Nitrosamines in Baby Bottle Nipples and Pacifiers: Occurrence, Migration, and Effect of Infant Formulas and Fruit Juices on in Vitro Formation of Nitrosamines under Simulated Gastric Conditions

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The results of a recent survey indicated that the levels of various volatile nitrosamines in baby bottle rubber nipples and pacifiers decreased significantly over that observed 1–2 years earlier. Of 42 samples analyzed, 26 were found to be negative and only 7 contained greater than 30 ppb total volatile nitrosamines (mainly, *N*-nitrosodimethylamine and *N*-nitrosodi-*n*-butylamine). Studies with some older nipples and pacifiers indicated that significant amounts of nitrosamines could migrate easily into artificial saliva, and additional amounts of nitrosamines could be formed upon acidification of such extracts for a brief period. However, various liquid infant foods such as infant formulas, orange juice, and apple juice significantly inhibited the formation of nitrosamines from amines leached-out from nipples under above-mentioned in vitro incubation tests. Cow's milk was less effective in this respect. The presence of various N-nitrosation inhibitors such as ascorbic acid, ascorbyl palmitate, and possibly phenolic compounds in these foods was thought to be responsible for this inhibitory action.

The first evidence of the presence of N-nitrosamines, simply called nitrosamines, in baby bottle rubber nipples and a few other rubber products was published by Ireland et al. (1980). Their study showed that elastomers compounded with accelerators or stabilizers derived from dialkylamines contained nitrosamines that were extractable into water. The nature of the nitrosamine detected depended on the type of the amine moiety of the accelerators or stabilizers used. A variety of nitrosamines (sometimes as many as five in one product) namely, *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA), *N*-nitrosomethylethylamine, *N*-nitrosomethylbutylamine, *N*-nitroso-di-*sec*-butylamine, *N*-nitrosodi-*n*-butylamine (NDBA), *N*-nitrosopiperidine (NPIP), *N*-nitrosopyrrolidine (NPYR), and *N*-nitrosomorpholine (NMOR), were detected. The raw polymer or the compounded and cured (without any added accelerator or stabilizer) polymer was usually negative. Since most nitrosamines are highly potent carcinogens in laboratory animals (Magee et al., 1976) and since neonates and infants using these products may be more sensitive to carcinogens than adults, these findings raised concerns as to the safety of these products.

Following the publication of the above report scientists at the German Cancer Research Center, Heidelberg, carried out a more thorough investigation of the occurrence of nitrosamines in baby bottle rubber nipples and pacifiers (Spiegelhalder and Preussmann, 1982). They showed that traces of the nitrosamines in these products could easily migrate to simulated saliva or milk. Considerable amounts of the corresponding amine precursors, which were present in the rubber, were also shown to migrate into the simulated saliva, thus raising the possibility of further formation of nitrosamines in vivo in the stomach of the infants sucking the nipples or the pacifiers. It was observed that the original concentration of the various nitrosamines present in the rubber additives (accelerators, stabilizers, etc.) did not account for all the nitrosamines found in the final products (nipples, pacifiers). This suggested that additional nitrosamines must have formed during processing or storage. *N*-Nitrosodiphenylamine, a retarder of rubber vulcanization and a strong trans nitrosating agent used in the manufacture of some rubber products, and NO_x in the air inside the plants were thought to be the nitrosating agents responsible for the formation of the nitrosamines. The German researchers suggested that the problem could be alleviated by (a) using amines that are either nonnitrosatable or that yield noncarcinogenic nitrosamines as rubber additives or (b) using a differnt type of accelerator or retarder to manufacture baby nipples and pacifiers. Several other investigators from Japan (Ishiwata et al., 1981), the U.S.A. (Havery and Fazio, 1982, 1983), and Sweden (Österdahl, 1983) also reported similar findings.

