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The Chronic Toxicity of Diphenylamine for Dogs

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When properly used, diphenylamine (DPA) prevents apple scald to a high degree, yet only minute residues of the compound (<8 ppm) remain on and in the treated fruit (Harvey and Clark, 1959; Bache *et al.*, 1962; Padfield and Clark, 1963). Two-year continued feedings of DPA to albino rats by Thomas *et al.* (1967) revealed no evidence of toxicity until the dietary level of 0.1% was reached. The results of similar experiments with dogs are reported below.

METHODS

Eight male and eight female purebred beagles, 8 months old, from four litters were selected for the experiment. Pairs of the same sex, their initial weights as closely as possible the same, were placed in cages, no cage mates being littermates. The animals were quartered in an air-conditioned room for the 737 days of the experiment.

DPA[2] was thoroughly dispersed in the diet by an electric mixer, at concentrations of 0.01, 0.10, and 1.0%. A commercial small kibble dog food, plus a vitamin-mineral supplement, in a 20 : 1 ratio, was the basic mixture during the first 119 days of the study. A 15 : 1 ratio was used during the remaining 618 days. Each dog in a dietary group received 168 g of its basic ration daily during the first period and 195 g daily during the second, the controls receiving like amounts. The feedings were moistened with warm water to make them more palatable, and 52 g of thawed (frozen) ground beef or lamb heart added as additional nutriment. At weekly intervals 10 ml of cod liver oil and a piece of raw oxtail were included. Except during certain studies of excretion, described below, water was available ad libitum. The four dogs on the 1.0% DPA diet rejected minor portions of their daily feedings during the first 6 days of the 2-year experiment. Because of their moisture content the residues were not weighed. A healthy, female control occasionally refused part of her meal. Otherwise, every dog consumed all its daily feeding voraciously.

Blood was drawn from the cephalic vein and oxalated, except that used for the preparation of smears. Hemoglobin was measured as acid hematin in a Klett-Summerson photometer equipped with a constant-voltage transformer. Tests of red cell fragility were conducted according to Daland and Worthely

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[2] Minimum purity by cryoscopy, 99.9%.

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(1935), blood glucose was determined by the Shaffer and Somogyi method (Horowitz, 1965), and liver function by the modified (Seligson *et al.*, 1957) technique of Rosenthal and White (1925). The phenolsulfonephthalein excretion measurement of kidney function (Rountree and Geraghty, 1912) was employed, and urine specific gravities were determined by pycnometer. Urines were tested for albumin by sulfosalicylic acid, and for glucose by glucose oxidase test strips. A saturated solution of $MgSO_4$ was administered intravenously to accomplish euthanasia.

RESULTS

Growth

Figures 1 and 2 show the growth of the dog pairs during the first 400 days of the experiment.[3] Two weeks later an outbreak of distemper occurred in

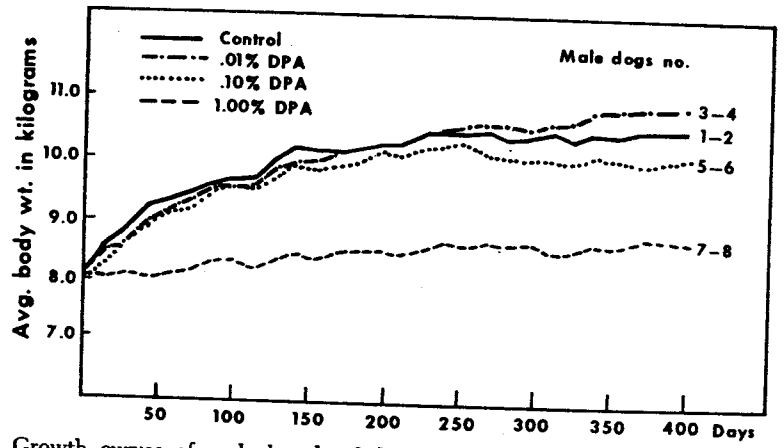

FIG. 1. Growth curves of male beagles fed dietary diphenylamine (DPA) for 400 days. Dogs 8 months old at zero time.

