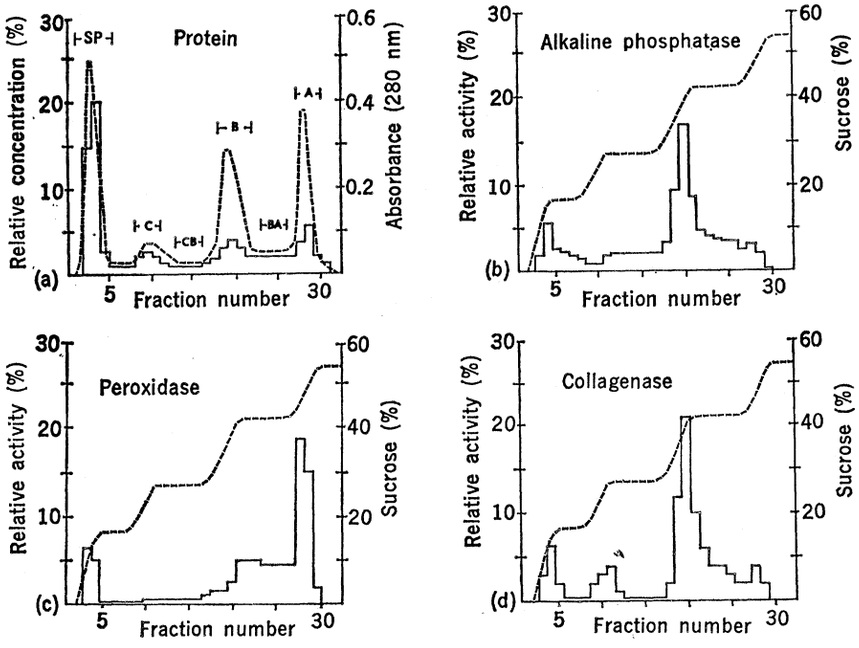

Fig. 1. Fractionation of leukocyte granules through a discontinuous sucrose gradient at 20,000 rev/min for 18 minutes. Absorbance was corrected for sucrose concentration. Protein was determined by the method of Lowry *et al.* (*11*). Peroxidase (c) was found at the heavy end of the gradient, and alkaline phosphatase (b) and collagenase (d) were consistently found together in the same region of the gradient. *SP*, Soluble phase; A, B, and C, regions of particle peaks; BA and CB, regions between peaks.

tion, which never exceeded 3 percent in reaction mixtures containing 0.5 μg of trypsin per microgram of collagen. A number of detergent preparations designed to release latent enzyme activity were tested and found to interfere with the collagenase assay. Freezing and thawing repeated ten times was used, therefore, to release granule enzyme activity before all assay procedures. Assays were performed either on individual 20-ml fractions collected from the rotor or on granule pellets resulting from centrifugation of fractions pooled as shown in Fig. 1a.

Fractionation of rabbit PMN leukocyte granules yielded the distribution patterns in Fig. 1. Intact granules were demonstrated in areas C, B, and A by phase microscopy. A few particles were noted in area BA. Myeloperoxidase and alkaline phosphatase assumed modal distributions consistent with those reported elsewhere (*5, 6*). Collagenase activity was consistently associated with the alkaline phosphatase–containing B granules (Fig. 1, area B). Assays of granule pellets from pooled fractions shown in Table 1 confirmed the strong correlation between collagenase and alkaline phosphatase activity.

Granules in PMN leukocytes appear to possess many of the properties of lysosomes (*14*), and leukocyte granules are responsible for intracellular digestion of materials engulfed by these cells (*14*). Free extracellular granules have also been described in inflamed connective tissue (*15*), wound healing sites (*16*), tuberculin sensitvity reactions (*17*), and delayed hypersensitivity and arthus reactions (*18*). We report the presence of two alkaline hydrolases, alkaline phosphatase and collagenase, in a well-defined cytoplasmic body characteristic of lysosomes. The collagenase derived from PMN leukocyte granules of man manifests a broad *p*H optimum of 7 to 9.5 (*2*). In the case of polymorphonuclear leukocytes, therefore, particular lysosomal-type granules are associated with enzymatic degradation of constituents at an acid *p*H, and other types appear to be concerned with degradation of constituents at neutral to alkaline *p*H.