In a previous publication we (Sen et al., 1984) reported the development of a simple and rapid method for the determination of volatile nitrosamines in various rubber nipples and pacifiers and confirmed some of the earlier findings regarding the occurrence of volatile nitrosamines in these products and the possible migration of the nitrosamines into liquid infant foods (formulas, fruit juices) and artificial saliva. The purpose of the present study was 2-fold: (1) To determine if the levels of volatile nitrosamines in various baby bottle rubber nipples and pacifiers available for sale to consumers in Canada have decreased from that observed 1–2 years earlier. (2) To investigate the effect of liquid infant foods on the formation of nitrosamines that might occur in babies' stomachs due to the interaction of salivary nitrite and ingested amines present in these rubber products. Although previous research from our laboratory as well as other laboratories suggested that such formation of nitrosamines in babies' stomachs was a possibility, the effect of food ingredients, which could have a significant catalytic or inhibitory effect on such formation, has not been investigated.

EXPERIMENTAL SECTION

(A) Samples. Since Canada is a minor producer of these products the rubber nipples and pacifiers used in this study were all imported from various countries. They were either purchased in the local retail outlets or picked up by the inspectors of Consumer and Corporate Affairs Canada. A few samples were obtained directly from distributors representing some foreign manufacturers. Milk, both the powdered (milk-based) and the liquid (soya-based) infant

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formulas, as well as the shelf-stable canned or bottled fruit juices were all purchased locally. The infant formulas were prepared as directed by the manufacturer.

(B) Reagents and Solvents. All reagents were of analytical grade and the solvents were of glass distilled variety. Each bottle of dichloromethane (Caledon Laboratories, Georgetown, Ontario) was tested for nitrosamine contamination (Sen and Seaman, 1981) before use. A complete reagent blank was also carried through the entire step of the method to ensure the absence of nitrosamine contamination. The volatile nitrosamine standards were purchased from Thermo Electron Corporation, Waltham, MA, and *N*-nitrosomethylphenylamine (NMPhA) and *N*-nitrosoethylphenylamine (NEPhA) standards were obtained as gifts from Prof. R. Preussmann.

(C) Gas–Liquid Chromatograph–Thermal Energy Analyzer (GLC–TEA). Two different setups were used: In one system a Varian gas chromatograph (Model 3700) interfaced with a table-top TEA (Model 543) and equipped with a 10 ft × $^1/_8$ in. o.d. Ni tubing and packed with 10% Carbowax 20M + 5% KOH on Chromosorb W (high performance) 100/120 mesh was used. A 6 in. × $^1/_8$ in. o.d. Ni column packed with 1% SE-30 on Chromosorb W (high performance) was connected in the front of the analytical column to act as a guard column. The GLC was operated at 150 °C under isothermal conditions, and the injector temperature was set at 180 °C. This system was mainly used for the survey of volatile nitrosamine contents of nipples and pacifiers. In the other system, a Varian gas chromatograph (Model Vista 6000) interfaced with a TEA (Model 502) and equipped with a 2.74 m × 2 mm (i.d.) coiled glass column and packed with 10% Carbowax 20M on Chromosorb W, HMDS, 60/80 mesh, was used. This was used for the analysis of volatile nitrosamines as well as NMPhA and NEPhA. It should be noted that to obtain optimum peak heights for NMPhA and NEPhA, an on-column injection technique using a glass column and a packing without any added alkali should be used. In the second system, the GLC analysis was started at 110 °C and then programmed to 150 °C at 4 °C/min. The injector and interface (between GLC and TEA) temperatures were 200 °C and 300 °C, respectively.

(D) High-Pressure Liquid Chromatographic–TEA (HPLC–TEA) Analysis. The identity of NEPhA in some samples and tests (e.g., total nitrosatable amine tests) was confirmed by HPLC–TEA analysis (Oettinger et al., 1975). The conditions used were as follows: Column, 250 mm × 4.6 mm (i.d.) Lichrosorb-Si60 (5 μm); solvent, 1.5% acetone in *n*-hexane; flow rate of solvent, 2 mL/min; TEA slush bath, acetone plus dry ice (−80 °C); sample size, 10–50 μL; attenuation, 8 or 16.

