

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

Volume No. 3

Chapter VII:	110 Week Toxicity Study in the Mouse
Chapter VIII:	A Supplemental Study of Dog Brains from a 106 Week Oral Toxicity Study
Chapter IX:	A Supplemental Study of Rat Brains from Two Tumorigenicity Studies
Chapter X:	Toxicological Evaluation in the Neonatal Rat
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Chapter XII:	A Sweetening Agent: Endocrine Studies
Chapter XIII:	Experiments in Mated and Pregnant Rhesus Monkeys
Chapter XIV:	An Evaluation of Embryotoxic and Teratogenic Potential in the Rabbit

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**UNIVERSITIES ASSOCIATED
FOR RESEARCH AND EDUCATION IN PATHOLOGY, INC.**

AUTHENTICATION REVIEW OF SELECTED MATERIALS
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.

November 18, 1978

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CHAPTER VII

E-76: 100-WEEK ORAL TOXICITY STUDY OF DIKETOPIPERAZINE IN THE MOUSE

INTRODUCTION

Searle Laboratories contracted with Hazleton Laboratories America, Inc., to conduct an oral toxicity study (P-T No. 985H71; Hazleton No. 700-260), to evaluate and characterize the safety during chronic administration to weanling mice by long term dietary feeding of the in vitro conversion product of aspartame, diketopiperazine (DKP; SC-19192). CD-1 (HaM/ICR Swiss) albino mice (Charles River Laboratories; Wilmington, Mass.) were used as the test animals. The oral toxicity trial commenced December 10, 1971 and terminated when sacrifice of mice began on January 15, 1974. The animals were divided into control, low, medium, and high dose groups. Data were to be collected on clinical observations, body weight changes, food consumption, compound consumption, survival, ophthalmoscopic observations, hematologic and clinical laboratory studies, tumor incidence, and necropsy studies. The methods will be detailed under the presentation of Results and Discussion.

General Experiment Design

Seventy-two males and 72 females were maintained in control groups and 36 males and 36 females in each of the three treatment groups, comprising a total of 360 animals. The diketopiperazine was manufactured by Searle Laboratories and supplied to Hazleton Laboratories in two lots, the first being received by Hazleton on November 23, 1971, and

the second on June 6, 1972. The first lot was fed from week 0 through week 51 and the second was fed from week 52 to 110. Each lot was considered 100% pure for the purposes of dosage calculation.

Personnel

The Searle protocol design committee for E-76 included:

Dr. Dutt.....Biostatistician
Dr. F. Saunders.....Biological Research Advisor
Dr. Ranney.....Drug Metabolism Representative
Dr. Polk.....Clinical Representative
Dr. McConnell.....Path-Tox Department Advisor

Dr. Rao, Searle Laboratories, although not a member of the protocol design committee, acted as the liaison between Searle Laboratories and Hazleton Laboratories for E-76. Personnel from Hazleton Laboratories, Inc. involved in E-76 included:

Dr. Reno.....Project Manager
Dr. Kwaipen.....Pathologist
S. Spruill.....Report Writer
M. Elliott.....Supervisor
Dr. Kundzins.....Ophthalmoscopic Examinations

The histopathologic diagnoses were made by Experimental Pathology Laboratories.

Experimental Animals and Conditions

The Charles River ICR Swiss mice were approximately four weeks of age at the start of the experiment. Animals were housed individually and provided with a complete powdered diet and free access to chlorinated water.

According to the animal receipt slip, mice were received on December 12, 1971, eight days prior to the start of the experiment, which allowed a seven day period of isolation before initiation of the experiment. Assignments to various groups were made using a stratified randomization by weight procedure.

General Comments on Protocol and Amendments

The specifications of the Searle protocol dated October 12, 1971, after being amended five times, are shown in Appendix VII-1 and discussed in VII-2. The statistical and computational procedures are mentioned in the protocol. In general, they called for computation of group means, standard error, and appropriate analysis of intergroup variance at each time interval.

Interim and Final Reports: The protocol requested Hazleton to provide the Director of the Path-Tox Department, Dr. McConnell (of Searle Laboratories), with a brief quarterly report on the first of January, April, July, and October. UAREP's request to Searle and Hazleton to see copies of these reports were answered by Hazleton with the statement that they had been provided to Searle.

Protocol Amendments: The initial protocol was amended eight times, with five being in the form of written amendments to the initial protocol and the remaining three communicated by some other means. The three changes made by Searle that were not supported by written amendments were: (1) deletion of all clinical chemistries, (2) change in the frequency of prothrombin time determinations, and (3) deletion of the pituitary weights from the experiment. On at least two occasions, Dr. Reno of Hazleton Laboratories issued memos prior to receiving the written amendment from Searle. Based on a review of dates of amendments and memos, these memos presumably were based on telephone conversations with Searle personnel. A more detailed discussion of the amendments of protocols and the changes in determinations for hematology and clinical chemistry is given in Appendix VII-2.

As in E-75, the experiment was extended beyond the initial 80 weeks planned. Although E-76 is entitled as a 110-week study, in various places reference is made to 110, 109, and 108 weeks and the terminal sacrifice of animals began at 108 weeks. Searle protocol amendment No. 5 requested that the title be changed to a 109 week study. (Appendix I, item D.)

RESULTS AND DISCUSSION

Clinical Observation

Morbidity-mortality observations were made daily. Motor and behavior activity were noted periodically. General physical external observations, including digital palpation for protruding tumor masses and noting body orifices and excrement, were carried out at week 5, week 10, and every 10 weeks thereafter. No special neurological observations were made. Clinical observations noted at the time of weighing were recorded weekly for weeks 0-4, every two weeks for weeks 5-12, and every 4 weeks for weeks 13 to 108. Prior information contained on computer output tapes was available. This information was numerically coded and reported with body weights and food consumption on the INTEC system (a computerized system for storage and retrieval of animal data utilized by Hazleton).

The clinical observations reflected that seven animals out of 360 were reported as escaping during the two year experimental period and two accidental deaths were reported. All other animals were accounted for, either at the time of necropsy of moribund or dead animals, or at the termination of the study. Two animals were reported as being replaced at week 4 because of cataracts, but they were not noted under the clinical observations for that interval. Replacement animals were given the identification numbers of the animals being replaced.

In a number of cases, clinical observations recorded on the INTEC computer printout were inconstant in reporting nodules on examination. Appendix VII-3 contains a complete listing of these variations. The individual performing the examination had knowledge of observations noted during previous examinations. Of the 360 animals and 34 intervals at which clinical observations were performed, only 15 animals were noted to have variations between one or more intervals.

A summary of palpable nodules, tissue masses, and/or swollen areas of body or legs contained in Entry Book E-76 (p 15) is presented along with UAREP's summary in Table 7-1. Observations from the eight animal groups reported were found to be essentially the same as those reported in the Entry Book. UAREP reported a total of 29 animals and HLA recorded 28. Appendix VII-3 contains a complete listing of all palpable nodules, tissue masses and/or swollen areas on an individual animal basis. The variations in intervals at which palpable lesions were observed and subsequently were not observed, are tabulated in Appendix VII-4. As discussed in Chapter IV, UAREP does not consider the apparent disappearance of a swelling as necessarily significant. We cannot differentiate between the disappearance of a physiologic process causing swelling and other problems of such observations.

On three occasions, animals were not reported as dying during an interval but recorded as necropsied during the same time period. One animal was reported missing at week 40, and another report showed it was necropsied the same day. Such minor discrepancies could be explained by the animal having been removed from its cage for purposes of necropsy early in the day on which it was necropsied without coordination of records.

Table 7-1
Clinical Observations of Palpable Nodules,
Tissue Masses, and Swollen Areas on Body or Legs

<u>Group</u>	Males		Females	
	<u>UAREP</u>	<u>HLA</u>	<u>UAREP</u>	<u>HLA</u>
1	9/72	9/72	0/72	0/72
2	4/36	4/36	1/36	1/36
3	6/36	6/36	2/36	2/36
4	4/35	3/35	3/37	3/37

Body Weight Gains

Body weight gains were determined on an individual animal basis weekly for weeks 0 through 4, every 2 weeks for weeks 4 through 12 and every 4 weeks for weeks 13 through 108. The computerized output was the earliest data source available for body weight gains. A description of the method used for weighing animals was not contained in the methods section of Entry Book E-76.

The computer output data for body weight gain was verified at various intervals by UAREP and found to be consistent with the information given in E-76, Table 1, Appendix pages 1-4. Graphs were prepared of body weight gains at various intervals to determine if there were apparent trends relating to the compound administered. No significant differences ($P < 0.05$) were found for body weight gains for the interval 0-24, 0-52, 0-108, and 52 to 108 weeks for the females or males with the exception of Group 3 males for the interval 0 through 24 which was significantly lower than the controls (Table 7-2). ANOVA, LSD and Newman-Keuls tests were performed.

Food Consumption

The basal diet utilized in E-76 was Wayne Laboratory Chow to which DKP was added for the various treatment groups. Individual food consumption for each mouse was recorded weekly on the INTEC Computer System for weeks 0 through 4, every other week for weeks 5 through 12 and every 4th week for weeks 13 to 108. The Entry Book did not indicate at what frequency fresh feed was given and feeders were cleaned. Procedures for

Table 7-2

Mean (\bar{x}) Body Weights, Standard Deviations (SD) and Percentage
Changes in Mice Surviving at Termination of Intervals Compared

<u>Males</u>		<u>Weight (in grams) at Week</u>				<u>% Change Over Indicated Interval</u>			
Group		0	24	52	108	0-24w	0-52w	0-108w	52-108w
1	\bar{x} 16.3	38.5	38.2	35.8	136.5	134.4	119.6	-6.3	
	SD ± 2.6	± 3.0	± 2.8	± 3.3					
2	\bar{x} 16.3	37.1	38.4	38.2	127.6	135.6	134.4	-0.5	
	SD ± 2.6	± 2.6	± 2.8	± 2.3					
3	\bar{x} 16.3	36.0	37.0	35.0	120.9	127.0	114.7	-5.4	
	SD ± 2.6	± 2.5	± 2.7	± 1.9					
4	\bar{x} 16.3	37.4	38.2	37.4	129.4	134.4	129.4	-2.1	
	SD ± 2.6	± 2.8	± 2.5	± 3.9					
<u>Females</u>									
1	\bar{x} 15.2	30.9	32.5	34.2	103.3	113.8	125.0	5.2	
	SD ± 1.7	± 2.2	± 3.3	± 4.0					
2	\bar{x} 15.2	31.2	32.8	33.2	105.3	115.8	118.4	1.2	
	SD ± 1.7	± 2.3	± 2.8	± 3.3					
3	\bar{x} 15.2	29.9	32.3	34.8	96.7	112.5	128.9	7.7	
	SD ± 1.8	± 1.9	± 1.9	± 1.9					
4	\bar{x} 15.2	30.7	33.5	32.7	102.0	120.4	115.1	-2.4	
	SD ± 1.8	± 2.1	± 2.4	± 3.2					

recording food consumption, body weight, and clinical observations were not outlined in Entry Book E-76. When Dr. Stowell of UAREP visited HLA and asked what type of procedure was used in recording food consumption, Dr. Reno said they had utilized an INTEC computerized system for recording food consumption as well as body weight and clinical observations for the duration of experiment E-76.

UAREP recalculated mean food consumption for each of the intervals for which INTEC computer printouts were available and found no discrepancies between the UAREP calculations and the values reported in the Entry Book for E-76. Food consumption records for the 32nd week interval were discarded due to the fact that there were a high number of negative food consumptions recorded in that interval. These unusual values had been detected by Hazleton and the food consumption redetermined and recorded at week 33 in Appendix Summary Table I, page 1, Entry Book E-76. No explanation of the relatively low and often negative food consumptions recorded during week 32 was made in Entry Book E-76.

UAREP recomputed the food consumptions for the interval 0 to 24, 0 to 52, and 0 to 108 and analyzed the food consumption for these intervals statistically. The results of these computations are contained in Table 7-3. Total mean food consumption of males in all treatment groups was found to be significantly lower than controls in the 0-24 and 0-52 week summaries (Table 7-3). This agrees with the observations made in the Entry Book. There was no significant difference between the treatment groups and controls in either males or females when the entire study (weeks 0-108) was considered. Total food consumption in Group 4 females was significantly higher than that of Groups 2 and 3 for both weeks 0 to 24 and 0 to 52 week summaries.

Table 7-3
Mean Food Consumption \pm Standard Deviation In Grams
For Intervals Indicated

Interval in Weeks \rightarrow	<u>Male Group</u>			<u>Female Group</u>		
	0-24	0-52	0-108	0-24	0-52	0-108
Group 1 (control)	463 \pm 32	740 \pm 46	1211 \pm 90	468 \pm 45	757 \pm 58	1268 \pm 99
Group 2	437 \pm 29	705 \pm 53	1211 \pm 83	461 \pm 44	747 \pm 72	1119 \pm 31
Group 3	437 \pm 35	705 \pm 52	1168 \pm 81	451 \pm 34	739 \pm 50	1243 \pm 70
Group 4	434 \pm 33	708 \pm 56	1231 \pm 68	485 \pm 50	780 \pm 62	1296 \pm 92
ANOVA	0.00	0.00	0.33	0.01	0.04	0.10
Statistical Interaction ($p < 0.05$)	(2 < 1) ^A	(2 < 1) ^A	Not Done	(4 > 2) ^B	(4 > 2) ^B	Not Done
	(3 < 1) ^A	(3 < 1) ^A		(4 > 3) ^A	(4 > 3) ^A	
	(4 < 1) ^A	(4 < 1) ^A				

Superscript A = significant by both LSD and Q ($p < 0.05$).

Superscript B = significant by LSD but not Q ($p < 0.05$).

Compound Consumption

Diketopiperazine was added to the basal diet (Wayne Laboratory Chow), and mixed in a twin shell Patterson-Kelley blender. Experimental treatment groups consisted of a control group with no added DKP, a low (.25 g/kg of body weight per day), a medium (0.5 g/kg body weight/day) and a high (1.0 g/kg body weight/day) group. On the basis of prior mean food consumption, compound concentration was adjusted weekly for weeks 0 through 4 and every 2 weeks for weeks 5 through 12 and every 4 weeks for week 13 through 108. UAREP is unaware of any quality control measures on the various batches of diet or on the compound itself. Conditions for storing the compound and mixing diets were not described in Entry Book E-76. Hazleton representatives stated that such diets were stored in containers with lids in refrigerated areas. Dr. Reno of Hazleton stated that controls had been run for other clients on the adequacy of mixing under comparable conditions and that they were considered satisfactory. Such data was regarded as privileged information to other clients and hence not available to UAREP. Since different compounds may have widely varying mixing characteristics, and inadequate mixing could represent a serious flaw in experiment design. UAREP offered its services to Searle to carry out independent tests of mixing of aspartame and of DKP, with the diet, using comparable equipment. Such tests were not run.

Appendix Table 1A (p 5) of Entry Book E-76 contains the mean daily compound consumption for each interval. Based on the information available for body weight and food consumption, UAREP recalculated compound consumption for the various intervals. These results, along with the compound consumption reported in Entry Book E-76 are shown in Appendix

VII-5. UAREP calculations agreed within 1% of those reported by Hazleton in Entry Book 76 in terms of mean values for all time intervals. Ten percent of the 162 comparisons of UAREP and HLA compound consumption computations for individual intervals differed by 5 to 8.5%.

Over the two year experimental period, nine individuals or combinations of individuals were involved in preparing the diet-compound required for experiment E-76. Ten individuals were responsible for computing the amount of compound required for the various treatment groups during the study. There were no subsamples taken of the diet after mixing for analysis to determine the concentration of DKP in any of the various test diets mixed. Throughout the experiment, the purity of the DKP was assumed to be 100%. UAREP was provided with no data on actual analytical determinations of the compound provided from Searle Laboratories to Hazleton for the experiment. The purity or dietary levels of DKP were not monitored during the experiment and compound consumption is based on calculated values, not actual values determined by analysis of diets.

Food consumption is calculated on weight change of containers; i.e., food removed from the container and eaten or scattered, less any urinary or fecal additions to the container. Hazleton claims these problems are minimized with their improved feeders; UAREP realizes that such problems can exist with some mice scattering some significant amounts of unconsumed food. This is probably not a major problem since such variation should have been averaged similarly between control and experimental groups. Hazleton has said animals wasting excessive amounts of food were recorded and not included in computing mean food

consumption. UAREP found no instances of this being recorded in HLA data in E-34 or E-76, which were the earliest and latest of the four experiments reviewed. UAREP has no reason to feel that high dose mice would scatter sweeter food more than controls.

Survival

Survival data for each of the treatment groups as well as the controls are given in Entry Book E-76, Table 1, Appendix pages 1-4, along with the food consumption and body weights. Survival was reported as a fraction with the number of animals surviving as the numerator, and the number of animals at risk as the denominator. Values recorded in the denominators of the various groups were not adjusted by Hazleton for mis-sexed animals or for the animals that escaped or were accidentally killed during the experiment. Appendix VII-6 gives the result of the UAREP analysis of survival.

Based on the survival data computed by UAREP, a graphic depiction of survival rates in various groups was prepared as mortality vs time and is shown in Figure 7-1A and 7-1B. The figures illustrate the fact that there is no apparent or consistent divergence in the mortality rate between groups.

Mean survival time in days as computed by UAREP and by HLA as contained in the Entry Book for E-76 is shown in Table 7-4B. There are some apparent differences in the HLA and UAREP data. The UAREP validation used death dates for each of the animals as reported on necropsy sheets in computing the means for survival times for the various groups. There was no statistically significant difference

Table 7-4

Comparison of HLA and UAREP Data for Survival for Groups of Mice in E-76

A. Mean Percentage Survival ± Standard Error at 108 Weeks			B. Mean Survival Time in Days	
Group	HLA Percent	UAREP Percent	HLA	UAREP
1M	34.8±5.7	32.9±6.3	597	613
2M	30.6±7.7	26.8±8.7	616	644
3M	38.9±8.2	36.5±8.7	628	638
4M	20.0±6.8	16.4±7.3	600	591
1F	33.9±5.7	31.4±6.2	581	593
2F	25.0±7.3	20.0±7.6	620	620
3F	16.7±6.3	11.9±6.1	621	632
4F	25.0±7.3	23.2±7.8	600	600

C. Percent Survival at Selected Intervals

Group	Weeks	Group 1		Group 2		Group 3		Group 4	
		HLA	UAREP	HLA	UAREP	HLA	UAREP	HLA*UAREP	
Male	13	100	100	100	100	97	97	97	100
	26	96	96	100	100	94	94	97	100
	52	85	89	86	92	89	89	83	89
	78	68	71	75	80	81	81	67	73
	91	56	58	58	62	75	75	44	46
	108	35	33	31	27	39	36	20	16
Female	13	97	97	97	97	100	100	100	100
	26	94	96	94	94	100	100	94	95
	52	82	86	92	92	94	94	89	89
	78	68	71	81	83	78	78	67	63
	91	56	58	58	61	50	50	56	57
	108	34	31	25	20	17	12	25	23

* HLA not corrected for mis-sexed animals.

UAREP calculations based on the number of animals at risk--including accidental deaths and animals reported as escaping, up to the time they were lost to the followup.

Figure 7-1A, Cumulative Percent Mortality in Male Mice in E-76

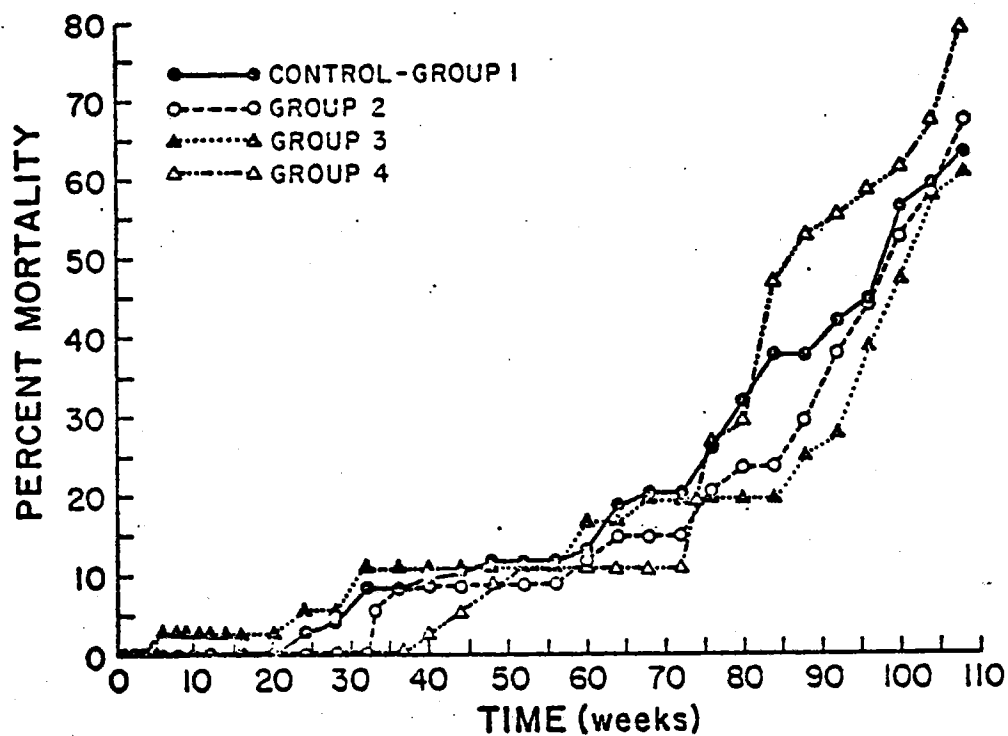
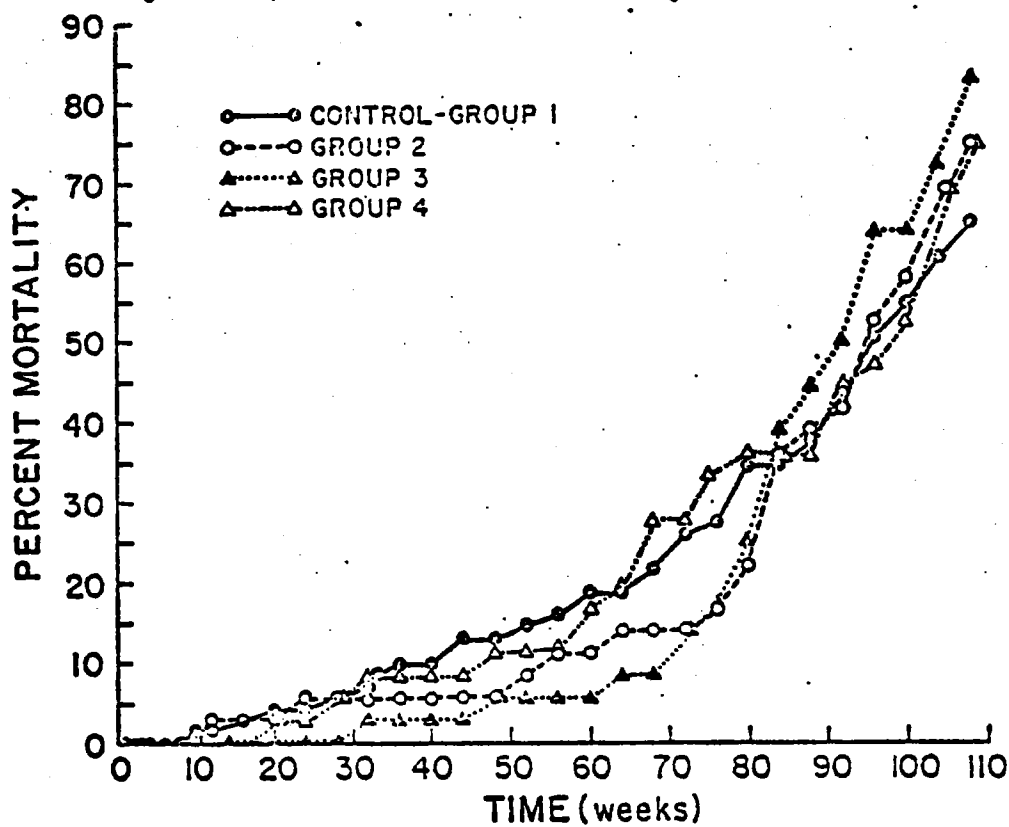


Figure 7-1B, Cumulative Percent Mortality in Female Mice in E-76



($P < 0.05$) found by ANOVA between the various treatment groups and the controls. A comparison of the HLA and UAREP mean percent survival for the various groups at 108 weeks is shown in Table 7-4A. A comparison of the percentage survival at selected intervals for HLA and UAREP is shown in Table 7-4C. The data in Table 7-4A and C are based on UAREP's life table analysis. The HLA data in Table 7-4C was taken from Figure 2A, page 22, E-76, except the 108 week figures which came from copies of the computer tapes used in their life table analysis. In general, one notes a fairly good degree of correlation at the earlier intervals, with increasing divergence occurring at the terminal interval. The reasons for this disagreement are not entirely clear, but may relate in some way to adjustment of data for animals which were missexed in Group 4 or some difference in consideration of counting animals that escaped or were lost to the study. Although HLA found a significant decrease in the survival of the mid-dose females, UAREP did not find any statistically significant difference in the survival of any of the groups of mice by life table analysis.

Clinical Laboratory Studies

Searle's initial protocol was designed to evaluate various hematologic and chemical components in blood and urine at 5, 10, 20, 40, 60 week intervals and at termination of the experiment. Determination of the clinical chemistry constituents was dependent upon the availability of microtechniques, which were not as micro as the available blood of individual mice at the time these experiments were done and hence, many of the desired tests had to be dropped. Hematology parameters were

determined at the above intervals, but clinical chemistries and urinalyses were deleted from the experiment by Hazleton Project Sheet No. 2 (Appendix VII-1, Item B) dated February 14, 1972, by which time the 5th and 10th week interval of the experiment had already passed. Entry Book E-76 did not contain data for these intervals for clinical chemistries or urinalyses, and they may have been deleted from the experiment prior to the time the memo was prepared, possibly by a verbal directive.

Additional clinical chemistries (insulin, serum ornithine carbamyl transferase, and protein electrophoresis) were added by amendments and subsequently deleted prior to sampling. Six days before the date of experiment termination, a number of clinical chemistry assays and urinalyses determinations were added for the terminal interval. Insulin was to be determined if sufficient serum was available, but since no results were reported, UAREP assumes that there was insufficient serum. A summary of hematology, urinalysis, and clinical chemistry results contained in the initial protocol and subsequent amendments, is given in the table in Appendix VII-2. Of the 137 possible determinations (parameters X intervals X groups X mice per group) included in the initial protocol or subsequent amendments, only 42 were actually done by the termination of the experiment and included in the final report.

Specimens for hematology, urinalysis, and clinical chemistry determinations were collected from individual animals, which the protocol specified should each receive subsequent postmortem workups (which was done). Unopette procedures were used for the collection of blood for hematology and clinical chemistries and sodium EDTA for specimens for

prothrombin times. The third page of Appendix VII-1, Item A lists the initial parameters and intervals for hematology, clinical chemistry, and urinalysis determinations.

The protocol specified that a simple randomization design was to be used in the experiment. Therefore, some might expect that random sampling of treatment groups would have been done for collection of blood samples, but the same six animals which were initially matched by random weight stratification among groups were used at each interval. The only time this was not the case was when an animal in a sampling group was removed from the experiment for some reason (moribund sacrifice, escape, or death from natural or accidental causes) and then the next animal in numerical order in that group was substituted for lost animal. A 0.25 ml sample of mixed arterial-venous blood was obtained from the tail by segmentation for the intervals 5 through 60 weeks. Terminal blood samples were obtained by arterial puncture, collecting 1.25 ml of blood from each mouse. The initial protocol specified that each 24 hour sample was to be collected using metabolism cages. However, this was changed and the volume of 0.15 ml of urine was collected at the termination of the experiment by evacuating the bladder at the time of necropsy. A 0.2 ml aliquot of serum from all animals alive at termination was provided by HLA to Searle Laboratories for their determination of L-phenylalanine.

To show a statistical significant difference based on the standard sample size of six animals (which in some instances of BUN was only two) the differences would have to be large.

Hematology - Hematologic determinations were performed at 5, 10, 20, 40, 60, and 108 week intervals. The same six mice were sampled throughout the experiment as long as they were available. When an animal was removed from the experiment because of escape, accidental death or sacrifice, the next animal in numerical order in that group was added to maintain the number of animals sampled at six. Statistical analysis of results by Hazleton were determined for hematocrits, hemoglobin, erythrocytes, and white blood cells. They compared treatment groups only against control groups by use of the t-test and no comparisons between treatment groups were evaluated statistically.

Based on the UAREP validation of the hematology data in Figure No. 4, pages 25-29 and Appendix Table No. 2, pages 9-53 of E-76, discrepancies were listed by UAREP in Appendix VII-8. In the 1240 hematology values reported in Entry Book E-76, 29 inconsequential rounding discrepancies and three transcriptional discrepancies were noted, two of which were due to a computational error. Hazleton reported 14 statistically significant results by use of the t-test of which 12 were confirmed by UAREP. In the other two instances the "t" value found by UAREP was slightly below the critical level for $P < 0.05$ as shown in Appendix VII-9. The results of UAREP's application of the Analysis of Variance, the Newman-Keul, and Least Significant Difference tests are reported in Appendix VII-9.

Although Hazleton did not carry out statistical analysis of the differential counts in Entry Book E-76, the results of UAREP's statistical analysis are shown in Appendix Table VII-10. In 20 comparisons,

the t-tests showed significant results at the 5% level but in only two instances, were the Analysis of Variance, Q test, LSD, and t-test all significant. This shows again that the t-test as compared with the Analysis of Variance, accompanied by the Q and LSD tests are measuring statistical significance in a somewhat different manner. Under these experimental conditions the aspartame consumption did not appear to have any consistent relationship to the hematologic parameters being measured.

The hematology data recorded in Appendix Table 2 of Entry Book E-76 (Appendix pp 6-45) were verified by UAREP. Two of the differential counts did not sum to 100.

Confidence intervals for the hematologic values were determined by UAREP based on the variance of the control groups and the minimum difference required for statistically significant difference to be noted was computed as shown in Appendix VII-11.

Clinical Chemistries - The initial protocol design included clinical chemistry parameters: BUN (Blood Urea Nitrogen), SGPT (Serum glutamic pyruvic transaminase), and alkaline phosphatase at the 5, 10, 20, 40, 60, and 80 (terminal) week intervals, which was subsequently changed to the terminal interval only. Samples for clinical chemistries were obtained from animals surviving at the 108 (110) week interval. Blood was collected by an abdominal aorta puncture at the time of sacrifice. The method used in handling samples was not described in Entry Book E-76.

BUN: In a number of instances, an insufficient quantity of blood was obtained, therefore the number of BUN determinations was inadequate at times as shown in Table 7-5. In half of the six treatment groups only

Table 7-5
Blood Urea Nitrogen Determinations

	<u>Male Mice</u>			<u>Female Mice</u>		
	No. Sampled	No.	BUN tests av. mg%	No. Sampled	No.	BUN tests av. mg%
Group 1	10	5	37.0	10	6	60.2
Group 2	10	5	35.4	7	4	45.5
Group 3	10	2	55.0	6	3	87.3
Group 4	6	2	66.5	7	2	44.0

two BUN determinations were made on from the six to 10 mice from which blood was drawn.

On page 30, the Entry Book states, "Blood urea nitrogen values for each animal including controls, were slightly to markedly elevated." Based on from two to six determinations per group (average of 3.6) as shown in Table 7-5, the significance of this statement may be questioned by some. Values in controls ranged from 26 to 106 mg%. Two of the three female groups had mean values of BUN in Table 7-5 lower than controls, whereas toxic or diseased conditions usually produce their changes in the direction of elevated BUN values.

SGPT and Alkaline Phosphatase: The SGPT activity in female Groups 2 and 3 was statistically significantly lower than their respective control groups. Five inconsequential rounding discrepancies were also noted in Appendix Table No. 3 of Entry Book E-76. They are listed in Appendix VII-13.

The Entry Book for E-76 on page 30 states, "A few control and treated mice exhibited high serum glutamic-pyruvic transaminase, or alkaline phosphatase values. There were no biologically meaningful differences between control and tested data." The normal range for glutamic pyruvic transaminase and alkaline phosphatase along with a low and high value reported in Entry Book E-76, are given in Table 7-7. Many of these values show a remarkably wide group range.

Table 7-6 summarizes the high incidence of tumors in the animals from which blood was collected at the terminal interval. Tumor incidence of the various treatment groups ranged from approximately 18% to 64% at the terminal interval. Admittedly, all mouse groups including controls, had tumors and were of comparable age. Such factors would tend to mask changes unless the groups were large--certainly larger than groups of two used for BUN. The BUN, SGPT, and alkaline phosphatase values as reported in Entry Book E-76 would be of limited value with respect to interpreting the effects of feeding diketopiperazine to mice due to the small number of sampled mice, their age, and their health at the time of sampling, as well as the high degree of variation associated with measurements of these various parameters.

Confidence intervals based on the variance observed in the control groups for BUN, SGPT, and alkaline phosphatase computed by UAREP are shown in Appendix VII-12.

Table 7-6

Tumor Incidence in Surviving Mice Based on Histopathologic
Diagnoses by UAREP

<u>Group</u>	<u>Surviving Male Mice with Tumors</u>		<u>Surviving Female Mice with Tumors</u>	
	No.	%	No.	%
1	14/22	64	9/19	47
2	2/11	18	4/7	57
3	5/14	36	3/6	50
4	3/6	50	2/9	22

Table 7-7

Range of Values Observed for Serum Glutamic Pyruvic
Transaminase and Alkaline Phosphatase

	<u>SGPT (RF)</u>	<u>Alkaline Phosphatase, K-A Units</u>
Group 1 (control)	28 - 70	6.8 - 35.6
Group 2 (0.25 g/kg/day)	23 - 45	9.4 - 39.9
Group 3 (0.50 g/kg/day)	22 - 35	6.5 - 35.6
Group 4 (1.00 g/kg/day)	23 - 59	15.2 - 21.3
Group 1 (control)	21 - 77	5.9 - 26.5
Group 2 (0.25 g/kg/day)	26 - 119	9.9 - 142.4
Group 3 (0.50 g/kg/day)	25 - 64	6.3 - 35.6
Group 4 (1.00 g/kg/day)	25 - 68	9.0 - 21.3

L-phenylalanine: The results shown in Table 7-8 indicate that there were no differences ($P < 0.05$) between the control and treatment groups for the males. The L-phenylalanine values in female treatment groups were all significantly lower ($P > 0.05$) than controls. Appendix VII-14 shows for SGPT and L-phenylalanine the five comparisons for which Hazleton found a positive t-test at the 5% significance level. UAREP confirmed all of these five tests of significance with the same test. If DKP were metabolized to L-phenylalanine, one would wonder why these values were not increased in the high treatment groups as an indication of absorption of DKP.

Table 7-8
Mean L-phenylalanine Results with Standard Deviation

	Male (mg/dl)	Female (mg/dl)
Group 1	1.78 \pm 0.27	2.32 \pm 0.28
Group 2	1.86 \pm 0.32	1.88 ^{S-} \pm 0.31
Group 3	1.62 \pm 0.14	1.65 ^{S-} \pm 0.33
Group 4	1.74 \pm 0.34	1.71 ^{S-} \pm 0.36

L-phenylalanine was only determined at the terminal interval, although the initial protocol stated that L-phenylalanine would be used as an indicator for the metabolism and absorption of diketopiperazine. Although it is well recognized that aspartame is metabolized to form L-phenylalanine, UAREP is not aware of the evidence that DKP also splits to form large amounts of L-phenylalanine in the serum.

The following statement is made on page 30 of E-76, "Serum L-phenylalanine determinations were unremarkable for male groups, but mean values for the three female treatment groups were significantly decreased; this finding is considered of little biologic significance since differences in individual values for the control and test animals were minor in degree." If L-phenylalanine was to be used as an indicator of metabolism and absorption of diketopiperazine, then the findings would seem to merit more discussion. Perhaps more significant findings would have emerged if samples for L-phenylalanine determinations had been drawn at various intervals throughout the experiment and not only at the termination of the experiment.

Urinalysis - Urine samples were collected from the bladder on an individual sample basis at the time of terminal necropsy. Parameters evaluated were pH, sugar, protein, occult blood, microscopic examination of sediment, and phenylketones. The Entry Book indicated that the results of terminal analysis were not remarkable.

Two transcriptional discrepancies (Table 7-9) were noted in the data contained in Entry Book Appendix Table No. 4, pages 52-53. There was also another problem of transcription of data from the earliest source made available to UAREP that compared to E-76, Table No. 4, page 52. Mouse 01112 had results recorded for pH, sugar, occult blood, and phenylketone. The entry n.g. was made in the category for epithelial cells and no entries were made in other categories for the microscopic examination or in the protein dipstick determination. The result for phenylketones was circled, but a line was drawn through the entire

column with the notation NSS, presumably for not sufficient sample. In the reported results for this mouse in Table 4 an "N" for No Test was entered in all categories except the phenylketones which contained an

Table 7-9

Transcriptional Discrepancies in Urinalysis in E-76, Appendix p. 52
When Compared to Earliest Data

Mouse No.	Group	Interval	Parameter	Incorrect Value	Correct Value
1288	2-F	110 week	Protein	1	2
1288	2-F	110 week	Phenylketone	2	1

entry of 1. No explanation was provided in the report as to why the value for phenylketones was reported, but not for other categories.

A summary of the adequacy of the urine samples is provided in Appendix VII-17. An average of 83% of the samples were tested for the 11 urine parameters evaluated, 14% were recorded as insufficient quantity, and 3% as no test. Considering the small amount of urine available in a mouse bladder at times, these results seemed good. UAREP agrees that under the experimental conditions applied, the values of urinalysis data do not show any significant effects of the diketopiperazine.

Overall Reliability of Laboratory Data

UAREP feels that its validation of the Searle studies has been more precise as it relates to the transcription, computing, and analysis of the data than as it relates to other important factors. Some

information on other factors could be gained by comparing and analyzing data from control animals under similar conditions. Experiment E-75 began on November 24, 1971 and E-76 on December 10, 1971. These two experiments, which started slightly more than two weeks apart, were similar in most respects except that mice in E-75 received aspartame as the test compound and in E-76 they received diketopiperazine. The control animals in the two experiments were obtained from the same source. Their housing, care, and the handling and analysis of their specimens were all very similar. Specimens were obtained for hematologic studies at intervals of 5, 20, 40, and either 104 or 110 weeks for analysis of hematocrit, hemoglobin, red blood count, and white blood count. The laboratory results for E-75 and E-76 were compared by UAREP with Analysis of Variance and when $P < 0.05$ was found, the Least Significant Difference and Newman-Keuls Q test were both applied at the $P < 0.05$. The four time intervals for the male controls and for the female controls provided eight opportunities for comparisons. All three statistical analyses were positive at the $P < 0.05$ for one of eight hematocrit studies, one of eight hemoglobins, three of eight RBCs and one of eight WBCs. One or more of the significant differences were observed at each of the four time intervals. Thus, for hematologic comparisons, six of the 32 group interactions, or 19%, were statistically significantly different whereas one would normally expect only 5%.

Clinical chemistry determinations for BUN, SGPT, alkaline phosphatase, and L-phenylalanine, were performed in E-75 and E-76 only at the terminal interval. Of the eight group interactions between control animal groups (four male and four female), only the phenylalanine

group comparison had ANOVA, Newman-Keul, and Least Significant Difference analyses all at $P < 0.05$.

Under ideal conditions (which seldom exist in experimental research), and with a sufficient sample size, one would expect 5% of such comparisons by chance alone to be statistically significant. The higher percentages observed in comparing the controls of these two experiments maintained under very similar conditions, could be interpreted by some to imply that all conditions were not perfect. The results do not permit us to assess the degree to which factors such as inherent variation in the animals, specimen handling and analysis, protocol design, or other unknown factors, were responsible for the statistically significant differences observed.

Ophthalmoscopic Examination

The initial protocol for E-76 called for ophthalmoscopic examinations to be performed at the 0, 20, 40, 60, and 80 week intervals. The results of the eye examinations were given in Appendix Table No. 5 (pp 57-60) and summarized in Figure No. 4 (p 31) of Entry Book E-76.

Hazleton Laboratories provided UAREP with the summaries of the eye examinations for the intervals 4, 21, 40, 61, and 108 weeks as prepared by Dr. Kundzins of the Hazleton Laboratories, and indicated that these summaries were all the backup materials that Hazleton had in reference to the eye examinations of the animals in E-76. It was noted when reviewing Appendix Table 5 of the Entry Book E-76 that additional information was contained in Table 5 that was not provided in the summaries submitted by Dr. Kundzins. Dr. Stowell of UAREP inquired from Dr. Reno of Hazleton Laboratories whether individual eye examination worksheets

could be provided for review. Dr. Reno indicated that individual eye examination worksheets were not used and the only reports they had concerning the eye examinations were the summary report as prepared by Dr. Kundzins. On a memo dated 9 January 1974, from Dr. Kundzins, which was the summary for the 109th week eye examinations, the following comment was made. "Identification numbers of the affected mice are on file in my office." This would indicate that additional information was once available concerning the eye examinations and possibly this could explain the source of additional information which was included in the Appendix Table No. 5 of Entry Book E-76 that was prepared by S. Spruill.

Table 7-10 contains a summary of the information that was not contained on the summary sheets, but subsequently reported in the final Entry Book report for E-76. The Entry Book E-76 made the following comment concerning the eye examination, "Conducted at week 4; the eyes of all 360 control and treated mice placed on study appeared grossly normal." The summary of the eye examinations as provided by Kundzins indicated that two animals were replaced because of cataracts detected at the 4th week. This was not reflected in the Entry Book, and new animals assumed the replaced animals' identification numbers. During the 60th week interval eye examination, three animals in Groups 2 were indicated to have severe or moderate cataracts, as reported in the eye examination summary for that interval. The Entry Book contains specific information on an individual animal basis, not contained in the summary, indicating which animals had severe or moderate cataracts. Also, at the 60th week interval examination, animal number 1308 was indicated to be number 1318. The Entry Book Appendix Table No. 5, pp 57-60 for E-76

Table 7-10
Discrepancies in Ophthalmoscopic Reports

<u>Interval (week)</u>	<u>Animal No.</u>	<u>Table No. 5, Entry Book E-76, Appendix pp. 67-70</u>	<u>Ophthalmoscopic Summary Provided UAREP</u>
4	--	All animals' eyes appeared normal.	Two animals had cataracts and were replaced.
60	1238	Left eye: severe cataract	Right eye: moderate or severe cataract.
60	1273	Right eye: severe cataract	Right eye: moderate or severe cataract.
60	1276	Left eye: moderate cataract	Left eye: moderate or severe cataract.
60	1308	1318	1308
110	All animals:	Listing of observations on individual animal basis.	Observations provided for groups with no individual observations given.

for interval 108 (110) week eye examinations gave a complete listing of individual observations made on animals at that interval. The summary sheet, as provided by Kundzins, only indicated the relative number of animals with each of the maladies for each of the various groups, and did not indicate the observed disorders on an individual animal basis at this interval. Figure No. 5 of the Entry Book E-76 also indicated that the examinations were made at the 20th, 40th, 60th, and 110th week intervals. In fact, the examinations were made at the 4th, 21st, 40th, 61st, and 108th week intervals which are close to the reported times. UAREP verified that all animals that had ophthalmoscopic observations reported on them were alive at the intervals at which observations were made.

Table 7-11 contains information relating to the terminal ophthalmoscopic examination of the various treatment groups, indicating the percentage of cataracts and corneal opacities observed. The following statement was made in the Results and Discussion Section of Entry Book E-76 (p 30), "At the termination, the percentage of animals with cataracts was increased for Group No. 2 and No. 4 as compared to the control, but was similar to Group No. 3 and the control group. This finding is considered incidental; the absolute number of affected animals in the treated group was relatively small and was similar to or less than in the controls; no dose response relationship was evident." Groups 2 and 4 showed a 13% and 17% higher incidence of cataracts than controls, while Group 3 was 5% lower than controls. All treated animals surviving to 108 weeks had a cataract incidence of 48%, which most

Table 7-11

Terminal Ophthalmoscopic Examination for E-76 for Combined
Males and Females, Unilateral and Bilateral Cataracts
and Unilateral and Bilateral Corneal Opacity

	<u>Cataracts</u> <u>(%)</u>	<u>Corneal Opacity</u> <u>%</u>
Control.	39.6	18.8
Group 2	52.6	26.3
Group 3	35.0	20.0
Group 4	56.2	6.2

scientists would consider to be more than a "relatively small number." UAREP's statistical analysis of the groups, however, revealed that the differences were not significant by Chi square test at the 5% level.

Necropsy

The protocol indicated that necropsies were to be performed under the following conditions: "Animals were exsanguinated following sodium pentobarbital anesthesia (Diabutal Diamond Laboratories, Inc., Des Moines, Iowa). Necropsies performed under the supervision of a pathologist, organ weights recorded and representative tissues preserved." When reviewing the initials on the individual necropsy sheets, it was found that the initials of 36 different individuals or combinations of individuals were found on the necropsy sheets. Of the 350 necropsied

animals, 232 of these were performed by seven individuals or combinations of individuals as indicated by the initialed necropsy sheets. UAREP was informed that a prosector technician and a clerical recorder initialed each necropsy sheet. The degree to which the supervising pathologist provided administration in absentia vs closely examining the necropsy organs of the mice is unknown to UAREP, since his initials were not clearly indicated on the necropsy forms.

Organ weights were recorded on an individual animal basis and tissues fixed for later microscopic evaluations as shown on page 3 of Appendix VII-1. The protocol further requested the histopathologic evaluation of all organs including any tissue masses of the control and high dose groups as well as two-thirds (24) of the medium dose group and 1/3 (12) of the low dose group.

Entry Book E-76 contains a summary of the body weights, organ weights, and organ/body weight ratios, in Appendix Table 7 (pp 72-77). A summary of the discrepancies UAREP found in Entry Book 76, Appendix Table 7 is contained in Appendix VII-16. There were 15 inconsequential rounding discrepancies noted, and one computational error.

UAREP's statistical evaluation of the organ weights and organ/body weight ratios as contained in Entry Book E-76, Appendix Table 1, confirmed the significant differences HLA found in five of six instances using the t-test (Appendix VII-17).

The ovary weights for Groups 1 vs 4 were indicated in the Entry Book to be significantly lower at the 5% level of probability. When UAREP re-evaluated the data, their t-test was not significant and the Analysis of Variance of the ovary weights indicated that there was no

significant difference even at the 10% level of probability. HLA did not analyze interactions between various treatment groups and UAREP did. For the female groups 2 < 3 and 2 < 4, thyroid weights and thyroid/body weight ratios were found to be significant at the 5% level of probability. The liver to body weight ratios for 2 < 4 and 2 < 4 were also significant.

The dates on which the mice in the various groups were sacrificed are shown in Table 7-12.

Table 7-12

Dates on Which Varying Numbers of Surviving Male (M) and Female (F) Rats were Sacrificed

Date	Group 1		Group 2		Group 3		Group 4		Total
	M	F	M	F	M	F	M	F	
1/15/74	10	0	10	0	10	0	6	0	36
1/16/74	2	10	1	7	0	6	0	9	35
1/17/74	10	9	0	0	4	0	0	0	23
Total sacrificed, 1/15 to 1/17	22	19	11	7	14	6	6	9	94

It is of interest that the majority of most of the groups of male rats were sacrificed on January 15, 1974 whereas the majority of the female rats in all groups were sacrificed on January 16 with the remainder of the animals being sacrificed on January 17. One each of female Group 1, female Group 2, and male Group 4 died on January 15, 1974. The plan for sacrifice of the animals in this experiment appeared somewhat better than in some of the other experiments.

Histopathologic Findings

The histopathologic review for E-76 was performed by the same UAREP pathologists and in the same manner as E-75. In general, the comments in Chapter 6 relating to the histopathology review of E-75 are pertinent to E-76. The considerations relative to the significance of discrepancies in the diagnosis of amyloid, fibrosis, and atrophy, explained in Chapter VI also apply to Chapter VII. The statements regarding discrepancies in retinal changes and the diagnosis of "retinal degeneration" in Chapter VI are equally relevant to E-76. Because UAREP felt that an adequate sampling had been done on other experiments, a comparative summary tabulation of non-neoplastic lesions was not prepared for E-76 as it was E-75 and E-33,34.

Tumors - A summary tabulation of total tumors by organ and type for each group is shown in Appendix VII-18 together with their presumed initial time of observation. The comments regarding the comparable appendices in Chapter VI (Appendices VI-11 and VI-12) also apply to Appendices VII-18 and VII-19. There was generally reasonably close agreement between the EPL and UAREP pathologists as to the total number of various types of tumors which were present in E-76. As in E-75, there was good agreement between UAREP and EPL data relating to the average number of tumors per animal, as shown in Appendix VII-18.

Appendix VII-20, gives information on six sections of tumors diagnosed by EPL for which no slide was made available to UAREP. If these slides had been available and received the same diagnostic interpretation, it would not have made any significant difference in the overall interpretation of results.

Statistical Analysis of Tumor Incidence - Hazleton performed their statistical analysis relative to tumor incidence according to six categories: any tumor, all malignant tumors, benign tumors, primary pulmonary tumors, vascular tumors, and lymphoreticular tumors. UAREP analyzed their diagnoses of histopathologically proven tumors by the same categories. Some of the categories for benign tumors for EPL are lower than UAREP because they did not include mice with benign tumors which also had a malignant tumor, whereas UAREP did.

Data for the probability of developing tumors as derived by UAREP and Hazleton, are shown in Appendix VII-22. Entry Book E-76 indicates that Hazleton employed a life table method of analysis which was followed by a t-test, although neither the Entry Book nor the protocols explained precisely how this was done. Copies of some of the computer tape data that Hazleton employed in their analysis were provided to UAREP. These tapes do not show specific information as to the number of animals with tumors at each of the varying time intervals. Although there was generally good agreement in the HLA and UAREP life table analysis data up to the interval of 90 or 100 weeks, there was generally a poorer agreement in the terminal weeks. The final figures at the termination of the experiment are the important ones that integrate the earlier data as shown in Appendix VII-22. These figures for tumor incidence do not agree closely. Hazleton tumor analysis data indicated significant decreases in Group 2 and 3 males in the number of mice with "any tumor"; a statistically significant decrease in Group 2 as compared with Group 1 males with "benign tumors"; a statistically significant decrease in Group 3 as compared with Group 1 primary lung tumors and a

significant decrease in Group 2 as compared with controls for lymphoreticular tumors. The only statistically significant difference that UAREP demonstrated was that Group 1 had a higher tumor incidence than Group 2 males for benign tumors. Neither UAREP nor HLA found any statistically significant difference in tumor incidence among the female mice of E-76. On the basis of UAREP's analysis of the data collected, there was no evidence that DKP was tumorigenic under the conditions of these experiments.

A listing of all the significant discrepancies in histopathologic diagnoses between EPL and UAREP interpretations is presented in Appendix VII-23. As mentioned previously, minor differences in degrees of lesions are not shown as discrepancies nor are relatively inconsequential diagnoses. All important diagnoses in which a discrepancy existed are shown. According to the definitions in Chapter II, only 20% are major discrepancies of non-neoplastic lesions and an additional 17% are significant in that they involve the difference between a benign tumor and hyperplasia. Considering the large number of microscopic sections involved, this does not represent an impressive list of discrepancies and there was no predilection for such discrepancies in any one group as compared with another.

Disease Related to Diketopiperazine

Neither HLA nor UAREP believed that the histopathologic evidence indicated that under conditions of these experiments, the ingestion of DKP was associated with consistently recognizable histopathologic lesions.

Discrepancies in Following Protocol Design and Correlation of Clinical vs Gross Necropsy vs Microscopic Observations -

Since the detailed of analysis of the clinical vs necropsy vs microscopic observations was done in E-33,34 without turning up significant discrepancies, such analysis was not repeated on E-76. In working with the data, there were no obvious problems relating to such correlation.

As shown in Appendix VII-24, a careful check was made by UAREP comparing the HLA figures and UAREP figures as to the number of tissues sectioned with respect to the numbers specified by the protocol. As in E-75, the protocol specified that all tissues would be sectioned for Group 1 and Group 4 and one-third or 12 animals sectioned for Group 2 and two-thirds, or 24, animals sectioned for Group 3. In general, the specifications for the protocol were carried out satisfactorily. As noted in E-76, thymic tissue in old mice is generally absent and small organs such as pituitary may not appear in the plane of section or be lost from the slide.

A detailed analysis indicated in Appendix VII-25 was made of animals which were not examined microscopically. Seven animals were missing and not accounted for. The notations relative to autolysis or absence of performing a necropsy are quoted directly from the individual necropsy sheets. In some instances, it was said that no necropsy was performed but checks were placed beside the various organs in the column for tissues taken. UAREP does not know whether organs were dissected after the carcass had been fixed, or whether the check marks were placed in the column inadvertently. The number of animals not available for microscopic examination did not occur predominantly in any treatment

group. Although the number is larger than one might desire, such problems are not always readily controlled. It is not believed that this would produce any bias in interpretation of experimental results.

CONCLUSIONS

This companion study to E-75 involved a long-term study of the potential toxicity of diketopiperazine in 360 mice. The animals were divided into groups similar to E-75 except that the low dose animals received .25 g DKP/kg body weight per day, the medium dose 0.5 g/kg body weight per day, and the high dose group 1.0 g DKP/kg body weight/day.

The summation of clinical observations of nodules and swellings as prepared by UAREP agreed closely with HLA.

For reasons that are not apparent, the details of the analysis of survival by UAREP did not agree closely with that of HLA. Both applied life table analysis but there were differences in percentage survival, especially at the terminal period. Although Hazleton found a significant decrease in the survival of the mid-dose females, UAREP did not find any statistically significant differences in survival for any of the groups of mice by life table analysis or Analysis of Variance.

The same parameters were evaluated for hematology and clinical chemistries at the same intervals as in E-75. Although there was considerable variability in some of the hematologic results, UAREP's validation revealed only inconsequential or minor discrepancies. Two of 14 statistically significant results according to HLA were not confirmed by UAREP using the same t-test.

There were variable results in the clinical chemistry determinations. Because of the small amount of blood available from the mice, three of the eight mean values for BUN were based on only two specimens. Only minor discrepancies were encountered in UAREP's validation of the

Hazleton data on clinical chemistry determinations.

Except for several minor transcriptional discrepancies, there were no problems encountered in UAREP's validation of the results of urinalysis.

A comparison of the hematology and clinical chemistry data on the control animals of E-75 and E-76 showed a considerably increased number of significant differences over what might be expected by chance alone. Since the animals came from the same source and the experiments were run within a few weeks of each other, this suggests a greater degree of variation than one might expect from normal biologic factors alone, although any detailed analysis of the causes of such variation by UAREP is not feasible.

Only minor discrepancies were noted by UAREP in its evaluation of the results of ophthalmoscopic examination. Although there was considerable difference in the average incidence of cataracts in various groups, the differences were not statistically significant by UAREP's analysis.

With the exception of the inability to confirm one statistically significant t-test in HLA's analysis of organ weights and organ to body ratios, further discrepancies noted were insignificant or minor in nature.

The sacrifice of animals during the 108th week of the experiment was carried out according to an evident plan.

Considering the large number of sections involved, there was generally good agreement between EPL and UAREP pathologists in their diagnosis of lesions. There was no evidence that the DKP produced signi-

ificantly more lesions in one group of animals than in another. As compared with control groups, Hazleton reported a decreased incidence of "any tumor," "benign tumors," "primary pulmonary tumors," and "lymphoreticular tumors," in one of each of the three treatment groups. The only statistically significant difference in tumors confirmed by UAREP related to the lower tumor incidence of benign tumors in Group 2 males as compared with controls. Although both Hazleton and UAREP used a life table method of analysis, there was some disagreement in the terminal figures for tumor incidence. The reasons for this difference are not evident.

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APPENDIX VII-1

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<i>Item I</i>	HLA Memo from Dr. Reno dated June 29, 1972
<i>Item J</i>	Searle Protocol Dated October 12, 1971
<i>Item K</i>	Searle Protocol Amendment No. 1
<i>Item L</i>	Searle Protocol Amendment No. 2
<i>Item M</i>	Searle Protocol Amendment No. 3
<i>Item N</i>	Searle Protocol Amendment No. 4
<i>Item O</i>	Searle Protocol Amendment No. 5

Item A

HAZLETON LABORATORIES PROJECT SHEET

851122

PROJECT SHEET NO. <u>1</u>		Path-Tox No. 985H73		PROJECT NO. <u>700-260</u>	
			PROJECT COORDINATOR Reno/Trutter	DATE November 17, 1971	
COMPOUND(S) SC-19192			LOT NO(S).	RECEIPT DATE	LH-NUMBER(S) 14091A
DIVISIONS PARTICIPATING Toxicology			DISTRIBUTION: CENTRAL FILE (2) EACH DIV. PARTICIPATING EACH DIV. DIRECTOR		Sponsor PROJ. COORD. DATA PROCESSING
PHYSICAL AND CHEMICAL PROPERTIES					
<div style="border: 1px solid black; padding: 5px; display: inline-block;"> RECEIVED NOV 22 1971 </div>					
SPECIAL PRECAUTIONS (INDICATE PERSONNEL HAZARDS, PROTECTIVE INSTRUCTIONS)					
CHRONIC TOXICOLOGY SECTION					
REFERENCE INFORMATION Searle protocol dated 10-12-71.					
PROGRESS REPORTS DUE Quarterly (Project Manager)		FINAL RPT DUE on completion	INITIALS FER/lgm	SIGNATURE (PROJ. COORDINATOR) <i>[Signature]</i>	
EXPERIMENTAL WORK to be performed in Small Animal Toxicology Section					
 <u>SC-19192: 80-Week Oral Toxicity Study in the Mouse</u>					
<u>Animal Groups and Dosage Levels</u> - Charles River ICR Swiss mice; four weeks of age at initiation:					
<u>Group No.</u>	<u>No. of Animals</u> male female		<u>Dosage Levels</u> grams/kg		
1 (Control)	72	72	0		
2	36	36	0.25		
3	36	36	0.5		
4	36	36	1.0		
 <u>General Observations</u>					
Morbidity - mortality: Daily					
Body weight - food consumption: Weekly for four weeks, biweekly for the next eight weeks, and every four weeks thereafter.					
Record pertinent observations as necessitated.					

10000

Project Sheet No. 1
Project No. 700-260 (851122)

- 2 -

November 17, 1971

Ophthalmoscopic Examination - At zero (or as soon as practical), 20, 40, 60, and 80 weeks.

Palpation for Tissue Masses - At zero, five, and 10 weeks, and every 10 weeks thereafter

Clinical Laboratory Studies - As per attached Page 3.

Termination - After 80 weeks of treatment.

Postmortem Procedures - For all animals found dead, killed in extremis, or sacrificed by design as per attached Page 4.

00002

Project Sheet No. 1
Project No. 700-260 (851122)
CLINICAL LABORATORY PROCEDURES*

- Page 3 -

PATH-TOX. PROJ. NO. 982172
November 17, 1971

Specimen collection: individual**
()/sex/level)

Blood: Unopettes for hematology. Serum for clin. chem. Na₂EDTA for protime.

Urine: 24 hour specimen collected in individual metabolism cages.