P. B. Robertson
R. B. Ryel, R. E. Taylor
K. W. Shyu, H. M. Fullmer
Institute of Dental Research, School of Dentistry, University of Alabama, Birmingham 35233

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Ascorbate-Nitrite Reaction: Possible Means of Blocking the Formation of Carcinogenic N-Nitroso Compounds

Abstract. *The formation of carcinogenic* N-*nitroso compounds by the chemical reaction between nitrous acid and oxytetracycline, morpholine, piperazine,* N-*methylaniline, methylurea, and (in some experiments) dimethylamine was blocked by ascorbic acid. The extent of blocking depended on the compound nitrosated and on the experimental conditions. Urea and ammonium sulfamate were less effective as blocking agents. The possibility of in vivo formation of carcinogenic* N-*nitroso compounds from drugs could be lessened by the combination of such drugs with the ascorbic acid.*

Most *N*-nitroso derivatives of secondary amines and amides are powerful carcinogens, which can act in several species (*1*). These derivatives might possibly be produced in the human stomach by the acid-catalyzed reaction between nitrite (*2*), which is present in some foods, and *N*-nitrosatable compounds,

which may be ingested as drugs, food additives, or natural constituents of food (*3*). As an experimental model for this hypothesis, tumors were induced in rats and mice by the long-term feeding of nitrite together with certain secondary amines (including the vermicide piperazine), alkylureas, and *N*-alkylcarbamates (*4*). The biological results are fairly well correlated with kinetic studies on the widely varying rates of the nitrosation reactions (*5*).

Since many drugs contain secondary amine, alkylurea, or *N*-alkylcarbamate groups, oral administration of these drugs might constitute a human health hazard, if nitrite-containing food were ingested at the same time. Examples of such drugs are piperazine, phenmetrazine, primaquine, pamaquine, physostigmine, synephrine, sulfanilylurea, and neohydrin. This possible hazard might be reduced by administering the drug together with a substance that preferentially reacts with and destroys any nitrite occurring in the stomach. We now present chemical data suggesting that ascorbic acid might be used for this purpose.

In addition to the classes of compound already mentioned, Lijinsky *et al.* (*6*) recently reported that certain drugs which contain tertiary amine or dialkylamide groups can be nitrosated to give dialkylnitrosamines. For example, reaction of the dimethylamino compound oxytetracycline with 0.30*M* nitrite at *p*H 4 and 37°C for 4 hours was reported to give a 65 percent yield of dimethylnitrosamine. We attempted to repeat this study on a commercial sample of oxytetracycline (Terramycin) and obtained negative results, but when pure oxytetracycline was used, we confirmed the published results. Our sample of Terramycin was compounded as a mixture containing 80 percent ascorbic acid, and when the same proportion of ascorbic acid was added to a sample of pure oxytetracycline and this was treated with nitrite, no nitrosation was observed. Further experiments were conducted in the presence and absence of ascorbate, and these confirmed that ascorbate almost completely blocked the nitrosation under conditions similar to those used by Lijinsky *et al.* (Table 1, experiments 1 and 2).

We then examined the effect of ascorbate on the nitrosation of five representative compounds whose nitrosation kinetics we have studied (*5*) (Table 1, experiments 2 to 21). Using a 2 : 1 ratio of ascorbate to nitrite, we found more than 98 percent blocking of nitrosation for morpholine and piperazine at all *p*H values tested. Blocking was only partial for *N*-methylaniline, but was fairly complete for methylurea. When the relatively slow nitrosation of dimethylamine was studied under conditions similar to those used for the other amines (reaction at 25°C with excess amine), blocking by ascorbate was fairly effective at *p*H 3 and 4, but at *p*H 2 there was little effect, and at *p*H 1, where the yield without ascorbate was only 0.042 percent, ascorbate increased the nitrosation to 0.076 percent (experiments 15 to 18). When dimethylamine was nitrosated under similar conditions to those used for the nitrosation of oxytetracycline, that is, prolonged reaction at 37°C with excess nitrite, the yield without ascorbate was much larger (2 to 82 percent) and more than 90 percent blocking was observed in the presence of ascorbate (experiments 19 to 21).