(E) GLC–High Resolution Mass Spectrometric Confirmation (GLC–MS). A VG Micromass ZAB-2F mass spectrometer, operating in the electron impact ionization mode and connected to a Varian (Model 3700) gas chromatograph, was used for GLC–MS confirmation. A fused silica column of either 22 m × 0.22 mm (i.d.) CP-Wax 51 (Chrompack Canada, film thickness, 0.08 μm) or a 30 m × 0.22 mm (i.d.) Durawax DX-4 (J&W Scientific Inc., Rancho Cordova, CA, film thickness, 0.25 micron) was used for the GLC analysis. A 0.5–1.0-μL aliquot of the sample extract was injected directly on the column. MS confirmation was carried out by repetitive exponential scanning (1 s/decade) in the mass range of m/z 15 to 350 as well as by selected ion monitoring of some appropriate fragment ions at a resolution of 1000 or 5000. Other operating conditions were as follows: Electron energy, 70 eV; ion source temperature, 150 °C; emission current, 100 μA; GC oven temperature, 120 °C for 3 min, then heated to 180 °C at 10 °C/min.; head pressure, 20 psi He. Prior to GLC–MS analysis, sample extracts were cleaned up by vacuum distillation (≈45 °C) and chromatography on basic alumina (Sen et al., 1979).

(F) Analysis of Nipples and Pacifiers for Volatile Nitrosamines Including NMPhA and NEPhA. The samples were cut into small pieces (≈10 mm^2) and a 2–5-g aliquot was analyzed by the Canadian dichloromethane (DCM) extraction method (Sen et al., 1984). Only changes introduced were those described above under section C. For samples which formed too viscous an extract for analysis by this method the technique of Havery and Fazio (1982) was used.

(G) Analysis of Nitrosamines in Liquid Infant Foods. A 20–100-g aliquot of fruit juices, milk, or infant formulas (after appropriate dilutions) was mixed with 100–200 mL of 3 N potassium hydroxide and 100 ng of *N*-nitroso-di-*n*-proplyamine (NDPA) internal standard. The mixture was analyzed by a low temperature vacuum distillation method reported previously (Sen et al., 1979). Because of the possible presence of NMPhA and NEPhA in some samples (those from migration studies) the GLC column had to be modified as mentioned under section C.

(H) Comparison of the Canadian DCM Extraction Method with the German Method. The Canadian method (Sen et al., 1984) measures only the levels of the preformed nitrosamines that are extractable into DCM. It does not measure migration of the nitrosamines to an aqueous system such as liquid foods or infant saliva, nor does it give any indication of the nitrosation potential of the amines present in these products. The German method (Spiegelhalder and Preussmann, 1982) is more appropriate in that regard as it serves as a measure of both the levels of the nitrosamines as well as of the amines that could migrate into "artificial saliva", such as an aqueous buffer containing traces of nitrite. The comparison of the two methods was carried out as follows: One-half portions of 1–3 nipples or pacifiers were cut into small pieces and analyzed for nitrosamine contents by the Canadian DCM extraction method (section F). The other halves (2–5 g) were cut into small pieces, mixed with 200 mL of artificial saliva (4.2 g of $NaHCO_3$, 0.5 g of NaCl, 0.2 g of K_2CO_3, and 0.005 g of $NaNO_2$ in 1 L of water) (Spiegelhalder and Preussmann, 1982) in a glass-stoppered flask, and the mixture shaken for 24 h in the dark at 40 °C. The sample was filtered and the filtrate made up to 250 mL with water. A 100-mL aliquot was made alkaline by adding 10 mL of 5 N NaOH, the mixture was extracted with three 50-mL portions of DCM, and the DCM extract was concentrated and analyzed for nitrosamines as described under section C. The amount of nitrosamines detected by this method gave a measure of the nitrosamines that could be extracted into or formed during extraction by an aqueous buffer containing traces of nitrite. For convenience, this will be called "buffer extractable-nitrosamines (BEN)".

