 20 male dogs are shown in Figure 3-3. The shaded area shows the range of the standard errors. The upper heavy line represents the mean values for the three littermates in the control male group. The lower heavy line represents the mean values for the three littermates in the high dose male group. Means of hemoglobin values in the two unrelated dogs in each group are plotted as the lighter lines which fall between the shaded area and the "littermate" line.

Table 3-13 shows red and white cell data using means calculated from the table of individual values in E-28. Means calculated from the data after the Coulter correction was made are shown also at the first four intervals. These data had not been corrected previously, according to UAREP's interpretation of the information available to us. Coulter Coincidence correction makes the red cell count approximately 11% higher.

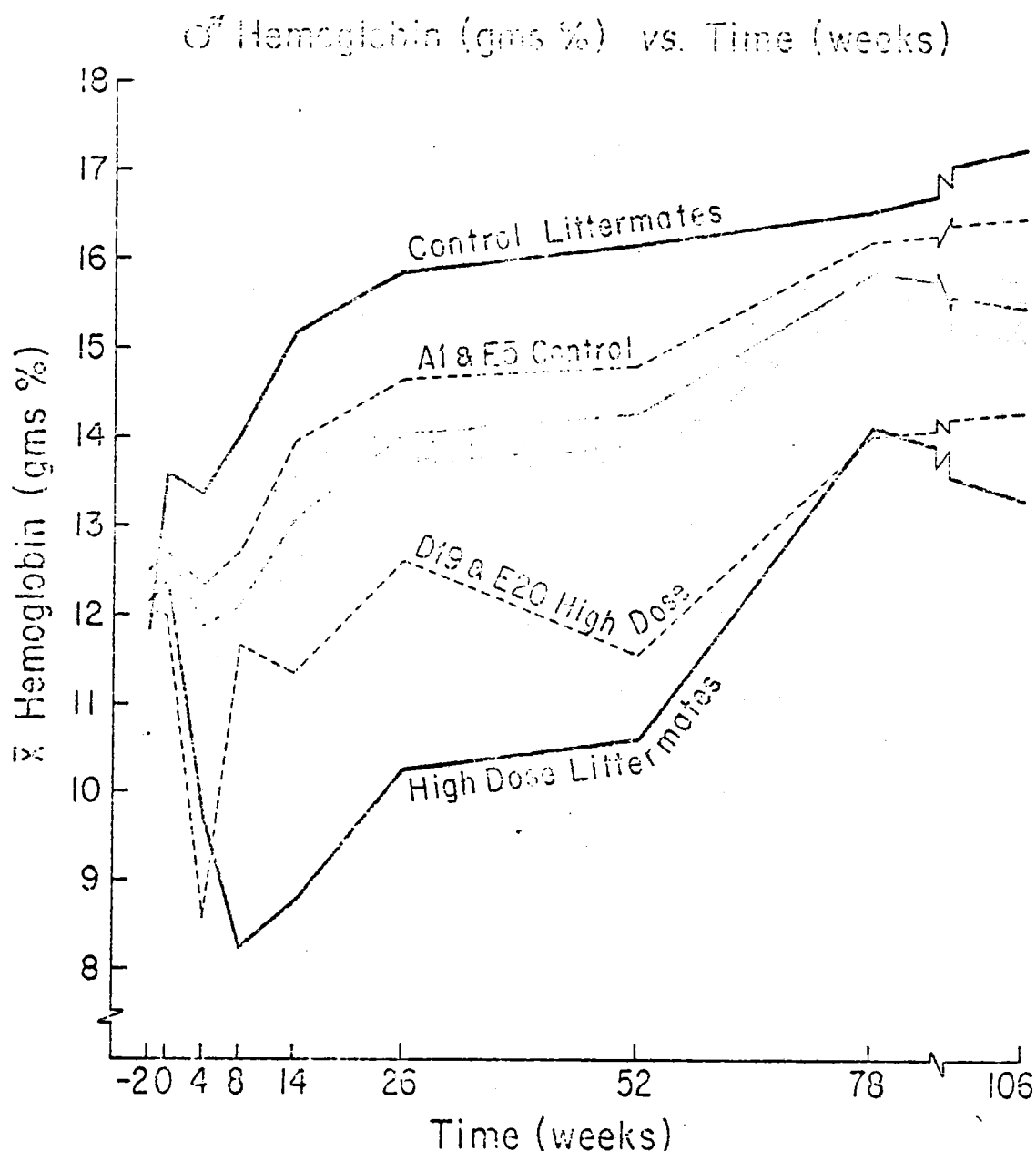


Figure 3-2: Hemoglobin (g%) in twenty male dogs during the two-year toxicity study.

The shaded area represents the standard error of the mean based on means and standard deviations derived from hemoglobin values for all the male dogs at each interval. The heavy uppermost line represents values from the three littermates in the control group. The next dashed line represents values from the two non-littermates in the control group. The heavy lowest line represents values from the three littermates in the high dose group. The lower dashed line represents values from the two non-littermates in the high dose group.

Differential smears: The only statistical analyses done on the differential counts by Searle was for the polymorphonuclear neutrophils. The summary table in the Entry Book (pp 26-29) shows significant differences in the high dose male group, which in week 14 was about 25% higher than the control group, and in week 52 was about 18% higher than the control group. In calculations using Analysis of Variance, we found that those groups mentioned above were not significantly different ($P > 0.05$). UAREP's t-test also was not significant at the 14 week interval while it was significant at 52 weeks. The high dose female group in week 4 was about 22% higher than the low dose group. This difference was significant when recalculated by UAREP using Analysis of Variance. The high dose female group at week 4 was 8% higher than the control female group. This difference was not significant by UAREP calculations. A fifty-two week summary of differential smear data (Appendix III-9) was evaluated by Dr. Hemm, and no treatment related changes were noted (Appendix III-10).

The hematology methods used were standard procedures. The only major problem we found was the lack of Coulter correction for red and white cell counts at the first four intervals. The Entry Book provides no extended series of normal values for Beagle blood values in Searle's experience, other than the five male and five female controls, which would have been helpful. Table 3-16 shows hematology normals for Beagles established in various laboratories (6, 18, 19, 20, 21). It should be noted that hemoglobin values for the high dose males, which were shown to be significantly lower than the control groups, also are

Table 3-16

Hematology Normals for Dogs

<u>Test</u>	<u>Age</u>	<u>Normals (6,19)</u>	<u>Schalm (21, p.193)</u>
RBC ($\times 10^6/\text{cmm}$)	Adults	6-9	5.5 - 8.5
	2-8 months ²⁰	5.3	
	38-212 days ²¹	5.0	
Hematocrit (%)	Adults	37-54	37-55
	38-212 days ²¹	37.8	
Hemoglobin (gm%)	Adults	12-18	12-18
	2-8 months ²¹	12.6	
WBC ($\times 10^3/\text{cmm}$)	6-12 months	12.7 - 13.8	6-17
	18-48 months	7-13	
Differential (%)			
Bands		0-4	0-3
Segmented		60-75	60-77
Lymphocytes		12-30	12-30
Monocytes		3-9	3-10
Eosinophils		2-10	2-10
Basophils		rare	rare
Prothrombin Time		9-12 seconds ¹⁸	
		8.0 - 8.5 seconds ²²	
MCV	Adults		60-77 μ^3
	38 - 212 days ²¹		76.3 μ^3
MCH	Adults	19.5-24.5 $\times 10^{-12}\text{g}$	
	38-212 days ²¹	18.6 $\times 10^{-12}\text{g}$	
MCHC	Adults	32-36 vol%	
	38-212 days ²¹	24.4 vol %	

lower than normal values for adult males. Hematocrit values for the high dose male groups are also below normal limits for adult dogs.

Coagulation: Prothombin times are all somewhat lower than the normal values of 9 to 12, given in reference 18. Summary Table 5 (pp 30-31, E-28) showed that prothrombin times in the low dose male group were significantly higher than the controls at week 52 and 106. UAREP's ANOVA calculations showed no significant difference at these points, while UAREP's t-test showed the low dose group was significantly different at week 52, but not at week 106 (Table 3-12). In the female dogs at week -1, prothrombin times in the high dose group were shown by Searle, using t-test, to be significantly higher than the control group. This was verified by UAREP's calculations using t-test as well as the LSD, but the Q test was not significant. At week 106, all the treated female groups were reported to be significantly higher than the control group. UAREP recalculations verified these findings.

In summary table 5 (Entry Book, pp 30-31) activated partial thromboplastin times in the control male group were significantly greater than the low and medium dose groups at week -1. Also in the males at week 4, the control group was significantly higher than the medium and low dose groups. In the females at week -1, the medium dose group was significantly higher than the control group. At week 14, activated partial thromboplastin time in the high dose female group was reported to be significantly higher than the control group, however, UAREP's calculations showed no significance. At week 52 the medium dose female group was reported to be higher than the control group, but UAREP's calculations again showed no significance.

UAREP's recalculations verified some of Searle's determinations. (Table 3-12).

Statistical Summary: Table 3-17 summarizes the various statistical comparisons of hematology data made by UAREP and Searle. In cases where data was corrected for any of the reasons discussed earlier, UAREP used the corrected data in its statistical calculations.

The percentages given in the "Percent Significant" column were obtained by dividing:

$$\frac{\text{total number significant}}{\text{total comparisons}}$$

The percent of significant comparisons is the same for UAREP's LSD and t-test, while UAREP's Newman-Keuls (Q) test yielded one percent fewer significant results.

Searle reported 15% significant results using the t-test.

Clinical Chemistries

General Comments: Abbreviations used in this section are listed in Table 3-18 and Chapter II, Appendix II-7.

In the absence of specific information in the Entry Book, we requested details from Searle on the processing of blood specimens before the samples were received by the chemistry and hematology laboratories. Dr. Bost states in his letter of April 24, 1978 (Appendix III-19) that samples were collected from fasting dogs between 8 and 9 a.m. Blood for hematology was collected in vacutainers containing EDTA • Na₂ which were placed in a "rotator" while subsequent samples were collected. They were delivered to the Hematology Laboratory immediately after collection.

Table 3-17

Summary of Statistical Comparisons of Hematology Data

	<u>Number Significant</u>	<u>Percent Significant</u>	<u>Total Comparisons</u>
LSD	77	11%	684
Q	65	10%	684
UAREP t	77	11%	684
Searle t	54	16%	342

Samples for chemistries were collected in heparinized vacutainers, centrifuged, plasma separated and sent to the Bioanalytical Lab. It is not noted in E-28 whether blood samples for chemistry were kept on ice during the collection of subsequent samples. If one or two people were drawing blood samples on 40 dogs it could take well over two hours. With this much time elapsing, it would be desirable to chill samples as they were drawn to slow down changes which might occur on standing at ambient temperature for a few hours. According to a phone conversation with Dr. Bost (May 11, 1978) blood samples were chilled immediately after collection.

The sequence of chemistry assays is shown in Table 3-19. This can be compared to Table 3-20 which relates to events in the toxicity study. The actual date of each week listed was calculated with the Hewlett-Packard 97 calendar program. Chemistry results were recorded at times ranging from a few days (in the case of week 26) to a month (in the case of week 14) later than specified in the protocol. UAREP is unable to ascertain if blood was obtained just prior to performing the chemistry assays, or if it was obtained at the time specified in the protocol and then stored in the interim.

We have obtained a partial list of equipment in use from November 1971 to February 1974. Enzymes were assayed on a Beckman DSA-560. The same instrument was used for total bilirubin, glucose oxidase, and BUN. Total cholesterol was assayed on an Autoanalyzer I using the ferric chloride procedure modified from Bloch, et al. L-phenylalanine was done by a manual method. Sodium and potassium were analyzed on the Perkin Elmer 403 atomic absorption spectrophotometer. Calcium was determined manually, as described in the reference adapted from Kingsley and Robnett given in the Entry report.

Table 3-18

List of Abbreviations in Chemistry Section

Alk P.....	Alkaline phosphatase
BSP.....	Bromsulphophthalein
BUN.....	Blood urea nitrogen
Ca.....	Calcium
Cl.....	Chloride
EDTA.....	Ethylenediaminetetracetic acid
FFA.....	Free fatty acids
Gluc.....	Glucose
GPT.....	Glutamic pyruvic transaminase
K.....	Potassium
Na.....	Sodium
OCT.....	Ornithine carbamyl transferase
TG.....	Triglyceride

Table 3-19
Summary of Chemistry Quality Control and List
of Available Laboratory Data (BA no.)

<u>Assay</u>	<u>BA no.</u>	<u>Week</u>	<u>Date</u>	<u>Technician Initials</u>	<u>Quality Control Evident</u>
BUN	6393	-1	3/18/70	BH	xxx
	6596	4	5/6/70	--	xxx
SGPT	6393	-1	3/18/70	NS	---
	6596	4	5/6/70	CC	---
	6726	8	6/4/70	CC	---
	7013	14	8/11/70	AK	---
	7240	26	10/2/70	GH	---
	7861	52	4/6/71	BH	---
Alkaline Phosphatase	6393	-1	3/18/70	NS	---
	6596	4	5/6/70	CW	---
	6726	8	6/4/70	CC	---
	7013	14	8/11/70	AK	---
	7240	26	10/1/70	--	xxx
	7861	52	4/6/71	AK	xxx
BSP	6596	4	5/6/70	BH	---
	6726	8	6/4/70	AK	---
	7013	14	8/11/70	WS	---
	7240	26	10/1/70	BH	---
	7861	52	4/6/71	AK	---
Bilirubin	6393	-1	3/18/70	CW/BH	xxx
	6596	4	5/6/70	CW/BH	xxx
	6726	8	6/4/70	AK/CC	xxx
	7013	14	8/11/70	AK/BH	xxx
	7240	26	10/1/70	FE/ER	xxx
	7861	52	4/6/71	K	xxx
Glucose	6726	8	6/4/70	BW	---
	7013	14	---	OS	xxx
	7240	26	10/1/70	FE	xxx
	7861	52	4/7/71	BH	---
Sodium & Potassium	6393	-1	3/18 or 19/70	--	---
	6596	4	5/7 or 8/70	--	---
	6726	8	6/5/70	--	xxx
	7013	14	8/11, 12, or 13/70	--	xxx
	7240	26	10/5/70	FE	xxx
	7861	52	4/7, 8 or 9/70	--	---
Chloride	6393	-1	3/19/70	CC	xxx
	6596	4	5/7/70	CC	xxx
	6726	8	6/5/70	AK	---
	7240	26	---	BH	xxx
	7861	52	4/14/71	AK	xxx
	9202 (partial)	106	4/18/72	--	---
Calcium	6393	-1	3/18/70	BH	xxx
	6596	4	5/7/70	CC	xxx
	6726	8	6/5/70	AK	---
	7013	14	---	BH	---
	7240	26	10/2/70	BH	xxx
	7861	52	4/6/71	FE	---
	8727	78	11/11/71	CC	---
	9202	106	4/12/70	FE	---
			4/17/70	BH	---

xxx = quality control was done
--- = quality control was not evident

Table 3-20
 Dates on Which Assays of Hematologic and Chemical
 Parameters Were Recorded

<u>Week</u>	<u>Calculated Date</u>	<u>Hematology</u>	<u>Chemistry</u>
-2	3/16/70	3/10 - 3/13	---
-1	3/23/70	---	3/18 - 3/19
0	3/30/70	3/18 - 3/19	---
4	4/27/70	4/27 - 5/1	5/6 - 5/8
8	5/25/70	5/25 - 5/28	6/4 - 6/5
14	7/6/70	7/6 - 7/10	8/11 - 8/13
26	9/28/70	9/25 - 9/29 (coag 10/8)	10/1 - 10/5
52	3/29/71	3/29 - 4/2	4/6 - 4/9 C1 ⁻ 4/14
78	9/27/71	8/1 - 8/8 (coag 10/1-10/20)	10/5 - 11/11
106	4/10/72	4/7 - 4/13	4/12 - 4/17

It was not possible to obtain from Searle the dates when equipment for automated analysis became operational in the laboratory. It is presumed that the dates of discontinuance of availability of raw data recording books corresponds to the installation of automated analytical methods.