Dahn *et al.* (*7*) studied the kinetics of the reaction between nitrous acid and ascorbic acid, in which dehydroascorbic acid and nitric oxide are formed, probably via the nitrite ester of the 3-hydroxy group. In 0.1 to 0.5*N* perchloric acid the main nitrosating species is protonated nitrous acid ($H_2NO_2^+$), whereas in more dilute acid (*p*H 1.5 to 5.0) the nitrosating species is nitrous anhydride (N_2O_3). These are also the nitrosating species for second-

Table 1. Effect of ascorbate on the yield of *N*-nitroso compounds. A solution of oxytetracycline (250 mg), $NaNO_2$ (500 mg), and acetic acid (1 ml) in water (to 23 ml) was adjusted to the desired *p*H with HCl and kept for 22 hours at 37°C in a 100-ml flask fitted with a loosely stoppered condenser; it was then made alkaline with solid NaOH and extracted twice with 75 ml of CH_2Cl_2. The CH_2Cl_2 solution was extracted with 14 ml of 0.1*N* HCl and analyzed for dimethylnitrosamine by gas-liquid chromatography (*6*). For the experiments with secondary amines (*14*), 5 ml of a solution of $NaNO_2$ in 0.05*M* sodium citrate was acidified to the desired *p*H with perchloric acid and was added at 25°C to a solution of the amine plus sodium ascorbate in 15 ml of 0.05*M* citrate (previously adjusted to the same *p*H with perchloric acid). The 20-ml reaction mixture was kept in a stoppered 50-ml conical flask for 5 to 60 minutes at 25°C in the dark. The reaction was stopped by making it alkaline, and solid NaCl was added (in the case of some amines). The nitroso derivative was extracted with CH_2Cl_2 or ether, and its absorbance at 358 to 369 nm was measured. When the absorbance was less than 0.10, the extract was dried over Na_2SO_4, some of the solvent was removed by a rotary evaporator, and another absorbance reading was taken. Results were calculated from the molar absorptivity (ϵ) of the nitroso derivative and corrected for losses due to incomplete extraction. Each experiment was performed in duplicate with and without blocking agent. The full spectra of the extracts at 320 to 450 nm were identical to those of the nitroso derivative. When amine, nitrite, or ascorbate were separately run through the complete procedure, the extract showed no absorbance. When an acidic solution of nitrite plus ascorbate was made alkaline, it turned yellow and then red, but extracts showed no absorption above 320 nm. Methylnitrosourea formation at 25°C was determined by absorbance of the aqueous solution at 400 nm (ϵ, 79) (*5*).

Exp. No.	Base (m*M*)	Nitrite (m*M*)	Ascorbate (m*M*)	*p*H	Time (min)	Yield from amine or urea (%)		Blocking (%)
						Without ascorbate	With ascorbate	
				Oxytetracycline				
1	21.6	315	475	2.8	1320	44	0	100
2	21.6	315	475	3.8	1320	63	0.045	99.9
				Morpholine				
3	25	50	100	2	30	20	0.48	98
4	25	50	100	3	30	65	0	100
5	25	50	100	4	30	34	0	100
				Piperazine				
6	10	10	20	1	60	35	0.58	98
7	10	10	20	2	20	44	1.2	98
8	10	10	20	3	10	56	0.33	99
9	10	10	20	4	15	54	0.24	99
				N-*Methylaniline*				
10	2.5	2.5	5	3	5	68	27	60
11	2.5	2.5	5	4	10	16	8.8	45
				Methylurea				
12	5	10	20	1	10	85	6.9	92
13	5	10	20	2	10	58	7.6	87
14	20	20	40	3	30	27	0	100
				Dimethylamine				
15	500	50	100	1	60	0.042	0.076	−80
16	500	50	100	2	60	0.27	0.29	−7
17	500	50	100	3	60	0.76	0.20	74
18	500	50	100	4	60	0.92	0.03	97
19	20	300	500	2	1440	2.2	0.17	92
20	20	300	500	2.8	1380	20	1.7	91
21	20	300	500	3.8	1380	82	1.4	98