Another 100-mL aliquot of buffer extract from the step described above was adjusted to pH 1 by adding 2 mL of 6 N HCl and mixed with 1 mL of $NaNO_2$ solution (500 μg/mL), the pH was checked again, and the mixture was kept for 30 min at room temperature. The nitrosation was stopped by adding 10 mL of 5 N NaOH solution and the mixture was extracted (with DCM) and analyzed for nitrosamines as above. This test gave a measure (not the actual concentration) of the "total nitrosatable amines (TNAm)", expressed as ppb (ng/g) of nitrosamine formed based on the weight of the nipple or pacifier used. A total of 10 ppm of $NaNO_2$ (based on the volume of the incu-

Table I. Levels of Volatile Nitrosamines in Recently Obtained Samples of Nipples and Pacifiers[a]

manufacturer or brands	no. of samples	levels, ppb				
		NDMA[b]	NDEA	NDBA	NPIP	NMOR
			Nipples			
A_1,[c] A_2[c]	2			negative[d]		
A_3	1	N[d]	5.1	N	N	N
A_4	1	N	6.1	N	N	N
B	2			negative		
C	3			negative		
D	1	N	N	379	N	N
E	3			negative		
F_3	1	1.4	N	104	17.7	N
H_1	1			negative		
H_2	1	6.6	N	12.7	N	N
H_3	1	10.1	N	3.5	N	N
R	1	13.0	N	105	N	N
S	1	15.0	N	N	N	N
			Nipple-Shield			
Y_1	1	N	N	135	N	5.7
Y_2	1			negative		
Y_3	1			negative		
			Pacifier			
A_5	1	N	N	112	N	N
E_1, E_2, E_3	3			negative		
F_4	1	N	N	6.5	N	N
F_5	1	6.9	N	55.9	N	N
F_3	1	4.2	N	18.0	N	N
L	1			negative		
M_1	1	N	N	57.7	N	N
M_2	1	N	N	25.0	N	N
N	1			negative		
Q–X	8			negative		
Z	1	N	N	7.3	N	N

[a] Sampled during Jan 12–Feb 10, 1984. [b] Abbreviations: NDMA = *N*-nitrosodimethylamine, NDEA = *N*-nitrosodiethylamine, NDBA = *N*-nitrosodi-*n*-butylamine, NPIP = nitrosopiperidine, NMOR = *N*-nitrosomorpholine. [c] Different subscript numbers indicate different samples of the same brand or same brand but manufactured in different countries. [d] N = negative (<1 ppb).

bation medium) was used in these tests compared with an approximate level of 20 ppm of nitrite used in the German method (Spiegelhalder and Preussmann, 1982). In a few preliminary experiments the effectiveness of 5 N NaOH in stopping the nitrosation reaction was compared to that of ascorbic acid and sulfamic acid. In all cases, the NaOH solution worked best (lowest amounts of nitrosamines formed), and, therefore, was selected in preference to the other two.

(I) Effect of Liquid Infant Foods on in Vitro Formation of Nitrosamines under Simulated Gastric Conditions. The experimental design was similar to that in section H (for BEN and TNAm) except that liquid infant foods were used instead of the aqueous buffer. In a typical experiment, cut or whole nipple pieces (1–5 g) were shaken for 1 h at 40 °C with 250 mL of a particular infant food (without any added nitrite), the mixture was filtered through glass wool, and a 100-mL aliquot of filtrate was analyzed for nitrosamines. This gave a measure of the nitrosamines that migrated into the liquid foods from the nipple (the food alone was also analyzed to determine the background level). Another 100-mL aliquot of the filtrate was adjusted to pH 1 with dilute HCl and mixed with 1 mL of $NaNO_2$ solution (1 mg/mL), and the mixture was kept for 30 min at room temperature. The sample was then mixed with 100 mL of 3 N KOH and the mixture was analyzed for volatile nitrosamines as described under section G. In a parallel control experiment, aqueous buffer was used instead of the liquid foods. The difference between the TNAm results obtained in control and test (with food) runs gave a measure of the inhibition of nitrosamine formation by the particular food. Since the levels of nitrosamines and that of the amine precursors may vary from nipple to nipple, it is important that portions of the same nipple or composites of several nipples should be used when comparing results from different experiments. In cases where the level of amine precursors in a nipple was high, one-quarter portion of the nipple was sufficient for an experiment. This allowed comparisons of results obtained from four experiments (e.g., control and three liquid foods).