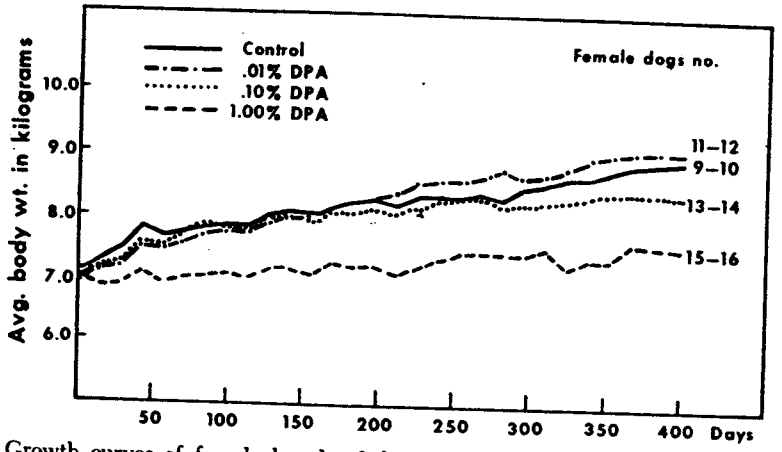

FIG. 2. Growth curves of female beagles fed dietary diphenylamine (DPA) for 400 days. Dogs 8 months old at zero time.

[3] Growth plots for the remainder of the two-year span reveal no additional pertinent information.

our dog colony.[4] Although the 16 animals used in this study had received live-virus vaccine when puppies, one female (No. 14, Fig. 2) died of the disease on day 434 and it was necessary to sacrifice two males (Nos. 3 and 5, Fig. 1) on the following day. (Significantly, these dogs were the three selected from one litter.)

Figures 1 and 2 show growth arrests of both sexes by the 0.1 and 1.0% DPA diets. Application of t tests to these four curves for the day 266 to day 400 period showed $p < 0.001$ at the 1.0% DPA concentration and < 0.005 at the 0.1% level. Since the food intakes were the same for all the dogs, except for the minor refusals noted above, the growth inhibitions can be entirely attributed to DPA. Calculations based upon the average weights of the dog pairs at 400 days show that those fed the 1.0% concentration had, by that time, consumed approximately the following amounts of DPA per kilogram of body weight: the males, 86 g; the females 99 g. As will be evident in the discussion to follow, such intakes, compared with the concentrations which protect apples from scald, are relatively enormous.

The eventual surpassing of each control curve by its respective 0.01% DPA curve can be partly attributed to the fact that, of the 8 dogs of each sex, the one which decidedly gained the most weight in 400 days was in the 0.01% group of that sex. Although the 0.01% curves might suggest a stimulation of the growth of dogs by low intakes of DPA it is more likely that the ultimate greater weights of the dogs fed the 0.01% diet were due to inherent differences of the dogs themselves. In any case, considerably more than two dogs per group would be required for an investigation of the matter. The variation in the weights with age and the caloric requirements of the pure-bred beagles in the colony from which the 16 dogs for the present study were obtained are subjects of a revealing study by Wolf *et al.* (1966).

Hematology

The marked variation of the blood picture of assorted normal dogs has been emphasized by Scarborough (1930–1931) and especially by Afonsky (1955). The timely studies of 2475 blood samples from 500 normal beagles during the first year of life by Andersen and Gee (1958) revealed the causes of some of the fluctuations.

Table 1 records the average hemoglobin concentrations and the red cell counts of the dogs on the days indicated and the averages of each group of four measurements. The anemia of the animals fed the 1.0% DPA diet is obvious. Subjecting the data of the 0.01 and 0.10% groups to t tests is precarious because of the fewer survivors. There do appear to be, however, progressive average decreases in hemoglobin and red cells with increases of DPA consumption. To conclude that a mild degree of anemia was caused by the prolonged ingestion of the 0.1% level is reasonable.

There was no disturbance of leukocyte counts attributable to DPA. A figure of 28,700 per cubic millimeter was obtained on day 225 in a female on the

[4] At this time all dogs in the colony received 10 ml of antiserum.