ary amines and alkylureas (*5*) [in the presence of bromide, iodide, and thiocyanate ions, the corresponding NOX species are active nitrosating species for *N*-nitrosation (*8*)]. Therefore, the blockage of *N*-nitrosation by ascorbate was probably due to competition for available nitrite, or to be precise, for N_2O_3 and $H_2NO_2^+$. Ascorbate anion is 240 times more rapidly nitrosated than ascorbic acid (pK_a, 4.29), due presumably to the greater nucleophilic activity of the anion. At *p*H 3 to 5, where the proportion of anion is significant, reaction with nitrite is so rapid that the formation of N_2O_3 is rate-limiting. This explains why ascorbate is more effective as a blocking agent at *p*H 3 to 4 than at *p*H 1 to 2.

Ascorbate had no effect on decomposition of the *N*-nitroso derivatives of methylurea and the amines listed in Table 1, indicating that its action in reducing or eliminating the formation of *N*-nitroso compounds is solely a consequence of the reaction with nitrite.

Nitric oxide produced by the ascorbate-nitrite reaction may combine with atmospheric oxygen to form nitrogen dioxide, some of which would reenter solution and react with water to generate equimolar amounts of nitrous and nitric acids. Thus up to half of the nitric oxide could potentially be reconverted to nitrite. The presence of excess ascorbate in our experiments served to minimize the effect of such reconversions on the extent of *N*-nitrosation.

The nitrosation kinetics for the amines studied is in accord with the relative effectiveness of ascorbate in blocking the nitrosation of these compounds. For example, *N*-methylaniline, like ascorbate, is nitrosated so rapidly that N_2O_3 formation is rate-limiting (*9*), so that the amine competes rather successfully with ascorbate for N_2O_3, and blocking is only partial. Morpholine and piperazine are nitrosated less rapidly (*5*), N_2O_3 formation is not rate-limiting, and it is therefore not surprising that these nitrosations were effectively blocked by ascorbate. Dimethylamine, which is nitrosated much slower than the other amines tested (*5*), was not effectively blocked by ascorbate under those conditions in which relatively little nitrosamine was formed even in the absence of ascorbate. When the reaction conditions were adjusted to increase the yield of nitrosamine, the blocking action of ascorbate was more pronounced. The reason for this behavior is not clear. Blocking of methylurea nitrosation was perhaps stronger than might be expected at *p*H 1, where the $H_2NO_2^+$ mechanism is predominant and methylurea is rapidly attacked (*5*).

Table 2. Effect of the blocking agents urea and ammonium sulfamate on the yield of nitrosomorpholine and mononitrosopiperazine. The experimental procedure followed that described for Table 1, except that urea or ammonium sulfamate was substituted for ascorbate.

Base (m*M*)	Nitrite (m*M*)	Blocking agent (m*M*)	*p*H	Time (min)	Yield from amine (%)			Blocking (%)	
					Without blocking agent	With urea	With ammonium sulfamate	Urea	Ammonium sulfamate
Morpholine									
25	50	100	1	45	7.3	0.4	0	95	100
25	50	100	2	30	25	18	0	24	100
25	50	100	3	30	56	55	0.6	2	99
25	50	100	4	30	32		9.3		71
Piperazine									
10	10	20	1	60	35	11	0	68	100
10	10	20	2	20	36	31	0	14	100
10	10	20	3	10	56	57	14	−2	75

The kinetics of oxytetracycline nitrosation have not yet been examined. Studies on other tertiary amines (*10*) suggest that nitrous acid first reacts with oxytetracycline to give dimethylamine, which is then nitrosated to give dimethylnitrosamine. Under similar conditions, ascorbate blocked the formation of dimethylnitrosamine more effectively from oxytetracycline (experiments 1 to 2) than from dimethylamine (experiments 19 to 21), suggesting that ascorbate reacts with nitrite sufficiently rapidly to prevent the formation of dimethylamine from oxytetracycline.