RESULTS AND DISCUSSION

The results of the recent survey, carried out during Jan–Feb, 1984, on the levels of volatile nitrosamines in rubber nipples, nipple shields, and pacifiers are presented in Table I. Of 42 samples analyzed, 26 were negative and only 7 contained greater than 30 ppb total volatile nitrosamines. Thus, a large proportion of the samples would pass the regulation recently introduced by Consumer and Corporate Affairs Canada that prohibits the sale of any such product containing greater than 30 ppb total volatile nitrosamines. In contrast, in an earlier survey carried out prior to Jan, 1983, nearly 100% of the 30 samples analyzed were found to be positive for nitrosamines, and a majority of them contained >30 ppb total volatile nitrosamines (Sen et al., 1984). It might be worth mentioning that as of Jan 1, 1985, it is proposed that the present Canadian regulation will be restricted further to a limit of 10 ppb.

Table II gives a few specific examples of trends in the levels of volatile nitrosamines as observed in the different surveys. As can be seen from these limited examples, there has been a dramatic decrease in the levels of nitrosamines in some brands but not in others (e.g., brand F). Unfortunately, such a year to year comparison of data was not possible for all brands because many of the brands analyzed in the latest survey were not monitored before. Nevertheless, the generally low average level of nitros-

Table II. Trends in the Levels of Volatile Nitrosamines in Specific Brands of Nipples and Pacifiers during the Past Few Years

	survey period					
	1982–83		July, 1983		Jan–Feb, 1984	
brand	n[b]	levels, ppb	n	levels, ppb	n	levels, ppb
			Nipples			
A	6	NDMA N[a]–4.0	2	(a) one completely negative[a]	4	two negative
		NDEA 4–20		(b) NPIP 5.6		(a) NDEA 5.1 (b) NDEA 6.1
		NDBA N–10				
		NPIP 37–146				
		NMOR N–86				
B	2	NDMA 28–30	1	negative	2	both negative
E	1	NDMA 9.4	1	negative	3	all negative
		NDEA 34.4				
F	5	NDMA N–47.7	2	one negative	1	NDMA 1.4
		NDEA 9–13		NDBA 158		NDBA 104
		NDBA 160–341		NPIP 10		NPIP 17.7
		NPIP 28–180				
		NMOR N–28				
			Pacifiers			
Q			1	negative	1	negative
R			1	NDMA 2.3	1	negative
				NDBA 6.5		
				NMOR 8.2		

[a] N = negative (<1 ppb for each nitrosamine). [b] n = number of samples.

amines observed in the latest survey (Table I) is highly encouraging. This general decline in the nitrosamine content of these products is attributed to the improvements in the rubber manufacturing process recently introduced by the rubber industry. Of the samples analyzed in the latest survey all of those made in Germany (total 13) were completely negative, suggesting that the manufacturers in that country have already taken the necessary corrective measures in eliminating nitrosamines from these products.

In addition to the normal volatile nitrosamines one brand (F) of nipples from the July, 1983 survey consistently contained NEPhA. One sample contained 165 ppb. Since animal studies have shown that NEPhA is a strong carcinogen (Magee et al., 1976), it might be advisable to include it in the future monitoring of nipples and pacifiers.

Table III gives a comparison of three sets (Canadian DCM extraction method, BEN and TNAm values by the German tests) of results for 14 samples of nipples and pacifiers procured during July–Nov, 1983. Several interesting observations can be made from these data. First of all, except for two products (brands F and A) none formed >200 ppb nitrosamines (TNAm) when artificial saliva extracts of these products were incubated under simulated gastric conditions. In other words, the concentration of amines in these samples was not high enough to form >200 ppb nitrosamines. This should be taken as an encouraging sign because these tests were carried out with products manufactured before July, 1983. Secondly, all the non-Latex products and those made in Germany (samples E, L, N, Q, R) gave negative results. And finally, the DCM extraction method, which is used both by the U.S. and Canada, can be slightly less sensitive than the German method in detecting potentially hazardous nipples or pacifiers. This was found to be the case in two samples (brands A and F). The test for TNAm is extremely important for products containing methylphenyl- or ethylphenylamines which nitrosate approximately 150 000 times faster than the strongly basic amines such as dimethylamine, diethylamine, or piperidine (Mirvish, 1975). In view of the high carcinogenicity of both NMPhA and NEPhA (Magee et al., 1976) and their ease of formation, future tests should include the determination of TNAm values. Our present study appears to be the first published report comparing the three methods for the determination of nitrosamines in rubber nipples and pacifiers.