TABLE 1

HEMOGLOBIN AND ERYTHROCYTES OF DOGS FED DIPHENYLAMINE (DPA)

Days on diet	Males: Number of survivors	Males: Average Hb (g/100 ml)	Males: Average RBC/mm³ × 10⁶	Females: Number of survivors	Females: Average Hb (g/100 ml)	Females: Average RBC/mm³ × 10⁶
			Controls			
224	2	14.09	8.06	2	15.17	8.08
395	2	15.16	7.16	2	15.93	8.10
591	2	15.46	7.33	2	14.39	6.63
724	2	16.53	8.20	2	17.30	8.40
Averages:		15.31	7.68		15.69	7.80
			0.01% DPA			
224	2	14.09	6.95	2	12.89	6.75
395	2	14.62	6.76	2	14.44	7.64
591	1	15.05	7.43	2	16.32	7.91
724	1	16.10	8.44	2	14.9	7.61
Averages:		14.96	7.39		14.65	7.47
			0.10% DPA			
224	2	13.35	7.58	2	12.76	6.94
395	2	13.93	7.08	2	14.16	7.75
591	1	15.18	7.21	1	14.50	7.54
724	1	14.38	6.75	1	14.72	7.75
Averages:		14.21	7.15		14.03	7.49
			1.0% DPA[a]			
224	2	10.08	5.20	2	10.80	5.04
395	2	12.31	5.90	2	12.09	5.87
591	2	11.97	5.93	2	12.25	4.98
724	2	11.85	5.69	2	12.03	5.88
Averages:		11.55	5.68		11.79	5.44

[a] Measurements of hemoglobin and red cells of this group and the controls on day 87 showed both sexes to be anemic.

1.0% diet, but the dog was devoid of gross symptoms. One of 20,450/mm³ was encountered on day 396 in the female in the 0.10% group which died of distemper on day 435. Otherwise, the values during the two years varied between 7200 and 17,950 cells per cubic millimeter, a normal range (Schalm, 1965; Schermer, 1967). The differential percentages also remained within normal limits.

After two years, erythrocyte fragility tests were performed on the 13 remaining dogs. An inspection of Table 2 shows the red cells of the animals fed the 1% DPA diet to have a moderately decreased resistance to hypotonicity.

Sulfobromophthalein (BSP) tests of liver function. The fasting dogs were

TABLE 2

ERTHYROCYTE FRAGILITY TESTS IN BEAGLE DOGS FED DIPHENYLAMINE (DPA)

Days on diet	Dog number	Percent DPA	Percent NaCl solution causing hemolysis			
			Complete hemolysis	Pronounced hemolysis	Definite hemolysis	Minimum hemolysis
			Males			
735	1	Control	0.26	0.34	0.42	0.46
735	2	0.01	0.26	0.32	0.42	0.44
735	4	0.01	0.26	0.32	0.42	0.46
735	6	0.10	0.28	0.34	0.42	0.46
735	7	1.0	0.32	0.32	0.52	0.56
735	8	1.0	0.32	0.36	0.52	0.56
			Females			
736	9	Control	0.26	0.32	0.42	0.46
736	10	Control	0.26	0.34	0.42	0.46
736	11	0.01	0.28	0.34	0.42	0.46
737	12	0.01	0.26	0.32	0.42	0.46
737	13	0.10	0.28	0.32	0.44	0.38
737	15	1.0	0.32	0.36	0.52	0.56
737	16	1.0	0.32	0.36	0.52	0.56

weighed and tranquilized with intramuscular doses (5 mg/lb) of a 5% aqueous solution of promazine hydrochloride. Thirty minutes later, BSP, at 5 mg/kg body weight, was administered intravenously. After 45 minutes, 10 ml volumes of blood were taken and the percentage of dye retention in duplicate samples of serum was determined, the agreement being excellent. Table 3 summarizes the results. In general, the degree of dye retention in-

TABLE 3

SULFOBROMOPHTHALEIN TESTS OF LIVER FUNCTION OF BEAGLE DOGS FED DIPHENYLAMINE (DPA)

Days on diet	Dog number	Sex	Percent DPA	BSP retention (%)	
					Average
626	1	M	Control	3.03	3.27
627	2	M	Control	3.52	
618	4	M	0.01	3.78	—
619	6	M	0.10	4.03	—
626	7	M	1.0	4.04	4.17
618	8	M	1.0	4.30	
624	9	F	Control	2.52	2.65
624	10	F	Control	2.78	
619	11	F	0.01	4.03	3.90
619	12	F	0.01	3.78	
624	13	F	0.10	1.01	—
627	15	F	1.0	5.50	4.90
627	16	F	1.0	4.30	

