peanuts; and F. G. Dollear who suggested glc analysis of volatile mold metabolites.

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Biological Effects of Sucrose Acetate Isobutyrate in Rodents and Dogs

Walter J. Krasavage,* George D. DiVincenzo, Bernard D. Astill, Robert L. Roudabush, and Clarence J. Terhaar

Sucrose acetate isobutyrate (SAIB), a suspending agent for essential oils in soft drinks, was studied in rodents and dogs. Oral and ip LD_{50}'s in rats and mice were in excess of 25 g/kg. SAIB produced no skin irritation or contact dermatitis in guinea pigs. Rats fed 5.0% for 95 days had slightly reduced body weights (males) and increased liver weights (females). No micropathology was associated with these changes nor were they reproducible. Subacute feeding to dogs increased liver weights and elevated serum alkaline phosphatase activities at levels of 0.6, 2.0, and 5.0%. Appropriate studies indicated that this increased SAP activity was liver derived. Indocyanine green clearance was unaffected in the rat, but was prolonged in the dog. Liver microsomes were unaffected in both the rat and the dog. It is concluded that SAIB is harmless to rats and causes only adaptive and functional changes in dogs which are reversible when the compound is withdrawn from the diet.

Sucrose acetate isobutyrate (SAIB) is a mixture of sucrose esters, about 95% esterified, having 2 mol of acetate and 6 mol of isobutyrate/mole of sucrose. It is a colorless, sticky, tasteless, highly viscous liquid with a molecular weight of 830–860 (Touey and Davis, 1960).

SAIB is currently being used as a suspending agent for certain oils in soft drinks and is a potential component of food packaging materials. Canadian soft drink manufacturers use 50 ppm of SAIB in combination with 15 ppm of brominated vegetable oil.

The composition of SAIB, an ester of sucrose with fatty acids of wide normal occurrence, suggests that it is an essentially innocuous substance when taken orally. In light of its possible extended use in soft drinks, the safety of SAIB was evaluated using acute toxicity studies in rats, mice, and guinea pigs and subacute feeding studies in rats and dogs, including studies of liver microsomal enzymes and indocyanine green clearance rates.

MATERIALS AND METHODS

Acute Toxicity Studies. A 50% solution of SAIB in corn oil was administered orally or intraperitoneally to determine these LD_{50}'s in rats and mice.

Five to 20 ml of a 20% solution of SAIB in acetone and corn oil (9:1) was held in contact with the depilated skin of guinea pigs by means of a gauze pad and rubber cuff for 24 hr to determine skin irritation.

Delayed sensitivity was tested using SAIB in acetone, dioxane, and guinea pig fat (7:2:1). Ten drops of this solution were applied to the depilated rump area of guinea pigs. Twenty-four and 48 hr later these areas were observed for primary irritation. After three applications in the next 5 days and a 3-week rest period, challenging doses were applied, to the right, and a week later, to the left shoulder area. Reactions were scored on a scale of 0 to 4 for erythema and edema.

Health and Safety Laboratory, Eastman Kodak Company, Rochester, New York 14650.

Subacute Toxicity Studies. Two subacute feeding studies were done in rats (no. I and II) and three subacute feeding studies were done in beagle dogs (no. I, II, and III). Dog Study I was carried out by the Food and Drug Research Laboratories (FDRL), Inc., Maspeth, N. Y. The other two dog studies and the rat studies were done in this laboratory. Another study (Rat Study III) was undertaken to determine indocyanine green plasma clearance rates at various intervals up to 36 days in male rats fed 4.0% SAIB in the diet.

RAT STUDIES

Rat Study I. Groups of 50 Sprague–Dawley (Holtzman) rats (25 males and 25 females) randomly assigned to two treatment and one control group were housed, five of each sex per cage, and fed 1.0, 5.0, or 0.0% SAIB in the diet for 95 days. Water was available *ad libitum*. SAIB was solubilized in acetone and incorporated into a basal diet of ground Purina Lab Chow. The mixtures were spread on shallow pans to allow the acetone to evaporate. A control diet, to which only acetone was added, was handled similarly.

Individual body weights and group feed consumption were recorded weekly, and hemoglobins, hematocrits, and white cell and differential counts were done on five animals from each group 3 days prior to feeding and on the 24th, 52nd, and 87th day of feeding.

On day 95, all animals were autopsied. Individual liver and kidney weights were recorded and tissue samples were processed for microscopic examination.