Table III. Comparison of DCM Extraction Method with the German Method

	levels of nitrosamines detected, ppb[a]		
	DCM	German method	
brand	method	BEN values[b]	TNAm levels[b]
	Nipples		
A_6[c]	N[d]	NDEA 38.1	NDEA 275
A_7	NPIP 5.6	NDEA 2.6	NDMA 4.3
			NDEA 2.6
			NDPA 5.1
A_8 (an old sample)	NPIP 32.5	NPIP 48.3	NPIP 121[e]
		NDEA 17.2	NDEA 401[e]
F_1	NDBA 158	NDBA 68	NDBA 749[e]
	NPIP 10	NPIP 16	NPIP 1035[e]
	NEPhA 165	NEPhA 310	NEPhA 98000[e]
			NDMA 1126[e]
F	NDBA 160	NDBA 115	NDBA 166
	NPIP 25	NPIP 32.4	NPIP 45
	NEPhA[e,f]	NDEA 11.4	NDEA 45.5
H_4	NDMA 6.9	NDMA 2.0	NDMA 48.9
			NDPA 16.1
H_5	NDMA 3.3	NDEA 2.0	NDEA 9.0
			NPIP 4.7
	Pacifiers		
F_2	N	NDEA 21.6	NDEA 926[e]
		NDBA 62.6	NDBA 5166[e]
E (non-Latex)	N	N	N
L (non-Latex)	N	N	N
N	N	N	NDMA 7.3
			NMOR 22.5
P (non-Latex)	N	N	N
Q (non-Latex)	N	N	N
R	N	N	N

[a] All results are based on the weight of the nipple or pacifier. Samples procured during July–Nov 1983. [b] For explanation, see text. [c] Different subscript numbers indicate different samples of same brand. [d] N = negative (detection limits: NDMA, NDEA 1 ppb; NDPA, NPIP, NMOR, 2–5 ppb; NEPhA, ≈10 ppb). [e] Confirmed by mass spectrometry. [f] Qualitatively confirmed only.

The results of the in vitro incubation experiments (Table III) suggest the possibility of formation of additional amounts of nitrosamines in babies' stomachs. Various food ingredients may also have pronounced catalytic or inhibitory effects on such formation. To investigate these possibilities, the effect of various liquid infant foods such as milk, formulas, and fruit juices (Table IV) on nitrosamine formation was studied by using two brands of nipples (F_1 and A) which earlier had consistently given positive results in such in vitro incubation tests. As can be seen from the data in Table IV, all the foods tested inhibited nitrosamine formation to varying degrees. None showed any catalytic effect. Both the infant formulas (one milk-based and the other soy-based) and all the fruit juices significantly inhibited the formation of all the nitrosamines—including that of NEPhA. Cow's milk (one homogenized milk and another containing 2% butter fat) was less effective for it inhibited the formation of NEPhA by only 15–37%. As an example, the detailed results obtained in the case of an orange juice are shown in Table V.

Most of the above experiments were carried out with nipples that had been boiled in water for 5 min before each test. Despite this, fairly high levels of nitrosamines were formed when aqueous buffer extracts of such nipples were

Table IV. Inhibition of Nitrosamine Formation by Various Liquid Infant Foods under Simulated Gastric Conditions[a]

nipple used	nitrosamine detected	percent inhibition[a] of nitrosamine formation				
		cow's milk	milk-based formula	soy-based formula	orange juice	apple juice
brand F_1	NDMA	96–100	97–100	100	100	100
	NDBA	26–63	65–99	100	94–100	100
	NPIP	30–90	100	100	100	100
	NEPhA	15–37	99–100	97	94–99	99
brand A	NDEA	*b*	97	100	99–100	100
	NPIP		91	100	94–100	100

[a] Compared to that formed in the absence of food. [b] Not tested.