Quality Control: On the basis of information supplied by Searle to UAREP, laboratory quality control during the course of the dog toxicity study appears to have been variable or lax. Table 3-19 gives a summary of the quality control we were able to identify in looking at the raw data. It appears that it was not laboratory policy at that time to run a quality control check with every batch of assays. Each assay interval had a "BA number." Table 3-19 shows that Calcium is the only assay for which UAREP received a full set of data. This is particularly true later in the study when we were told that a conversion to computerized output was in process.

Statistics: Searle's statistical analysis was done by transforming the data to logarithms. Treatment groups were compared to control data by t-test using logarithms and the means were reported as geometric means. This was done because of the tendency of the chemistry data to be "skewed." This approach seems rather cumbersome, and is probably unnecessary. Snedecor and Cochran (25) say, on page 276: "In a single classification with equal N, various mathematical studies agree in showing that the F-test (Analysis of Variance) is little affected by moderate non-normality." Biostatistics by Goldstein (10) was also used as a general reference.

UAREP's validation was done using both arithmetic and geometric means with Analysis of Variance and t-test comparing not only treatment groups against controls, but also treatment groups with each other.

Summary of Specific Methods: References for the chemistry methods used are given on pages 58-59 (E-28). Kits from Fermco were used to determine glutamic pyruvic transaminase, alkaline phosphatase, and blood urea nitrogen. The Fermco Company did not answer our requests for information on these methods, but we finally obtained copies from Searle. The Canalco disc electrophoresis method, used for electrophoresis of lipoproteins, was also obtained from Searle. Most of the remaining methods are fairly standard procedures, although some have been replaced by automated or semi-automated methods in recent years. The reference given for insulin was a reference for inulin (E-28, p 58, ref 18).

Alkaline Phosphatase: Plasma alkaline phosphatase is normally reported to one decimal place as on page 76 in the Entry Book. Means in the summary table should also have had one decimal place instead of two (E-28, pp 33-34). Few significant differences were reported for males or females in any interval. However, in the table of individual values on page 76 in the Entry Book, week -1 was higher than all the other intervals (mean = 6.0). Week 26 was lower than week -1 by 72% (mean = 1.7). It is known that in young dogs (19), alkaline phosphatase values can be elevated during the rapid growth phase, which might account for the elevated values found at week -1. However, it is difficult to account for the lower values at week 26, as compared to all the other intervals.

Raw data that we have available includes the assays from week -1 to week 52 (Table 3-19). The notebooks indicate readings were taken at 30 minutes. Normally, a plasma control is run with each unknown and it is not evident from the raw data that plasma controls were run at any time. This could cause the results to be generally higher than they would be otherwise. However, as shown in Table 3-21, most of the alkaline phosphatase results are within normal limits. Serum or heparinized plasma should be used because oxalate, citrate, and EDTA inhibit the reaction (14). The enzyme is stable for 7 days at 4 degrees C (13). Calculation of results involves subtracting the plasma control value from the plasma unknown value. Since plasma controls were not recorded, it was difficult to evaluate the validity of the test as reported.

Plasma Glutamic Pyruvic Transminase: In summary Table 6 (E-28, p 34), under week 78, the means for GPT in seven out of the eight values are very low. Zero values were reported for eight animals at week 78 on page 75 (E-28). In spite of the zero values, five was the number of determinations used to obtain these means since there was apparently no reason to discard the zero results. The large LSD of $\pm 1563.0\%$ is a result of this spread. In the laboratory notebook, the sample for dog D19HM at week 26 was noted as lost. In the table on page 75 (E-28), however, a value of 13.2 units is shown for that animal. At the same interval in the laboratory book, dog E20HM was recorded as having 92 units of GPT in plasma. This result was circled and a repeat requested. The repeat assay also had a result of 92 (back-up data book N26378). In the table on page 75 (E-28), a value of 24.7 units is shown for this animal, but the source of this value is unknown to UAREP.

Table 3-21
Normal Blood Chemistry Values for Dogs

Alkaline Phosphatase.....	1.8 - 7.8 Bodansky Units (6) Female
	3.8 Bodansky Units, Male
GPT.....	21.1 \pm 7.3 R-F units, beagles (21)
BSP.....	<5% retained in 30 minutes (19)
Total Bilirubin.....	0.09 - 0.41 mg/100 ml Male beagles (26)
	0.08 - 0.48 mg/100 ml Female beagles (26)
BUN.....	7.8 - 16.7 mg/100 ml, Male beagles (26)
	7.1 - 17.9 mg/100 ml Female beagles (26)
Glucose.....	55-99 mg% Male beagles (26)
	45-99 mg% Female beagles (26)
Sodium.....	141 - 153 mEq/L Male beagles (26)
	142 - 152 mEq/L Female beagles (26)
Potassium.....	4.2 - 5.2 mEq/L Male beagles (26)
	4.3 - 5.5 mEq/L Female beagles (26)
Calcium.....	5.53 \pm 0.30 mEq/L Male beagles (21)
	5.51 \pm 0.31 mEq/L Female beagles (21)
Chloride.....	102-118 mEq/L Male and Female beagles (3)
Cholesterol.....	107-325 mg% Male beagles (26)
	116-380 mg% Female beagles (26)
Albumin/Globulin Ratio.....	0.93 - 1.21 (19)
Lipoprotein.....	820.23% \pm 5.46 (5)
	α 79.76% \pm 5.46 (5)
FFA.....	0.2 - 0.8 mEq/L (19)
Na/Cl Ratio.....	1.29 - 1.38 (see text)
Ornithine Carbonyl	
Transferase (human).....	0.17 IU/l
Phenylalanine.....	0.58 - 1.74 mg% (dog - race not indicated) (1)

Adults Unless Otherwise Indicated

One of the periodic summaries during the study (Appendix III-5), done at week 26, mentioned problems with GPT which were attributed to specimen handling or laboratory methodology. Zero values are unusual in this assay, although they can occur. Other factors such as inadequately cleaned glassware can sometimes cause low results. Although GPT values are normally reported in whole numbers, they are reported to the first decimal place in the table on page 75 (E-28). They are also reported to the first decimal place in the summary tables (E-28, pp 33-34).

Since erythrocytes contain about 3-5 times more GPT than plasma, grossly hemolyzed samples should not be used, as they would yield erroneously high results. There is no indication that any hemolyzed samples were used in this toxicity study. Samples are stable up to one week in the refrigerator (13).

At the beginning of the study, the mean in the control males was 12 and the mean for control females was 7, whereas at the end, the control males were 43 and the control females were 35. The values were 4 to 6 times higher at the end of the study than at the beginning. Overall means for combined males and females for an entire interval remained between 7 and 9 for the first three intervals. At week 14, the mean was 24, week 26 was 31, week 52 was 18, week 78 was 11, and week 106 was 34. This upward trend is difficult to interpret. The coefficient of variation of data means for the various weeks is so great that the upward trend may have little real meaning. Also at week 106 all the group E values (1 dog of 5 in each group) were 1.5-3.0 times higher than other values in the group. These 8 dogs were all done on day 5 of the final week. This result is difficult to interpret other than suggesting a problem either in specimen handling or laboratory methodology, as was suggested by Searle early in the study (Appendix III-5).

BSP (bromsulfophthalein): Individual values for BSP are found on page 17 (E-28). Entire groups of values seem to trend higher in the last four to five intervals. For example, the mean for the control males at week -1 was 0.128 mg% and the mean for the control females was 0.189 mg %. At week 106, the control male mean was 0.8 mg% and the control female mean was 0.3 mg%.

BSP is a standard test for liver function in veterinary practice (6,19). The usual dose is 5 mg/kg body weight with a blood sample taken at 30 minutes after injection of the dye. Color is developed at alkaline pH. The control reading is taken on plasma buffered at acid pH so that the dye remains colorless. The method in reference 15 of E-28 calls for this sort of plasma blank to be run (23). Blanks were recorded in the lab notebooks for only the first two intervals. At week 52 no standard was recorded. At weeks 14, 26, and 52 only results were recorded, so it is impossible to know if blanks were done, and the calculations can not be checked. If blanks were not done, that could help account for the trend to higher results reported at these times.

According to Dr. Bost from Searle, the amount of dye used was 0.2 ml per kg body weight (Appendix III-20). In other words, if the standard dye (50 mg/ml) was used, 10 mg/kg dye was injected. We are unable to interpret the units of mg% in which the BSP results were reported since the usual way of reporting BSP is as percent of dye retained in the plasma after a given length of time. This is a clearance test to determine liver function. If the liver is functioning normally, more than 95% of the dye should be removed from the plasma after 30 minutes.

Assuming that the information we have on the amount of dye given is correct, we have calculated the percent of dye retained as shown in Table 3-22. The equation used is in a footnote to Table 3-22. Moderate to severe lipemia can interfere with the test, which is, therefore, normally done in a fasting stage. Four borderline values (over 5% retention) are underlined in Table 3-22, as well as one of 11.35%.

Chloride: According to the available laboratory notebooks, there is no evidence that plasma blanks were done as described in the reference method (E-28, p 58, ref 14). More than one method was in use at Searle during this study (personal communication from Judy Schmal who was supervisor of Searle's Clinical Chemistry Laboratory during the latter part of this study). The silver nitrate macrotitration was used at one time. At week 52, a run of chlorides was done which apparently used no plasma blanks or standards, however, Versatol, a quality control serum, was run. No readings were supplied to us for week 52--only final results with a mean of 135 mEq/L. Later, the same samples were run again, and lower results (mean of 116 mEq/L), a difference of 16%, were obtained which were used in the Entry Book. In the table of individual values for chloride results (E-28, p 84), it can be seen that weeks 78 and 106 show much higher values than the other intervals. Raw data are available for weeks -1, 4, 8, 26, 52, and part of 106 (Table 3-19). At week 106, some samples were run on April 18, 1972. Data for the remainder of the run are not available. Values for week 106 (E-28, p 84) are very high and have a sodium/chloride ratio of approximately 1, as opposed to the usual 1.37-1.41 (Table 3-23). The raw data we have for that interval shows values of 110 to 115 which are normal. It is not understood why these values were not used in the final report, rather than the abnormally high ones.

Table 3-22
Percentage BSP Retention in Male Dogs

Control	Week -1	Week 14	Week 52	Week 78	Week 106
A1CM	0.60	1.80	2.60	4.95	5.00*
B2CM	0.75	1.85	2.75	6.75*	0.70
C3CM	1.10	1.05	3.15	4.00	1.25
D4CM	0.55	1.40	3.50	2.70	1.40
E5CM	0.40	1.10	2.75	1.80	1.75
<u>Low Dose</u>					
A6LM	0.75	1.70	3.00	4.05	3.20
B7LM	0.60	1.75	3.65	4.85	1.80
C8LM	0.45	0.90	1.95	1.80	1.10
D9LM	1.00	0.60	3.45	2.05	1.35
E10LM	1.25	0.95	3.20	2.50	1.60
<u>Medium Dose</u>					
A11MM	0.80	1.35	2.15	1.80	0.50
B12MM	1.10	1.30	4.20	2.85	1.65
C13MM	1.25	1.05	2.30	2.70	1.40
D14MM	0.45	0.90	3.20	3.25	0.90
E15MM	0.95	0.80	2.25	1.35	1.35
<u>High Dose</u>					
A16HM	1.15	1.20	3.15	3.05	2.90
B17HM	1.15	1.37	2.70	1.95	0.85
C18HM	1.35	1.00	2.45	2.30	0.80
D19HM	0.85	0.85	3.40	2.15	0.80
E20HM	0.85	0.90	2.50	1.10	1.15

Data in Table 3-22 based on the following calculation:

$$\% \text{ BSP retained after 30 minutes} = \frac{\text{Kg body wt} \times .05 \times 1000}{100} \times \text{mg\%} \times \frac{10}{10 \times \text{kg body weight}} \times 100$$

which simplifies to: .05 x mg% x 100

taking 5% of the body weight as the plasma volume and based on a dose of 0.2 ml (10 mg) dye per kg body weight (personal communication from Dr. Bost of Searle).

*Values greater than or equal to 5% are underlined.

Table 3-23

Means of Sodium/Chloride Ratios

Week	-1	4	8	14	26	52	78	106
<u>Control</u>								
Males	1.39	1.41	1.37	1.26	1.29	1.31	1.11	0.99
Females	1.41	1.37	1.38	1.23	1.31	1.34	1.14	0.96
<u>Low Dose</u>								
Males	1.37	1.39	1.38	1.21	1.29	1.31	1.13	0.99
Females	1.41	1.38	1.39	1.23	1.32	1.34	1.14	0.95
<u>Medium Dose</u>								
Males	1.39	1.39	1.38	1.22	1.28	1.36	1.27	1.01
Females	1.42	1.36	1.38	1.24	1.33	1.35	1.12	0.98
<u>High Dose</u>								
Males	1.39	1.39	1.39	1.24	1.28	1.37	1.21	0.99
Females	1.45	1.38	1.37	1.23	1.31	1.36	1.14	0.97

The high values at weeks 78 and 106 are difficult to interpret. Chloride can be elevated by a large amount of hemolysis. There is no reason to think hemolysis was a problem in all the samples at weeks 78 and 106, especially since we found the data indicating that at least some of the samples at week 106 were actually in the "normal" range.

Sodium and Potassium: The sodium and potassium run for week 4 is labeled "Rat Plasma" in the laboratory notebook, but includes 40 samples. Results shown in the notebook are the ones given in the table of individual values on pages 81 and 82 (E-28), so we assume that these results are for the dog study. In week -1 data, there were a number of samples marked to be repeated. They were repeated and exactly the same numbers were obtained. It was not evident why the repeat studies were ordered since they were all in the same range as the values which were not repeated.