Rat Study II. Three experiments were run concurrently to elucidate and complement data obtained in Rat Study I. Two-hundred-and-eighty (140 males and 140 females) albino rats (Sprague–Dawley, Carworth Farms, Inc.) were randomly divided into 14 treatment groups of each sex with ten animals per group. Five of each sex were housed in wire-bottom cages and fed SAIB at concentrations of 1.0, 2.0, or 4.0% (w/w) in ground Purina Laboratory Chow supplemented with 5.0% Mazola Corn Oil (w/w). The groups of ten rats per sex were fed their respective experi-

Table I. SAIB (Rat Study II). Liver Weights and Mean Microsomal Enzyme Activity[a] of Rats Fed SAIB

Dose, %	Days on diet	Body wt, g	Liver		G-6-PTase[b]	*p*-NDase[c]	B-G-Tase[d]
			Wt, g	Body wt, %			
			Males				
4.0	28	240	7.79	3.25	0.83	235	50.5
		(206–267)[e]	(6.50–8.71)	(3.09–3.48)	(0.70–1.02)	(200–260)	(40.2–55.8)
0.0	28	247	7.81	3.15	1.03	266	44.8
		(230–269)	(6.75–8.90)	(2.94–3.35)	(0.80–1.26)	(177–344)	(24.2–61.5)
4.0	56	311	8.76	2.81	1.23	289	30.5
		(266–366)	(6.45–11.01)	(2.43–3.06)	(0.68–1.52)	(195–367)	(22.7–34.0)
2.0	56	289	8.10	2.79	N.D.[f]	N.D.	N.D.
		(242–339)	(5.26–9.90)	(2.43–3.11)			
4.0 + 0.0	28 + 28	328	9.05	2.76	N.D.	N.D.	N.D.
		(278–353)	(7.24–10.06)	(2.54–2.88)			
0.0	56	316	8.51	2.69	1.40	299	31.0
		(245–361)	(5.84–10.5)	(2.35–2.97)	(0.54–2.66)	(233–377)	(24.7–38.5)
			Females				
4.0	28	166	5.01	3.02	0.61	165	55.0
		(157–175)[e]	(4.51–5.46)	(2.85–3.27)	(0.41–0.82)	(117–202)	(48.7–62.8)
0.0	28	171	5.03	2.93	0.93	157	45.7
		(154–186)	(4.36–5.73)	(2.72–3.14)	(0.46–1.58)	(127–200)	(35.0–59.3)
4.0	56	211	5.58	2.65	0.66	282	46.1
		(190–229)	(5.0–6.26)	(2.41–2.80)	(0.41–1.10)	(200–326)	(33.0–56.3)
2.0	56	208	5.46	2.62	0.67	228	N.D.[f]
		(190–241)	(4.66–6.38)	(2.45–2.77)	(0.55–0.76)	(210–277)	
4.0 + 0.0	28 + 28	200	5.14	2.57	0.59	251	N.D.
		(189–215)	(4.71–5.76)	(2.42–2.71)	(0.47–0.73)	(177–317)	
0.0	56	213	5.70	2.68	0.77	196	50.6
		(190–227)	(5.04–6.30)	(2.52–2.86)	(0.59–0.93)	(150–227)	(29.3–69.1)

[a] Values are duplicate determinations of pooled pairs from ten rats per group. [b] μMPi × 15 min^{-1} × mg $protein^{-1}$. [c] $\Delta OD \times 10^{-5} \times min^{-1}$ × mg $protein^{-1}$. [d] $\Delta OD \times 10^{-4} \times min^{-1}$ × mg $protein^{-1}$. [e] Numbers in parentheses are extremes. [f] No data.

mental diets for 28 or 56 days continuously or for 28 days followed or preceded by 28 days on a control diet. Two groups of each sex were fed the control diet containing corn oil only for 28 or 56 days continuously. Individual body weights and group feed consumption were recorded at weekly intervals. At the termination of their respective feeding periods, all animals were sacrificed by decapitation. Blood was collected for serum alkaline phosphatase (SAP), ornithine carbamyl transferase (OCT), blood urea nitrogen (BUN), triglyceride, cholesterol, and glucose determinations. Animals were autopsied and examined for gross pathology, livers were weighed for organ weight comparisons, and a small sample of each was fixed for microscopic examination. The remainder of the liver was processed for microsomal enzyme assay.

Rat Study III. Groups of male rats (Sprague–Dawley) were fed an experimental diet of ground Purina Laboratory Chow containing 4.0% SAIB and 5.0% Mazola Corn Oil or a control diet of ground chow plus 5.0% corn oil only. Indocyanine green (ICG) clearance rates were determined on two rats from each group on days 1, 3, 5, 8, 10, 22, 26, and 36 after feeding began. The carotid artery was cannulated or, more usually, the exterior jugular veins were exposed with the animal under pentobarbital anesthesia. ICG was injected iv at dosages of 4 or 6 mg/kg. Heparinized blood samples were collected before and at 3, 6, and 9 min after injection. After centrifugation, aliquots of the plasma were processed and the absorbance of the standard, blank, and postinjection samples was read on a Beckman DU spectrophotometer at 805 nm. The values, as mg % of dye, were plotted against time on two cycle semilog graph paper and a visually fitted straight line was extrapolated to zero time. The value at zero time was reduced by half; this point was found on the fitted line and the corresponding time was read as the plasma clearance half time ($T\frac{1}{2}$).

DOG FEEDING STUDIES

Dog Study I (FDRL, Inc.) consisted of 36 purebred beagles (18 males and 18 females) divided into three treatment groups of four dogs of each sex and a fourth group (controls) of six dogs of each sex.

SAIB, at concentrations of 0.20, 0.60, and 2.00%, in 6.0% cottonseed oil was incorporated into a basal diet of New Purina Dog Meal. These diets and the control diet were fed to the appropriate groups for 12 weeks. The control diet contained 6.0% cottonseed oil and no SAIB.