16. HEMATOLOGY

Parameter	No./sex/ level	Rx interval (wks)
Hematocrit.....	6	5,10,20,40,60,80
Hemoglobin.....	6	"
Total RBC.....	6	"
Total WBC.....	6	"
Differential.....	6	"
Reticulocyte.....		
Platelets.....		
Coagulation (L-W).	4	80
Pro. time.....	6	80
Activ. PTT.....		
Urinary sugar.....		

17. URINALYSIS

Parameter	No./sex/ level	Rx interval (wks)
Sp. gravity.....	6	5,10,20,40,60,80
Bili-Labstix.....		
pH, Bilirubin, Protein, Sugar, Ketones, Blood.	6	"
Urobilinogen.....		
Microscopic.....	6	"
Phenylketones***	6	"

18. CLINICAL CHEMISTRY****

Parameter	No./sex/ level	Rx interval (wks)	Parameter	No./sex/ level	Rx interval (wks)
BUN.....	6	5,10,20,40,60,80	GPT.....	6	5,10,20,40,60
Uric acid.....			GOT.....	6	"
Glucose.....	6	"	AP.....	6	"
Sodium.....	6	80	BSP.....		
Potassium.....	6	80	Bilirubin.....	6	80
Calcium.....	6	80	OCT.....		
Fibrinogen.....			CPK.....		
Total			Serum		
Serum Protein.....	6	80	Phenylalanine.....	6	5,10,20,40,80
Serum Cholesterol	6	5, 10, 20, 40,60,80			

* Report actual pre-Rx specimen collection(s) as negative number (wks). Clin. lab workup done preferably on those animals receiving complete postmortem workup.

** Mice used for clinical lab. work should likewise receive postmortem workup as indicated.

*** C57 group only. Do all groups if H group is positive.

**** C5 parameters will be prioritized after receiving the information on availability of methods.

00003

PROTOCOL FOR A PRECLINICAL SAFETY STUDY OF SC-19192

Project Sheet No. 1

Project No. 700-260 (851122)

- Page 4 -

PATH-TOX. PROJ. NO 9851173

November 17, 1971

19) PHARMACOLOGIC EFFECTS
of compound absorption:

Evaluation of the following parameters provides evidence

Serum phenylalanine levels.

20) POSTMORTEM PROCEDURES

TISSUES	A Wt.	B Fix	C (Micro)			
			L	M	H	C
Stomach		X	12	24	36	72
Small intestine		X	12	24	36	72
Large intestine		X	12	24	36	72
Lung		X	12	24	36	72
Heart	X	X	12	24	36	72
Kidney	X	X	12	24	36	72
Liver	X	X	12	24	36	72
Gall bladder		X	12	24	36	72
Spleen		X	12	24	36	72
Pancreas		X	12	24	36	72
Pituitary	X	X	12	24	36	72
Thyroid	X	X	12	24	36	72
Adrenal	X	X	12	24	36	72
Gonad	X	X	12	24	36	72
Uterus/sem.v.	X	X	12	24	36	72
Vagina/prostate	M	X	12	24	36	72
Mammary gland R 4&5		X	—	—	36	72
Brain; 2 levels		X	—	—	36	72
Spinal cord		X	—	—	36	72
Nerve (brachial pl.) with muscle		X	—	—	36	72
Eye Right		X	—	—	36	72
Urinary bladder		X	36	36	36	72
Salivary gland (mand)		X	—	—	36	72
Lymph node (Mesent.)		X	—	—	36	72
Thymus		X	—	—	36	72
Bone marrow (femoral)		X	—	—	36	72
Rib junction		X	—	—	36	72
Skin		—	—	—	—	—
Unusual lesions		X	36	36	36	72
Usual lesions		X	36	36	36	72

A -- The organs weighed from each animal.

B -- The tissues preserved from each animal.

C -- Tissues examined microscopically from the indicated no. of animals of each sex.

Additional postmortem procedures:

09004

Item B

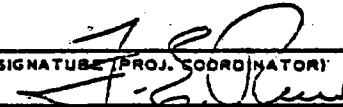
HAZLETON LABORATORIES PROJECT SHEET

851122

PROJECT SHEET NO. <u>2</u>		Path-Tox No. 985H73		PROJECT NO. <u>700-260</u>	
		PROJECT COORDINATOR Reno/Trutter		DATE February 14, 1972	
COMPOUND(S) SC-19192 (DKP)		LOT NO(S). 1R	RECEIPT DATE 11/23/71	LH-NUMBER(S) 14,091A	
DIVISIONS PARTICIPATING Toxicology		DISTRIBUTION: CENTRAL FILE (2) EACH DIV. PARTICIPATING EACH DIV. DIRECTOR		SPONSOR PROJ. COORD. DATA PROCESSING	
PHYSICAL AND CHEMICAL PROPERTIES					
SPECIAL PRECAUTIONS (INDICATE PERSONNEL HAZARDS, PROTECTIVE INSTRUCTIONS)					
REFERENCE INFORMATION: Searle protocol dated 10/12/71.					
PROGRESS REPORTS DUE Quarterly (Project Manager)		FINAL REPT DUE on completion	INITIALS FER:lgm	SIGNATURE (PROJ. COORDINATOR) <i>F. E. Reno</i>	
EXPERIMENTAL WORK to be performed in Small Animal Toxicology Section					
<p><u>SC-19192: 80-Week Oral Toxicity Study in the Mouse</u></p> <p><u>Modified Protocol</u></p> <p>Delete clinical chemistry and urine analysis until further advised.</p>					
<p style="text-align: right;">RECEIVED</p> <p style="text-align: right;">FEB 16 1972</p> <p style="text-align: right;">CHRONIC TOXICOLOGY SECTION</p> <p style="text-align: right;">00005</p>					
HAZLETON LABORATORIES FORM NO. 121 REVISED 1-67					

Item C

HAZLETON LABORATORIES PROJECT SHEET

PROJECT SHEET NO. <u>3</u>		P-T No. 985H73		PROJECT NO. <u>700-260</u>	
			PROJECT COORDINATOR Reno/Trutter	DATE June 5, 1973	
COMPOUND(S) SC-19192			LOT NO(S).	RECEIPT DATE	LH-NUMBER(S) 14,091
DIVISIONS PARTICIPATING Toxicology			DISTRIBUTION: CENTRAL FILE (2) EACH DIV. PARTICIPATING EACH DIV. DIRECTOR Sponsor PROJ. COORD. DATA PROCESSING		
PHYSICAL AND CHEMICAL PROPERTIES					
SPECIAL PRECAUTIONS (INDICATE PERSONNEL HAZARDS, PROTECTIVE INSTRUCTIONS)					
REFERENCE INFORMATION Searle protocol dated 10/12/71					
PROGRESS REPORTS DUE Quarterly (Project Manager)		FINAL REPT DUE on compl.	INITIALS FER:da	SIGNATURE (PROJ. COORDINATOR) 	
EXPERIMENTAL WORK to be performed in Small Animal Toxicology					
<u>Amended Protocol</u> Duration of this study will be extended. Termination of study will be performed only when the survival rate of either sex in the <u>control group</u> declines to 25% (18/72).					
RECEIVED JUN 6 1973 CHRONIC TOXICOLOGY SECTION 000006					

Item D

HAZLETON LABORATORIES PROJECT SHEET

PROJECT SHEET NO. <u>4</u>		P-T No. 985H73		PROJECT NO. <u>700-260</u>	
			PROJECT COORDINATOR Reno/Trutter	DATE August 24, 1973	
COMPOUND(S) SC-19192			LOT NO(S).	RECEIPT DATE	LN-NUMBER(S)
DIVISIONS PARTICIPATING Toxicology			DISTRIBUTION: CENTRAL FILE (2) EACH DIV. PARTICIPATING EACH DIV. DIRECTOR		Sponsor PROJ. COORD. DATA PROCESSING
PHYSICAL AND CHEMICAL PROPERTIES <div style="text-align: right; font-size: 1.5em; font-weight: bold;">RECEIVED</div> <div style="text-align: right;">AUG 27 1973</div>					
SPECIAL PRECAUTIONS (INDICATE PERSONNEL HAZARDS, PROTECTIVE INSTRUCTIONS) <div style="text-align: right;">CHRONIC TOXICOLOGY SECTION</div>					
REFERENCE INFORMATION Searle Protocol dated 8-20-73					
PROGRESS REPORTS DUE Quarterly (Project Manager)		FINAL REPT DUE on compl.	INITIALS FER:da	SIGNATURE (PROJ. COORDINATOR) <i>F. E. Reno / J. Trutter</i>	
EXPERIMENTAL WORK to be performed in Small Animal Toxicology					
<u>Protocol Amendment No. 3 - Clinical Laboratory measurements</u>					
<p>Please make the following additions to the clinical chemistry section for terminal bleeding in all groups (6/sex/group).</p> <ol style="list-style-type: none"> 1) Serum Insulin (Radioimmunoassay). 2) Serum Ornithine Carbamyl Transferase (low priority). 3) Serum Protein Electrophoresis. 					
<u>Protocol Amendment No. 4 - Histopathology</u>					
<p>Brain and urinary bladder will be examined from each animal on the study.</p> <p><u>Brain:</u> In order to perform a thorough histopathologic evaluation of brain to detect intracranial microscopic tumors, eight coronal slices, 2-4 mm thickness, will be examined grossly and embedded. These slices will be numbered 1 thru 8 from cranial to caudal, and one section of each slice will be examined microscopically.</p> <p>A schematic representation of any neural tumor and identification of the block, animal and path no. will be included in the report.</p> <p>Tumor data from the brain will be evaluated in two ways:</p> <ol style="list-style-type: none"> a) taking into consideration all the 8 sections from each animal; b) eliminating the data from sections 1 and 8. In other words, use the data from sections 2, 3, 4, 5, 6 and 7 from each animal. 					
00007					

Project Sheet No. 4
Project No. 700-260
P-T No. 985H73

- 2 -

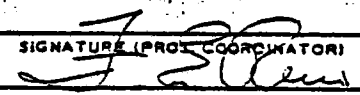
August 24, 1973

Urinary Bladder: At necropsy urinary bladder will be slightly distended by injecting neutral buffered formalin into the lumen through the wall. Fixed urinary bladder will be halved longitudinally, examined grossly, both hemispheres embedded, and two longitudinal sections cut from each hemisphere with approximately 50 microns between each section. Hence, four transverse sections from each urinary bladder would be examined microscopically.

00008

Item E

HAZLETON LABORATORIES PROJECT SHEET

PROJECT SHEET NO. <u>5</u>		P-T No. 985H73		PROJECT NO. <u>700-260</u>	
		PROJECT COORDINATOR F.E. Reno		DATE January 11, 1973 <u>4</u>	
COMPOUND(S) SC-19192		LOT NO(S).		RECEIPT DATE	LN-NUMBER(S)
DIVISIONS PARTICIPATING Toxicology		DISTRIBUTION: CENTRAL FILE (2) EACH DIV. PARTICIPATING EACH DIV. DIRECTOR		SPONSOR PROJ. COORD. DATA PROCESSING	
PHYSICAL AND CHEMICAL PROPERTIES					
<div style="border: 1px solid black; padding: 5px; display: inline-block;"> RECEIVED JAN 14 1974 </div>					
SPECIAL PRECAUTIONS (INDICATE PERSONNEL HAZARDS, PROTECTIVE INSTRUCTIONS)					
CHRONIC TOXICOLOGY SECTION					
REFERENCE INFORMATION Searle Protocol dated 1-2-74					
PROGRESS REPORTS DUE Quarterly (project coordinator)		FINAL REPT DUE on completion	INITIALS FER:et	SIGNATURE (PROJ. COORDINATOR) 	
EXPERIMENTAL WORK to be performed in Small Animal Toxicology					
SC-19192: 109 Week Oral Tumorigenicity Study in the Mouse					
This study will be terminated after 109 weeks of treatment. The following supersedes all previous amendments.					
1. <u>Clinical Laboratory Measurements</u> - to be performed on a maximum of 10 per sex per group at terminal sacrifice. <ol style="list-style-type: none"> Hematology: hemoglobin, total RBC, total WBC, differential, and hematocrit Urine analysis: Microscopic and phenyl ketones; other conventional measurements if possible. Clinical Chemistry: GPT, AP, BUN, and L-phenylalanine* (Insulin determinations will be performed if sufficient serum is obtained.) 					
*To be performed by Searle Laboratories; 0.2 ml of frozen serum will be shipped to the sponsor for L- phe measurements.					
03009					

Project Sheet No. 5
Project No. 700-260

- 2 -

January 14, 1974

2. Postmortem brain tissue processing - As per Dr. Voelker's memo to Dr. Reno, dated August 28, 1973, the brain from each animal will be trimmed grossly into 5 transverse blocks; each block will be embedded in a single paraffin block and 6 tissue sections will be prepared. Sections 1 and 6 will be stained and read microscopically; the remaining 4 sections will be saved unstained for future use, as necessary. All information gained from reading slides no. 1 and 6 will be included in the study report. As a separate document, the incidence of brain tumors in slide 1 and in slide 6 will be recorded separately and made available to Searle Laboratories for future reference in planning brain tissue evaluation in mouse tumorigenicity studies. Thus, this SC-19192 mouse study will provide diagnostic information from two tissue sections from each of five transverse blocks of brain from each animal.

00010



Item F

HAZLETON LABORATORIES

TO: Minner/Petrovics

CC: Trutter

DATE: July 5, 1972

Technical Data File-

700-247; 700-248;

700-259; 700-260 .

FILE:

Cottrell

SUBJECT: Pituitary Organ Weights

FROM: Reno *FER*

BLDG.

ROOM:

Pituitary weights from mice will no longer be required on any 700-studies currently underway. This will preclude any mechanical damage to the organ. However, histological sections will still be prepared as required.

FER:dma



- 770 -
Item G
HAZEN LABORATORIES

TO	FILE	CC	John E. Hinner	DATE	May 22, 1970
	Project Nos.		✓ Vets. & Youth	FILE	
	700-259 & 700-260		Contractor		
			Control		
SUBJECT:	Project Extension			FROM:	Kenn
				BLOC	ROOM

In a telephone conversation with Dr. E.S. Rao from Searle Laboratories, I was advised that the duration of the above referenced projects would be extended. The actual duration is open-ended, with each study to be terminated when the mortality of either sex in the control or high dose group reaches 40%.

/et

03012



HAZLETON LABORATORIES

TO:

✓ Minner/Petrovics

CC: Johnson
Trutter
Cottrell

DATE: May 31, 1973

FILE:

SUBJECT:

Final Decision, Termination of
Project Nos. 700-259 and 700-260

FROM: Reno

BLDG.

ROOM:

All previous instructions regarding the termination of these studies are hereby cancelled.

Per instructions from Dr. McConnell these studies are to be terminated only when the survival of either sex in the control group reaches 25% (18/72).

Present data indicates that at Week 79 the survival in the control groups are averaging 70%. I would therefore venture that the studies will be extended approximately two months beyond their intended duration.

/et

Item I



AVOID VERBAL ORDERS

TO: ☒ Inner/ ☐ Miller/Protocol Bldg. _____ Mail Sta. _____ Date _____

FROM: _____ Bldg. _____ Mail Sta. _____ Ext. _____

SUBJECT: 770-250

Hematology: At the next clinical interval (Week 40) and at subsequent intervals as indicated in Project Sheet No. 1, please run prothrombin time determinations, if possible. This determination was done at Weeks five and (10, but not at Week 20.)

STEMS 99 REV. 9-67

UAREP's interpretation of the above:

6-29-72

Hematology: At the next clinical interval (week 40) and at subsequent intervals as indicated in Project Sheet No. 1, please run prothrombin time determinations, if possible. This determination was done at Weeks five and (10, but not at Week 20.)

00014

Item J

FINAL PROTOCOL FOR A PRECLINICAL SAFETY STUDY OF SC-19192

COMMERCIAL LAB PROJ. NO. _____

AMENDED (1)(2)(3)(4)(5) PATH-TOX PROJ. NO. 985H73

0051

- 1) Protocol finalized 10-12-71 Treatment initiated 12-10-71 Animals terminated _____ Final report _____ es fi
- 2) Cpd. needed (kg): Total 10.3 First 4 wks. 0.5Kg Ordered _____ Del'vy _____ es fi
- 3) Study title & objectives: SC-19192: 80 week oral toxicity study in the mouse P-T no. 983H7.
An evaluation of safety during chronic administration to weanling mice, for FAP submission to enable marketing of SC-18862 as a food additive in the UK.
- 4) Species, strain, sex, (M,F): Charles River, FCR Swiss Age (wk) at Rx start: 4
- 5) Rx duration (wks): 80 Route & Freq. of admin.: Continuous oral ad lib
- 6) Mode of admin.: Compound admixed w/v in diet.
- 7) Drug-vehicle mixture stability analysis; Rx wks.: (schedule being devised)
- 8) Est. daily human (50 kg.) dose & route: 7.5 MPK daily orally (assumes 25% decomposition of 30 MPK SC-18862 dose to 27 Kg. human).
- 9) Dose levels (MPK daily): Control 0; Low 0.25; Med. 0.5; High 1.0
- 10) Multiple of human dose: 0; 33; 67; 133
- 11) No. & sex of animals/level: 72 M; 36 M; 36 M; 36 M
72 F; 36 F; 36 F; 36 F
- 12) Total animals required: 360
- 13) Housing & basal diet: Individual; Mouse complete diet; powdered; chlorinated water.
- 14) General observations (frequency; wks)
Morbidity-mortality: Observe daily.
Motor & behavioral activity: Periodically as needed.
Body weight: Weekly up to 4 weeks; biweekly for the next 8 weeks & once every 4 wks thereafter.
Food consumption & dose adjust.: Concurrent with body weight interval.
Additional observations: Record pertinent observations.
- 15) Physical examination (frequency; wks)
Gen'l external features, incl. body orifices & excrement: 5, 10 & every 10 wks thereafter
Limited neurological: -- Detailed neurological: --
Ophthalmoscopic and/or slit lamp: At 0, 20, 40, 60, and 80 weeks.
Digital palpation for protruding tissue masses: Pre-Rx; 5, 10 & every 10 wks thereafter.
Body temperature (rectal): --
Blood pressure and/or ECG: --

KCP
10/12/71Fig 1116
10-17

Page 2

16-18

CLINICAL LABORATORY PROCEDURES*

PATH-TOX. PROJ. NO. 985173

0052

Specimen collection: individual**
(/sex/level)

Blood: Unopettes for hematology. Serum for clin. chem. Na₂EDTA for proteins.

Urine: 24 hour specimen collected in individual metabolism cages.

16. HEMATOLOGY

Parameter	No./sex/ level	Rx interval (wks)
Hematocrit.....	6	5,10,20,40,60,80
Hemoglobin.....	6	"
Total RBC.....	6	"
Total WBC.....	6	"
Differential.....	6	"
Reticulocyte.....		
Platelets.....		
Coagulation (L-W).	6	80
Pro. time.....	6	80
Activ. PTT.....		
Barrow smear.....		
.....		
.....		

17. URINALYSIS

Parameter	No./sex/ level	Rx interval (wks)
Sp. gravity.....	6	5,10,20,40,60,80
Bili-Labstix.....		
pH, Bilirubin, Protein, Sugar, Ketones, Blood.	6	"
Urobilinogen.....		
Microscopic.....	6	"
Phenylketones***	6	"

18. CLINICAL CHEMISTRY****

Parameter	No./sex/ level	Rx interval (wks)	Parameter	No./sex/ level	Rx interval (wks)
BUN.....	6	5,10,20,40,60,80	GPT.....	6	5,10,20,40,60,80
Uric acid.....			GOT.....	6	"
Glucose.....	6	"	AP.....	6	"
Sodium.....	6	80	BSP.....		
Potassium.....	6	80	Bilirubin.....	6	80
Calcium.....	6	80	OCT.....		
Fibrinogen.....			CPK.....		
Total			Serum		
Serum Protein.....	6	80	Phenylalanine.....	6	5,10,20,40,60,
Serum Cholesterol	6	5, 10, 20, 40,60,80			

* Report actual pre-Rx specimen collection(s) as negative number (wks). Clin. lab workup done preferably on those animals receiving complete postmortem workup.

** Mice used for clinical lab. work should likewise receive postmortem workup as indicated.

*** C&H group only. Do all groups if H group is positive.

**** CC parameters will be prioritised after receiving the information on availability of micro methods.

- 19) PHARMACOLOGIC EFFECTS Evaluation of the following parameters provides evidence of compound absorption:

Serum phenylalanine levels.

20) POSTMORTEM PROCEDURES

TISSUES	A Wt.	B. Fix	C (Micro)			
			L	M	H	C
Stomach		X	12	24	36	72
Small intestine		X	12	24	36	72
Large intestine		X	12	24	36	72
Lung		X	12	24	36	72
Heart	X	X	12	24	36	72
Kidney	X	X	12	24	36	72
Liver	X	X	12	24	36	72
Gall bladder		X	12	24	36	72
Spleen		X	12	24	36	72
Pancreas		X	12	24	36	72
Pituitary	X	X	12	24	36	72
Thyroid	X	X	12	24	36	72
Adrenal	X	X	12	24	36	72
Gonad	X	X	12	24	36	72
Uterus/scn.v.	X	X	12	24	36	72
Vagina/prostate	M	X	12	24	36	72
Mammary gland R 4&5		X	--	--	36	72
Brain; 2 levels		X	--	--	36	72
Spinal cord		X	--	--	36	72
Nerve with muscle (brachial pl.)		X	--	--	36	72
Eye Right		X	--	--	36	72
Urinary bladder		X	36	36	36	72
Salivary gland (mand)		X	--	--	36	72
Lymph node (Mesent.)		X	--	--	36	72
Thymus		X	--	--	36	72
Bone marrow (femoral)		X	--	--	36	72
Rib junction		X	--	--	36	72
Skin		--	--	--	--	--
Unusual lesions		X	36	36	36	72
Usual lesions		X	36	36	36	72

A -- The organs weighed from each animal.

B -- The tissues preserved from each animal.

C -- Tissues examined microscopically from the indicated no. of animals of each sex.

Additional postmortem procedures:

21) STATISTICAL EVALUATION OF DATA: PROCEDURES USED

- a) Body wt. change; food & drug consumption:
Group mean \pm S. E.; appropriate analysis of intergroup variance at each time interval.
- b) Clinical laboratory values:
Group mean \pm S. E.; appropriate analysis of intergroup at each time interval.
- c) Incidence and onset of neoplasms:
Mean incidence and appropriate analysis of intergroup variance at termination.
- d) Randomization procedures:
Simple randomization.

22) INTERIM AND FINAL STUDY REPORTS

The sponsor (Director; Path-Tox Dept) requires a brief quarterly report relating statistically significant changes in items 16, 17, and 18 with a general statement on items 14, 15 and 20, by or on the 1st of Jan., April, July, and October; serious adverse findings are to be reported immediately.

Protocol Distribution List

Design Committee Members:

- 1) Dr. Sammeta (Biostatistician)
- 2) Dr. F. Saunders (Biol. Res. Adviser)
- 3) Dr. Ranney (Drug Metab. Rep.)
- 4) Dr. Polk (Clinical represent.)
- 5) Dr. Rao (P-T Dept. monitor)
- 6) Dr. McConnell (P-T Dept. adviser)

Technical Staff:

- 1) _____ (Path. Lab)
- 2) _____ (Autopsy Lab)
- 3) _____ (Bio-Anal. Lab)
- 4) _____ (Gen'l Tox. Lab)
- 5) _____ (Hematology Lab)
- 6) _____ (Pathologist)

Item K

May 21, 1973

0050

MEMO TO: Sweetener Preclinical Safety Protocol Design Committee Members:

Dr. Dutt (Biostatistician)
Dr. F. Saunders (Bio. Res. Director)
Dr. Ranney (Metab. Rep.)
Dr. Polk (Clin. Rep)
Dr. McConnell (P-T Dept. Advisor)

COPY TO: Dr. Reno (Hazleton)

FROM: Dr. Rao

SUBJECT: SC-19192: 80 Week Oral Toxicity Study in the Mouse; P-T 985H73.
Protocol Amendment No. 1.

This eighty week study is due to terminate in June 1973. It was primarily designed to evaluate the tumorigenic potential of SC-19192. This could best be evaluated by exposing the animals to the agent for the maximum duration possible without unduly reducing the survival of animals. For this reason, we decided to extend or continue the study until the mortality of either sex in the control or high dose groups reaches 40%. Study would be terminated at that time. Further modification of this study may arise based on the May 24-25 meeting of NAS-NRC Carcinogenicity Conference.

K. S. Rao

K. S. Rao, Ph. D.

KSR:dv

0049

Item L

May 31, 1973

MEMO TO: Sweetener Preclinical Safety Protocol Design Committee Members

Dr. Dutt (Biostatistician)
Dr. F. Saunders (Biol. Res. Director)
Dr. Ranney (Metab. Rep.)
Dr. Polk (Clin. Rep.)
Dr. McConnell (P-T Dept. Advisor)

COPY TO: Dr. Reno (Hazleton)

FROM: Dr. Rao

SUBJECT: SC-19192: 80 Week Oral Toxicity Study in the Mouse; P-T 985H73.
Protocol Amendment No. 2.

Based on the information acquired at the NAS-NRC Carcinogenicity Conference (May 1973, New York), it was decided to continue the above study until the survival rate declines to 25% in either sex in the control group. In terms of number of animals, this means 18 control mice or more per sex.

K. S. Rao

K. S. Rao

KSR:lg

RR mcl
5-31-73

6048

Item M

August 20, 1973

MEMO TO: Sweetner Preclinical Safety Protocol Design Committee Members:

- 1) Dr. Dutt (Biostatistician)
- 2) Dr. F. Saunders (Biol. Res. Advisor)
- 3) Dr. Ranney (Drug Metab. Rep.)
- 4) Dr. Polk (Clinical Rep.)
- 5) Dr. McConnell (P-T Dept. Advisor)

COPY TO: Dr. Reno (Hazleton Lab)

FROM: Dr. Rao

SUBJECT: SC-19192: Oral Tumorigenic Study in the Mouse; P-T 985H73;
Protocol Amendment No. 3. Clinical laboratory measurements.

Please make the following additions to the clinical chemistry section for terminal bleeding in all groups (6/sex/group).

- 1) Serum Insulin (Radioimmunoassay).
- 2) Serum Ornithine Carbamyl Transferase (low priority).
- 3) Serum Protein Electrophoresis.

K. S. Rao

KSR:lg

Reg Metab
8-20-73

Item N

High File
0047

August 20, 1973

MEMO TO: Sweetener Preclinical Safety Protocol Design Committee Members:

- 1) Dr. Dutt (Biostatistician)
- 2) Dr. F. Saunders (Biol. Res. Advisor)
- 3) Dr. Ranney (Drug Metab. Rep.)
- 4) Dr. Polk (Clinical Rep.)
- 5) Dr. McConnell (P-T Dept. Advisor)

COPY TO: Dr. Reno (Hazleton Lab.)

FROM: Dr. Rao

SUBJECT: SC-19192: Oral Tumorigenic Study in the Mouse; P-T 985H73.

Protocol Amendment No. 4. Histopathology.

Brain and urinary bladder will be examined from each animal on the study.

Brain: In order to perform a thorough histopathologic evaluation of brain to detect intracranial microscopic tumors, eight coronal slices, 2-4 mm thickness, will be examined grossly and embedded. These slices will be numbered 1 thru 8 from craniad to caudad, and one section of each slice will be examined microscopically.

A schematic representation of any neural tumor and identification of the block, animal and path no. will be included in the report.

Tumor data from the brain will be evaluated in two ways:

- a) taking into consideration all the 8 sections from each animal;
- b) eliminating the data from sections 1 and 8. In other words, use the data from sections 2, 3, 4, 5, 6 and 7 from each animal.

The objective of the latter procedure is to assess the accuracy of evaluating brain tissue from 6 vs. 8 sections, for implementation in future tumorigenic studies.

Urinary Bladder: At necropsy urinary bladder will be slightly distended by injecting neutral buffered formalin into the lumen through the wall. Fixed urinary bladder will be halved longitudinally, examined grossly, both hemispheres embedded, and two longitudinal sections cut from each hemisphere with approximately 50 microns between each section. Hence, four transverse sections from each urinary bladder would be examined microscopically.

S. Rao

K. S. Rao

KSR:ja

Ranney
8-30-73

Item 0

0046

January 2, 1974

MEMO TO: Sweetener Preclinical Safety Studies Protocol Design Committee

Dr. Dutt (Biostatistician)
Dr. F. Saunders (Biological Research Advisor)
Dr. Ranney (Drug Metabolism Representative)
Dr. Polk (Clinical Representative)
Dr. McConnell (P-T Dept. Advisor)

COPY TO: Dr. F. Reno; Hazleton Laboratories

FROM: Dr. Rao

SUBJECT: SC-19192: Oral Tumorigenicity Study in the Mouse; P-T 985H73.
Protocol Amendment No. 5.

1) Clinical laboratory measurements. Please make the following measurements on all survivors at terminal sacrifice:

- a. Hematology: hemoglobin, total RBC, total WBC, differential, and hematocrit.
- b. Urinalysis: Microscopic, and phenylketones*; other conventional measurements if possible.
- c. Clinical chemistry: GPT, AP, BUN, insulin*, L-phenylalanine* (Searle Laboratories).

Hazleton Laboratories will ship 0.2 ml of frozen serum to Searle Laboratories for performance of the I-phe measurement; other measurements will be performed at Hazleton Laboratories.

The above supersedes all previous amendments regarding clinical laboratory procedures.

2) Postmortem brain tissue processing. As per Dr. Voelker's memo to Dr. Reno, dated 28 Aug 73, the brain from each animal will be trimmed grossly into 5 transverse blocks: each block will be embedded in a single paraffin block and 6 tissue sections will be prepared. Sections 1 and 6 will be stained and read microscopically; the remaining 4 sections will be saved unstained for future use, as necessary. All information gained from reading slides no. 1 and 6 will be included in the study report. As a separate document, the incidence of brain tumors in slide 1 and in slide 6 will be recorded separately and made available to Searle Laboratories for future reference in planning brain tissue evaluation in mouse tumorigenicity studies. Thus, this SC-19192 mouse study will provide diagnostic information from two tissue sections from each of five transverse blocks of brain from each animal.

3) Terminate the study after 109 weeks of treatment. Modify the study title to read as follows: SC-19192: 109 Week Oral Tumorigenicity Study in the Mouse: P-T 985H73.

K. S. Rao

K. S. Rao

KSR:jm

* 6 mice/sex: C & H groups only, unless latter not unremarkable.

RgM
1-8-4

APPENDIX VII-2

Appendix VII-1 contains the initial Hazleton Laboratory Project Sheet No. 1 (Item A), together with five amendments (Items B, C, D, and E). Item J is the complete Searle protocol which has Dr. Rao's initials and the date of October 12, 1971. This protocol indicates that there have been five amendments and these are included as items K, L, M, N, and O. The agreement between the Hazleton and Searle amendments and memoranda follows the evolution of the protocol design of E-76 somewhat more closely than for a number of the other Hazleton experiments.

There were many changes in the clinical laboratory plans during the course of carrying out the experiments for E-76. Some of these are summarized in Table A, which follows. This shows the number of determinations originally planned for each parameter together with the number actually carried out.

APPENDIX VII-2A

TABLE A

SUMMARY OF DETERMINATIONS FOR HEMATOLOGY AND CLINICAL CHEMISTRY
PLANNED AND DONE

	5w	10w	20w	40w	60w	T
<u>Hematology</u>						
Hematocrit	6/6	6/6	6/6	6/6	6/6	6/6-10
Hemoglobin	6/6	6/6	6/6	6/6	6/6	6/6-10
RBC	6/6	6/6	6/6	6/6	6/6	6/6-10
WBC	6/6	6/6	6/6	6/6	6/6	6/6-10
Differential	6/6	6/6	6/6	6/6	6/6	6/6-10
Prothrombin	0/6	0/6	0/0	6/6	6/6	6/0
<u>Clinical Chemistry</u>						
BUN	6/0	6/0	6/0	6/0	6/0	6/2-5*
Glucose	6/0	6/0	6/0	6/0	6/0	6/0
Cholesterol	6/0	6/0	6/0	6/0	6/0	6/0
SGPT	6/0	6/0	6/0	6/0	6/0	6/6-10
SGOT	6/0	6/0	6/0	6/0	6/0	6/0
Alk. phosphatase	6/0	6/0	6/0	6/0	6/0	6/4-10*
Phenylalanine	6/0	6/0	6/0	6/0	6/0	6/3-10*
Na						6/0
K						6/0
Ca						6/0
Total Protein						6/0
Bilirubin						6/0
Insulin						6/0
OCT						6/0
Electrophoresis						6/0

Under each time interval, the figure on the left is the number of determinations initially or subsequently planned and the number on the right is the number actually done for each sex and each of four groups. T, terminal interval was initially planned for 80 weeks and subsequently changed to 108. The initial protocol stated that clinical chemistry parameters would be prioritized after receiving information on available micromethods.

* Numbers on right are less than six due to insufficient blood for test in at least one group.

APPENDIX VII-3

INDIVIDUAL LISTING OF MICE WITH PALPABLE NODULES, TISSUE MASSES,
AND SWOLLEN AREAS ON BODY OR LEGS

<u>Group</u>	<u>Mouse No.</u>	<u>Interval (week)</u>	<u>Group</u>	<u>Mouse No.</u>	<u>Interval (week)</u>
1M	1090	88, 108	3M	1318	84, 92
1M	1107	104	3M	1323	72, 80, 92
1M	1112	88, 104	3M	1326	104
1M	1116	104	3M	1327	96
1M	1124	88, 92	3M	1331	104
1M	1128	88, 96, 108	3F	1351	84
1M	1129	88, 92	3F	1370	88
1M	1135	104	4M	1374	108
1M	1148	72	4M	1380	84
2M	1230	84, 92	4M	1396	96, 100, 104
2M	1239	96	4M	1408	80, 92, 96
2M	1245	104, 108	4F	1410	84, 92, 104
2M	1259	104	4F	1426	84
2F	1285	92, 96	4F	1433	100
3M	1309	108			

APPENDIX VII-4

SUMMARY OF VARIATIONS AS REPORTED IN CLINICAL OBSERVATIONS MADE
FOR TISSUE MASSES AND NODULES FOR E-76

<u>Group</u>	<u>Animal Number</u>	<u>Observation</u>
1M	1107	Swelling reported, week 104--both hind legs Swelling not reported, week 108
	1117	Small firm nodule reported, week 104--lower midline Nodule not reported, week 108
	1128	Small firm nodule reported, week 88--left inguinal Nodule not reported, week 100
	1129	Small firm nodule reported, week 88--right inguinal Nodule not reported, week 96
1F	1168	Left eye protruding, reported, week 92 Protruding not reported, week 96
	1183	Enlargement reported, week 104--right hind paw Enlargement not reported, week 108
	1210	Protruding vagina reported, week 104 Protruding vagina not reported, week 108
2M	1239	Small firm nodule reported, week 96--left inguinal Nodule not reported, week 100
	1249	Sores and swelling reported, week 104--tail Sores and swelling not reported, week 108
2F	1274	Swelling reported, week 64--mouth Swelling not reported, week 68

Appendix VII-4
page 2

<u>Group</u>	<u>Animal Number</u>	<u>Observation</u>
2F	1285	Small firm nodule reported, week 96--vagina Nodule not reported, week 96 Small firm nodule reported, week 96--right inguinal Nodule not reported, week 100
3M	1318	Small firm nodule reported, week 84--left inguinal Nodule not reported, week 88 Small soft nodule reported, week 92--left inguinal Nodule not reported, week 96
	1323	Firm nodule reported, week 64--chest Nodule not reported, week 76
	1326	Small firm nodule reported, week 104--lower midline Nodule not reported, week 108
	1331	Small firm nodule reported, week 104--left inguinal Nodule not reported, week 108
3F	1351	Tissue mass reported, week 64--vagina Tissue mass not reported, week 68 Red protruding small tissue mass reported, week 84--vagina Tissue mass not reported, week 88
4F	1410	Red, swollen protruding vagina, reported, week 84 Protruding vagina not reported, week 96 Red, swollen vagina reported, week 100 Red, swollen vagina not reported, week 108

APPENDIX VII-5

COMPARISON OF HLA AND UAREP COMPUTATIONS OF TOTAL TERMINAL

MEAN (\bar{x}) \pm STANDARD DEVIATION (SD) OF DKP IN GRAMS/KILOGRAMS BODY WEIGHT AND AT INTERVALS UP TO 108 WEEKS

Intended Dose \bar{x} SD	HLA			UAREP		
	Group 2M	Group 3M	Group 4M	Group 2M	Group 3M	Group 4M
	.250 ±.037	.500 ±.092	1.000 ±.152	.250 ±.039	.500 ±.087	1.000 ±.144
Interval in weeks						
0-4	.271	.562	1.132	.278	.568	1.147
4-8	.245	.477	.962	.246	.478	.963
8-12	.229	.470	.924	.236	.480	.953
12-16	.239	.439	1.006	.240	.446	.967
16-20	.242	.431	.828	.242	.453	.892
20-24	.233	.501	.973	.239	.502	.959
24-28	.284	.547	.993	.287	.551	1.012
28-33	.224	.476	.952	.221	.472	.951
33-36	.258	.559	1.223	.274	.576	1.243
36-40	.229	.423	.813	.226	.398	.783
40-44	.301	.586	1.354	.304	.597	1.337
44-48	.191	.515	.897	.187	.560	.931
48-52	.231	.365	.847	.232	.363	.848
52-56	.221	.382	.792	.216	.392	.806
56-60	.359	.839	1.397	.369	.814	1.335
60-64	.229	.456	1.091	.229	.458	1.065
64-68	.223	.435	.901	.229	.447	.926
68-72	.226	.491	.859	.238	.495	.885
72-76	.261	.466	.922	.250	.466	.916
76-80	.265	.579	1.058	.265	.567	1.074
80-84	.298	.528	1.079	.297	.536	1.111
84-88	.186	.407	.971	.188	.419	.931
88-92	.290	.549	.988	.293	.532	.997
92-96	.261	.495	.993	.253	.497	.967
96-100	.275	.582	1.168	.272	.569	1.137
100-104	.204	.441	.887	.208	.459	.853
104-108	.276	.560	1.033	.273	.530	1.077

APPENDIX VII-5 (cont.)
page 2

Intended Dose	HLA			UAREP		
	Group 2F	Group 3F	Group 4F	Group 2F	Group 3F	Group 4F
	\bar{x} SD	\bar{x} SD	\bar{x} SD	\bar{x} SD	\bar{x} SD	\bar{x} SD
	.250 ±.046	.500 ±.086	1.000 ±.168	.250 ±.049	.500 ±.076	1.000 ±.164
Interval in weeks						
0-4	.268	.567	1.157	.286	.575	1.168
4-8	.243	.488	.992	.242	.488	.992
8-12	.234	.469	.959	.248	.488	.967
12-16	.233	.466	.906	.224	.440	.916
16-20	.216	.440	.937	.225	.460	1.003
20-24	.292	.497	.907	.294	.518	.885
24-28	.236	.540	.844	.237	.512	.866
28-33	.232	.474	1.073	.234	.496	1.020
33-36	.271	.545	1.196	.275	.528	1.278
36-40	.206	.419	.822	.204	.436	.833
40-44	.301	.578	1.157	.308	.567	1.135
44-48	.232	.507	.987	.238	.511	1.009
48-52	.220	.376	.914	.209	.391	.862
52-56	.171	.378	.624	.175	.379	.670
56-60	.409	.777	1.558	.427	.751	1.526
60-64	.206	.461	1.013	.198	.468	1.003
64-68	.231	.432	.870	.238	.448	.898
68-72	.236	.458	.960	.241	.465	.902
72-76	.232	.486	.929	.227	.486	.979
76-80	.278	.571	1.115	.282	.552	1.111
80-84	.304	.543	.970	.300	.546	1.024
84-88	.200	.475	.895	.200	.461	.900
88-92	.247	.452	1.183	.260	.470	1.218
92-96	.257	.473	1.075	.244	.485	1.002
96-100	.285	.634	.967	.281	.583	.919
100-104	.206	.415	.932	.212	.433	.945
104-108	.293	.618	1.089	.268	.618	1.041

APPENDIX VII-6

UAREP SURVIVAL SUMMARY FOR E-76¹

Week	Male Groups			
	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>
0	72/72	36/36	36/36	35/35
1	72/72	36/36	36/36	35/35
2	72/72	36/36	36/36	35/35
3	72/72	36/36	36/36	35/35
4	72/72	36/36	36/36	35/35
6	72/72	36/36	35/36	35/35
8	72/72	36/36	35/36	34/34
10	72/72	36/36	35/36	34/34
12	72/72	36/36	35/36	34/34
16	72/72	36/36	35/36	34/34
20	72/72	36/36	35/36	34/34
24	70/72	36/36	34/36	34/34
28	69/72	36/36	34/36	34/34
32	66/72	36/36	32/36	34/34
33	66/72	34/36	32/36	34/34
36	66/72	33/36	32/36	34/34
40	65/72	32/35	32/36	33/34
44	62/69	32/35	32/36	32/34
48	61/69	31/34	32/36	30/33
52	61/69	31/34	32/36	29/33
56	61/69	31/34	32/36	29/33
60	60/69	30/34	30/36	29/33
64	56/69	29/34	30/36	29/33
68	55/69	29/34	29/36	29/33
72	55/69	29/34	29/36	28/33
76	51/69	27/34	29/36	24/33
80	47/69	26/34	29/36	23/33
84	43/69	26/34	29/36	17/33
88	43/69	24/34	27/36	15/33
92	40/69	21/34	26/36	14/33
96	38/69	19/34	22/36	13/33
100	30/69	16/34	19/36	12/33
104	28/69	14/34	15/36	10/33
108	25/69	11/34	14/36	6/33

Week	Female Groups			
	1	2	3	4
0	72/72	36/36	36/36	37/37
1	72/72	36/36	36/36	37/37
2	72/72	36/36	36/36	37/37
3	72/72	36/36	36/36	37/37
4	72/72	36/36	36/36	37/37
6	71/71	36/36	36/36	37/37
8	71/71	36/36	36/36	37/37
10	70/71	36/36	36/36	37/37
12	70/71	35/36	36/36	37/37
16	69/71	35/36	36/36	37/37
20	68/71	35/36	36/36	36/37
24	68/71	34/36	36/36	36/37
28	67/71	34/36	36/36	35/37
32	66/71	34/36	35/36	34/37
33	65/71	34/36	35/36	34/37
36	64/71	34/36	35/36	34/37
40	64/71	34/36	35/36	34/37
44	60/69	34/36	35/36	34/37
48	60/69	34/36	34/36	33/37
52	59/69	33/36	34/36	33/37
56	58/69	32/36	34/36	33/37
60	56/69	32/36	34/36	31/37
64	56/69	31/36	33/36	30/37
68	54/69	31/36	33/36	27/37
72	51/69	30/36	31/36	27/37
76	50/69	30/36	29/36	25/37
80	45/69	29/36	27/36	24/37
84	45/69	23/36	22/36	24/37
88	43/69	22/36	20/36	24/37
92	39/69	21/36	18/36	21/37
96	34/69	17/36	13/36	20/37
100	31/69	15/36	13/36	18/37
104	27/69	12/36	10/36	12/37
108	24/69	9/36	6/36	10/37

1 - Numerator is the number of surviving animals. Denominator is the number of animals at risk excepting animals that escaped or were accidentally killed.

APPENDIX VII-7A

FEMALE MICE IN E-76 FROM WHICH BLOOD WAS COLLECTED FOR HEMATOLOGY (H) OR CLINICAL CHEMISTRY (C) SPECIMENS AT INTERVALS OF 5, 10, 20, 40, 60, AND 110 WEEKS

	Interval (weeks)					
	5	10	20	40	60	110
Group 1F	01157H →	157H →	157H D	164H →	164H D	165HC
	01158H →	158H →	158H →	158H →	158H →	158H
	01159H →	159H →	159H →	159H →	159H D	168HC
	01160H →	160H →	160H →	160H →	160H →	160HC
	01161H →	161H D	163H →	163H →	163H D	172HC
	01162H →	162H →	162H →	162H →	162H D	173HC
						176HC
						180HC
						183HC
						184HC
Group 2F						185 C
	01265H →	265H →	265H →	265H →	265H D	272HC
	01266H →	266H →	266H →	266H →	266H D	275HC
	01267H →	267H →	267H →	267H →	267H D	284HC
	01268H →	268H →	268H →	268H →	268H D	285HC
	01269H →	269H →	269H →	269H →	269H →	269H
	01270H →	270H →	270H →	270H →	270H D	286HC
						288HC
						295HC
Group 3F	01337H →	337H →	337H →	337H →	337H D	347HC
	01338H →	338H →	338H →	338H →	338H D	349HC
	01339H →	339H →	339H →	339H →	339H D	350HC
	01340H →	340H →	340H →	340H →	340H D	354HC
	01341H →	341H →	341H →	341H →	341H →	341HC
	01342H →	342H →	342H →	342H →	342H D	361HC
Group 4F	01409H →	409H →	409H →	409H →	409H D	416HC
	01410H →	410H →	410H →	410H →	410H →	410HC
	01411H →	411H →	411H →	411H →	411H →	411HC
	01412H →	412H →	412H →	412H →	412H D	424HC
	01413H →	413H →	413H →	413H →	413H →	413HC
	01414H →	414H →	414H →	414H →	414H D	426HC
						427HC
						429H
						443H

D means animal died and was replaced

APPENDIX VII-78

MALE MICE IN E-76 FROM WHICH BLOOD WAS COLLECTED FOR HEMATOLOGY (H) OR CLINICAL CHEMISTRY (C) SPECIMENS AT INTERVALS OF 5, 10, 20, 40, 60, AND 110 WEEKS

		<u>Interval (weeks)</u>					
		<u>5</u>	<u>10</u>	<u>20</u>	<u>40</u>	<u>60</u>	<u>110</u>
Group 1M	01085H →	085H →	085H →	085H →	085H →	085H →	085HC
	01086H →	086H →	086H →	086H →	086H →	086H D	096HC
	01087H →	087H →	087H →	087H →	087H →	087H →	087HC
	01088H →	088H →	088H →	088H →	088H →	088H →	088HC
	01089H →	089H →	089H →	089H →	089H →	089H →	089HC
	01090H →	090H →	090H →	090H →	090H →	090H D	103HC
							107HC
							110HC
							112HC
							116HC
Group 2H	01229H →	229H →	229H →	229H →	229H →	229H →	229HC
	01230H →	230H →	230H →	230H →	230H →	230H D	240HC
	01231H →	231H →	231H →	231H →	231H →	231H D	241HC
	01232H →	232H →	232H →	232H →	232H →	232H D	249HC
	01233H →	233H →	233H →	233H →	233H →	233H D	255HC
	01234H →	234H →	234H →	234H →	234H →	234H →	234HC
							257HC
							258HC
							260HC
							263HC
Group 3M	01301H →	301H →	301H →	301H →	301H →	301H D	309HC
	01302H →	302H →	302H →	302H →	302H →	302H D	313HC
	01303H →	303H →	303H →	303H →	303H →	303H D	314HC
	01034H →	304H →	304H →	304H →	304H →	304H D	317HC
	01305H →	305H →	305H →	305H →	305H →	305H D	318HC
	01306H →	306H →	306H →	306H D	307H →	307H D	324HC
							326HC
Group 4H	01373H →	373H →	373H →	373H →	373H →	373H D	381HC
	01374H →	374H →	374H →	374H →	374H →	374H →	374HC
	01375H →	375H →	375H →	375H →	375H →	375H D	387HC
	01376H →	376H →	376H →	376H →	376H →	376H →	376HC
	01377H →	377H →	377H →	377H →	377H →	377H D	398HC
	01378H →	378H →	378H D	379H →	379H →	379H D	401H
							404HC

D means animal died and was replaced

APPENDIX VII-8

DISCREPANCIES NOTED IN HEMATOLOGICAL VALUES BETWEEN ENTRY BOOK FIGURE

No. 4, p 25-28 AND APPENDIX TABLE No. 2, p 9-53

Interval (weeks)	Group	Parameter	HLA Value	Type of Discrepancy	UAREP Value
5	2M	Hgb (\bar{x})	17.4	C due to T	17.28
5	2M	Hgb (S.D.)	± 0.7	C due to T	0.564
5	2M	WBC	± 4.3	R	4.2 (4.255)
5	3M	Pro-Time	9.3	R	9.2 (9.250)
10	4M	RBC	S^+	ST	Appendix VII-8
10	4M	WBC	20.1	R	20.0 (20.05)
10	4M	WBC	S^+	ST	Appendix VII-8
10	3M	Pro-Time	S^+	ST	Appendix VII-8
20	3M	Hct (S.D.)	± 1.0	R	0.9 (.949)
20	3M	WBC (S.D.)	± 4.1	R	4.0 (4.047)
40	3M	Hct	2.9	R	2.8 (2.695)
40	3M	RBC	S^+	ST	Appendix VII-8
40	1M	Pro-Time	8.5	R	8.4 (8.450)
40	4M	RBC	S^+	ST	Appendix VII-8
40	4M	WBC	S^+	ST	Appendix VII-8
60	1M	Pro-Time	8.3	R	8.2 (8.250)
60	2M	RBC	8.95	R	8.96 (8.955)
60	3M	Pro-Time	± 0.5	R	0.4 (.458)
60	4M	WBC	15.5	R	15.45 (15.450)
60	4M	Pro-Time	8.3	R	8.2 (8.251)
110	3M	RBC	± 0.71	R	0.70 (.7050)
5	1F	Pro-Time	8.9	R	8.8 (8.850)
5	2F	Hgb	17.1	R	17.0 (17.05)
5	3F	Pro-Time	8.9	R	8.8 (8.850)
5	4F	RBC	± 0.29	R	0.28 (.2855)
10	1F	Pro-Time	± 0.7	T	0.6
10	2F	Hct	50.3	R	50.2 (50.250)
10	3F	Hgb	S^-	ST	Appendix VII-8
20	1F	WBC	± 3.9	R	3.8 (3.845)
20	2F	WBC	± 2.8	R	2.7 (2.746)
20	4F	Hct	50.3	R	50.2 (50.250)
20	3F	Hgb	S^+	ST	Appendix VII-8
40	2F	WBC	17.1	R	17.0 (17.050)

Appendix VII-8
continued, page two

<u>Interval (weeks)</u>	<u>Group</u>	<u>Parameter</u>	<u>HLA Value</u>	<u>Type of Discrepancy</u>	<u>UAREP Value</u>
40	2F	Pro-Time	8.7	R	8.6 (8.650)
40	2F	Hct	± 2.3	R	2.2 (2.253)
40	3F	Pro-Time	S ⁻	ST	Appendix VII-8
60	2F	Hgb	± 0.9	R	0.8 (.850)
110	2F	RBC (\bar{x})	7.31	R	7.32 (7.3150)
110	2F	RBC (S.D.)	± 0.75	R	0.74 (.7457)
110	3F	RBC	7.59	R	7.60 (7.595)
110	3F	WBC	9.7	R	9.6 (9.650)

APPENDIX VII-9

COMPARISON OF UAREP AND HLA STATISTICALLY SIGNIFICANT

GROUP DIFFERENCES IN HEMATOLOGY PARAMETERS

Parameter	Inter- val	Sex	ANOVA	Groups	Q	LSI	UAREP t-test	t-test value	HLA t-test
HGB	10	F	.22	1>3	ND	ND	N	(2.20)	S ⁻
	20	F	.14	1<3	ND	ND	S	3.10	S ⁺
RBC	5	F	.00	1>3	S	S	N	--	N
				1>4	N	S	S	2.36	S ⁻
				2>3	S	S	S	3.65	ND
				2>4	S	S	S	5.40	ND
	10	M	.06	1<2	ND	ND	N	(2.16)	N
				1<3	ND	ND	N	--	N
				1<4	ND	ND	S	2.05	S ⁺
	40	M	.06	1<3	ND	ND	S	2.92	S ⁺
				1<4	ND	ND	S	2.32	S ⁺
WBC	5	F	.17	1<2	ND	ND	S	2.47	S ⁺
	10	M	.04	1<4	S	S	S	2.46	S ⁻
				3<4	N	S	ND	--	ND
	20	F	.01	1<2	S	S	S	2.03	S ⁺
				2>3	S	S	S	4.74	ND
				2>4	S	S	N	(2.21)	ND
	40	M	.18	1<4	ND	ND	S	2.86	S ⁺
	110	F	.00	1<4	S	S	S	3.57	S ⁺
				2<4	S	S	S	2.61	ND
				3<4	S	S	S	2.81	ND
Pro- time	5	M	.04	1<3	N	S	S	2.53	S ⁺
				3>4	N	S	S	2.81	ND
	10	M	.04	1<3	N	S	N	(2.22)	S ⁺
				1<4	N	N	S	2.34	S ⁺
				2<3	N	S	N	(2.14)	ND
				2<4	N	N	N	(2.20)	ND

ANOVA indicates the exact probability that all group means are equal, based upon the F test for Analysis of Variance. UAREP applied the Analysis of Variance at a difference of $P < 0.05$. When values less than 0.05 were obtained the least significant difference (LSD) and Newman-Keuls (N) tests were run. If the F statistic was greater than 0.05, we did not do the LSD or Q tests because we accept the hypothesis (at the 5% level) that all the means being compared are equal. All ANOVA values of .00 in this report indicate less than 1% chance that means are equal.

S means $P < 0.05$

N means $P > 0.05$

UAREP used the two tailed t-test with 8 degrees of freedom, $t_{0.05} = 2.306$. HLA has not specified their critical value for $t_{0.05}$. They compared experimental groups with controls whereas UAREP did intergroup analysis comparison of all groups.

ND = not done

APPENDIX VII-10

STATISTICALLY SIGNIFICANT GROUP DIFFERENCES IN HEMATOLOGY DIFFERENTIAL COUNT DATA

Parameter	Interval	Sex	ANOVA	Groups	Q	LSD	UAREP t-test	t-test value
Segmented Polys	5	M	0.12	1<2	ND	ND	S	2.25
		F	0.05	1<2	N	S	S	2.86
				2<4	N	S	N	2.17
	10	F	0.10	2<4	ND	ND	S	2.69
	40	F	0.11	1>4	ND	ND	S	3.74
	110	F	0.05	1>3	N	S	N	1.99
Lymphocyte	5	M	0.08	1>2	ND	ND	S	2.35
				1>4	ND	ND	S	2.93
		F	0.04	1>2	N	S	S	2.85
				1>3	N	S	S	2.35
				2<4	N	S	N	2.14
	10	M	0.11	2<3	ND	ND	S	2.80
		F	0.03	1>2	N	S	N	2.06
				2<3	N	S	N	1.85
				2<4	S	S	S	2.70
	40	F	0.11	1<4	ND	ND	S	3.74
	60	F	0.05	1<2	N	S	N	1.87
				1<4	N	S	S	2.24
	110	F	0.04	1<3	N	S	N	2.07
				1<4	N	S	S	2.34
Monocyte	10	M	0.09	1>2	ND	ND	S	2.71
				1>3	ND	ND	S	2.50
	60	F	0.02	1>4	S	S	S	4.44
				2>4	N	S	N	1.94
Eosinophil	110	F	0.15	2<4	ND	ND	S	2.55
	10	F	0.09	2>3	ND	ND	S	2.87
	110	F	0.19	1>2	ND	ND	S	2.42

ANOVA indicates the exact probability that all group means are equal, based upon the F test for Analysis of Variance. UAREP applied the Analysis of Variance at a difference of $p < 0.05$. When values less than 0.05 were obtained the least significant difference (LSD) and Newman-Keuls (Q) tests were run. If the F statistic was greater than 0.05, we did not do the LSD or Q tests because we accept the hypothesis (at the 5% level) that all the means being compared are equal.

S means $p < 0.05$.

N means $p > 0.05$.

UAREP used the two tailed t-test with 8 degrees of freedom, $t_{0.05} = 2.306$. HLA has not specified their critical value for $t_{0.05}$. They compared experimental groups with controls whereas UAREP did intergroup analysis comparison of all groups.

ND = not done

APPENDIX VII-11

CONFIDENCE INTERVALS FOR HEMATOLOGY

Interval (weeks)	Sex	Parameter	Group Means				Confidence Interval	
			1	2	3	4	Low	High
5	Males	Hct	53.5	55.1	52.2	53.3	49.4	57.6
		Hgb	16.7	17.3	16.9	16.4	16.1	17.3
		RBC	8.38	8.47	8.76	8.62	7.31	9.45
		WBC	19.0	19.6	20.2	21.6	14.0	24.0
	Females	Hct	54.8	53.0	52.5	53.7	52.5	57.1
		Hgb	17.7	17.0	17.2	17.5	16.9	18.6
		RBC	8.02	8.51	7.34	7.25	7.23	8.81
		WBC	14.8	19.2*	18.0	18.4*	11.7	18.0
10	Males	Hct	49.6	50.0	49.8	49.7	48.2	51.0
		Hgb	15.2	16.0	15.2	14.8	13.7	16.7
		RBC	8.65	9.33	9.46*	9.57*	7.95	9.35
		WBC	15.8	17.5	16.4	20.1*	12.4	19.2
	Females	Hct	51.3	50.2	49.1	50.4	47.6	55.0
		Hgb	16.3	16.0	15.7*	16.0	16.0	16.6
		RBC	9.68	9.72	9.43	9.98	9.15	10.21
		WBC	19.7	19.4	19.6	17.6	16.0	23.4
20	Males	Hct	48.0	48.9	49.0	49.0	46.4	49.6
		Hgb	16.5	16.5	16.2	16.5	15.8	17.2
		RBC	7.48	7.99	8.66*	7.95	6.32	8.64
		WBC	17.2	17.9	18.2	19.0	12.4	22.0
	Females	Hct	50.2	50.7	51.7	50.2	46.9	53.4
		Hgb	16.4	16.8	17.1	16.6	15.9	16.9
		RBC	8.50	8.66	8.51	8.51	8.14	8.86
		WBC	14.3	19.8*	12.6	15.4	10.2	18.3
40	Males	Hct	46.5	46.8	46.8	47.0	44.1	48.9
		Hgb	15.3	15.5	15.6	15.4	14.6	16.0
		RBC	8.27	8.89	9.24*	8.97*	7.59	8.95
		WBC	14.0	18.4	17.2	21.2	9.9	18.1
	Females	Hct	47.7	47.8	47.3	48.6	44.7	50.6
		Hgb	15.5	15.7	15.4	15.9	14.5	16.4
		RBC	8.47	8.11	8.42	8.69	8.21	8.73
		WBC	13.2	17.0*	13.6	17.4*	9.7	16.8

Appendix VII-11
continued, page two

Interval (weeks)	Sex	Parameter	Group Means				Confidence Interval	
			1	2	3	4	Low	High
60	Males	Hct	46.0	46.8	47.7	46.6	42.6	49.4
		Hgb	15.5	15.2	15.1	15.6	14.8	16.1
		RBC	8.55	9.00	8.87	8.58	7.68	9.42
		WBC	14.5	15.5	13.5	15.4	8.7	20.3
	Females	Hct	47.7	49.4	46.4	47.5	45.2	50.1
		Hgb	15.5	16.0	15.4	15.7	14.8	16.1
		RBC	8.72	9.64*	9.29	8.88	8.09	9.35
		WBC	13.1	13.7	12.3	14.3	8.3	17.9
110	Males	Hct	44.0	42.4	44.8	42.9	39.6	48.3
		Hgb	14.5	13.9	15.0	14.4	12.9	16.2
		RBC	8.21	7.87	8.82	7.89	7.35	9.07
		WBC	16.6	14.0	11.3	14.0	9.7	23.4
	Females	Hct	41.8	41.6	41.6	40.9	39.2	44.4
		Hgb	14.0	14.1	14.0	13.8	13.1	14.8
		RBC	7.22	7.32	7.60	7.48	6.50	7.94
		WBC	9.8	10.7	9.6	17.8	7.9	11.7

* denotes mean values outside confidence interval

APPENDIX VII-12

BUN, SGPT AND ALKALINE PHOSPHATASE CONFIDENCE

INTERVAL FOR E-76

<u>Sex</u>	<u>Parameter</u>	<u>Control Means</u>				<u>Confidence Interval</u>	
		<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>Low</u>	<u>High</u>
Males	BUN(mg%)	37.0	35.4	55.0	66.5	16.2	57.8
Females		60.2	45.5	87.3	44.0	35.5	84.9
Males	SGPT(R-F)	40.0	43.8	37.8	39.5	25.9	54.1
Females		44.8	30.7	27.7	37.0	35.8	53.8
Males	Alk.	14.6	31.8	16.0	15.9	9.7	19.4
Females	Phos. (K-A)	16.2	16.3	18.9	16.2	10.4	21.9

APPENDIX VII-13

DISCREPANCIES NOTED BY UAREP IN BLOOD CHEMISTRY VALUES IN APPENDIX
TABLE NO. 3, PAGES 46-49 AND FIGURES NO. 4A,
PAGE 29 OF ENTRY BOOK E-76

<u>Group</u>	<u>Interval (weeks)</u>	<u>Para- meter</u>	<u>Searle Value</u>	<u>UAREP Value</u>	<u>Type of Discrepancy</u>
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Data from Appendix Table No. 3

1F	110	Alk. Phos.	±8.047	8.046 (8.0465)	R
3F	110	SGPT	±4.89	4.88 (4.885)	R
4F	110	SGPT	±13.15	13.14 (13.145)	R

Data from Results & discussion Summary
Figure No. 4A

1F	110	Alk. Phos.	±8.1	8.0 (8.04)	R
3F	110	BUN	±33.7	33.6 (33.65)	R

R=Rounding Discrepancy, all of which are inconsequential, involve
standard deviations and would not alter interpretation of results.