At week 78, animal C8LM was recorded as QNS in the individual potassium values (E-28, p 82). However, in the laboratory book the sample is recorded as lost. The same week, animal C13MM is shown as QNS and the laboratory book notes that no potassium was run.

Blood Urea Nitrogen: Values for BUN are shown on page 79 (E-28). It can be seen that the values for the first two intervals (the only ones for which we have lab data) are approximately three times higher than those shown at the other intervals. The Sigma method (24) recommends running five points on a standard curve, and reporting whole numbers rather than tenths of units. It is not apparent in the laboratory notebooks what

instrument was used to obtain the readings. We have no indication that standard curves were run. A factor of "x 5 + 10" is shown in the calculation; its source is not clear. Versatol A was run as quality control check.

Glucose: Glucose is generally reported in whole numbers rather than tenths as was done in E-28 (p 80). In the glucose oxidase method (8) which was used, catechols can interfere (reduce color) with the chromagen by acting as hydrogen donors for peroxidase. Uric acid, ascorbic acid, glutathione, cysteine, bilirubin, and thymol can also act as reducing agents, or otherwise interfere with color development in this method. Chlorine from tap water contamination can cause increases in color development.

Calcium: It should be pointed out that these results are reported in milliequivalents/liter (16). Many people are accustomed to seeing calcium reported in mg/100 milliliters. The conversion factor for changing milligrams per 100 ml to milliequivalents/liter is .499 (7). At week 106, most of the assays were done on April 12. However, a number of them were done on April 17. Since the necropsies were done from April 10 to April 14, one wonders how the samples were preserved in the meantime. Storage of plasma (cold or frozen) should not cause any loss of calcium as long as it was separated from the cells soon after collection (13). In the table of individual values, it should also be noted that the values for the entire interval at week 14 appear to be decreased by about 1/3.

Phenylalanine: L-phenylalanine in plasma was assayed only at week 106 (Appendix III-16). The highest blood levels probably would have been observed at about 1 to 3 weeks of treatment (15). Rats fed a diet containing 3.5% L-phenylalanine showed a fall in phenylalanine hydroxylase levels in liver in the first 24 hours. At four weeks the enzyme activity in liver was 59% of control values. After 16 weeks it had risen to about 75% of control values.

A memo dated February 21, 1972 from Dr. Rao to Dr. Ranney (Appendix III-15) expressed interest in monitoring phenylalanine hydroxylase levels in liver at the end of the dog study. However, nothing further appears to have been done about this enzyme assay.

Rats given a diet of 5% DL-phenylalanine had a normal weight gain while those given 5% L-phenylalanine had poor weight gain (28). Frequent feedings of the rats were necessary to produce high phenylalanine levels in plasma, leading to a urinary increase. In this dog study (E-28), fluctuation in the plasma levels of L-phenylalanine due to the single daily feeding of the compound may explain why urine levels were mostly negative, even in the high dose dogs. Urinary levels would depend upon when the sample was collected in relation to feeding.

Dietary percentages of aspartame as well as those of the L-phenylalanine moiety of the aspartame molecule which were fed in this study are given in Table 3-24. Percentages increased during the study as the dogs gained weight and more aspartame per day was added to maintain dosage. The dogs in the high dose group were receiving more than 5% L-phenylalanine after 26 weeks of the study. If the dogs were affected by this amount of L-phenylalanine in a manner similar to the rats mentioned above, the diminished food consumption and weight gain in the high dose groups might be explained.

Table 3-24

Percentages of Aspartame and L-phenylalanine*
in Daily Diet During the Dog Toxicity Study

	low dose group (1g/kg)	medium dose group (2g/kg)	high dose group (4g/kg)
Aspartame (start→final)	1.5→2.7%	3.0→7.2%	6.7→11.8
L-phenylalanine (start→final)	0.82→1.5%	1.6→3.9%	3.7→6.5%

*Based on aspartame containing 55% L-phenylalanine (E-28, p 32)

Depression of food intake in response to imbalances in amino acids (including phenylalanine) recurs in the literature (2, 3, 4, 9, 11, 12, and 14) although the mechanism of action of this effect is not well explained.

FFA (free fatty acids): The method used was not employed as widely as the Trout, Estes, Friedburg method (27) of microtitration which many lipid research laboratories used at the time of the study. Free fatty acids were measured only in the terminal samples. Individual values range from 263 to 874 and are shown on page 87 of E-28. The table reports these units to be milliequivalents per liter, whereas they probably are microequivalents per liter. Normal values for fasting dogs in milliequivalents per liter would be 0.2 to 0.8 (Table 3-21).

It would have been interesting in the evaluation of lipogenic potential of aspartame if FFA had been assayed at the beginning of the study as well as at selected intervals throughout. Comparison of treated groups with the control group indicates little difference at the end of the study.

Lipoprotein electrophoresis: It was decided on September 10, 1971 (Appendix III-11) after the study had been underway for six months, to include a lipid panel consisting of free cholesterol, cholesterol esters, triglycerides, free fatty acids, and lipoprotein electrophoresis. A Canalco kit employing disc type polyacrylamide gel electrophoresis was used to separate the lipoprotein fractions. Varying degrees of separation were achieved. In this method, VLDL (very low density lipoproteins)

migrate as a post-beta band. Chylomicrons stay at the interface between the stacking gel and the running gel (origin). The densitometer scans rarely showed a baseline before the first peak, so one can not tell exactly where the peak begins. Also, the base line is off scale in many cases. Total percentages shown by the densitometer rarely add up to exactly 100. Smearing occurred frequently, which could be attributed to technique or absorptive state of the animal. In extracting information from these scans, Searle considered the largest number as alpha lipoprotein corresponding to the fastest migrating and highest peak. Even though there might be a sizeable shoulder on the peak, it was ignored. The highest number in the beta region was taken as the beta band even if there was a series of small peaks in that region.

Sodium EDTA is the anticoagulant of choice for plasma to be assayed for lipoprotein fractions. According to Dr. Bost (Appendix III-19), heparinized vacutainers were used for the collection of all chemistry samples. The Canalco method (E-28, p 58, No. 20) specifies use of sodium EDTA to achieve clean separation of the fractions. Lipoproteins are denatured by freezing, so plasma which has been frozen can also be a cause of poor separation. It is not known whether any of these samples had been frozen before electrophoresis was done.

The statistical computations at Searle employed arithmetic means when analyzing the data on lipoprotein fractions. This use of arithmetic means seems inconsistent since geometric means were reported in the statistical analyses of all of the other chemistries assayed. UAREP found that the choice of arithmetic or geometric means did not alter the statistical significance in this case.

Total Bilirubin: Individual values for the bilirubin results are found on page 78 (E-28). Low results are usually reported as < 0.2 mg%. At the last two intervals all the bilirubins were less than 0.2 mg% and, therefore, they were reported to the second decimal place. In the summary tables (E-28, pp 35-36) bilirubin is reported to three decimal places, which is unnecessary. The low values reported at the last two intervals make one wonder about the possibility of some laboratory problem--since, in each case, they are consistent throughout the entire interval. On the basis of the available laboratory data we are unable to evaluate the possibility that these are actually "direct" bilirubin values and total bilirubin was not completed.

A serum blank should be done and set to zero, and each sample read against its own blank (13). A 2 microgram per ml standard should be used. A number of factors affect this determination. Temperature affects the rate of color development, i.e., color develops faster at 37 degrees C than at 25 degrees C. Prolonged contact with erythrocytes should be avoided; they can adsorb bilirubin, as much as 0.7 mg per 10 ml of cells. Hemolysis causes low results. Bilirubin is destroyed by light and may be reduced up to 50% in one hour in sunlight, whereas samples can be kept frozen in the dark up to three months.

Triglycerides: Controls increased between 78 and 106 weeks as shown by values on pages 85 and 86 (E-28). In the male control group, at 106 weeks, values were double what they were at 78 weeks, while in the female group they were almost triple. Results in all groups at 106 weeks were noticeably higher than the ones at 78 weeks. The data were

Table 3-25
continued

Parameter	Week	Sex	ANOVA	Group	LSD	Q	UAREP t	t Value	Searle t
Calcium (cont'd)	14	M	0.18	1>2	ND	ND	N	---	S
		M		1>3	ND	ND	N	---	S
		F	0.10	1>4	ND	ND	N	(2.236)	N
	52	F		2>4	ND	ND	N	(2.236)	ND
		M	0.00	1>4	S	S	S	6.560	S
		M		2>4	S	S	S	4.159	ND
		M		1>3	S	S	S	4.521	S
		M		3>4	S	S	S	2.737	ND
		M		1>2	S	S	S	2.780	N
	78	M	0.38	1>4	ND	ND	S	2.452	N
Chloride	-1	M	0.10	2>3	ND	ND	S	2.581	ND
	8	M	0.06	1>3	ND	ND	S	2.909	S
		M		1>4	ND	ND	N	---	S
	14	M	0.10	1<2	ND	ND	S	2.610	S
		M		1<3	ND	ND	S	2.540	S
	52	M	0.04	2>4	S	N	S	3.714	ND
		M		3>4	S	N	S	2.444	ND
		M		1<2	S	N	N	---	N
		F	0.34	1<4	ND	ND	N	---	S
	78	M	0.03	2>3	S	N	S	2.547	ND
		M		1>3	S	N	S	2.354	S
		M		2>4	S	N	S	2.424	ND
BSP		M		1>4	N	N	N	---	S
	-1	M	ND	1<4	ND	ND	S	2.578	S
	14	M		1>3	ND	ND	N	---	S
		M		1>4	ND	ND	N	---	S
		F		1<2	ND	ND	S	5.097	S
		F		1<3	ND	ND	S	5.556	S
		F		1<4	ND	ND	S	3.516	S
	26	M		1>4	ND	ND	S	2.668	S
	78	M		1>3	ND	ND	N	---	S
		M		1>4	ND	ND	N	---	S
	106	M		1>3	ND	ND	N	---	S
		M		1>4	ND	ND	N	---	S
TG	78	F	0.22	1<4	ND	ND	N	---	S
Insulin	78	M	0.01	1<4	S	S	S	3.540	S
Lipoprotein	78	F	0.08	1<3	ND	ND	S	2.440	N
Bilirubin	-1	M	0.08	2<3	ND	ND	N	---	ND
		M		3>4	ND	ND	N	---	ND
	4	F	0.08	2<3	ND	ND	S	3.191	ND
	8	M	0.02	1>4	S	S	S	6.080	N
		M		1>3	S	S	S	2.850	N
FFA	106	M	0.07	1>3	ND	ND	S	2.560	N

ND = Not Done

ANOVA indicates the exact probability that all group means are equal upon the F test for Analysis of Variance (ANOVA).

LSD (Least Significant Difference) S = significant at $P < 0.05$; N = $P > 0.05$.

Q (Newman-Keuls test) S = significant at $P < 0.05$; N = $P > 0.05$.

UAREP used the two-tailed t-test with 8 degrees of freedom, $t_{.05} = 2.306$, to compare each treatment group against the control group.

UAREP used ANOVA to perform comparisons of all groups at a given interval.

All ANOVA values of 0.00 in this report indicate less than 1% chance that the means being compared are equal.

so variable that UAREP's statistical calculations showed no significance. Since the animals were fasting when the samples were drawn, it is difficult to interpret the variations in data.

Statistical Summary: Table 3-25 compares significant differences in chemistries reported in the Entry Book with those found by UAREP calculations of ANOVA. The ANOVA method compares each group with every other group, e.g., each treatment group was compared against the control group as well as against the other two treatment groups.

The t-test used by Searle compares each treatment group against the control.

Table 3-26 summarizes the various statistical comparisons of chemistry data made by UAREP and Searle. In cases where data were corrected for any of the reasons discussed earlier, UAREP used the corrected data in its statistical calculations.

Percentages given in "Percent Significant" column were obtained by dividing:

$$\frac{\text{total number significant}}{\text{total comparisons}}$$

The percent of significant comparisons is similar in the LSD and Q summary. UAREP t-test yielded 6% significant results while the Searle t-test showed 10% significant.

Table 3-26

Summary of Statistical Comparisons of Blood Chemistry Data

	<u>Number Significant</u>	<u>Percent Significant</u>	<u>Total Comparisons</u>
LSD	42	3% (3.5)	1184
Q	31	3% (2.6)	1184
UAREP t	70	6%	1184
Searle t	57	10%	592

Urinalysis

Collection: Spontaneously voided urine was collected from drainage pans underneath the cage. Cages were not the metabolic type (Appendix III-19). In the protocol dated 3/30/70 (Appendix III-3), the use of bladder expression was suggested, which might indicate an ongoing problem with urine sample collection. Urine specimen handling was not explained in any detail. It is not mentioned how the specimens were preserved, the time of day they were collected, or at what time of day they were assayed. According to Dr. Bost's letter (Appendix III-19), the urine samples were collected as cage run-off and assayed in the mornings. The earliest urinalysis data available to UAREP are recorded on intermediate work sheets.

Specific Gravity: Specific gravity (E-28, p 89) was determined with a total solids meter. The upper limit of normal for specific gravity is considered 1.050 in dogs (9, 16). Some total solids meters read as high as 1.050, while many of them only read to 1.035. Some of the data on individual dogs, especially in the first two intervals, were as high as 1.070. We have no basis to judge the extent to which this concentration was due to evaporation or contamination, and no way to determine how the high values were read on the meter. Table 3-27 gives means and standard deviations for specific gravity data. These data were compared by Analysis of Variance, LSD, and Q tests. Only the comparisons among the females were statistically significant. At week -2, the medium dose females showed a significantly higher specific gravity than the low dose group. At week 106, the medium dose females showed significantly lower values than the low dose group.

Table 3-27
Urinalysis Specific Gravity Data (Means \pm Standard Deviation) on Groups of Dogs

Week	Controls		Low Dose		Medium Dose		High Dose	
	Males	Females	Males	Females	Males	Females	Males	Females
-2	1.029 \pm 0.009	1.041 \pm 0.020	1.055 \pm 0.019	1.026 \pm 0.007	1.045 \pm 0.027	1.054 \pm 0.017	1.041 \pm 0.023	1.053 \pm 0.031
4	1.027 \pm 0.007	1.020 \pm 0.005	1.031 \pm 0.008	1.034 \pm 0.012	1.025 \pm 0.025	1.024 \pm 0.012	1.032 \pm 0.017	1.022 \pm 0.009
14	1.028 \pm 0.007	1.028 \pm 0.011	1.017 \pm 0.008	1.027 \pm 0.008	1.024 \pm 0.012	1.029 \pm 0.004	1.023 \pm 0.004	1.035 \pm 0.026
26	1.035 \pm 0.014	1.031 \pm 0.013	1.024 \pm 0.019	1.037 \pm 0.010	1.037 \pm 0.025	1.032 \pm 0.012	1.033 \pm 0.016	1.028 \pm 0.016
52	1.029 \pm 0.016	1.029 \pm 0.009	1.029 \pm 0.008	1.035 \pm 0.007	1.023 \pm 0.010	1.029 \pm 0.007	1.033 \pm 0.013	1.026 \pm 0.007
78	1.029 \pm 0.007	1.026 \pm 0.006	1.033 \pm 0.007	1.026 \pm 0.009	1.031 \pm 0.005	1.029 \pm 0.004	1.031 \pm 0.002	1.030 \pm 0.007
106	1.025 \pm 0.006	1.025 \pm 0.005	1.025 \pm 0.005	1.026 \pm 0.008	1.016 \pm 0.010	1.018 \pm 0.002	1.015 \pm 0.009	1.022 \pm 0.004

Specific gravity data from the 20 males was combined at weeks -2, 52, and 106. The three groups were compared by Analysis of Variance and LSD. Data from week -2 were significantly higher than the other two intervals. Most of the abnormally high (> 1.050) values were reported for week -2 before any treatments were started. UAREP offers no explanation for this.