Hemoglobins, hematocrits, white cell and differential counts, blood glucose, lactic dehydrogenase (LDH), BUN, SAP, and complete urinalyses were done on all animals twice prior to and at the end of the 12-week feeding period. Neurological reflexes, food intake, and body weights were recorded weekly. At termination, all dogs were sacrificed by exsanguination while under pentobarbital anesthesia and autopsied. Samples of all tissues were fixed in formalin. All tissues from the high dose (2.0%) and control group and only the liver and kidneys from the dogs in the 0.2 and 0.6 groups were processed and examined for histological changes. The livers, kidneys, spleen, gonads, adrenals, pituitaries, and brains of all animals were weighed for organ weight comparisons.

Dog Study II was designed to ascertain if recorded effects of SAIB on serum alkaline phosphatase and relative liver weights were reversible. Six male beagle dogs approximately 6 years old were acclimated to a diet of ground Purina Laboratory Chow containing 5.0% (w/w) corn oil for 3 weeks. All dogs were then fed an experimental diet which contained 5.0% SAIB for 28 days. The control diet was fed for the next 57 days and on day 86 four of the dogs were returned to the SAIB diet and ICG plasma clearance rates and SAP determinations were done 24 and 48 hr later. The study was terminated 3 days later.

In Dog Study III, ten purebred male beagles 11 to 13

Table II. Mean ICG Plasma Clearance Times ($T_{1/2}$ min) in Rats and Dogs Fed SAIB

Days on experiment	Rats[a] Mean accumulated dose, mg/kg	Rats[a] Treated	Rats[a] Controls	Dog study II[b] Mean accumulated dose mg/kg	Dog study II[b] Treated	Dog study III[c] Mean accumulated dose, mg/kg	Dog study III[c] Treated	Dog study III[c] Controls
1	2497	3.1 (3.0–3.2)[d]	2.8 (2.8–2.7)					
3	7758	2.9 (2.8–3.0)	2.6 (2.4–2.7)					
5	14,181	2.9 (2.5–3.2)	2.8 (2.7–2.8)					
8	18,852	3.2 (3.1–3.3)	2.7 (2.5–2.8)					
10	24,025	4.2 (3.8–4.6)	3.1 (3.1)					
22	57,400	3.2 (2.9–3.5)	4.0 (3.6–4.2)	16,511	27.0 (17.0–40.0)	28,758	21.5 (21.0–22.0)	7.0 (6.0–8.0)
26	88,128	2.7 (2.5–2.8)	3.1 (3.0–3.1)					
28				Dogs returned to control diet				
36	128,812	3.6 (3.5–3.7)	2.8 (2.5–3.0)					
42				0	9.0 (8.0–9.5)	52,795	35.5 (34.5–36.0)	12.5 (7.0–16.0)
63				0	6.5 (6.0–8.0)	83,869	24.5 (21.0–26.0)	6.0 (5.5–7.0)
85				0	6.5 (4.5–9.5)	112,357	29.0 (20.0–40.0)	10.5 (9.0–13.0)
86				Dogs returned to SAIB diet (5.0%)				
87				1250	18.0 (12.5–24.5)			

[a] Values are means of two rats per group per time period except for Day 22, which values are means of three rats per group. [b] Values are means of four dogs. [c] Values are means of three dogs per group. [d] Numbers in parentheses are extremes.

months of age and weighing an average of 11 kg were randomly divided into two groups of five dogs each. One group was fed 5.0% SAIB plus 5% corn oil in ground Purina Laboratory Chow and the other group was fed a control diet. These diets were fed in 250-g quantities per day for 91 days. Three weeks after Study III began, a treated animal was removed from the experiment because of progressive deterioration in its condition, diagnosed at autopsy as a preexistent leukemia. No data from this animal are reported here.

In Dog Studies II and III, hematocrits, hemoglobins, white cell and differential counts, SGOT, blood glucose, BUN, serum protein, SAP, and OCT determinations were done at least twice prior to and at weekly intervals throughout the feeding period. Triglyceride and cholesterol determinations were done weekly in Dog Study II and at termination in Dog Study III. Indocyanine green plasma clearance rates were determined at 3-week intervals. Physical appearance, behavior, food consumption, and body weight were recorded daily throughout both Dog Studies II and III. Serum bilirubin determinations were done after 7 weeks of feeding in Dog Study III only.

In both Studies II and III, all dogs were sacrificed with CO_2; livers and kidneys were weighed and tissues from all organ systems were processed for microscopic examination. Liver tissues in Dog Study III were specially stained for glycogen content, samples were processed for microsomal enzyme assay, and liver, kidney, bone, bile, and scrapings of the intestinal mucosa were analyzed for tissue alkaline phosphatase activity.

Microsomal Enzyme Studies. Liver microsomal assays were done on livers pooled in pairs from Rat Study II and on livers from all dogs in Dog Study III. Total liver protein, glycogen, and phosopholipid concentrations were also measured.