creased with the dose of DPA, with the noteworthy exception of the 1.0% retention by dog No. 13, fed the 0.10% diet for 624 days. Regression coefficients for both the male and female series were not significant. However, since lesions and increased liver weights were found only in the four dogs fed the 1.0% DPA level (below), it is logical to conclude that the test did detect some liver damage. The control percentages in Table 3, are somewhat higher than those for normal dogs reported by others employing a 45-minute collection period. Moses *et al.* (1948) and Larson and Morrill (1960), using dogs whose breeds and ages are not specifically correlated with the individual results, show the retention of 5 mg/kg doses of BSP at 45 minutes to be, respectively, <1.0% and <1.0 to 1.5%. Cornelius (see Cornelius and Kaneko, 1963), who used the same dose, presents the retention curves of six normal dogs, the final blood samples being taken between 20 and 30 minutes. Extrapolation of the curves to 45 minutes gives percentages roughly between 1.5 and 2.5%. Cornelius states: "Differences in methods have caused a wide variation of dye retention values for normal dogs."

Blood Glucose

The averages of duplicate determinations of the blood sugar concentrations of fasting dogs, on the days indicated, are listed in Table 4. The values all lie within normal limits (Spector, 1956; Cornelius and Kaneko, 1963).

TABLE 4

BLOOD GLUCOSE OF DOGS FED DIPHENYLAMINE (DPA)

	Males			Females		
Percent DPA	Dog number	Days on diet	Blood glucose[a] (mg %)	Dog number	Days on diet	Blood glucose[a] (mg %)
Control	1	730	80	9	731	70
Control	2	730	76	10	731	64
0.01	4	730	70	11	731	70
0.01	—	—	—	12	731	82
0.10	6	730	68	13	731	84
1.00	7	730	78	15	731	100
1.0	8	730	76	16	731	96

[a] Averages of duplicate determinations. Numbers 3, 5, and 14 were distemper casualties. Mg% = milligrams per 100 ml of blood.

Kidney Function Tests

In the phenolsulfonephthalein (PSP) test of kidney function, elimination of the dye is independent of urine volume, provided there is an adequate fluid intake. The latter was assured by giving each (fasting) dog 600 ml of warm consommé soup 1 hour after it had received an intramuscular injection of promazine hydrochloride (5 mg/lb in volumes of 2.0–2.5 ml). The soup was always completely consumed by each animal. A half-hour later, the tranquilized dog was anesthetized by an intravenous dose of sodium pentobarbi-

tal, 20–26 mg/kg (stock: 64 mg/ml), as required for moderate anesthesia.

Catheterization was performed with aseptic technique, the urine was saved for albumin and glucose tests, and, in the case of the males, the retention catheter was clamped off. Exactly 1.0 ml of an aqueous PSP solution (6 mg) was injected intramuscularly, and the time was recorded. One hour and 10 minutes later the bladder was emptied and the specimen saved. (This was the 1-hour sample, since it takes about 10 minutes for PSP excretion to begin.) After an additional hour, the bladder was again emptied, the urine saved, and the catheter withdrawn. For the females, a speculum and a metal catheter were used, the dog being placed on her abdomen. The catheter was inserted with the aid of a head lamp and withdrawn after each specimen was obtained. Otherwise, the procedure was the same as for the males. The anesthetic did not reduce 2-hour urine excretions below normal values.

After the dilution of each sample to 1 liter, and the development of full color by sodium hydroxide addition, the solutions were filtered through paper and the dye present was measured, in duplicate, photometrically against a standard PSP solution containing 6 mg of the compound per liter. The results are listed in Table 5.

None of the above values indicate a repression of kidney function, nor, as we shall see in the pathology section below, were any morphological changes found in the kidneys of these dogs at autopsy other than a mild increase in kidney weight and some hemosiderosis. The liver, however, normally retains some of the dye, as well as excreting a portion, during a 2-hour test. In the four control animals these functions accounted for about 24% of the dye ad-

TABLE 5

PHENOLSULFONEPHTHALEIN TESTS OF KIDNEY FUNCTION OF BEAGLE DOGS FED DIPHENYLAMINE (DPA)