Chemistry: Protein, pH, glucose, acetone, occult blood, phenylketones, and urobilinogen were assayed using appropriate dipsticks. Normal pH in dog urine ranges from 4.5 to 7.0. Several of the pH results shown (E-28, p 90) are higher than 7 and up to 9. Plausible explanations could include abnormal urine, problems in reading dipsticks, the use of outdated dipsticks, delay in urine sample analyses and/or contamination with residue of detergents or disinfectants from cage washing procedures. In addition to being generally higher than the other results (Table 3-28), week 14 data for pH was unusual in that four groups had no variance at all, i.e., all results in those groups were identical with each other. Dogs on dry-type diets tend to have alkaline urine (9, 16); it is the degree of alkalinity that is in question. Protein results (E-28, p 91) for week 14 had a large number of high results. Tests for glucose (E-28, p 91) acetone (E-28, p 97), and occult blood (E-28, p 97) were all negative throughout the study. Phenylketones (E-28, p 98) were essentially negative throughout the study, except in week 52 when there were occasional results of 15 mg% in all the dosage groups. Positive results in the high dosage group were scattered through all the intervals. Urobilinogen (E-28, p 99) was negative for the two intervals studied, weeks 78 and 105.

Table 3-28
Urinalysis pH Data
Means + Standard Deviations

Week	Controls		Low Dose		Medium Dose		High Dose	
	Males	Females	Males	Females	Males	Females	Males	Females
-2	6.8+1.0	7.6+0.9	6.6+0.9	6.6+ 0.5	6.6+0.9	7.0+1.4	7.4+0.5	7.2+0.8
4	7.4+1.1	7.0+1.2	7.2+1.1	7.2+0.8	7.6+0.9	8.4+1.3	7.4+1.1	7.8+0.8
14	9.0+0.0	9.0+0.0	8.8+0.4	8.2+0.8	9.0+0.0	8.8+0.4	8.2+1.3	9.0+0.0
26	8.3+0.4	8.5+0.0	8.4+0.5	7.8+1.2	8.6+0.4	8.6+ 0.4	8.0+0.7	8.5+0.3
52	7.9+1.3	6.4+0.5	6.5+0.5	7.2+1.0	6.7+0.8	7.3+1.3	6.7+0.4	6.5+0.5
78	8.5+0.9	8.5+0.5	7.6+1.5	7.8+1.4	8.0+1.4	8.9+0.2	8.7+0.4	8.7+0.4
106	6.4+0.5	6.7+0.8	6.9+0.4	6.5+0.5	6.6+0.8	6.7+1.4	6.8+0.8	6.5+0.5

Sediment: Urine sediment was examined for red blood cells, white blood cells, casts, bacteria, and crystals. The number of red blood cells per high power field at week 14 (Appendix III-24) was remarkable in that all groups were much higher than at the other intervals even though occult blood tests were negative. We cannot explain this, although refractile bodies which occur in urine sediments have been misinterpreted as red blood cells by less experienced persons. Alternatively, since the animals were approximately nine months old at week 14, many of the females could have been in heat at this time. However, this explanation fails to account for the large number of males which also had greatly increased numbers of RBC/hpf.

White blood cells per high power field at week -1 (Appendix III-25) are generally higher than all the intervals in controls as well as groups to receive aspartame but are within acceptable limits. Week 52, 78, and 106 were normal and unremarkable. Casts, bacteria, and crystals (E-28, pp 94-96) were generally unremarkable.

Necropsy; Gross Diagnosis; Organ Weights; Organ/Body Weight Ratios

Necropsy - The eight group A animals (one from each treatment group - see page 20) were necropsied on 4/10/72. Group B animals were necropsied 4/11/72 and so on (Table 3-11). A sample necropsy report and list of tissues sectioned are shown in Appendix III-26. The quality of

sections and stains at the time of UAREP review was still good, except for Oil Red O which normally deteriorates over the years. Nineteen of the necropsies were performed by Dr. McConnell, and 21 were done by (RDH) (Table 3-11). The description of gross findings was generally quite brief. Copies of gross descriptions were available to MBR and UAREP pathologists reviewing slides.

Organ Weights - Transcription of organ weights from necropsy sheets to report table was verified without any discrepancies. The testes weight for animal E-15 included only the right side. However, when means were calculated this value was included as though it comprised both sides as did the weights from other animals. Statistical analysis of the organ weight data by t-test as well as ANOVA showed no statistically significant differences. These findings by UAREP agree with those reported by Searle in the Entry Book.

Organ/Body Weight Ratios - Ratios were calculated (Table 3-29). Statistical comparison of the various groups by ANOVA showed no significant differences by the Q test. However, the kidney/body weight ratios in the high and medium dose male groups were significantly higher ($P < 0.05$) by the LSD test. The biological significance of this, if any, is unclear.

Table 3-29
Final Organ to Body Weight Ratios (g/kg)

<u>Male</u>								
Animal	Heart	Liver	Kidney	Adrenals	Thyroid	Pituitary x 10 ³	Testes	Prostate
Control								
1	7.18	25.92	5.01	.09	.12	5.81	1.49	.91
2	6.27	24.38	5.19	.09	.08	5.49	1.58	1.29
3	6.55	23.56	4.96	.08	.09	6.96	1.41	1.59
4	7.07	26.16	6.11	.09	.06	7.02	1.48	.94
5	8.79	26.10	6.51	.11	.13	8.98	2.67	1.19
Low								
6	8.52	23.00	5.67	.11	.09	9.81	2.46	.88
7	6.57	23.36	5.11	.12	.07	5.81	2.27	.55
8	7.67	26.96	5.55	.10	.11	7.29	2.52	1.24
9	10.48	29.99	6.94	.11	.09	6.62	2.04	1.31
10	7.03	23.69	5.02	.09	.08	5.12	2.15	.81
Medium								
11	9.69	33.49	7.40	.13	.16	11.16	2.34	.92
12	7.69	24.18	5.08	.08	.08	6.04	2.18	.83
13	9.06	26.86	6.53	.15	.12	8.74	2.15	.95
14	9.05	25.15	6.51	.12	.09	7.48	1.97	.96
15	7.78	25.76	5.86	.10	.09	7.16	.79*	1.15
High								
16	10.71	26.05	9.36	.09	.09	7.14	1.69	.74
17	7.73	26.28	5.72	.11	.11	6.85	1.73	1.67
18	7.88	29.20	9.54	.11	.10	8.62	1.83	.68
19	8.70	27.74	5.91	.10	.11	7.46	1.75	1.64
20	8.16	24.70	6.52	.10	.09	5.48	2.45	1.98

*right side only

Final Organ to Body Weight Ratios (g/kg)

<u>Female</u>								
Animal	Heart	Liver	Kidney	Adrenals	Thyroid	Pituitary x 10 ³	Ovaries	Uterus
Control								
21	7.22	23.96	4.84	.12	.10	8.11	.08	.68
22	9.80	27.76	6.60	.13	.10	8.51	.18	1.18
23	9.97	35.16	7.65	.19	.17	13.21	.17	.72
24	6.93	29.28	4.17	.11	.11	6.93	.08	.34
25	8.13	25.24	4.95	.14	.10	7.76	.09	.40
Low								
26	6.18	34.88	4.72	.13	.10	7.47	.12	1.48
27	7.68	26.54	5.04	.13	.12	9.12	.10	.57
28	6.80	23.48	5.03	.12	.11	6.79	.11	.42
29	7.26	27.51	4.63	.11	.10	8.90	.12	.60
30	7.54	24.34	5.13	.18	.06	10.05	.11	.91
Medium								
31	7.06	18.79	4.65	.14	.07	7.78	.07	.34
32	6.92	29.16	4.27	.16	.08	7.76	.22	1.75
33	6.82	28.26	4.56	.08	.14	9.17	.08	.51
34	8.10	26.31	5.36	.10	.07	7.31	.19	1.47
35	8.12	27.55	5.77	.14	.09	9.33	.16	2.53
High								
36	7.76	31.38	5.53	.12	.09	7.56	.15	2.32
37	7.92	35.95	6.17	.14	.09	6.96	.21	2.38
38	8.50	28.92	5.44	.14	.22	9.22	.21	2.10
39	8.51	26.83	7.31	.19	.10	11.23	.12	.62
40	7.69	30.27	4.88	.14	.08	9.80	.09	.54

Histopathology

Histopathology diagnoses were made in the Department of Pathology at Microscopy for Biological Research, Ltd., in Albany, New York.

In reviewing the slides on E-28, UAREP consultant Dr. Jack E. Moulton was asked to use the same system that MBR had used and not to grade the lesions. Using such systems, one would expect that a pathologist who made a diagnosis on the basis of a few inflammatory cells might not have that diagnosis initially confirmed by another pathologist who required a more significant number of inflammatory cells before making such a diagnosis. This, in fact, occurred because Dr. Moulton generally did not diagnose questionable, very slight, or inconsequential degrees of a condition. With one exception, there was no significant disagreement in any of the slides on the control dogs. On the control animal 91992, slide 16, MBR diagnosed chronic inflammation of the prostate. In his initial diagnostic review, Dr. Moulton did not notice the one small area containing inflammation, but in the re-review with Dr. Stowell, it was agreed that it was sufficient to warrant a diagnosis of chronic inflammation of the prostate. In the great majority of instances in which Dr. Moulton had not confirmed an MBR diagnosis of inflammation, his detailed notes recorded presence of a few inflammatory cells. The difference in criteria required by different pathologists to make a diagnosis is well illustrated by the fact that a diagnosis of chronic inflammation of the liver was recorded by MBR in control animals in 10 of 10 vs UCD 4 of 10; in the high dose group in 10 of 10 vs UCD 1 of 10; in the medium dose group in 6 of 9 vs 1 of 9; and in the low dosage group MBR diagnosed 7 of 10 in contrast to Dr. Moulton's diagnosis of 3 of 10. With the exception of slides of the central nervous system, there was no significant discrepancy in diagnoses in the high and

medium dose group of dogs between UCD and MBR. The findings in the brains will be discussed in more detail in Chapter VIII dealing with E-86.

In the low dose group, Dr. Moulton made a diagnosis of inflammation of neurohypophysis which had not been made by MBR. Independent rereview of the slides agreed with Dr. Moulton. Out of the 40 dogs, MBR made a diagnosis of fibrocystic disease of the mammary gland on only 3 of the low dose females. Dr. Moulton recognized the dilated ducts but did not concur in the diagnostic terminology. In the review of slides by Microscopy for Biological Research (MBR), lesions were indicated as present or absent and not graded on a scale of 1-5 as was done by Experimental Pathology Laboratories (EPL) in other histopathologic studies reviewed by UAREP. Since fat soluble lipid stains such as Oil Red O fade, precipitate, and migrate on standing, UAREP did not attempt review of these slides. Lipid is normally present in the proximal convoluted tubule of the dog.

According to the Entry Book: "All kidney sections showed a positive Oil Red O in the distal convoluted tubules with the exception of pathology slides Nos. 91999, 92001, 92002, and 92003 which correspond to high dose males Nos. A16HM, C18HM, D19HM, and E20HM, respectively, which were negative. . . None of these conditions were sex or dose related."

Thus, with the exception of the neuropathology slides (Chapter VIII), in the 1171 tissue sections there was a significant difference in the initial diagnoses reported by MBR and by UAREP in one instance of chronic inflammation of the prostate and in one instance of inflammation of the neurohypophysis. None of the histopathologic diagnoses made in E-28 were dose related or represented biologically significant differences between various groups of dogs.

CONCLUSIONS

This is a substantial two year toxicity study involving the examination of many pertinent parameters in 40 dogs at three dosage levels of aspartame. A general summary of the discrepancies and their relative importance is included in Appendix III-23. Such a long term experiment involves more than 100,000 bits of data and sources of information. The opportunities for attention to detail and precise work range from tremendous to overwhelming.

UAREP validation of data handling showed 0-0.4% transcriptional errors, 0-1.8% computational discrepancies, some inconsequential rounding discrepancies, and a variety of other problems. Few of these would individually introduce more than a 1% quantitative difference in result, and none would alter the overall results by more than 5%.

In some respects, the E-28 report was not particularly well written. UAREP had to request considerable additional information from Searle for its validation work.

UAREP has pointed out a number of problems encountered in evaluating this toxicity study.

It was difficult for UAREP to understand how the dogs were randomized. Two problems were encountered. First, there were three groups of five animals in which three out of the five were littermates, and five groups in which two out of three were littermates. Second, the animals in the high dose groups were statistically significantly heavier than the controls at the start of the study.

Laboratory notebooks recording hematology data gave no indication that erythrocyte and leukocyte counts at the first four intervals had been corrected for the Coulter Coincidence Factor. In fact, there were numbers reported for total leukocyte counts, which would be impossible if the Coulter correction had been made.

The bromsulfophthalein (BSP) determination was also difficult to interpret. Information in the Entry Book was lacking so that UAREP had to contact Searle to ascertain the amount of dye used and the conditions of the test. The results of the BSP determination were reported in an unconventional manner as mgm%. It was necessary for UAREP to construct an equation to calculate the amount of dye remaining in the plasma after thirty minutes. The actual conduct of the assay in the laboratory was dubious. UAREP also questions whether a mildly toxic substance such as BSP should have been administered eight times to the animals over the course of two years.

Chloride data given in the Entry Book at weeks 78 and 106 were considered unlikely in well dogs. UAREP found laboratory data on chlorides at week 106 for half of the dogs which were within normal limits, but these were not used in the Entry Book.

The high incidence of red cells in urine sediments reported at week 14 was of sufficient magnitude to be apparent in voided urine. It is difficult to correlate this finding with the negative occult blood results reported at the same time.