The microsomal enzymes studied were *p*-nitroanisole demethylase (*p*-NDase), glucose-6-phosphatase (G-6PTase), and bilirubin-β-D-glucuronyl transferase (B-G-Tase). The specific activities of these enzymes were determined according to methods described elsewhere by Krasavage *et al.* (1972).

Alkaline Phosphatase Studies. Alkaline phosphatase activity of serum or tissue extracts was assayed by the automated procedure of Morgenstern *et al.* (1965).

Polyacrylamide gels were prepared according to the method of Clarke (1964). The gels containing alkaline phosphatase activity were incubated at 37° for 120 min in culture tubes containing 0.3% (w/v) α-naphthyl phosphate dissolved in 0.05 *M* carbonate–bicarbonate buffer at pH 9.8. Alkaline phosphatase liberates α-naphthol, which is subsequently complexed with a buffered solution of 0.1% (w/v) fast blue RR. Brown bands appear in the areas of AP activity.

Tissue extracts were prepared from thawed samples by a procedure modified from the methods of Morton (1954) and Nagode *et al.* (1969). Two grams of each tissue, with the exception of bone, were homogenized in 9 vol of glass-distilled water. 1-Butanol was added to the homogenate so that the final solution contained 20% (v/v) 1-butanol. This mixture was stirred for 30 min at room temperature and centrifuged at 2000 × *g* for 1 hr. The clear supernatant was removed and the residue was reextracted. The supernatants were pooled, adjusted to volume, and stored at 0–2°.

Seventy-five to 100 g of bone (from the region encompassing the distal epiphysis of the femur and the proximal

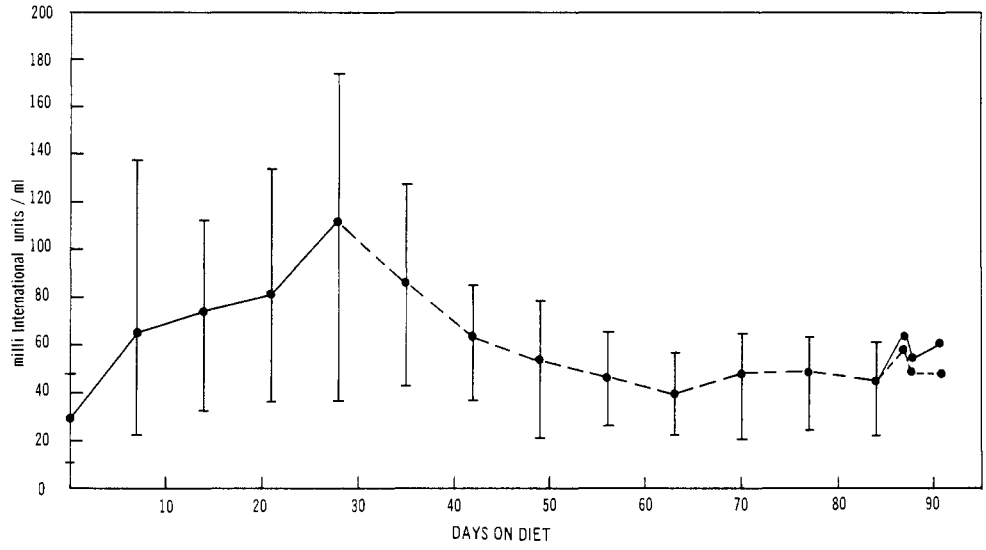

Figure 1. Dog Study II, mean serum alkaline phosphatase of dogs fed 5.0% SAIB in the diet. ⧳, mean and extremes of six values; ●– – –●, control; and ●——●, 5.0% SAIB.

epiphysis of the tibia) were ground in a Waring blender and the fragments were stirred for 1 hr with approximately 2 vol of 20% (v/v) aqueous 1-butanol. The extract was centrifuged and the supernatant was collected as described above.

1-Butanol extracts were analyzed for AP activity and subjected to polyacrylamide gel electrophoresis for isoenzymic characterization (Nagode *et al.*, 1969). When serum or tissue extracts are subjected to the proper conditions, a characteristic proportion of isoenzymic inactivation results for each organ specific isoenzyme. AP isoenzymic inactivation was induced by the following conditions.

Heat Inactivation. Sera and tissue extracts were incubated at 56° for 15 min, immediately cooled, and subsequently analyzed for alkaline phosphatase activity.

Urea Inactivation. Equal volumes of 6 *M* urea were added to sera and tissue extracts (physiological saline replaced urea for controls), which were then incubated at 37° for 10 min and immediately analyzed for AP activity.

L-*Phenylalanine Inhibition.* D- and L-phenylalanine were prepared, 25 m*M*, in buffered *p*-nitrophenylphosphate solution, and inhibition of AP activity due to the L form was compared to the activity of the D form.

RESULTS

Acute Toxicity Studies. Oral doses of SAIB as high as 25.6 g/kg caused no mortality in mice and killed only 1/7 of the rats. When this dose was administered ip, 2/7 of each species died. Initial symptoms were weakness, diarrhea, rough coat, and food refusal, after which all survivors appeared normal, ate, and gained weight. The animals that succumbed died 3–4 days after dosing. SAIB held in contact with the depilated skin of guinea pigs caused only a slight transient irritation. In a routine skin sensitization test, guinea pigs failed to show any increased reactivity to SAIB.