Dog number	Percent DPA	Days on diet	Percent excretion of 6 mg PSP			
			1st hour	2nd hour	Total (2 hours)	
			Males			
						Average
1	Control	647	53.0	20.9	73.9	75.8
2	Control	651	48.7	29.1	77.8	
4	0.01	651	71.7	14.4	86.1	—
6	0.10	676	40.8	24.2	65.0	—
7	1.0	687	43.5	35.2	78.7	86.0
8	1.0	652	75.0	18.4	93.4	
			Females			
9	Control	628	48.7	28.1	76.8	77.0
10	Control	625	48.5	28.8	77.3	
11	0.01	607	48.3	33.4	81.7	74.2
12	0.01	607	55.3	11.4	66.7	
13	0.10	613	59.3	30.3	89.6	—
15	1.0	612	57.2	13.8	71.0	73.8
16	1.0	612	55.8	20.8	76.6	

ministered. Thus it is possible that the 93% excretion rate of male No. 8 (1% DPA) is indicative of some *liver* damage. At autopsy (day 736) this dog's liver showed marked peripherolobular fatty change, but no more than that of its cage mate, No. 7, which excreted 79% of the dye. The fat in the liver of female No. 13 (0.1% DPA) was not in excess of normal at autopsy (day 737), although her excretion rate was virtually 90%. Evidence of a mild hepatitis was present, however. This was the dog whose BSP retention (Table 3) was but 1.0%. No pathological basis was found to indicate that the 86% excretion rate of male No. 4 (0.01% DPA) was abnormal. The average excretion, during the first hour, of 11 healthy dogs between 9 months and 5 years of age was found by Gleiser (1941) to be 69.1%.

The urine specimens obtained by catheter prior to the PSP tests (see above) were tested for glucose with glucose oxidase test tapes and for albumin with 3% sulfosalicylic acid. All tests were negative.

Urine Volumes and Specific Gravities

For this study the dogs were placed in individual urine-collecting cages and deprived of water overnight. At about 8:30 AM each was given 500 ml of warm consommé soup containing a few pieces of raw heart muscle. Following the prompt consumption of this mixture, each was fed its regular daily diet to which 150 ml of water was added, making the total fluid intake for the ensuing 24 hours, 650 ml. Urine was collected at 3 PM that day and at 9 the following morning, filtered through paper, and measured in a graduated cylinder; the specific gravities were measured with a pycnometer.

In Table 6 data concerning the specimens collected from the two male controls and the two males receiving the 1.0% DPA diet are presented. All urines were collected on the same eight 24-hour periods (each divided as explained above) between days 692 and 732. In the table the mean values of the data for each dog are shown: milliliters of urine per kilogram and specific gravities, for each of the two time periods of the 8 collection days.

TABLE 6

VOLUMES AND SPECIFIC GRAVITIES OF URINE OF MALE DOGS FED DIPHENYLAMINE (DPA)[a]

Dog number	Urine (ml/kg body weight)		Specific gravity	
	9 AM–3 PM	3 PM–9 AM	9 AM–3 PM	3 PM–9 AM
		Controls		
1	34.58	13.68	1.011	1.032
2	29.62	13.73	1.010	1.032
Average:	32.10	13.70	1.010	1.032
		1.0% DPA		
7	27.68	13.50	1.011	1.039
8	31.85	13.23	1.012	1.038
Average:	29.76	13.36	1.011	1.038

[a] Eight 24-hour collections were made per dog between day 692 and day 732.

Thus each average recorded for the pairs of dogs was obtained from 16 measurements.

Subjection of the urine volume data (Table 6) to *t* tests showed no significant differences in excretion by the two groups. Besides, all the specific gravities, the averages shown as well as the 64 individual measurements, were well within the normal limits for dogs: 1.001–1.060 [Dukes, 1955; Bloom, 1960; Bentinck-Smith (see Cornelius and Kaneko, 1963)]. Moreover, although the experimental animals had each consumed very close to 1.4 kg of DPA in almost two years, their kidneys had lost none of their ability to concentrate urine during the night. A similar experiment with the two control females and the two fed 1.0% DPA, with four urine collections between day 677 and day 734, produced similar results.

Pathology

After two years, all dogs, regardless of DPA consumptions, were in general good health, high spirited, and active. At autopsy, they were found to be free of parasites and intercurrent disease. No neoplasms were encountered. All lesions discovered were in the two males and two females fed the 1.0% DPA diet. These consisted, in order of severity, of (1) a peripherolobular fatty change in the liver accompanied by a marked increase in liver weight and in ether-extractable lipids, (2) mild hemosiderosis of the spleen, kidneys, and bone marrow, and (3) a mild increase in kidney weight without morphologic change other than hemosiderosis. In addition to the marked fatty hepatic change noted, there were slight amounts of intracellular bilirubin. Lesser degrees of hemosiderosis occurred in other control and treated dogs. The process was not severe enough to be manifest by obvious cellular changes in the bone marrow. The "breakpoint" in the response of the dog to DPA thus appears to lie between 0.1% and 1.0% of the diet. At autopsy an increase in both splenic and heart size was suspected, but weight comparisons were inconclusive.