APPENDIX VII-14

COMPARISON OF UAREP AND HLA STATISTICALLY SIGNIFICANT GROUP DIFFERENCES
IN CLINICAL CHEMISTRY DETERMINATIONS

Parameter	Inter- val	Sex	ANOVA	Groups	Q	LSD	UAREP t-test	t-test value	HLA t-test
SGPT	110	F	.02	1>2	N	S	S	2.62	S ⁺
				1>3	S	S	S	3.16	S ⁺
L-Phenyl- alanine	110	F	.00	1>2	N	N	S	3.03	S ⁺
				1>3	N	S	S	4.26	S ⁺
				1>4	N	S	S	3.22	S ⁺

ANOVA indicates the exact probability that all group means are equal, based upon the F test for Analysis of Variance. UAREP applied the Analysis of Variance at a difference of $p < 0.05$. When values less than 0.05 were obtained the least significant difference (LSD) and Newman-Keuls (Q) tests were run. If the F statistic was greater than 0.05, we did not do the LSD or Q tests because we accept the hypothesis (at the 5% level) that all the means being compared are equal.

S means $p < 0.05$.

N means $p > 0.05$.

UAREP used the two tailed t-test with 8 degrees of freedom, $t_{0.05} = 2.306$. HLA has not specified their critical value for $t_{0.05}$. They compared experimental groups with controls whereas UAREP did intergroup analysis comparison of all groups.

ND = not done

APPENDIX VII-15
ANALYSIS OF URINE DATA

	Total No. available samples	Samples determined No.	%	Samples insufficient quantity No.	%	Samples not tested No.	%
pH	62	52	84%	7	11%	3	5%
Sugar	62	52	84%	6	10%	4	6%
Protein	62	55	89%	5	8%	2	3%
Occult Blood	62	56	91%	4	6%	2	3%
Phenyl- ketones	62	62	100%	0	0%	0	0%
RBC	62	48	78%	12	19%	2	3%
WBC	62	48	78%	12	19%	2	3%
Epithelial cell	62	48	78%	12	19%	2	3%
Amorphous	62	48	78%	12	19%	2	3%
Crystals	62	48	78%	12	19%	2	3%
Bacteria	62	48	78%	12	19%	2	3%
TOTAL:	682	438	83%	94	14%	23	3%

The number of animal samples per group was:

Group 1	Male	10
Group 1	Female	9
Group 2	Male	10
Group 2	Female	6
Group 3	Male	9
Group 3	Female	5
Group 4	Male	6
Group 4	Female	7

APPENDIX VII-16

DISCREPANCIES NOTED BY UAREP IN ORGAN AND ORGAN TO BODY WEIGHT
RATIOS IN APPENDIX TABLE 7, ENTRY BOOK E-76, PAGES 72-76

<u>Parameter</u>	<u>Group</u>	<u>HLA Value</u>	<u>Type of Discrepancy</u>	<u>UAREP Value</u>
Thyroid Weight (mean)	1F	0.007	R	0.008 (0.0075)
Thyroid Ratio (mean)	1F	0.03	R	0.02 (0.025)
Heart Ratio (S.D.)	1M	0.14	R	0.13 (0.133)
Heart Ratio (mean)	2M	0.68	R	0.69 (0.687)
Heart Weight (mean)	4M	0.05	R	0.04 (0.045)
Heart Ratio (S.D.)	3F	0.12	R	0.13 (0.126)
Liver Weight (S.D.)	3M	0.39	R	0.38 (0.385)
Kidney Ratio (mean)	2M	2.3	R	2.4 (2.36)
Adrenal Ratio (S.D.)	4M	0.015	R	0.014 (0.0145)
Adrenal Ratio (S.D.)	2F	0.009	R	0.01 (0.0096)
Adrenal Ratio (S.D.)	3F	0.006	R	0.007 (0.0067)
Prostate Weight (S.D.)	1M	0.03	R	0.04 (0.035)
Ovaries Weights (S.D.)	1F	0.082	R	0.081 (0.0814)
Ovaries Ratio (mean)	1F	0.35	R	0.36 (0.355)
Ovaries Ratio (S.D.)	3F	0.12	R	0.13 (0.127)
Ovaries Weight	4F		ST	See Appendix VII-17
Uterus Ratio	3F	3.1	C	3.0 (2.985)

APPENDIX VII-17

COMPARISON OF UAREP AND HLA STATISTICALLY SIGNIFICANT ORGAN WEIGHT
AND ORGAN TO BODY WEIGHT RATIOS

<u>Parameter</u>	<u>Sex</u>	<u>ANOVA</u>	<u>Groups</u>	<u>Q</u>	<u>LSD</u>	<u>UAREP t-test</u>	<u>t-test Value</u>	<u>HLA t-test</u>
Body Weight	M	.020	1<2	N	N	S	2.50	S ⁺
Thyroid Weight	F	.0003	1<3	S	S	S	4.44	S ⁺
			1<4	S	S	S	3.90	S ⁺
			2<3	S	S	S	2.77	N.D.
			2<4	S	S	S	2.54	N.D.
Thyroid Ratio	F	.001	1<3	S	S	S	3.63	S ⁺
			1<4	S	S	S	4.03	S ⁺
			2<3	S	S	N	----	N.D.
			2<4	S	S	S	2.44	N.D.
Liver Ratio	F	.040	3<4	S	S	S	3.07	N.D.
			2<4	N	S	N	----	N.D.
Ovary Weight	F	.363	1>4	N.D.	N.D.	N	----	S ⁻

S means $P < 0.05$, N means $P > 0.05$, and ND = not done

ANOVA indicates the exact probability that all group means are equal, based upon the F test for Analysis of Variance. UAREP applied The Analysis of Variance at a difference of $P < 0.05$. When values less than 0.05 were obtained the least significant difference (LSD) and Newman-Keuls (Q) tests were run. If the F statistic was greater than 0.05, we did not do the LSD or Q test because we accept the hypothesis (at the 5% level) that all the means being compared are equal.

UAREP used the two tailed t-test with 8 degrees of freedom, $t_{0.05} = 2.306$. HLA has not specified their critical value for $t_{0.05}$. They compared experimental groups with controls whereas UAREP did intergroup analysis comparison of all groups.

APPENDIX VII-18

COMPARISON OF TOTAL NUMBERS OF MICE WITH DIAGNOSES OF SPECIFIC TUMORS

MADE BY UAREP (U) AND EPL (E)

[illegible]

Appendix VII-18
continued, page 2

	Male Groups								Female Groups							
	Group 1		Group 2		Group 3		Group 4		Group 1		Group 2		Group 3		Group 4	
	U	E	U	E	U	E	U	E	U	E	U	E	U	E	U	E
Lymph node	62	48	5	5	5	4	29	28	63	51	12	5	14	5	32	27
Angioma	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0
Angiosarcoma	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Testis	65	65	12	13	24	24	31	32								
Interstitial cell tumor	1	2	0	0	0	0	0	0	Not applicable							
Brain	65	65	32	32	32	34	31	32	67	67	34	34	34	34	36	35
Meningioma	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Ovary									54	56	19	21	27	28	33	30
Granulosa cell tumor			Not applicable						0	0	1	1	1	1	0	0
Uterus									60	62	21	22	30	30	33	35
Leiomyoma									2	2	1	1	1	1	0	0
Leiomyosarcoma									1	2	0	0	2	2	1	1
Angioma									1	2	1	1	0	0	0	2
Angiosarcoma			Not applicable						0	0	0	1	0	0	0	0
Endometrial polyp									2	1	0	0	1	1	0	0
Carcinoma									0	1	0	0	0	0	0	0
Vagina									44	56	6	11	16	20	26	30
Carcinoma			Not applicable						1	2	0	0	0	0	0	0
Skin/Tissue Mass																
Sarcoma	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Poorly differentiated tumor	1	1	0	0	0	0	0	0	0	1	0	0	0	0	1	1
All Organs																
Lymphoma	7	8	3	2	2	2	1	1	11	9	6	5	6	6	8	7
Mesothelioma	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Metastatic tumor, primary site not determined	0	0	0	0	0	0	0	0	2	1	0	1	0	0	0	0
Total tumors	39	43	11	10	10	10	9	7	28	*30	16	15	17	*16	13	12
Original EPL totals		42		10		10		7		28		15		15		12
Average number of tumors per animal	.6	.6	.3	.3	.3	.3	.3	.2	.4	.4	.4	.4	.5	.4	.35	.3

^d-a discrepancy relating to counting of Angiosarcoma on animal 01-135, Group 1M. Figure No. 9, page 42, E-76 lists "Spleen and Liver-Angiosarcoma" with a footnote explaining that a primary site was not identified. UAREP chose to assume that the tumors were primary in the organs listed.

* totals differ because UAREP counted endometrial polyps, EPL did not.

APPENDIX VII-19

MICE WITH TUMORS HISTOLOGICALLY PROVEN BY UAREP WITH WEEK
OF PRESUMED INITIAL OBSERVATION OF TUMOR

<u>Group</u>	<u>Path Number</u>	<u>Animal Number</u>	<u>Tumor Type</u>	<u>Time (weeks)</u>
1M	100-322	01-085	Testis - Interstitial cell tumor	110
1M	100-323	01-087	Liver - Neoplastic nodule	110
			Lung - Alveolar adenoma	110
1M	100-324	01-088	Lung - Adenocarcinoma	110
1M	100-326	01-091	Lung - Alveolar adenoma	110
1M	100-327	01-092	Lymphoma	110
1M	100-329	01-103	Lung - Alveolar adenoma	110
1M	100-332	01-111	Spleen - Angioma	110
1M	100-333	01-112	Lung - Alveolar adenoma	110
1M	100-335	01-119	Lymphoma	110
1M	100-336	01-123	Lung - Alveolar adenoma	110
1M	100-339	01-145	Lung - Alveolar adenoma	110
1M	100-340	01-146	Liver - Hepatocellular carcinoma	110
			Liver - Poorly differentiated tumor, possibly lymphoma	110
			Lung - Alveolar adenoma	110
1M	100-341	01-152	Thyroid - Parathyroid adenoma	110
1M	100-342	01-154	Thyroid - Parathyroid adenoma	110
			Lung - Alveolar adenoma	110
			Lymphoma	110
1M	100-345	01-090	Lung - Alveolar adenoma	109

Appendix VII-19
(cont'd) page 2

<u>Group</u>	<u>Path Number</u>	<u>Animal Number</u>	<u>Tumor Type</u>	<u>Time (weeks)</u>
1M	100-346	01-093	Lymphoma	104
1M	100-347	01-095	Lung - Alveolar adenoma	108
1M	100-350	01-099	Lung - Alveolar adenoma	104
1M	100-351	01-100	Lung - Alveolar adenoma	76
1M	100-353	01-102	Lymphoma	61
1M	100-354	01-105	Lung - Alveolar adenoma	100
			Lymph node - Angiosarcoma	100
1M	100-357	01-109	Lung - Alveolar adenoma	76
1M	100-363	01-121	Kidney - Adenoma	104
1M	100-367	01-130	Lung - Alveolar adenoma	80
			Lymphoma	80
1M	100-368	01-131	Lung - Alveolar adenoma	109
1M	100-370	01-133	Mesothelioma	80
1M	100-372	01-135	Liver - Angioma	106
			Spleen - Angioma	106
1M	100-373	01-136	Liver - Neoplastic nodule	100
1M	100-374	01-137	Lung - Alveolar adenoma	91
1M	100-386	01-153	Lung - Alveolar adenoma	67
1M	100-387	01-155	Lymphoma	107
2M	100-427	01-249	Lung - Alveolar adenoma	110
			Stomach - Adenoma	110
2M	100-428	01-255	Liver - Hepatocellular carcinoma	110
			Lung - Alveolar adenoma	110

Appendix VII-19
(cont'd) page 3

<u>Group</u>	<u>Path Number</u>	<u>Animal Number</u>	<u>Tumor Type</u>	<u>Time (weeks)</u>
2M	100-439	01-237	Lymphoma	31
2M	100-441	01-244	Liver - Angioma	99
2M	100-444	01-248	Lymphoma	74
2M	100-451	01-261	Lymphoma	34
2M	100-452	01-262	Lung - Adenocarcinoma	91
			Lung - Alveolar adenoma	91
3M	100-456	01-308	Lung - Alveolar adenoma	110
3M	100-461	01-318	Lung - Alveolar adenoma	110
3M	100-462	01-324	Liver - Neoplastic nodule	110
3M	100-465	01-331	Lung - Alveolar adenoma	110
3M	100-466	01-332	Liver - Hepatocellular carcinoma	110
3M	100-467	01-301	Liver - Neoplastic nodule	97
3M	100-470	01-311	Lymphoma	95
3M	100-475	01-330	Lung - Alveolar adenoma	101
3M	100-483	01-319	Liver - Neoplastic nodule	85
3M	100-485	01-322	Lymphoma	57
4M	100-391	01-387	Lung - Adenocarcinoma	110
4M	100-392	01-398	Lung - Adenocarcinoma	110
			Lung - Alveolar adenoma	110
4M	100-393	01-404	Liver - Neoplastic nodule	110
4M	100-395	01-375	Salivary gland - Adenoma	96
4M	100-396	01-377	Lung - Alveolar adenoma	82
4M	100-407	01-390	Thyroid - Adenoma	87

<u>Group</u>	<u>Path Number</u>	<u>Animal Number</u>	<u>Tumor Type</u>	<u>Time (weeks)</u>
4M	100-416	01-401	Lung - Alveolar adenoma	110
4M	100-419	01-405	Lymphoma	51
1F	100-489	01-164	Lymphoma	110
1F	100-491	01-168	Uterus - Endometrial polyp	110
1F	100-492	01-170	Uterus - Endometrial polyp	110
			Lymphoma	110
1F	100-494	01-173	Lymphoma	110
1F	100-496	01-180	Vagina - Carcinoma	110
			Uterus - Leiomyosarcoma	110
1F	100-498	01-184	Lung - Alveolar adenoma	110
1F	100-500	01-187	Spleen - Angioma	110
1F	100-503	01-215	Uterus - Leiomyoma	110
1F	100-504	01-218	Lung - Alveolar adenoma	110
1F	100-512	01-163	Lymphoma	92
1F	100-516	01-174	Metastatic carcinoma, primary site not determined	65
1F	100-520	01-179	Lymphoma	55
1F	100-521	01-181	Lymphoma	99
1F	100-526	01-191	Lung - Alveolar adenoma	92
1F	100-529	01-194	Metastatic tumor, primary site not determined	103
1F	100-530	01-195	Uterus - Leiomyoma	60
1F	100-532	01-197	Lymphoma	68
1F	100-534	01-199	Lymphoma	49
1F	100-535	01-200	Uterus - Angioma	91
1F	100-544	01-211	Lymphoma	109

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(cont'd) page 5

<u>Group</u>	<u>Path Number</u>	<u>Animal Number</u>	<u>Tumor Type</u>	<u>Time (weeks)</u>
1F	100-546	01-213	Lung - Alveolar adenoma	108
1F	100-547	01-216	Lymphoma	104
1F	100-548	01-217	Liver - Neoplastic nodule	109
1F	100-550	01-222	Lung - Adenocarcinoma	101
			Pancreas - Spindle cell tumor	101
1F	100-552	01-224	Lymphoma	84
2F	100-591	01-272	Ovary - Granulosa cell tumor	110
			Uterus - Angioma	110
2F	100-592	01-275	Lung - Alveolar adenoma	110
2F	100-594	01-285	Lung - Alveolar adenoma	110
2F	100-596	01-288	Uterus - Leiomyoma	110
2F	100-598	01-265	Lung - Alveolar adenoma	107
2F	100-600	01-269	Lymphoma	110
2F	100-601	01-294	Spleen - Angioma	107
2F	100-602	01-300	Lymphoma	107
2F	100-603	01-266	Lymphoma	103
2F	100-604	01-268	Stomach - Carcinoma	82
2F	100-609	01-277	Pancreas - Malignant tumor	101
2F	100-616	01-289	Lymphoma	52
2F	100-620	01-293	Stomach - Malignant tumor	72
2F	100-621	01-296	Lymphoma	101
2F	100-624	01-299	Lymphoma	78
3F	100-626	01-347	Pancreas - Islet cell tumor	110

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(cont'd) page 6

<u>Group</u>	<u>Path Number</u>	<u>Animal Number</u>	<u>Tumor Type</u>	<u>Time (weeks)</u>
3F	100-627	01-349	Uterus - Endometrial polyp	110
3F	100-629	01-354	Ovary - Granulosa cell tumor	110
3F	100-633	01-344	Uterus - Leiomyosarcoma	107
3F	100-634	01-352	Lung - Alveolar adenoma	108
			Uterus - Leiomyoma	108
3F	100-635	01-362	Lung - Alveolar adenoma	103
3F	100-640	01-353	Spleen - Angiosarcoma	101
3F	100-641	01-356	Lymphoma	93
3F	100-643	01-365	Lymphoma	83
3F	100-644	01-367	Uterus - Leiomyosarcoma	95
			Lymphoma	95
3F	100-648	01-346	Lung - Alveolar adenoma	82
3F	100-650	01-338	Spleen Angiosarcoma	74
3F	100-651	01-340	Lymphoma	71
3F	100-652	01-348	Lymphoma	79
3F	100-657	01-366	Lymphoma	61
4F	100-556	01-410	Uterus - Leiomyosarcoma	110
4F	100-559	01-416	Lung - Alveolar adenoma	110
4F	100-565	01-391	Lymph node - Angioma	104
4F	100-569	01-415	Lung - Alveolar adenoma	104
4F	100-573	01-420	Lymphoma	68
4F	100-575	01-422	Lymphoma	56
4F	100-576	01-423	Lymphoma	80
4F	100-578	01-428	Lymphoma	89

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(cont'd) page 7

<u>Group</u>	<u>Path Number</u>	<u>Animal Number</u>	<u>Tumor Type</u>	<u>Time (weeks)</u>
4F	100-585	01-437	Skin - Angioma	25
4F	100-586	01-438	Lymphoma	102
			Tissue mass (skin) - Undiffer- entiated sarcoma	100
4F	100-588	01-440	Lymphoma	106
4F	100-589	01-442	Lymphoma	100
4F	100-399	01-380	Lymphoma	84

UAREP recognized that data on the time of onset of tumors is grossly approximate when using criteria either (a) of time of sacrifice or death with proven tumor not previously recognized, or (b) first date of clinical observation of swelling subsequently confirmed histologically as a tumor. Better data is not available.

APPENDIX VII-20

LISTING OF MISSING SECTIONS WITH TUMOR

DIAGNOSES BY EPL

Group	Path No.	Animal No.	Organ	UAREP	EPL
2M	100-429	01-257	Liver	No section	Angiosarcoma
2M	100-429	01-257	Lung	No section	Adenoma
4M	100-390	01-381	Liver	No section	Angioma
4M	100-418	01-403	Liver	No section	Angiosarcoma
1F	100-490	01-165	Uterus	No section	Carcinoma
1F	100-498	01-184	Uterus	No section	Angioma

APPENDIX VII-21

NUMBERS OF MALE AND FEMALE MICE WITH HISTOLOGICALLY PROVEN TUMORS
AS DIAGNOSED BY UAREP (U) AND EPL (E)

	Male Groups							
	1		2		3		4	
	U	E	U	E	U	E	U	E
Any tumor	31	32	7	7	10	10	8	7
All malignant tumors	10	15	5	4	3	5	3	4
Benign tumors	24	17	4	3	7	5	6	3
Primary pulmonary tumors	18	19	3	4	4	4	4	4
Vascular tumors	1	4	1	2	0	1	0	2
Lymphoreticular tumors	8	8	3	2	2	2	1	1

Female Groups								
Any tumor	25	24	15	14	15	14	13	12
All malignant tumors	15	17	9	9	9	9	9	9
Benign tumors	11	7	6	5	6	5	4	3
Primary pulmonary tumors	5	3	3	3	3	2	2	1
Vascular tumors	2	5	2	3	2	2	2	2
Lymphoreticular tumors	11	9	6	5	6	6	8	7

EPL data is from Figure No. 10, page 52 of Entry Book E-76. UAREP data is based on Appendix VII-18 and VII-19. HLA figures for benign tumors are lower than UAREP's because they are based on animals with only a benign tumor whereas UAREP figures include mice with both a benign and malignant tumor.

APPENDIX VII-22

COMPARISON OF COMPUTATIONS BY UAREP AND HLA OF PROBABILITIES OF TUMOR
INCIDENCE IN MALE AND FEMALE MICE RECEIVING ASPARTAME
OR SERVING AS CONTROLS

Group	Male				Significance P<0.05 HLA UAREP	Female				Significance P<0.05 HLA UAREP
	HLA	UAREP	HLA	UAREP		HLA	UAREP	HLA	UAREP	
	P	P	[N]	[N]		P	P	[N]	[N]	
Any Tumor										
1	76.6	87.4	41.8	35.5		64.8	72.1	37.0	34.7	
2	38.6	36.4	18.1	19.2	1vs2	78.1	83.3	17.9	18.0	
3	48.5	55.5	20.6	18.0	1vs3	69.9	78.6	20.0	19.1	
4	63.3	56.9	11.1	14.1		52.8	65.6	22.7	19.8	
Benign Tumors										
1	51.5	76.4	33.0	31.4		24.3	45.1	28.8	24.4	
2	18.1	30.0	16.6	13.3	1vs2 p=.04	50.4	57.1	9.9	10.5	
3	29.8	46.1	16.8	15.2		42.5	57.4	11.8	10.4	
4	32.2	47.4	9.3	12.7		20.4	33.5	14.7	11.9	
All Malignant Tumors										
1	41.6	41.4	36.1	24.1		48.0	49.1	35.4	30.6	
2	23.3	23.9	17.2	20.9		47.0	51.6	19.1	17.4	
3	23.2	16.6	21.6	18.1		45.5	45.3	19.8	19.9	
4	37.5	29.1	10.7	10.3		40.6	46.8	22.2	19.2	
Primary Lung Tumors										
1	56.1	64.4	33.9	27.9		11.7	24.9	25.6	42.7	
2	30.5	26.2	13.1	11.4		33.3	33.3	9.0	9.0	
3	25.5	34.8	15.7	11.5	1vs3	12.5	29.7	16.0	10.1	
4	45.7	40.6	8.8	9.8		7.6	22.2	13.2	9.0	
Lymphoreticular Tumors										
1	24.7	35.8	32.4	22.3		25.0	36.6	36.0	30.0	
2	6.2	9.1	32.3	33.0	1vs2	30.3	45.7	16.5	13.1	
3	7.1	7.3	28.2	27.4		23.4	22.9	25.6	26.2	
4	3.3	3.3	30.3	30.3		29.4	39.5	24.1	20.2	
Vascular Tumors										
1	13.3	6.5	30.1	15.4		19.3	8.8	25.9	22.7	
2	14.2	5.1	14.1	19.6		24.7	23.5	12.1	8.5	
3	5.7	0	17.5	0		10.9	17.1	18.3	11.7	
4	21.0	0	9.5	0		13.8	14.2	14.5	14.1	

Only group comparisons which are statistically significant are shown under the appropriate columns

P= calculated probability, X100, of developing a tumor during the total test period

[N]=estimate of "effective number" of animals on test over the entire period [number of tumor bearing mice/P]

APPENDIX VII-23
SIGNIFICANT DISCREPANCIES BETWEEN HISTOPATHOLOGIC DIAGNOSES
BY UAREP AND EPL ON E-76

Group	Animal Number	Path Number	Organ	EPL Diagnosis	UAREP Diagnosis
1M	01-087	100-323	Liver	Hepatoma, malignant	Hepatoma, benign
1M	01-088	100-324	Lung	Adenoma	Adenocarcinoma
1M	01-090	100-345	Liver	0	Hyperplasia 5, necrosis 5
1M	01-095	100-347	Testis	0	Inflammation 3
1M	01-101	100-352	Spleen	Angioma	Congestion 2
1M	01-107	100-330	Skeletal muscle	Myositis 3	X
1M	01-108	100-356	Small intestine	Mucosal erosion 4	0
1M	01-119	100-335	Liver Testis	Hepatoma, malignant Interstitial cell tumor	0 0
1M	01-121	100-363	Kidney	Adenocarcinoma	Adenoma
1M	01-124	100-365	Lymph node	Reticulum cell sarcoma	Hyperplasia 5
1M	01-133	100-370	Urinary Bladder Testis	X Mesothelioma	Squamous metaplasia 3 Interstitial cell hyperplasia 3
1M	01-135	100-372	Liver Spleen	Angiosarcoma Angiosarcoma	Angioma Angioma
1M	01-136	100-373	Liver	Hepatoma, malignant	Hepatoma, benign
1M	01-137	100-374	Liver	X	Cytomegaly 3, hyperplasia 3
1M	01-141	100-378	Eye Testis	Dacryoadenitis 3 0	X Interstitial cell hyperplasia 3
1M	01-145	100-339	Eye	Keratitis 3	X
1M	01-146	100-340	Kidney	Adenocarcinoma	Metastatic tumor
1M	01-152	100-341	Thyroid	X	Parathyroid adenoma
1M	01-154	100-342	Thyroid	Inflammation 2	Parathyroid adenoma
1M	01-155	100-387	Adrenal	Cortical adenoma	Subcapsular hyperplasia 3
1F	01-157	100-507	Lung	X	Congestion/edema 4
1F	01-158	100-508	Stomach	X	Inflammation 3
1F	01-164	100-409	Lymph node Bone marrow	Angioma Osteomyelitis 3	0 X
1F	01-166	100-513	Uterus	0	Cystic hyperplasia 3
1F	01-168	100-491	Spleen	X	Reticuloendothelial cell hyperplasia 4
1F	01-169	100-514	Lymph node	X	Hyperplasia 5
1F	01-170	100-492	Uterus	Glandular hyperplasia 3	Polyp
1F	01-171	100-515	Pancreas	0	Atrophy/fibrosis 5
1F	01-173	100-494	Tissue mass (lymph node)	Undifferentiated sarcoma	Lymphoma

X indicates section was unremarkable
0 indicates that no comparable diagnosis was recorded
1-5 degrees of severity of diagnosis as follows:
1 minimal
2 slight
3 moderate
4 moderately severe/high
5 severe/high

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cont. page 2

Group	Animal No.	Path No.	Organ	EPL Diagnosis	UAREP Diagnosis
1F	01-174	100-516	Brain	Meningioma	Metastatic carcinoma
1F	01-193	100-528	Heart	X	Fibrosis 3
1F	01-194	100-529	Lung	Epithelial hyperplasia 3	Metastatic tumor
1F	01-200	100-535	Lung	X	Congestion/edema 4
1F	01-203	100-537	Lung	X	Congestion/edema 4
1F	01-204	100-538	Uterus	Leiomyosarcoma	0
1F	01-212	100-545	Uterus	Endometriosis	0
1F	01-213	100-546	Lung	Epithelial hyperplasia 2	Adenoma
1F	01-216	100-547	Heart	Angioma	0
1F	01-217	100-548	Liver	Hepatoma, malignant	Hepatoma, benign
1F	01-222	100-550	Pancreas Lung Skeletal muscle	X Metastatic tumor Sarcoma	Spindle cell tumor Adenocarcinoma Metastatic tumor
1F	01-224	100-552	Lymph node	Lymphoid hyperplasia 4	Lymphoma
1F	01-228	100-506	Vagina	Carcinoma	X
2M	01-241	100-425	Heart	X	Amyloid 3, fibrosis 3
2M	01-244	100-441	Liver	0	Cytomegaly 4, diffuse hyperplasia 4
2M	01-253	100-447	Liver	0	Cytomegaly 3, diffuse hyperplasia 3
2M	01-261	100-451	Lymph node	Lymphoid hyperplasia 3	Lymphoma
2F	01-266	100-603	Lymph node	0	Lymphoma
2F	01-276	100-608	Uterus	Angiosarcoma	0
2F	01-277	100-609	Pancreas Uterus	0 Endometriosis 3	Malignant tumor 0
2F	01-285	100-594	Heart	0	Hamartoma
2F	01-291	100-618	Stomach	0	Ulceration/inflammation 5
2F	01-293	100-620	Stomach	Metastatic tumor	Malignant tumor, possibly lymphoma
3M	01-301	100-467	Liver	Nodular hyperplasia 3	Neoplastic nodule
3M	01-303	100-469	Lung	X	Congestion/edema 4
3M	01-311	100-470	Lung	X	Congestion/edema 4
3M	01-318	100-461	Liver	X	Cytomegaly 3
3M	01-319	100-483	Lung Liver	X Hepatoma, malignant	Congestion/edema 4 Hepatoma, benign
3M	01-321	100-472	Lung	X	Congestion/edema 4
3M	01-324	100-462	Liver	Hepatoma, malignant	Hepatoma, benign
3M	01-327	100-473	Skeletal muscle	0	Myositis 3
3M	01-330	100-475	Testis	Hyperspermatogenesis 5	Interstitial cell hyperplasia 4
3F	01-352	100-634	Lung	X	Alveolar adenoma
3F	01-353	100-640	Spleen	Angioma	Angiosarcoma
3F	01-357	100-654	Spleen	Lymphoid depletion 4	X
3F	01-361	100-630	Urinary Bladder	Inflammation 3	0
3F	01-371	100-659	Uterus	0	Hemorrhage/thrombosis 4
4M	01-375	100-395	Salivary gland	0	Adenoma
4M	01-377	100-396	Testis	Hyperspermatogenesis 3 Interstitial cell hyperplasia 3	0 0

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cont. page 3

Group	Animal No.	Path No.	Organ	EPL Diagnosis	UAREP Diagnosis
4M	01-390	100-407	Thyroid	Hyperplasia 3	Adenoma
4M	01-392	100-408	Liver	0	Necrosis 3
4M	01-404	100-393	Liver	0	Hepatoma, benign
4F	01-391	100-565	Lymph node	Hemorrhage 3	Angioma
4F	01-414	100-568	Uterus	Angioma	Hemorrhage/thrombosis 4
4F	01-421	100-574	Spleen	Lymphoid hyperplasia 3	Extramedullary hematopoiesis 4, lymphoid depletion 4
4F	01-431	100-579	Kidney	0	Amyloid 5
4F	01-432	100-580	Uterus	0 Angioma	Endometritis 4 Hemorrhage/thrombosis 2
4F	01-437	100-585	Skin	0	Angioma
4F	01-438	100-586	Lymph node	0	Lymphoma

APPENDIX VII-24

RATIOS OF TISSUES CUT TO TISSUES SPECIFIED
BY EXPERIMENT PROTOCOL* E-76

	Group 1		Group 2		Group 3		Group 4	
	M	F	M	F	M	F	M	F
Total animals available at start of experiment	72	72	36	36	36	35	36	37
Protocol specified sections	72	72	12	12	24	24	36	36
Available mice at termination	66	68	32	34	34	35	32	35
Brain	65/66	67/68	32/32	34/34	34/34	34/35	32/32	35/35
Pituitary	47/66	54/68	10/12	12/12	15/24	20/24	20/32	19/35
Spinal cord	61/66	63/68	0/0	0/0	0/0	0/0	30/32	32/35
Eye	63/66	67/68	0/0	0/0	0/0	0/0	32/32	35/35
Salivary gland	66/66	65/68	1/0	4/0	0/0	0/0	31/32	33/35
Thyroid	61/66	54/68	12/12	11/12	23/24	22/24	32/32	29/35
Lung	66/66	67/68	13/12	16/12	25/24	24/24	32/32	35/35
Heart	66/66	66/68	13/12	12/12	25/24	24/24	32/32	35/35
Liver	66/66	68/68	15/12	18/12	25/24	25/24	32/32	34/35
Gall bladder	53/66	58/68	11/12	10/12	22/24	20/24	24/32	30/35
Spleen	63/66	62/68	17/12	19/12	26/24	30/24	30/32	35/35
Kidney	66/66	67/68	13/12	16/12	30/24	27/24	32/32	35/35
Adrenal	59/66	65/68	12/12	12/12	24/24	24/24	29/32	34/35
Stomach	66/66	67/68	14/12	20/12	26/24	29/24	32/32	35/35
Pancreas	66/66	64/68	14/12	13/12	24/24	25/24	30/32	33/35
Small intestine	63/66	66/68	15/12	14/12	25/24	25/24	31/32	34/35
Large intestine	66/66	65/68	13/12	12/12	25/24	26/24	31/32	34/35
Lymph node (mesenteric)	48/66	51/68	5/0	5/0	4/0	5/0	28/32	27/35

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(cont'd) page 2

	Group 1		Group 2		Group 3		Group 4	
	M	F	M	F	M	F	M	F
Urinary bladder	65/66	64/68	32/32	33/34	33/34	34/35	31/32	34/35
Gonad	65/66	56/68	13/12	21/12	24/24	2 8/24	32/32	32/35
Prostate/Uterus	59/66	62/68	14/12	22/12	21/24	30/24	30/32	35/35
Seminal vesicle/ Vagina	66/66	56/68	13/12	11/12	23/24	20/24	31/32	30/35
Mammary gland	66/66	65/68	1/12	0/12	0/24	0/24	31/32	34/35
Bone	66/66	68/68	0/0	0/0	0/0	0/0	32/32	35/35
Bone Marrow	65/66	68/68	0/0	1/0	0/0	0/0	32/32	35/35
Skeletal Muscle	65/66	65/68	0/0	1/0	1/0	0/0	31/32	34/35
Nerve	65/66	65/68	0/0	0/0	0/0	0/0	31/32	34/35
Thymus	0/66	0/68	0/0	0/0	0/0	0/0	0/32	0/35

* Appendix VII-1 shows the histopathology protocol specifications for E-76

APPENDIX VII-25

ANIMALS IN E-76, WHICH WERE NOT EXAMINED MICROSCOPICALLY

<u>Group</u>	<u>Mouse No.</u>	<u>Necropsy Comments</u>	<u>Tissues Taken</u>
1M	01094	Animal missing	---
	01104	Animal missing	---
	01120	No observations can be made due to advanced autolysis.	Yes
	01125	Due to very advanced autolysis, no gross observations to be made.	Yes
	01127	Animal missing	---
	01150	No necropsy performed due to very advanced autolysis. Animal preserved in its entirety.	No
2M	01238	No necropsy performed due to advanced autolysis. Animal preserved in entirety.	Yes**
	01239	No necropsy performed due to very advanced autolysis. Animal preserved in entirety.	Yes**
	01242	No necropsy performed due to very advanced autolysis. Animal preserved in its entirety.	Yes**
	01250	Animal missing	--
3M	01323	No necropsy performed due to very advanced autolysis. Preserved in its entirety.	No**

Appendix VII-25
(cont'd) page 2

<u>Group</u>	<u>Mouse No.</u>	<u>Necropsy Comments</u>	<u>Tissues Taken</u>
3M	01334	Due to advanced autolysis, no gross observations made. Whole animal preserved.	No**
	01373	No necropsy performed due to very advanced autolysis of animal. Animal preserved in entirety	No**
4M	01400	No observations made due to very advanced autolysis. Mouse taken whole.	Yes
	01406	Animal missing	---
1F	01167	Animal missing	---
	01202	Due to very advanced autolysis, all tissue preserved with carcass.	Yes
	01214	No necropsy performed due to advanced autolysis. Animal preserved in its entirety.	Yes
	01221	Animal missing	---
2F	01270	No necropsy performed due to very advanced autolysis. Animal preserved in its entirety.	No
	01278	No necropsy performed due to very advanced autolysis. Animal preserved in entirety.	No**
3F	01343	No necropsy performed due to very advanced autolysis. Animal preserved in entirety.	No**

Appendix VII-25
(cont'd) page 3

<u>Group</u>	<u>Mouse No.</u>	<u>Necropsy Comments</u>	<u>Tissues Taken</u>
4F	01430	No necropsy performed due to very advanced autolysis of animal. Preserved in entirety.	No**
	01441	No necropsy performed due to very advanced autolysis. Animal preserved in entirety.	No**

The individual animal necropsy sheets (see Appendix VI-30 for examples) contain a column entitled "Tissues taken". The organs intended to have tissues taken at necropsy are precoded. The prosector is to check tissue removed for fixation. Whether organs were checked on the sheet is shown by Yes or No.

**Indicates that "Carcass" was added and checked under "Tissues taken" column on the necropsy sheet.

CHAPTER VIII

E-86: A SUPPLEMENTAL STUDY OF DOG BRAINS FROM A 106-WEEK

ORAL TOXICITY STUDY

Because of Searle's concern that some of the lesions initially reported in the dog brains by Microscopy for Biologic Research (MBR) on E-28 might be of some significance, Searle had additional blocks of brains sectioned. Six of the dogs were reported as showing minimal, focal, subependymal proliferation of glial cells in the third ventricle. The brain sections were submitted to Dr. J. R. M. Innes on July 30, 1973, and he returned a report to Dr. R. G. McConnell on August 30, 1973, which was submitted to FDA as Entry 86. The dog brain slides were initially reviewed for UAREP by Dr. Jack Moulton, without knowledge of the specific findings of MBR or Dr. Innes. Dr. Moulton concluded that the dog brain slides showed no significant pathologic changes relating to abnormal proliferation of cells. In some of the sections the sub-commissural body and pineal gland were identified as potential sources of confusion to others.

Because of the potential importance of any brain tumors that might be related to compound administration, UAREP convened a panel of consultant neuropathologists on August 8 and 9, 1978 in the Department of Pathology at the University of Maryland School of Medicine in Baltimore. The panel that met with Dr. Stowell included Drs. Julio H. Garcia, Jan E. Leestma, John Strandberg, and F. Stephen Vogel. Dr. Vogel prepared the following report for UAREP.

This review was conducted without knowledge by the consultants as to the identities of individual case material relative to their origins from animals in treated or control groups. The selection of this material had been made by UAREP representatives based upon the occurrence of discrepancies or discussions that had arisen in previous reviews by MBR and by Dr. Innes. Subsequent identification of this material was as follows: pathology specimen 92005, high dose; 92009 or 92010 and 92011 or 92012, both medium dose; 92024 or 92026, 92027 or 92028, and 92021 or 92022, all three low dose. (Some of the specimens were listed as one of two numbers because two brain specimens have been placed in the same jar for storage purposes and therefore it was not known to which of the two animals a particular specimen related.) The issue raised by MBR relative to the above material was "minimal focal subependymal proliferation of the glial cells in the third ventricle." Cases 92001 and 92002, both high dose, were reviewed because of MBR's notation of "focal ependymal proliferations." Cases 91996, (control) 92001, 92002, and 92015 or 92016, all medium dose, were examined because of Dr. Innes' concern that they showed circumventricular organs (subcommissural or subfornical organs).

The histology slides had been prepared from brains sectioned coronally in multiple (standard) planes from the frontal pole to the medulla and cerebellum. No spinal tissues were included. Preparations were of reasonably good technical quality and were stained with hematoxylin and eosin, with selected sections of higher quality stained by luxol fast blue or periodic acid-Schiff.

To summarize this review, the consultants observed no histologic evidence of significant alterations in any of the brains. This evaluation was concerned with the possible presence of neoplastic lesions, inflammatory processes (in meninges or cerebrum, as indicated by either acute cellular exudates or chronic glial proliferations), vascular alterations (vasculitis or occlusive vascular disorders, or manifestations thereof as infarcts or hemorrhages), demyelinating states, and disturbances in the number, architectonics or cytologic appearance of neurons.

Since the circumventricular organs became an issue in previous reviews, the nature of these organs will be commented upon briefly. Several sections among the reviewed material contained portions of the subcommissural organ, the pineal, the subfornical organ and areas of prominence of the subependymal plate (residium of the embryonic germinal mantle).

The so-called circumventricular organs are a number of specialized structures occupying various locations in, beneath, and as evaginations of the ventricular or ependymal surfaces. They have been conveniently classified in relation to their embryonic origin as:

- 1) modifications of the ependyma (e.g. choroid plexus, subcommissural organ, paraphysis, pineal organ),
- 2) modifications of the subependymal tissues with high vascularization (area postrema, intercolumnar, or subfornical organ, supraoptic crest), and
- 3) differentiation of deep subependymal wall (e.g. neurohypophysis).

Subcommissural Organ

This organ is more prominent in lower phylogenetic orders, but is present in the dog as a specialized structure in the diencephalic-mesencephalic roof of the third ventricle, lying upon the posterior commissure between the cerebral aqueduct posteriorly and the pineal recess anteriorly. It is a defined structure in man only during embryonic development (first trimester).

In the dog, the subcommissural organ appears as a discrete region of specialized, ciliated, ependyma, stratified to a thickness of several cells and sometimes made prominent by their arrangement into rosettes. Special stains, particularly chrome-alum-hematoxylin or PAS, show these cells to contain secretory products, cystine-rich mucopolysaccharides. In lower animals, notably the fish and Amphioxus, a strand or fiber, "Reissner's fiber" is appended to the surface of the subcommissural organ and hangs free as it passes down the ventricular aquiduct, fourth ventricle, and spinal canal to terminate in the "caudal mass" near the filum terminale. Electron microscopy has confirmed the secretory activity of the subcommissural organ, by the presence of secretory granules, and has strengthened the impression that Reissner's fiber is extruded secretions rather than a flagellum. The fiber is inconspicuous in the dog and was not identified in the present material.

The organ is generally viewed in mammals, such as the dog, as an ancestral homologue of certain hypothalamic neurosecretory centers, such as the supraoptic nuclei with their product of antidiuretic hormone or the pineal, whose current role in the production of melatonin and other regulatory hormones has only recently come under investigation.

There is usually a prominence of the "spongioblastic" cells immediately beneath the modified ependyma of the subcommissural organ. This was particularly evident in the present material from the dog, and was attributed to the angle of the histologic sections rather than to any pathologic alteration. This subependymal zone shows some prominence of sinusoidal vascularization by capillaries, of the same nature, but less conspicuous than in the area postrema or subfornical organ.

In summary, the subcommissural organ was present in the sections from several of the dogs (92015, 92021, 92028). Its presence is not abnormal in this species and its appearance showed no deviation from normal.

The subcommissural organ is rudimentary or non-existent in adult man and for these many reasons this issue has no immediate relevance to the present study as it might relate to man.

Subfornical Organ (intercolumnar tubercle)

This organ is identified as a focal region of differentiation of the subependyma situated between the anterior border of the paraphysial arch (tela choroidea) and the lamina terminalis in the roof of the third ventricle. It occupies a position beneath the fornix (thus its name) and the margins of the foramina of Monro. Its structure suggests a homology with the area postrema, in that both possess rich vascularization by sinusoidal vessels. The blood brain barrier is notably lowered in both instances. The blood supply to the subfornical organ is massive, being derived from the branches of the anterior and posterior

choroidal arteries and the anterior cerebral (the subfornical branch). The function of the subfornical organ remains in doubt, yet peculiarities of its structure (shared with the area postrema and supraoptic crest) indicate that it is likely to play a role in cerebral metabolism. Transmission of electrolytes, or their absorption, perhaps with a sophisticated role in their regulation, have been functions attributed to this organ.

The subfornical organ was identified in several animals of the present study (92021 and 92027). It was interpreted as having a normal structure in each instance.

Subependymal Mantle

In several sections there was prominence of the subependymal "spongioblastic" layer particularly in the lateral walls of the lateral ventricles. This was viewed as a normal finding in the dog.

This structure is a residual of the embryonic germinal mantle, from which are derived the neuroblasts and spongioblasts that migrate outward through the developing cerebrum to populate it with nerve cells, astrocytes and oligodendroglia. There is normally a residuum of spongioblasts in the subependymal regions of the adult brain. It is somewhat more prominent in the dog than man. This prominence in this material was interpreted as normal.

Subependymal Gliosis

In all animals in which the mid-brain was presented for study, there was a population of fibrillary astrocytes in the subependymal region about the aqueduct of Sylvius. In some instances this was nodular and caused sessile elevations of the ependyma. In no instance was the lumen of an aqueduct impinged upon (to the contrary, it regularly appeared large). The presence of these astrocytes was unaccompanied by inflammation or necrosis and the ependymal layer was intact. The finding was interpreted as a normal variant for the dog.

SUMMARY STATEMENTS

- 1) The subcommissural organ is presented as an identifiable structure in this material and should not be misinterpreted as a pathologic lesion.
- 2) The subfornical organ is well shown in several animals of this study and has a normal morphology.
- 3) The presence of subependymal astroglia and spongioblasts is normal in the dog. Their presence had no apparent relevance to the purported "hydrocephalus" of previous reviewers.
- 4) No significant pathologic lesions were noted in the brains of these dogs related to the feeding of aspartame.

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1. Kühlenbeck, H. The Central Nervous System of Vertebrates, Vol. 3,
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CHAPTER IX

E-87: A SUPPLEMENTAL STUDY OF RAT BRAINS FROM TWO TUMOROGENICITY STUDIES, E-33,34 AND E-70

This Entry to the Food and Drug Administration was submitted by Searle on the basis of a report from Dr. J. R. M. Innes. Because of the occurrence of certain glial tumors in the rats in these two series of experiments, sections of brains of all rats were submitted to Dr. Innes for his evaluation. In his report he only mentioned specimens which had a primary intracranial tumor. UAREP submitted to its review panel of neuropathology experts, not only the slides on which EPL or Dr. Innes had diagnosed a brain tumor, but also certain other slides on which UAREP had diagnosed a brain tumor which had not been diagnosed by EPL or Dr. Innes.

Neuropathologic Diagnosis - The review was conducted by Drs. Julio H. Garcia, Jan E. Leestma, John Strandberg, and F. Stephen Vogel, with Dr. Robert E. Stowell, in the Department of Pathology, University of Maryland, on August 8-9, 1978. The review group had no previous knowledge of the identity of the case material with prior experimental protocols, although they were told in general the treatment which had been given to the animals. The following four pages of the report were prepared by Dr. Stephen Vogel.

The histologic sections had been chosen by representatives of UAREP on the basis of occurrence of central nervous system lesions or prior

discrepancies in their identification. They were derived from two studies, distinguished on the histologic slides by the prefix number 64- and 71-, and totaled 18 and 10 cases respectively. The pathology numbers prefixed 64- related to Entry 33,34, and those with the prefix 71- related to Entry 70. The preparations were in the coronal plane through the entire cerebrum or cerebellum and brain stem. No spinal tissue was included. Tissues were stained with hematoxylin and eosin, and were of good technical quality.

The issue focused principally upon the occurrence and classification of primary brain tumors, as is portrayed in Table IX-1.

The nomenclature of the classification employed by the consultant group conformed closely with that proposed recently by the World Health Organization, which, in turn, does not differ significantly from those proposed by Bailey and Cushing (1), Russell and Rubinstein (2), Berger and Vogel (3), and the one stated to have been used by the previous reviewers.

There were 16 examples of neoplasia encountered among the 18 rats from E-33,34. Of those, 10 were astroglial in nature, and all, being well differentiated, were classified as astrocytomas. One neoplasm (64-712) was composed of oligodendroglia. A lesion (64-881) that extensively involved the cerebellum was formed of extremely anaplastic spindle cells that were interpreted, in the absence of special stains, as a mixture of neuroblasts and mesenchymal cells, hence the designation medulloblastoma/meningeal sarcoma. In three cases (64-879, 64-901, 64-977), the meninges at the base of the brain were involved by tumor presumably derived from the pituitary. One case (64-900) showed a diffuse meningoencephalitis. Two cases (64-777 and 64-662) showed no

significant lesion in the sections provided for review.

Among the 10 rat brains examined from Entry 70, there were eight examples of neoplasia. These were represented by seven lesions of astroglial origin, all termed astrocytomas. However, a notable degree of anaplasia was exhibited by one (71-701). One tumor (71-581) was considered to be of ependymal origin. Two cases (71-686 and 71-660) had no significant lesions in the slides reviewed.

Addressing the nature of the neoplasms in both series, it is to be noted that there was a very high predominance of astrocytomas. These occurred in widespread distribution. Most were large lesions involving the deeper portions of a single hemisphere; case 64-715 was massive whereas 64-777 was largely restricted to one side of the thalamus. The thalamus was also involved in 71-526. Among these gliomas, there was one oligodendroglioma (64-712) and one ependymoma (71-581). This ratio of other gliomas to the 17 astrocytomas is not dissimilar to that in man.

A discrepancy exists between the incidence of ependymomas as recorded by Dr. Innes and that of this review group. This discrepancy underscores the difficulty of absolute identification of individual gliomas without the aid of special techniques that often include elaborate histologic stains and electron microscopy. The present reviewers interpreted the lesions as predominantly astrocytic, rather than ependymal for the following reasons.

- 1) At low magnification, the tumor cells were in patternless array, rather than demonstrating the perivascular haloes and occasional rosettes or pseudorosettes of the ependymoma.
- 2) At high magnification, most areas of most lesions were rich in neuroglial fibers, imparting a fibrillary quality to the matrix. Ependymomas may present dense glial processes but this feature is usually focal rather than diffuse.
- 3) The anaplastic cells had nuclei which were rounded or slightly elongated, whereas ependymal cell nuclei are characteristically elongate. The chromatin material within astrocytic nuclei is generally more finely particulate than in ependymal cells; such was the case in these lesions.

The review group viewed the lesion in 64-881 with ambivalence, appreciating that it was extremely anaplastic. Dr. Innes termed the lesion a sarcoma; EPL, a meningioma; and the UAREP pathologists at the University of Maryland, a medulloblastoma. The committee took no issue with the diagnosis of meningial sarcoma; however, a question was raised concerning the possibility of a medulloblastoma. This latter possibility rests upon the presence of pleomorphic, hyperchromatic nuclei in patternless sheets, with very scant cytoplasm. Rosettes were not observed.

A notable discrepancy existed in the diagnosis of meningioma by Dr. Innes and EPL on case 71-686, since no lesion was observed in the slides presented to the review committee. In view of the recognized

competence of Dr. Innes as a neuropathologist and the highly distinctive features of a meningioma, it was concluded that the present reviewers did not have the same slide as that reviewed by Dr. Innes.

In a similar vein, it needs to be pointed out that some of the case material shown to the UAREP review panel was not brought to the attention of Dr. Innes, as indicated in Table 9-1.

Slides on seven of these cases were not initially made available to UAREP. These slides were located at EPL with some difficulty. It is possible that there might have been an additional slide which contained a meningioma, as diagnosed by Dr. Innes and by EPL, but which was not transmitted to UAREP with the other missing slides.

Summary - The series of rat brains contained a number of unequivocal examples of neoplasia, principally primary gliomas. These neoplasms were predominantly astrocytomas and although generally well differentiated, were infiltrative and sometimes massive. Examples of oligodendroglial and ependymal lesions were present. The brain was impinged upon by contiguous tumors from the pituitary in several of the cases.

Survival Data - E-87 presents survival data at termination for rats in E-33,34 (Appendix IX-1) and E-70 (Appendix IX-2) which were reproduced from E-33, p 23, Figure 2 and E-70, p 22, Figure 2. There is an inconsequential discrepancy in that the mean survival time of control females is shown as 649 in E-70 and 648 in E-87. The other discrepancies in survival data between UAREP and HLA are discussed in Chapters IV and V.

Table 9-1

UAREP Neuropathology Consultant's Review of Rat Brains for E-33,34 & E-70

Group	Animal No.	Path No.	EPL Diagnosis	Innes Diagnosis	UAREP Diagnosis
<u>E-33,34</u>					
1M	83-651	64-603	no lesion (d)	no lesion (d)	astrocytoma
2M	83-745	64-775	---	astrocytoma	astrocytoma
2M	83-750	64-777	---	astrocytoma with ependymal components	no lesion
2F	83-769	64-989	---	astrocytoma	astrocytoma
2F	83-766	65-011	---	astrocytoma with ependymal components	astrocytoma
3M	83-837	64-764	---	astrocytoma with ependymal components	astrocytoma
3F	83-861	64-977	---	---	involved by pituitary carcinoma
4M	83-888	64-712	---	oligodendroglioma	oligodendroglioma
4M	83-892	64-713	---	ependymoma (d)	astrocytoma
4M	83-895	64-715	---	ependymoma (d)	astrocytoma
4M	83-919	64-707	---	astrocytoma with ependymal components	astrocytoma
4F	83-934	64-926	ependymoma (d)	ependymoma (d)	astrocytoma
5M	83-996	64-662	meningioma (d)	---	no lesion
5F	84-007	64-879	no lesion (d)	---	involved by pituitary carcinoma
5F	84-010	64-881	meningioma (d)	sarcoma (meningeal)	medulloblastoma/meningeal sarcoma
5F	84-019	64-888	astrocytoma/glioma	glioma, unclassified	astrocytoma
5F	84-033	64-900	meningioma (d)	---	meningoencephalitis
5F	84-034	64-901	no lesion (d)	---	involved by pituitary carcinoma
<u>E-70</u>					
1M	90-818	71-465	astrocytoma	astrocytoma	astrocytoma
1M	90-819	71-466	astrocytoma	astrocytoma	astrocytoma
1M	90-838	71-502	astrocytoma	astrocytoma	astrocytoma
1F	90-876	71-635	astrocytoma	astrocytoma	astrocytoma
1F	90-927	71-660	metastatic carcinoma (d)	---	no tumor
2M	90-943	71-581	ependymoma	ependymoma	ependymoma
2M	90-967	71-600	glioma/astrocytoma	astrocytoma	astrocytoma
2F	90-969	71-701	glioma/astrocytoma	astrocytoma	astrocytoma
3M	91-016	71-526	astrocytoma	astrocytoma	astrocytoma
3F	91-061	71-686	meningioma (d)	meningioma (d)	no lesion

(d) indicates significant discrepancy with UAREP diagnosis.

At the time of initial EPL review, there were no brain sections on E-33,34 rats in groups 2, 3, and 4, and no sections on group 2 rats in E-70, unless such brains showed gross lesions. Subsequently, the brains were sectioned on all rats and reviewed by Dr. Innes, but he only reported on tumors of glial and meningeal origin.

UAREP agrees with the conclusion of E-87 that differences in survival time were not a significant factor in differences in brain tumor incidence in E-33,34--but for a different reason. E-87, p 4 states, "Treated group mean survival rates per sex were comparable to concurrent control values except in the very high dose female group (no. 5). It is unlikely that the statistically significant reduction observed in the latter group exerted a meaningful effect on tumor incidence, since tumors in the lower dose groups occurred more often in males than in females and were not observed in Group 5 males. Thus, enhanced survival of females in this group would not be likely to increase the tumor incidence." As discussed in Chapter IV, the Group 5 females had an apparently reduced survival time because all survivors were sacrificed at 102 weeks instead of 104, and the rats which survived 102 weeks were omitted by Hazleton Laboratories from computation of the group's mean survival time.

Brain Tumor Incidence - E-87, page 4, states relative to E-33,34, "These results suggest an apparent treatment related, but not dosage or sex related occurrence of intracranial neoplasm. The overall incidence of such neoplasms in this two year study was 2.7%. In controls the incidence was zero; in treated rats it was 3.7%."

On page 6 of E-87 relative to E-70, it states, "These results indicate a random occurrence of intracranial neoplasms, unrelated to treatment, dosage, or sex. The overall occurrence of such neoplasms in this lifetime toxicity study was 3.2%. In controls the incidence was 3.3%; in treated rats it was 3.1%."

Because of the small number of tumors involved, UAREP feels it is appropriate to pool the results of E-33,34 and E-70 as follows:

Dosage of Aspartame	0	1-2 gm/kg	4-8 gm/kg	0-8 Total
tumors in males	4/120	4/120	5/120	13/360
tumors in females	1/120	3/120	3/120	7/360
tumors in M & F	5/240	7/240	8/240	20/720

The above results did not include one case (71-686) diagnosed as meningioma by Dr. Innes and EPL, on which the UAREP slide was normal. This incidence of glial tumors (2.6%) appears high but approaches that observed by others. UAREP applied a life table analysis to these tumors and found no significant differences at the 5% level between the controls and treatment groups. The incidence in males (3.6%) is higher than in females (1.9%). UAREP concludes there is no significant difference in these tumors in relation to sex, treatment, or dosage.

REFERENCES

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3. Burger, P. C. and Vogel, F. S. Surgical Pathology of the Nervous System and its Coverings. John Wiley & Sons. N. Y. 1976.

CHAPTER IX
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E-33,34 and E-70

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APPENDIX IX-1

TABLE 1

SC-18862: 104 WEEK ORAL TOXICITY STUDY IN THE RAT
P-T No. 838H71
(HLI 700-233)

Survival Data at Termination

Group No.	Test Level g/kg/day	Percent Survival \pm S.E.		Mean Survival Time Days	
		males	females	males	females
1	0 (Control)	38.4 \pm 6.3	46.7 \pm 6.5	569	657
2	1	45.0 \pm 7.9	57.5 \pm 7.9	629	663
3	2	52.5 \pm 7.9	50.0 \pm 8.0	636	640
4	4	57.5 \pm 7.9	35.0 \pm 7.6 ^{S-}	666	613
5	8	52.5 \pm 7.9	25.0 \pm 6.9 ^{S-}	651	423

S.E. = Standard Error.

S- = Significantly lower than control at $p < 0.05$.