Regarding the histopathological examination, there was a significant difference in the initial diagnoses reported by MBR and by UAREP in one instance of chronic inflammation of the prostate and in one instance of inflammation of the neurohypophysis. None of the histopathologic diagnoses made were dose related or represented biologically significant differences among the various groups of dogs.

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CHAPTER III
LIST OF TABLES

- 3-1 Ages of Dogs at Start of Experiment in E-28
- 3-2 Distribution of Littermates in E-28
- 3-3 Body Weights (kg) at Start (Week 0) of Study
- 3-4 Discrepancies in Reporting Body Weights When Drug Dosage
 Records, "Raw" Data and Entry Book are Compared
- 3-5 Substantial Variation in Individual Body Weights - Data
 From Appendix Table I pages 60-64, E-28
- 3-6 Body Weight Gain (kg) Weeks 0 to 26 of Study E-28
- 3-7 Percent Body Weight Gain Weeks 0 to 26
- 3-8 Total Food Consumption (kg) Weeks 1 to 26
- 3-9 Total Food Consumption (kg) Weeks 1 to 10
- 3-10 Compound Consumption g/kg
- 3-11 Dates of Necropsies and Ophthalmoscopic Exams as Verified
- 3-12 Statistical Significant Differences by Three Methods
 Between Control and Treatment Groups in Comparison of
 Various Hematologic Parameters
- 3-13 Examples of Comparison of Means for RBC ($\times 10^6/\text{cmm}$) and
 WBC ($\times 10^3/\text{cmm}$) using Corrected and Uncorrected Data
- 3-14 Examples of Discrepancies in Hematology Data From
 Appendix Table 2, E-28
- 3-15 Wintrobe Indices SC-18862: 106 Week Oral Toxicity Study
 in The Dog
- 3-16 Hematology Normals for Dogs
- 3-17 Summary of Statistical Comparisons of Hematology Data

Chapter III, Tables
continued

- 3-18 List of Abbreviations in Chemistry Section
- 3-19 Summary of Chemistry Quality Control and List of
Available Laboratory Data (BA No.)
- 3-20 Dates on Which Assays of Hematologic and Chemical
Parameters Were Recorded
- 3-21 Normal Blood Chemistry Values for Dogs
- 3-22 Percentage BSP Retention in Male Dogs
- 3-23 Means of Sodium/Chloride Ratios
- 3-24 Percentages of Aspartame and L-phenylalanine in Daily
Diet During the Dog Toxicity Study
- 3-25 Comparison of Statistical Difference in Chemistry Data
(based on geometric means)
- 3-26 Summary of Statistical Comparisons of Blood Chemistry Data
- 3-27 Urinalysis Specific Gravity Data (Means \pm Standard Deviation)
on Groups of Dogs
- 3-28 Urinalysis pH Data Means \pm Standard Deviations
- 3-29 Final Organ to Body Weight Ratios (g/kg)

Figures

- 3-1 Body weight gain (kg) in individual animals, weeks 0-26
- 3-2 Graph of Hgb for 20 males \pm SEM with littermates also shown

APPENDICES

Background Papers Relating to E-28

- III-1 March 13, 1970. Screening Test on 4 dogs of consumption of diet containing Purina Dog Meal plus 4 g/kg (anticipated high dose) of aspartame.
- III-2 March 26, 1970. Anticipated daily Feeding Schedule for 26 week dog study.
- III-3 March 30, 1970. Protocol amendment: stating grouping of dogs by dose level; feeding one to three times a day as required; Wayne Lab-blox for basal diet.
- III-4 April 6, 1970. Feeding schedule effective 4-16-70 (study day 17 onward).
- III-5 June 24, 1970. Interim progress review (12, 17, 20, 26, 40 weeks). Evaluation of body weight changes, food consumption, nutritional status, appearance, behavior, elimination and laboratory results.
- III-6 September 28, 1970. Memo: McConnell to Martinez, et al.
Subject: SC-18862; 6 month toxicity study in the dog; P-T No. 855S70, protocol amendment, extending study indefinitely.

- III-7 October 29, 1970. Memo: McConnell to Martinez, et al.
Subject: SC-18862 toxicity studies, protocol amendment; 26 week dog study; P-T No. 855S70; extends study to 104 weeks; increases compound requirement; sets further lab work at 52, 78, and 104 weeks with termination date set at 4/1/72.
- III-8 May 28, 1971. Memo: McConnell to Hemm.
Subject: Request for special review of certain hematology data.
- III-9 52 week differential smear data summary.
- III-10 June 11, 1971. Memo: Hemm to McConnell.
Subject: 52 week differential smear data.
- III-11 September 10, 1971. Memo: Polk to McConnell.
Subject: Information needed from preclinical studies to aid in overall clinical development of SC-18862. Notes on necessity of evaluating glucogenic, lipogenic, and caloric potential of aspartame.
- III-12 October 1, 1971. Memo: McConnell to Polk.
Subject: Response to 9-10-71 memo. Notes on proposed methods of obtaining the data discussed in III-15.
- III-13 October 19, 1971. Memo: Rao to Youkilis.
Subject: Ophthalmoscopic examination of 2 siblings of dogs in the 104 week study; observation of pups obtained from the parents of dogs B12MM and E40HF.

- III-14 October 22, 1971. Memo: Rao to McConnell.
Subject: Ophthalmoscopic examination of two full sibs of dogs with bilateral cataract.
- III-15 February 21, 1972. Memo: Rao to Ranney.
Subject: 104 week toxicity study in dog; particular mention of evaluation of phenylalanine hydroxylase activity.
- III-16 April 7, 1972. Memo: Rao to protocol design committee and technical staff.
Subject: Protocol amendment No. 4. Clinical laboratory measurements. Requests addition of insulin, phenylalanine, OCT, protein electrophoresis, and total protein determinations in serum at terminal bleeding.
- III-17 August 19, 1974. Memo: Rao to McConnell.
Subject: Correction in released report. Data in the graph in Figure 2 of the report was incorrectly labeled. A corrected copy was to be included in the file copy of the report.
- III-18 Uncorrected and corrected versions of graph in Figure 2 of the report.
- III-19 April 24, 1978. Letter from Bost answering questions on personnel, methods for collecting, processing, and storing blood samples, collection and processing of urine samples.
- III-20 Letter from Bost June 29, 1978 verifying amount of BSP used; statistical method used in E-19; two-tailed t-test used in E-28.

- III-21 (2 pages) Experiment code number, tattoo number, date whelped, age at start of experiment, tattoo numbers of dam and sire.
- III-22 Batch numbers of aspartame, number of days used, percent DKP.
- III-23 Summary of data bits and discrepancies.
- III-24 Copy of red blood cells per high power field (RBC/hpf) data from urinalysis assays.
- III-25 Copy of white blood cells per high power field (WBC/hpf) data from urinalysis assays.
- III-26 Necropsy form and tissues fixed and sectioned.

APPENDIX III-1

From 7/1/3-13-5

COPY-502 Diet Screen Test on 4 Dogs

Dose: 4 g/Kg

Type and Amount of Feed: Purina Dog Meal (purchased); 350 gm/dog
Compound formulation administered: mixed into the feed with 180 ml water.

Frequency of Comp administration: once daily (semi-albik)

After 7 days to 4 dogs:

all dogs are eating above ration
c 4 gm/Kg SC-18862 added;

Get proper dishes ordered with Mon-
Dietary diet c Harris; 170 gm meat. - ?

Mix pur diet + drug; administer
once daily to each dog ^{separately} on
Group X. Feed ~~pur~~ pur diet +
drug once daily in late PM;
feed meat supplement once daily
in AM.

APPENDIX III-2

Proj. No. 855570

COPY - 18862; 26 wks. dog study.
2 dogs; 20 ♂, 20 ♀.

	♂	♀
\bar{X} Body wt \pm S.E. (3-25-0)	6.17 \pm 0.13	5.12 \pm 0.1
\bar{X} Age on Rx day 1: (3-30-0)	154 \pm 5*	156 \pm 7

Feeding schedule:

200 gms. of Purina meal (dry) or
Purina meal-SC-18862 mixture (dry)
to each dog at about 9 AM; ^{approx. amount + 1/2} presume
each dog consumes entire amount
within 1 hr. Then, after 1 hr, give
250 gms of Wayne Dog Pellets (dry)
(dry) per dog. After 2 additional
hours, remove dish of dog pellets,
weigh, record & calculate food
consumed (gms), and discard
uneaten food.

Dosage calculation:

Group \bar{X} body wt will be calculated
weekly initially, for each sex. ~~Being~~
Drug-diet conc! will be prepared for
each group based on \bar{X} body wt, and
each dog based thus. Individual
drug.

* ♂ group
70 days
each in
Groups.

gm/kg) will be calculated
2; remaining 3 are
are distributed one
, medium, & high dose

2/26-0

APPENDIX III-3

Protocol Amendment (3/30/70)

2 pages

amendments 123

est. date due

est. 222

Delivery date requested. *By - 10/20/68*

Duration of study (wks): 24

Species, strain, sex, & age at Rx start: *Doc. B. 1000. MF. 4-6 months*

Compound formulation administered: *Compound with lead (see page 2)*

Route & frequency of expd administration: *Quo. - three times a day as ordered*

Dose levels (daily): control — ; low 1 ; medium 2 ; high 4 *

No. & sex of animals per dose level: 5M, 5F Total no. of animals needed: 40

Housing & feed: individual 59 cages; drug diet 100 mg 100 mg 100 mg

GP-K	1	2	4
% of dist	2.5	5	10
Rat			
Mouse			
Dog **	L	M	H

weight of the high dose group: 70.5 kg

duration of the study $\times \frac{18.2}{18.2}$ days

12.740

summation of daily dose; all groups. X 7 gms

Total compound required for study 89180 gms

spillage; add 15% 13,377.2ms

Total cnd to order for the study: \$2557.50**

EXAMINATIONS AND OBSERVATIONS

General Observations: only at 200x

Then a Harvest. Food & Land Consumption

Physical Examination:

Pre-X1: There are 11/15/80

* Raise high door only
after 7 hrs, if
cleared floor.

** 7 daily feed intake - 335
on 40 gm/kg/day

7 bolunt. for \rightarrow cpd. cell
lation \Rightarrow 7 Kg.

~~***~~ No allwarks for ins.
high dose gelif.

17. Kg Cpdl/m² for 6 mo.

Eye examination: cooked eye only

Louise

Optimizing - accepted

רמת גן: 1991

PROJ. NO. 855 570

A PRECLINICAL SAFETY STUDY ON SC-

18862

PG 2

COP

Rotation of diet and "drugs"?

Practical

Each dog, irrespective of sex, age, or sex, receives 3.5% daily of dry Wayne Lab-Blox in pellet or purr for except that this amount will be increased for the control dogs in amt equivalent to the caloric value of the medium dose level (approx 56 cal/gm). Suitable, practical means of administration are being tested. The feeding of these dogs is to be done by the Path- & Dept.

HEMATOLOGY:

Specimen collection procedures--

Na₂EDTA for hemogram

Citrate for coagulogram } IV puncture

PARAMETER	Frequency repeated	
	No./group	Treatment weeks
PCV	5	
Hb	5	
Rbc.....	5	-2, 4, 13, 26
Wbc	5	
Diff	5	-2, 4, 13, 26
Retic.....	5	
Platelets		
Coagulation (L-W)		
Pro time.....	5	-2, 4, 13, 26
Act. PT	5	-2, 4, 13, 26
Bone marrow smears	5	26 #

*To be read on request only.

URINALYSIS:

Specimen collection procedures--

Cage floor pan drainage - ?
Try bladder expression.

PARAMETER	Frequency repeated	
	No./group	Treatment weeks
Sp. grav.	5	
pH	5	
Bilirubin.....	5	-2, 4, 13, 26
Protein	5	
Sugar.....	5	
Ketones	5	
Blood	5	
Microscopic.....	5	-2, 13, 26, 4
Phosphatase	5	-2, 4, 13, 26

CLINICAL CHEMISTRY:

Specimen collection procedures--

IV puncture, heparin anticoagulant

PARAMETER	Frequency repeated	
	No./group	Treatment weeks
BUN	5	-2, 4, 13, 26
Uric acid	5	-2, 4, 13, 26
GPT.....	5	-2, 4, 13, 26
GOT	5	
AP	5	
BSP.....	5	
Bilirubin	5	
Glucose	5	
Na.....	5	-2, 4, 13, 26
K	5	
Ca	5	
Cl.....	5	

MISC. CLINICAL LAB WORK:

Extended frequency of hematology, cl. chem + urinalysis submission:
- 52, 78 + 104 wks

APPENDIX III-A

COPY

62; 2-6 WK dog study; Proj. No. 555370
40 dogs; 20 ♂, 20 ♀; Rx started: 3/20, 31, 4/1-3/70

Feeding Schedule: (effective 4/6/70; study day 17 onward)

1st Feeding: 200 gms of Purina meal (dry) or Purina meal - SC-15562 mixture (dry) given to each dog at about 9 AM; supplement + H₂O ad lib.
After 2 hrs, each cage is examined. & note

2nd Feeding: A 2nd feeding of 200 gms of Wayne Dog pellets (dry) is given to each dog which has consumed entire amount of meal-SC-15562 mixture. After 3 1/2 - 4 additional hours, dish of dog pellets is removed and weighed; food consumed (gms) is calculated and recorded and uneaten food is discarded.

Dogs which do not consume the 1st feeding after 2 hrs but able to consume entire amount by 4:30 PM, are likewise given 200 gms of Wayne Dog pellets. After overnight and before the following day's feeding, the dish of dog pellets is removed and weighed, and food consumed (gms) is calculated and recorded; uneaten food is discarded.

initial feeding of drug-diet
Dogs which have not consumed ~~feeding~~ by 4:30 PM, are left with some food overnight. Uneaten food after overnight is calculated and recorded.

Dosage Calculations (effective 4/6/70, 1 week after start of test)

Based on individual body weight.

APPENDIX III-5

Interim progress review at twelve, seventeen, twenty, twenty-six and forty weeks, including evaluation of body weight changes, food consumption, nutritional status, appearance, behavior, elimination, and laboratory results.