Since urea and ammonium sulfamate (*11*) also react rapidly with nitrite, the effect of these substances on the nitrosation of piperazine and morpholine was examined (Table 2). Urea was relatively ineffective, especially at higher *p*H. Ammonium sulfamate was even more effective than ascorbate at *p*H 1 and 2, but was relatively ineffective at *p*H 3 and 4. The *p*H of the fasted stomach can reach 0.9, but when a meal is ingested the *p*H of the food is lowered gradually over 1 to 2 hours until it reaches 1.5 to 2.0 (*12*). Hence ascorbate has the advantage over the other agents in that it acts efficiently at the higher *p*H values, and it could react with nitrite in the stomach before the *p*H is lowered to where extensive reaction with amines or ureas might occur.

Although under some conditions the nitrosation of dimethylamine was actually increased by ascorbate, the chemical results on the whole support the suggestion that drugs which can be *N*-nitrosated could be combined with ascorbate before they are administered. This would apply whenever nitrosation of the drug in vivo was likely to occur and combination with ascorbate would reduce this potential hazard without affecting the drug action. Furthermore, addition of ascorbate to certain foods containing nitrite or nitrosatable compounds might be worth considering. In fact, small amounts of ascorbate are often added to nitrite-preserved meat to increase the nitric oxide available for formation of the red nitrosomyoglobin color (*13*).

SIDNEY S. MIRVISH
LAWRENCE WALLCAVE
MICHAEL EAGEN, PHILIPPE SHUBIK
Eppley Institute for Research in Cancer, University of Nebraska Medical Center, Omaha 68105

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14. For each amine, the amount of reagents added at the end of reaction, the volume of CH_2Cl_2 or ether, the wavelength (and the molar absorptivity) where absorbance was measured, the extraction efficiency, and the nitrosamine formed were as follows: morpholine, 2 ml of 5*N* NaOH, 2 × 10 ml CH_2Cl_2, 358 nm (106), 94 percent, nitrosomorpholine; piperazine, 4 ml of 5 NaOH and 2 g of NaCl, 4 × 15 ml of CH_2Cl_2, 358 nm (114), 86 percent, mononitrosopiperazine [kinetic studies (*5*) show that mono- and not dinitrosopiperazine is the main initial product]; *N*-methylaniline, 15 ml of saturated sodium tetraborate solution adjusted to *p*H 9, 3 × 10 ml of CH_2Cl_2, 369 nm (227), 100 percent, *N*-methyl-*N*-nitrosoaniline; dimethylamine (experiments 15 to 18), 5 ml of 5*N* NaOH and 2.5 g of NaCl, 4 × 35 ml of ether, 358 nm (119), 80 percent, dimethylnitrosamine; dimethylamine (experiments 19 to 21), temperature (37°C) and other reaction conditions as for oxytetracycline experiments, workup as for experiments 15 to 18.
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Decreased RNA Polymerase Activity in Mammalian Zinc Deficiency

Abstract. *The activity of DNA-dependent RNA polymerase has been measured in liver nuclei from suckling rats nursed by zinc-deficient dams, or by controls that were either pair-fed or given free access to the diet. In the zinc-deficient pups, the activity of the enzyme did not increase; it fell after the tenth day of life.*

Zinc is an essential nutrient that appears necessary for the synthesis of nucleic acids (*1–3*) and protein (*4*). The manner in which it influences these processes is unknown. Its requirement for the action of various metalloenzymes is well known (*5*), and its influence on the activity of mammalian (*1*) and *Escherichia coli* (*6*) DNA polymerase has been reported. While zinc may play a role in the maintenance of the conformation of nucleic acids (*7*), and zinc nutriture of the animal influences the sucrose density gradient profile of liver polysomes as well as the incorporation of uridine into those polysomes (*8*), it is far from clear how these latter events take place. Therefore, the influence of zinc deficiency on the activity of DNA-dependent RNA polymerase in nuclei of liver cells has been assessed in suckling rats.