Subacute Toxicity Tests. *Rats.* In Rat Study I, the only differences between treated animals and controls were at the high dose level. The males fed 5.0% SAIB for 95 days had a lower mean body weight than the controls (420 *vs.* 437 g) at the end of the feeding period. Also, a very slight increase was seen in the relative and absolute liver weights of the females fed 5.0% SAIB (3.52 *vs.* 3.35% and 8.7 *vs.* 8.1 g). Neither of these changes was associated with any micropathology or other untoward effects.

In Rat Study II, SAIB fed at concentrations of 1.0, 2.0,

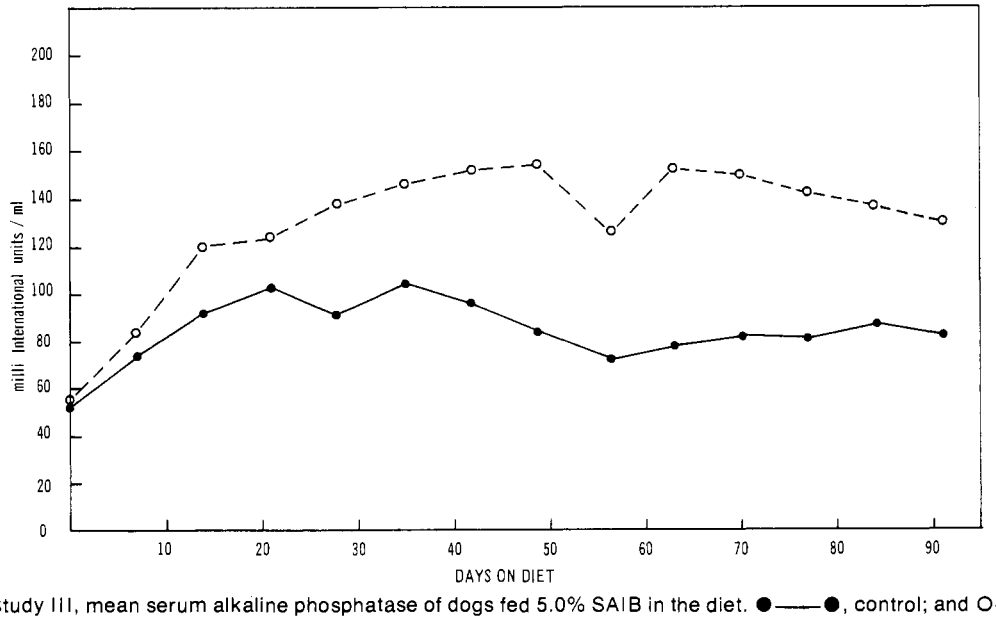

Figure 2. Dog Study III, mean serum alkaline phosphatase of dogs fed 5.0% SAIB in the diet. ●——●, control; and O– – –O, 5.0% SAIB.

Table III. SAIB (Dog Study I) (FDRL). Liver Weight Comparisons and Serum Alkaline Phosphatase in Dogs

Dose group	Body wt, kg	Liver		Mean SAP (units)[c]	
		Wt, g	%	Pre-exp	12 weeks
		Males			
0.2%	9.9	344.5	3.48	1.4	2.0
	(7.2–12.2)[a]	(237.6–418.3)	(2.35–4.81)	(0.8–1.9)	(0.9–2.9)
0.6%	7.8	283.0	3.63[b]	2.1	2.2
	(5.7–10.2)	(196.0–383.3)	(3.15–4.16)	(1.3–3.3)	(1.4–2.5)
2.0%	8.2	327.2	3.99[b]	2.3	5.3[b]
	(7.1–9.2)	(284.3–362.6)	(3.64–4.65)	(1.8–2.8)	(3.6–6.6)
0.0%	10.2	287.6	2.82	1.8	1.3
	(6.1–14.1)	(212.8–365.6)	(2.19–3.62)	(1.3–2.5)	(0.8–1.7)
		Females			
0.2%	8.4	226.0	2.69	1.9	1.5
	(6.9–9.1)	(213.6–229.6)	(2.52–3.10)	(1.7–2.1)	(1.1–1.8)
0.6%	7.8	276.9	3.55[b]	2.5	2.7
	(7.1–8.6)	(235.7–354.3)	(2.94–4.66)	(2.1–3.1)	(2.1–3.6)
2.0%	8.3	306.3	3.69[b]	2.1	4.5[b]
	(7.3–10.3)	(278.8–322.5)	(2.74–4.28)	(1.2–2.9)	(2.4–7.3)
0.0%	8.4	235.2	2.80	2.3	1.8
	(5.3–12.5)	(146.3–366.5)	(2.55–2.97)	(1.6–3.3)	(0.9–2.4)

[a] Numbers in parentheses are extremes. [b] Statistically significant $p \leq 0.05$. [c] Methods of Babson *et al.* (1966).

and 4.0% in the diet for 28 or 56 days continuously or for 28 days followed or preceded by 28 days of a control diet caused no toxicologically significant effects. Body weight gain and feed consumption did not differ between the treated animals and the controls. There was no mortality or morbidity. SAP, OCT, triglyceride, cholesterol, and BUN levels showed no significant changes. Gross pathology at autopsy was negative and liver weights (absolute and relative) were normal.