(5 pages)

Proj. No. 85557A

Preclinical Safety Study on SC- 1886.2 (DJA)

INTERIM PROGRESS REVIEW

Days on study: Males ±84; Females ±84

(12 wks) 6/24/70

General Observations:

Parameters	MALE					FEMALE				
	C	L	M	H	1/2	C	L	M	H	1/2
Drug doses actually recd. (mg)										
% deviation from desired dose										
% body wt. change (base: Pre-1st body wt.)	↑60%	↑56%	↑44%	↑38%		↑51%	↑48%	↑45%	↑39%	
Food consumption (g/day)	>200	>200	>200	>200		>200	>200	>200	>200	
Nutritional status- mortality rate	u; 0/5	u; 0/5	u; 0/5	u; 0/5		u; 0/5	u; 0/5	u; 0/5	u; 0/5	
Appearance	u	u	u	u		u	u	u	u	
Behavior	u	u	u	u		u	u	u	u	
Elimination	u	u	u	u		u	u	u	u	

r = remarkable; u = unremarkable

EVIDENCE OF DRUG ABSORPTION:

Physical Exams Including Eye:

CLINICAL PATHOLOGY:

Hematology: (See separate sheets)

Clinical Chemistry: (See separate sheets)

Urinalysis:

SUMMARY AFTER _____ WEEKS OF TREATMENT (_____):

Proj. No. 025010

Preclinical Safety Study on SC- 18862 (55g)

INTERIM PROGRESS REVIEW

Days on study: Males ±119; Females ±119
(~17wks)

7/28/70

General Observations:

Rx 119

112

119

119

119

119

119

Parameters	MALE				FEMALE			
	C	L	M	H	C	L	M	H
Drug doses actually recd. (mg)								
% deviation from desired dose								
Body wt. changes (Base & Pre-R body wt.)	↑72.2	↑69.3	↑49.4	↑42.4	↑70.3	↑61.0	↑73.7	↑40.4
Food consumption (g/day)	>200	>200	>200	>200	>200	>200	>200	>200
Nutritional status- mortality rate	u; 0/5	u; 0/5	u; 0/5	u; 0/5	u; 0/5	u; 0/5	u; 0/5	u; 0/5
Appearance	u	u	u	u	u	u	u	u
Behavior	u	u	u	u	u	u	u	u
Elimination	u	u	u	u	u	u	u	u

r = remarkable; u = unremarkable

EVIDENCE of DRUG ABSORPTION:

Physical Exams Including Eye:

CLINICAL PATHOLOGY:

Hematology:

Clinical Chemistry:

Urinalysis: Phenylketones - all negative at 14 wks post Rx

SUMMARY AFTER _____ WEEKS OF TREATMENT (____):

NO. 555310

Preclinical Safety Study on SU-10000 (1207)

INTERIM PROGRESS REVIEW

Days on study: Males ~140; Females ~140
(~20 wks)

General Observations:

8/19/70

Parameters	MALE				FEMALE			
	C	L	M	H	C	L	M	H
Drug doses actually recd. (mg)								
% deviation from desired dose								
Body wt. change (Base: Pre-study body wt)	84.8	74.0	55.5	46.4	75.8	66.8	78.7	43.4
Food consumption (g/day)	>200	>200	>200	>200	>200	>200	>200	>200
Functional lesions - Mortality Rate	u; 0/5	u; 0/5	u; 0/5	u; 0/5	u; 0/5	u; 0/5	u; 0/5	u; 0/5
Appearance	u	u	u	u	u	u	u	u
Behavior	u	u	u	u	u	u	u	u
Elimination	u	u	u	u	u	u	u	u

r = remarkable; u = unremarkable

EVIDENCE OF DRUG ABSORPTION:

Physical Exams Including Eye:

CLINICAL PATHOLOGY:

Hematology: (see separate sheets)

Clinical Chemistry: (see separate sheets)

Urinalysis:

SUMMARY AFTER _____ WEEKS OF TREATMENT (date):

Preclinical Safety Study on SC- 18362

INTERIM PROGRESS REVIEW

Days on study: Males 182; Females 182
(26 wks)

9/24/70

General Observations:

Parameters	MALE				FEMALE			
	C	L	M	H	C	L	M	H
Drug doses actually recd. (mg)								
% deviation from desired dose								
Body wt. change (Base: Pre-17 body wt.)	493.3	479.93	456.52	451.54	452.26	471.20	458.59	442.36
Food consumption (g/day)	>200g.	>200g.	>200g.	>200g.	>200g.	>200g.	>200g.	>200g.
Nutritional status- mortality rate	u, 0/5	u, 0/5	u, 0/5	u, 0/5	u, 0/5	u, 0/5	u, 0/5	u, 0/5
Appearance	u	u	u	u	u	u	u	u
Behavior	u	u	u	u	u	u	u	u
Elimination	u	u	u	u	u	u	u	u

r = remarkable; u = unremarkable

EVIDENCE of DRUG ABSORPTION:

Physical Exams Including Eye: See separate report

CLINICAL PATHOLOGY:

Hematology: unremarkable

Clinical Chemistry: OK; lab methodology on specimens handling has caused 1 in 4 pt to be general

Urinalysis: unremarkable

SUMMARY AFTER 76 WEEKS OF TREATMENT (182): unremarkable except eye report a-v. Body wt gain decrease, is definitely a dose-related effect in both sexes. Changes in behavior are not significant.
H-3-0

Proj. No. 955570

Preclinical Safety Study on SC- 18852

INTERIM PROGRESS REVIEW

Days on study: Males 250 ; Females 780
(40 weeks)
1/26/71

General Observations:

Parameters	MALE				FEMALE			
	C	L	M	H	C	L	M	H
Drug doses actually received (mg)								
% deviation from desired dose								
% body wt. change (base: Pre-study body wt.)	104.81	131.31	160.70	156.79	116.69	180.00	180.60	149.30
Food consumption (g/day)	>200g.	>200g.	>200g.	>200g.	>200g.	>200g.	>200g.	>200g.
Nutritional status - mortality rate	11	11	11	11	11	11	11	11
Appearance	11	11	11	11	11	11	11	11
Behavior	11	11	11	11	11	11	11	11
Elimination	11	11	11	11	11	11	11	11

r = remarkable; u = unremarkable

EVIDENCE of DRUG ABSORPTION: _____

Physical Exams Including Eye: _____

CLINICAL PATHOLOGY:

Hematology: _____

Clinical Chemistry: _____

Urinalysis: _____

SUMMARY AFTER _____ WEEKS OF TREATMENT (_____): _____

APPENDIX III-6

COPY

September 23, 1970

~~MEMO~~ → Mr. Martinez
Dr. Huttsell
Dr. Henn
Miss Brausch
Mrs. Mayer

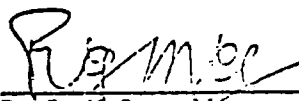
FROM: Dr. McConnell

SUBJECT: SC-18362; 6 month toxicity study in the dog; P-T No. 855370;
protocol amendment.

This study will be continued indefinitely, with treatment at the present dosage levels continued. Autopsy as scheduled for the week of September 23, 1970 is thus cancelled.

Clinical pathology as indicated for termination will be performed for this present 26 week interval, so specimens should be drawn accordingly.

A decision in the near future will provide a specific date for termination of this study.


R. G. McConnell

RCMcC:sg

APPENDIX III-7

October 29, 1970

30PY

→: Mr. Martinez
Mrs. Mayer
Dr. Hutsell
Dr. Youkilis
Dr. Moe

FROM: Dr. McConnell

SUBJECT: SC-13362 toxicity studies; Protocol amendment;
26 week dog study; P-T No. 655870

This study was originally scheduled for 26 week duration. It is tentatively extended to 104 weeks duration, with termination date of 4-1-72 and final report date of 7-1-72.

Body weight gain requires an increase of 40% in estimated monthly compound requirement. 24. kg/month will be needed during remaining 1970 and during 1971.

The profile of clinical lab work (clinical chemistry, hematology, urinalysis) as indicated in the original protocol should be repeated at 52, 78 and 104 week intervals. An ophthalmoscopic examination should be performed at these same intervals, and additionally as required. Autopsy procedures remain unchanged.


R. G. McConnell

RGMcC:sg

COPY

May 28, 1971

MEMO TO: Dr. Hemm

COPY TO: ✓ Mr. Martinez

FROM: Dr. McConnell

SUBJECT: SC-18862; 104 week dog toxicology study
P-T 855S70;
Request for special review of
certain hematology data

This study finished 52 weeks Rx about 1 April; clinical laboratory data from that interval is now available and has been reviewed. Would you please make a special review of the 52 week differential smear data and provide an opinion on presence of treatment related changes? Also whether protocol changes (hematology) are indicated? Data is available in the Toxicology Lab. Remaining observational and clinical lab data are unremarkable. Please check on current screening data regarding immunosuppressive or similar activity of this cpd. Seems that Larry Hayes was once working with it at very high dose levels.



R. G. McConnell

RGMcC:dsh

COPY

APPENDIX III-9

P-T 855870: 52 Week Differential Smear Data

	<u>Segs</u>	<u>Lymphs</u>	<u>Mon.</u>	<u>Eos.</u>	<u>Bas.</u>	<u>Comments:</u>
Control						{ C3CM - 1 NRBC/100 cells E5CM - 2% Atypical Lymph D24CF - 1 NRBC/100 WBC
Male	58.2	32.4	3.6	5.6	1.0	
Female	62.4	30.4	4.0	3.4	1.5	
Low Dose						{ D9LM - eosinophilia A26LF - Polychromasia 2% Atypical Lym D29LF - 1 NRBC/100 WBC
Male	56.2	26.4	4.6	10.8	5.0	
Female	63.8	27.8	4.2	3.4	2.0	
Medium Dose						{ A31MF + polychromatocytosis + anisocytosis + poikilocytosis
Male	61.4	30.0	5.0	4.4	1.0	
Female	72.6	19.8	2.2	4.8	3.0	
High Dose						{ A16HM - Hypochromia & polychromasia C18HM - + Target cell + Anisocytosis + poikilocytosis D19HM - 2 NRBC/100 WBC C38HF - 1 NRBC/100 WBC D39HF - 2 NRBC/100 WBC
Male	71.0	18.2	3.6	6.8	2.0	
Female	62.6	28.8	4.3	4.4	4.0	

COPY

APPENDIX III-10

June 11, 1971

MEMO TO: Dr. McConnell

FROM: Dr. Hemm

SUBJECT: P-T 855S70 - 52 week differential smear data

← File

Review of the 52 week SC-18862 dog toxicity study differential smear data reveals no evidence of treatment related changes. Mean differential white cell percentages are roughly equal in control and treated groups. A table of 52 week mean differential smear data is attached. Sporadic instances of aberrant red cell morphology are present for some dogs, reflecting a slightly impaired erythron which was frequently evident prior to treatment. A single dog (D9LM) exhibited notable eosinophilia, possibly resulting from parasitic infestation. Atypical lymphocytes (2%) were noted in 2 dogs; the number of atypical lymphocytes apparent in these two dogs are not outside the limits of variability seen in the dog and other species. These were recorded because the atypism exceeded borderline qualitative criteria and probably because of recent interest in atypical lymphocytes. I am presently recording and classifying the incidence of atypical lymphocytes occurring in various laboratory species.

canine → Dr. Hayes has found SC-18862 active in the Jerne plaque assay at 500 mg/kg; SC-19192 was active at 200 mg/kg.

I do not feel protocol changes for evaluation of the hematologic system are indicated.

R. D. Hemm
R. D. Hemm

RDH:dsh
Attachment

APPENDIX III-11

September 10, 1971

MEMORANDUM TO: Dr. McConnell

COPY TO: Dr. Gwinn
Dr. Roe
Dr. Stewart

FROM: Dr. Polk

SUBJECT: Information needed from preclinical studies to aid in overall clinical development of SC-13362 (APM).

During the meeting of the Toxicology Protocol Design Committee for APM on 9/8/71 several matters pertaining to preclinical development of APM were discussed. It seems that perhaps it would be desirable to set down in writing a few particular items which will be needed from our standpoint for the clinical development of this compound.

1. Glucogenic potential. The effects of APM on serum glucose levels and insulin secretion in animals would be very helpful data. Our clinical diabetic consultant has suggested these..
2. Lipogenic potential. Specifically, the effects of APM on serum lipids including free cholesterol, cholesterol esters, triglycerides, free fatty acids, and if possible, lipoprotein electrophoresis.
3. Caloric potential. I am not sure whether this particular item belongs in a memo to the Division of Biological Research, but if it does not belong here I would appreciate a suggestion as to whom I should contact for this. Basically, we need to know the number of calories per gram or smaller units produced by APM.


RCP

meb

APPENDIX III-12

127674 &
21-10-71

October 1, 1971

MEMO TO: Dr. Polk

COPY TO: ~~Dr. F. Saunders~~
Dr. Rao
Dr. Cook
Dr. Ranney

FROM: Dr. McConnell

SUBJECT: SC-18362 (APM) preclinical safety studies protocols;
response to your memo dated 9-10-71.

The three separate items discussed in your memo (copy attached) are being handled as follows:

1. Glucogenic potential.
Serum glucose levels are being measured periodically in the dog and rat chronic studies. Data on insulin secretion in treated animal is not being generated in toxicology studies, but is or will be developed by Dr. R. Saunders in the Pharmacology Dept. We will, if possible, measure insulin secretion in animals treated chronically with this agent if you consider this necessary to support clinical studies.
2. Lipogenic potential.
Total cholesterol, FFA, and triglycerides have been added as a routine measurement henceforth on a 104 week dog study now in its 75th week.
If total cholesterol is affected, the fractions (free & esterified) will then be measured. We will gain similar information in additional species in the near future. Lipoprotein electrophoresis will likewise be performed once in the dog study, and repeated as indicated. Serum protein electrophoresis is scheduled terminally in the 104 week rat study.
3. Caloric potential.
I will request that Dr. Ranney respond to this question. A great deal of relevant information on amino acids is contained in the several volumes on Protein Metabolism, edited by Dr. H. Munro.

Called
Ranney
the
Ranney

Attachment
RCMc:pm

↓

R. G. McConnell
R. G. McConnell

protein = 4 cal/gm
alcohol = 7 cal/gm
Since the sum of these yields 10.3 cal/gm,
 $4 \times .9 = 3.6$ cal from dipyrrol
 $7 \times .1 = .7$ " " alcohol
Sum total = 4.3 cal per gram ! DER

COPY

APPENDIX III-13

October 19, 1971

MEMO TO: Dr. Youkilis

COPY TO: Dr. McConnell
Mr. T. Martinez ✓

FROM: Dr. Rao

SUBJECT: Ophthalmoscopic examination of 2 siblings of dogs in the
104 week study of SC-18862 (P.T. No. 855S72).

Two pups from the same parents as of B12MM and E40HF dogs which
are in the above experiment have been received today. I request these
pups be examined ophthalmoscopically for the presence or development
of lesions in their eyes every 4 weeks until otherwise notified.
These pups will be maintained on control basal diet during the entire
period of observation.

K. S. Rao

K. S. Rao

KSR:ml

APPENDIX III-14

COPY

October 22, 1971

MEMO TO: Dr. McConnell

COPY TO: Dr. Youkilis
Mr. Martinez

FROM: Dr. Rao

SUBJECT: Ophthalmoscopic examination of 2 full sibs of dogs with bilateral cataract in the 104 week dog study of SC-18862 (P-T No. 855S72).

The above two male pups received on October 19 were examined by Dr. Magrane for possible eye lesions. It is gratifying to note that one male pup had cataract in the left eye only, involving temporal portion of the lens. Ophthalmoscopic examination of these pups will be performed at every two weeks instead of four weeks as mentioned earlier.