DISCUSSION

A brief comparison of the effects of 2-year continued feedings of DPA to dogs and rats (Thomas *et al.*, 1967) is in order. The growth of each species was first arrested at the 0.1% concentration. The inhibitions at 1.0% were pronounced, although the effect on rats at this level was partly due to lesser food intakes. Anemia occurred in both species, the degree at comparable periods of 1% DPA consumption being considerably less in rats. No disturbances in leukocyte counts or in differential percentages were encountered, nor did DPA cause proteinuria or glycosuria in either animal. Lesions caused by DPA in rats were limited to the urinary tract, there being interstitial nephritis and a cystic dilatation of renal tubules containing proteinaceous fluid or blood-derived pigment. The latter sometimes accumulated in the renal pelvis or bladder, accompanied by hyperplasia or metaplasia. The threshold for the above abnormalities was in the region of the 0.1% dietary level of DPA. In contrast, we have seen that all the lesions discovered in dogs were in those fed the 1.0% diet, the greatest being a pronounced fatty

change in the liver, hemosiderosis in the spleen, bone marrow, and kidneys and a mild increase in kidney weight.

Aside from the likelihood that innate differences of the two species were responsible for those results above which are in contrast, it is well to remember that, at 2 years of age, the rat has begun to show senile changes, whereas the dog is in its prime for a much longer period.

But, in any case, the amounts of DPA fed to the two species in the experiments described, though normal for their purpose, are astronomical when compared to the DPA residues of entire apples well-protected from scald. For example, concerning their determinations of the DPA remaining in the peelings and pulp of apples so protected, Harvey and Clark (1959) state: "The results here show that when apples were treated by a wet process, the immediate residues were as high as 7 to 8 ppm, but that after storage periods of 3 to 7 months the residues were not greater than 3 ppm. A statistical treatment of the results after storage indicates the chances of obtaining more than 3 ppm by any of the methods used are remote."

No evidence of toxicity to dogs or rats (Thomas *et al.*, 1967) was encountered during continued feedings of 0.01% DPA diets for two years. By that time the two female dogs fed that diet averaged 9.09 kg in weight, each having consumed 14 g of the compound (1.54 g/kg). If a human, then weighing 60 kg, could, after two years, have achieved the same intake of DPA by eating apples having a residue of 3 ppm, he would have been obliged to consume about 92 lb of the fruit every day during that period. This preposterous figure plus the assumption that the effects of the chemical on man are reasonably similar to those on dogs and rats attest to the very wide margin of safety to the public when it consumes apples protected from scald by the proper use of DPA of the high degree of purity employed in the above studies.

SUMMARY

Diphenylamine (DPA), mixed with an adequate diet in concentrations of 0.01, 0.10, and 1.0%, was fed daily to male and female beagles for two years. Growth of both sexes was arrested by the 0.1 and 1.0% diets, the total two-year DPA intakes being, respectively, 0.14 and approximately 1.4 kg per animal. A pronounced anemia developed in the dogs on the 1.0% diet, one of mild degree appearing at the 0.1% level. Leukocyte numbers and differential percentages remained normal. After two years the erythrocytes of the dogs on the 1.0% diet showed a moderately decreased resistance to hypotonicity. Sulfobromophthalein tests of liver function from day 618 to day 627 indicated a moderate degree of liver damage in the dogs fed the 1.0% diet. Blood glucose concentrations were all within normal limits. Phenolsulfonephthalein tests of kidney function gave normal values, and the urines of the animals were negative for albumin and glucose. Twenty-four-hour urine collections between days 692 and 732 from individual dogs on controlled water intakes showed no significant difference between the excretion rates of the controls and those on the 1.0% DPA diet. All the urine specific gravities were within normal limits. All lesions discovered were in the dogs fed the 1.0% diet, these being a peripherolobular fatty change in the liver with a marked increase in liver weight and ether-extractable lipids; a mild hemosiderosis of the spleen, kidneys, and bone marrow; and a slight increase in kidney weight. If it be assumed that high-purity DPA, like that used in the foregoing experiments, has a toxicity threshold for man similar to that for dogs, any health hazard to the consumer of apples protected from scald by the proper use of such DPA should be extremely remote.

ACKNOWLEDGMENT

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Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

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