Feeding a biotin-enriched diet containing sprayed egg white (20 percent by weight) (*3*) to pregnant dams from the 18th day of gestation through the 16th day of the neonatal period produced zinc deficiency in their pups. Pair feeding of the zinc-supplemented dams (160 μg every other day, by intraperitoneal injection) also resulted in growth failure in their pups. The zinc supplementation was sufficient to maintain normal growth in pups nursed by dams given free access to an adequate diet (Fig. 1). Similar observations have been reported by Mutch and Hurley (*9*), who also found that the concentration of zinc in milk from zinc-deficient dams is decreased along with a decrease in total volume. In contrast, they found that milk from pair-fed dams has increased concentrations of zinc and protein while the volume is decreased. Growth retardation in pups nursed by pair-fed dams therefore reflects the fewer calories available to the pups as a consequence of maternal starvation.

The litters of the dams were sampled at intervals, the sampled pups were decapitated, and their livers were excised and pooled (3 to 11 pups per assay, with the usual number being 5 or 6) The effect of zinc deficiency, starvation, and free access to food on the activity of nuclear DNA-dependent RNA polymerase was measured. The method of Widnell and Tata (*10*) was used to prepare the nuclear suspensions. Enzyme activity was assayed as described by Weiss (*11*). The [^{14}C]adenosine triphosphate incorporated into RNA formed on the nuclear DNA was assayed by liquid scintillation counting and was reported in relation to the amount of deoxyribose (*12*) present. By duplicate assays, these techniques were found to have a high degree of reproducibility; in addition, the activity of the enzyme from pups of the same age and treatment was also similar.

Our studies indicate that starvation (pair feeding) did not inhibit the activity of the enzyme while zinc deprivation did (Table 1). In fact, the activity of the enzyme showed a steady decline in the pups deficient in zinc from day 10 of life ($P < .001$).

These observations are the first, of which we are aware, to show the requirement of zinc for the activity of nuclear DNA-dependent RNA polym-

Table 1. Effect of zinc deficiency on activity of liver nuclear DNA-dependent RNA polymerase of suckling rats. The activity of the enzyme rose very little from birth to day 10 of life and then decreased. Pair feeding did not suppress the activity of the enzyme.

Age (days)	Carbon-14 per milligram of deoxyribose (count min^{-1} mg^{-1} ml^{-1})		
	Zinc-deficient	Controls	
		Pair-fed	Free access to food
2	83.0*	81.5	78.6
4	85.9*		90.9
5		91.5	
6	85.0	87.0	94.8
7			103.5
8		98.9	105.0*
10	91.2	111.3*	
11			115.9*
12	80.4	114.4*	112.8
14	70.0	112.1	115.4†
15	64.8	111.1*	112.8*
16	60.4	107.8	107.4

* Average of two sets of pups. † Average of three sets of pups.

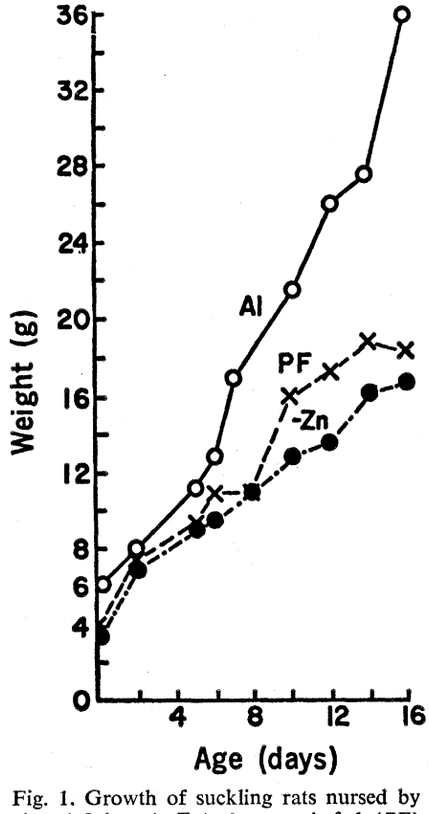

Fig. 1. Growth of suckling rats nursed by zinc deficient (*—Zn*) dams, pair-fed (*PF*) dams, and dams given free access to a normal diet (*Al*). Both zinc-deficient and pair-fed pups showed poor weight gain.