Distribution of Intracranial Neoplasms

Animal No.	Sex	Survival	Diagnosis
		Time Days*	

GROUP NO. 1 - 0 G/KG (Control); 60 ♂, 60 ♀

None

GROUP NO. 2 - 1 G/KG; 40 ♂, 40 ♀

83745	Male	728	Astrocytoma
83750	Male	728	Astrocytoma with ependymal components
83766	Female	483	Astrocytoma with ependymal components
83769	Female	728	Astrocytoma

GROUP NO. 3 - 2 G/KG; 40 ♂, 40 ♀

83837	Male	539	Astrocytoma with ependymal components
-------	------	-----	---------------------------------------

GROUP NO. 4 - 4 G/KG; 40 ♂, 40 ♀

83888	Male	420	Oligodendroglioma
83892	Male	343	Ependymoma
83895	Male	700	Ependymoma
83919	Male	728	Astrocytoma with ependymal components
83934	Female	595	Ependymoma

GROUP NO. 5 - 8 G/KG; 40 ♂, 40 ♀

84010	Female	91	Sarcoma (Meningeal)
84019	Female	469	Glioma - unclassified

* Week of Survival X 7.

APPENDIX IX-2

TABLE 2

SC-18862: LIFE-TIME TOXICITY STUDY IN THE RAT
P-T No. 892H72
(HLI 700-240)

Survival Data at Termination					
Group No.	Test Level g/kg/day	Percent Survival S.E.		Mean Survival Time - Days	
		males	females	males	females
1	0 (Control)	41.7±6.4	48.4±6.5	643	648
2	2	50.0±8.0	45.0±7.9	654	632
3	4	57.5±7.9	52.5±7.9	650	661

S.E. = Standard Error.

No statistically significant differences in survival.

Distribution of Intracranial Neoplasms			
Animal No.	Sex	Survival Time Days*	Diagnosis
GROUP NO. 1 - 0 G/KG (Control); 60 , 60			
90818	Male	728	Astrocytoma
90319	Male	728	Astrocytoma
90838	Male	714	Astrocytoma
90876	Female	595	Astrocytoma
GROUP NO. 2 - 2 G/KG; 40 , 40			
90343	Male	378	Ependymoma
90967	Male	630	Astrocytoma
90969	Female	728	Astrocytoma
GROUP NO. 3 - 4 G/KG; 40 , 40			
91016	Male	728	Astrocytoma
91061	Female	595	Meningeoma

* Week of survival X 7.

APPENDIX IX-3

DISTRIBUTION BY SEX AND ASPARTAME TREATMENT OF ANIMALS WITH
INTRACRANIAL PRIMARY TUMORS IN E-33,34 AND E-70

APM (g/kg) → 0		E-33,34		4gm	6-8gm	Total by Sex
		1gm	2gm			
Group	1	2	3	4	5	
Male	83-651=1/60	83-745=1/40	83-837=1/40	83-888 } 83-892 } 83-895 } 83-919 } =4/40	--- 0/40	7/220
Female	--- 0/60	83-766 } 83-769 } =2/40	--- 0/40	83-934=1/40	84-010 } 84-019 } =2/40	5/220
M&F	1/120	3/80	1/80	5/80	2/80	12/440
		4/160		7/160		
		E-70				
Group	1	2	3			
Male	90-818 } 90-819 } =3/60 90-833 }	90-943 } 90-967 } =2/40	91-016=1/40			6/140
Female	90-876=1/60	90-969=1/40	0/40			2/140
M&F	4/120	3/80	1/80			8/280
		E-33,34 and E-70 combined				
Total Male	4/120	4/120		5/120		13/360
Total Female	1/120	3/120		3/120		7/360
M&F	5/240	7/240		8/240		20/720

CHAPTER X

E-9: TOXICOLOGICAL EVALUATION OF ASPARTAME IN THE NEONATAL RAT

This study (Searle Pathology-Toxicology No. 893H71) was performed at Hazleton Laboratories America (HLA Project 700-241) with rats from F_{2A} litters of the Two-Generation Reproduction Study discussed in Chapter XI. This project was designed to evaluate and characterize the effects of aspartame on hematological and biochemical parameters as well as on tissues of rats one through 21 days of age.

Twenty male and 20 female pups (five culled litters) were included in each group: control, low dose (2 g/kg) and high dose (4 g/kg). Ten pups from each group (2 per litter) were killed at 24 hours and 5, 15, and 21 days postpartum, and clinical laboratory studies and histopathology on selected tissues were performed. Dosage levels mentioned above were administered to the maternal animals and were available to the pups through the mother's milk. The report (E-9, p 3) raises, but does not comment on, the important question of whether or not aspartame as such would be present in the mother's milk. The report states: "The test material was added to the feed of the mothers in the test groups at the indicated dosage levels and was available to the pups through the milk of the mother, if present therein." (See also Appendix X-1). Diets containing appropriate amount of compound were available ad libitum to both mothers and pups. As the pups approached weaning age (21 days) they may have been eating some of the diet.

Personnel involved in this project were:

Dr. Frederick E. Reno, Project Manager, Toxicology Department

Dr. John F. Ferrell, Consultant Pathologist

Animals used were Charles River cesarean-derived rats (as described in Chapter XI). The first parental generation (grandparents of the rats used in this study) was obtained from Charles River Breeding Laboratories, Inc.

Animals to be killed were weighed, weights were recorded, and then an overdose of sodium pentobarbital was given. At twenty-four hours postpartum, ten pups (two per litter) from each treatment group were killed and gross necropsy was performed. Out of 24 tissues which were sampled and fixed (see Appendix X-1, p 2), the five examined microscopically were the heart, liver, kidney, stomach, and urinary bladder. One control pup which was found dead at four days postpartum was preserved whole in 10% neutral buffered formalin. Under the circumstances such fixation is appropriate for such small animals.

At 5, 15, and 21 days postpartum, ten pups from each group were killed and blood was taken from the abdominal aorta for clinical laboratory studies, gross necropsies were performed, and histopathology was done on selected tissues.

Hematology assays to be done (at 5, 15, and 21 days) included hematocrit, hemoglobin, erythrocyte count, and total and differential leukocyte counts.

Blood chemistries done at 15 and 21 days postpartum included fasting blood sugar, blood urea nitrogen, total serum protein, total serum bilirubin, serum glutamic-pyruvic transaminase, and serum alkaline phosphatase.

RESULTS AND DISCUSSION

A major problem in this study occurred in presentation of the data for white blood cell counts in rats killed at day 5. The white blood cell counts reported in the Entry Book were not corrected for the large numbers of normally occurring nucleated red cells in these young rats. These nucleated red cells had been counted when the differentials were done and the white counts corrected accordingly, but the data was overlooked at the bottom of the worksheets (Appendix X-2).

Table 10-1 lists white cell data in animals killed at five days and shows the often sizeable effect of the corrections.

Table 10-2 gives a statistical summary of the hematological comparisons for both the uncorrected white blood cell count data and the data after correction for presence of nucleated red cells. As the report (E-9) points out the control values of white cell counts were so variable that interpretation of the significance of changes is difficult. In summarizing the statistical results (Table 10-2) UAREP found the white counts in the control group were less than those in the low dose group at day 5, while the white counts in the low dose group were depressed in 15 and 21 day rats, and white counts in the high dose group were depressed in 15 and 21 day rats. There were no significant changes in hematocrit, hemoglobin, total erythrocyte counts, glucose, BUN, SGPT, alkaline phosphatase, total bilirubin, or total protein. The differential leukocyte counts also showed no changes.

Individual data on terminal body weights (E-9, p 22) showed no statistically significant differences among the various groups.

Comparison of Corrected and Uncorrected White Blood Cell Counts
on Rats Five Days Postpartum

<u>Group</u>	<u>Animal Number</u>	<u>Sex</u>	<u>Uncorrected White Cell Count</u>	<u>White Cell Count Corrected for NRBC*</u>
1	87-769	M	12.7	7.9
	87-771	M	42.7	8.5**
	87-775	M	5.1	4.1
	87-776	M	11.0	7.1
	87-786	M	9.7	5.0
2	87-799	M	10.3	7.8
	87-801	M	21.8	21.8
	87-803	M	21.6	15.0
	87-808	M	6.9	5.6
	87-809	M	4.9	3.4
3	87-829	M	9.7	4.9
	87-832	M	7.5	4.5
	87-833	M	4.8	3.5
	87-834	M	10.5	4.8
	87-840	M	22.8	14.4
1	87-769	F	4.0	3.5
	87-771	F	7.1	0.8**
	87-775	F	5.9	4.7
	87-776	F	8.7	4.1
	87-786	F	7.5	2.6

Table 10-1
(cont'd) page two

<u>Group</u>	<u>Animal Number</u>	<u>Sex</u>	<u>Uncorrected White Cell Count</u>	<u>White Cell Count Corrected for NRBC*</u>
2	87-799	F	6.7	5.3
	87-801	F	8.2	8.2
	87-803	F	8.8	7.6
	87-808	F	9.5	3.0
	87-809	F	7.0	5.9
3	87-829	F	10.5	5.4
	87-832	F	9.9	4.5
	87-833	F	6.0	3.1
	87-834	F	6.3	4.2
	87-840	F	11.6	6.4

*NRBC=nucleated red blood cells

**data does not show on data sheets provided UAREP; only in report
E-9

Table 10-2
Statistical Summary of Hematological Comparisons
in the Neonatal Rat

Parameters	Interval	Sex	ANOVA	Groups	LSD	Q	UAREP t-test	Value t-test	HLA t-test
WBC*	5	F	0.05	1<2	S	S	N	(2.04)	ND
WBC*	15	M	0.02	1>2	S	S	S	2.45	ND
				1>3	S	S	S	3.03	ND
WBC	15	M	0.01	1>2	S	S	S	2.71	S
				1>3	S	S	S	3.11	S
WBC	21	F	0.00	1>2	S	S	S	3.59	S
				1>3	S	S	S	2.86	S
WBC*	21	F	0.01	1>2	S	S	S	2.8	ND
				1>3	S	S	N	2.2	ND

* = values corrected for presence of nucleated RBC's

$t_{.05}$ at 7 degrees of freedom = 2.36; at 8 degrees of freedom $t_{.05}$ = 2.31

ND = not done with corrected values.

ANOVA indicates the probability ($P < 0.05$) that all group means are equal based upon the F test for Analysis of Variance (ANOVA).

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

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

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

Sexing of newborn rats is difficult. The report says (E-9, p 4): "pups evenly divided as to sex if possible. . . ." There were three instances of pups which were mis-sexed: animal 87-803 (Path No. 61-298) was originally grouped as a male and proved to be female at necropsy; animals 87-799 (Path No. 61-324 and 61-325) were grouped as one male and one female but were both found to be males at necropsy; animals 87-801 (Path No. 61-326; 61-327) originally grouped as one male and one female were shown to be both female at necropsy.

Histopathology

All the histopathology slides pertaining to the E-9 experiment were reviewed by a UAREP pathologist without knowledge of the findings contained in the final report on E-9. Selected slides were then reviewed by another pathologist who concurred in the diagnoses. There were no significant discrepancies in diagnoses between UAREP and EPL and all findings described by EPL pathologists were confirmed.

A change in the nuclei of the tubular cells of the inner renal cortex was described by EPL as consisting of mild or moderate vesiculation and hypertrophy occurring in the kidneys of 15 day old high dose group of both males and females, and to a lesser extent in the 21 day old high dose group. The 21 day old female low dose group showed minimal changes of the same nature in several animals. There was one 15 day control male that also showed a minimal degree of the same changes, but none of the female control animals had any detectable renal tubular cell alterations. These findings were all confirmed by UAREP with only minimal differences in the description of the severity and extent of the

changes. As an additional confirmatory procedure, all the kidney slides from the original experiment were rereviewed in a random manner with the identifying labels covered and similar results were obtained.

The lesion appears to involve the tubular cell nuclei of the outer stripe of the outer medulla. It consists of enlargement and vesiculation of the nuclei with alteration of the usual chromatin pattern. Mitotic figures are frequent. The change involves large areas of the outer medulla-inner cortical zone and is seen initially as a less densely stained and slightly more basophilic area. The nuclear changes became apparent upon higher magnification. It is interesting to note that the change is first observed in animals 15 days of age. This is about the same time that weaning occurs, and the rats began to ingest aspartame. The change became less noticeable at 21 days of age when eating is well established.

In an attempt to define and study this renal alteration more fully, additional kidneys were examined microscopically from rats 28 to 30 days of age which had been similarly exposed to aspartame (Chapter XI). EPL and UAREP pathologists found no changes in these kidneys comparable to those previously described in 15 day and 21 day old animals. However, UAREP did note that the manner of sectioning was such that the inner cortical zone was quite small in area on these additional slides from 28-30 day old rats, as compared with the 15-21 day old animals. Findings such as focal inflammation, distended tubules, and small cortical cysts were noted by both EPL and UAREP. No significant discrepancies were found.

The significance of these renal changes is unclear. It appears to occur most markedly at the time of weaning, when oral ingestion of

aspartame would be initiated. Presumably, the aspartame ingested by the mothers is metabolized to phenylalanine, aspartic acid, and other products in the intestine, liver, and other organs. Thus, aspartame would not be expected to appear in the mothers' milk. The report does not discuss this or present any evidence as to the levels of phenylalanine or aspartic acid in lactating rats in plasma and milk. Because of the sensitivity of maturing neonatal tissues to toxic damage, this work is of potential interest.

CONCLUSIONS

The only statistically significant changes were the depressed white blood cell counts which occurred in the treatment groups. The use of white counts which were not corrected for nucleated red cells did not materially affect the outcome of the statistical analyses.

Since aspartame is split by peptidases in the gut into phenylalanine and aspartic acid (E-28, p 32) and metabolized further in the liver and other organs, aspartame is unlikely to be present as such in maternal milk. Information was not available for correlation of phenylalanine levels in maternal plasma or milk with changes in plasma or tissues of the neonates.

UAREP agreed with the EPL histopathologic diagnoses including the nuclear changes in renal tubular cells which were described in the 15 and 21 day old rats. We also agree that these changes appear to be related to aspartame treatment.

CHAPTER X
LIST OF TABLES

- 10-1 Comparison of Corrected and Uncorrected White Blood Cell Counts
on Rats 5 Days Postpartum
- 10-2 Statistical Summary of Hematological Comparisons in the Neonatal
Rat

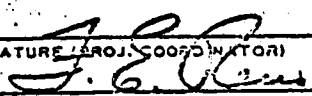
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X-2 Hematology Data Work Sheet for Group 1, 2, and 3 Neonatal Rats	861

APPENDIX X-1

HAZLETON LABORATORIES PROJECT SHEET

850969

PROJECT SHEET NO. <u>1</u>		PROJECT NO. <u>700-241</u>													
		PROJECT COORDINATOR Reno/Trutter	DATE March 19, 1971												
COMPOUND(S) SC-18862	LOT NO(S).	RECEIPT DATE 7-9-70	LN-NUMBER(S) 12,237K												
DIVISIONS PARTICIPATING Toxicology	DISTRIBUTION: CENTRAL FILE (2) EACH DIV. PARTICIPATING EACH DIV. DIRECTOR		Sponsor PROJ. COORD. DATA PROCESSING												
PHYSICAL AND CHEMICAL PROPERTIES		<div style="border: 1px solid black; padding: 5px; display: inline-block;"> RECEIVED MAR 26 1971 </div>													
SPECIAL PRECAUTIONS (INDICATE PERSONNEL HAZARDS, PROTECTIVE INSTRUCTIONS)		CHRONIC TOXICOLOGY SECTION													
REFERENCE INFORMATION															
PROGRESS REPORTS DUE	FINAL REPT DUE On Completion	INITIALS EER:rh	SIGNATURE (PROJ. COORDINATOR) 												
EXPERIMENTAL WORK to be performed in Small Animal Toxicology Section															
<u>Postpartum Clinical Pathology Study</u>															
<p><u>Objective</u> - The purpose of this study is to evaluate the effects of SC-18862 on hematological and biochemical parameters and on tissues of rats one through 21 days of age.</p> <p><u>Animal Groups</u> - The rats for this study will be selected from the F2A litters produced in the Two-Generation Reproduction Study, Hazleton Laboratories Project No. 700-239. After the F2A litters have been culled to eight pups per litter, 40 pups per group (five litters per group) will be utilized for evaluation of clinical-pathology parameters. The animals will be as follows:</p> <table border="1" style="margin-left: auto; margin-right: auto; border-collapse: collapse;"> <thead> <tr> <th style="text-align: center;">Group No.</th> <th style="text-align: center;">No. of Animals males and females</th> <th style="text-align: center;">Dose Levels* gm/kg b.w. of mother</th> </tr> </thead> <tbody> <tr> <td style="text-align: center;">1 (Control)</td> <td style="text-align: center;">40</td> <td style="text-align: center;">0</td> </tr> <tr> <td style="text-align: center;">2</td> <td style="text-align: center;">40</td> <td style="text-align: center;">2</td> </tr> <tr> <td style="text-align: center;">3</td> <td style="text-align: center;">40</td> <td style="text-align: center;">4</td> </tr> </tbody> </table> <p>*The test material will be added to the feed of the mothers and will be available to the pups through the milk, if present therein.</p> <p><u>Maintenance</u> - The individual litters will be maintained in the nesting boxes with their respective mothers.</p>				Group No.	No. of Animals males and females	Dose Levels* gm/kg b.w. of mother	1 (Control)	40	0	2	40	2	3	40	4
Group No.	No. of Animals males and females	Dose Levels* gm/kg b.w. of mother													
1 (Control)	40	0													
2	40	2													
3	40	4													
00001															

Project Sheet No. 1
Project No. 700-241 (850969)

- 2 -

March 19, 1971

Observations - These pups will be observed for compound effect on lactation, growth, and mortality (the same observations as made during the weaning period for the remaining F_{2A} litters on Project No. 700-239).

In addition, during the weaning period selection of pups for clinical laboratory studies and tissue preservation will be as follows:

At 24-hours postpartum - 10 pups from each group (two pups per each litter) will be sacrificed and tissues preserved.

At five, 15, and 21 days postpartum - 10 pups from each group (two pups per each litter) at each interval will be sacrificed, blood will be taken for clinical laboratory studies, and tissues will be preserved.

The pups selected for sacrifice from each group at each of the above intervals should be evenly divided as to sex if possible.

Necropsy - All pups at time of sacrifice will be subjected to gross necropsy.

Clinical Laboratory Studies* - At five, 15, and 21 days, the following studies will be performed on 10 pups per each group:

Hematology

hematocrit
hemoglobin
erythrocyte count

total leukocyte count
differential leukocyte count

Clinical Biochemistry

fasting blood sugar
blood urea nitrogen
total serum protein
total serum bilirubin

serum glutamic-pyruvic transaminase
serum alkaline phosphatase

*Sacrifice pups to take blood

Tissue Preservation - Preserve the following tissues from each pup in 10% neutral buffered formalin:

brain
pituitary
spinal cord
eye
thyroid
skin

lung
heart
liver
spleen
kidney
bone

adrenal
stomach
pancreas
small intestine
large intestine
bone marrow

lymph node
urinary bladder
testis
ovary
uterus
nerve with muscle

00002

Project Sheet No. 1
Project No. 700-241 (850969)

- 3 -

March 19, 1971

Histopathological Examination - To be performed on the following tissues from each pup:

heart
liver
kidney

stomach
urinary bladder

Report - At termination of the study, the results will be reported in full.

.0003

APPENDIX X-2

HEMATOLOGY

Project No. 700-241 Coordinator DR. RENO Species RAT Date 4-6-53
 Interval 5 DAY Group No. 1 Requested By CHRONIC

SEX MAL ✓SEX FEMALE ✓

ANIMAL NO. →	87-769	87-775	87-776	87-786			87-769	87-775	87-776	87-786	
CHEMISTRY LAB. NO. +	757009	757010	757011	757012			757013	757014	757015	757016	
DETERM. +											
SED. RATE mm/hr											
* CELL VOL. %	410	330	390	380			38.5	330	370	39.5	
* HGB %/100 ml.	11.9	8.8	9.9	9.3			9.3	8.5	9.0	10.6	
PLATELETS x 10 ³ /cm ³											
RETICS %											
* RBC x 10 ⁶ /cm ³	3.46	2.27	2.82	2.69			3.17	2.41	2.33	2.70	
WBC x 10 ³ /cm ³	12.7	5.1	11.0	9.7			4.0	5.9	8.7	7.5	
MY + ME %	0	0	0	0			0	0	0	0	
JU + BA %	11	11	14	0			6	11	0	3	
SEG %	8	16	30	16			34	26	32	24	
LYMPH %	88	83	66	84			60	70	66	70	
MONO %	1	0	0	0			0	2	0	2	
EOSIN %	1	0	0	0			0	1	2	1	
BASO %	10	0	0	0			0	0	0	0	
COAG. TIME min. sec.							487-786				
PRO. TIME sec.	#87-7750	#87-7760	#87-7761	#87-7762			#87-7763	#87-7764	#87-7765	#87-7766	
#87-7690 - lab. ant. polychromasia, <i>Leishmania</i> spp. #87-7860 - lab. ant. polychromasia, <i>Leishmania</i> spp. #87-7690 - lab. ant. polychromasia, <i>Leishmania</i> spp. #87-7750 - lab. ant. polychromasia, <i>Leishmania</i> spp.											
COMMENTS: Blood was started at 9:45 - stopped at 11:45, then completed at 12:30. #87-7690 - plasma <i>Leishmania</i> #87-7690 - 59 NRBCs, WBC = 7.9; #87-7750 - 22 NRBCs, WBC = 4.1 #87-7760 - 53 NRBCs, WBC = 7.1; #87-7860 - 92 NRBCs, WBC = 5.0 #87-7690 - 13 NRBCs, WBC = 3.5; #87-7750 - 24 NRBCs, WBC = 4.7 #87-7760 - 112 NRBCs, WBC = 4.1; #87-7860 - 182 NRBCs, WBC = 2.6											
.0005											

HEMATOLOGY

Project No. 700-241 Coordinator DR. RENO Species RAT Date 4-6-51
Interval 5 DAY Group No. 2 Requested By CHRONIC

SEX MALE ✓

SEX FEMALE ✓

ANIMAL NO. →	87799 ♂	87803 ♂	87809 ♂				87799 ♀	87803 ♀	87809 ♀			
CHEMISTRY LAB. NO. →	751017	751018	751019				751020	751021	751022			
DETERM. +												
SED. RATE mm/hr												
* CELL VOL.	330	380	340				350	37.5	350			
* $\frac{RBC}{100 \text{ ml}}$	8.8	9.9	9.0				9.0	9.9	10.3			
PLATELETS $\times 10^3/\text{cm}^3$												
RETICS												
* $\frac{RBC}{10^6/\text{cm}^3}$	2.66	2.86	2.37				2.63	2.61	2.60			
WBC $\times 10^3/\text{cm}^3$	10.3	21.6	4.9				6.7	8.8	7.0			
DIFFERENTIAL												
MY + ME	0	0	0				0	0	0			
JU + BA	0	1	0				2	3	2			
SEG	15	23	26				20	29	16			
LYMPH	85	76	72				78	64	81			
MONO	0	0	1				0	4	0			
EOSIN	0	0	1				0	0	1			
BASO	0	0	10				0	0	0			
COAG. TIME min. sec.												
PRO. TIME sec.												
COMMENTS:	<p>#87-799 ♂, 87-803 ♂, 87-809 ♂ - mod. polythromb., sm. ant. ^{large spinules} stippling</p> <p>#87-799 ♀ - sm. ant. polythromb.</p> <p>#87-803 ♀ - la. ant. polythromb., mod. bas. stippling</p> <p>#87-809 ♀ - la. ant. polythromb., sm. ant. bas. stippling</p>											
COMMENTS:	<p>#87-799 ♂ - 32 NRBC/400, WBC = 7.8; #87-803 ♂ - 44 NRBC/400, WBC 15.0</p> <p>#87-809 ♂ - 44 NRBC/400, WBC = 3.4; #87-799 ♀ - 26 NRBC/400, WBC = 5.3</p> <p>#87-803 ♀ - 15 NRBC/400, WBC = 7.6; #87-809 ♀ - 18 NRBC/400, WBC = 5.9</p>											

00008

HEMATOLOGY

Project No. 100-241 Coordinator DR. RENO Species RAT Date 4-6-71
Interval 5 DAY Group No. 3 Requested By CHRONIC

SEX MALE ✓

SEX FEMALE ✓

ANIMAL NO. →	87-829 ♂	87-832 ♂	87-833 ♂	87-834 ♂	87-840 ♂	87-829 ♀	87-832 ♀	87-833 ♀	87-834 ♀	87-840 ♀
CHEMISTRY LAB. NO. →	751023	751024	751025	751026	751027	751028	751029	751030	751031	751032
DETERM. + SED. RATE mm/hr										
* CELL VOL. %	36.0	34.5	29.0	29.0	30.0	34.5	38.0	34.5	30.0	33.0
* $\frac{HGB}{100} \pm 1$	9.0	10.6	8.9	8.2	8.8	8.2	9.9	9.3	7.7	9.3
PLATELETS $\times 10^3/cm^3$										
RETICS %										
* $\frac{RBC}{10^6/cm^3}$	2.61	2.05	2.39	2.28	2.27	2.25	2.68	2.67	2.25	2.36
* $\frac{WBC}{10^3/cm^3}$	9.7	7.5	4.8	10.5	22.8	10.5	9.9	6.0	6.3	11.6
DIFFERENTIAL										
MY + ME %	0	0	0	0	0	0	0	0	0	0
JU + BA %	1	13	1.6	12	13	4	13	13	4	13
SEG %	14	22	20	24	22	34	24	15	52	39
LYMPH %	83	71	74	74	75	60	71	81	44	55
* MONO %	1	1	3	0	0	0	1	2	1	0
EOSIN %	1	1	0	0	0	1	0	0	0	1
BAZO %	10	1	1	0	10	1	0	0	0	10
COAG. TIME min. sec.										
PRO. TIME sec.	#87-829 ♂	#87-833 ♂	#87-834 ♂	#87-840 ♂	#87-829 ♀	#87-832 ♀				
87-833 ♀, #87-834 ♀, 87-840 ♀ - mod. ant. polychromasia, small ant. basophilic stippling										
#87-832 - mod. polychrom., mod. bas. stippling										
COMMENTS: #87-829 ♂ - 96 NRBC, ^{corrected} WBC=4.9; #87-832 ♂ - 66 NRBC, ^{con.} WBC=4.5										
#87-833 ♂ - 36 NRBC, ^{con.} WBC=3.5; #87-834 ♂ - 116 NRBC, ^{con.} WBC=4.8										
#87-840 ♂ - 58 NRBC, ^{con.} WBC=4.4; #87-829 ♀ - 92 NRBC, ^{con.} WBC=5.4										
#87-832 ♀ - 116 NRBC, ^{con.} WBC=4.5; #87-833 ♀ - 92 NRBC, ^{con.} WBC=3.1										
#87-834 ♀ - 50 NRBC, ^{con.} WBC=4.2; #87-840 ♀ - 81 NRBC, ^{con.} WBC=6.4										
Null										

CHAPTER XI

E-11: TWO-GENERATION REPRODUCTION STUDY IN RATS

INTRODUCTION

This two generation study was designed to evaluate and characterize the effects of aspartame on reproductive performance of albino Charles River cesarean-derived (CRcd) rats obtained from Charles River Breeding Laboratories, Inc. The project sheets (Appendix XI-1) describe experimental design. The study began on September 1, 1970 and ended with weaning of the last litter on May 10, 1971. Hazleton Laboratories America (HLA) carried out the studies (HLA Project No. 700-239) and submitted their report to Searle on September 8, 1971.

Animals of each sex were randomized by a stratified body weight procedure. There were 12 males and 24 females in each of the three groups--control, 2 mg/kg, and 4 mg/kg aspartame.

Aspartame was mixed with Purina Laboratory Chow in a twin-shell blender in quantities calculated to provide the appropriate dietary level for the treated groups. Food and water were available ad libitum throughout the study. Fresh diets were prepared weekly. Starting dosages were adjusted on the basis of group mean body weights and food consumption for each sex determined during the pretreatment period. This dose was used until week 4 when body weights and food consumption were determined again. Those results were used until week 9 when new

determinations were made and compound requirements were increased and used until the termination of the study.

Before breeding, parental generation animals were maintained in individual wire cages and fed the appropriate diet for nine weeks until they were 90-100 days of age. Individual body weights, food consumption, and general observations were recorded initially and at four and nine weeks.

Among the criteria used by HLA to evaluate compound effects were survival, body weights, food consumption, physical appearance, and behavior of the parental generations, as well as indices of fertility, gestation, live birth, and lactation. Offspring were evaluated for litter size, appearance, behavior, body weights, and growth. Gross necropsies were performed on selected weanlings.

Reproductive Phases

First Breeding Phase: One male and two females of appropriate treatment group were placed in a breeding cage. During the three-week breeding period, males were rotated among the females at weekly intervals.

First Filial Generation (F_{1A} litters): After mating, males were returned to their individual cages, and females were placed in individual nesting boxes. Each litter was culled at twenty-four hours after birth and reduced to a maximum of 10 pups to be nursed.

After a 21 day nursing period, ten males and twenty females from each group were used as the second parental generation. Another 120 pups (60 of each sex) from the control group and 40 of each sex from

each of the treatment groups were utilized for a Lifetime Toxicity Study of aspartame (HLA Project No. 700-240, E-70; see Chapter V). Necropsies were performed on a number of excess weanlings prior to discard.

Second Breeding Phase: After the nine week prebreeding treatment period, the P_2 animals were bred in the same manner as the first parent group.

Second Filial Generation (F_{2A} litters): Observations were made and recorded as before with the exception that litters were culled to a maximum of eight pups to be nursed. Five F_{2A} litters per group were utilized for Project 700-241 Toxicological Evaluation of SC-18862 in the Neonatal Rat (E-9). The remaining litters per group were used in evaluation of F_{2A} weaning data. Necropsies were performed on ten pups of each sex per group.

Terminal Studies

Necropsies were not scheduled for any of the parental generations unless indicated by results of the study.

Necropsies were performed on approximately one-third of the pups in each F_{2A} litter at weaning and on whichever F_{1A} weanlings were not needed for the special study.

Tissue Preservation: Tissues from F_{2A} pups were preserved in 10% neutral buffered formalin and held for possible future examination-- brain, pituitary, eye, thyroid, lung, heart, liver, spleen, kidney, adrenal, stomach, pancreas, small intestine, large intestine, urinary bladder, testis, ovary, bone, bone marrow, and unusual lesions. Histo-pathologic diagnoses of kidney sections from ten male and ten female

rats in each group were reviewed in connection with observations reported in E-9 (See Chapter X).

Analysis of Data: The reproduction indices were:

$$\text{fertility index} = \frac{\text{number of pregnancies}}{\text{number of females mated}}$$

$$\text{gestation index} = \frac{\text{number of fullterm litters born}}{\text{number of pregnancies observed}}$$

$$\text{live birth index} = \frac{\text{number of pups alive at 24 hours}}{\text{number of pups at birth}}$$

$$\text{lactation index} = \frac{\text{number of pups weaned}}{\text{number of pups nursed}}$$

Statistical analysis of pup body weight data 24 hours postpartum and at weaning was done by t-test at the 5% level. The report did not state whether a one- or two-tailed test was used.

Personnel involved in preparation of this report included Dr. Frederick E. Reno, Project Manager, and Dr. D. C. Jessup, Project Coordinator.

RESULTS AND DISCUSSION

As stated in the project sheets (Appendix XI-1, p 3), the report was to include information on experiment design, physical appearance and behavior, and effects of aspartame on body weight, food consumption and survival of parental generations.

Experiment design is explained in detail in Entry Book E-11. There is no discussion of physical appearance and behavior in this report. Approximately 1500 bits of data were included in the Entry Book, and UAREP made 144 statistical comparisons. There are data in the report for individual body weights of the parental generation rats (E-11, pp 14-25). Survival data can be found in the report (E-11, pp 10-13). No information on individual food consumption was given either in the Entry Book or in the back-up data sent to UAREP. The only information concerning food consumption sent to UAREP gives means and range of consumption (high and low values) at weeks four and nine. There are computer sheets that note individual animals which had food consumption or body weight values outside 95% confidence limits for animals of the same sex and group at the indicated interval. UAREP feels that complete data on food consumption at weekly intervals, if not daily, would have been desirable considering, first, that the rats were in a rapid growth phase; and second, that the higher dose level of aspartame causes appetite depression in several species (E-28, dog; E-70, rat; E-33,34, rat). An amount of L-phenylalanine equivalent to that found in the high dose of aspartame has also been shown to cause appetite depression and diminished weight gain in rats (1).

The project sheets also stated that data reported on progeny would include:

- number and gross appearance of offspring
- number born alive
- number weaned
- lactation indices
- birth and weaning weights
- fertility indices
- gestation indices
- live birth indices

This information was summarized in the report (E-11, pp 26-27).

Tables were to be furnished showing data collected from each breeding phase, mean body weights, weight ranges, food consumption, and survival data for parents. These data were given in Table No. 1 (E-11, pp 10-13).

Statistical evaluation of the following were to be included: survival of parental generations, fertility indices, gestation indices, live birth indices, lactation indices, and weights of pups. Statistical analysis of pup body weight data comparing control and test groups was done by t-test at the 5% level. The other parameters were shown in Table 2 (E-11, pp 26-27) but a statistical evaluation was not presented.

UAREP's summary of statistical analyses is given in Tables 11-1 and 11-2.

The first and second filial generations in the high dose groups had body weights at weaning which were significantly lower than the respective control values. The pups in the high dose groups were also noticeably

Table 11-1
Statistical Summary of Mean Litter Weights

Generation	Interval	Sex	ANOVA	Group	LSD	Q	UAREP t-test	t Value	HLA t-test
P ₂ F ₂	24 hrs.	M	0.06	2>3	ND	ND	S	2.21	ND
P ₁ F ₁	Weaning	M	0.01	1>3	S	S	S	2.45	S ⁻
				2>3	S	S	S	2.33	ND
		F	0.00	1>3	S	S	S	3.40	S ⁻
				2>3	S	S	S	2.35	ND
P ₂ F ₂		M	0.00	1>3	S	S	S	3.47	S ⁻
				2>3	S	S	S	2.58	ND
		F	0.00	1>2	S	S	N	--	N
				1>3	S	S	S	4.90	S ⁻
				2>3	S	S	S	2.62	ND

S means P < 0.05

N means P > 0.05

ND means Not Done

LSD and Q were not done if the Analysis of Variance (ANOVA) table showed P > 0.05

Table 11-2
Statistical Summary of Parental Body Weights

Parameter	Interval (week)	Sex	ANOVA	Group	LSD	Q	UAREP t-test	t value	HLA t-test
Body Wt.	0	P ₁ M	0.05	1vs2vs3	N	N	N	---	ND
	0	P ₂ F	0.00	1>2	S	S	N	---	ND
				1>3	S	S	S	4.07	ND
				2>3	S	S	ND		ND
	9		0.06	1>2	ND	ND	S	2.24	ND
	terminal		0.01	1>2	S	S	S	2.86	ND
				1>3	S	S	S	2.61	ND

$t_{.05} = 2.02$ @ 37 degrees of freedom

P₁ = First generation parents

P₂ = Second generation parents

S means $P < 0.05$

N means $P > 0.05$

ND means Not Done

LSD and Q were not done if the ANOVA table showed $P > 0.05$.

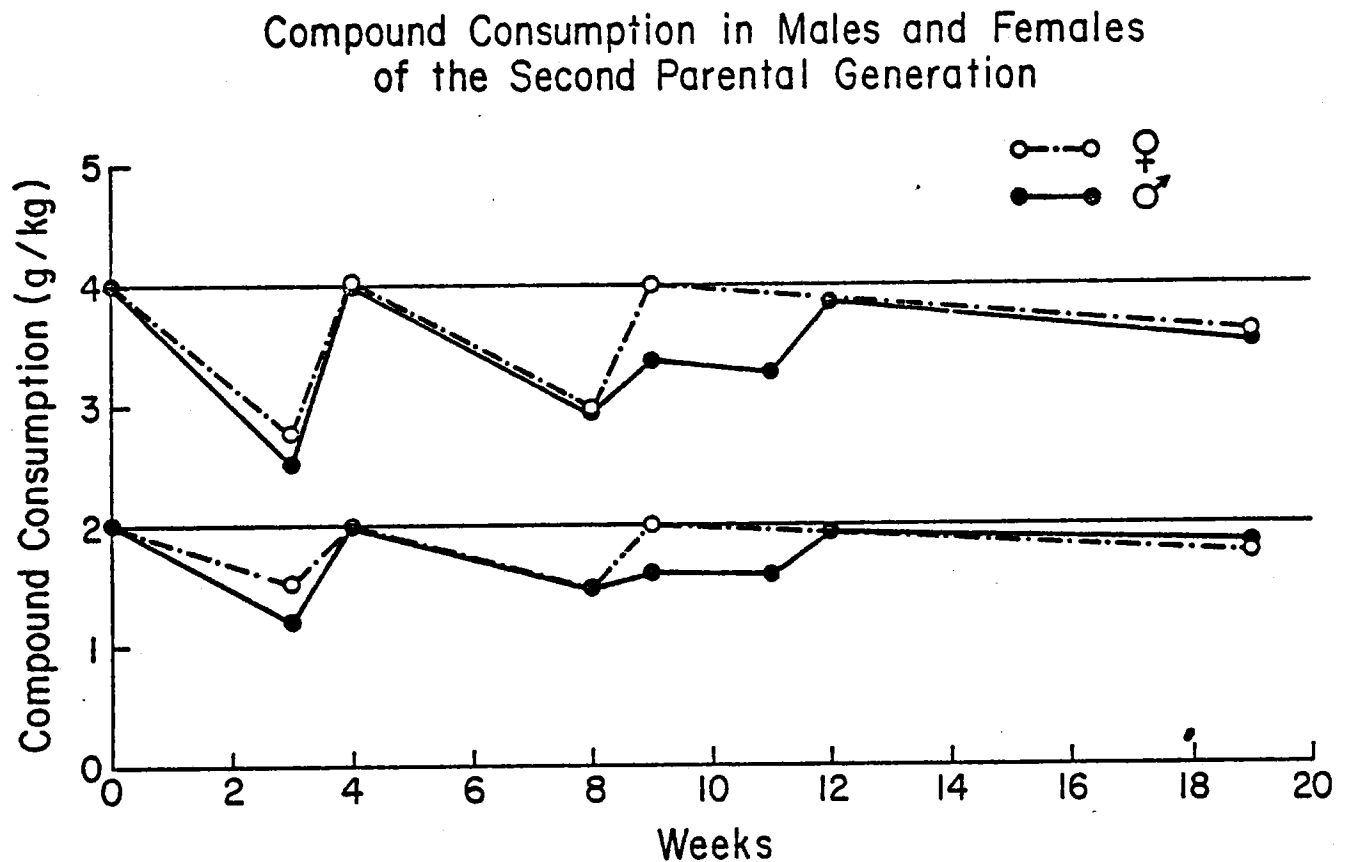
smaller than those in the control or low dose groups. In addition, the low dose (2 g/kg) female pups of the second generation had significantly lower mean litter weights than the controls.

Table 11-2 summarizes UAREP's statistical review of parental body weights. The only significant differences in parental body weights occurred in the second parental generation of females. At birth the high dose (4 g/kg) group was lower than both the control and 2 g/kg dose groups by ANOVA. At nine weeks the low dose (2 g/kg) group was lower than the controls by t-test but not LSD or Q. Terminal weighing showed both treatment groups to be statistically lower than the controls.

Compound consumption for each generation is shown in Figures 11-1 and 11-2. In Figure 11-1, it can be seen that compound consumption in both treatment groups in the first generation of parents was as much as 25% lower than specified in the protocol at various points during the study. Figure 11-2 shows that in the second parental generation compound consumption was as much as 38% lower than specified in the protocol between 0 and 4 weeks, and up to 25% lower at times during the remainder of the study.

UAREP validated and agreed with HLA data on fertility, gestation, live birth, and lactation indices.

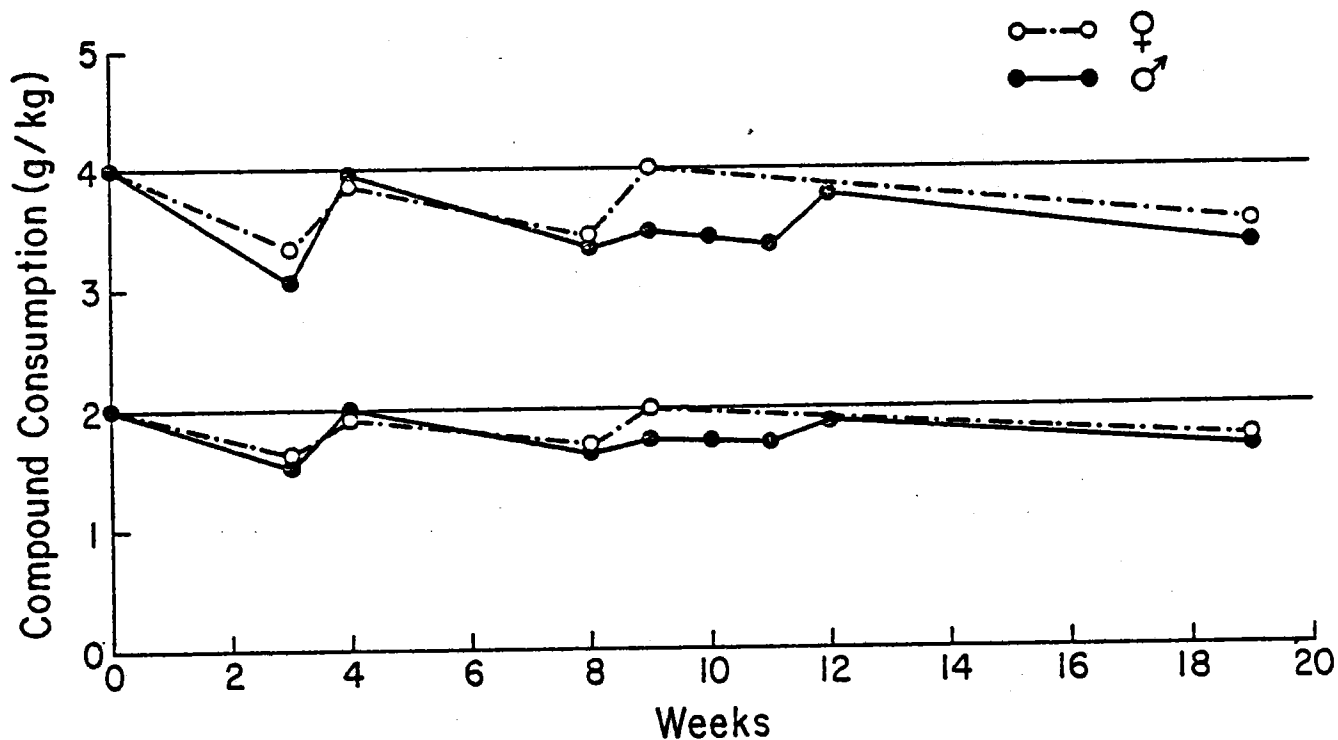
Figure 11-1



The above graph was constructed by pro-rating changes in body weight and food consumption which occurred between weeks 0-4, 4-9, and 9-19 (termination) for the first parental generation. Compound consumption was calculated on the basis of these data and plotted to give an indication of the probable fluctuations in compound consumption which occurred between adjustments of amount of compound in the diet.

Figure 11-2

Compound Consumption in Males and Females
of the First Parental Generation



The above graph was constructed by pro-rating changes in body weight and food consumption which occurred between weeks 0-4, 4-9, and 9-19 (termination) for the second parental generation. Compound consumption was calculated on the basis of these data and plotted to give an indication of the probable fluctuations in compound consumption which occurred between adjustments of amount of compound in the diet.

CONCLUSIONS

UAREP was generally in agreement with the findings as set forth in this report (E-11). However, UAREP found by Analysis of Variance that the mean litter weights of the low dose female offspring of the second generation were significantly lower than the controls (Table 11-1). Both HLA and UAREP t-tests were not significant for this comparison, while they were significant in other comparisons made by both HLA and UAREP.

The incidence of discrepancies or problems encountered by UAREP was less in this than in most of the other reports selected for review by UAREP. UAREP did note that consumption of aspartame was from 25 to 38% lower than planned at some stages of the experiment.

REFERENCES

1. Wang, H. L. and Waisman, H. A. Experimental phenylketonuria in rats. Proc. Soc. Exp. Biol. Med. 108:332, 1961.

APPENDICES

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HAZLETON LABORATORIES PROJECT SHEET

850754

PROJECT SHEET NO. <u>1</u>		PROJECT NO. <u>700-239</u>	
PROJECT COORDINATOR <u>Jessup/Reno</u>		DATE <u>July 14, 1970</u>	
COMPOUND(S) <u>SC-18862</u>	LOT NO(S)	RECEIPT DATE <u>7-9-70</u>	LH-NUMBER(S) <u>12, 237K</u>
DIVISIONS PARTICIPATING <u>Toxicology</u>	DISTRIBUTION: CENTRAL FILE (2) EACH DIV. PARTICIPATING EACH DIV. DIRECTOR		
SPONSOR PROJ. COORD. DATA PROCESSING			

PHYSICAL AND CHEMICAL PROPERTIES

SPECIAL PRECAUTIONS (INDICATE PERSONNEL HAZARDS, PROTECTIVE INSTRUCTIONS)

REFERENCE INFORMATION

PROGRESS REPORTS DUE	FINAL REPT DUE on completion	INITIALS DCJ:mtg	SIGNATURE OF PROJ. COORDINATOR <i>[Signature]</i>
----------------------	---------------------------------	---------------------	--

EXPERIMENTAL WORK to be performed in Small Animal Toxicology Section

Two-Generation Reproduction Study - Rats

Animal Groups - Sufficient weanling albino rats will be used to provide at least 20 pregnancies per group. The animals will be selected at random and placed in the following groups:

Group No.	No. of Animals		Dietary Levels gm/kg
	male	female	
1 (Control)	12	24	0
2	12	24	2
3	12	24	4

Group No. 1 will serve as a control group and will be treated in the same manner as the other groups, except that no test material will be administered.

The rats in each group will be individually housed.

Water and appropriate diets will be freely available during the course of the study.

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JUL 17 1970

CHRONIC TOXICOLOGY SECTION

Project Sheet No. 1
Project No. 700-239

- 2 -

July 14, 1970

Diet Preparation - The basal laboratory diet will consist of a commercial laboratory ration. The test material will be incorporated in the basal diet on a weight-per-weight basis and mixed in a twin-shell blender to provide the appropriate dietary level for each group. Fresh diets will be prepared every week.

Breeding - The P_1 generation will be individually housed and fed the appropriate diet until the animals are approximately 100 days of age or for 60 days prior to breeding. At the end of this period, one male and two females will be placed in each breeding cage for 21 days. The males will be rotated once weekly with the females in their respective groups.

The males will be returned to their individual cages after this period, and the females will be placed in individual nesting boxes until the F_{1A} litters are weaned.

At birth, each litter will be culled to produce 10 young per litter per group. Treatment of the mothers will continue through to weaning of the young.

At weaning of the F_{1A} litters, 10 males and 20 females will be randomly selected from each of Groups No. 1, No. 2, and No. 3; and they will be individually housed. These animals, the P_2 generation, will be fed the appropriate diets until they are caged at approximately 100 days of age.

The remaining weanlings from each group of the F_{1A} litters (approximately 170 rats) will be utilized for the Lifetime Toxicity Study to be performed as Hazleton Laboratories' Project No. 700-240.

The P_2 generation first litters (F_{2A}) will be culled to eight pups and weaned as were the F_{1A} litters. Fifteen of the expected 20 F_{2A} litters per group will be utilized for the evaluation of weaning data.

The remaining five F_{2A} litters per group will be utilized for the Clinical Pathology Study to be performed as Hazleton Laboratories' Project No. 700-241.

Observations - Individual body weights will be recorded for the P_1 generation initially and at four and nine weeks. Food consumption will be recorded at four and nine weeks. The same data will be recorded for the P_2 generation initially and at four and nine weeks.

For each individual litter, F_{1A} and F_{2A} , the following records will be maintained:

number of conceptions	stillbirths
litter size	deaths
live births	number of pups weaned

Mean weights of pups by sex will be recorded 24 hours after birth and at the time of weaning. Records will also be maintained of any gross abnormalities in the pups.

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Project Sheet No. 1
Project No. 700-239

- 3 -

July 14, 1970

Necropsies - Necropsies will not be performed on any of the parental (P_1 and P_2) generations unless the results of the study so dictate.

Necropsies will be performed on approximately one-third of the pups in each F_{2A} litter at weaning and on the weanlings from F_{1A} which are not needed for the special study.

Histopathological Examination - Tissues will not be preserved or examined from the parental generations, P_1 and P_2 , or the F_{1A} litter unless the results of the study so dictate.

The following tissues from 10 male and 10 female weanling pups chosen at random from the F_{2A} litters of the control and each test group will be held for possible future examination:

brain
pituitary
eye
thyroids
lung
heart
liver
spleen
kidneys
adrenals

stomach
pancreas
small intestine
large intestine
urinary bladder
gonads
bone
bone marrow
unusual lesions

Analysis and Report - The report will include the following:

experimental design
physical appearance
behavior

effects on body weight, food consumption, and survival of parental generations

- Data reported on progeny will include:

number and gross appearance
of offspring
number born alive
number weaned
lactation indices

birth and weaning weights
fertility indices
gestation indices
live birth indices

- Statistical evaluation:

survival of parental
generations
fertility indices
gestation indices

live birth indices
lactation indices
weights of pups

- Tables will be furnished showing:

data collected from each
breeding phase
mean body weights

weight ranges
food consumption
survival data for parents

00003

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HAZLETON LABORATORIES PROJECT SHEET

850754

PROJECT SHEET NO. <u>1</u>		PROJECT NO. <u>700-239</u>	
		PROJECT COORDINATOR <u>Jessup/Reno</u>	DATE <u>July 14, 1970</u>
COMPOUND(S) <u>SC-18862</u>	LOT NO(S).	RECEIPT DATE <u>7-9-70</u>	LH-NUMBER(S) <u>12,237K</u>
DIVISIONS PARTICIPATING <u>Toxicology</u>	DISTRIBUTION: CENTRAL FILE (2) <u>Sponsor</u> EACH DIV. PARTICIPATING <u>PROJ. COORD.</u> EACH DIV. DIRECTOR <u>DATA PROCESSING</u>		
PHYSICAL AND CHEMICAL PROPERTIES			
SPECIAL PRECAUTIONS (INDICATE PERSONNEL HAZARDS, PROTECTIVE INSTRUCTIONS)			
REFERENCE INFORMATION			
PROGRESS REPORTS DUE	FINAL REPT DUE on completion	INITIALS DCJ:mtg	SIGNATURE OF COORDINATOR <i>[Signature]</i>

EXPERIMENTAL WORK to be performed in Small Animal Toxicology Section

Two-Generation Reproduction Study - Rats

Animal Groups - Sufficient weanling albino rats will be used to provide at least 20 pregnancies per group. The animals will be selected at random and placed in the following groups:

Group No.	No. of Animals		Dietary Levels gm/kg
	male	female	
1 (Control)	12	24	0
2	12	24	2
3	12	24	4

Group No. 1 will serve as a control group and will be treated in the same manner as the other groups, except that no test material will be administered.

The rats in each group will be individually housed.

Water and appropriate diets will be freely available during the course of the study.

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CHRONIC TOXICOLOGY SECTION

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Project Sheet No. 1
Project No. 700-239

- 2 -

July 14, 1970

Diet Preparation - The basal laboratory diet will consist of a commercial laboratory ration. The test material will be incorporated in the basal diet on a weight-per-weight basis and mixed in a twin-shell blender to provide the appropriate dietary level for each group. Fresh diets will be prepared every week.

Breeding - The P_1 generation will be individually housed and fed the appropriate diet until the animals are approximately 100 days of age or for 60 days prior to breeding. At the end of this period, one male and two females will be placed in each breeding cage for 21 days. The males will be rotated once weekly with the females in their respective groups.

The males will be returned to their individual cages after this period, and the females will be placed in individual nesting boxes until the F_{1A} litters are weaned.

At birth, each litter will be culled to produce 10 young per litter per group. Treatment of the mothers will continue through to weaning of the young.

At weaning of the F_{1A} litters, 10 males and 20 females will be randomly selected from each of Groups No. 1, No. 2, and No. 3; and they will be individually housed. These animals, the P_2 generation, will be fed the appropriate diets until they are mated at approximately 100 days of age.

The remaining weanlings from each group of the F_{1A} litters (approximately 170 rats) will be utilized for the Lifetime Toxicity Study to be performed as Hazleton Laboratories' Project No. 700-240.

The P_2 generation first litters (F_{2A}) will be culled to eight pups and weaned as were the F_{1A} litters. Fifteen of the expected 20 F_{2A} litters per group will be utilized for the evaluation of weaning data.

The remaining five F_{2A} litters per group will be utilized for the Clinical Pathology Study to be performed as Hazleton Laboratories' Project No. 700-241.

Observations - Individual body weights will be recorded for the P_1 generation initially and at four and nine weeks. Food consumption will be recorded at four and nine weeks. The same data will be recorded for the P_2 generation initially and at four and nine weeks.

For each individual litter, F_{1A} and F_{2A} , the following records will be maintained:

number of conceptions	stillbirths
litter size	deaths
live births	number of pups weaned

Mean weights of pups by sex will be recorded 24 hours after birth and at the time of weaning. Records will also be maintained of any gross abnormalities in the pups.

00002

Project Sheet No. 1
Project No. 700-239

- 3 -

July 14, 1970

Necropsies - Necropsies will not be performed on any of the parental (P_1 and P_2) generations unless the results of the study so dictate.

Necropsies will be performed on approximately one-third of the pups in each F_{2A} litter at weaning and on the weanlings from F_{1A} which are not needed for the special study.

Histopathological Examination - Tissues will not be preserved or examined from the parental generations, P_1 and P_2 , or the F_{1A} litter unless the results of the study so dictate.

The following tissues from 10 male and 10 female weanling pups chosen at random from the F_{2A} litters of the control and each test group will be held for possible future examination:

brain	stomach
pituitary	pancreas
eye	small intestine
thyroids	large intestine
lung	urinary bladder
heart	gonads
liver	bone
spleen	bone marrow
kidneys	unusual lesions
adrenals	

Analysis and Report - The report will include the following:

experimental design	effects on body weight, food consumption, and survival of parental generations
physical appearance	
behavior	

- Data reported on progeny will include:

number and gross appearance of offspring	birth and weaning weights
number born alive	fertility indices
number weaned	gestation indices
lactation indices	live birth indices

- Statistical evaluation:

survival of parental generations	live birth indices
fertility indices	lactation indices
gestation indices	weights of pups

- Tables will be furnished showing:

data collected from each breeding phase	weight ranges
mean body weights	food consumption
	survival data for parents

00003

CHAPTER XII

E-19: A SWEETENING AGENT: ENDOCRINE STUDIES

INTRODUCTION

This brief report covers a battery of screening tests of aspartame most of which, according to Dr. Nutting, Director of Endocrinology at Searle, were used routinely for compounds undergoing pre-clinical testing. There were seven hormone related tests and six physiologic response tests.

Estrogenic Activity (Mouse Uterine Weight Assay); 24a2: Estrogenic activity of aspartame (SC-18862) and DKP (SC-19192) was determined by measuring increases in the uterine weights of immature (22-25 day old) mice. Control animals (oil only) were run as well as those given estrone as a reference standard. Results are summarized in Table 1 (E-19, p 2).

Estrogen Antagonism (Mouse Uterine Weight Assay); 24a2(-): Estrogen antagonism was determined in immature (22-25 day old) female mice again by measuring uterine weights. However, in this test all the mice were given 0.3 μ g estrone, and estrone plus progesterone was used as the reference standard. The role of progesterone in this test is to block the response of estrogen sensitive tissues. Results are summarized in Table 2 (E-19, p 4).

Progesterone-like Activity (Clauberg and Uterine Carbonic Anhydrase Assays);

24b2: Progesterone-like activity was measured in immature female rabbits primed for six days with 5 µg estradiol-17β. Both McPhail score and carbonic anhydrase concentration in the endometrium were measured. The McPhail score grades the arborization of the luminal epithelium of the uterus from +1 to +4 (a response of +1 indicates an unstimulated uterus). Proliferation of the glandular epithelium in estrogen-primed rabbits is brought about by progesterone. An increase in carbonic anhydrase activity indicates a progesterone-like response. Results are summarized in Table 3 (E-19, p 6).

Progesterone Antagonism (Clauberg and Uterine Carbonic Anhydrase Assays);

24b2(-): Progesterone antagonism is checked by the same assays. Test compound is given along with progesterone. Reduction in arborization and/or carbonic anhydrase activity indicates progesterone antagonism. Estrone in conjunction with progesterone inhibits the uterine response to progesterone, and is used as a reference for progesterone antagonism. Results are summarized in Table 4 (E-19, p 9).

Androgenic-Myotrophic Activity (Castrate Rat Test); 24c1, 9a1:

Androgenic-myotrophic activity was tested in castrated immature male rats by measuring the weights of the seminal vesicles, ventral prostate and levator ani muscle. A dose of compound is rated active for androgenicity if it produces a significant ($P < 0.01$) increase in the weight of the seminal vesicles and ventral prostate compared to the controls. Compound was considered active androgenically if it possessed a potency

greater than 25% of the methyl testosterone effect. Anabolic activity was based on a potency greater than 100% of the methyl testosterone effect. Increase in levator ani muscle weight compared to controls is taken as an index of myotrophic activity. Methyl testosterone was used as the reference standard. Results are summarized in Table 5 (E-19, p 11).

Androgen Antagonism (Castrate Rat Test); 24c1(-), 9a1(-): Androgen antagonism was assayed in the same manner using 0.5 mg testosterone propionate alone or in conjunction with the test compounds. An anti-androgen should prevent the increase in weights of seminal vesicles, ventral prostate and/or levator ani muscle in response to testosterone. Results are summarized in Table 6 (E-19, p 13).

Cortisone-like Activity (Neoglycogenic Activity); 21a1: Neoglycogenic activity was measured by assay of glycogen deposition in livers of fasted, adrenalectomized rats. Rats were given corn oil (controls), aspartame, or DKP intragastrically. Cortisone acetate was given subcutaneously. A compound is rated active if it produces a significant increase ($P < 0.01$) in total liver glycogen. The assay is based on the depletion of liver glycogen in fasting, which, in the intact animal, is overcome to some extent by the conversion of fats and proteins to glucose. Adrenalectomy prevents this response while glucocorticoid replacement restores it. Results are summarized in Table 7 (E-19, p 15).

Effect on Fertility (Rats, Post-Ovulatory; Hamsters, Post-Ovulatory);

24d1, 24d2: Antifertility effects were checked in rats and hamsters. Rats were treated with corn oil (controls), aspartame, DKP or estrone from day 1 to day 7 post coitum. The number of normal appearing implantation sites were noted at sacrifice on day 15 of gestation. Table 8 (E-19, p 16) summarizes these results.

Hamsters were grouped in the same way and treated from days 1-5 post coitum. They were killed on gestation day 6; corpora lutea and fetuses were counted and their condition noted. Implantation rate was determined as $\frac{\text{total sites} \times 100}{\text{total corpora lutea}}$. Table 9 (E-19, p 18) summarizes these results.