Dr. Youkilis will also be taking pictures of the lens of these pups on a continuing basis to get an idea of the progression of cortical cataract with time.

K. S. Rao
K. S. Rao

KSR:dsh

February 21, 1972

MEMO TO: Dr. Ranney

COPY TO: Dr. Saunders
Dr. McConnell

FROM: Dr. Rao

SUBJECT: SC-18862: 104 week toxicity study in the dog.
P.T. No. 855S72.

As you are aware, we are carrying out a two year toxicity study in the dog. The dogs from the above study will be sacrificed beginning April 10 through 14, 1972. If you need blood or any other tissues from these dogs, you are free to use them. I was particularly interested in evaluating the phenylalanine hydroxylase enzyme in the livers of these dogs chronically administered with APM. Please let me know in advance, if you are planning to use any of the tissues from the above study for your work.

K. S. Rao

K. S. Rao

APPENDIX III-16

April 7, 1972

MEMO TO: Protocol Design Committee
Members:

Technical Staff:

- | | |
|--------------------------------------|----------------------------------|
| 1) Dr. Sammeta (Biostatistician) | 1) Mrs. Sprenger (Path. Lab) |
| 2) Dr. F. Saunders (Biol. Res. Dir.) | 2) Mr. Martinez (Autopsy Lab) |
| 3) Dr. Rannoy (Drug Metab. Rep.) | 3) Dr. Hutsell (Bio-Anal. Lab) |
| 4) Dr. Polk (Clinical represent.) | 4) Dr. Rao (Gen'l Tox. Lab) |
| 5) Dr. Rao (P-T Dept. monitor) | 5) Mrs. Perkins (Hematology Lab) |
| 6) Dr. McConnell (P-T Dept. adviser) | 6) Dr. McConnell (Pathologist) |

FROM: Dr. Rao

SUBJECT: SC-18862: 104 week oral toxicity study in the dog; P-T 855S70;

Protocol Amendment No. 4. Clinical Laboratory Measurements.

Please make the following additions to the clinical chemistry section for terminal bleeding in all groups.

- 1) Serum Insulin
- 2) Serum Phenylalanine
- 3) Ornithine Carbamyl Transferase
- 4) Serum protein electrophoresis
- 5) Serum total protein.

K. S. Rao.
K. S. Rao

KSR:dsh

Revised 4-7-72

RECEIVED

AUG 2

August 19, 1974

MEMO TO: Dr. McConnell

FROM: Dr. Rao

SUBJECT: SC-18862: 106 Week Oral Toxicity Study in the Dog; P-T 855S70.
Correction in Released Report.

In the above report, the following MINOR errors were detected on page 15, figure 2:

<u>Error</u>	<u>Correction</u>
Control	Medium
Low	Control
High	Low
Medium	High

A corrected copy of figure 2 is attached.

The interpretation of data in the report was based on the group mean body weights presented in Table 1. Hence, the errors detected in figure 2 will not alter the conclusions drawn in the report.

It is my personal opinion that a copy of this memo be filed with the final report in the P-T Department file and there is no need for wide dissemination of this information.

K. S. Rao.
K. S. Rao, P-T Monitor

KSR:ck
Attachment

APPENDIX III-18

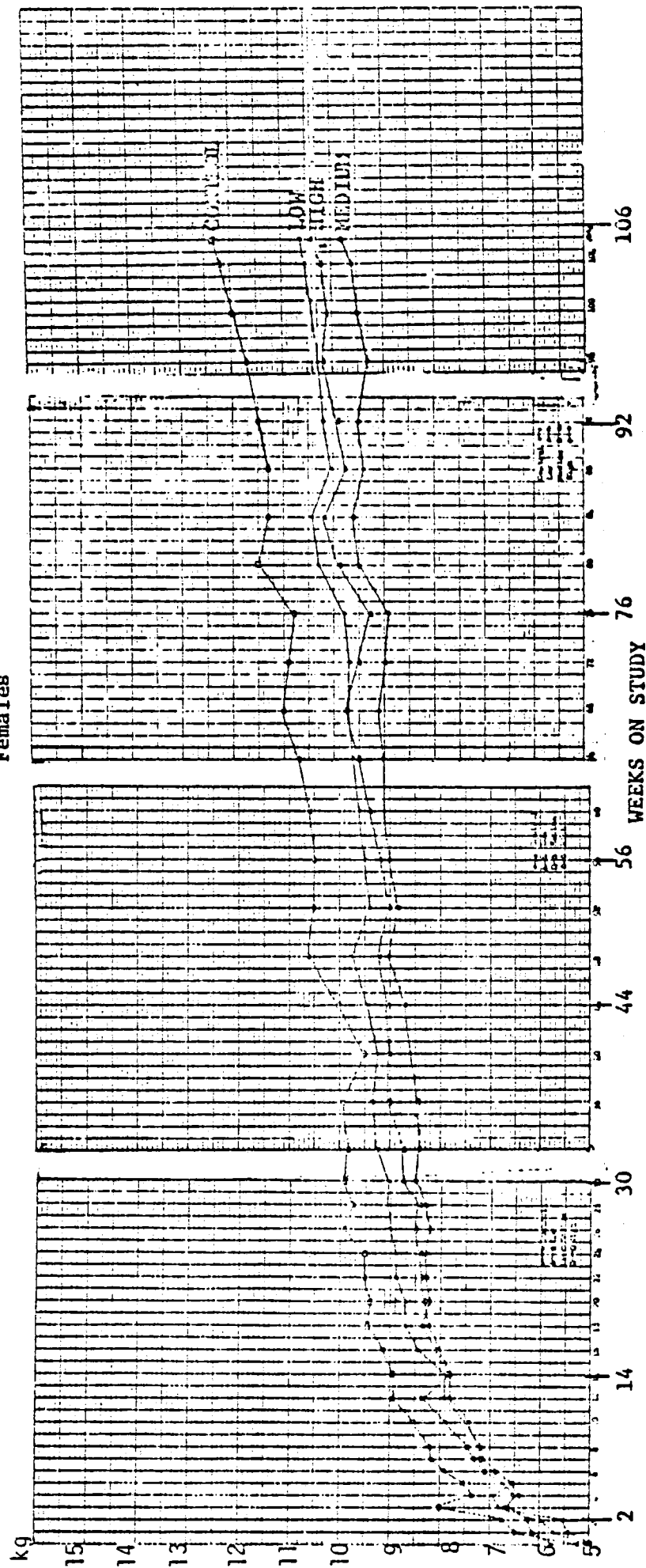
Corrected and uncorrected versions of the graph in Figure 2, E-28, p 16. The uncorrected version was in the original report. Dr. Rao then corrected the labeling error (Appendix III-17) which was subsequently included in the Entry Book.

Figure 2

SC-18862: 106 WEEK ORAL TOXICITY STUDY IN THE DOG

Mean Body Weight (kg)

Females

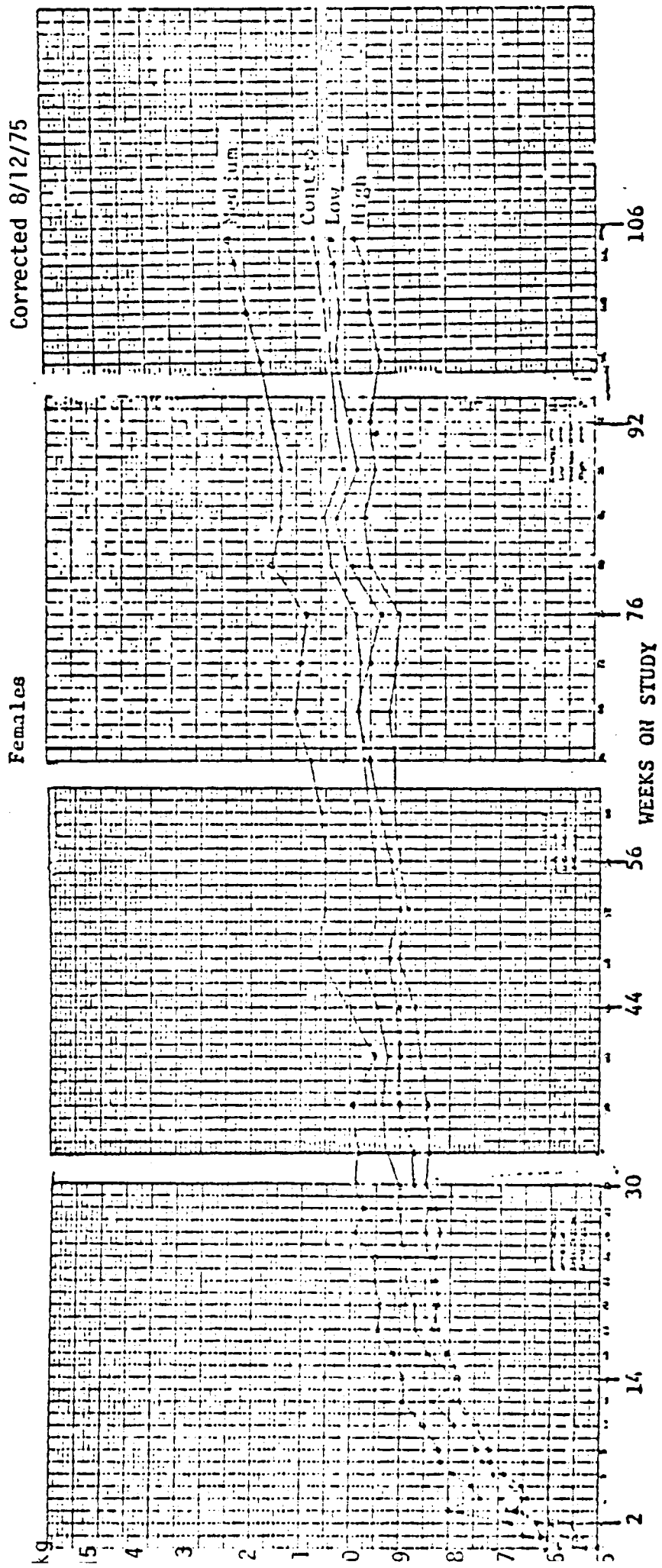


- Control
- △ Low
- Medium
- ◇ High

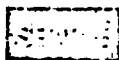
Figure 2

SC-18862: 106 WEEK ORAL TOXICITY STUDY IN THE DOG

Mean Body Weight (kg)



APPENDIX III-19



Searle Laboratories

Division of G. D. Searle & Co.
Box 5110
Chicago, Illinois 60680
Telephone (312) 982-7000

April 24, 1978

Dr. Robert Stowell
University of California, Davis
Department of Pathology
School of Medicine
Davis, California 95616

Dear Dr. Stowell:

Reference your letter of March 31, 1978.

1. Study E-90 was submitted October 28, 1975.

2. Supervisors during the period March, 1970, to April, 1972:

Clinical Chemistry	Dr. Thomas Hutsell, Ph.D.
Hematology	Ms. Denise Perkins
Animal Quarters	Dr. T. H. Harris, D.V.M.

3. Personnel involved in blood collection (March, 1970, - April, 1972):

Tony Martinez	Res. Asst., Supervisor	B.A. in Biology, with Searle since 1960.
Jan (Bial) Prah	Technician	Medical technician; with Searle since 1965.
David Kie	Technician	B.A. in Biology; with Searle since April, 1968.
Bart Tangonan	Technician	College degree (Philippines); with Searle since November, 1968.
Philip Muellner	Technician	High school degree; with Searle since July, 1970.
K. S. Rao	Supervisor; not involved in collecting blood samples	Ph.D.; with Searle since June, 1971.

App. III-19
page 2

Dr. Robert Stowell
April 24, 1978
Page Two

4. Time of day of collection of blood samples:

Approximately 8:00a.m., prior to feeding at 9:00a.m.

5. Fasting:

Animals were without food from 4:00p.m. to 9:00a.m.

6. Steps involved in processing samples:

Hematology Lab - blood samples were collected in vacutainers with Na₂EDTA for hemogram analyses. For coagulogram, vacutainers with citrate were used. Vacutainers filled with blood were placed in a "rotator" (tube inverting equipment) during the blood collection. These samples were delivered to the Hematology Lab immediately after blood collection.

Clinical Chemistry Lab (Bioanalytical) - blood samples were collected in heparinized vacutainers, centrifuged, plasma was separated and immediately submitted to Bioanalytical.

7. How and for how long were samples stored before assay?

Hematology samples were not stored.

8. Were the dogs housed in metabolism cages?

No. Urine samples were collected in the back of the stainless steel dog cages as run-off.

9. Time of day of urine sample collection:

Approximately 8:00a.m., before blood collection.

App. III-19
page 3

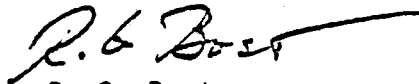
Dr. Robert Stowell
April 24, 1978
Page Three

10. How long were urine samples stored before assay?

The standard procedure did not include storage. If samples had to be stored, they were stored refrigerated for not more than three hours.

Also included are methods for the Canalco Electrophoresis of Lipoproteins in Serum, Insulin Test, Alkaline Phosphatase, and Blood Urea Nitrogen.

I trust this will be helpful. If you have additional questions; please let me know.



R. G. Bost
Director, Food Products
Regulatory Affairs

RGB:js

Enclosures

**Searle Laboratories**

Division of G. D. Searle & Co.
Box 5110
Chicago, Illinois 60680
Telephone (312) 982-7000

June 29, 1978

Dr. Joan Mayerle
Dept. of Pathology
School of Medicine
University of California
Davis, California 95616

Dear Joan:

Enclosed is some information which you had requested. I hope that you find this to be helpful. The following items are included:

- ..Information on BSP and Chloride Assays (attached).
- ..BSP determinations in E-28 were performed using a 5% BSP solution. The animals received 0.2 cc/kg to provide a dose of 10 mg/kg.
- ..In E-19, Wilcoxon's rank sum test was used in all assays.
- ..For E-28, a two-tailed "t" test was used to determine statistical significance.

Please let me know if additional information is required.

Sincerely,

A handwritten signature in cursive script, reading "R. G. Bost", followed by a horizontal line.

Robert G. Bost

RGB:ct
Encs.

INFORMATION ON BSP AND CHLORIDE ASSAYS
(1970 - 1972)

There are no implementation dates for the DSA methods for BSP and Cl listed in our files. Methods most probably run during the time period 1970-1972 are listed below.

Parameter	Description of Method	Data Format
1. BSP	a) Manual - Colorimetric. Reference: Seligson, D., Clinical Chemistry 3:638 (1957)	a) Spiral notebook
	b) Beckman DSA - colorimetric	b) DSA sheets; possible spiral notebooks
2. Chloride	a) Manual - AgNO ₃ titration	a) Spiral notebooks
	b) Manual - colorimetric Reference: Schoenfeld, R.G., and C. J. Lewellen. A Colorimetric Method for Determination of Serum Chloride. Clin. Chem. <u>10</u> , No. 6, p. 533 (1964)	b) Spiral notebooks
	c) Beckman DSA - Colorimetric Reference: Schoenfeld (as in b)	c) DSA sheets; possible spiral notebooks

APPENDIX III-21

Experiment code numbers and tattoo numbers of dogs, date whelped,
age at start of experiment, and tattoo numbers of dam and sire.