Table V. Some Detailed Results of Brand F on the Inhibition by Orange Juice of Nitrosamine Formation from Amines in Nipples

expt	nitrosamines detected, ng/g nipple[a]			
	NDMA	NDBA	NPIP	NEPhA
(1) juice (blank)	N[b]	N	N	N
(2) migration from nipple into juice (no nitrite)	N	38	17	N
(3) acidification of no. 2 and incubation with 10 ppm nitrite	2	64	10	400
amount formed in step 3	2	26	−7[c]	400
(4) migration from nipple into artificial saliva (no nitrite)	N	40	3	2
(5) acidification of no. 4 and incubation with 10 ppm nitrite	570	1516	451	7100
amount formed in step 5	570	1476	448	7098
% inhibition by orange juice	99.6	98.2	100	94.4

[a] Brand F_1. [b] N = negative (detection limit, 1–10 ng/g or ng/mL). [c] Assumed to be zero.

Table VI. Results of Experiments Using a Boiled Whole Nipple[a]

expt[b]	nitrosamine detected, ng/g nipple			
	NDMA	NDBA	NPIP	NEPhA
(1) migration from nipple into milk-based formula	N	13	6	N
(2) as no. 1, then acidification and incubation with 10 ppm nitrite	6	35	8	2
(3) migration from nipple into orange juice	N	10	3	N
(4) as no. 3, then acidification and incubation with 10 ppm nitrite	N	9	4	126
(5) migration from nipple into artificial saliva	N	12	trace	N
(6) as no. 5, then acidification and incubation with 10 ppm nitrite	226	76	162	2200

[a] Brand F_1. The nipple was boiled for 5 min in water before each experiment. [b] The same nipple was used over and over again in the order shown.

incubated for 30 min at pH 1 with 10 ppm nitrite. Hence a single boiling did not completely remove the amine precursors from the nipples. Also, experiments were carried out in which a whole nipple (instead of the cut pieces) was used over and over again for 6 consecutive experiments with 5-min boiling in water before each test. The data (Table VI) indicated that even after 6 boilings significant levels of amine precursors remained in the nipple as evidenced by the high values of TNAm obtained in the last experiment (no. 6). Obviously, some amines were leached out. The results suggested that considerably more boilings in water would be needed to bring down the amine concentrations to a negligible level. Low TNAm values obtained with formula (experiment no. 2) and orange juice (experiment no. 4) compared to that obtained in the control (experiment no. 6) again confirmed the excellent inhibitory capacity of these liquid foods.

The inhibitory effect of the above food items on nitrosamine formation could be attributed to the presence of various nitrosation inhibitors (e.g., ascorbic acid, ascorbyl palmitate, α-tocopherol, phenolic compounds) in these foods. These compounds either occur naturally in these products (Health and Welfare Canada, 1979; Pignatelli et al., 1980; Sebrell, Jr., and Harris, 1972) or are present as additives as indicated on the labels. Ascorbic acid, but not α-tocopherol, at a concentration normally present in fruit juice effectively inhibited the formation of nitrosamines from amines leached out from nipples. Therefore, it is highly likely that both ascorbic acid and its ester (ascorbyl palmitate) present in many of the liquid foods tested were responsible for the inhibitory effects observed. Lower inhibitory effect of cow's milk than that of the fruit juices or of the infant formulas might have been due to its lower vitamin C contents (Health and Welfare Canada, 1979). The role of phenolic compounds or other inhibitors in these systems is yet to be investigated. Work along these lines are continuing.

It was concluded from these studies that the health hazard, if any, that could arise due to the formation of additional amounts of nitrosamines in babies' stomachs from ingested amines in rubber nipples might not be as great as thought previously beacuse of the modulating effect of food ingredients which are ingested at the same time. However, significant amounts of nitrosamines, especially NDBA and NEPhA, might be formed in the absence of nitrosation inhibitors. Therefore, efforts to reduce the levels of both the nitrosamines and the amine precursors in these rubber products should continue. It is hoped that concerted efforts by the industry, government regulators, and various research institutions will help achieve this goal in the near future.