Pituitary Regulation (Ovarian Compensatory Hypertrophy); 20a4: Effects

of aspartame and DKP on ovarian compensatory hypertrophy (anterior pituitary activity) in hemicastrated adult female rats treated for 14 days were tested. Suppression of hypertrophy occurred in rats given norethynodrel which were used as a reference standard. Results are summarized in Table 10 (E-19, p 20).

Anti-inflammatory Activity (Foot Edema Test); 27a5B: Anti-inflammatory

(hydrocortisone-like) activity was tested by inducing an inflammatory reaction in the hind feet of male rats. A small amount of a 1% solution of carrageenin was injected into the plantar surface of the hind feet. This induces a local inflammatory response manifested as edematous swelling in the surrounding tissue. One hour prior to the carrageenin

injection the rats were given saline (controls), aspartame, DKP, or hydrocortisone. Volume displacement of the hind feet was measured. Suppression of swelling was considered significant at the 5% level ($P < 0.05$). Results are summarized in Table 11 (E-19, p 22).

Anti-inflammatory Activity (Cotton Wad Granuloma Formation); 27a3:

Further anti-inflammatory activity was assessed by measuring granuloma formation in response to cotton pellet implants. This was done in rats which were adrenalectomized the day before implantation. Test compounds were administered the day of the implants and the following day. Forty-eight hours after pellet implantation, the pellets and their surrounding granuloma were dissected, dried, and weighed. Treatment with hydrocortisone prevented the growth of granuloma tissue on these pellets. Results are summarized in Table 12 (E-19, p 23).

Anti-inflammatory Activity (Chronic Polyarthrititis); 27a7A: The effects of the test compounds on chronic polyarthrititis were checked. Intact young (130-150g) male rats were sensitized by injection of a suspension of killed Mycobacterium butyricum in oil. Test compounds were administered for 19 days beginning on the day of inoculation. Controls were given only saline. Animals were sacrificed 24 hours after the last injection and rear ankle volumes were determined. Hydrocortisone was used as the standard. Results are summarized in Table 13 (E-19, p 25).

Immunosuppressive Activity (Jerne Plaque) - Immunosuppressive activity in mice was determined by the Jerne Plaque Test. This is a measurement of the number of specific antibody producing cells in spleens of mice which have been sensitized to sheep erythrocytes. The level of circulating hemolysin is reflected by the number of sensitized cells in the spleen. Proliferation of the hemolysin producing spleen cells that appear after sensitization to sheep cells is inhibited by immunosuppressive agents. Table 14 summarizes these results (E 19, p 28).

General Comments

Erhard F. Nutting, Ph.D., Director of Endocrinology for Searle, is the only personnel member mentioned in the E-19 Report.

As indicated above, experimental animals included albino mice, Sprague-Dawley rats, rabbits and hamsters of unknown strain, and C57B1 mice. Conditions under which the animals were maintained were not discussed.

It was necessary for UAREP to request clarification of data sheets, and to ask Dr. Nutting to indicate which data sheets of the large number sent were actually pertinent to the study in hand. The copies sent were difficult to read and interpret.

Except for the Jerne Plaque assay, there is no mention of the statistical methods used. There is also no explanation of the data in most of the tables. A check of the data sheets indicates that means were used in most of the tables but standard deviations were not included as an indication of the precision of the assays.

Dr. Nutting explained to Dr. Stowell that the details of these routine procedures were carried out by his technical staff, and he saw only the results they presented to him.

Test compounds (aspartame and DKP) were administered intragastrically presumably to duplicate the anticipated route of human exposure. However, the report says, imprecisely, that the compounds were "dissolved or suspended in corn oil." It does not specify which. Neither would correspond to conditions of human consumption, which would usually be in an aqueous vehicle such as soft drinks. UAREP has no information whether the test compounds are soluble in oil, or how slowly they would be absorbed if "dissolved or suspended in corn oil." The volumes and concentrations of all compounds used were not indicated in the Report. It is well known that a specific amount of compound admitted in a large volume may be much less effective than the identical quantity given in a smaller volume (1).

Dr. Woolley noted that in most cases exposure to test compounds was of short duration ranging from one hour to seven days. Exceptions were the pituitary gonadotrophin assay which ran for 14 days, the androgenic-myotrophic studies which ran for 20 days, and the chronic polyarthrititis assay which ran for 19 days.

The volumes in which test compounds aspartame and DKP were administered do not follow a logical pattern (Table 12-1). If larger volumes were used with larger doses, one would assume the solubility of the compounds necessitated use of the larger volumes. However, volume size is erratic in relation to amount of compound. For example, amounts less than 20 mg were in 0.1 ml, 20 mg was put in 1.0 ml while 30 mg was in

Table 12-1
Variations in Volume of Vehicle Used for Different
Dosages of Aspartame and DKP

E-19 Table	species	mg aspartame	injection volume (ml)	mg DKP	injection volume (ml)
1	mouse	1350	0.1	1350	0.1
		450	0.1	450	0.1
2	mouse	1350	0.1	1350	0.1
		450	0.1	450	0.1
3	rabbit	300	1.0	300	1.0
4	rabbit	300	1.0	300	1.0
5	rat	350	0.4	350	0.4
		50	0.2	50	0.2
6	rat	350	0.1	350	0.1
		50	0.2	50	0.2
7	rat	45	0.1	45	0.1
8	rat	60	0.4	60	0.4
9	hamster	30	0.4	30	0.4
10	rat	60	0.5	60	0.5
		10	0.1		
		2	0.1		
11	rat	36	1.0	36	1.0
12	rat	65	2.0	65	0.5
		20	1.0	32	0.5
13	rat	60	1.0	60	1.0

Aspartame and DKP were administered orally "dissolved or
suspended in oil."

0.4 ml. Sixty-five milligrams was "dissolved or suspended" in 2.0 ml, while 300 mg was in 1.0 ml. Three hundred fifty mg was administered in 0.1 ml in the work for Table 6 (E-19) and 0.4 ml in the assays for Table 5. Sixty mg was administered in three different volumes at various times; 0.4 ml for Table 8, 0.5 ml for Table 10, and 1.0 ml for Table 13. It is difficult to understand why in the assays for Table 6 (E-19, p 13) the smaller amounts (50 mg) of DKP and aspartame were given in 0.2 ml while the 350 mg doses were given in 0.1 ml.

It is well known that the volume in which a given amount of a substance is administered can change its effectiveness to a large degree. For example, the decrease in cholinesterase activity in plasma of newborn rats was 3.1 times greater when 8 mg/kg parathion was administered in 0.01 ml than in 0.1 ml (1), at 2 hours after intraperitoneal injection. Inhibition of cholinesterase in brains of newborn rats was 3.9 times greater two hours after the same injections.

Table 12-2 lists the assay worksheets by Table number. In some cases the elapsed time between the first and last worksheets was quite large. For example, in Table 12 the control data was collected over a period of 2.2 years. In Table 10, the norethynodrel 100 μ g data was collected over a period of 2.5 years. The table shows other gaps of time for data derived for the same experiment ranging from a few days to 64 weeks. When data was collected over a span of 2.5 years, the animals might have changed due to change of vendors or some other conditions. Data collected at different times of the year may be quite different. Animal experiments are usually set up so that controls and

TABLE 12-2
Pertinent vs Extraneous Data Sheets

E-19	No. of Pertinent Pages	Searle Category	No. of Extraneous Pages	Compounds	Dates Assays Performed	Time Elapsed Between First and Last Assays (wks)
Table 1	5	24a2	10	all compounds run	10-21-71	0
Table 2	22	24a2(-)	3	control	8-5-71 8-19-71 10-21-71 8-10-72	53
				aspartame, 450 µg	8-5-71 8-10-72	53
				DKP, 450 µg	8-5-71 8-10-72	53
				progesterone, 200 µg	3-5-71 8-19-71 10-21-71 8-10-72	53
				progesterone, 100 µg	3-5-71 8-19-71 10-21-71 8-10-72	53
Table 3	12	24b2	3	control	10-21-71 11-9-71 1-13-72 5-9-72	30
				progesterone, 50 µg	9-28-71* 10-21-71 10-26-71*	4
				progesterone, 20 µg	1-13-72 5-9-72	16
Table 4	10	24b2(-)	0	control	10-12-71 11-23-71 1-18-72 2-29-72	20
				estrone, 2mg	11-23-71 2-29-72 3-20-72*	18
Table 5	9	24c1 9a1	17	control	8-16-71 10-27-71	10
Table 6	9	24c1 (-) 9a1 (-)	3	control	10-6-71 10-27-71 11-29-71	7
				aspartame, 50 mg	10-6-71 11-29-71	7
				DKP, 50 mg	10-6-71 11-29-71	7
Table 7	5	21a1	0	all compounds run	2-14-72	0
Table 8	4	24d1	28	control	8-24-71 8-25-71	0
				aspartame DKP	8-24-71 8-24-71	
				estrone, 2 µg estrone, 4 µg	2-17-70* 2-17-70*	
Table 9	11	24d2	0	control	10-6-71 10-25-71	3
				aspartame DKP	10-22-71* 10-25-71	
				estrone, 20 µg	9-23-71* 10-6-71	2
				estrone, 10 µg	9-23-71 10-6-71*	2
Table 10	15	20a4	13	control	10-8-71	0
				aspartame, 60 mg	10-3-71	0
				aspartame, 10 mg	11-21-69* 6-5-70*	28
				aspartame, 2 mg	4-25-69*	
				DKP, 60 mg	10-3-71	
				norethynodrel, 100 µg	4-25-69* 7-24-70*	128
				norethynodrel, 50 µg	10-8-71 5-2-69* 9-26-69*	64
				norethynodrel, 20 µg	7-24-70* 10-11-69* 6-5-70* 7-24-70*	38

Table 12-2 (cont.)
page two

Pertinent vs Extraneous Data Sheets

	No. of Pertinent Pages	Searle Category	No. of Extraneous Pages	Compounds	Dates Assays Performed	Time Elapsed Between First and Last Assays (wks)
Table 11	18	27A5b	4	control	7-13-71 7-23-71 8-3-71 8-10-71 8-12-71 8-18-71 9-8-71	8
				aspartame	8-3-71	0
				DKP	8-3-71	0
				cortisol, 10 mg	7-28-71 8-12-71 9-3-71	6
				cortisol, 5 mg	7-28-71 8-12-71 8-18-71 9-8-71	6
				cortisol, 2 mg	3-18-71	3
Table 12	24	27a3	18	control	12-18-69 8-6-71 9-3-71 1-14-72 3-9-72	116
				aspartame, 65 mg**	8-6-71	0
				aspartame, 20 mg	12-18-69	0
				DKP, 65 mg	8-16-71 9-3-71 1-14-72 3-9-72	21 0
				DKP, 32 mg	3-9-72	
				cortisol, 1 mg	8-6-71 9-3-71 1-14-72 3-9-72	31 7
Table 13	7	27a7a	5	control	8-23-71	0
				aspartame	8-23-71	
				DKP	3-23-71	
				cortisol, 0.5 mg	8-23-71	
				cortisol, 1 mg	8-23-71	
				cortisol, 2 mg	8-23-71	

* no simultaneously run controls

The Searle category is the designation used on their data sheets, and is also used in this chapter.

all dose levels are run concurrently. Table 12-2 shows that in E-19 many sequential runs of animals were incomplete. Data for which there were no concurrently run controls are starred with an asterisk in the table.

RESULTS AND DISCUSSION

Estrogenic Activity; 24a2: Assay of estrogenic activity (E-19, table 1) was done in 21 day old female albino mice maintained on synthetic estrogen-free diet. The use of an estrogen-free diet was good procedure, but the means of ascertaining that the diet was indeed estrogen-free, or any further description of the diet, were not specified. The composition of the data should have been stated.

The doses of aspartame and DKP used (according to Table 1, p 2, E-19) were about 90 mg/kg (or 900 mg/kg depending on whether dosage on worksheet or in Entry Book is correct) compared to the maximum anticipated human consumption of 30 mg/kg a day. The doses of test compounds given in the Entry Book (E-19, p 2) appear to be 0.1 X those recorded on the worksheets. A coefficient of variation (Table 12-3) of more than 10% indicates data which are rather variable and probably not "normal" in distribution (2).

Mice receiving aspartame and DKP showed less than 10% increase in uterine weight over the control animals. These increases were not statistically significant, while the increases in uterine weight effected by both 0.1 and 0.3 μ g estrone were significant at $P < 0.01$ by UAREP calculations using Wilcoxon Rank Sum Method, and agreed with the reported results.

Estrogen Antagonism; 24a2(-): This assay was similar to the preceding one in all respects except that all animals were given 0.3 μ g estrone subcutaneously (Table 12-4). Mice given 1.35 mg aspartame had uteri

Table 12-3

Summary of Uterine Weight Data From Estrogen-Like Activity Assay

Compound	Total Dose (ug)	N	Uterine Weight (mg)	UAREP Calculation $\bar{x} \pm SD$	CV
Control	--	8	9.1	9.1 ± 1.1	12.1%
aspartame	1350	10	9.4	9.4 ± 2.3	24.5%
DKP	1350	10	10.0	10.1 ± 1.3	12.9%
Estrone	0.3	9	69.2	69.2 ± 11.3	16.3%
	0.1	9	23.6	23.6 ± 4.0	16.9%

The worksheet indicates ten times larger doses of aspartame and DKP than the E-19 report.

CV = coefficient of variation = $\frac{\text{standard deviation}}{\text{mean}} \times 100$

averaging 9.1% less in weight than those of the controls. Those given 1.35 mg DKP had uteri averaging 9.3% less in weight than those of the controls. These results were not statistically significant, but could suggest a degree of estrogen antagonism. It should be noted that this dose is about three times (could be 30X depending on whether worksheet or Entry Book records the correct dosage) the maximum anticipated human consumption per day. As in the foregoing experiment, the test compounds were given for three days. The coefficients of variation on these data ranged from 16.8% to 40.1%, the latter indicating a rather large degree of variability. In two cases (see Table 12-4) UAREP found N (number of test animals) to be one less than the report showed and in one case UAREP determined N was one greater than shown in the report.

Progesterone-like Activity; 24b2: The description of this test again states imprecisely: "Compounds, dissolved or suspended in corn oil, were administered for 5 days. . . ." Table 3 (E-19, p 6) states that aspartame and DKP were given to the rabbits buccally. Progesterone, used as a reference standard, was injected subcutaneously, as was the priming dose of estradiol-17 β . Degree of arborization in the uteri was determined by histologic examination. It was quantified on a scale of +1 to +4, and listed as "McPhail Score."

Carbonic anhydrase concentration (in μ g/100 mg wet weight of uterus) was 18.2 in the rabbits given 300 mg/kg aspartame for five days following the priming dose of estradiol (Table 12-5). This was 50.4% greater than the control result of 12.1, and only 4.9% less than the animals given 0.02 mg of progesterone. Some might feel that a 50%

Table 12-4

Summary of Uterine Weight Data From Estrogen Antagonism Assay

<u>Antagonist</u>	<u>Total Dose (ug)</u>	<u>N</u>	<u>Uterine Weight (mg)</u>	<u>UAREP calcu- lation ($\bar{x} \pm SD$)</u>	<u>CV</u>
None	--	38(37)	64.0	63.1 \pm 17.2	27.3%
aspartame	1350	10	58.2	58.1 \pm 10.0	17.2%
	450	20	56.8	66.8 \pm 19.8	29.6%
DKP	1350	10	58.0	58.0 \pm 11.9	20.5%
	450	20	63.7	63.7 \pm 25.5	40.1%
Progesterone	200	39(38)	25.2	26.8 \pm 4.6	17.1%
	100	39	33.3	33.9 \pm 5.7	16.8%
	50	38(39)	41.2	42.1 \pm 9.6	22.7%

Numbers in parenthesis indicate "N" values UAREP determined from data sheets.

Above doses of test compounds are 0.1 X that shown on the worksheets.

CV is coefficient of variation.

Table 12-5
Summary of Data on Carbonic Anhydrase Activity
In Measuring Progesterone-Like Activity

Compound	Daily Dose (mg)	N	Searle Carbonic Anhydrase Concentration	UAREP Calculation $\bar{x} \pm SD$	CV
Control	--	11	12.1	12.0 \pm 4.9	40.8%
aspartame	300	4	18.2	18.2 \pm 6.6	36.3%
DKP	300	4	13.4	13.4 \pm 8.2	61.2%
Progesterone	0.1	11(10)	69.5	68.2 \pm 19.7	28.9%
	0.05	12	40.5	40.4 \pm 16.3	40.3%
	0.02	8	19.1	19.5 \pm 8.0	41.0%

Number in parenthesis represents UAREP "N" if different from Searle.

CV is coefficient of variation.

Carbonic Anhydrase Concentration is in μ g/100mg wet weight of uterus.

change suggests some degree of progesterone-like activity; however, Searle said aspartame was devoid of progesterone properties and caused no increase.

Only four rabbits were given aspartame and DKP, whereas there were eleven controls; and eleven, twelve, and eight rabbits given 0.1, 0.05, and 0.02 mg of progesterone respectively. UAREP and Searle found the 0.1 mg progesterone uteri were significantly different ($P < 0.01$) from controls.

Progesterone Antagonism; 24b2(-): This test was conducted in the same manner as the previous one, except that all animals were given 0.1 mg progesterone, in addition to the priming dose of estradiol-17 β . The problems of dosage administration mentioned in the previous section apply to this assay as well. Again, there were four rabbits given aspartame, DKP, or 1 μ g estrone (reference standard). There were 15 control rabbits. Twelve were given 2 μ g estrone as reference standard (Table 12-6). UAREP is unable to account for these large differences in numbers of animals used. Scientists usually attempt to have similar numbers of animals in control and test groups.

No standard errors or statistical summaries were reported on the data for this or the previous assay. UAREP found no statistically significant differences in the progesterone antagonism data (E-19, p 9, Table 4). Significance may have been obscured by variability of the data (as shown by coefficients of variation in Table 12-6).

However, the four rabbits which received 300 mg/kg DKP had uteri with 40.6% less carbonic anhydrase (CA) activity than the controls. The

Table 12-6
Summary of Data on Carbonic Anhydrase Activity
In Measuring Progesterone Antagonism

Antagonist	Dose (mg)	N	Searle Carbonic Anhydrase Concentration	UAREP Calculation $\bar{x} \pm SD$	CV
None	--	15	57.3	59.7 \pm 27.4	45.9%
aspartame	300	4	56.8	56.8 \pm 46.1	81.2%
DKP	300	4	34.0	34.0 \pm 11.2	32.9%
Estrone	.002	12(14)	23.6	20.5 \pm 8.2	40.0%
	.001	4	91.5	91.5 \pm 12.5	13.7%

Number in parenthesis represents UAREP "N" value which was different from Searle.

CV is coefficient of variation.

twelve animals which received 2 µg estrone had uteri with 58.8% less CA activity than the controls. This could indicate some degree of progesterone antagonism. The report states (E-19, p 8) "the observed decrease in uterine carbonic anhydrase appears to be a spurious test result without physiological significance since the McPhail score, recorded for the same tissue, was not decreased."

The test compound doses in this and the previous assay were about ten times the maximum anticipated human consumption, but the duration of exposure was only six days.

Androgenic-Myotrophic Activity; 24c1 and 9a1: The assay summary says: "The test compounds, dissolved or suspended in corn oil," were administered daily for 7 days. The aspartame, DKP and methyl testosterone were administered intragastrically.

There were 20 control animals (Table 12-7) while there were only six animals given test doses of aspartame and DKP which recomputed on a weight basis would be at 300 and 2100 mg/kg levels.

Seminal vesicle weights in rats at the 350 mg (2100 mg/kg) dose of aspartame were 8.8% less than those in the control group. Prostates at this dose of aspartame weighed 15% less than those of the controls, while prostates in the DKP animals at the same dose weighed 13% less than controls. Seminal vesicles in the animals which received 350 mg of DKP were 32.9% lighter than in the controls. Although these effects were not statistically significant, they suggest a slight degree of antiandrogenicity which possibly could have biological significance. The average coefficient of variation ($\frac{\text{standard deviation}}{\text{mean}} \times 100$) was 19.1% for the seminal vesicles, 16.7% for the ventral prostate and 20.0%

Table 12-7

Summary of Data on Androgenic-Myotrophic Activity

Seminal Vesicle

	<u>Control</u>	<u>350mg</u>	<u>aspartame</u> <u>50mg</u>	<u>350mg</u>	<u>DKP</u> <u>50mg</u>	<u>Methyl Testosterone</u> <u>60mg</u>	<u>10mg</u>
N	18 (20)	6	8	6	8(6)	8	8
UAREP (mg)							
$\bar{x} \pm SD$	9.1 \pm 2.0	8.3 \pm 1.9	10.3 \pm 2.6	6.1 \pm 0.8	9.2 \pm 1.2	50.2 \pm 6.5	19.8 \pm 5.2
CV	22.0%	22.9%	25.2%	13.1%	13.0%	12.9%	26.3%

Ventral Prostate

N	20	6(7)	8	6	8	8	8
UAREP (mg)							
$\bar{x} \pm SD$	10.0 \pm 1.8	8.5 \pm 1.1	11.6 \pm 1.1	8.7 \pm 1.4	11.6 \pm 1.8	62.4 \pm 12.1	24.2 \pm 5.8
CV	18.0%	12.9%	9.5%	16.1%	15.5%	19.4%	24.0%

Levator Ani Muscle

N	20	6(7)	8	6	8	8	8
UAREP (mg)							
$\bar{x} \pm SD$	45.5 \pm 10.8	49.2 \pm 9.5	46.1 \pm 8.8	49.2 \pm 10.0	49.1 \pm 13.3	80.5 \pm 10.0	54.9 \pm 10.3
CV	23.7%	19.3%	19.1%	20.3%	27.1%	12.4%	18.8%

CV is coefficient of variation.

Numbers in parenthesis represent UAREP's N which was different from Searle's.

UAREP's recalculations of the means agreed with Searle's data in Table 5 (E-19, p.11).

for the levator ani muscle. Such variable data could obscure statistical significance.

Myotrophic activity was assessed by weighing the levator ani muscle. The animals on aspartame and DKP (at the 350 mg level) both showed an 8.1% increase over controls in weight of this muscle, indicating a mild anabolic effect of both compounds.

UAREP's statistical evaluation of these data agreed with that of Searle, that the response to methyl testosterone was significantly higher ($P < 0.01$) than controls for all parameters at both doses with one exception; the levator ani muscle weight was not significantly different from controls in animals receiving 10 mg methyl testosterone.

Androgen Antagonism; 24b1(-): Testosterone propionate (0.5 mg) was administered intramuscularly to castrated male rats. Twenty controls were done. There were seven rats in the 350 mg dose group testing aspartame or DKP, and fifteen rats in the lower dosage (50 mg) groups. The test compounds were given in addition to testosterone (Table 12-8).

UAREP's statistical evaluation agreed with Searle's that the data showed no statistically significant differences in anti-androgenic or anti-metabolic activities. At the 350 mg levels of aspartame and DKP, some might feel that the decrease of 21 and 22% suggest the possibility of a degree of androgen antagonism. However, the report states that aspartame and DKP were "devoid of anti-androgenic and anti-myotrophic properties" (E-19, p 12).

Table 12-8

Summary of Anti-androgenic and Anti-myotrophic Activity Data

	Control	aspartame		DKP	
		350mg	50mg	350mg	50mg
<u>Seminal Vesicle</u>					
N	20	7	15	7	15
Wt (mg)	85.5	67.9(20.6%+)	80.3(6.17%+)	67.0(21.6%+)	75.3(11.9%+)
UAREP (mg)					
$\bar{x} \pm SD$	85.5±16.4	67.9±12.6	80.3±20.2	67.0±16.0	75.3±21.1
CV	19.2%	18.6%	25.2%	23.9%	28.0%
<u>Ventral Prostate</u>					
N	20	7	15	7	15
Wt (mg)	72.6	67.5(7.0%+)	74.3(2.3%+)	70.0(3.6%+)	73.1(0.72%+)
UAREP (mg)					
$\bar{x} \pm SD$	72.6±15.5	67.5±19.7	74.3±10.7	70.0±24.6	73.1±21.6
CV	21.3%	29.2%	14.4%	35.1%	29.5%
<u>Levator Ani Muscle</u>					
N	20	7	15	7(6)	15
Wt (mg)	81.5	74.7(8.3%+)	75.8(6.9%+)	82.0(0.6%+)	78.7(3.4%+)
UAREP (mg)					
$\bar{x} \pm SD$	81.5±10.6	74.7±12.7	75.7±13.2	82.0±14.0	78.7±12.7
CV	13.0%	17.0%	17.4%	17.1%	16.1%

UAREP "N" in parenthesis represents a discrepancy with the Searle data.

CV is coefficient of variation.

Cortisone-like Activity; 21a1: When compared with controls, aspartame and DKP (225 mg/kg) appeared to have no effect on glycogen deposition in livers of fasted, adrenalectomized rats (Table 12-9). Cortisone acetate (0.5 mg) increased glycogen deposition about thirty-five fold. This increase was statistically significant at $p < 0.01$. UAREP's statistical evaluation agreed with Searle's. Cortisone acetate (0.2 mg) also increased glycogen deposition, but the difference was not statistically significant. The variability of the cortisone data with coefficients of variation over 100% may have obscured statistical significance in the 0.2 mg group.

Effect on Fertility (Rats, postovulatory); 24d1: Five test animals were used for aspartame and DKP in this assay as opposed to ten in the control and standard groups (Table 12-10). Aspartame and DKP were given intragastrically; the reference standard, estrone, was administered subcutaneously. The report states that aspartame and DKP "did not exhibit anti-fertility activity" (E-19, p 14). Table 8 (E-19, p 16) shows that one of the five rats given aspartame did not get pregnant. Table 12-10 also notes that animals treated with aspartame had a 50% lower fertility rate. However, UAREP would not promote aspartame for anti-fertility effects on the basis of these few observations in tables 12-10 or 12-11.

Effect on Fertility (Hamsters, postovulatory); 24d2: This assay was essentially the same as the previous one, except that the animals were killed on gestation day 6. Implantation rate was determined as

$$\frac{\text{total sites}}{\text{total corpora lutea}} \times 100.$$

Table 12-9

Summary of Data on Glucocorticoid-Like Activity

By Measurement of Glycogen Formation in Liver of Adrenalectomized Rats

Compound	Total Dose (mg)	N	Glycogen UAREP	CV	Glycogen per 10 g body wt, Searle (mg)	Glycogen per 10 g body wt, UAREP (mg)	Glycogen per 10 g body wt, CV
control	--	9	0.55±0.10	18.2%	0.04	0.04±0.01	25.0%
aspartame	45.0	9	0.47±0.18	38.3%	0.03	0.03±0.01	33.3%
DKP	45.0	9	0.50±0.09(8)	18.0%	0.03	0.03±0.01(7)	33.33%
cortisone	0.5	10	20.0±20.0	100.01%	1.41	1.41±1.4	99.3%
acetate	0.2	10	8.0 ± 8.3	103.7%	0.53	0.53±0.54	101.9%

N in parenthesis indicates the total number of samples UAREP used in its calculations

because of data crossed out on worksheets.

CV is coefficient of variation.

Table 12-10
Summary of Number of Implantation Sites
in Rats Treated from Day 1 to Day 7
Post Coitum

Compound	Dose (mg)	N	Normal Implantation Sites	Average Sites per Animal	Average Abnormal Sites per Animal	Average Scars per Animal
Control	--	10	121	12.1	0	4
aspartame	60	5	33	6.6	0	4
DKP	60	5	53	10.6	0	1
Estrone	4	10	0	0	0	0
	2	10	32	3.2	2	18

Fifteen animals were included in each of the control and standard animal groups, while five were in each of the test groups (Table 12-11).

There were 18% fewer normal implantation sites in the DKP animals than in the controls of aspartame-treated hamsters, and 19% more abnormal corpora lutea. The report states (E-19, p 17): "Thus, SC-18862 (aspartame) and SC-19192 (DKP) did not exhibit antifertility activity as measured in this test."

Pituitary Regulation (Ovarian Compensatory Hypertrophy); 20a4: Removal of one ovary from young rats stimulates pituitary gonadotrophin secretion resulting in hypertrophy of the remaining ovary. The assay measures inhibition of pituitary gonadotrophin secretion. Table 10 (E-19, p 20), which shows the data from this assay, seems to have a number of errors. The doses are off by a factor of 1000; that is, they are listed as milligrams when microgram quantities were actually used. Only the largest dose (60 mg) was used in testing DKP while three levels (60, 10, and 2 mg) were used in testing aspartame. C minus T is presumed to mean "control" minus "treated" ovarian weights, but the numbers listed in the table in several cases cannot be derived from the data given. The data listed in the "percent inhibition" column cannot be calculated from any of the data given in the table. UAREP was, therefore, unable to check the calculations or the statistical evaluation in this table. However, noting that the mean ovarian weights in the ten control rats were 52.96 mg and those of the twenty-nine animals given 20 µg norethynodrel were 51.1 mg, one is puzzled that the difference between them of 1.86 mg as shown in Table 10 of E-19 could possibly be significant (Table 12-12). Data for ovarian weights from an intact control group,

Table 12-11
Summary of Hamster Fertility Data

Compounds	Dose Volume (ml)	Dose (mg)	N	Normal Implantation Sites (Searle)	Normal Implantation Sites (UAREP)	Normal corpora lutea (UAREP)
Control	0.2	--	15	99.4%	99.5%	93.6%
aspartame	0.4	30	5	97.5%	97.5%	94.0%
DKP	0.4	30	5	82.1%	82.1%	74.6%
Estrone	0.2	20	15	0.0%	0	13.8%
	0.2	10	15	24.1%	31.5%	55.1%

Table 12-12

Summary of Ovarian Weight Data in Response to Hemicastration

Compound	Dose (mg)	N	Searle Ovarian Weight (mg)	UAREP Ovarian Weight ($\bar{x} \pm SD$)	CV
Control	--	10	52.96	53±10	18.9%
Aspartame	60	10	52.99	53±12	22.6%
	10	19	56.10	56±9	16.1%
	2	10	56.90	57±12	21.0%
DKP	60	9	52.18	52±6	11.5%
Norethynodrel	0.1	30	42.1	42±8	19.0%
	0.05	29	44.0	44±5	11.4%
	0.02	29	51.1	52±7	13.5%

or for the weights of the ovaries that were removed should have been included to enable one to do the calculations indicated (E-19, p 30, ref 17).

Anti-inflammatory Activity (Foot Edema Test); 27a5B: According to Dr. Woolley, rats are usually adrenalectomized for such tests, and two "control" groups should be included: one group which received nothing and another group which received cargeenin. In this way a better idea is given of the magnitude of the increase in volume caused by the irritant.

Table 11 (E-19, p 22) reports the volume of the hind feet ranging from 58.9 to 65.2 ml which seems large. No explanation is given to account for these figures. The numbers of rats used in the various groups are unusual. For example, there were 63 control rats, 8 rats given aspartame, 8 given DKP, 27 given 10 mg hydrocortisone, 36 given 5 mg hydrocortisone, and 18 rats given 2 mg hydrocortisone (Table 12-13). The hind feet of the rats which received 10 mg hydrocortisone displaced 58.9 ml. This was significantly less than the controls at $P < 0.05$. Hind feet of the rats which received 5 mg hydrocortisone displaced 59.5 ml, a difference of 0.6 ml from the 10 mg dose, and this result was not statistically different from the controls. UAREP's statistical evaluation agreed with Searle's.

Anti-inflammatory Activity (Cotton Wad Granuloma Formation); 27a3: The rats used in this assay were adrenalectomized. Unequal numbers of rats were used in the various groups as follows: control group-57; two aspar-

Table 12-13
Summary of Data on Foot Edema (Cortisone-Like Activity)

Compound	Dose (mg)	N	Volume Displacement (Searle Table)	UAREP Calculations				
				L	CV(L)	R	CV(R)	Combined CV (Combined)
Control	--	63	65.24	32.6 ± 3.0	9.2%	32.6 ± 2.5	7.6%	65.3 ± 5.3 8.1%
aspartame	36	8	62.31	30.8 ± 2.2	7.1%	31.5 ± 2.2	7.0%	62.3 ± 4.2 6.7%
DKP	36	8	63.13	31.3 ± 1.7	5.4%	31.8 ± 1.7	5.3%	63.1 ± 3.1 4.9%
Hydrocortisone	10	27	58.87	30.0 ± 2.0	6.6%	29.7 ± 2.1	7.1%	58.9 ± 4.1 6.9%
	5	36	59.51	29.6 ± 2.3	7.7%	29.9 ± 2.6	8.7%	59.5 ± 4.7 7.9%
	2	18	62.94	31.7 ± 3.4	10.7%	31.3 ± 3.2	10.2%	62.9 ± 6.4 10.1%

tame groups, 6 each; DKP, 65 mg group-21; DKP, 32 mg group-10; and hydrocortisone, 1 mg group-48 (Table 12-14).

Rats given aspartame showed little difference from the controls. Rats given 65 mg DKP developed granuloma tissue which was significantly less than the controls ($P < 0.05$). While the rats given 32 mg DKP developed granuloma tissue which was significantly greater than the controls ($P < 0.05$) by Analysis of Variance. The diminished granuloma formation (a difference of 9.3 mg) in rats which received 65 mg DKP is considered "minimal (anti-inflammatory) reduction" (E-19, p 21) even though it is statistically significant, while the increase in granuloma formation at the 32 mg level of DKP (a difference of 18.5 mg) is not even mentioned. One mg of hydrocortisone resulted in 30.5 mg less granulation tissue formation and was also statistically significant ($P < 0.05$) by Wilcoxon Rank Sum Method.

The report does not explain the meaning of either "adjusted median weight" of pellets, or "the results were computed using median values and taking into account the performance of each technician" (E-19, p 21).

Anti-inflammatory Activity (Chronic Polyarthrititis); 27a7A: Numbers of rats used in the various groups were similar: control group-18; all test groups-11 or 12 (Table 13, p 25, E-19). Volume displacement of the rear ankle joints of the rats was measured but the units are not given in the table. Based on the previous table, one could assume the displacement is expressed in milliliters (ml). Sixty mg (400 mg/kg) of aspartame and DKP were given to the rats. There was an insignificant 1.4 ml increase

Table 12-14
Summary of Data on Granuloma Formation

Compound	Dose (mg)	N	Median wts of pellets Searle (mg)	UAREP Calculation pellet wt (mg)	CV
Control	--	57	82.5	85.5 \pm 16.0	18.7%
aspartame	65	6	84.9	88.8 \pm 13.0	14.6%
	20	6	80.4	80.9 \pm 9.1	11.2%
DKP	65	21	73.2	75.5 \pm 12.9	17.1%
	32	10	101.0	101.0 \pm 13.5	13.3%
Hydrocortisone	1.0	48	52.0	55.2 \pm 9.3	16.8%

CV is coefficient of variation.

in total ankle volume between aspartame and control rats (Table 12-15), while the rats on DKP showed a 5 ml increase. Hind limb volumes of 50 ml seem large for rats in the 100-200 g weight range. The data appear quite variable (CV, Table 12-15) in this assay.

The column headed "percent reduction" is not explained in E-19, Table 13, p 25). UAREP's calculation yielded results as follows:

		<u>Percent Reduction</u>	
	<u>Dose</u>	<u>UAREP</u>	<u>Searle</u>
Hydrocortisone	2.0 mg	28.7%	73.2%
	1.0 mg	23.1%	58.3%
	0.5 mg	18.9%	47.8%

UAREP was unable to duplicate Searle's calculations of percent reduction using the data available.

Immunosuppressive Activity (Jerne Plaque) - The data as presented in Table 14 (E-19, p 28) are difficult to interpret. The backup data available to UAREP do not entirely agree with the data in Table 14. UAREP did not receive raw data on the Jerne Plaque assay until the third set of data was requested. Data on experiment VI were not included. When data from the other five experiments were compared (a total of 49 comparisons), there were 14 data sets whose means did not agree with UAREP's calculations by amounts greater than could be accounted for by rounding differences. Data are presented with five significant numbers when it seems that two are all that are justified. The saline control

Table 12-15
Summary of Data on Chronic Polyarthrititis

Compound	Dose (mg)	N	Searle Table Volume Displace- ment	UAREP Calculations Left	CV(L)	Right	CV(R)	Total Volume	CV(Total)
Control	--	18	49.8	26.2 ± 7.6	29.0%	23.7 ± 6.3	26.6%	49.8 ± 12.5	25.1%
aspartame	60	12	51.2	25.7 ± 6.4	24.9%	25.5 ± 7.4	29.0%	51.2 ± 13.0	25.4%
DKP	60	11	54.8	26.6 ± 5.7	21.4%	28.2 ± 6.8	24.1%	54.8 ± 11.5	21.0%
Hydro- cortisone	2	12	35.5	17.3 ± 1.4	8.7%	18.0 ± 2.6	14.4%	35.5 ± 3.8	10.7%
	1	11	38.3	19.4 ± 5.5	28.3%	18.9 ± 3.8	20.1%	38.3 ± 8.6	22.4%
	0.5	11	40.4	19.8 ± 4.4	22.2%	20.5 ± 6.1	29.7%	40.4 ± 10.4	25.7%

CV is coefficient of variation.

data run at various times varies from 23407 to 47512 (Table 12-16), a difference of 24105 (more than double). Each test group was compared with a concurrently run control group, although one would not expect well-run reliable tests to have that much variation in the controls. Such variable data are difficult to interpret and show no consistent pattern of change.

TABLE 12-16

Summary of Data on Assay of Immunosuppressive Activity of Aspartame

Compound	Dose (mg)	N	Searle Table	UAREP Calculation	CV
Exp I					
Control	--	12	23407	23407:5470	23.4%
aspartame	125	14	24953	24953:4591	18.4%
DKP	125	18	20979	20979:1532	7.3%
Phenylalanine	125	14	23453	23453:2222	9.5%
Aspartic Acid	125	12	21330	21331:2338	11.0%
aspartame	250	16	22183	22184:2930	13.2%
DKP	250	18	21175	21175:1702	8.0%
Phenylalanine	250	16	21320	21321:2407	11.3%
Aspartic Acid	250	14	20957	20958:2708	12.9%
aspartame	500	16	22733	22957:479	2.1%
DKP	500	18	20637	20638:1555	7.5%
Phenylalanine	500	14	21936	21936:2411	11.0%
Aspartic acid	500	16	21776	21767:1997	9.2%
Exp II					
Control	--	16	39344	39469:6017	15.2%
aspartame	250	18	30780	30780:10848	35.2%
DKP	250	12	30973	40890:10230	25.1%
Phenylalanine	250	12	32025	32027:12274	38.2%
Aspartic Acid	250	12	36358	36358:4414	12.1%
aspartame	500	16	23561	23686:2555	10.8%
DKP	500	14	34947	34948:8257	23.6%
Phenylalanine	500	14	28541	28541:7739	27.1%
Aspartic Acid	500	14	29132	29133:11274	38.7%
aspartame	750	18	23488	23489:4581	19.5%
DKP	750	16	36420	35471:6125	17.3%
Phenylalanine	750	14	35030	35030:6342	18.1%
Aspartic Acid	750	12	41215	41216:6126	14.9%
Exp. III					
Control	--	10	35705	35705:4057	11.4%
aspartame	50	10	28950	29950:5053	16.9%
DKP	50	10	36486	36486:5170	14.2%
Phenylalanine	50	8	35481	35481:5160	14.5%
Aspartic Acid	50	10	32601	32619:4358	13.4%
aspartame	125	10	32452	32452:4139	12.8%
DKP	125	10	34113	34113:5157	15.1%
Phenylalanine	125	6	32203	32203:6308	19.6%
Aspartic Acid	125	8	37332	37332:5529	14.8%
aspartame	250	10	35982	35972:6047	16.8%
DKP	250	8	36146	36146:2736	7.6%
Phenylalanine	250	8	34568	34569:3056	8.8%
Aspartic Acid	250	8	36586	36586:7367	20.1%
Exp IV					
Control	--	10	42197	43275:4936	11.5%
aspartame	500	10	42048	42197:4594	11.6%
DKP	500	10	42096	42096:7219	42.3%
aspartame	750	12	43275	42048:3639	8.7%
DKP	750	10	47812	47807:7546	15.8%
Exp V					
Control	--	8	43053	44179:5240	11.9%
aspartame	50	12	42573	42412:2239	5.4%
DKP	125	12	44540	44540:7332	15.8%
	250	10	41615	41616:3505	7.4%

CV is Coefficient of Variation

CONCLUSIONS

This Entry Book contains a variety of screening tests said to be routinely used by Searle in searching for evidence of endocrine or general physiologic effects. Some of the tests seem to be used so routinely that they are carried out with less precision or documentation than some might desire. In some respects, protocol design is not clearly indicated. In some experiments, data collected over a wide range of time as compared with variable numbers of animals and shows some overlapping spread of results with relatively high coefficients of variation.

This report was difficult for UAREP to reconstruct and interpret. At times results noted as being statistically significant may be dismissed as "minimal activity" while results of notable magnitude, but not statistically significant, are not mentioned at all.

Searle sent 334 raw data sheets of which 151 pertained to the report in question. Eventually, it was necessary to ask the project director, Dr. Nutting, to identify the pertinent data sheets.

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CHAPTER XII

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APPENDIX XII-1

Category: 24a2 E. F. Nutting	Gonads and Reproductive Organs: Estrogenic; Mouse.
Animal Used:	Mouse, female, immature (22-25 days old), no anesthesia, no pretreatment.
Organ or Tissue Involved:	Uterus.
Route of Test Drug Administration:	Subcutaneous in oil.
Initial Screening Dose:	1000 μ g, total dose in 3 days. (Compounds inactive at this dose have less than 0.01% the activity of estrone.)
Reference Standard:	Estrone.
Activity Measurement and Interpretation:	Increase in uterine weight in 72 hours. Activity expressed as percent of estrone activity.
Basis of Assay:	Estrogens cause an increase in the weight of the uterus of the immature mouse. The changes induced in women by estrogenic treatment are similar to those found in lower animals.
Clinical Correlation:	The estrogens so found have been used widely in medicine to combat postmenopausal or postcastration syndromes, to suppress lactation, and to overcome certain types of menstrual disturbances.

(Revised 5-26-61)

APPENDIX XII-2

Category: 24a2(-) E. F. Nutting	Gonads and reproductive organs: Estrogen antagonistic; mouse.
Animal Used:	Mouse, female, immature (22-25 days old), no anesthesia, no pretreatment.
Route of Test Drug Administration:	Subcutaneous in oil daily.
Initial Screening Dose:	One mg. total dose in 3 days. Compounds inactive at this dose have less than 5-10% the activity of progesterone.
Reference Standard:	Progesterone, given simultaneously with 0.3 μ g estrone/mouse total dose.
Activity Measurement and Interpretation:	Estrone-stimulated uterine growth can be antagonized by the simultaneous administration of progesterone. Activity is expressed in per cent of progesterone activity as evaluated by comparison of dose response lines.
Basis of Assay:	Progesterone will prevent the uterus of intact female mice from growing under estrogen stimulation.
Clinical Correlation:	Estrogens as well as androgens, progestins, corticoids and other estrogen antagonists have been found to be of value in the treatment of various menstrual dysfunctions. It is not possible at this time to know whether these favorable therapeutic results can be ascribed to direct hormonal action, or to the fact that these materials are antagonists of the more potent estrogens. This test concentrates on the antagonism side of this problem. In addition, a number of estrogen-dependent tumors have been found to be responsive to estrogen antagonists.
Comment:	This test is essentially similar to 24a4(-).

(Revised 7-5-61)

APPENDIX XII-3

Category: 24b2 R. L. Elton	Gonads and Reproductive Organs; Progesterone, Clauberg Assay.
Animal Used:	Immature, intact female rabbits, primed with 5 µg estradiol-17β for 6 days.
Organ or Tissue Involved:	Uterus; histological examination of uterine endometrium.
Route of Test Drug Administration:	Subcutaneously or buccally in oil. Test compound daily for 5 days.
Reference Standard:	Progesterone.
Activity Measurement and Interpretation:	The degree of arborization of the luminal epithelium of the uterus is graded from +1 to +4. An average rating of +2 represents minimal activity (MED progesterone subcutaneously = 0.05 mg/rabbit/day) while an average rating of +3.5 to +4 represents maximal activity (produced by progesterone given at 0.1 mg/day subcutaneously). Unless stated to the contrary, potency estimates will be made by comparing doses of the standard and test compound that produce minimal activity. Compounds having less than 5% the activity of progesterone will be classified inactive.
Basis of Assay:	Arborization (proliferation) of glandular epithelium in estrogen-primed rabbits is brought about by progesterone.
Clinical Correlation:	Progesterone, or a functional corpus luteum, is a factor in normal uterine development in the menstrual cycle and maintenance of pregnancy. Development and function of mammary tissue is also influenced by progestins.

APPENDIX XII-4

Category: 24b2(-) R. L. Elton	Gonads and Reproductive Organs; Anti-Progesterone, Rabbit.
Animal Used:	Immature, intact female rabbits primed with 5 μ g estradiol-17 β daily for 6 days.
Organ or Tissue Involved:	Uterus; histological examination of uterine endometrium.
Route of Test Drug Administration:	Subcutaneously in oil. Test compound is given with progesterone (0.1 mg/day) in a single injection daily for 5 days.
Reference Standard:	None.
Activity Measurement and Interpretation:	Progesterone, at a subcutaneous dose of 0.1 mg/day, produces arborization of the luminal epithelium of the uterus which, when graded, averages about +3.5 (see 24b2). Estrone, mixed with this dose of progesterone, inhibits the uterine response to progesterone. Test compounds are considered active if they reduce uterine arborization.
Basis of Assay:	Reduction of progesterone-induced uterine arborization.
Clinical Correlation:	Agents that inhibit progesterone-dependent phenomena (i.e., tubal development and passage of fertilized ova, development and function of the uterus and Fallopian tubes, or the implantation process) may be of use in controlling fertility.

2-12-60

APPENDIX XII-5

Category: 24cl Endocrine Screening	Gonads and Reproductive Organs: Androgenic, Rat.
Animal Used:	Rat, male immature, castrated at 22-23 days of age, 20 days before treatment.
Organ or Tissue Involved:	Ventral prostate and seminal vesicle.
Route of Test Drug Administration:	Intramuscularly or intragastrically in oil daily.
Initial Screening Dose:	5 mg IM or 15 mg IG total dose in 7 days.
Reference Standard:	Testosterone propionate is the standard for intramuscular administration; methyltestosterone for intragastric administration.
Activity Measurement and Interpretation:	A <u>dose of compound</u> is rated active if it produces a significant ($p < 0.01$) increase in the weight of the seminal vesicles. A <u>compound</u> administered IM is rated active if it possesses a potency > 1% testosterone propionate. A <u>compound</u> administered IG is rated active if it possesses a potency > 25% methyltestosterone. IM potencies are based on ratios of minimally active doses, whereas IG potencies are calculated from dose-response lines.
Basis of Assay:	The male sex hormones stimulate the development of the seminal vesicles and ventral prostate glands as indicated by weight increase above controls. Estrogens cause enlargement of the seminal vesicles but not of the ventral prostate.
Clinical Correlation:	The known androgens produce effects in man, qualitatively similar to those observed in lower animals. Quantitative relations between compounds, so far as they have been explored, also appear to be comparable.

APPENDIX XII-6

Category: 9a1
Endocrine Screening

Musculo-skeletal and Articular: anabolic, rat.

Animal Used:

Rat, male, 150 grams, castrated at 22-23 days,
20 days before first injection.

Organ or Tissue
Involved:

Levator ani muscle.

Route of Test Drug
Administration:

Intramuscularly or intragastrically in oil daily.

Initial Screening Dose: 5 mg IM or 15 mg IG total dose in 7 days.

Reference Standard:

Testosterone propionate is the standard for intra-
muscular administration; methyltestosterone for
intragastric administration.

Activity Measurement
and Interpretation:

A dose of compound is rated active if it produces
a significant ($P < 0.01$) increase in the weight
of the levator ani muscle. A compound administered
IM is rated active if it possesses a potency $>$
4% testosterone propionate. A compound administered
IG is rated active if it possesses a potency $>$ 100%
methyltestosterone. IM potencies are based on
ratios of minimally active doses, whereas IG potencies
are calculated from dose response lines.

Revised 12-1-65

Basis of Assay:

During a period of anabolism with nitrogen retention,
the muscle weight increases. The levator ani has
been found to be a particularly good measure of this
response. Male sex hormones stimulate anabolism in
human beings and levator ani weights in castrate animals.

Clinical Correlation:

Testosterone propionate and methyltestosterone
produce anabolic responses in man and cause an increase
in the weight of the levator ani muscle in rats.

APPENDIX XII-7

Category: 24c1(-) E. F. Nutting	Gonads and Reproductive Organs; Anti-Androgenic, Rat.
Animal Used:	Rat, male, castrated at 22-23 days of age, 20 days before first injection.
Organ or Tissue Involved:	Seminal vesicles and ventral prostate.
Route of Test Drug Administration:	Intramuscular (or oral if indicated) in oil.
Initial Screening Dose:	5 mg total dose in 7 days. Testosterone propionate at a total dose of 0.5 mg. (IM) is administered concurrently.
Reference Standard:	None.
Activity Measurement and Interpretation:	Decrease in organ weight compared to testosterone propionate alone.
Basis of Assay:	In the castrate, testosterone propionate causes an increase in weight of the above organs. An anti- androgen should prevent this increase.
Clinical Correlation:	None at present. Follow-up should include study in prostatic hypertrophy and atherosclerosis.

(Revised 5-26-61)

APPENDIX XII-8

Category: 9a1(-) E. F. Nutting	Musculo-skeletal and articular: Anti-anabolic, Rat.
Animal Used:	Rat, male, castrated at 22 to 23 days of age, 20 days before the first injection.
Organ or Tissue Involved:	Levator ani muscle.
Route of Test Drug Administration:	Intramuscular or oral in oil.
Initial Screening	5 mg total dose in seven days. Testosterone propionate at a total dose of 0.5 mg (IM) is administered concurrently.
Reference Standard:	None.
Activity Measurement and Interpretation:	Decrease in organ weight compared to testosterone propionate alone.
Basis of Assay:	Androgens are anabolic. They also increase the weight of the levator ani muscle.
Clinical Correlation:	None.

(Revised 5-26-61)

APPENDIX XII-9

Category: 21a1 Endocrine Screening	Adrenal Cortex; Neoglycogenesis, Rat.
Animal Used:	Rat, male 120-160 gm, adrenalectomized at 5 days. High protein diet. Normal saline as drinking fluid. Fasted 24 hours.
Organ or Tissue Involved:	Liver.
Route of Test Drug Administration:	Subcutaneous.
Initial Screening Dose:	10 mg total dose per rat divided into 4 doses at 1 1/2 to 2 hour intervals.
Reference Standard:	Cortisone acetate.
Activity Measurement and Interpretation:	Total liver glycogen is measured 6 to 8 hours after the first injection. A dose of compound is rated active if it produces a significant ($P < 0.01$) increase in total liver glycogen. A compound is rated active if it is active at the screening dose of 10 mg or less; thus it would have $\geq 0.5\%$ the potency of cortisone acetate.
Basis of Assay:	Fasting depletes the liver of glycogen but in the intact animal, the liver is able to convert fats and proteins to glucose and maintain glycogen reserves, although at a reduced level. Adrenalectomy prevents the response but the administration of glucocorticoids restores this function.
Clinical Correlation:	The glucocorticoids which are most effective in causing deposition of liver glycogen in this test are those most effective in human diseases such as rheumatoid arthritis.

APPENDIX XII-10

Category: 24d1 Endocrine Screening	Gonads and reproductive organs; antifertility, rat.
Animals Used:	Female rats during first 15 days after mating.
Organ or Tissue Involved:	Reproductive system.
Route of Test Drug Administration:	Subcutaneous or intragastric.
Initial Screening Dose:	4 mg/day, daily for 7 days. Compounds inactive at this dose subcutaneously, have a potency of less than 0.1% that of estrone.
Reference Standard:	None. All estrogens are active. Estrone ED ₅₀ is 3 mcgm subcutaneously and 360 mcgm intra-gastrically.
Activity Measurement and Interpretation:	Normally about 95% of mated animals become pregnant. (Pregnant = 1 or more "normal" fetuses). Groups that have less than 50% pregnant rats are considered active. The ED ₅₀ is the dose which reduces the number of rats with normal size fetuses to 50%.
Basis of Assay:	During the first week after mating, pregnancy can be prevented by a number of mechanisms. This test does not differentiate among these mechanisms.
Clinical Correlation:	Essentially none at present.

Revised 5/1/70.

APPENDIX XII-11

Category: 24d2 Endocrine Screening	Gonads and reproductive organs: anti-fertility, hamster.
Animal Used:	Female hamster during the first 6 days after mating.
Organ or Tissue Involved:	Reproductive System.
Route of Test Drug Administration:	Subcutaneous or intragastric.
Initial Screening Dose:	5 mg/day, daily for 5 days. Compounds inactive at this dose have a potency of less than 0.2% that of subcutaneously administered estrone.
Reference Standard:	None. All estrogens are active but activity does not correlate with estrogenicity. Estrone ED ₅₀ , 11 mcgm subcutaneously and 820 mcgm intragastrically.
Activity Measurement and Interpretation:	A dose of compound is rated active if the implantation rate (total sites x 100/total corpora lutea) is 50% or less. Long term control data show that untreated groups have an implantation rate of from 53 to 100% with an average of 87%.
Basis of Assay: 2/1/70	Post ovulatory treatment may prevent fecundity in several ways. "Active" compounds may vary in their mechanism of action but their net effect is to prevent implantation while certain other compounds, classed as inactive, may permit implantation but interfere with fetal development.
Clinical Correlation:	Essentially none at present.

APPENDIX XII-12

Category: 20a4 Endocrine Screening	Pituitary: Anterior; Ovarian Compensatory Hypertrophy, Rat.
Animal Used:	Rat, female, unilaterally ovariectomized, 72 days old, approximately 200 grams.
Organ or tissue Involved:	Ovary
Route of test drug Administration:	Variable
Initial screening dose:	1000 µg daily for 14 days.
Reference Standard:	Norethynodrel administered subcutaneously.
Activity Measurement and Interpretation:	A <u>dose of compound</u> is rated active if it produces a significant ($P < 0.05$) decrease in the weight of the ovary. A <u>compound</u> is rated active if it is active at the screening dose of 1000 µg; thus it would have $\geq 3.2\%$ of the potency of norethynodrel.
Basis of Assay:	Unilateral ovariectomy in the rat results in a compensatory increase in weight of the remaining ovary. The physiology of this compensatory mechanism is poorly understood but presumably is due to lowered ovarian steroid production and consequential elevation of circulating gonadotrophin.
2/1/66	Substances classed as pituitary inhibitors in tests with parabiotic rats and assays measuring the content of FSH and LH in the pituitary glands of rats also prevent the compensatory growth of the remaining ovary following hemicastration. All known estrogens are very active in this test.
Clinical Correlation:	In humans, the administration of certain estrogens, progestins, and androgens results in a decrease of circulating gonadotrophin as measured in the plasma and urine. These steroids are also active in this test.

APPENDIX XII-13

Category: 27a5B Endocrine Screening	Tissue reaction: anti-inflammatory, carrageenin induced foot edema, rat.
Animal Used:	Rat, male, intact, 120 gram.
Organ or Tissue Involved:	Hind feet injected with carrageenin 1 hour after administration of drug.
Route of Test Drug Administration:	Subcutaneous or intragastric in saline, oil, or propylene glycol.
Initial Screening Dose:	25 mg total dose subcutaneously in one injection. Active compounds receive further testing intragastrically at 5.0 mg and lower.
Reference Standard:	Hydrocortisone administered intragastrically.
Activity Measurement and Interpretation:	A dose of compound is rated active if it causes a significant decrease ($P < 0.05$) in the volume of the feet six hours after administration. The M.E.D. for Hydrocortisone is 2 mg intragastrically and 0.8 mg subcutaneously.
Basis of Assay:	Introduction of carrageenin into the foot induces a local inflammatory response manifested as an edematous swelling in the surrounding tissue. Hydrocortisone, Butazolidin, aspirin, and Indomethacin inhibit the extent of the swelling and are therefore considered to be anti-inflammatory agents.
Clinical Correlation:	These same compounds show anti-inflammatory activity in the treatment of rheumatoid arthritis and of so-called collagen diseases in man.

7/1/69

APPENDIX XII-14

Category: 27a3 Endocrinology Screening	Tissue reaction: Anti-inflammatory, Cotton was granuloma, Rat.
Animal Used:	Rat, male, 180-220 gm; adrenalectomy 1 day prior to implantation.
Organ or Tissue Involved:	Subcutaneous connective tissue (granuloma) surrounding implanted cotton pellets.
Route of Test Drug Administration:	Intragastric or subcutaneous.
Initial Screening Dose:	20 mg daily for 2 days.
Reference Standard and Dose:	None; 0.5 mg hydrocortisone administered <u>subcutaneously</u> will be found active 95% of the time (0.2 mg has borderline activity); 10 mg Sodium Butazolidin (sodium phenylbutazone) administered <u>intragastrically</u> will be found active 95% of the time.
Activity Measurement and Interpretation:	A compound is rated active if it causes a significant decrease ($P < .05$) in the adjusted median weights of granuloma tissue encapsulating implanted cotton pellets.
Basis of Assay:	When a foreign material, such as cotton, is subcutaneously introduced into an animal, it becomes encapsulated with connective tissue, forming a granuloma. This is a manifestation of a local inflammatory response. Cortisone, hydrocortisone and Butazolidin will inhibit the formation of granuloma tissue and from this standpoint are considered to be anti-inflammatory agents.
Revised: 5/1/68	
Clinical Correlation:	In general, corticosteroids and Butazolidin which show anti-inflammatory activity in this test are also effective in treatment of so-called collagen diseases in man, including rheumatoid arthritis.

APPENDIX XII-15

Category: 27a7A Endocrine Screening	Tissue reaction: Anti-inflammatory; chronic prophylactic polyarthritis, <u>Mycobacterium butyricum</u> induced hypersensitivity, 20 day test, rat.
Animal Used:	Rat, male intact, 160-180 gms, treated intradermally with <u>M. butyricum</u> in paraffin oil.
Organ or Tissue Involved:	Hind limbs which become swollen as a result of the hypersensitivity.
Route of Test Drug Administration:	Intragastric or subcutaneous.
Initial Screening Dose:	5 mg per day IG for 19 days beginning on the day of <u>M. butyricum</u> inoculation. Prior to 6/23/71 the route of administration was SC.
Reference Standard:	None. The minimum effective dose of Sodium Butazolidin or hydrocortisone administered intragastrically or subcutaneously is 0.5-1.0 mg.
Activity Measurement and Interpretation:	A dose of compound is rated active if it causes a significant decrease ($P \leq 0.05$) in the volume displacement of the hind paws of treated animals as compared to controls.
Basis of Assay:	An intradermal injection of killed <u>Mycobacterium butyricum</u> suspended in paraffin oil causes a generalized hypersensitivity which becomes manifest beginning about 10 days after inoculation as an edematous swelling of the extremities, particularly the hind legs. Joint degeneration resembles histologically that seen in rheumatoid arthritis.
6/23/71	
Clinical Correlation:	Anti-inflammatory drugs and immuno-suppressive agents inhibit the development of this swelling. These same drugs are used in the treatment of rheumatoid arthritis and other connective tissue diseases in man. These diseases are suspect of being auto-immune in nature.