(2 pages)

SC-17762 Chronic Dog Study
DT-755573

COPY

	Tattoo No.	Date Whelped	Age at Start of Test	Dam	Sire
A 21 LF	BT9	10-21-69	6 mos	HIO6	RANDALL
B 22 CF	CJT9	10-26-69	6 mos	ECJ8	FXM9
C 23 CF	CBT9	10-28-69	6 mos	EBN7	HY15
D 24 CF	COT9	10-28-69	6 mos	MKH4	CII6
E 25 CF	CPT9	10-28-69	6 mos	MKH4	CII6
A 26 LF	DCT9	10-29-69	6 mos	FLO6	ACQ4
B 27 LF	BST9	10-21-69	6 1/2 mos	HIO6	RANDALL
C 28 LF	BKT9	10-21-69	6 1/2 mos	DHR6	ANIL6
D 29 LF	BUT9	10-21-69	6 1/2 mos	HIO6	RANDALL
E 30 LF	BCT9	10-18-69	6 1/2 mos	DWP6	ACQ4
A 31 MF	BDT9	10-18-69	6 1/2 mos	DWP6	ACQ4
B 32 MF	BBT9	10-18-69	6 1/2 mos	DWP6	ACQ4
C 33 MF	CWT9	10-28-69	6 mos	RANDALL-2	RANDALL-1
D 34 MF	CUT9	10-28-69	6 mos	RANDALL-2	RANDALL-1
E 35 MF	AVT9	10-17-69	6 1/2 mos	DYLR	CII6
A 36 HF	AWT9	10-17-69	6 1/2 mos	DYLR	CII6
B 37 HF	BHT9	10-21-69	6 1/2 mos	DHR6	ANIL
C 38 HF	BGT9	10-21-69	6 1/2 mos	DHR6	ANIL6
D 39 HF	BJT9	10-21-69	6 1/2 mos	DHR6	ANIL
E 40 HF	ALV9	11-2-69	6 mos	AJ-FX4-	J

APPENDIX III-22

Batch numbers of aspartame used, number of days used, percent DKP.

106 Week Toxicity Study in the Dog;

P-T no. 855570

COPY

	Lot No.	Approx. No. of Dogs Used	% DKP (SDs)
15	74050	30	0.0
30	75010	56	0.0
11	75040B	25	0.0
14.3	75070A	29	0.0
10	75080B	18	0.3
10	75080A	22	0.3
15	75090B	26	0.1
14	75100B	27	0.5
~15	{75120 75090B}	25	{1.3 0.1}
~25	{75130A 75140C}	37	{0.4 0.8}
25	75150B	52	0.4
25	76030A	38	1.0
25	76040D	47	0.7
25	76060A	41	0.8
25	56082B	36	0.5
25	56147B	47	1.0
25	56117A	32	<1.0
25	56117B	42	<1.0
25	56067B	39	1.0
25	56147C		1.0

APPENDIX III-23

DISCREPANCY SUMMARY

Body Weight, Feed, and Compound Consumption - A number of errors were made in transcribing body weights from the raw data sheets to the table of individual values in the Appendix (E-28, pp 60-64). There were 54 rounding discrepancies and 6 transcription errors made in addition to the apparent transposition of weights noted in Table 3-5.

There were 1640 bits of raw data on body weights. These were processed in different ways, so that a total of 7973 bits of information were handled in evaluating the body weight data.

A memo from Rao in 1974 (Appendix III-17) notes that the graph of mean body weight for the female dogs (E-28, Fig. 2) was in error. Both versions are attached (Appendix III-18). They were replotted (Fig. 3-4), by UAREP. A number of points were off by a few tenths in the original plots.

Food and compound consumption involved a total of 88,890 data bits and 427,130 bits of data checked.

Hematology - There were 3280 bits of original data in this part of the dog study. There were 38,689 bits of data handled in the recalculation and re-evaluation processes.

UAREP recorded a total of 54 rounding discrepancies and 15 transcription errors. The apparent lack of correction for Coulter Coincidence, discussed earlier, resulted in the total of 276 discrepancies.

The means listed in the data summary for hematology (E-28, tables 3-5) contained 13 rounding discrepancies, 58 computational discrepancies which were not related to the Coulter correction problem and 57 computational discrepancies which resulted from the lack of Coulter correction.

Clinical Chemistry - There were 4160 bits of data examined. A major portion of the computation errors occurred in the A/G calculations. Method problems occurred in the chloride determinations at week 106 where all of the values reported were too high, but raw data for 12 of them were normal. Chlorides for week 78 were also too high, but raw data was not available to check for that interval.

Problems with significant figures have been discussed under the appropriate assay headings.

Percentages represent the total for each discrepancy divided by the total data bits examined.

Transcription	10	0.2%
Computation	44	1.0%
Rounding	38	0.9%
Sample QNS	15	0.4%
Method Problem (chloride)	28	0.7%
Lost Specimen	12	0.3%

App III-23
page 3

Discrepancies in individual bits of data were also categorized according to the differences between Searle and UAREP calculations:

0-1.0%

1.1 - 5.0%

>5.1%

In the chemistry data, there were 41 discrepancies less than 1%, 33 between 1.1 - 5.0%, and 32 greater than 5.1%.

In the hematology data there were 1 less than 1%, 171 between 1.1 - 5.0%, and 169 greater than 5.1%.

App III-23
page 4

SUMMARY OF DATA BITS AND MINOR DISCREPANCIES

	initial data bits	total data checks	Discrepancies				
			trans- cription	compu- tation	rounding	method problem	other problems
Terminal body weights and autopsy checks	360	4,843					
Food & Compound Consumption	38,390	427,130					
Two-year body weight record	1,640	7,273	6(0.2%)		54(3.3%)		
Hematology	3,280	38,689	15(0.4%)	58(1.3%)	67(2.0%)		333 ^B
Clinical Chemistry	4,160	42,928	10(0.2%)	44(1.0%)	38(0.9%)	28 ^C (0.7%)	15 ^A (0.4%) 12 ^D (0.3%)
Urinalysis	3,920	7,840					
Necropsy	40	2,643					
Histopathology Tissues	1,171						

$$\% \text{discrepancy} = \frac{N}{\text{total bits}} \times 100$$

^AQNS

^BCoulter coincidence correction

^CChloride method weeks 78, 106

^DLost Specimens

Appendix Table 6 (cont.)

SC-18862: 106 WEEK ORAL TOXICITY STUDY IN THE DOG

Urinalysis
(Individual Values)

Treatment and Dog No.	RBC /HPF					
	-2	Weeks of Treatment				
		14	26	52	78	106
Control						
A1CM	0-2	20-30	0	0	0	0
B2CM	2-4	9-10	0	0	0	0
C3CM	0-1	0	0	0	0	0
D4CM	0	400+	0	0	0	0
E5CM	8-10	400+	0	0	0	0
A21CF	6-8	0-2	0	0-3	0	0
B22CF	1-3	2-3	0	0	0	0
C23CF	8-10	400+	0	0	0	2-3
D24CF	8-10	1000+	0	0	0	0
E25CF	0	500-600	0	0	0	0
Low Dose						
A6LM	4-6	400+	0	0	0	0
B7LM	0-1	9-10	0	0	0	0
C8LM	1-3	400+	0	0	0	0
D9LM	1-3	400+	0	0	0	0
E10LM	4-6	200-300	2-4	0	0	0
A26LF	2-4	0-1	0	0	0	0
B27LF	0-1	0-2	0	0	0	0
C28LF	3-5	40-60	0	0	0	0
D29LF	3-5	400+	0	0	0	0
E30LF	0	1000+	0	0	0	0
Medium Dose						
A11MM	3-5	4-5	0	0	0	2-3
B12MM	1-3	0	0	0-1	0	2-3
C13MM	0-1	200+	0	0	0	0
D14MM	0-1	200-300	0	0-1	0	0
E15MM	4-6	1000+	0	0	0	0
A31MF	0-1	10-15	0	0	0	0
B32MF	2-4	4-5	0	4-6	0	0
C33MF	2-4	400+	0	0	0	0
D34MF	0-1	200-300	0	3-4	0	0
E35MF	1-3	1000+	2-3	0	0	0
High Dose						
A16HM	4-6	0	0	0	0	0
B17HM	0	0	0	0	0	0
C18HM	15-20	100-200	0	0	0	0
D19HM	0-1	200-300	0	0	0	0
E20HM	8-10	1000+	0	0	0	0
A36HF	0-1	0	0	0-4	0	0
B37HF	3-5	10-15	0	0	0	0
C38HF	3-5	400+	0	0	0	0
D39HF	12-15	1000+	0	0	0	0
E40HF	10-12	100-200	0	0	0	0

APPENDIX III-25

Appendix Table 6 (cont.)

SC-18862: 106 WEEK ORAL TOXICITY STUDY IN THE DOG

Urinalysis

(Individual Values)

Treatment and Dog No.	WBC /HPF					
	Weeks of Treatment					
	-2	14	26	52	78	106
Control						
A1CM	5-7	0	3-4	0-2	0	0
B2CM	6-8	0	8-10	2-3	2-3	0
C3CM	1-3	0	10-12	0	0	0-5
D4CM	0-1	0	0	0	0	0
E5CM	30-35	0	10-20	0	0-2	0
A21CF	50-60	0	40-50	0	0	0
B22CF	15-20	0-1	6-8	4-5	0	0
C23CF	35-40	0-1	0	0	0	0
D24CF	15-20	15-20	10-20	0	0	0
E25CF	4-6	4-5	0	0	0	0
Low Dose						
A6LM	25-30	4-5	5-6	0	0	0
B7LM	0	1-2	2-3	0	0	0
C8LM	50-60	2-3	2-3	0	2-5	0
D9LM	8-10	5-6	0-4	1-2	0	0
E10LM	1-3	9-10	7-8	0-2	0	0
A26LF	14-16	0-1	2-3	0	0	0
B27LF	4-6	0-2	70-80	0	0	0
C28LF	4-6	5-10	2-3	0	0	0
D29LF	12-15	0-1	0	0	0	0
E30LF	2-4	4-5	1-2	0	0	0
Medium Dose						
A11MM	40-45	0-1	0-1	0	5-10	5-10
B12MM	4-6	0	5-6	0	0	10-20
C13MM	0-2	5-6	2-3	0	0	5-10
D14MM	0-1	0-1	6-7	2-3	0	0
E15MM	1-3	4-5	2-3	0	0	0
A31MF	4-6	4-5	2-3	0	0	0
B32MF	70-80	5-7	10-12	5-10	0	0
C33MF	4-6	4-5	2-3	0	0	0
D34MF	2-4	5-6	3-4	1-2	0	0
E35MF	20-25	0	10-12	0-1	0	0
High Dose						
A16HM	8-10	0	16-20	2-3	0	0
B17HM	0-1	0	10-12	0	0	0
C18HM	3-5	4-5	0	0	0	0
D19HM	1-3	0-1	0-4	0	0	0
E20HM	12-15	10-15	0	4-6	2-3	0
A36HF	10-12	0	2-3	0	0	0
B37HF	70-80	0-1	30-40	0	0	0
C38HF	12-14	2-3	5-6	0	0	0
D39HF	25-30	0	8-10	0	0	0
E40HF	20-25	9-10	0	0	0	0

APPENDIX III-26

NECROPSY FORM AND TISSUES FIXED AND SECTIONED

Part one of the form lists the tissues (indicated by a check) which were submitted for microscopic examination. All tissues were fixed in formalin except testis (in Bouin) and pituitary and bone marrow (in Zenker's).

(2 pages)

AUTOPSY SUBMISSION SHEET

STUDY SC-18862: 196w. Chr. Dog CAGE NO. B2017 MASTER NO. _____ PATH. NO. 91990

Sex ♂ Kc (Kd) Sr _____ PROJECT NO. 855570 No. days on study 742

Date Autopsied 4/11/72 Time: Death to tissue fix. 5-10 Hr. (min) Euthanasia Sodium Pentobarbital

Fixative: nb Formalin all else Zenker's (acetic) Pituitary Bouin Other _____

DIGESTIVE gm*			CIRCULATORY gm*			HEMOPOIETIC		
✓	Mouth		✓	Heart	122 8	✓	Blood	<u>Plasma</u>
✓	salivary gl. (mand)			Arteries (Aorta)		✓	Marrow (femur, ilium) sect/	
✓	Stomach			Veins		+	Spleen	
✓	Intestine, sm.			Lymphatics		✓	Lymph nodes (axilla, meser)	
✓	Intestine, lg.							
X	Liver*	399 8		NERVOUS			ENDOCRINE gm*	
✓	Pancreas		✓	Brain		✓	Thymus	
✓	Peritoneum		✓	Spinal cord		✓	Pituitary	670
✓	Gallbladder		✓	Peripheral n. (sciatic)		✓	Thyroid	1 34
	REPRODUCTIVE			Meninges			Parathyroid	
✓	Gonads	25 85		RESPIRATORY		✓	Adrenal	1 44
	Genitalia, ext.			Trachea			TISSUE	
	Genitalia, int.		X	Lungs			Skin	
	Uterus			Pleura		✓	Skeletal muscle (diaphragm)	
	Vagina					✓	Bone (femur, rib, skull)	
	Accessory glands		✓	URINARY			SENSE ORGANS	
	Sem. Vesicle		✓	Kidneys	85 16	✓	Eyes Both	
✓	Prostate, v.	21 12		Bladder			Ears	
✓	Mammary gl.	4-5R+L						

✓ = Specimen submitted for micro. * Recorded weights are for fresh ~~fixed~~ organs.

X = Specimen submitted for micro & gross abnormality described.

PERTINENT CLINICAL HISTORY: negative see over

EXTERNAL EXAMINATION:

Body weight (kg) day 0 5.95 At autopsy 16.4 (↑175.2%)

Nutritional status: emaciated poor good obese None

Skin Hair (-) Both eyes (-) Palpable SC masses

Hair (-) Mammary glands (-)

Teeth (-) Ext. Genitalia (-)

GROSS FINDINGS & DIAGNOSIS:

Lung - Single 1 mm. dia. firm grey nodule on R. diaphragmatic and L. apical lobe.

Spleen - Multiple (3) 2 x 5 mm. tan ovoid slightly raised capsular plaques on cephalad margin of anterior pole.

Liver - Single 3mm. dia. discrete red focus of capsular discoloration on ventral surface of L. lateral lobe.

All other organs examined were grossly normal and unremarkable.

RDH