Results of the liver microsomal enzyme assays done on selected groups of male and female rats in this study, along with liver weights, are presented in Table I. No significant elevation of hepatic microsomal *p*-NDase activity or of B-G-Tase activity was seen. However, there was a slight depression of G-6-PTase activity.

Indocyanine Green Clearance in Rats. ICG plasma clearance rates in rats that had ingested average accumulative doses of about 2500 to 130,000 mg/kg of SAIB were not significantly different from those of the control animals. These results contrast with those in the dog (see below and Table II).

Dog Studies. The only differences from controls for male and female beagles fed 0.2, 0.6, and 2.0% SAIB in their diet for 12 weeks (Dog Study I) were slightly increased SAP levels in the 2.0% animals and increased relative liver weights in the dogs fed 0.6 and 2.0% SAIB (Table III).

The significant results seen in dogs fed 5.0% SAIB for 4 weeks (Dog Study II) are presented in Figure 1 and Table II. These animals had moderate increases in SAP (Figure 1) and a prolongation of ICG plasma clearance by the liver (Table II). Within 5 weeks after the withdrawal of SAIB from the diet, SAP activity had returned to near normal and appeared to level off. The ICG clearance rate was within normal range 2 weeks after the compound was removed from the diet.

After feeding the control diet for 8 weeks, four of the dogs were again fed 5.0% (1250 mg/kg) SAIB and 24 hr later SAP and ICG clearance determinations were done. The ICG clearance rate was significantly prolonged, while the SAP did not appear to be affected.

In Dog Study III, the dogs fed SAIB showed a moderate elevation in SAP (Figure 2), a definite prolongation of indocyanine green clearance (Table II), and statistically significant increases in relative and absolute liver weights (Table IV). Liver glycogen and phospholipid content showed apparent increases, while liver protein was slightly decreased (Table V). The liver microsomal assays showed a slight questionable decrease in G-6-PTase and B-G-Tase activity, with no effect on *p*-NDase activity (Table IV).

Disk electrophoresis, isoenzyme inactivation studies, and analyses of tissue alkaline phosphatase of liver, kidney, bone, scrapings of intestinal mucosa, and bile suggest that the elevation of SAP in Dog Study III was related to the liver isoenzyme.

Electrophoresis revealed two distinct areas of AP activity, identified as liver and bile AP. The color development was more intense in the liver region of the experimental animals than in the controls, suggesting a greater liver isoenzyme activity. This was confirmed when the tissues were analyzed for AP activity. The liver content of AP was increased twofold in the treated animals compared to the controls (Table VI).

Isoenzyme inactivation studies also showed that the ele-

Table IV. SAIB (Dog Study III) (HSL). Liver Weights and Mean Microsomal Enzyme Activity of Dogs Fed SAIB

Dose	Body wt, kg	Liver		Microsomal enzyme activity		
		Wt, g	% Body wt	G-6-PTase[a]	*p*-NDase[b]	B-G-Tase[c]
5% SAIB	9.2	429[e]	4.68[e]	3.29	464	4.4
	(8.6–9.8)[d]	(408–449)	(4.16–5.22)	(2.44–4.59)	(227–577)	(3.3–7.2)
Control	9.9	370	3.77	4.19	450	6.6
	(8.6–11.4)	(337–400)	(3.51–3.92)	(3.34–4.68)	(380–577)	(4.0–8.7)

[a] Specific activity is μMPi × 15 min^{-1} × mg $protein^{-1}$. [b] Specific activity is $\Delta OD \times 10^{-5} \times min^{-1} \times$ mg $protein^{-1}$. [c] Specific activity is $\Delta OD \times 10^{-4} \times min^{-1} \times$ mg $protein^{-1}$. [d] Values in parentheses are extremes. [e] Statistically significant $p \leq 0.05$.

Table V. SAIB (Dog Study III) (HSL). Hepatic Tests in Dogs Fed 5.0% SAIB

Dose	Hepatic protein[a]	Phospho-lipids[b]	Liver glycogen[a]
5% SAIB	286	796	30.73
	(265–321)[c]	(636–913)	(12.0–61.5)
Control	300	516	12.20
	(281–319)	(483–550)	(6.28–18.2)

[a] Milligrams per gram of tissue. [b] Micrograms P per gram of tissue. [c] Values in parentheses are extremes.

Table VI. SAIB (Dog Study III) (HSL). Tissue Alkaline Phosphatase Activity[a] in Dogs Fed 5% SAIB

Tissue	Control	5% SAIB
Liver	5.74	13.70
	(2.31–8.60)[b]	(5.95–24.9)
Bone	3.04	3.53
	(2.4–3.6)	(2.9–4.0)
Kidney cortex	36.1	41.2
	(29.7–43.3)	(34.7–47.3)
Intestinal mucosa	171	176
	(90–209)	(85.5–240)
Bile[c]	408	361
	(157–799)	(61.2–682)

[a] Activity is IU/g of tissue (wet weight). [b] Values in parentheses are extremes. [c] Activity is IU/ml.

vation in SAP activity in the SAIB-fed dogs was of hepatic origin. When the sera were subjected to urea, heat, and L-phenylalanine inhibition, essentially no difference was seen between the treated and control animals.