CHAPTER XIII

E-88: EXPERIMENTS IN MATED AND PREGNANT RHESUS MONKEYS;

A COMPILATION OF AVAILABLE FRAGMENTARY DATA

I. Background and Origin of Material

Dr. Harry Waisman was Professor of Pediatrics at the University of Wisconsin and a nationally recognized primatologist with a deep interest in research on birth defects in children. He served as a consultant to Searle Laboratories and carried out experiments for Searle, one of which was submitted to the FDA as E-32, carried their Pathology-Toxicology number 856ot70, and was entitled "52 Week Oral Toxicity Study in the Infant Monkey."

Following Dr. Waisman's death, some fragmentary research observations on a number of other monkeys were found in the process of cleaning out his laboratory and office. It is said that these animals were not a part of the PT856ot70 study. Rather, the data appeared to be the kind of preliminary, incomplete, exploratory observations that are not infrequently made by scientists to ascertain whether a problem is worth studying in more detail with a properly prepared and planned protocol. In their "effort to make available all technical information regarding aspartame. . . whether scientifically meaningful or not" (Appendix XIII-1), this fragmentary, incomplete material was gathered together and submitted to the FDA as E-88. Searle says that these preliminary experiments were unknown to them prior to receiving the materials from Wisconsin, and that these studies were not specifically supported by Searle.

The Primate Center at the University of Wisconsin is one of seven regional primate research centers sponsored by the National Institutes of Health. The initial emphasis of this Center concerned itself with a number of facets of maternal and infant behavior, and many of the monkeys there showed abnormal social adaptation. Nevertheless, in order to achieve maximum utilization of these valuable non-human primates, such animals were occasionally used for other unrelated experiments. It is stated that some of these socially maladjusted females were used by Dr. Waisman, and in their particular environment, because of poor maternal care for their offspring, a somewhat higher than usual infant mortality was noted.

The variable nature of the data on the eight adult and one newborn monkeys in E-88 is shown in Table 13-1. The data consist of five pages of graphs on six monkeys, one table of data on one monkey, 10 pages of SMA 12/60 charts on five monkeys, and three pages of a necropsy report on the neonatal offspring of one.

Table 13-1
E-88 Monkeys and Available Data

Monkey Number and Status	Days After Mated		Data Available				Data page in E-88
	SMA 12/60	APM g/kg/day	Body wt.	APM	Formula Intake	Serum Dal	
A23 preg. stillbirth	34,56,115	138 day, max 1.8	+	+	+	+	5-8
879 preg. control	24,56,112	0	+	0	+	0	5,9-11
B24, aborted 65 da	21,56	38 da, 8 da 2.5	+	+	+	+	13-15
830 not preg.	30	0	0	0	0	0	18
831 not preg.	51	0	0	0	0	0	19
863 not preg.	0	39 da, max 1.6	+	+	+	+	17
836 aborted 40 da	0	55 da, max 0.6	+	+	+	+	16
A39 del. N38	0	41 day, max 3	++	++	++	++	12,29
N32 newborn of A39	0	0	++	0	0	0	25-27,29

Table 13-1 provides information regarding the pregnancy status of eight monkeys in E-88, the number of days after mating on which SMA 12/60 charts were provided, and, if graphs were available, the number of days aspartame (APM) was administered and some information regarding its dosage in g/kg/day. B24 received over 2.5 g/kg/day on only eight days; the figure for consumption of aspartame in g/kg/day for A-23, 863, 836, and A-39 represent the maximum dosages they received. The days of aspartame administration represent days prior to termination of pregnancy. The ++ signs for A-39 indicate the availability of tabular data (page 29 of E-88 book) whereas the + signs under "data available" indicate information in graphs reproduced in E-88 on pages 5, 12, 13, 16, and 17.

Admittedly, attempts to summarize information from such available data are far from precise. We do not know who prepared the graphs or raw data and one can only estimate some of the values in Table 13-1. There are some data provided in the table on page 29 of E-88, but there is generally poor agreement between that data and the graphed information on page 12. The table indicates that Similac was started on the 74th day of pregnancy; the graph suggests day 80. The graph shows intake of Similac in cc/kg/day but the data in the table generally do not agree with the graph. The body weights in the table do not precisely fit the graph in Figure 8. In a Searle internal memorandum dated April 10, 1975, Dr. K. S. Rao states, "The attached graph indicates that this monkey received SC-18862 beginning 112 days of gestation. However, the raw data on actual intake of SC-18862 do not show any consumption of APM on 112th day of gestation. Hence, I indicated in the report that this monkey received SC-18862 beginning 119 days of gestation, for which data are available." The table on page 29 shows .55 g/kg APM at 127 days, but no figures are indicated for 112 or 119 days. UAREP would suggest that it is possible that monkey A39 received aspartame for between 33 and 48 days.

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II. Analysis and Critique of E-88 Report

The sequence of events in the formulation of the E-88 report is outlined in Appendix XIII-1. For the most part, the background information indicates that the Searle staff attached little significance to this miscellaneous collection of data.

Dr. McIlreath's transmittal letter to FDA dated June 19, 1975 is straightforward and states in part, "It is a compilation of this additional fragmentary data. It constitutes all of the data from the late Dr. Waisman's laboratory of which we are aware. The studies from which these data are derived were conducted without our knowledge or support. They appear to have been exploratory in nature."

A. Dr. Wagner's Critique

This two page analysis of the report was included in E-88. Dr. Bernard M. Wagner makes a brief summary of the data and raises pertinent questions regarding the lack of necessary and desirable information. He criticizes the use of terms "stillbirth" and "abortion" in the context

of the sparse data available. He concludes that ". . . the experimental design and evaluation of data are incomplete and not suitable for the scientific community." After this crisp and critical scientific approach, he then includes the statement, "The summary presented on page 33 (subsequently became page 22), of the report is fully supported by the information made available."

B. Text of Report

Pages 1-3 of the E-88 report present straightforward statements disclaiming that Searle Laboratories had any role in planning or was even aware of these experiments. "These data apparently reflect an exploratory effort perhaps to ascertain information for future studies. It is readily apparent that these data failed to establish or refute any given scientific hypothesis. All raw data records available to the authors are reproduced in this report."

It should be noted that none of the nine pages of data which were transmitted to Searle via Dr. Harper on 1-9-75 was included in the report, although four of the monkeys were noted there (see Appendix XIII-2). However, these written notes were of such a raw and disorganized nature that it is questionable whether they would be meaningful to most readers.

On page 2 of E-88, after quoting 15 criteria of data which should have been supplied but were not, the statement is made that for these reasons ". . . the data reported are not sufficient to establish the effects of huge quantities of SC-18862 in the pregnant Rhesus monkey. This report is submitted simply to comply with the obligation of pro-

viding all the information known to us regarding this agent." This is a sound critique of information that would have been necessary to provide meaningful data in this situation.

Some of the copies of graphs and tables submitted by Searle to FDA are reproduced as copies which can not be easily read and therefore are not subject to interpretation. This is true of Figure 8 and page 29 of E-88. Figures 1, 9, 12, and 13 of the E-88 report are most clearly depicted on photographic copies that became available to UAREP from the Searle files at the time FDA removed its seals. These show much more information, by means of color coding, (See Appendix XIII-3) than is readily apparent on the black and white Xerox copies. It is of some interest that the data contained in these graphs apparently are not entirely duplicated in the raw data from the Searle raw data files; nor are these figures included in the listing of raw data submitted by the University of Wisconsin to Searle, although that is presumed to be their source. In response to a query regarding the source of the data in these graphs, Dr. McIlreath expressed the opinion that they were prepared by people at the University of Wisconsin. Whatever the source of the data, the information contained is virtually the only information included in the Entry data that has any potential significance, and even that significance is questionable.

Ten figures in E-88 present the results of SMA 12/60 analysis of Searle specimens for total protein, albumin, calcium, inorganic phosphates, cholesterol, glucose, blood urea nitrogen, uric acid, creatinine, total bilirubin, alkaline phosphatase, and glutamic oxaloacetic transaminase. Figures 2, 3, and 4 present the results on monkey A-23 at 34 days, 56 days, and 115 days of pregnancy. Figures 5, 6, and 7

similarly present the results on monkey 879 for 25 days, 56 days, and 112 days pregnancy. Figures 10 and 11 give the results on monkey B-24 for 21 days and 156 days pregnancy. Figures 14 and 15 give similar results for monkey 830 at 30 days and monkey 831 at 51 days pregnancy, although as subsequent notes indicate, neither animal was pregnant. The laboratory results shown are essentially within normal limits and Searle wasted no effort in attempting to interpret or dwell upon the significance of any of these findings. On pages 20 and 21, the Entry report presents a reasonable statement of many of the problems in attempting to interpret such data.

The last paragraph on page 21 of the E-88 report states: "It was apparent that feeding SC-18862 throughout the entire gestation period (monkey No. A-23) resulted in an elevation of the maternal serum L-phenylalanine level comparable to the increases observed by Kerr, et al² when feeding approximately equimolar quantities of L-phenylalanine. Also, the birth weight of the neonates (A-23 and B-24) are comparable to those obtained by Kerr, et al. when feeding L-phenylalanine. Thus, it would appear that in monkeys exposed to SC-18862 in large amounts throughout the entire gestation period, and proceeding to term delivery, no adverse effects other than those produced by feeding the L-phenylalanine only were observed." (See Appendix XIII-3 and 4).

C. Conclusions of the Report

The conclusions as stated on page 22 of E-88 depict an exercise in semantics to which many scientists studying the E-88 report would take some degree of exception. It states, "The following generalizations appear to be consistent with the data presented," followed by four statements:

"1. Administration of SC-18862 to pregnant monkeys at dose levels up to 3.8 g/kg/day did not adversely affect the maternal appetite (formula intake) or body weight, nor did it induce seizures." A review of the details on the four pregnant monkeys shows the following: A-23 was on aspartame for 138 days at 1.8 g/kg/day or less and the pregnancy resulted in a "stillbirth." E-24 was on aspartame for 39 days before she aborted, and the level of aspartame was above 2.6 g/kg/day for 8 days. Before she aborted, Monkey 836 received aspartame for 25 days at a maximum consumption of 0.6 g/kg/day. Monkey A-39 received aspartame for approximately 41 days before delivery. Her average consumption during this time was 2.0 g/kg/day with a maximum of 3 g/kg/day. Thus, three of the monkeys that were pregnant received the compound for 25, 39, and 41 days and one monkey for 138 days of a usual term of pregnancy of 168 ± 4 days. A statement that administration ". . . to pregnant monkeys at dose levels up to 3.8 g/kg/day" is misleading in that only one monkey received such dosage and that for only one interval. To many it would seem apparent that the very small number (four) of monkeys at test and the time of exposure with only one exceeding 41 days, do not justify drawing any conclusions. It is true that the formula intake and body weight, as presented in the graph in the absence of supporting raw data, did not show a consistent variation.

The second conclusion on page 22 was "The administration of SC-18862 to pregnant monkeys during the major portion of the gestation period did not produce obvious anatomic malformations in the term fetus." Since only one monkey received treatment for more than 41 days, some would question the implication that pregnant monkeys were treated

over a major portion of the gestation period (168 days). Again, only one offspring was examined, and that by autopsy techniques, which would only show the most obvious of anatomical malformations. It would seem that this series of one observation, as evidenced in these experiments, would surely not justify conclusions as to the teratogenic potential of aspartame. The number of animals and duration of exposure would be considered by most (as they were in earlier memos by Drs. McConnell and Rao) to be inadequate to justify any conclusions.

Conclusion number 3 states: "Administration of high doses of SC-18862 did produce significant elevation of maternal serum phenylalanine levels, roughly comparable to those produced by administering approximately equal quantities of L-phenylalanine." Except for the extremely small number of animals involved, the data give some credence to this conclusion.

Conclusion number 4 states: "The alleged premature termination of pregnancy (abortion) in the SC-18862 treated monkey was not associated with notable elevation of maternal serum phenylalanine levels. The two alleged abortions reported involved monkeys with serum phenylalanine levels within the normal range. This infers that the abortions may be incidental and not directly related to the SC-18862 administration." Again, one is concerned with a series of two pregnancies that resulted in abortion, without information as to the incidence of spontaneous abortions in this colony. Many scientists would feel that the number of animals is insufficient to merit any conclusion.

Pages 25, 26, and 27 provide such information as is available on necropsy 70:53 on animal N-38 (the offspring of A-39) performed by Dr.

Paik. The report notes that there was minimal myocardial hemorrhage and intracytoplasmic vacuolization in the liver, but suggests that the primary cause of death at 3 days of age may relate to inadequate post-natal care partially relating to a socially maladjusted mother. There is one obvious minor discrepancy in the necropsy report. On page 25, the sex of the infant is twice referred to as male, whereas the histologic description of the ovary on page 27 is said to be "not remarkable." This discrepancy was noted on the copy of the report by Dr. Rao of the Searle staff on 3-17-75.

III. UAREP Conclusions on Review of E-88

It is the opinion of UAREP that this is an inconsequential report based on woefully inadequate and confusing data obtained from an inadequate number of animals. As written, the report appears to attempt to provide something for everyone of different views. Many scientists reviewing this report would feel that 28 of the 29 pages are plausible, but that the one page of conclusions (page 22) goes too far in attempting to glean some conclusions from data which elsewhere in the report are properly recognized as being inadequate.

CHAPTER XIII
LIST OF TABLES

13-1 E-88 Monkeys and Available Data

LIST OF APPENDICES

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XIII-1 Sequence of Events in Formulation of the E-88 Report and Inventory of Data from Wisconsin	954
XIII-2 Discussion of Nine Pages of Data Transmitted by Dr. A.E. Harper, University of Wisconsin, to Dr. Fred McIlreath of Searle Laboratories on January 9, 1975	958

APPENDIX XIII-1

SEQUENCE OF EVENTS IN FORMULATION OF THE E-88 REPORT AND INVENTORY OF
DATA FROM WISCONSIN

Experimental observations on these animals ranged from December 3, 1970 to March 17, 1971.

May 13, 1970 - Dr. Steven Paik performed a necropsy on the offspring N-38 of mother A-39.

June 23, 1971 - Dr. John Davenport, Director of the Wisconsin Primate Center transmitted materials to Searle with data relating to PT2560t70 (E-32). This material was acknowledged to consist of a total of 5 pages, reproduced in E-88 as autopsy number 70:53, N-38 on pages 25, 26, and 27; Figure 8, entitled "A-39 Chart of Body Weight and Aspartyl Phenylalanine Consumption" on page 15; and Table of Data on A-39 on page 29. In juxtaposition to these pages in the Searle raw data book were 10 pages of SMA-12/60 data reports on several presumably pregnant Rhesus monkeys with dates of determination ranging from 12-3-70 to 3-17-71.

January 9, 1975 - A. E. Harper, Professor of Biochemistry and Nutritional Sciences at the University of Wisconsin transmitted 9 pages of notes to Dr. McIlreath.

January 13, 1975 - Materials from Dr. Harper received by Regulatory Affairs at Searle.

January 14, 1975 - Above materials received, with a note to Bob (McConnell) from F. (McIlreath).

January 15, 1975 - Memorandum from McConnell and Rao addressed to file, PT856ot70, Volume 5, relating to 9 pages of fragmentary data. This stated ". . . wider distribution appears to serve no scientific purpose." (The Pathology Toxicology number PT856ot70 for E-32 was presumably used because there was no prior P-T project for these observations.)

April 7, 1975 - Searle transmittal form indicates that material (for E-88) was sent to the typist.

April 10, 1975 - Handwritten memorandum by Dr. Rao indicates that he interpreted the administration of SC-18862 beginning on 119 days of gestation instead of 112 days as indicated on some of the materials received.

Undated Memorandum (presumably prepared in April, 1975) from McConnell to Aspartame raw data file: PT856ot70; Volume 5. This memorandum concludes: "Since these data are fragmentary and inconclusive and provide no evidence of an adverse effect of aspartame, further dissemination appears to serve no useful purpose."

April 29, 1975 - Dr. Bernard M. Wagner, Professor of Pathology, College of Physicians and Surgeons, Columbia University, New York, and Director,

Department of Laboratories of Beekman-Downtown Hospital, wrote a personal note to Dr. McConnell criticizing the draft of the report for E-88 and making suggestions for its improvement.

May 5, 1975 - Dr. Bernard M. Wagner submitted a formal report to Dr. McConnell which was, generally highly critical of the scientific merit of the data, yet he agreed in general with the conclusions of the report. The E-88 report was subsequently edited considerably and reduced from 40 to 29 pages in length. No copy of the 40 page report was submitted by Searle to UAREP and none was requested by UAREP, because only the 29 page report was submitted by Searle to FDA.

May 14, 1975 - Memorandum from McConnell to McIlreath entitled, "Prompt distribution of the attached document entitled SC-18862: EXPERIMENTS IN MATED AND PREGNANT RHESUS MONKEYS." The memorandum stated in part, "In our effort to make available all technical information regarding aspartame known to us, whether scientifically meaningful or not, we have compiled the attached document. . . Acquiring the information compiled was notably complicated as Dr. Waisman died several years ago. The scientific benefit is nil, and the effort expended was considerable. Please distribute the document promptly."

June 16, 1975 - McConnell memorandum says, "This report was completed in final form on 11 April 1975 and was signed and dated by the two authors; it was subsequently reviewed critically and commented on by several authoritative non-Searle staff consultants, as requested by Searle Laboratories management. The report was re-submitted for appropriate distribution on 16 June 1975. The decision was made by the authors to eliminate any of the report data." (The only available authoritative non-Searle staff consultant from whom comments were supplied to UAREP was from Dr. Wagner.)

June 16, 1975 - Report re-submitted for distribution.

June 19, 1975 - Dr. Fred McIlreath, Director of Regulatory Affairs Department for Searle, transmitted report to FDA.

APPENDIX XIII-2

DISCUSSION OF NINE PAGES OF DATA TRANSMITTED BY
DR. A. E. HARPER, UNIVERSITY OF WISCONSIN, TO DR. FRED McILREATH
OF SEARLE LABORATORIES ON JANUARY 9, 1975

The appended pages consist of the following items:	Page
A. Memo to Aspartame "Raw Data File P-T No. 856ot70; Volume 5," dated January 15, 1975.	960
B. Note apparently to Bob (McConnell) from F. (McIlreath) time stamped January 14, 1975.	961
C. Note from A. E. Harper to F. J. McIlreath dated January 9, 1975.	962
D. Copy of long-hand notes regarding various animals.	963
E. Tabulated data on E-65 and E-56 dated 12-29-69.	965
F. Duplicate copy of item E.	966
G. Graph on Monkey E-27, Aspartyl-phenylalanine methyl ester.	967
H. Graph on Monkey B-56, Aspartyl-phenylalanine methyl ester.	968
I. Graph on Monkey D-43, Aspartyl-phenylalanine methyl ester.	969
J. Graph on Monkey E-65, Aspartyl-phenylalanine methyl ester.	970
K. Long-hand written data on Monkeys A-23, 879, 830, 831.	971
L. Long-hand written data on several monkeys.	973
M. Table comparing monkey data in E-88, 9 pages, E-32 and 2 publications.	975

The papers on items D-L are folded the same as Dr. Harper's letter to McIlreath and presumably they were all transmitted together. The two names circled, the underlining of "but not pregnant" on item D, the arrows to lines on item G-J, and the underlining on "but not pregnant" on item K, were all marked in red ink on the original copy by some unidentified individual.

Because the copies as reproduced here lose something over the copies originally transmitted, UAREP's interpretation of the information on items D, K, and L is interspersed with items D*, K*, and L*.

On item D, 15 different monkeys are indicated, of which four near the middle of the page, are identifiable as being included in E-88. (A-23, 830, 831, 879). Six (on the lower half of the page) are in E-32 (M-34, M-38, P-53, P-60, M-64, M-79), and five animals are in neither E-88 or E-32 (E-27, D-43, B-56, E-65, 141). Data on the phenylalanine increase and tryptophan decrease on the first four of these five monkeys are given in the graphs and table on sheets E-I. The data presented on sheets E, F, G, H, I, and J on animals E-27, E-43, E-56, and E-65 do not pertain to any animals identifiable in E-88 or E-32. On item K, all four animals are in E-88 and there are no notations on other animals. On item L, there are three animals in E-88 (A-23, 830, 831), and four animals in E-32 (34, 38, P-53, 79). In the absence of dates and more detailed information, one can only surmise from items D and L that someone unknown was making notations regarding the animals in both E-88 and E-32 at the same time. In other words, since Searle states that they were unaware that the experiments with aspartame on the animals in E-88 had been undertaken, it appears probable that there were at least five other monkeys in addition to the animals in E-88 and E-32, which were being given aspartame by a Wisconsin investigator.

Item A

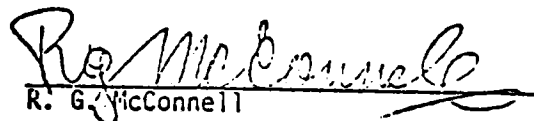
January 15, 1975

MEMO TO: Aspartame "Raw Data" File; P-T No. 856ot70; Volume 5. ✓
COPY TO: F. McIlreath
A. Harper; Univ. of Wisconsin
FROM: R. G. McConnell
K. S. Rao
SUBJECT: Attached note from A. Harper to F. McIlreath and subsequent
nine pages of notations and/or data from the late H. Waisman's
laboratory records.

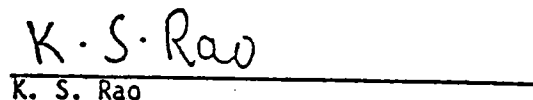
The attached information was procured by Dr. Harper, upon request by Dr. McIlreath, from records available to him presumably at the University of Wisconsin. The intent was to ascertain that all available data on the Aspartame infant monkey study (P-T No. 856ot70) was, in fact, known to Searle Laboratories and available in our records.

We have reviewed these new "data" and find that, based on animal numbers indicated, none of the data is part of the study listed above. It would appear that these data are of a rangefinding type, and some were generated prior to implementation of the above infant monkey study, and some during the study. Both pregnant and non-pregnant animals appear to have been employed, and probably constitute some exploratory work conceived and conducted unilaterally by Dr. Waisman. At the time of Dr. Waisman's death Searle Laboratories had not, to my knowledge, entered into any agreement that he study Aspartame in pregnant monkeys.

We will maintain these data on file, but due to their fragmentary nature, wider distribution appears to serve no scientific purpose. Please let me know if you do not agree with this conclusion. *(Spec. Copies)*


R. G. McConnell

RGMCC:jm
Attachment


K. S. Rao

Item B
A. E. Harper, Ph.D.
Professor of Biochemistry and Nutritional Sciences

RECEIVED
JAN 13 1975
REGULATORY AFFAIRS
3447 Edgemoor Parkway
Madison, Wisconsin 53705

1/9/75

Dr. F. J. McCreath
Searle Laboratories
Chicago, Ill.

Dear Fred,

Here are the notes from the Weismann
study. They are not very illuminating, but
they do give the numbers of the animals

Sincerely,
A.E.H.

RECEIVED
JAN 14 1975
PATHOL DEPT.

RECEIVED

JAN 14 1975

PATH-TOX DEPT.
GEARLE LABORATORIES

Bob

Alf. was able to
obtain these notes
from the Primate Center
U of Wisc

It appears to be
new data!!

7

<

Item D

~~11-58~~

2-4994
2-2727

Shurt/km?

B 56 photo 1/2
E 27 - photo 1/2
E 65 photo 1/2
D 43 - photo 1/2

23 pages
18/12/19
279 - page control
n. 830 on airport file
but not page
n. 831

David Lyons
Port. Police
Adm. Serv. Div.
N. Adelaide, S. Australia
5006

newborn ~~is~~ ~~in~~ ~~the~~ ~~file~~

under
Davenport's control

p. 53
DOB 8-28-70
not normal
weak legs
neck and back
birth damage

Gertie
Schaffner
263-3519

scissors
accidents
34
38
79 - animal behavior
but he
ground in
M. 79

Antony
mastered
P 60

M 79 } Has been sent to Seawater M 64 5/10/15

Item D *

~~M-38~~

2-4994
2-2727

Short term?

B-56 phenylalanine increased; tryptophan decreased
E 27 phenylalanine increased; tryptophan decreased
E 65 phenylalanine increased; tryptophan decreased
D 43 phenylalanine increased; tryptophan decreased

A 23 pregnant
1 g/kg/day

879 - pregnant control

? 830 on aspartyl phenylalanine

but not pregnant

? 831 but not pregnant

Newborn - ~~offspring~~ probably not

? p 53

David Lyons
Dept. Paediatrics
Adelaide Child. Hosp.
N. Adelaide, S. Australia
5006

under
Davenport's control

DOB 8-28-70

not normal
weak legs
needs hand feeding
birth damage?

but o.k.

grand mal seizures

M 34
M 38 dead

? seizure activity

P 79
? M7 also had to be hand fed

P60

Autopsy
Material
sent to Searle

M 79
M 141 HCE lengthy low

M64
M79 (HC and
lengthy low

- 12-29-69

Item E

	L-φ ₂	L-T _{grain}	L-T _{hydrocarbon}
E-65 0	1.9	1.2	.968
1 h	6.6	2.7	.817
2	12.4	3.8	.650
3	18.4	4.3	.594
4	12.5	4.3	.550
6	8.4	3.0	.550

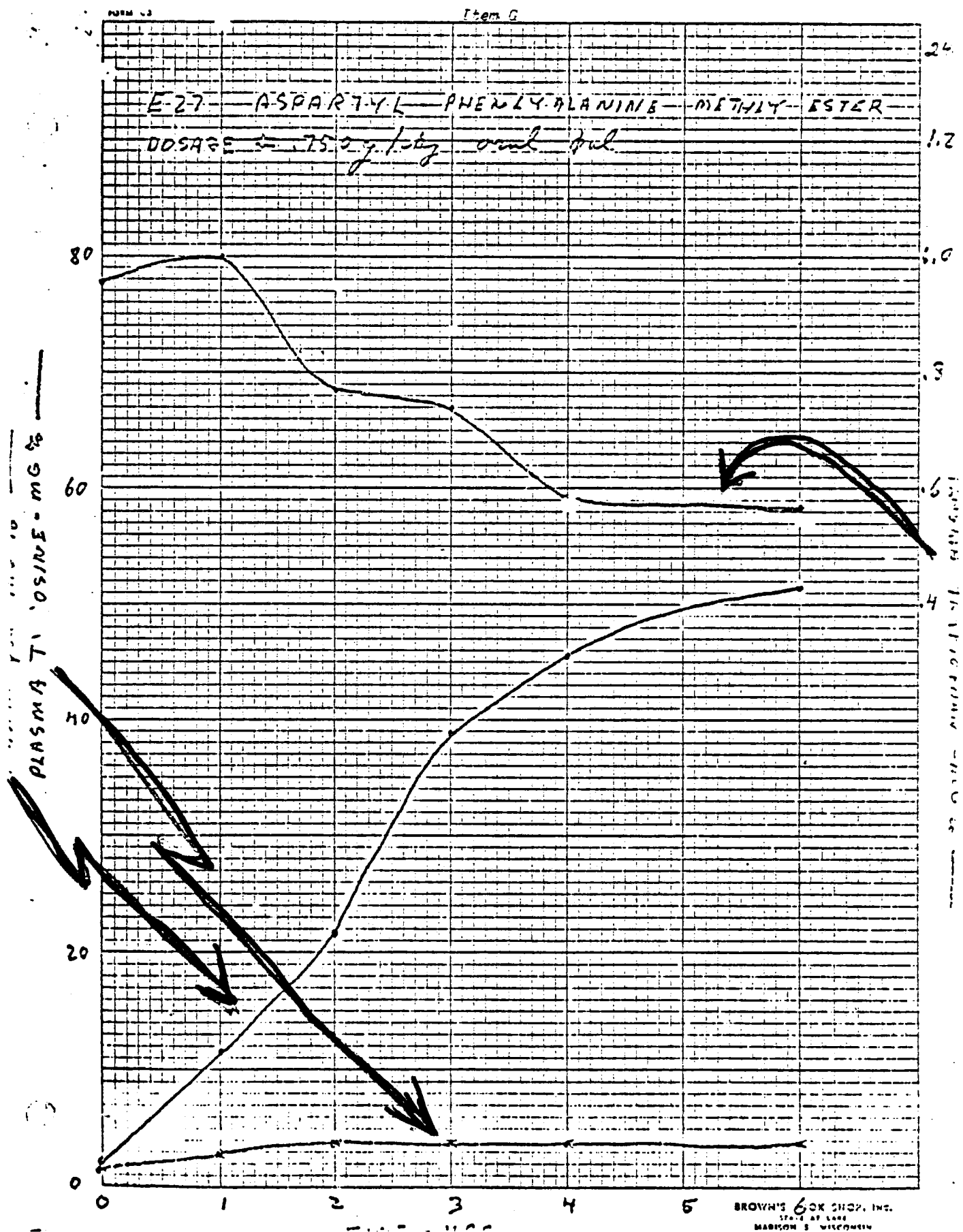
B-560	1.4	1.2	.928
1	21.8	2.9	.917
2	22.3	4.5	.617
3	29.5	5.2	.565
4	33.4	5.2	.582
6	45.1	4.9	.417

- 12-29-59

Item F

	L-φ ₂	L-T _{grain}	L-T _{myofibrillar}
E-650	1.9	1.2	.968
1 h	6.6	2.9	.817
2	12.4	3.8	.650
3	18.4	4.3	.594
4	12.5	4.3	.550
6	8.4	3.0	.550

B-560	1.4	1.2	.928
1	21.8	2.9	.717
2	22.3	4.5	.617
3	29.5	5.2	.565
4	33.4	5.2	.582
6	45.1	4.9	.417

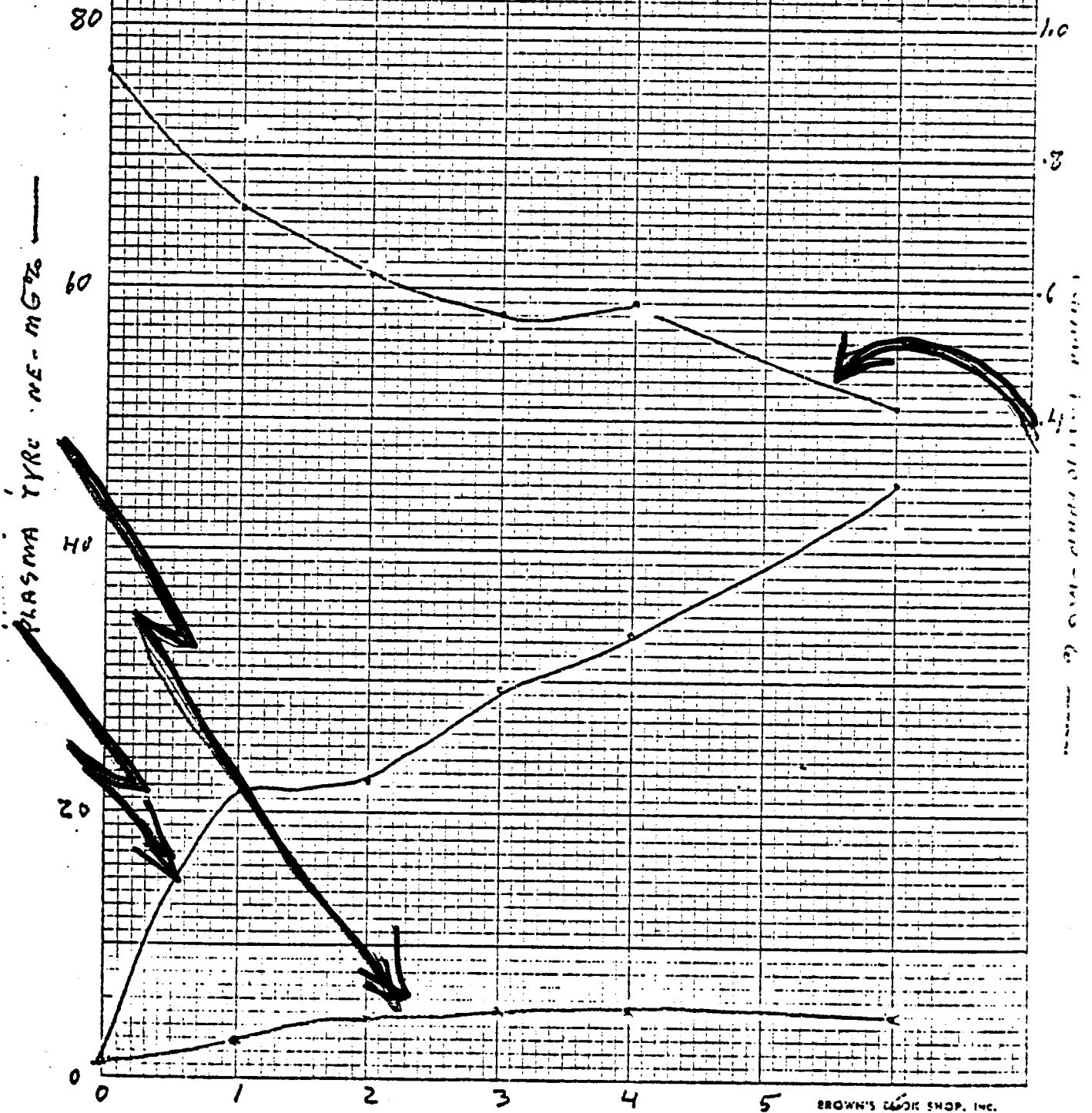


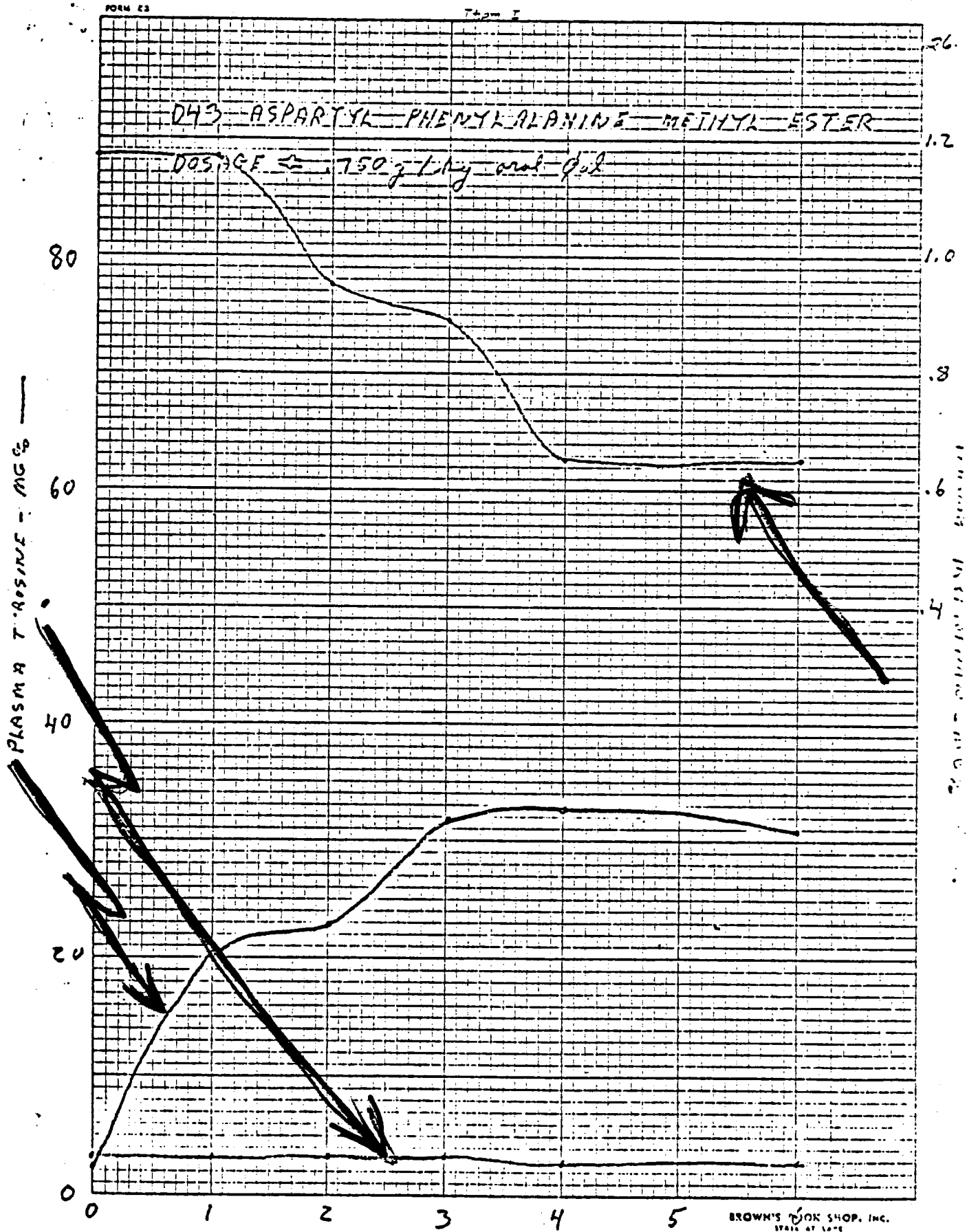
FORM 63

Item H

B5.6 ASPARTIC PHENYLALANINE METHYL ESTER

DOSEAGE 0.750 g / 2g oral Pul





FORM C3

Item J

E65 - ASPARTIC - PHENYLALANINE - METHYL - ESTER

DOSEAGE = 750 mg Oral qid

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1.3

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113

"A. 23

- Haven 'sporth'

Item K

$\approx 0.11 \text{ gm/cc}$

Volume intake = 700

$\therefore 700 \times 11$

Herbst = 7 kg

$\therefore 1 \text{ gm/Kg/day}$

but increased damage to the given
week means time of prog.

879

• must take in fat.

were before for security of

५३०

- on asphalt but not paving

231

12-18-70

A-23 Given aspartyl phenylalanine 0.11 gm/cc

Volume intake = 700 cc

700 x .11.

Her wt = 7 kg

1 gm/kg/day

but increased dosage to be given with increased
time of pregnancy

879 - won't take aspartyl phenylalanine
. will be pregnant control for aspartyl phenylalanine

830 - on aspartyl but not pregnant

831 - on aspartyl but not pregnant

Item L
 1st asp d'at file
 Total Summation 2-255/1000

500 f 30 - 3000 gm/cc -
 (8. Kg)
 50 f 31
 (8. Kg)
 0000/kg A-23 -- 022 gm/cc
 (8. Kg)
 Kg

8-53 - 203 - 8/28/70 - 2000 g to 2000 gm
 2000 gm to 2000 gm
 2000 gm to 2000 gm

34 2nd row (under 2nd row of 2nd row)
 38 - 1st row (under 1st row of 2nd row)
 79 2nd row (under 2nd row of 2nd row)

Put in Aspartylphenylalanine
file

Total Sweetener
concentration
22.88/1000 cc.

<u>Intake</u>	Pregnant Females	Item L*
550	830	- 3 mgm/cc
	(8.0 kg)	
650	831	
	(8.0 kg)	
1000 cc/day	A-23	.022 gm/cc
	(8.00 kg)	

P-53 - DOB 8/28/70 - needs to be hand fed
may be birth damage
not normal

weak legs
mother's first pregnancy

34

38 - grand mal (when caught and disturbed)
& or when replaced
79 sleep (only when disturbed)

Item M

Source of Data on Monkeys in Five Sources

<u>E-88</u>	<u>9 pages Data</u>	<u>E-32</u>	<u>Article 1965</u>	<u>Article 1968</u>
		N-14	A08	B4
			A18	
<u>A-23</u>	<u>A-23</u>		<u>A-23</u>	<u>A-23</u>
<u>B-24</u>			A-24	<u>B-24</u>
			A-32	
	<u>M-34</u>	<u>M-34</u>		
<u>A-39</u>			<u>A-39</u>	
				B-39
<u>830</u>	<u>830</u>			
<u>831</u>	<u>831</u>			
	<u>P60</u>	<u>P60</u>		
836	<u>M64</u>	<u>M64</u>		
863	<u>M79</u>	<u>M79</u>		
<u>879</u>	<u>879</u>			
	B-56			
	E-27			
	D-43			
	E-65			

Animals with data in more than one source are underlined.

Nine pages of data were transmitted from Wisconsin to Searle on January 9, 1975 with implication that they related to E-32. M-34, P-60, M-64, and M-79 related to E-32 and not E-88; whereas A-23, 830, 831 and 879 related to E-88 and not E-32. Monkeys A23 and A39 in E-88 were apparently utilized in earlier published work, as indicated.

Waisman, H. A., and Harlow, H. F., Science 147:686, 1965.

Kerr, G. R., Chamove, A. S., Harlow, H. F. and Waisman, H. A., Pediatrics 42:27, 1968.

APPENDIX XIII-3

Figures 1, 9, 12, and 13 from Entry 88 Report do not convey much of the information that is shown in colors in the original. Therefore, these colored graphs are reproduced. Figure 8 and page 29 have been reproduced from the originals with a writing over of numbers and other illegible data.

An initial check on the available data and the contents of the five graphs in E-88 (Figures 1, 9, 12, 13, and 8) indicates that the data presented in the graphs can only be reproduced to a limited extent from other available data. Some points on Figure 8 can be checked, but other points do not check precisely. The raw data used to produce most of the information in Figures 1, 9, 12, and 13 are not available. Such limited data as are available do not precisely reproduce all points on the graphs. According to Dr. McIlreath, the original graphs were supplied by the University of Wisconsin. Because the UAREP reviewers agree with the repeated comments made by Searle personnel that the data are inadequate to warrant conclusions, only a limited amount of time was expended in attempts to check for more detailed data.

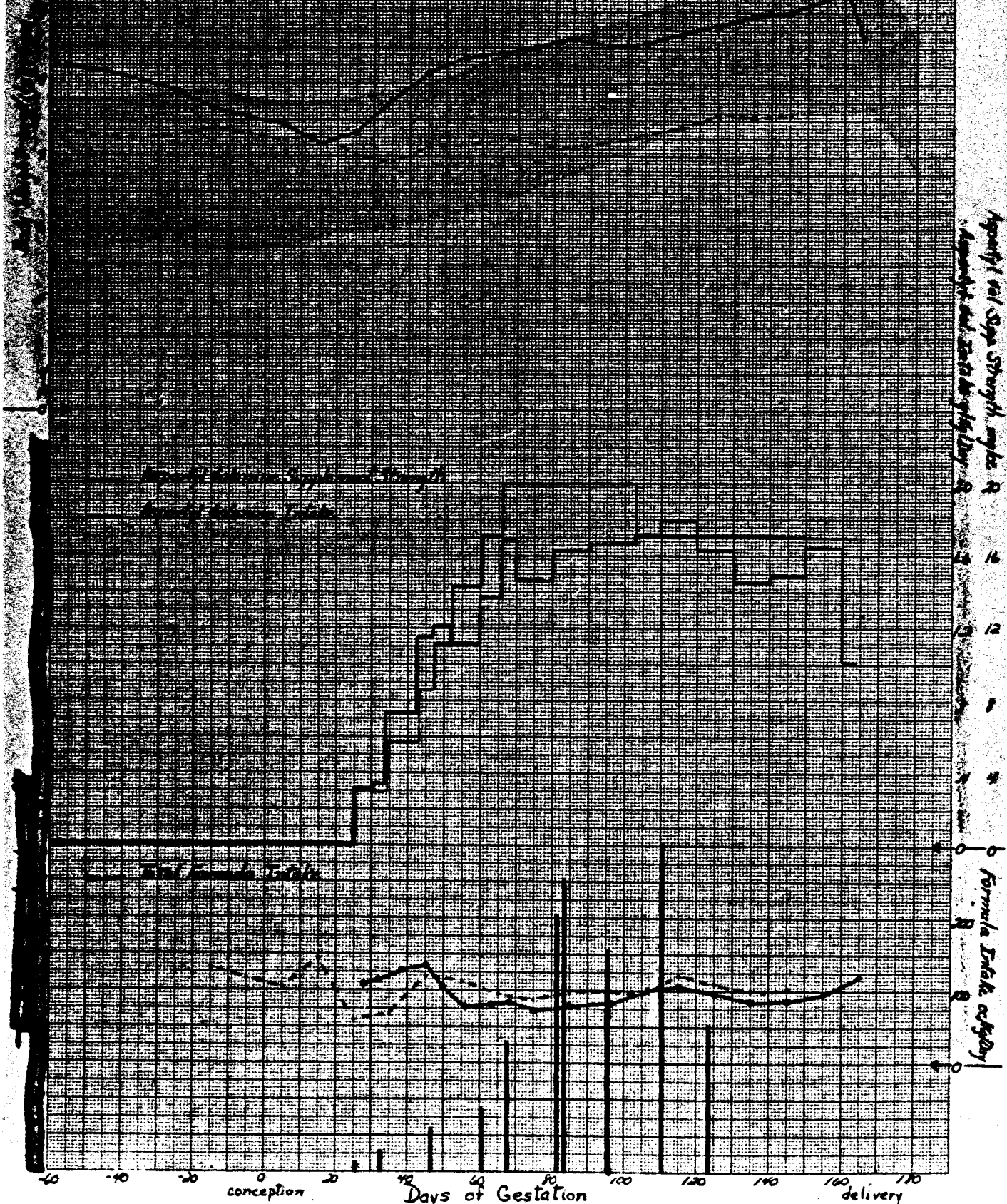
Mom A23 Prenatal Aspartyl Phenylalanine Supplement to Similac

Mom A23 This Fed Similac to A23's Formula Intake

Day Weight at Day 90 (Gest 90)

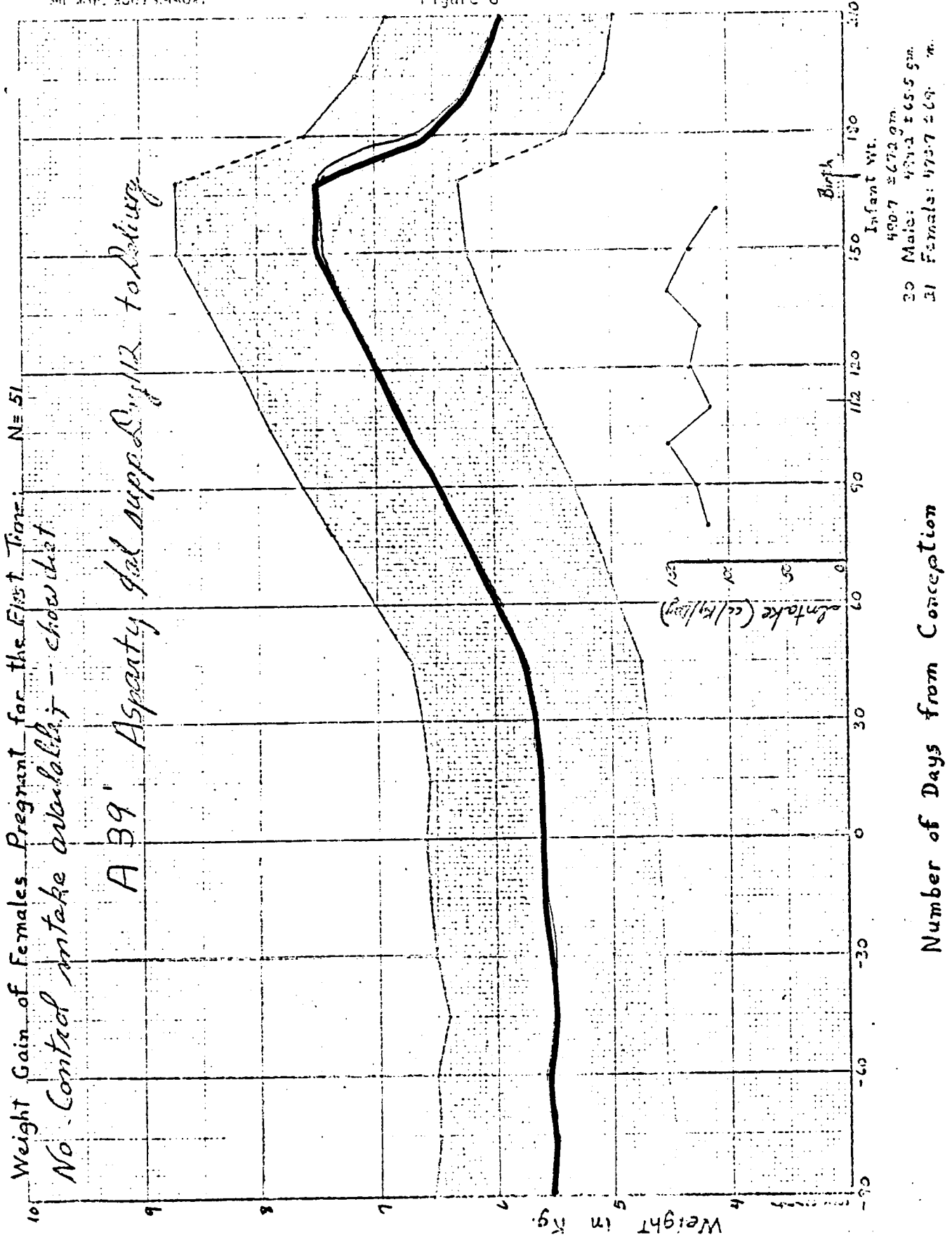
A23 Fed Similac (Child had 123 days 2 weight 3115 gm)

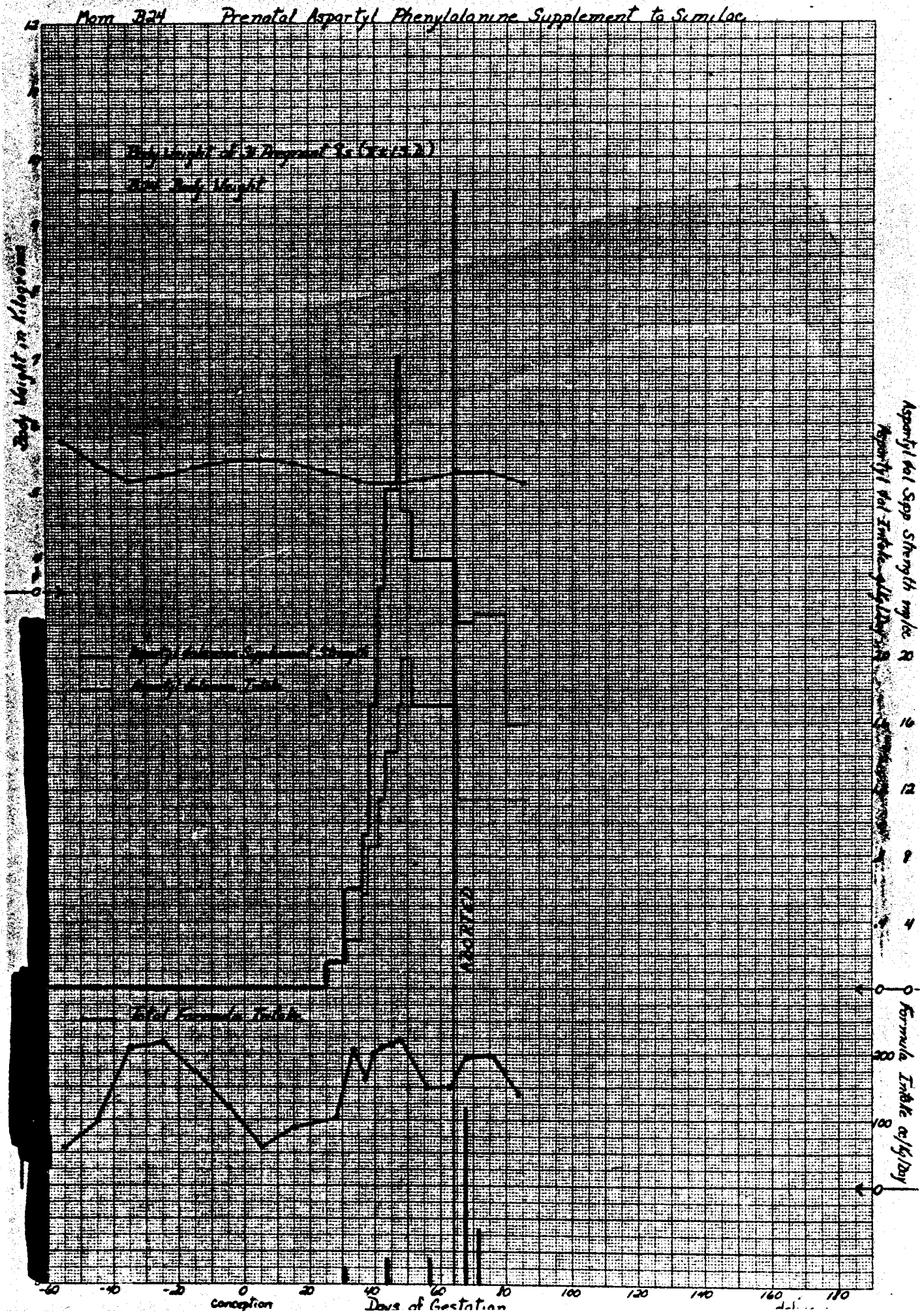
A23 Fed Aspartyl (Child had 123 days 2 weight 3115 gm)

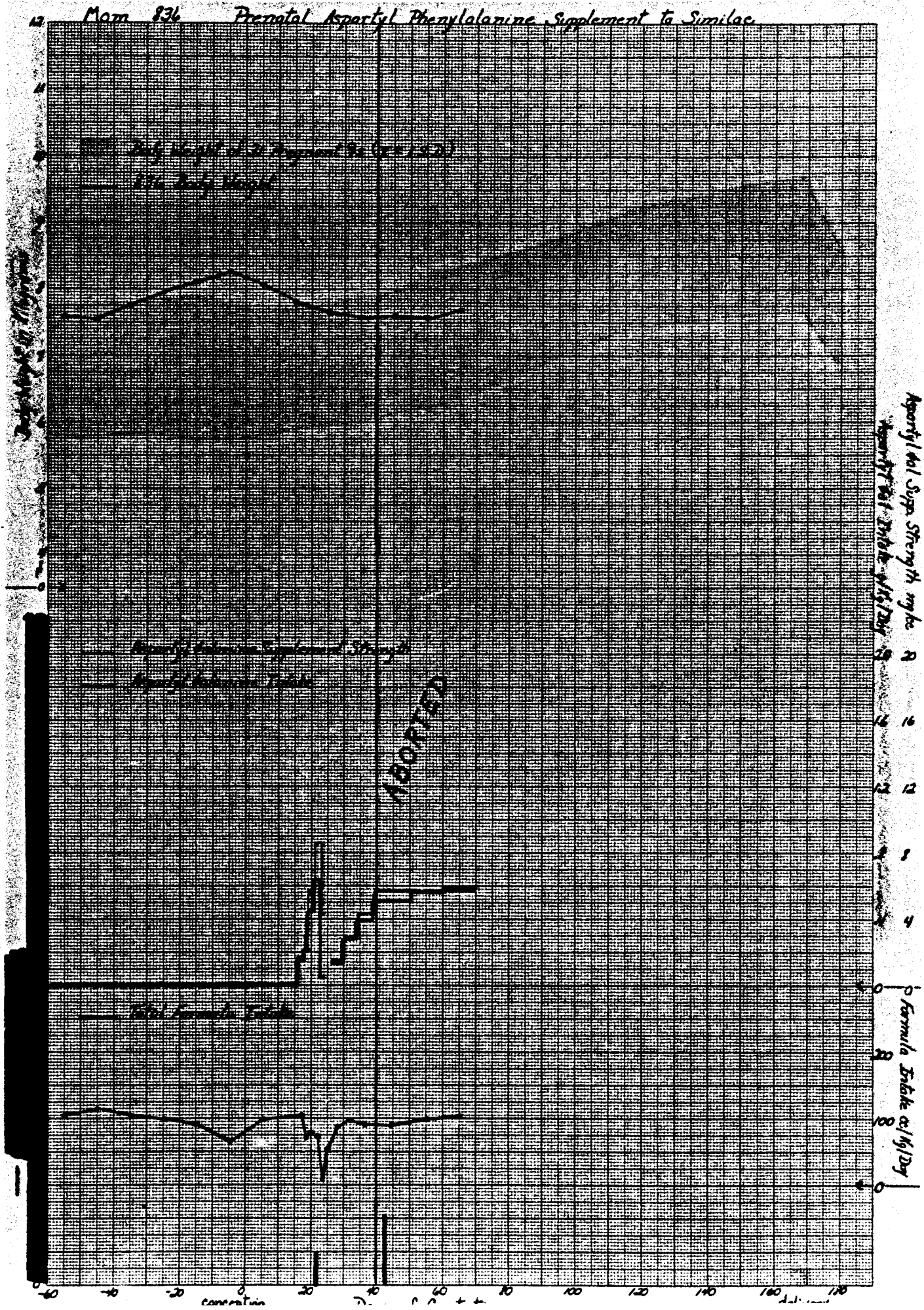


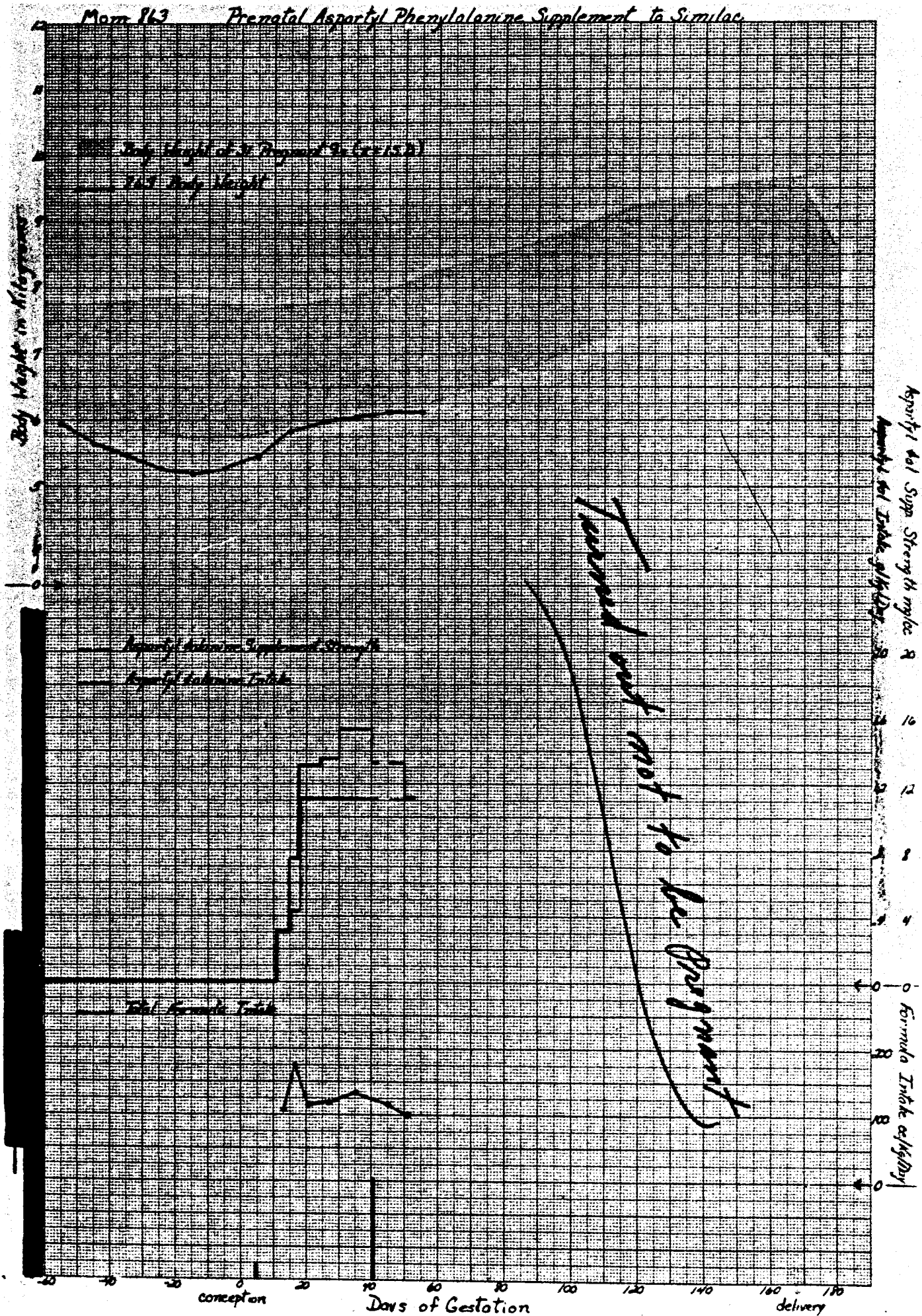
15

Figure 3









Start of the Third Transmitter.
A.39

-29-

BLOOD DATA - - BORN: / / - - FROM DAY - - DIED: / /

Code	Date	Age	Last Intake before Drawing		Fasting	24 hr. Intake	Date Prior to Drawing	Strength	Height	g/Kg	blood level	Type	le.
	195	Days	8 hrs.	4 hrs.	hrs.	cc		g/cc	Kg	Day	mg%		
1000	5-1	72 days			100		100			-	9.2		
1001	5-1	72 days		100	100	100	100	100		-			
1002	5-4	75 days		100	100	100	100	100		-			
1003	5-12	78 days		100	100	100	100	100		-	1		
1004	5-20	87 days		100	100	100	100	100		-			
1005	5-27	94 days	100	100	100	100	100	100		-	1.5	1.6	
1006	6-4	101 days	100	100	100	100	100	100		-	1.7	1.9	
1007	6-11	108 days	100	100	100	100	100	100		-	2.4	2.4	
1008	6-15	112 days	100	100	100	100	100	100		-	2.7	3.3	
1009	6-18	115 days	100	100	100	100	100	100		-	13.7	4.2	
1010	6-21	118 days	100	100	100	100	100	100		-	2.5	2.5	
1011	6-21	118 days	100	100	100	100	100	100		-	2.3	4.7	
1012	6-21	118 days	100	100	100	100	100	100		-	2.0	2.8	
1013	6-21	118 days	100	100	100	100	100	100		-	2.0	2.8	

offspring N38 weighed 440gms at birth 5-10-70
 Gestation 160 Days)

P S F = Fimiled

APPENDIX XIII-4

ROLE OF ASPARTAME IN MATERNAL SERUM L-PHENYLALANINE

The statements on page 21 of E-88 are of interest in a number of respects. Of the nine pregnancies reported by Kerr, Chemove, Harlow and Waisman, three were from A23 and three from B24, and both monkeys are presumed to be the same pregnant females as in E-88. The mean phenylalanine as determined by 55 observations in three pregnancies of A23 was 34.6 mg %, whereas an average of 10 serum phenylalanine determinations on A23 in E-88 was 20.8 mg%. Similarly, according to the published paper, 54 determinations in three pregnancies of B23 showed the mean serum phenylalanine to be 19.9 mg %, as contrasted with an average of 106 mg % based on 5 determinations in E-88. In the case of A-39 in E-88, eight determinations gave a mean serum phenylalanine level of 5.8 mg%. The serum phenylalanine levels in A23 and B24 apparently are in the 50 to 60% percentile range of those in the published paper. Only one of the four pregnant monkeys in E-88 was on an aspartame regimen for more than 40 days of the normal gestation of 168 days. Two of the four pregnant monkeys aborted at 40 and 65 days and one was said to have a "stillbirth" at term, whereas the offspring of the remaining monkey, A39, died within three days of delivery. The scientific data in E-88 do does not justify the statement: "It would appear that in monkeys exposed to SC18862 in large amounts throughout the entire gestation period and proceeding to term delivery that no adverse effects other than those produced by feeding the L-phenylalanine only were observed."