Safety Note! Since most nitrosamines are carcinogenic to laboratory animals proper precautions should be taken while working with these compounds.

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The pH-Stat Method for Assessing Protein Digestibility: An Evaluation

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With the use of a microprocessor controlled pH stat, factors affecting the digestibility of proteins were studied. Linear hydrolysis was obtained with a substrate concentration of 3 mg/mL and an enzyme–substrate ratio of 1:128 and 1:100 for pancreatin and trypsin, respectively. Salt ($>10^{-3}$ M) reduced proteolysis of soy glycinin (11S) but had little effect on bovine serum albumin (BSA) or casein digestibility. Under optimum conditions the pH stat was sensitive to trypsin inhibitor (TI), and linearity in rates of digestion of 11S was sensitive to the TI–trypsin ratio rather than total trypsin or TI concentration. This may complicate the use of the pH stat as a routine method for determining TI activity. Extensive reduction of the disulfide bonds in bovine serum albumin enhanced proteolysis of BSA but upon reoxidation digestibility was reduced.

INTRODUCTION

Many proteins, e.g., oilseed proteins, are thermally treated to desolventize and to improve their potential nutritional value (Rackis, 1974; Anderson et al., 1979; Liener, 1979), and proteins may also be modified to improve specific functional properties (Kinsella and Shetty, 1979). Because digestibility is an important factor affecting amino acid availability (Kakade, 1974), in vitro digestibility tests are useful in evaluating the effect of such treatments on bioavailability. The important aspects of in vitro digestibility tests were summarized by Mauron (1973), Hackler (1975), Evans and Witty (1978), and Milhalyi (1978).

Several in vitro digestibility studies of proteins are based on the initial proteolysis rates (Saunders et al., 1973; Maga et al., 1973; Hsu et al., 1977). Hsu et al. (1977) reported good correlations between the pH drop after 10 min of proteolysis and apparent in vivo digestibility. However, significant discrepancies due to buffering and differing pK values of amino groups may invalidate this approach (O'Hare et al., 1984). Proteolysis can also be monitored continuously in a pH stat at constant pH, as proposed by Milhalyi (1978), and thus, enzymatic activity is not affected by pH changes during proteolysis. The number of peptide bonds cleaved are directly proportional to alkali consumption, and the results generally agree with results obtained from chemical tests, e.g., ninhydrin and DTNB (Von Hippel et al., 1960; Samuelson and Li, 1964). Theoretical and experimental aspects of the pH-stat method were discussed in detail by Milhalyi (1978).

Nutritional evaluations of proteins based on the pH-stat method have been reported (Richardson and Catsimpoolas, 1979; Lynch et al., 1977; Stinson and Snyder, 1980; Hill et al., 1982). Richardson and Catsimpoolas (1979) used the pH-stat method to monitor the effects of heat treatment on the initial rates of trypsin proteolysis of soy protein. They observed decreased digestibility of a minimally heat treated soy protein sample. Lynch et al. (1977) determined the effects of chemical modification on the tryptic hydrolysis of soy proteins fractions. Stinson and Snyder (1980) and Hill et al. (1982) measured the initial rate of tryptic hydrolysis of heat treated soy meal samples at several trypsin concentrations to assess residual trypsin inhibitor (TI) activity. Several experimental problems and inconsistencies were encountered, e.g., the initial proteolysis rate showed an unusual decrease with soy meal protein concentration (Stinson and Snyder, 1980) and when initial rates were plotted against added trypsin, shifts in intercepts were not proportional to the amount of TI activity present in the samples.

Emanating from an interest in monitoring the effects of protein modification and conformational changes on digestibility of proteins (Shetty and Kinsella, 1982, 1982a) and a need to assess the sensitivity of the in vitro proteolysis method to modifications of quaternary and tertiary structure of proteins, we investigated the experimental and practical aspects of the pH-stat method in more detail. The effects of protein concentration, substrate–enzyme ratio, pH, ionic strength, and interfering food components (e.g., TI and fatty acids), and protein modification on

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