All other statistics recorded in this study, as in the previous dog studies, were normal.

DISCUSSION

Acute toxicity studies, po and ip, in rats and mice at dose levels up to 25.6 g/kg indicate that SAIB is essentially nontoxic by these routes of administration. It is also not a sensitizer and did not cause irritation when applied to the skin of guinea pigs.

An initial subacute (95-day) feeding study in rats revealed possible body and liver weight changes; however, these results were not reproduced in a study designed to repeat these changes and study their reversibility. This second study showed that SAIB was without adverse effects when fed to both male and female rats. This is in agreement with studies in rats done at higher doses by Procter *et al.* (1972).

In contrast to the rat, dogs fed SAIB (5.0%) in the diet showed significant changes in serum alkaline phosphatase, indocyanine green plasma clearance time by the liver, and absolute and relative liver weights, all of which were increased when compared to the controls. However, the study designed to detect reversibility of effects did show that the prolonged ICG clearance rate and increase in SAP had returned to the normal range within 5 weeks after the compound was withdrawn from the diet. This study also revealed that the increased ICG clearance time occurred within 24 hr after a single feeding of SAIB and that the SAP levels of these dogs were not affected 24 or 48 hr later. None of these changes were associated with any micropathology and other liver function tests, and nonliver-related statistics were normal in these animals. Procter *et al.* (1972) also reported increased SAP and liver weights with delayed clearance of bromosulfophthalein, all of which were reversible when SAIB was withdrawn from the diet.

Our studies also revealed that the elevated SAP seen in the dog was related to the liver isoenzyme. Disk electrophoretic studies detected only two bands of alkaline phosphatase activity in the serum of control and treated dogs. These bands were identical in both groups and identified as liver and bile alkaline phosphatase. The liver band in the sera of the treated dogs was more intensely colored than the control sera, suggesting that the liver alkaline phosphatase component of serum from the experimental dogs possessed greater alkaline phosphatase activity. Isoenzyme inactivation substantiated the disk electrophoretic observations and tissue analysis revealed that the liver content of alkaline phosphatase in the experimental dog was twice that of the control animals.

Liver microsomal enzymes indicative of oxidation (*p*-NDase) and conjugation (B-G-Tase) showed no consistent patterns in either the dog or rat which could be interpreted as evidence of an increased activity. If SAIB were capable of inducing enzyme activity, the dose level and duration of feeding used in this study were judged sufficient to produce induction based on such well-known microsomal enzyme inducers as phenobarbital and zoxazolamine (Conney, 1967). It thus appears unlikely that SAIB will induce liver microsomal drug metabolizing enzymes under use conditions and it should not affect the handling of other foreign compounds by the liver. The slight depression of G-6-PTase seen in both species appears to be independent of the elevated liver weights in the dog, since it also occurred to the same extent in the rats without any increase in liver weight. The elevated liver phospholipid level in the dog can be correlated with the increase in smooth endoplasmic reticulum reported in dogs fed SAIB (Procter *et al.*, 1972); such membrane proliferation is commonly accompanied by increased phospholipid syntheses (Conney, 1967).

The absence of any untoward effects in rats and the low order of effects in the dog is in agreement with our *a priori* suggestion that SAIB is an innocuous substance. Fate and disposition studies with [^{14}C]SAIB show that the rat uses SAIB extensively as a food, with extensive elimination of the labeled $^{14}CO_2$ (Reynolds *et al.*, 1972), whereas the dog absorbs much less of the intake and appears to show a different distribution of metabolic products in the urine. The implications of these differing modes of disposition and elimination are currently being studied. Humans utilize [^{14}C]SAIB extensively as a food, eliminating much of the intake as $^{14}CO_2$, and in this respect show a considerable similarity to rats.

The changes seen in the dogs fed SAIB are considered to be adaptive and functional in nature and appear to have no toxicological significance. These studies and the fate and disposition studies reveal that the dog and rat respond differently when ingesting SAIB, indicating a species difference, a difference also reported by Procter *et al.* (1972). The fate and disposition studies (Reynolds *et al.*, 1972) further indicate that the rat and human utilize and eliminate SAIB in a similar manner and therefore the rat, not the dog, would appear to be the species of choice when evaluating the safety of SAIB for human usage.

The data from the studies reported here and those reported by Procter *et al.* (1972), together with the reported wide human usage of SAIB outside the United States without any apparent incident, indicate that the level of intake arising from the small quantities of SAIB proposed for usage as a suspending agent in soft drinks is not hazardous.

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Effects of Heat *in situ* on Electrophoretic Patterns of Reserve Proteins and Enzymes in Dormant Peanuts (*Arachis hypogaea* L.)