The literature contains numerous references to the deleterious effects of feeding large amounts of L-phenylalanine to pregnant monkeys.

After aspartame is administered to monkeys at doses of 16 or 60 mg/kg for 10 days, there is no effect on the disappearance of intravenously administered ^{14}C phenylalanine from the plasma. Such doses of aspartame do not modify phenylalanine metabolism in monkeys (J. Nutr. 103:1460, 1973). Feeding excess L-phenylalanine to pregnant rhesus monkeys produced infants with low birth weight with significant reduction in learning behavior (Pediatrics 42:27, 1968).

In other Searle studies submitted to FDA as Entry-32, aspartame was administered orally to newborn Rhesus monkeys with mean daily dosage levels of 0.97, 3.01, and 3.62 g/kg. These levels are said to be multiples of 32, 100, and 120 times the estimated maximal human daily intake of 30 mg/kg/day for a 27 kg child. The animals in the two higher dosage groups experienced grand mal convulsions after 200+ days of treatment, said to be similar to those induced in monkeys by feeding L-phenylalanine alone in equimolar quantities (Science 147:685, 1965). The occurrence of these seizures coincided with the attainment of high serum phenylalanine levels. Food intake and growth rate were mildly reduced by aspartame administration. The higher dosages of aspartame produced significant increases in serum phenylalanine levels as well as phenylketonuria.

ADDENDUM to Appendix XIII-4

In commenting on the last sentence of the last page of Appendix XIII-4, Dr. Bost of Searle stated, "Searle is unaware of any good evidence that phenylketonuria can be produced simply by loading experimental animals with phenylalanine. Does the last statement have support in the scientific literature?" On the page referred to in the report, UAREP quoted two papers by Waisman and colleagues as well as the E-32 report to FDA written by Drs. McConnell and Rao of the Searle staff, after Dr. Waisman's death. Page 37 of E-32 is attached. Among the voluminous papers Searle provided UAREP, were a series of 12 reprints on the metabolism of aspartame. Three of them dealt with research on phenylketonuria in rats and monkeys fed large amounts of phenylalanine. Other papers, in reviewing the literature, made reference to these three as well as other articles on this subject. Thus, UAREP is unable to explain Dr. Bost's statement which is not supported by the work of former Searle employees and their consultants.

REFERENCES

- Boggs, D. E. and Waisman, H. A. Effect on the Offspring of Female Rats Fed Phenylalanine. Life Sci. 8:373, 1962.
- Boggs, D. E. and Waisman, H. A. Influence of Excess Dietary Phenylalanine on Pregnant Rats and their Fetuses. Proc. Soc. Exp. Biol. Med. 115:407, 1964.
- Boggs, D. E. and Waisman, H. A. Biochemical Correlates in Rats with Phenylketonuria. Arch. Biochem. Biophysics 106:307, 1964.
- Waisman, H. A. and Harlow, H. F. Experimental Phenylketonuria in Infant Monkeys. Science, 147:685, 1965.

measurements at birth in the latter two. The head circumference of all other monkeys was within normal range. The body length of all treated monkeys was within the historical control range.

Hematology and clinical chemistry parameters were generally unremarkable in treated animals, as compared with data from historical control animals of the same age from the same laboratory. No biologically significant alterations were observed except, as mentioned earlier, there was a significant increase in serum phenylalanine and tyrosine levels at the medium and high dose levels. Urinalysis parameters were generally unremarkable, except for a significant excretion of phenylketones in both medium and high dose groups after 6 months. This increase coincided with the increase of serum phenylalanine levels. Thus, the SC-13862 treated monkeys exhibited increased serum phenylalanine levels, increased urinary phenylketone levels, and episodes of grand mal seizures in relation to the phenylalanine moiety of the compound administered. At the low dose level (1 g/kg/day), none of the above alterations were observed through 30 weeks of treatment, at which point the study terminated.

It is concluded that dietary administration of SC-13862 to infant monkeys starting at birth and continuing for 30 consecutive weeks at approximately 1 g/kg/day, caused no biologically meaningful alterations in physical or behavioral findings or in clinical laboratory parameters. At higher dosages a significant increase in serum phenylalanine and tyrosine levels, an increase in urinary phenylketone excretion and episodes of grand mal type seizure activity were observed at this point, and continued through the 52 weeks of treatment. Both the nature and magnitude of the changes observed were comparable to historical positive control animals fed equivalent quantities of L-phenylalanine alone.

CHAPTER XIV

E-90: AN EVALUATION OF EMBRYOTOXIC AND TERATOGENIC POTENTIAL OF ASPARTAME IN THE RABBIT

INTRODUCTION

Searle has submitted to the FDA a number of studies on the embryotoxic and teratogenic potential of aspartame in several species. At a meeting on November 8, 1977 the FDA approved Searle's request that E-90 be reviewed in lieu of three earlier studies.

This study, performed at Searle Laboratories, (Pathology-Toxicology Project No. 1201), was designed to evaluate the embryotoxic and teratogenic potential of aspartame in the pregnant rabbit. The experiments began on January 20, 1975. Aspartame was administered by gavage during fetal organogenesis (gestation days 6-18). L-phenylalanine and L-aspartic acid, the principal constituents of the aspartame molecule, were administered to two additional groups of pregnant rabbits so the effects of these compounds could also be evaluated.

The protocol dated January 14, 1975 (Appendix XIV-1) notes this is a segment II study performed to support marketing of aspartame as a food additive in the USA, UK, Canada, and Europe.

Three hundred nulliparous female New Zealand White rabbits, 32-44 weeks of age, were used. They were housed individually in stainless steel cages and provided with Purina Rabbit Chow Special No. 5430 and tap water ad libitum. Animals were acclimatized for at least a month

prior to the beginning of the study. Animal quarters were air-conditioned and maintained at 72°F, with a twelve hour photoperiod provided by fluorescent lighting.

Test compounds were administered intragastrically in divided doses twice daily at an interval of three hours. Appropriate amounts of aspartame, L-phenylalanine or L-aspartic acid were suspended on a w/volume basis in a vehicle containing 1% (V/V) aqueous Tween-80 and 0.5% carboxymethylcellulose (W/V). Doses were prepared fresh each day and equal volumes (20 ml/kg) were administered to all animals. Doses were used within 4-6 hours and stored at 10°C in the interim. Searle stated that the preparation is stable under these conditions. Control animals received a comparable volume of vehicle only, also given twice daily.

Artificial insemination was carried out using pooled semen collected from twenty proven fertile males of the same strain maintained "in-house." Semen was evaluated microscopically under low power for abundance and motility of spermatozoa. Pooled samples were diluted 1:9 with warm isotonic saline. Females were inseminated with 0.7-1.0 ml of diluted semen "containing not less than 20 million sperm." The protocol (Appendix XIV-1) says "containing not less than 10 million sperm." Females were then immediately injected (via marginal ear vein) with 1.5 mg/kg pituitary luteinizing hormone to induce ovulation.

Maternal animals that died or were sacrificed during the study were necropsied, but histopathologic examination was not done. Surviving animals were killed by air embolism on gestation day 28 and an immediate

laparotomy was performed. The ovaries, uterus, and major organs were examined in situ. Fetal swellings and resorption sites and their relative positions in the uterine horns were recorded. Uterine horns were opened and fetal viability determined.

All fetuses were removed and weighed to the nearest 0.1 gm. Crown-rump distance (CRD) was measured. The fetuses were examined for gross abnormalities prior to fixation. Then following a random selection process half the fetuses of a litter were preserved in Bouin's solution to be examined by the free-hand razor blade sectioning technique of Wilson. The remaining half of each litter was preserved in 95% aqueous ethanol before evisceration and processing of the skeletons by Alizarin Red S staining and clearing technique. Fetal viscera were examined grossly during the evisceration step. A detailed description of procedures used in fetal examination is given in Appendix XIV-3.

Personnel involved in this teratology study were:

Design Committee:

Dr. Dutt (Biometrics Advisor)
Dr. F. Saunders (Biological Research Advisor)
Dr. Ranney (Metabolism Monitor)
Dr. Polk (Clinical Monitor)
Dr. Rao (Pathology-Toxicology Department Monitor)
Dr. McConnell (Pathology-Toxicology Department Monitor)
Dr. Bernard L. Oser (Path-Tox Department Project Advisor)
Dr. P. D. Klimstra (Vice-President, Preclinical R&D)

Technical Staff:

Dr. Vondruska (Teratology Laboratory)

Alan L. Mitchell (Teratology Laboratory)

Raymond E. Schroeder (Teratology Laboratory)

Toxicology Coordinating Committee Members

Dr. J. Clifford (United Kingdom)

Prof. M. Brunaud (France)

Dr. R. McConnell (USA)

Because of the fragile nature of the embryo specimens, UAREP arranged to have them re-examined in the Searle Laboratories by UAREP employee Dr. Anthony J. Steffek, who worked without consultation with Searle employees. Dr. Steffek had no knowledge of Searle findings during his work. His findings were compared with those of Searle by Dr. Andrew Hendrickx in Davis, California. Dr. Steffek then reviewed all specimens in which there was any discrepancy between his findings and those of Searle with UAREP consultant, Dr. Larry Ross, who also had no knowledge of Searle findings. Dr. Steffek and Dr. Ross agreed on all their findings, which were transmitted to Dr. Hendrickx who prepared the final analysis and tables.

RESULTS AND DISCUSSION

General Comments

A memo dated October 24, 1975 (Appendix XIV-2) notes that Searle had found that rabbit liver in vitro does not metabolize L-phenylalanine to tyrosine as rapidly as mouse or rat liver under the same conditions. The resulting high plasma levels of phenylalanine may have been responsible for reduced food consumption in the high dose group.

The high dose of aspartame (2 g/kg) was half (or less) the amount used as high dose in a number of other studies validated by UAREP. This 2 g/kg dose is 66X the maximum anticipated human consumption. The table on page 3 in the Entry Book shows the dosages (g/kg) of all the test compounds used in this study. The amounts of L-phenylalanine and L-aspartic acid used were the reverse of what they should have been. Based on L-phenylalanine comprising 55% of the aspartame molecule the amount used should have been 1.1 g/kg while the amount of L-aspartic acid should have been 0.82 g/kg. The effects of this error can be seen in Figure 3 (p 17, E-90) where maternal food consumption in the L-phenylalanine group falls between that of the high dose group and the others. As was noted by Searle (p 1, E-90), these doses of L-phenylalanine and L-aspartic acid were 75% and 135% respectively, of the amounts present in the high dose of aspartame.

Food consumption was monitored daily during gestation. At the time the animals were handled for compound administration they were also examined for morbidity, mortality, and behavioral irregularities.

A summary of animals which died and were necropsied prior to gestation day 28, as well as cause of death, is given in Table 14-1.

Figure 14-1 compares food consumption in the various groups of maternal animals. In addition, the high dose animals are separated into those which aborted and those which did not abort. There is a somewhat different exposition of these data shown in Figures 3 and 4 in the Entry Book (E-90, pp 17-19). UAREP's graph does not show the high-dose non-aborting group overlapping the L-phenylalanine group, but rather falling between it and the high dose group as a whole. Food consumption in the high dose animals which aborted, as well as the high dose group as a whole, fell to extremely low levels during the treatment period. Food consumption in these groups ranged as low as 5% of that in the control group. Figure 14-2 compares data on food consumption in the non-pregnant animals from the various treatment groups. These data are quite variable. The only distinctive item is that the high dose group is consistently below all the other groups for most of the treatment period (days 7-14).

Figure 3 (E-90, p 17) summarizes daily mean food consumption for each group during the treatment period. A statistical summary of Searle and UAREP findings on food consumption in animals known to be pregnant is shown in Table 14-2. As indicated in the footnote to Table 14-2, UAREP divided the high dose group into animals that aborted and animals that did not abort. These data were included in the Analysis of Variance, LSD, and Newman-Keuls tests as separate groups in addition to the high dose group as a whole. There were 82 t-test comparisons made by both Searle and UAREP. In 62 cases (76%) UAREP's findings agreed with Searle's while there was disagreement in 20 instances (24%). UAREP did

Table 14-1

Summary of Diagnoses Based on Gross Necropsy Findings
in Sex Groups of Rabbits

<u>Cause of Death</u>	<u>Control</u>	<u>Low Dose</u>	<u>Medium Dose</u>	<u>High Dose</u>	<u>L-phe</u>	<u>L-asp</u>
Pneumonia	1	-	-	2	1	1
Purulent Pneumonia & Pyothorax	1	2	-	-	2	-
P. Pneumonia, Pyothorax & Peritonitis	-	-	-	1	-	-
Faulty Administ- tration of Test Material	1	2	1	-	2	-
Tracheal Obstruction	-	-	-	-	-	1
Undetermined	1	-	-	1	-	-
Total	4	4	1	4	5	2

L-phe = L-phenylalanine

L-asp = L-aspartic acid

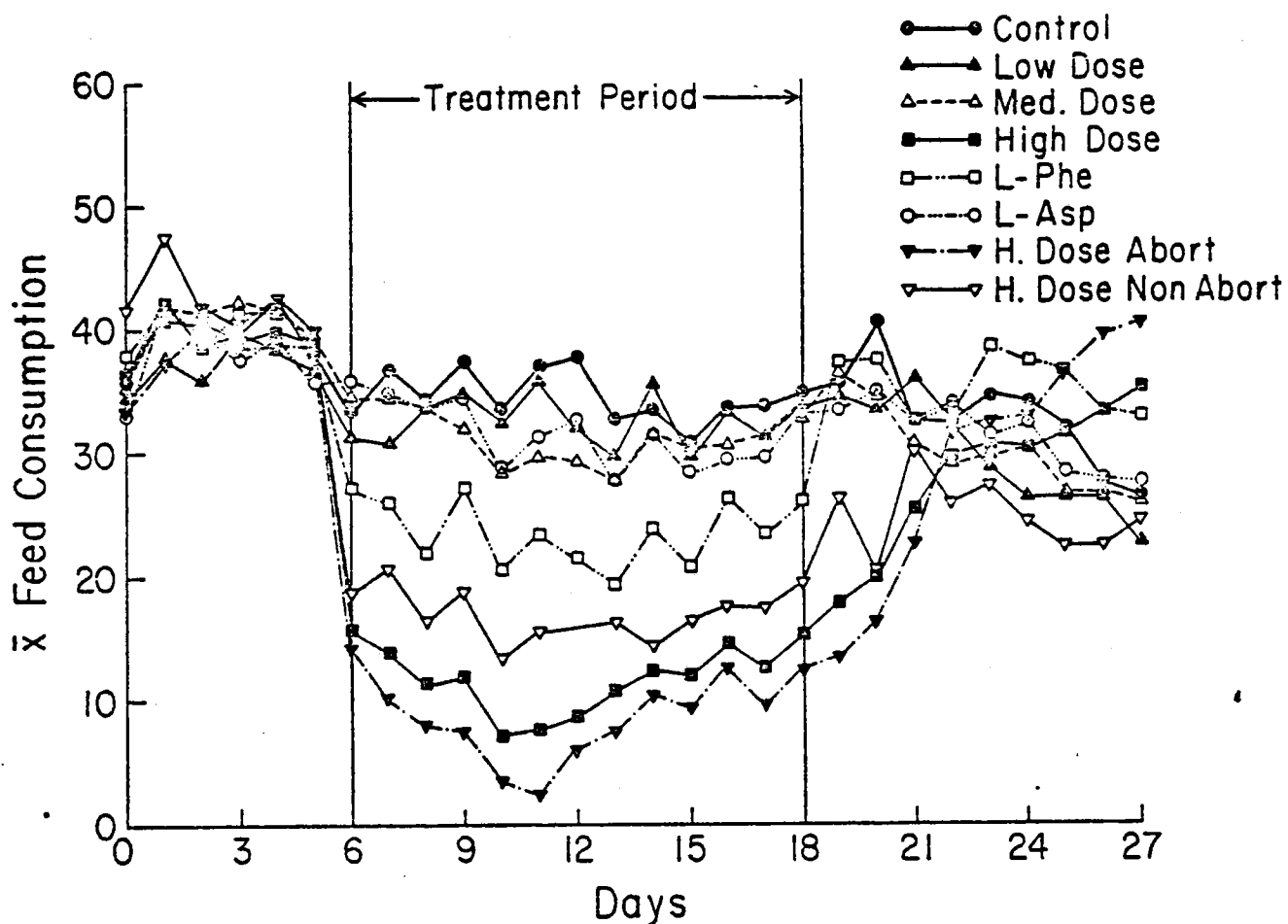


Figure 14-1: Graph showing mean food consumption in grams of food per kilogram body weight per day of rabbits which were pregnant. Groups include control, low dose aspartame (0.5 gm/kg), medium dose aspartame (2.0 gm/kg), L-phenylalanine (0.82 g/kg) and L-aspartic acid (1.10 g/kg). The high dose group was further separated into rabbits which aborted (H. Dose Abort) and rabbits which did not abort (H. Dose Non Abort).

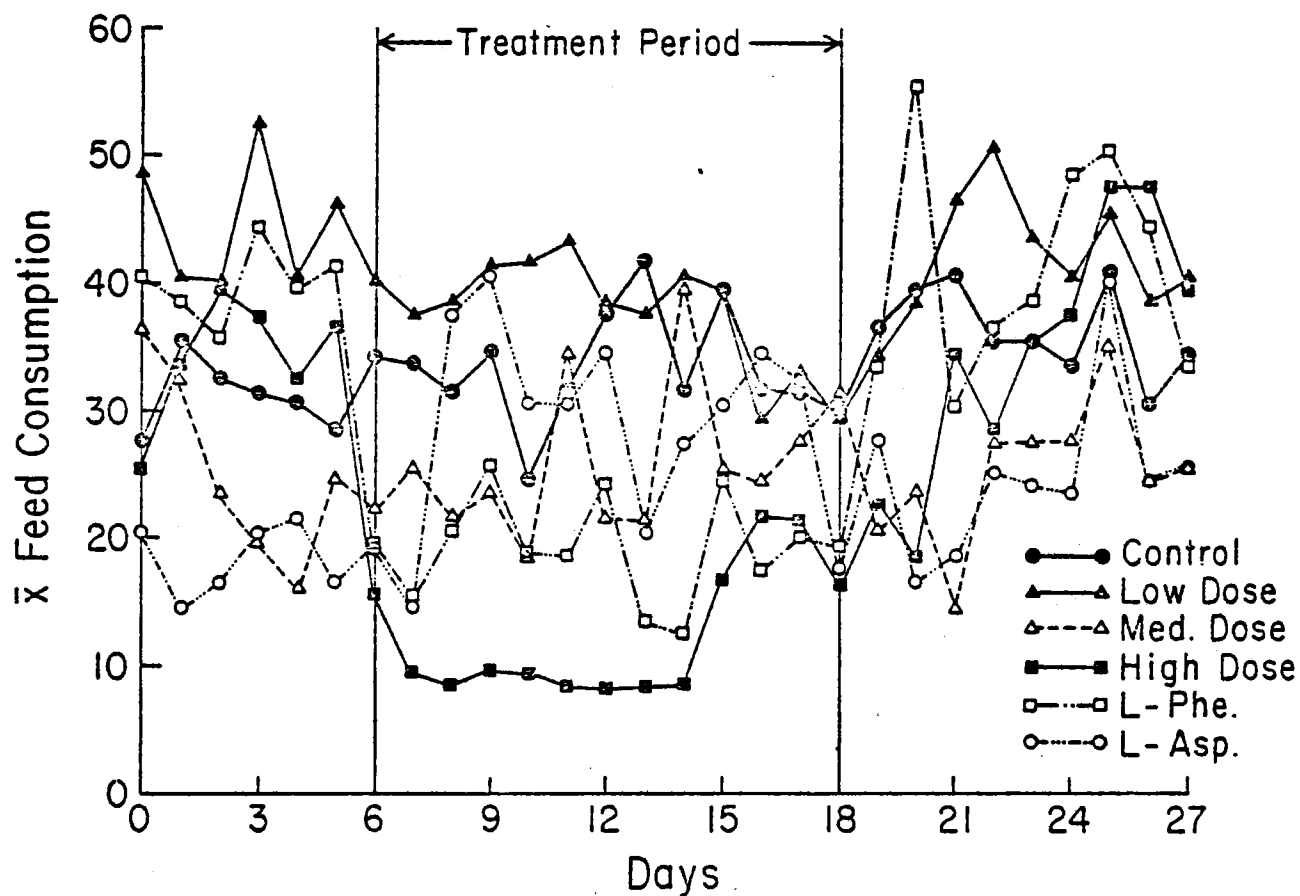


Figure 14-2: Graph showing mean food consumption (in grams of food per kilogram body weight per day) of rabbits which were not pregnant. Groups include control, low dose aspartame (0.5 g/kg), medium dose aspartame (1.0 g/kg), high dose aspartame (2.0 g/kg), L-phenylalanine (0.82 g/kg) and L-aspartic acid (1.10 g/kg).

Table 14-2
Statistical Summary of Searle and UAREP Findings on Food Consumption
(in g/kg Body Weight) in Animals Known to be Pregnant

Interval Days	ANOVA	Group	LSD	Q	UAREP t-test	t-test Value	Searle t-test
0	0.53	1 < 5	ND	ND	N	--	S
		5 > 6	ND	ND	N	2.03	S
3	0.63	5 > 6	ND	ND	N	--	S
6	0.00	1 > 4	S	S	S	5.48	S
		1 > 5	S	S	S	3.46	S
		1 > 6	S	S	S	5.30	S
		1 > 7	S	N	N	--	S
		2 > 4	S	S	ND		ND
		2 > 5	S	S	ND		ND
		2 > 6	S	S	ND		ND
		3 > 4	S	S	ND		ND
		3 > 5	S	S	ND		ND
		3 > 6	S	S	ND		ND
		3 > 7	S	N	ND		ND
		4 < 7	S	S	ND		ND
		4 < 8	S	S	ND		ND
		5 < 7	S	S	ND		ND
		5 < 8	S	S	ND		ND
		6 < 7	S	S	ND		ND
		6 < 8	S	S	ND		ND
		7 < 8	S	S	ND		ND
7	0.00	1 > 2	S	N	N	--	N
		1 > 4	S	S	S	5.89	S
		1 > 5	S	S	S	3.15	S
		1 > 6	S	S	S	6.67	S
		1 > 7	S	S	S	2.51	S
		2 > 4	S	S	ND		ND
		2 > 5	S	S	ND		ND
		2 > 6	S	S	ND		ND
		3 > 4	S	S	ND		ND
		3 > 5	S	S	ND		ND
		3 > 6	S	S	ND		ND
		3 > 7	S	S	ND		ND
		4 < 5	S	S	ND		ND
		4 < 7	S	S	ND		ND
		4 < 8	S	S	ND		ND
		5 > 6	S	S	S	2.32	N
		5 < 8	S	S	ND		ND
		6 < 7	S	S	ND		ND
		6 < 8	S	S	ND		ND
		7 < 8	S	S	ND		ND
8	0.00	1 > 4	S	S	S	5.64	S
		1 > 5	S	S	S	4.68	S
		1 > 6	S	S	S	8.01	S
		1 > 7	S	S	S	3.32	S
		2 > 4	S	S	ND		ND
		2 > 5	S	S	ND		ND
		2 > 6	S	S	ND		ND
		2 > 7	S	S	ND		ND
		3 > 4	S	S	ND		ND
		3 > 5	S	S	ND		ND
		3 > 6	S	S	ND		ND
		3 > 7	S	S	ND		ND
		4 < 7	S	S	ND		ND
		4 < 8	S	S	ND		ND
		5 > 6	S	S	S	2.54	S
		5 < 8	S	S	ND		ND
		6 < 7	S	S	ND		ND
		6 < 8	S	S	ND		ND
		7 < 8	S	S	ND		ND

Table 14-1 (continued)
page two

Interval Days	ANCOVA	Group	LSD	Q	DAPEP t-test	t-test Value	Spearle t-test
9	0.00	1 > 4	S	S	S	5.33	S
		1 > 5	S	S	S	3.64	S
		1 > 6	S	S	S	7.56	S
		1 > 7	S	S	N	--	S
		2 > 4	S	S	NO		NO
		2 > 5	S	S	NO		NO
		2 > 6	S	S	NO		NO
		2 > 7	S	N	NO		NO
		3 > 4	S	S	NO		NO
		3 > 5	S	S	NO		NO
		3 > 6	S	S	NO		NO
		4 < 7	S	S	NO		NO
		4 < 8	S	S	NO		NO
		5 > 6	S	S	S	2.93	S
		5 < 7	S	S	NO		NO
		5 < 8	S	S	NO		NO
		6 < 7	S	S	NO		NO
		6 < 8	S	S	NO		NO
		7 < 8	S	N	NO		NO
10	0.00	1 > 4	S	S	S	8.65	S
		1 > 5	S	S	S	5.65	S
		1 > 6	S	S	S	10.91	S
		1 > 7	S	S	S	4.08	S
		2 > 4	S	S	NO		NO
		2 > 5	S	S	NO		NO
		2 > 6	S	S	NO		NO
		2 > 7	S	S	NO		NO
		3 > 4	S	S	NO		NO
		3 > 5	S	S	NO		NO
		3 > 6	S	S	NO		NO
		3 > 7	S	S	NO		NO
		4 < 5	S	S	NO		NO
		4 < 7	S	S	NO		NO
		4 < 8	S	S	NO		NO
		5 > 6	S	S	S	3.01	N
		5 < 7	S	S	NO		NO
		5 < 8	S	S	NO		NO
		6 < 7	S	S	NO		NO
		6 < 8	S	S	NO		NO
		7 < 8	S	S	NO		NO
11	0.00	1 > 3	S	S	S	2.28	N
		1 > 4	S	S	S	8.54	S
		1 > 5	S	S	S	4.20	S
		1 > 6	S	S	S	11.65	S
		1 > 7	S	S	S	3.02	S
		2 > 4	S	S	NO		NO
		2 > 5	S	S	NO		NO
		2 > 6	S	S	NO		NO
		2 > 7	S	S	NO		NO
		3 > 4	S	S	NO		NO
		3 > 5	S	S	NO		NO
		3 > 6	S	S	NO		NO
		4 < 5	S	S	NO		NO
		4 < 7	S	S	NO		NO
		4 < 8	S	S	NO		NO
		5 > 6	S	S	S	3.65	S
		5 < 7	S	S	NO		NO
		5 < 8	S	S	NO		NO
		6 < 7	S	S	NO		NO
		6 < 8	S	S	NO		NO
		7 < 8	S	S	NO		NO

Table 14-1 (continued)
page three

Interval Days	ANOVA	Group	LSD	F	UNREP t-test	t-test value	Searle t-test
12	0.00	1 > 3	N	N	S	3.53	S
		1 > 4	S	S	S	9.93	S
		1 > 5	S	S	S	6.53	S
		1 > 6	S	S	S	10.19	S
		1 > 7	S	S	S	4.26	S
		2 > 4	S	S	NO		NO
		2 > 5	S	S	NO		NO
		2 > 6	S	S	NO		NO
		3 > 4	S	S	NO		NO
		3 > 5	S	S	NO		NO
		3 > 6	S	S	NO		NO
		4 < 7	S	N	NO		NO
		4 < 8	S	S	NO		NO
		5 > 6	N	N	N	--	S
		5 < 3	S	S	NO		NO
		6 < 7	S	S	NO		NO
		6 < 8	S	S	NO		NO
		7 < 3	S	N	NO		NO
13	0.00	1 > 4	S	S	S	6.39	S
		1 > 5	S	S	S	3.75	N
		1 > 6	S	S	S	6.84	S
		1 > 7	S	S	S	3.74	S
		2 > 4	S	S	NO		NO
		2 > 5	S	S	NO		NO
		2 > 6	S	S	NO		NO
		2 > 7	S	S	NO		NO
		3 > 4	S	S	NO		NO
		3 > 5	S	S	NO		NO
		3 > 6	S	S	NO		NO
		3 > 7	S	S	NO		NO
		4 < 7	S	S	NO		NO
		4 < 8	S	S	NO		NO
		5 > 6	S	S	S	2.05	S
		5 < 8	S	S	NO		NO
		6 < 7	S	S	NO		NO
		6 < 8	S	S	NO		NO
		7 < 8	S	S	NO		NO
14	0.00	1 > 4	S	S	S	5.18	S
		1 > 5	S	S	S	3.36	S
		1 > 6	S	S	S	5.21	S
		1 > 7	S	N	N	--	S
		2 > 4	S	S	NO		NO
		2 > 5	S	S	NO		NO
		2 > 6	S	S	NO		NO
		2 > 7	S	S	NO		NO
		3 > 4	S	S	NO		NO
		3 > 5	S	S	NO		NO
		3 > 6	S	S	NO		NO
		4 < 7	S	S	NO		NO
		4 < 8	S	S	NO		NO
		5 < 7	S	S	NO		NO
		5 < 8	S	S	NO		NO
		6 < 7	S	S	NO		NO
		6 < 8	S	S	NO		NO
15	0.00	1 > 4	S	S	S	4.79	S
		1 > 5	S	S	S	2.52	N
		1 > 6	S	S	S	5.07	S
		1 > 7	S	S	S	2.53	S
		2 > 4	S	S	NO		NO
		2 > 5	S	S	NO		NO
		2 > 6	S	S	NO		NO
		2 > 7	S	S	NO		NO
		3 > 4	S	S	NO		NO
		3 > 5	S	S	NO		NO
		3 > 6	S	S	NO		NO
		3 > 7	S	S	NO		NO
		4 < 7	S	S	NO		NO
		4 < 8	S	S	NO		NO
		5 > 6	S	N	N	--	N
		5 < 3	S	S	NO		NO
		6 < 7	S	S	NO		NO
		6 < 8	S	S	NO		NO
		7 < 8	S	S	NO		NO

Table 11-2 (continued)
page four

Interval Days	ANOVA	Group	LSD	Q	DAFEP t-test	t-test Value	Scaris t-test
16	0.00	1 > 4	S	S	S	4.40	S
		1 > 5	S	S	S	2.99	N
		1 > 6	S	S	S	4.12	S
		1 > 7	S	N	N	--	S
		2 > 4	S	S	NO		NO
		2 > 5	S	S	NO		NO
		2 > 6	S	S	NO		NO
		2 > 7	S	N	NO		NO
		3 > 4	S	S	NO		NO
		3 > 5	S	S	NO		NO
		3 > 6	S	S	NO		NO
		4 < 7	S	S	NO		NO
		4 < 8	S	S	NO		NO
		5 < 7	S	S	NO		NO
		5 < 8	S	S	NO		NO
		6 < 7	S	S	NO		NO
		6 < 8	S	S	NO		NO
17	0.00	1 > 4	S	S	S	5.26	S
		1 > 5	S	S	S	2.83	S
		1 > 6	S	S	S	5.97	S
		1 > 7	S	S	S	2.30	S
		2 > 4	S	S	NO		NO
		2 > 5	S	S	NO		NO
		2 > 6	S	S	NO		NO
		2 > 7	S	S	NO		NO
		3 > 4	S	S	NO		NO
		3 > 5	S	S	NO		NO
		3 > 6	S	S	NO		NO
		3 > 7	S	S	NO		NO
		4 < 7	S	S	NO		NO
		4 < 8	S	S	NO		NO
		5 > 6	S	N	N	--	N
		5 < 8	S	S	NO		NO
		6 < 7	S	S	NO		NO
		6 < 8	S	S	NO		NO
18	0.00	1 > 4	S	S	S	4.96	S
		1 > 5	S	S	S	2.73	S
		1 > 6	S	S	S	4.97	S
		1 > 7	S	N	S	2.04	S
		2 > 4	S	S	NO		NO
		2 > 5	S	S	NO		NO
		2 > 6	S	S	NO		NO
		3 > 4	S	S	NO		NO
		3 > 5	S	S	NO		NO
		3 > 6	S	S	NO		NO
		4 < 7	S	S	NO		NO
		4 < 8	S	S	NO		NO
		5 > 6	N	N	N	--	S
		5 < 8	S	S	NO		NO
		6 < 7	S	S	NO		NO
		6 < 8	S	S	NO		NO
19	0.00	1 > 4	S	S	S	4.27	S
		1 > 5	S	N	N	--	N
		1 > 6	S	S	S	5.27	S
		2 > 4	S	S	NO		NO
		2 > 5	S	N	NO		NO
		2 > 6	S	S	NO		NO
		3 > 4	S	S	NO		NO
		3 > 5	S	N	NO		NO
		3 > 6	S	S	NO		NO
		4 < 5	S	S	NO		NO
		4 < 7	S	S	NO		NO
		4 < 8	S	S	NO		NO
		5 > 6	S	S	S	2.08	N
		5 < 7	S	S	NO		NO
		6 < 7	S	S	NO		NO
		6 < 8	S	S	NO		NO

Table 14-2 (continued)
page five

Interval Days	ANOVA	Group	LSD	Q	UAREP t-test	t-test Value	Searle t-test
20	0.00	1 > 4	S	S	S	3.58	S
		1 > 5	S	S	N	--	N
		1 > 6	S	S	S	3.97	S
		2 > 4	S	S	ND		ND
		2 > 6	S	S	ND		ND
		3 > 4	S	S	ND		ND
		3 > 5	S	N	ND		ND
		3 > 6	S	S	ND		ND
		4 < 7	S	S	ND		ND
		4 < 8	S	S	ND		ND
		5 > 6	S	N	N	--	N
		5 < 7	S	S	ND		ND
		5 < 8	S	N	ND		ND
		6 < 7	S	S	ND		ND
		6 < 8	S	S	ND		ND
26	0.00	1 < 6	S	S	S	2.60	ND
		2 < 4	S	N	ND		ND
		2 < 6	S	S	ND		ND
		2 < 7	S	N	ND		ND
		3 < 4	S	N	ND		ND
		3 < 6	S	S	ND		ND
		3 < 7	S	N	ND		ND
		4 > 5	S	S	ND		ND
		5 < 6	S	S	S	2.90	ND
		5 < 7	S	S	ND		ND
		6 > 8	S	S	ND		ND
27	0.00	1 < 4	S	S	N	(1.99)	S
		1 < 6	S	S	S	2.99	S
		1 < 7	S	N	N	--	S
		2 < 4	S	S	ND		ND
		2 < 6	S	N	ND		ND
		2 < 7	S	S	ND		ND
		3 < 4	S	S	ND		ND
		3 < 6	S	S	ND		ND
		3 < 7	S	N	ND		ND
		4 > 5	S	S	ND		ND
		4 > 8	S	S	ND		ND
		5 < 6	S	S	S	2.90	S
		5 < 7	S	N	ND		ND
		6 > 7	S	S	ND		ND
		6 > 8	S	S	ND		ND

Group 1 = Control
Group 2 = Low Dose
Group 3 = Medium Dose
Group 4 = High Dose
Group 5 = High Dose rabbits which did not abort
Group 6 = High Dose rabbits which aborted
Group 7 = L-phenylalanine
Group 8 = L-aspartic acid

ANOVA indicates the exact probability that all group means are equal, based upon the F test for Analysis of Variance. UAREP applied the Analysis of Variance at a difference of $p < 0.05$. When values less than 0.05 were obtained the least significant difference (LSD) and Newman-Keuls (Q) tests were run. If the F statistic was greater than 0.05, we did not do the LSD and Q tests because we accept the hypothesis (at the 5% level) that all the means being compared are equal. Throughout this report, all ANOVA values of 0.00 indicate less than 1% chance that means are equal.

S means significant ($P < 0.05$).

N means not significant ($P > 0.05$)

ND means not done by UAREP because they were not done by Searle

a total of 3024 statistical comparisons on the food consumption data.

Food consumption decreased from 10 to 25% in control, low, medium, and L-aspartic acid groups in response to the twice daily gastric intubation. Food consumption in the high dose group was decreased by 65 to 75% during the treatment period and was statistically significantly different than the control group on each day of treatment. Similarly, food consumption in the group treated with L-phenylalanine was decreased by 40 to 50% during the treatment period and was significantly less than the control group on each treatment day.

When data for the high dose animals which aborted were compared with the high dose animals which did not abort, food consumption was lower than the controls in both groups. It was significantly lower by Analysis of Variance (versus controls) on 17 days in the high dose aborted group and on 12 days (versus controls) in the high dose non-aborting group.

A graph of mean body weight data in the various groups is shown in Figure 1 (E-90, p 13). The high dose group was significantly lower than the controls from treatment day 13 through 28. A statistical summary of these data is given in Table 14-3. The animals which aborted in the high dose group also had body weights which were significantly lower than the controls ($P < 0.05$) by UAREP t-test for the same period (days 13 through 28). In addition, body weights of the animals which aborted in the high dose group were significantly lower ($P < 0.05$) by UAREP t-test (days 15 through 28). It should be noted that the high dose aborting and non-aborting groups' body weights remained significantly lower than the controls until sacrifice at gestation day 28 even though

Table 14-3

Statistical Summary of Maternal Body

Weight Comparisons

Day	ANOVA	Group	LSD	Q	UAREP t-Test	t Value	Searle t-Test
13	0.00	1>4	N	N	S	2.49	S
		1>7	N	N	S	3.09	S
		6>7	S	N	ND	--	ND
15	0.00	1>4	N	N	S	3.09	S
		1>7	N	N	S	3.79	S
		4<6	S	N	ND	--	ND
		6>7	S	N	ND	--	ND
18	0.00	1>4	N	N	S	4.21	S
		1>7	N	N	S	5.23	S
		6>7	S	S	ND	--	ND
		7<8	N	N	S	3.54	S
22	0.00	1>4	S	N	S	4.51	S
		1>7	S	S	S	5.48	S
		2>4	S	N	ND	--	ND
		2>7	S	S	ND	--	ND
		3>4	S	N	ND	--	ND
		3>7	S	S	ND	--	ND
		4<6	S	S	ND	--	ND
		5>7	S	N	ND	--	ND
		6>7	S	S	ND	--	ND
28	0.00	7<8	S	S	S	3.67	S
		1>4	S	N	S	4.12	S
		1>7	S	N	S	4.98	S
		2>4	S	N	ND	--	ND
		2>7	S	N	ND	--	ND
		3>4	S	N	ND	--	ND
		3>7	S	N	ND	--	ND
		4<6	S	S	ND	--	ND
		5>7	S	N	ND	--	ND
		6>7	S	S	ND	--	ND
		7<8	N	N	S	2.56	S

t .05 = 2.04 with 31 degrees of freedom
(aborting versus non-aborting)

1.99 with 64 degrees of freedom
(aborting versus controls)

Group 1 = controls
2 = low dose
3 = medium dose
4 = high dose
5 = L-phenylalanine
6 = L-aspartic acid
7 = high dose aborting
8 = high dose non-aborting

treatment was stopped at day 18. This is in contrast to the data on food consumption in which these groups both recovered to or above control levels by day 22 (4 days after cessation of treatment). UAREP's statistical findings were in complete agreement with Searle's on the body weight comparisons.

There was also decreased food consumption and reduced body weight in the L-phenylalanine group, but since the amount of L-phenylalanine was equivalent to only 75% of that received in the aspartame molecule by the high dose group, this lessened effect would be expected.

Body weight data from non-pregnant rabbits were sorted into appropriate treatment groups and analyzed statistically. Table 14-4 gives a statistical summary of UAREP's findings on body weights of non-pregnant rabbits. There are some consistent differences during the treatment period between high dose animals versus controls and L-phenylalanine animals versus controls. Other significant differences occurring before day 6 when treatment began are difficult to explain.

The numbers of animals in each group which were not pregnant are as follows:

<u>Group</u>	<u>Number of Rabbits</u>	<u>Percent of Group</u>
1. Control	8/46	17
2. Low Dose	4/46	9
3. Medium Dose	4/49	8
4. High Dose	13/46	28
5. L-phenylalanine	11/45	24
6. L-aspartic acid	6/48	13

Table 14-4

Statistical Summary of UAREP Findings on Food Consumption
in g/kg Body Weight of Non-Pregnant Animals

<u>Interval</u> <u>(in weeks)</u>	<u>ANOVA</u>	<u>Group</u>	<u>LSD</u>	<u>Q</u>
0	0.01	1<2	S	N
		2>4	S	S
		2>6	S	S
		3>6	S	N
		4<5	S	N
		5>6	S	N
6	0.03	2>4	S	N
		2>5	S	N
		2>6	S	N
7	0.00	1>4	S	S
		1>5	S	S
		1>6	S	S
		2>4	S	S
		2>5	S	S
		2>6	S	S
		3>4	N	S
8	0.00	1>4	S	S
		2>3	N	S
		2>4	S	S
		2>5	S	S
		3<6	N	S
		4<6	S	S
		5<6	N	S

Table 14-4 (cont.)
page 2

<u>Interval</u> <u>(in weeks)</u>	<u>ANOVA</u>	<u>Group</u>	<u>LSD</u>	<u>Q</u>
9	0.04	2>4	S	N
		4<6	S	N
10	0.02	2>3	S	N
		2>4	S	N
		2>5	S	N
		4<6	S	N
		1>4	S	N
11	0.00	2>4	S	S
		2>5	S	N
		3>4	S	S
		1>4	S	N
12	0.01	2>4	S	N
		4<6	S	N
		1>4	S	S
13	0.01	1>5	S	N
		2>4	S	N
		2>5	S	N
		1>4	S	N
14	0.01	2>4	S	S
		2>5	S	S
		3>4	S	S
		3>5	S	N
		1>4	S	N

Table 14-4 cont.
page 3

<u>Interval</u> <u>(in weeks)</u>	<u>ANOVA</u>	<u>Group</u>	<u>LSD</u>	<u>Q</u>
26	0.00	1<4	S	N
		3<4	S	N
		3<5	S	N
		4>5	S	N
		5>6	S	N

Refer to footnotes in Table 14-2.

These data suggest that the high dose of aspartame and L-phenylalanine did have some effect on fertility.

The number of pups per litter was 8.2 in the high dose aborting group and 10.6 in the high dose non-aborting group. There were 8.0 pups per litter in the control group and 7.2 in the L-phenylalanine group. This parameter was not affected by treatment.

Some clinical observations are listed in Appendix XIV-4. Table 14-1 gives causes of death of the twenty animals that died before day 28 of gestation. Detailed autopsy reports for these twenty animals are in Appendix II of E-90.

Comprehensive litter examination data are given in Appendix III, E-90. These reports include maternal animal number, method of delivery, location and number of implantations, and number of resorptions. Also given are fetal identification numbers, viability information, weight, crown to rump distance, sex, external and soft tissue examination data, and skeletal examination data.

In 237 instances (3%) out of 8400 food consumption entries, UAREP found that the Entry Book reported "ND = no data" for food consumption while the raw data showed "negative consumption" or rather a gain in weight of the food container during a twenty-four hour period. There were 309 instances (4%) in which the Entry Book reported "ND" and UAREP found data. UAREP has no explanation for either situation, although it is recognized that rabbits tend to eat their own feces instead of food.

Data entries on computer printouts of statistical analyses sent to UAREP were verified. Statistical calculations were spot-checked and

UAREP agreed with most of the results (Table 14-5). However, in the Chi-square calculation for male-female sex distribution it was impossible for UAREP to duplicate the value for Group 3 on the printout. UAREP duplicated the other four chi-square values in this set exactly, but got 0.6491 for Group 3 rather than 25.7753. The former value would not be significant, while the latter one was. UAREP is unable to explain this disagreement.

Teratology Findings

Significant problems were encountered when UAREP consultant Dr. Anthony Steffek attempted to review the body cross sections of the fetuses. The slices were packed into jars with relatively narrow necks, and some specimens were partially dried out. When slices were removed from the jars they tended to disintegrate. On the basis of examining approximately 200 fetuses it was Dr. Steffek's opinion that reviewing such material would be difficult, and he was concerned about the potential inaccuracy of any comparisons that might be made.

Searle was promptly advised of the existence of this problem. UAREP's request to Searle for any information on the number and condition of these specimens was unanswered. Searle was formally advised of UAREP's decision not to examine the fetal body cross sections on May 12, 1978.

Occasional artifactual problems were encountered by Dr. Steffek in his review of the cleared skeletal specimens but he attempted to differentiate between loss of structure through extraneous trauma versus congenital defects.

Table 14-5
Chi Square (χ^2) Check of Computer Printout

Parameter	Group No. vs. Control	UAREP	Searle	Significance
Number of fetuses	2	0.39	0.39	
	3	0.01	0.01	
	4	can't read		
	5	input		
	6	0.01	0.01	
Number of fetuses only vs. number of fetuses plus resorptions	2	0.00	0.00	
	3	1.36	1.36	
	4	0.33	0.33	
	5	1.12	1.12	
	6	0.06	0.06	
Fetal sex distribution	2	0.398	0.398	P = 0.01*
	3	0.001	0.001	
	4	0.649	25.775	
	5	0.009	0.009	
	6	0.667	0.667	
Survival data	2	0.136	0.136	
	3	0.842	0.842	
	4	0.136	0.136	
	5	0.000	0.000	
	6	0.177	0.177	
Conception data	2	0.161	0.161	
	3	0.632	0.632	
	4	1.581	1.581	
	5	0.141	0.141	
	6	0.446	0.446	
Abortion data	2	0.002	0.002	P = 0.01
	3	not run		
	4	36.12	36.12	
	5	2.70	2.70	
	6	not run		
Premature delivery data	2	0.610	0.610	
	3	0.006	0.006	
	4	0.393	0.393	
	5	0.169	0.169	
	6	0.561	0.561	
Abortion and premature delivery data	2	0.006	0.006	P = 0.01
	3	0.006	0.006	
	4	29.518	29.518	
	5	1.609	1.609	
	6	0.561	0.561	
Rib pairs	2	4.619	4.619	P = 0.05
	3	2.659	2.659	
Sixth centra absent	2	28.295	28.295	P = 0.01
	3	0.912	0.912	

* disagreement between Searle and UAREP

On their visit to Searle Laboratories on June 9, 1978, Dr. Dale Brooks and Dr. Robert E. Stowell discussed a number of matters regarding the experiments in E-90 with Mr. Alan Mitchell. Ray Schroeder, who had also been involved in the project, had moved from the Chicago area. Neither he nor Dr. Vondruska (who is no longer employed by Searle) were available for interview by UAREP.

A memo dated May 6, 1975 (Appendix XIV-5) from Dr. Vondruska to Drs. Dutt and Schmidt summarizes the fetal malformation and categorizes them into major and minor groups.

A memo dated May 19, 1975 (Appendix XIV-6) from M. Schmidt to Dr. Vondruska gives tables and a brief description of the statistical findings regarding the fetal malformation data.

Dr. Andrew G. Hendrickx, a UAREP consultant in teratology, compiled the following tables and text comparing Steffek's findings to Searle's. In an accompanying letter (Appendix XIV-7) Dr. Hendrickx mentions the problems caused by the poor condition of the visceral cross section material which made it impossible to evaluate.

UAREP Teratology Summary

Significance of Discrepancies in Searle/UAREP Findings - Table 14-6

gives a summary of discrepancies and concurrence in Searle and UAREP findings in examination of fetal skeletal preparations. Dr. Hendrickx thinks the discrepancies in the Searle/UAREP findings do not constitute a basis for changing Searle's interpretation of their results, primarily because seven out of 10 discrepancies (Table 14-6) were characterized by

Table 14-6
Summary of Discrepancies and Concurrence
in Searle and UAREP Findings

<u>Groups</u>	<u>No. of Fetuses</u>			
	<u>Total Malformations Reported</u>	<u>Examined</u> ¹	<u>Concurrence</u>	<u>Discrepancies</u> ²
Control	8	8	5	3
Low Dose	7	7	6	1
Medium Dose	10	10	8	2
High Dose	9	9	8	1
L-Phe	14	14	11	3
L-Asp	14	14	14	0
	—	—	—	—
	62	62	52	10
			83.9%	16.1%

¹ Includes skeletal malformations found at fetal external examination by Searle (Appendix I, Table 7) but not detected (or expected to be detected) by UAREP in the skeletal exam (Alizarin Red).

² See Table 14-7 for details. The above compilation includes the discrepancies in the Searle and UAREP findings.

identification of additional minor malformations by UAREP in fetuses which were previously reported by Searle to be malformed. He feels it is equally important that the remaining three discrepancies were essentially equally distributed in the experimental and control groups.

Discrepancies in Description of Skeletal Anomalies - Table 14-7 gives a comparison of Searle and UAREP findings of skeletal anomalies which were reviewed by Drs. Steffek, Ross, and Hendrickx (Appendix XIV-7, 8, 11). Supernumerary nasal sutures are cited in the Searle findings as minor malformations of individual fetuses in four of five experimental groups and in the controls (E-90, Appendix I, Table 7). They are also included in Table 8 (E-90, Appendix I) as part of the Summary of Fetal Skeletal Examination Data. However, no reference to this condition is presented in the Glossary. In spite of this apparent omission, the occurrence of supernumerary nasal sutures must be regarded as a minor malformation. The failure of Searle to identify this minor malformation in three specimens--11102F (Control), 21110F (Low Dose), and 32812F (Medium Dose)--constitutes a discrepancy in comparing the Searle vs UAREP findings. This has been tallied accordingly (Table 14-7).

Adequacy of Skeletal Examination - Table 14-8 lists and discusses the discrepancies in UAREP's findings of skeletal anomalies which Dr. Hendrickx considers artifactual. In general, it is Dr. Hendrickx' opinion that the examination of the 801 skeletal specimens is adequate to evaluate the accuracy of the Searle teratological diagnosis. His

Table 14-7
Discrepancies Between Searle and UAREP in the Teratologic
Findings of Skeletal Anomalies

Treatment Group	Fetal No.	Malformation	
		<u>Searle</u>	<u>UAREP</u> ¹
Control	11102 F	None	Supernumerary nasal sutures
Control	12407 F	a) Fused ribs (R), 3rd to 4th and 5th to 6th b) Fused 3rd and 4th thoracic vertebral centra c) Hypoplastic 5th thoracic vertebral centrum d) Fused transverse process (R), 3rd to 4th thoracic vertebrae e) Hypoplastic transverse process (R) of 6th cervical vertebrae and agenesis of transverse process and centrum	a and b findings same as Searle. But c, d & e not found, plus extra frontal suture with wide fontanelle
Control	14302 F	7th cervical ribs (B)	None
Low dose	21110 F	None	Supernumerary nasal sutures
Medium dose	32302 M	Agenesis of right transverse process and centrum of 13th thoracic vertebra	Same as Searle finding, plus only 11 ribs on right side
Medium dose	32812 F	None	Supernumerary nasal sutures
High dose	43001 M	Split interparietal bone	Same as Searle finding, plus split second sternbrae
L-Phe	25603 M	Short tail	Same as Searle finding, plus scrambled caudal vertebrae ²
L-Phe	27803 M	Supernumerary nasal suture Hypoplasia of the 3rd cervical vertebral centrum	Supernumerary nasal suture only
L-Phe	29204 M	Supernumerary nasal suture	None

¹ Animals 11102 (Control), 21110 (Low dose) and 32812 (Medium dose) appear only in Appendix I, Table 5 but do not appear in Appendix III (Comprehensive Litter Examination Report) and have been added to the above table.

² Listed in Entry Book E-90, Appendix I, Table 8 as "Fused caudal vertebrae."

Table 14-8
Artifactual Discrepancies in the Teratologic
Findings of Skeletal Anomalies

Treatment Group	Fetal No.	Malformation		
		<u>Searle</u>	<u>UAREP</u>	<u>Comment</u>
Medium Dose	34806 F	Supernumerary nasal suture	Irregular nasal suture and small bone spur between cervical vertebrae 6 & 7 (right side)	Nasal suture malformation considered identical; bone spur considered artifact; therefore, no discrepancies exist in Searle/UAREP findings
High Dose	43001 M	Omphalocele Split inter-parietal bone	Same as Searle finding, plus split second sternbrae 4 sternbrae	4 sternbrae considered artifact; therefore, only real discrepancy is split second sternbrae
L-Asp	39008 M	Split 5th sternbrae	Same as Searle finding, plus short tail	Short tail considered artifact; therefore, no discrepancy exists in Searle/UAREP findings

opinion is based on the fact that in the course of doing the skeletal staining procedure, major soft tissue anomalies are recognizable. In fact, the majority of the soft tissue malformations listed in Table 7 (E-90, Appendix I) were detected during the fetal external examination. Furthermore, it is readily apparent that the majority of the malformations occurred in the skeletal system, body wall or brain; and in each instance these defects are best detected by external examination or after skeletal staining. However, the inability to examine the body cross-sections raises the possibility that soft tissue defects such as internal hydrocephalus could have been overlooked and therefore could have occurred more or less often than reported by Searle within one or more groups. Nevertheless, he believes the examination of the skeletal specimens alone constitutes an adequate indication of the accuracy of the findings by Searle.

General Comments - In a letter dated October 22, 1975 from Dr. Morgareidge to Dr. Oser (Appendix XIV-9), many of the problems involved with using laboratory rabbits are discussed. Among these is the difficulty in obtaining genetically homogeneous strains. In Dr. Morgareidge's experience shipments of "virgin" females often arrived with a 10-15% pregnancy rate and many animals showing signs of enteritis and/or coccidiosis. He also mentioned problems with highly variable background incidence of spontaneous resorptions, abortions, fetal deaths, and frank malformations. A number of other problems with rabbits were mentioned, and he questioned whether rabbits were desirable as test animals in

teratology studies designed for detection of borderline teratogenic activity.

In a memo dated August 12, 1975 (Appendix XIV-10) this rabbit study (E-90) and a mouse teratology study (not sent to UAREP) are discussed in the light of a consultation with Dr. A. K. Palmer of Huntingdon Research Center in England. Dr. Palmer felt that E-90 was a much improved study over previous rabbit teratology studies done by Searle. He felt it was adequate to demonstrate lack of teratogenic activity in the rabbit. In his opinion the various effects noted in the 2 g/kg aspartame group and in the L-phenylalanine group were the result of reduced maternal food consumption and not teratogenic effects per se. He commented on a number of other items, and felt the Analysis of Variance would have been preferable to the t-test for comparing maternal body weight and food consumption.

CONCLUSIONS

Three hundred rabbits were used in this study, fifty in each group. Aspartame was administered at three different dosage levels. In addition, L-phenylalanine and L-aspartic acid, the major components of the aspartame molecule, were administered in amounts which were meant to be, but were not, equivalent to what the high dose group received.

UAREP's teratology review was conducted without prior knowledge of Searle's findings. Although it was impossible to examine the fetal body cross sections, UAREP's teratology consultants found generally good agreement in examination of the cleared specimens for skeletal anomalies. Seven out of ten discrepancies were minor malformations identified in fetuses which were previously reported by Searle to be malformed. UAREP felt that this was an adequate indication of the accuracy of Searle's findings.

There were a number of discrepancies in recording and/or reporting the data on food consumption. There were also some discrepancies in transcription of body weights.

In statistical analysis of food consumption, UAREP's t-test data agreed with Searle's in 62 instances (76%). In the statistical evaluation of body weight data, there was complete agreement.

UAREP checked 44 of the statistical results shown on computer printout and agreed in all but one instance.