Dempsey L. Thomas[1] and Navin J. Neucere*

The effects of heat on general (soluble) proteins and several enzymes in wet- and dry-roasted peanuts were determined by starch gel electrophoresis. Staining of general proteins was more obvious in extracts of seed wet-roasted at 155° than in extracts of seed dry-roasted at that temperature. No apparent migrational differences were observed for α-arachin with increasing temperatures. New fast moving bands did not appear at higher temperatures, but the migrations of proteins near the origin were slightly different from that in the control. Wet-roasting reduced enzyme activity more than dry-roasting for all enzymes assayed; most enzymes exhibited some activity in peanuts dry-roasted up to 130°, but differential thermostability of isoenzymes was observed. Aqueous extracts of seeds defatted with acetone–hexane showed general protein and enzyme electrophoretic patterns similar to extracts defatted by centrifugation of native seed.

The effects of heat on peanut proteins heated at 145° for 1 hr were investigated by chromatography on DEAE-cellulose, polyacrylamide gel electrophoresis, immunoelectrophoresis, and ultracentrifugation (Neucere *et al.*, 1969). Immunochemical and other analyses indicated that the major reserve globulin, α-arachin, remained antigenic during the heating process but showed a reduction in its solubility and diffusion coefficient in agar. The effects of heating *in situ* at several different temperatures on the general proteins of imbibed and dry peanut seed were studied by disk electrophoresis and immunochemistry (Neucere, 1972). The disk gels showed greater differences in protein migration after dry heat than after wet heat. Immunoelectrophoretic patterns indicated that α-arachin maintained antigenicity in both wet- and dry-heated seeds but other proteins were progressively inactivated antigenically.

Most work with heat sensitivity of isoenzymes has been performed *in vitro* with whole extracts of cells or tissues or with purified isoenzymes. Studies of animal tissues showed considerable variations in thermal stability of lactate dehydrogenase (LDH) isoenzymes (Fondy *et al.*, 1964; Knudsen *et al.*, 1970; Vesell *et al.*, 1968). Crude cell-free extracts from *Bacillus subtilis* heated at 60 and 70° for 30 min showed marked differences in stability of two bands of malate dehydrogenase (MDH) (Antohi *et al.*, 1970). Other studies of maize and HeLa cells showed differential heat sensitivities for acid phosphatase isoenzymes (Efron, 1970; Tan and Aw, 1971).

The aim of this report was to investigate, by starch gel electrophoresis, the general effects of heat *in situ* on the major proteins and several common enzymes in soaked and dry peanut seed.

EXPERIMENTAL SECTION

Preparation of Heated Samples and Controls. The heat treatment was described by Neucere (1972) and is briefly discussed here. One-year-old seeds of *Arachis hypogaea* L. (Virginia 56-R, 1968 crop) were roasted either dry or after full imbibition. One kilogram of dry seed was soaked 16 hr in distilled water at 25°, blotted, and placed for 1 hr in a shallow pan in a forced air temperature-regulated oven preset to designated temperatures (°C). Each batch of seeds was extracted with hexane–acetone (1:1) at a ratio of 1 g of tissue per 3 ml of solvent at 5°. Based on 0.1 g of meal per ml extracted at room temperature in phosphate buffer, pH 7.8, ionic strength 0.2, the following amounts of protein were solubilized from each sample (Table I).

The controls used in this study (samples 11 and 12) were prepared from acetone–hexane defatted seeds that were unheated and from full-fat unheated seeds; the same phosphate buffer was used for extraction. All extracts were frozen and stored at −20° before experimentation.

Preparation of α-Arachin. α-Arachin was partially purified from crude phosphate buffer extracts of peanut cotyledons by cold-precipitation (2°) according to Neucere (1969).

Application of Samples in Gels. To increase protein quantities for each sample, the application paper was dipped twice into the extract with drying between each dipping before inserting the sample into the gel. The dipping-drying procedure did not alter the banding patterns but effectively increased protein concentration; this procedure with α-arachin is discussed later.

Electrophoresis. Procedures for horizontal starch gel electrophoresis were similar to those of Smithies (1955) and Poulik (1957) and were described previously (Thomas and Brown, 1970).

Enzyme Assays. To demonstrate nonspecific α-esterases (α-EST), the starch slices were incubated 2 hr at 25° in 100 ml of 0.2 *M* phosphate buffer, pH 6.0, containing 75 mg of Fast Blue RR salt, 1.5 ml of 1% α-naphthyl acetate in acetone–water 1:1 (v/v), and 10 ml of 1-propanol (absolute). Leucine aminopeptidase (LAP) was detected by incubating the gels 2 hr in 100 ml of 0.2 *M* phosphate buffer, pH 4.4, containing 20 mg of L-leucyl-β-naphthylamide HCl, and 25 mg of Black Salt K. The solution for the malate dehydrogenase (MDH) assay consisted of 100 ml of 0.1 *M* Trisma Base buffer, pH 8.5, 3 ml of neutralized 2 *M* *dl*-malic acid, 50 mg of β-diphosphopyridine nu-

Southern Regional Research Laboratories, Agricultural Research Service, U. S. Department of Agriculture, New Orleans, Louisiana 70179.
[1] Present address: Department of Biological Sciences, Louisiana State University in New Orleans, Lake Front, New Orleans, Louisiana 70122.