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APPENDIX XIV-1

K. L. Rao FINAL PHASE II FOR A PRECLINICAL SAFETY STUDY OF SC-12862 ¹²⁻⁹⁻⁷⁵

AMENDED 1 2 3 4 5 Cost (\$) Est. R&D X Non-R&D PATH-TOR PROJ. NO. 120157

1) Protocol finalized 14 Jan 75 Treatment initiated Animals terminated Final report est. fir

Compound Total Ordered Deliver est. firm

2) needed (kg): First 4 wks Ordered Deliver est. firm

3) Study title & objectives: SC-12862: An Evaluation of Embryotoxic and Teratogenic Potential in the Rabbit; P-T No. 1201575. Segment II study performed to support marketing of SC-12862 as a food additive in the USA, UK, Canada, and Europe.

4) Species, strain, sex (M,F): Rabbit; New Zealand White; F Age (wk) at Rx start: 35 ± 6

5) Route & frequency of cpl. administration:

Premating period: Not applicable

Mating period: Not applicable

Gestation & lactation period: Intragastric in divided doses, twice daily at an interval of three hours. Administer compound from gestation day 6 through 16; a 13 day period. Control animals receive vehicle only in comparable volume twice daily as the test animals.

6) Mode of admin.: Appropriate concentrations of SC-12862, L-phenylalanine or L-aspartic acid, suspensions (w/v) in 1% aqueous Tween-80 solution (v/v) are prepared fresh each day. Administer equal volumes (ml/kg) to all animals. (Max. conc. to be used-?)

7) Drug-vehicle mixture stability analysis: Rx wks: Prepare fresh daily and use within 4-6 hrs during this period store at 10°C. Preparation is stable under these conditions.

8) Est. max. daily human dose & route: 50 mg/kg (for 27 kg child) orally in divided doses.

9) Dose levels (mg/kg daily): Control ; Low ; Med ; High

10) Multiple of human dose: ; ; ;

11) No. & sex of animals/level: Male ; SEE PAGE 1a ; ;

Female ; ; ;

12) Total inseminated animals required: 100

13) Housing & basal diet: Individually housed in stainless steel cages. Purina Rabbit Chow and tap water ad libitum.

KSP
12-9-75
1-13-75

RPM
1-14-75

0001

Page 1a

PROTOCOL FOR A PRECLINICAL SAFETY STUDY OF SC-18862
PATH-TOX PROJ. NO. 1201S75

Treatment Groups, Dosage and No. of Animals

Treatment Group	Dosage g/kg/day	No. Rabbits
Control	0	50
SC-18862 (Low Dose)	0.5	50
SC-18862 (Medium Dose)	1.0	50
SC-18862 (High Dose)	2.0	50
L-Phenylalanine [†]	0.82	50
L-Aspartic acid [†]	1.10	50

[†]Analytical grade (Ajinomoto Co., Inc.).

14) General Observations (frequency; wks)

Morbidity-mortality: Daily.

Motor & behavioral activity: Observe daily.

Body weight: Periodically during gestation; day 0, 3, 6, 10, 13, 15, 18, 22, 29

Food consumption & dose adjust.: Daily; dosage adjusted on day 10

Additional observations: None.

15) Mating Procedure: Artificial insemination: semen, collected from several proven fertile males maintained in-house, is evaluated qualitatively under a microscope (low power) for relative abundance and motility of spermatozoa. Several of the best samples are then pooled and diluted with warm isotonic sodium chloride solution (9 parts saline solution:1 part semen). Each female is then inseminated with 0.7-1.0 ml of diluted semen containing not less than 10 million sperm. Immediately following insemination, females are injected with 0.3 cc (1.5 MPK) of pituitary luteinizing hormone (PLH; Armour Pharmaceutical Co., Omaha, Nebraska) via the marginal ear vein to induce ovulation.

16) Terminal Examination: On day 28 of gestation the animals are sacrificed by air embolism and immediately laparotomized. Ovaries and uterus are examined in situ; the uterine horns are exposed and fetal swellings and resorption sites are counted and relative positions are recorded. The uterine horns are then opened and fetal viability determined on the basis of respiratory movements, skin color and movements of the extremities and head, etc.

17) Litter Examination: In utero litters; all fetuses are removed and weighed to the nearest 100 mg, crown-rump distance measured and placed in appropriate fixative.

18) Fetal Examination: All fetuses are examined for gross abnormalities prior to fixation; then, employing a random fetus selection procedure, the following is done:

1) One-half of each litter is preserved in Bouin's solution to be examined for visceral abnormalities by the free-hand, razor blade sectioning technique of Wilson;

2) Remaining one-half of each litter is preserved in 95% aqueous ethanol for processing of fetal skeletons by the Alizarin Red S skeletal staining technique. Fetal viscera are examined grossly during the evisceration step of the staining process.

NOTE: Fetuses with externally evident malformations may be selected by the investigator for either skeletal or visceral examination, as deemed appropriate, prior to randomly selecting the externally unremarkable fetuses.

20) Statistical evaluation of data: Procedures used:

0004

Body weight data (maternal and fetal); food consumption.

Group means \pm S.E. Mean comparisons are made by employing the Student's t-test with $p < 0.05$.

Conception rate, survival rate (maternal and neonatal) and litter incidence data:

Chi-square analysis ($p < 0.05$) employing appropriate modifications for reduced sample sizes when necessary.

Litter size and resorption data:

Means \pm S.E. Group means compared by employing Student's t-test ($p < 0.05$). Resorption data: possible employment of rank sum comparisons.

Incidence of fetal abnormalities:

Unusual anatomical findings among the fetuses will be categorized as major or minor malformations or as anatomical variants using the attached glossary as a guide (derived from a Huntingdon Research Centre publication entitled, Effects of Drugs on Reproductive Processes, October 1968). Both the individual fetal and litter incidence of individual and combined malformations shall be analyzed using the Fisher Exact Test ($p < 0.05$). Analysis shall be performed for major and minor malformations separately and combined.

Randomization procedures:

Simple randomization procedures.

21) INTERIM AND FINAL STUDY REPORTS

The sponsor (Director, Path-Tox Dept.) requires a brief quarterly report relating statistically significant changes in items 14, 17, 18 and 19 by or on the 1st of Jan., April, July and October; serious adverse findings are to be reported immediately. Tabulation of data and the general format of the report shall follow that of the included sample pages.

22) PROTOCOL DISTRIBUTION LIST

Design Committee Members:

- | | |
|--------------------|------------------------|
| 1) Dr. Dutt | (Biometrics Advisor) |
| 2) Dr. F. Saunders | (Biol. Res. Advisor) |
| 3) Dr. Ranney | (Metabolism Monitor) |
| 4) Dr. Polk | (Clinical Monitor) |
| 5) _____ | (Formulations Advisor) |
| 6) Dr. Rao | (P-T Dept. Monitor) |
| 7) Dr. McConnell | (P-T Dept. Advisor) |

Toxicology Coordinating Committee Members:

- 1) Dr. J. Clifford (U.K.)
- 2) Prof. M. Brunaud (France)
- 3) Dr. R. McConnell (U.S.A.)

Technical Staff:

- 1) Dr. Vondruska (Teratology Lab.)

October 24, 1975

MEMORANDUM

1218 G-1

TO: J. Potts

FROM: J. Vondruska

SUBJECT: Summary of Segment II Teratology Studies with
Aspartame (SC-18862) and Diketopiperazine (SC-19192)

The comprehensive toxicological investigations of the sweetening agent, aspartame, that have been undertaken to establish its safety under conditions of use, include a series of reproduction and teratological studies in experimental animals. The species that have yielded significant results have been rats and mice, although rabbits have also been employed. The most recent mouse teratology study is included herewith.

In the case of the rabbit, difficulties were encountered with the administration of test dosages due either to non-acceptance of the diets in which they were incorporated, or to resistance to intragastric intubation during the dosing period, i.e., the period of fetal organogenesis. These factors adversely affected the outcome of pregnancy in the control as well as test rabbits.

Interference with the normal course of pregnancy due to malnutrition has been reported in rabbits and in other animal species, and is one of the problems that have accounted for the variability of rabbits in teratological studies. Because of these and other factors, the usefulness of rabbits for teratology testing as a model for humans has been seriously questioned by experts in the field. In fact, many experts have concluded that the rabbit is not an appropriate model for the teratology testing of food additives. Nevertheless, repeated efforts were made to complete a satisfactory teratological test in this species and the final results of our latest test are included herewith.

Studies with Rats

Teratological studies were performed in which rats were fed diets containing the test materials, aspartame, the diketopiperazine (SC-19192) and a 3:1 mixture of the two, from the 6th through the 15th day (Segment II) of gestation.

The dosage levels provided 2.0 and 4.1 g/kg body weight of aspartame, 0.5, 1.0 and 2.0 g/kg of SC-19192, and 1.0, 2.0, 3.0 g/kg of the mixture. Unsupplemented rat rations were fed to control group. Fetuses were delivered by hysterotomy on the 20th day of pregnancy. The usual parameters, viz the number of implantations, resorptions, fetal deaths and the weight and length of the fetuses, were recorded. Examination of the fetuses for soft tissue and skeletal malformations were made and the findings classified, using Palmer's method as a guide.

No evidence of embryotoxicity, fetotoxicity, impaired fetal growth or teratogenicity were found upon statistical comparison of the responses of the treated with the control groups.

Studies with Mice

Sexually mature pregnant mice were fed diets containing aspartame which provided dose levels of 1.4, 2.7 and 5.7 g/kg from the 6th to 15th days of gestation. The fetuses were delivered by hysterotomy following maternal sacrifice on gestation day 18 and the number of implantations, resorptions, fetal deaths and the weight and length of the fetuses were recorded. The fetuses were examined for soft tissue and skeletal malformations and the findings classified, using Palmer's work as a guide.

No evidence of embryotoxicity, fetotoxicity, impaired fetal growth or teratogenicity were found upon statistical comparison of the responses of the treated with the control groups.

Studies with Rabbits

Despite earlier difficulties with the rabbit as a subject for teratological investigations, including poor acceptance of diets containing aspartame and resistance to gastric intubation, which in turn affected the outcome of pregnancy in all groups, a final large study was attempted with Aspartame and its two amino acid constituents. The purpose of this study was to attempt to apply experience acquired in earlier rabbit studies to determine whether or not a definitive study could be done in this species.

Aspartame was administered at doses of 0.5, 1.0 and 2.0 g/kg to groups of 50 artificially inseminated rabbits from post-insemination days 6 through 18. L-phenylalanine and L-aspartic acid were administered in a like manner at doses of 0.82 and 1.1 g/kg, respectively. Because of the poor aqueous solubility of aspartame, volumes of approximately 40 ml were administered twice daily to all animals.

Body weight and food consumption were recorded throughout gestation until sacrifice at post-insemination day 28. At hysterotomy, the numbers of implantations, resorptions, fetal deaths and the weight and length of the fetuses were recorded. The fetuses were then examined for soft tissue and skeletal malformations and the findings classified, using Palmer's work as a guide.

Administration of up to 1.0 g/kg of aspartame or 1.1 g/kg of aspartic acid by gastric intubation to pregnant rabbits produced no biologically meaningful or statistically significant differences in maternal body weight or food consumption data, litter losses, litter sizes, resorption rates, fetal sizes or malformation incidences.

Intubation of 2.0 g/kg of aspartame or 0.82 g/kg of L-phenylalanine reduced maternal food consumption in a manner that was approximately proportional to the amount of L-phenylalanine received in excess of that contained in a 1.0 g/kg dose of aspartame (0.55 g/kg). In those instances where food consumption was sufficiently reduced, significant maternal weight loss also occurred. The poor maternal nutrition and weight loss was associated with fetal wastage, which was expressed as abortion of the litter, total litter resorption or resorption of individual fetuses. The more severe types of fetal wastage were associated with greater degrees of maternal malnutrition. Those fetuses from malnourished mothers, which survived until hysterotomy were significantly smaller and the proportions of these fetuses that were malformed with significantly increased.


No teratogenic effects were observed in rabbits at doses of up to 1.0 g/kg of aspartame. A meaningful evaluation of teratogenicity at the 2.0 g/kg dose of aspartame in the rabbit was confounded as a result of the compromised maternal nutrition, and interpretation of these data were not possible.

Finally, unpublished work in our laboratories has demonstrated that the in vitro rabbit liver does not metabolize phenylalanine to tyrosine as rapidly as the livers of the rat or the mouse. Other work has demonstrated that pregnant female rabbits given high doses of L-phenylalanine (0.82 g/kg) resulted in greatly elevated blood concentrations of phenylalanine whereas tyrosine levels were not elevated to the same extent. It would appear that the persistence of the increased blood concentration of L-phenylalanine may have been responsible for the reluctance of the rabbit to consume adequate amounts of the basal diet.

Summary

In summary, satisfactory teratology studies have been performed in the rat and mouse, using doses of up to 4.1 and 5.7 g/kg, respectively. No teratogenic effects were observed in these two species.

The present rabbit teratology study demonstrated, again, the difficulty of designing and carrying out a reliable teratology study in this species using either dietary administration or gastric intubation. This question of the reliability of the rabbit for teratology effects of food additives has been questioned by experts and is addressed separately.


J. F. Vondruska

SCW

APPENDIX XIV-3

Terminal observations.

On gestation day 28 all females were sacrificed and the abdominal cavity opened. The ovaries and uterine horns were exposed, and the number of corpora lutea, fetuses and resorption sites recorded. Additionally, the relative position of each implantation along the uterine horns was recorded. Subsequently, fetuses were removed from the uterus and fetal viability was determined by respiratory movements, skin color, and movements of the extremities and head.

Fetal external examination. The weight and length (crown-rump distance, CRD) of each fetus were recorded. Each fetus was then given a thorough gross examination for external malformations prior to being euthanized and preserved intact in a fixative solution. Fetuses designated for soft-tissue examination were preserved in Bouin's solution. Fetuses designated for skeletal examination were preserved in 95% (v/v) aqueous ethyl alcohol.

Fetal soft tissue examination. Approximately one-half of the fetuses from each litter were fixed in Bouin's solution for subsequent examination by the free hand sectioning technique of Wilson² as follows:

Head: Five parallel tissue slices, approximately 5 mm thick, were prepared in a transverse plane and were examined for malformations of the palate, nasal cavities, eyes and brain.

Thorax: Five parallel slices, approximately 2 mm thick, were made in a transverse plane starting at the shoulders and proceeding caudally to the diaphragm. These slices were examined for malformations of the thymus, heart, lungs, esophagus, trachea, diaphragm and major blood vessels.

Abdomen: One slice was made approximately 8 mm caudal to the diaphragm and was examined for malformations of the liver, stomach and spinal cord. The next slice was made at the level of the right kidney and was examined for malformations of the kidney, liver, stomach, spleen and spinal cord. A final slice was made at the level of the left kidney and was examined for malformations of the spinal cord, aorta and renal pelvis. Structures found in the pelvic cavity (i.e., ureters, bladder and reproductive tract) were examined in situ after removal of the intestine.

Tissue slices were examined under a dissecting microscope (7X). All tissue slices from control and treated fetuses were then transferred to amber glass bottles filled with 70% (v/v) aqueous ethyl alcohol for temporary storage.

Fetal skeletal examination. The remaining fetuses (approximately one-half of each litter) were preserved in 95% (v/v) aqueous ethyl alcohol for subsequent skeletal staining by the Alizarin Red S staining technique.³





Following gross examination of the internal organs, these fetuses were eviscerated, and the remaining soft tissues macerated and cleared in 5.0% aqueous potassium hydroxide solution. Skeletal structures were then stained with Alizarin Red S and the stained preparations were stored in 100% glycerin (to which several crystals of thymol were added to reduce microbial growth). Such preparations were examined under a dissecting microscope (7X) for malformations. Additionally, the number and size of sternebrae, degree of closure of cranial ossifications, number of metacarpals, metatarsals and corresponding phalanges, etc., were recorded. All treated and control skeletal preparations were then placed in temporary storage.

APPENDIX XIV-4

CAGE TAG LABELS

<u>Animal Number</u>	<u>Group</u>	<u>Handwritten Information on Cage Tag Labels</u>
101	Control	114140; 21-65-02
102		Got approxiately 1/2 of dose; ? 2 nd IG skipped
107		White discharge and blood 2/16, 2/18, 2/19, 2/24, & 2/25 - above delivered 1 fetus
108		White mucus and blood discharge 2/16
110		Blood in pan 2/27
111		Threw the food on the floor
113		Skipped 2 nd IG 2/16
125		Delivered 6 viable pups 2/17/75, right side lungs - pyothorax 5 3 sites of placental attachment
126		Delivered 8 viable pups 3/17/75 4 5 no unusual path. findings
127		White discharge 2/27, 3/3 N.P. 3 5 uterine infections, horns swollen
133		Thick white discharge 3/7
136		Blood in pan 2/28
143		White discharge 3/20 & 3/31
202	Low Dose	Mean animal
250		Blood in pan 4/1/75 & 4/2/75
301	Medium Dose	114140; 21-65-02
302		Mean animal
313		Dropped on 2/14
332		IG skipped 3/6

<u>Animal Number</u>	<u>Group</u>	<u>Handwritten Information on Cage Tag Labels</u>
333	Medium Dose (cont'd)	Mean animal
335		Blood in pan 3/24, 3/25; 3/25 5 fetuses, 7 placental remnants; (?), Blood 3/26, 3/27
342		Blood in pan 3/17
?		N.P. No ovul.
?		R-4 Not pregnant, ovulated, L-7
405	High Dose	2/17/75 Thick blood on pan 2/18/75 & 2/19/75
406		Blood in pan 2/13/75 & 2/14/75 & 2/16
407		Blood in pan 2/19, 2/20, & 2/21
409		Blood in pan 2/16, 2/19, 2/20, & 2/21
411		Blood 2/19/75, 3 placental remnants 2/20; 5 placental remnants 2/21
413		Blood 2/24, 2/26 + placental remnant
415		Blood 2/20, 2/21, 2/24, & 2/26
416		Blood 2/26, 2/27, & 2/28
417		Thick white discharge 2/18 with some blood; white discharge 2/25, 2/27, & 2/28; white discharge blood in pan 2/20 & 2/21
418		Blood in pan 2/27, 2/28, 3/3, & 3/6
419		Thick white discharge 2/20, 2/21, 2/24, 3/3, 3/6, 3/7, & 3/10
420		Blood in pan 2/28, 3/6, & 3/7 mean animal
422		Blood in pan 3/6, 3/7
423		Blood in pan 3/6
426		Blood in pan 3/6

<u>Animal Number</u>	<u>Group</u>	<u>Handwritten Information on Cage Tag Labels</u>
?	High Dose (cont'd)	5  7 all sites of former placental attachment
?		5  8
427		Blood in pan 3/13
431		Blood in pan 3/10
433		2 nd IG skipped
434		Blood in pan 3/19/75, 3/20, & 3/21
435		Blood in pan 3/19 & 3/20
437		Blood in pan 3/22, 3/23, & 3/24
439		Blood in pan 3/22, 3/23, 3/24, & 3/26
440		Blood 3/26; 2 placental remnants and blood 3/27; blood 3/31
441		Blood 3/26, mean animal
442		Small amount blood 3/27, mean animal 8  3-7 sites of former placental attachment
444		Blood 3/31
445		Blood in pan 3/31 & 4/1
447		Blood in pan and 4 placental remnants 4/4/75
251	L-Phe	114140; 21-65-02
253		8  3
254		No weight taken 2/14/75 may have been missing since then, Missing 2/19/75; papers were changed 2/17/75 and since then no fecal material or food spill was evident on the paper could have dissappeared anytime
264		Blood in pan 2/14
266		White discharge 2/18 & 2/19
268		Blood 2/26 & 2/27; Blood with 2 placental remnants 2/28

<u>Animal Number</u>	<u>Group</u>	<u>Handwritten Information on Cage Tag Labels</u>
269	L-Phe (cont'd)	Thick white discharge 2/18, 3/3, & 3/6; 2 fetal remnants 3/9; 3 fetal remnats 3/10, Blood
275		White discharge 2/25
277		Blood in pan 3/18
296		This one urinated on your head when reinserted into cage
298		Blood in pan 4/1, 4/2, 4/4 & 4/7/75
354	L-ASP	Skipped sun 2 nd IG
355		Mean animal
356		Perforated IG tube, Sun 1 st IGing compound and lost about 2cc of
357		Abcess under neck 2/18/75
361		Skipped both IGs Sun
362		Mean animal
386		White discharge 3/6
388		Mean animal
389		Mean animal
391		Mean animal
392		Thick white discharge 3/11, 3/13, 3/16, & 3/18
398		I dropped this one 4/11/75
301		White discharge 3/20, mean animal

APPENDIX XIV-5

May 6, 1975

MEMO TO: J. Dutt
M. L. Schmidt

COPY TO: R. G. McConnell
K. S. Rao

FROM: J. F. Vondruska

SUBJECT: Statistical analyses of fetal malformation data from Segment II rabbit study with SC-18862; P-T 1201.

Please test the incidence of major and minor fetal malformations both by litter and by fetal incidence using the Fisher's Exact Test. The survival, conception, fetal recovery and resorption data was included in my memo of 4-13-75 and will not be repeated. A few changes in the fetal recovery and major malformations may be noted.

The abnormalities which were detected in this study and classified as major malformations are:

Test Group	Litters Examined	Fetuses Examined	Litter Number	Litter Size	Fetus Number & Sex	Malformation
Vehicle Control	35	279	128	9	12809M	Hydrocephalus*
Low Dose	40	339	220	6	22006M	Spina bifida
Medium Dose	44	344	314	10	31409M	Diaphragmatic hernia*
			348	11	34811F	Umbilical hernia
High Dose	9	75	412	10	41202M	Cleft palate
			430	7	43001M	Omphalocele
					43002M	Cleft palate
					43006F	Cleft palate
L-Phe	26	186	264	11	26407F	Omphalocele, arthrogryposis, unilateral renal agenesis and vertebral defects
			270	5	27001F	Cleft palate
			279	5	27901M	Omphalocele, sternal defects and syndactylous oligodactyly.
			281	5	28105M	Omphalocele, oligodactyly and vertebral defect
			284	11	28409F	Umbilical hernia
			292	4	29201M	Retinal folding*
					29203F	Omphalocele, unilateral amelia and sternal defects

- continued -

May 6, 1975
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Test Group	Litters Examined	Fetuses Examined	Litter Number	Litter Size	Fetus Number & Sex	Malformation
L-Asp	43	337	✓ 352	10	35208F	Omphalocele, cyclopia, microencephaly, astomia, absence of nares and philtrum and diaphragmatic hernia
			375	7	37502F	Hydrocephalus*
					37507F	Arthrogryposis
			390	11	39004M	Umbilical hernia + <i>skull defects</i> JFV

*These malformations were detected at soft tissue examination. Approximately one-half of each litter was examined for soft tissue malformations and one-half for skeletal malformations.

The incidences of major malformations are summarized below. All of the fetuses were viable at the time of hysterotomy.

Test Group	Litters Affected Litters Examined	Fetuses Affected Fetuses Examined	Fetuses Affected With:			
			Hydrocephalus	Cleft Palate	Ventral Midline Defects	Other
Vehicle Control	1/35	1/279	1	0	0	0
Low Dose	1/40	1/339	0	0	0	1
Medium Dose	2/44	2/344	0	0	2	0
High Dose.	2/9	4/75	0	3	1	0
L-Phe	6/26	7/186	0	1	5	1
L-Asp	3/43	4/337	1	0	2	1

Not included in the above tabulation is any data collected on prematurely delivered or aborted fetuses or late fetal resorptions. These fetuses are examined where possible, but are not included in the tabulations. These examinations represented a total of about 30 fetuses and were generally unremarkable. One late resorption recovered at hysterotomy from L-Phe female No. 289 had an umbilical hernia. The seven littermates were viable and unremarkable. This malformation is in addition to those on the above table.

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The minor malformations which were detected in this study are presented below. Except as noted, these malformations were detected at skeletal examination, and represent approximately one-half of the fetuses.

Test Group	Litters Examined	Fetuses Examined	Litter Number	# Fetuses in Litter Examined	Fetus Number & Sex	Malformation
Control	35	139	124	6	12407F	Vertebral & rib defects (fused ribs, vertebral centra & transverse processes, hypoplastic centra and transverse processes)
			132	5	13210M	Split 5th sternebra
			143	4	14302F	Vertebral & rib defects (7th cervical ribs)
					14304M	Vertebral & rib defect (7th cervical ribs, fused ribs & transverse processes)
					14306F	Vertebral & rib defects (7th cervical ribs)
Low Dose	40	169	150	5	15002M	Split 5th sternebra
			220	3	22001F	Fused 4th & 5th sternebrae
			226	5	22603M	Split 2nd sternebra
			232	4	23206F	Supernumary nasal sutures
			242	3	24206F	Split 5th sternebra
			243	4	24302M	Vertebral & rib defects (Split 2nd & 3rd vertebral centra)
Medium Dose	44	172	315	5	31503F	Fused 3rd, 4th & 5th sternebrae
			323	4	32302M	Vertebral & rib defects (agenesis of 13th thoracic vertebral transverse process and centrum.
			331	4	33108M	Supernumary nasal sutures
			336	5	33609F	Split 5th sternebra
			345	4	34504M	Split 5th sternebra
			347	6	34710M	Supernumary nasal sutures
			348	5	34806F	Supernumary nasal sutures

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Test Group	Litters Examined	Fetuses Examined	Litter Number	# Fetuses in Litter Examined	Fetus Number & Sex	Malformation
High Dose	9	36	✓ 408	2	40803F	Vertebral & rib defects (hypoplastic transverse process & centrum & fused ribs)
			✓ 426	11	42608F*	Short tail
			✓ 431	4	43103M	Split 2nd sternebra
					43105F	Fused 2nd, 3rd & 4th sternebrae
			✓ 443	2	44302M	Split 3rd sternebra
L-Phe	26	87	✓ 256	3	25602F*	Short kinked tail
					25603M*	Short tail
			✓ 261	2	26103F	Additional sternebrae
			✓ 264	11	26407F**	No tail
			✓ 278	2	28703M	Supernumary nasal sutures vertebral & rib defects (hypoplastic centrum)
			292	3	29202F	Fused 1st - 5th sternebrae
					29204M	Supernumary nasal sutures
			✓ 297	4	29706F	Vertebral & rib defects (Fused ribs)
L-Asp	43	160	✓ 353	5	35304F	Fused 4th & 5th sternebrae
			✓ 373	4	37302F	Skull closure grading 3
					37304F	Skull closure grading 3
					37306M	Skull closure grading 3
					37308F	Skull closure grading 3
			✓ 374	1	37401M	Skull closure grading 3
			✓ 382	4	38208F	Vertebral & rib defects (agenesis of transverse process and centrum)
			✓ 387	2	38704M	Fused 3rd, 4th & 5th sternebrae
			✓ 390	6	39008M	Split 5th sternebra
			✓ 396	4	39604F	Supernumary nasal sutures

*Minor malformations which were detected at hysterotomy. The total population for these malformations is the entire test group, and not only those fetuses examined for skeletal abnormalities.

**This fetus also had multiple major malformations.

*high 0
tail m - sup. nasal sut. *
39008M Split 5th sternebra
39604F Supernumary nasal sutures
Cerv. vert*

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The incidences of minor malformations are summarized below. All of the affected fetuses were viable at the time of hysterotomy. For purposes of this table, the Fetuses Examined are only those subjected to skeletal examination (fetuses with short tails detected at external examination are not included).

Test Group	<u>Litters Affected</u> <u>Litters Examined</u>	<u>Fetuses Affected</u> <u>Fetuses Examined</u>
Vehicle Control	4/35	6/139
Low Dose	5/40	5/169
Medium Dose	7/44	7/172
High Dose	4 3/9	5 4/36
L-Phe	5 4/26	7 3/87
L-Asp	5 7/43	11 18/160

The breakdown of various types of minor malformations is as follows (Fetus/Litters).

Test Group	Vertebral and Rib Defects	Split Sternebrae	Fused Sternebrae	Additional Sternebrae	Supernumerary Nasal Sutures	Skull Closure Grade 3	Short Tail
Control	4/2	2/2	0/0	0/0	0/0	0/0	0/0
Low Dose	1/1	2/2	1/1	0/0	1/1	0/0	0/0
Medium Dose	1/1	2/2	1/1	0/0	3/3	0/0	0/0
High Dose	1/1	2 2/3	1/1	0/0	2 1/1	0/0	1/1
L-Phe	2/2	0/0 5FV	1/1	1/1	2/2	0/0	3/2
L-Asp	2 1/1 3FV	1/1	2/2	0/0	2 1/1 3FV	5/2	0/0

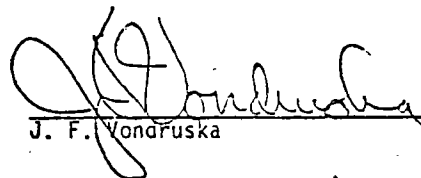
If the above data for each test group are totaled, they may not agree with the previous table due to the addition of the short tailed fetuses, one fetus with two different types of malformations, or one litter having two fetuses with two different malformations.

All fetal malformation data is summarized below. Categories include major malformations, minor malformations and all malformations. The data are presented as Fetuses Affected/Fetuses Examined or Litters Affected/Litters Examined.

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Test Group	Major Malformation Incidence		Minor Malformation Incidence		Total Malformation Incidence	
	by Fetus	by Litter	by Fetus	by Litter	by Fetus	by Litter
Vehicle Control	1/279	1/35	6/279	4/35	7/279	5/35
Low Dose	1/339	1/40	5/339	5/40	6/339	5/40
Medium Dose	2/344	2/44	7/344	7/44	9/344	8/44
High Dose	4/75	2/9	5/75	4/9	9/75	6/9
L-Phe	7/186	6/26	7 18/186	5 26	14 18/186	10 26
L-Asp	4/337	3/43	10/337	7/43	14/337	9/43
Historical Control	11/1717	11/233	18/1717	18/233	29/1717	26/233

Also included in the above table is the historical control incidence of major and minor malformations in the rabbit in our laboratory. Please compare the incidence of major malformations, minor malformations and major and minor malformations combined with the vehicle control and the historical control data. Also compare the incidence of the more prominent individual defects such as cleft palate and the ventral midline defects.


J. F. Vondruska

/dd

Handwritten notes:
- 2/25 - 2/25 - 2/25
- 1/11 - 1/11 - 1/11
- 1/11 - 1/11 - 1/11
- 1/11 - 1/11 - 1/11

May 19, 1975

To: J. Vondruska

From: M. Schmidt

Subject: STATISTICAL ANALYSIS OF FETAL MAJOR AND MINOR MALFORMATIONS
FROM SEGMENT II RABBIT TERATOLOGY STUDY WITH SC-18862

Attached are the results of the Fisher Exact Tests on malformations for the Segment II Rabbit Teratology Study with SC-18862. Tables I and II give results for major malformations using the litter and fetus as units of measure respectively. The first set of tests in each table is on the number of malformations occurring regardless of its specific type. The number of malformations in the L-Phe group is significantly higher than the control when either the litter or fetus is used. In the High dose group only when the number of fetuses is used is there a statistically significant difference.¹ For all other groups there are no statistically significant differences regardless of the unit of measure.

When looking at specific major malformations there are no significant differences between the controls and treated groups for incidence of hydrocephalus. For cleft palate only the High Dose incidence is significantly higher than the control. The L-Phe group has a significantly higher incidence of ventral midline defects than the control group regardless of the unit of measure.

¹In a previous memo (4/28/75) there was also a significant difference when the litter was the unit of measure. This reversal is due to the change in incidence in the vehicle control group (0/35 to 1/35).

In Tables III and IV the results of the Fisher Exact Tests on incidence of minor malformations are given using the litter and fetus as the units of measure respectively. When all minor malformations are considered together, the only statistically significant differences occur between the controls and High Dose levels. This is true both for the litter and fetus as units of measure. All other comparisons to control are not significant. When specific minor malformations are considered, there are only two cases of a significant difference occurring. That is, when the fetus is the unit, the incidence of Skull Closure Grade 3 is significantly higher in the L-Asp group than in the control and the incidence of Supermammary Nasal Sutures is higher in the High Dose group than in control. It might be pointed out that in these two tables are also a number of cases where the incidence of minor malformations is less in the treated than in the control.

Tables V and VI look at the incidence of both major and minor malformations in this study as compared to the Historical Control. For major malformations the incidence is significantly higher in the L-Phe group than in the Historical Control both when the litter and the fetus are used as units of measure. For the High Dose group the incidence is higher only when the fetus is used as the measuring unit.

For minor malformations, both the High Dose and L-Phe groups have significantly higher incidences of malformations than the Historical Controls. This is true for both the litter and fetus as units of measure. Also, the L-Asp incidence rate is higher than this control when the fetus is used.

If the malformations are grouped together (both minor and major), the High Dose and L-Phe are significantly higher than the Historical Control for both the litter and fetus measuring units. And again, the L-Phe Group has a significantly higher incidence of malformations per fetus than the Historical Control.

Tables VII and VIII give the results of the tests using the vehicle control group for the comparisons. The major alone and minor alone comparisons are also given in Tables I, II and III, IV respectively. To briefly iterate, the High Dose group has significantly higher incidence of major malformations than the vehicle control when fetus is used and L-Phe group is significantly higher for both units of measure. For minor malformations, the High Dose group is significantly different for both units. If the malformations are combined one finds that both the High Dose and L-Phe group have higher incidences of malformations than the vehicle control. This is true both for the litter and for the fetus.

Mary Lou
MLS/maj

Table I
Fisher Exact Tests
Vehicle Control Comparisons (Litter)
Segment II Rabbit Study SC-18862

Malformation	Group	No. of Litters With Malf.	No. of Litters Without Malf.	Total	p-value
Major	Vehicle Control	1	34	35	
	Low Dose	1	39	40	0.719 NS ⁺
	Medium Dose	2	42	44	0.586 NS
	High Dose	2	7	9	0.102 NS
	L-Phe	6	20	26	0.020 *
	L-Asp	3	40	43	0.389 NS
Hydrocephalus	Vehicle Control	1	34	35	
	Low Dose	0	40	40	0.467 NS ⁺
	Medium Dose	0	44	44	0.443 NS ⁺
	High Dose	0	9	9	0.796 NS ⁺
	L-Phe	0	26	26	0.574 NS ⁺
	L-Asp	1	42	43	0.699 NS ⁺
Cleft Palate	Vehicle Control	0	35	35	
	Low Dose	0	40	40	--
	Medium Dose	0	44	44	--
	High Dose	3 2	6 7	9	0.006 ** 0.025 ⁺
	L-Phe	1	25	26	0.426 NS 3FV
	L-Asp	0	43	43	--
Ventral Midline Defects	Vehicle Control	0	35	35	
	Low Dose	0	40	40	--
	Medium Dose	2	42	44	0.307 NS
	High Dose	1	8	9	0.205 NS
	L-Phe	5	21	26	0.011 *
	L-Asp	2	41	43	0.301 NS

NS Not statistically significant at 5% level

* Statistically significant at 5% level

** Statistically significant at 1% level

+ Control incidence of malformations higher than treated

No p-value given in cases where there were zero occurrences in control and treated.

Table II
Fisher Exact Tests
Vehicle Control Comparisons (Fetus)
Segment II Rabbit Study SC-18862

Malformation	Group	No. of Fetuses With Malf.	No. of Fetuses Without Malf.	Total	p-value
Major	Vehicle Control	1	278	279	
	Low Dose	1	338	339	0.699 NS ⁺
	Medium Dose	2	342	344	0.578 NS
	High Dose	4	71	75	0.008 **
	L-Phe	7	179	186	0.008 **
	L-Asp	4	333	337	0.251 NS
Hydrocephalus	Vehicle Control	1	278	279	
	Low Dose	0	339	339	0.452 NS ⁺
	Medium Dose	0	344	344	0.448 NS ⁺
	High Dose	0	75	75	0.788 NS ⁺
	L-Phe	0	186	186	0.600 NS ⁺
	L-Asp	1	336	337	0.701 NS ⁺
Cleft Palate	Vehicle Control	0	279	279	
	Low Dose	0	339	339	--
	Medium Dose	0	344	344	--
	High Dose	3	72	75	0.009 **
	L-Phe	1	185	186	0.400 NS
	L-Asp	0	337	337	--
Ventral Midline Defects	Vehicle Control	0	279	279	
	Low Dose	0	339	339	--
	Medium Dose	2	342	344	0.305 NS
	High Dose	1	74	75	0.212 NS
	L-Phe	5	181	186	0.010 **
	L-Asp	2	335	337	0.299 NS

NS Not statistically significant at 5% level

* Statistically significant at 5% level

** Statistically significant at 1% level

+ Control incidence of malformations higher than treated

No p-value given in cases where there were zero occurrences in control and treated.

Table III
Fisher Exact Tests
Vehicle Control Comparisons (Litter)
Segment II Rabbit Study SC-18862

Malformation	Group	No. of Litters With Malf.	No. of Litters Without Malf.	Total	p-value
Minor	Vehicle Control	4	31	35	
	Low Dose	5	35	40	0.586 NS
	Medium Dose	7	37	44	0.408 NS
	High Dose	4	5	9	0.042 *
	L-Phe	4 5	22 21	26	0.467 NS 0.311 NS
	L-Asp	7	36	43	0.397 NS 3 FU.
Vertebrae and Rib Defects	Vehicle Control	2	33	35	
	Low Dose	1	39	40	0.449 NS ⁺
	Medium Dose	1	43	44	0.414 NS ⁺
	High Dose	1	8	9	0.506 NS
	L-Phe	2	24	26	0.574 NS
	L-Asp	1	42	43	0.422 NS ⁺
Split Sternebrae	Vehicle Control	2	33	35	
	Low Dose	2	38	40	0.640 NS ⁺
	Medium Dose	2	42	44	0.601 NS ⁺
	High Dose	3	6	9	0.050 NS
	L-Phe	0	26	26	0.325 NS
	L-Asp	1	42	43	0.422 NS ⁺
Fused Sternebrae	Vehicle Control	0	35	35	
	Low Dose	1	39	40	0.533 NS
	Medium Dose	1	43	44	0.557 NS
	High Dose	1	8	9	0.205 NS
	L-Phe	1	25	26	0.426 NS
	L-Asp	2	41	43	0.288 NS
Additional Sternebrae	Vehicle Control	0	35	35	
	Low Dose	0	40	40	--
	Medium Dose	0	44	44	--
	High Dose	0	9	9	--
	L-Phe	1	25	26	0.426 NS
	L-Asp	0	43	43	--

Table III (continued)

Malformation	Group	No. of Litters With Malf.	No. of Litters Without Malf.	Total	p-value
Supernumary Nasal Sutures	Vehicle Control	0	35	35	
	Low Dose	1	39	40	0.533 NS
	Medium Dose	3	41	44	0.168 NS
	High Dose	1	8	9	0.204 NS
	L-Phe	2	24	26	0.178 NS
	L-Asp	1	42	43	0.551 NS
Skull Closure Grade 3	Vehicle Control	0	35	35	
	Low Dose	0	40	40	--
	Medium Dose	0	44	44	--
	High Dose	0	9	9	--
	L-Phe	0	26	26	--
	L-Asp	2	41	43	0.301 NS
Short Tail	Vehicle Control	0	35	35	
	Low Dose	0	40	40	--
	Medium Dose	0	44	44	--
	High Dose	1	8	9	0.205 NS
	L-Phe	2	24	26	0.178 NS
	L-Asp	0	43	43	--

NS Not statistically significant at 5% level

* Statistically significant at 5% level

** Statistically significant at 1% level

+ Control incidence of malformations higher than treated

No p-value given in cases where there were zero occurrence in control and treated.

Table IV
Fisher Exact Tests
Vehicle Control Comparisons (Fetus)
Segment II Rabbit Study SC-18862

Malformation	Group	No. of Fetuses With Malf.	No. of Fetuses Without Malf.	Total	p-value
Minor	Vehicle Control	6	133	139	0.368 NS ⁺
	Low Dose	5	164	169	0.566 NS ⁺
	Medium Dose	7	165	172	0.606 **
	High Dose	7 5	29 31	36	0.425 NS
	L-Phe	5 7	82 80	87	0.243 NS
	L-Asp	11 0 3FU	149 150 3FU	160	0.317 NS
Vertebrae and Rib Defects	Vehicle Control	4	135	139	0.131 NS ⁺
	Low Dose	1	168	169	0.126 NS ⁺
	Medium Dose	1	171	172	0.727 NS ⁺
	High Dose	1	35	36	0.576 NS ⁺
	L-Phe	2	85	87	0.922 NS ⁺
	L-Asp	2	158	160	
Split Sternebrae	Vehicle Control	2	137	139	0.612 NS ⁺
	Low Dose	2	167	169	0.605 NS ⁺
	Medium Dose	2	170	172	0.060 NS
	High Dose	3	33	36	0.377 NS ⁺
	L-Phe	0	87	87	0.447 NS ⁺
	L-Asp	1	159	160	
Fused Sternebrae	Vehicle Control	0	139	139	0.549 NS
	Low Dose	1	168	169	0.553 NS
	Medium Dose	1	171	172	0.206 NS
	High Dose	1	35	36	0.385 NS
	L-Phe	1	86	87	0.286 NS
	L-Asp	2	158	160	
Additional Sternebrae	Vehicle Control	0	139	139	--
	Low Dose	0	169	169	--
	Medium Dose	0	172	172	--
	High Dose	0	36	36	--
	L-Phe	1	86	87	0.385 NS
	L-Asp	0	160	160	--

Table IV (continued)

Malformation	Group	No. of Fetuses With Malf.	No. of Fetuses Without Malf.	Total	p-value
Supernumary Nasal Sutures	Vehicle Control	0	139	139	
	Low Dose	1	168	169	0.549 NS
	Medium Dose	3	169	172	0.168 NS
	High Dose	2	34	36	0.041 *
	L-Phe	2	85	87	0.147 NS
	L-Asp	2	158	160	0.286 NS
Skull Closure Grade 3	Vehicle Control	0	139	139	
	Low Dose	0	169	169	--
	Medium Dose	0	172	172	--
	High Dose	0	36	36	--
	L-Phe	0	87	87	--
	L-Asp	5	155	160	0.043 *
Short Tail	Vehicle Control	0	139	139	
	Low Dose	0	169	169	--
	Medium Dose	0	172	172	--
	High Dose	1	35	36	0.206 NS
	L-Phe	3	84	87	0.056 NS
	L-Asp	0	160	160	--

NS Not statistically significant at 5% level

* Statistically significant at 5% level

** Statistically significant at 1% level

+ Control incidence of malformations higher than treated

No p-value given in cases where there were zero occurrence in control and treated.

Table V
Fisher Exact Tests
Historical Control Comparisons (Litter)
Segment II Rabbit Study SC-18862

Malformation	Group	No. of Litters With Malf.	No. of Litters Without Malf.	Total	p-value
Major	Historical Control	11	222	233	
	Vehicle Control	1	34	35	0.519 NS ⁺
	Low Dose	1	39	40	0.453 NS ⁺
	Medium Dose	2	42	44	0.659 NS ⁺
	High Dose	2	7	9	0.078 NS
	L-Phe	6	20	26	0.003 **
	L-Asp	3	40	43	0.376 NS
Minor	Historical Control	18	215	233	
	Vehicle Control	4	31	35	0.319 NS
	Low Dose	5	35	40	0.233 NS
	Medium Dose	7	37	44	0.079 NS
	High Dose	5	4	9	0.001 ** 0.05 NS
	L-Phe	6	20	26	0.022 * 0.06 NS
	L-Asp	7	36	43	0.072 NS
Combined Major and Minor	Historical Control	26	207	233	
	Vehicle Control	5	30	35	0.380 NS
	Low Dose	5	35	40	0.489 NS
	Medium Dose	8	36	44	0.147 NS
	High Dose	7	2	9	0.001 ** 0.05 NS
	L-Phe	11	15	26	<0.001 ** OK
	L-Asp	9	34	43	0.070 NS 3 FV

NS Not statistically significant at 5% level

* Statistically significant at 5% level

** Statistically significant at 1% level

+ Control incidence of malformations higher than treated

No p-value given in cases where there were zero occurrences in control and treated.

Table VI
Fisher Exact Tests
Historical Control Comparisons (Fetus)
Segment II Rabbit Study SC-18862

Malformation	Group	No. of Fetuses With Malf.	No. of Fetuses Without Malf.	Total	p-value
Major	Historical Control	11	1706	1717	
	Vehicle Control	1	278	279	0.484 NS ⁺
	Low Dose	1	338	339	0.387 NS ⁺
	Medium Dose	2	342	344	0.627 NS ⁺
	High Dose	4	71	75	0.003 **
	L-Phe	7	179	186	0.001 **
	L-Asp	4	333	337	0.222 NS
Minor	Historical Control	18	1699	1717	
	Vehicle Control	6	273	279	0.107 NS
	Low Dose	5	334	339	0.326 NS
	Medium Dose	7	337	344	0.109 NS
	High Dose	6 5	69 70	75	<0.001 ** 0.001 **
	L-Phe	8 7	178 179	186	0.002 ** 0.001 **
	L-Asp	11 10	326 327	337	0.004 ** 0.010 **
Combined Major and Minor	Historical Control	29	1688	1717	
	Vehicle Control	7	272	279	0.230 NS
	Low Dose	6	333	339	0.529 NS
	Medium Dose	9	335	344	0.170 NS
	High Dose	10 9	65 66	75	<0.001 ** 0.001 **
	L-Phe	15 14	171 172	186	<0.001 ** same
	L-Asp	14 14	323 324	337	0.006 ** 3FV

NS Not statistically significant at 5% level

* Statistically significant at 5% level

** Statistically significant at 1% level

+ Control incidence of malformations higher than treated

No p-value given in cases where there were zero occurrences in control and treated.

Table VII
Fisher Exact Tests
Vehicle Control Comparisons (Litter)
Segment II Rabbit Study SC-18862

Malformation	Group	No. of Litters With Malf.	No. of Litters Without Malf.	Total	p-value
Major	Vehicle Control	1	34	35	
	Low Dose	1	39	40	0.719 NS ⁺
	Medium Dose	2	42	44	0.586 NS
	High Dose	2	7	9	0.102 NS
	L-Phe	6	20	26	0.020 *
	L-Asp	3	40	43	0.389 NS
Minor	Vehicle Control	4	31	35	
	Low Dose	5	35	40	0.586 NS
	Medium Dose	7	37	44	0.408 NS
	High Dose	4	5	9	0.042 *
	L-Phe	4 5	22 21	26	0.467 NS 0.311 NS
	L-Asp	7	36	43	0.397 NS
Combined Major and Minor	Vehicle Control	5	30	35	
	Low Dose	5	35	40	0.543 NS ⁺
	Medium Dose	8	36	44	0.440 NS
	High Dose	7 6	2 3	9	0.001 ** 0.001 ^{NS}
	L-Phe	11 10	15 14	26	0.015 * 0.031 ^{NS}
	L-Asp	9 JFV	34 JFV	43	0.324 NS JFV

NS Not statistically significant at 5% level

* Statistically significant at 5% level

** Statistically significant at 1% level

+ Control incidence of malformations higher than treated

No p-value given in cases where there were zero occurrences in control and treated.

Table VIII
Fisher Exact Tests
Vehicle Control Comparisons (Fetus)
Segment II Rabbit Study SC-18862

Malformation	Group	No. of Fetus With Malf.	No. of Fetus Without Malf.	Total	p-value
Major	Vehicle Control	1	278	279	
	Low Dose	1	338	339	0.699 NS ⁺
	Medium Dose	2	342	344	0.578 NS
	High Dose	4	71	75	0.008 **
	L-Phe	7	179	186	0.008 **
	L-Asp	4	333	337	0.251 NS
Minor	Vehicle Control	6	133	139	
	Low Dose	5	164	169	0.368 NS ⁺
	Medium Dose	7	165	172	0.566 NS ⁺
	High Dose	7 5	29 31	36	0.005 ** 0.150 NS
	L-Phe	5 7	82 80	87	0.425 NS 0.189 NS
	L-Asp	11 10	149 150	160	0.243 NS 0.13 NS
Combined Major and Minor	Vehicle Control	7	272	279	
	Low Dose	6	333	339	0.359 NS ⁺
	Medium Dose	9	335	344	0.571 NS
	High Dose	10 9	65 66	75	0.001 ** 0.002 **
	L-Phe	15 14	171 172	186	0.001 ** 0.011 **
	L-Asp	14 3FV	123 323 3FV	337	0.185 NS 3FV

NS Not statistically significant at 5% level

* Statistically significant at 5% level

** Statistically significant at 1% level

+ Control incidence of malformations higher than treated

No p-value given in cases where there were zero occurrences in control and treated.

16 August 1978

Dr. Robert E. Stowell
Department of Pathology
School of Medicine
Med. Sci. 1-A
Davis Campus

RE: Review of E-90

Dear Bob:

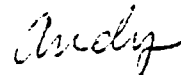
In response to your request of July 18, 1978 I have compiled the following data:

1. Table (A) summarizing concurrence/nonconcurrence (discrepancies) in the teratologic findings of Searle and UAREP.
2. Table (B) itemizing the nonconcurrence by Searle and UAREP and the discrepancies in their respective findings.
3. Table (C) of artifactual findings.

In addition, Tables 7 & 8 (Appendix I) have been changed to account for the discrepancies tallied in Table B. Similarly, Tables 3 & 4 have been changed to account for these findings.

You will also find attached statements concerning my opinion about the findings of the supernumerary nasal sutures, interpretation of results by Searle in light of the discrepancies found in the review by UAREP, and the possible limitations of UAREP findings because of the unsatisfactory condition of the visceral cross-section material.

Sincerely,



Andrew G. Hendrickx, Ph.D.
Research Physiologist

AGH:cmr
Enclosure

APPENDIX XIV-8

18 May 1978

Dr. Robert E. Stowell
Department of Pathology
School of Medicine
Med. Sci. 1-A
Davis Campus

RE: An Evaluation of Embryotoxic and
Teratogenic Potential in the Rabbit

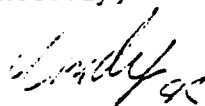
Dear Bob:

I have recently completed a review of the notes made by Dr. A. J. Steffek on his observations of the fetal skeletal preparations. After completing this review I compared his findings to the report submitted by Searle. The results of this comparison can be summarized as follows: 53 of the 59 abnormal fetuses reported by Searle were in the group recently reviewed by Steffek (skeletal preparations).

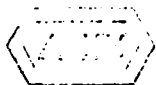
Dr. Steffek found that the defects in 30 (56.6%) of the 53 fetuses were identical to those reported by Searle. In eight (15.1%) fetuses each investigator found different defects; and in eight (15.1%) fetuses each investigator found some common defects in addition to several dissimilar anomalies. Steffek failed to find defects in six of the fetuses, primarily because he made no attempt to identify cleft palate or spine bifida, and failed to recognize the presence of cervical ribs and was not specific in identifying vertebral defects, consequently, he overlooked several vertebral defects. Steffek also found that one fetus was too damaged (decapitated and dismembered) to make an analysis.

In addition to the above observations, Steffek also recorded minor defects in 51 specimens. They are being reexamined by Dr. Steffek (see accompanying letter) to determine if they are in fact defects or deviations from the norm.

Sincerely,


Andrew G. Hendrickx, Ph.D.
Research Physiologist

AGH:cmr
Enclosure



FOOD AND DRUG

Research LABORATORIES, INC.

October 22, 1975

Dr. Bernard L. Oser
B. L. Oser Associates, Inc.
108-18 Queens Blvd.
Forest Hills, New York 11375

COPY

OCT 29 1975

R & D CENTRAL FILE

Dear Ben:

Pursuant to your request of October 21st, I will try to indicate in the following paragraphs some of the conclusions drawn from our experiences with the use of rabbits in the teratology assay. As you know, this experience was gained in the course of assays conducted under contract for the Food & Drug Administration in an attempt to evaluate the teratologic potential of a series of substances selected from the so-called GRAS list (21 CFR 121.101). Out of a total of some 90-odd materials submitted, 58 were tested in four laboratory species; namely, mice, rats, hamsters, and rabbits. The general method entailed the administration of the test material at four dosage-levels by gavage in a suitable vehicle (if needed) from the 6th to the 18th day of gestation (in rabbits) using a sufficient number of artificially inseminated does to ensure the survival of at least 10 pregnant animals to the 29th day. This usually required the use of groups of not less than 15 animals per dosage level to allow for losses due to accidental deaths and/or mortality due to other extraneous factors and approximately an 85 per cent nidation rate. In many cases, the total number of does used per test group exceeded 20. Comparable groups of controls (sham treated and positive teratogen-dosed) were included with each pair of test materials run concurrently.

Thus, over-all, nearly 5000 rabbits were employed in these studies along with approximately double that number of mice, rats, and hamsters. The following conclusions are based on this experience although it should be understood that they are presented here as the personal observations of the writer and do not necessarily represent those of others who collaborated in the work.

(1) During the period covered by this work, the only feasible supply of animals was that purchased from commercial dealers who, for the most part, purchase rabbits from independent breeders whose stocks are genetically heterogeneous and whose husbandry practices often leave much to be desired. As a consequence, shipments of "virgin"

COPY

Dr. Bernard L. Oser
Page 2

10/10/53

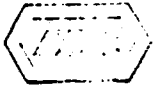
210 CENTRAL FILE

females were often delivered to the Laboratory in which 10 to 15 per cent of the animals were already pregnant and with many showing signs of infection with enteritis and/or coccidiosis. A two to four week quarantine period was mandatory to permit some assurance that carefully selected animals would be suitable for group assignment. Even so, unexplained mortality during the early stages of the procedure remained a constant source of trouble.

(2) This lack of control over uniformity of the test animals contributed greatly to the already high level of inherent biological variability characteristic of the reproductive processes, especially. This was more apparent in comparison with the relative uniformity obtained with mice and rats. The latter were from highly inbred strains of known origin.

(3) The limitation of group size to 10 pregnant does was, of course, dictated by both cost and space considerations. However, coupled with the normally fewer number of fetuses per litter, the over-all number of pups available for evaluation terminally severely limited the reliability of the final data. This had to be judged against a background of a highly variable incidence of spontaneous resorptions, premature deliveries (abortions), fetal deaths, and frank malformations. Available statistical techniques were ineffective in determining the significance of differences between test and control groups with so few degrees of freedom. In some cases, apparently significant differences were obviously due to large discrepancies between sequential control data.

(4) Maternal morbidity and mortality was also a continuing problem with rabbits as compared with the other species which was not obviously related to treatments. These effects may have been correlated with the comparatively greater degree of trauma associated with gastric intubation in rabbits which tend to struggle more violently than do the other species. Spontaneous abortion during the third trimester of pregnancy is also common in rabbit colonies. This tends to confuse the interpretation of signs associated with the toxicity of the test compound to the maternal organism. Furthermore, the practice of attempting to administer the highest tolerated dose of any compound is bound to result in confusion with respect to differentiation between maternal and fetal effects.



C O P Y

JUN 10 1972

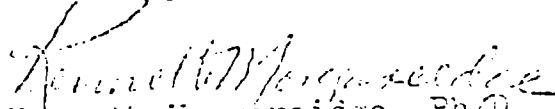
U.S. CENTRAL FILE

Dr. Bernard L. Oser
Page 3.

In view of the above, it was concluded by the Scientists of the Food and Drug Administration and concurred in by those of FDRL's Staff, that the use of rabbits in teratologic studies of potential food additives and GRAS substances was no longer to be recommended. This is not to infer that this species is not susceptible to truly potent teratogens such as 2-amino nicotinamide, which was employed throughout these studies as the positive control agent. However, for all of the reasons cited above, the rabbit was determined to be unreliable for use in screening studies aimed at the detection of borderline teratogenic activity.

I trust that the foregoing will be found responsive to your questions. If I can be of further assistance, please do not hesitate to call.

Cordially,


Kenneth Morgareidge, Ph.D.
Science Advisor

APPENDIX XIV-10

August 12, 1975

MEMO TO: File
FROM: J. F. Vondruska
RE: Conversation with Tony Palmer regarding P-T 1201 and P-T 1218, two recent teratology studies with Aspartame.

COPY TO: R. G. McConnell
J. Pendry
J. Potts

Dr. A. K. Palmer of Huntingdon Research Centre phoned on the morning of 12 August 1975 to give me his preliminary impressions of the rabbit and mouse study reports which were recently delivered to him by Ms. Marian Perkins.

He feels that the mouse study, P-T 1218, is a good study and adequately demonstrates the absence of any teratogenic activity or maternal toxicity of Aspartame in the Charles River cd mouse. The poorly ossified supra-occipital bone which was present in a significant number of medium dose fetuses is a function of the fetal age, and should not be considered to be biologically meaningful in this study.

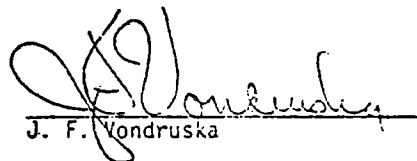
The rabbit study, P-T 1201, is a much improved study over those done earlier and is more than adequate to demonstrate lack of teratogenic activity in the rabbit from his point of view. He feels that the effects seen at the 2.0 g/kg Aspartame and at the L-phenylalanine levels are a result of reduced maternal food consumption and not a teratogenic effect per se. The items upon which he chose to comment at this time were minor, and generally consisted of differences in style of writing and presentation of data, and include:

1. Indicate whether the Fisher Exact test which was used for comparing the incidence of malformations was one-tailed or two-tailed. (The two-tailed test was used).
2. Since the effects seen in the high dose and L-phenylalanine groups were similar, it would be reasonable to combine the abortion and total litter resorption data for these two groups for statistical analysis.
3. They consider abortion or total litter resorption to be in the same category of total litter loss and combine them for statistical comparison. Another similar type of approach they might use is to compare the total numbers of females in each group having viable fetuses at term.
4. They would use the analysis of variance for comparing maternal body weight and food consumption data rather than the Student's t-test.

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5. The maternal food consumption graphs might be confusing since we do not present food consumption data for day 5, the last day before dosing. It is possible that a casual observer might get the impression that food consumption begins to drop off after gestation day 3. Daily food consumption is presented in the appendix and the course of events is explained in the text.

Dr. Palmer indicated that he was going on holiday for awhile and would prepare a formal written report upon his return in early September. He is not familiar with the Permutation Test which was used to compare the distribution of litters with resorptions, and asked for more information on this test. The references which we made to partial or complete food restriction as a cause of fetal malformations in the rat and mouse are valid and the same situation exists for the rabbit. He has indicated that he will provide us with a personal communication type of reference for this statement since it does not appear anywhere in the literature that we know of, and he has data to support that statement.


J. F. Vondruska

JFV:s

APPENDIX XIV-11

COMPARISON OF DR. STEFFEK'S OBSERVATIONS TO THOSE REPORTED BY
SEARLE ON MALFORMED FETUSES IN THE SKELETAL PREPARATION SERIES

Groups	No. of Fetuses (Observations)*						Total
	Same	Different	Same plus Different	No Defects	Damaged	Exam.	
Control	3	1	1	1		6	7
Low Dose	4	2				6	6
Medium Dose	4	2	2			8	9
High Dose	4	1	1	1		7	9
L-Phenyl	5	2	1	4	1	13	14
L-Aspar	10		3			13	14
Subtotal	30	8	8	6	1	53	59
(%)	30/53 (56.6%)	8/53 (15.1%)	8/53 (15.1%)	6/53 (11.3%)			

*Soft tissue defects reported by Searle were not included in this compilation although in one case it constituted the basis for a difference in malformations detected.