and VI P5D8 and VIII P4AF10 monoclones should be capable of blocking virus neutralization. To test this, KiSV (C3H MMTV) was first incubated with a 1:100 dilution of either VI P5D8 or VIII P4AF10 for 30 minutes at 37°C and then with serial twofold dilutions of VII P2G6 monoclone. Virus-antiserum mixtures were added to duplicate dishes containing indicator cells and incubated for 7 to 10 days at which time the number of foci was quantitated. As a control, the nonneutralizing, noncompeting monoclone XVII P5F8 was used in the first incubation period. The results are presented in Fig. 3. As expected, only monoclones VI P5D8 and VIII P4AF10 were able to block the neutralizing capacity of monoclone VII P2G6. In contrast, monoclone XVII P5F8 has no effect on the neutralizing capacity of VII P2G6. The results show that the percentage blocking increases as the concentration for each blocking monoclone increases relative to that for VII P2G6.

The use of monoclonal antibodies for topographical analysis represents an important new approach for identifying the mechanism of antibody-mediated virus neutralization and the generation of the nonneutralized fraction. Although the nonneutralized fraction has been observed with other viruses and polyvalent antiserums, to our knowledge this is the first direct demonstration that this occurs by way of blocking antibodies. This mechanism must now be considered in studying chronic viral infections in which infectious virus persists in the presence of neutralizing antibodies. Examples of chronic viral infections in humans that fit into this category are chronic viral hepatitis, subacute sclerosing panencephalitis (measles virus), and Epstein-Barr virus infections. The results of this study should be applicable for modifying such disease states. Finally, in designing future vaccination programs with purified antigens, fragments of antigens, and adjuvants, the potential for generating blocking antibodies should be strongly considered.

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Evaluation of Nitrate Synthesis by Intestinal Microorganisms in vivo

Abstract. *The nitrate balance of germfree and conventional rats was assessed to determine whether the intestinal flora produces nitrate in vivo. The results indicate that there can be excess nitrate in the urine of germfree as well as conventional rats. This nitrate is apparently of host origin, and the presence of intestinal flora decreases the output of nitrate in urine.*

It is now widely accepted that *N*-nitroso compounds, whether of exogenous or endogenous origin, may be involved in the etiology of several types of human cancer (*1*). The pharmacokinetics of nitrite and nitrate are important considerations, since these compounds, under appropriate conditions, can nitrosate amines and amides to form the corresponding *N*-nitroso compounds.

Tannenbaum and his colleagues have reported that humans (*2*) and rats (*3*) have more nitrate in their urine than they ingest in their diet and that nitrate and nitrite are present in the intestinal contents of humans (*2*). To explain the excess urinary nitrate and the presence of these ions in the intestinal contents, it was proposed (*2*) that heterotrophic microorganisms in the intestinal tract synthesize nitrate and nitrite from reduced nitrogenous compounds by a process termed heterotrophic intestinal nitrification (HIN). In support of this concept, it was reported (*4*) that microorganisms isolated from the human intestinal tract are able to oxidize certain nitrogenous compounds to nitrite.

Heterotrophic intestinal nitrification, if it occurs, is important for the following reasons.

1) Oxidation of organic or inorganic nitrogenous compounds to nitrite or nitrate by heterotrophic bacteria in the intestinal tract has not previously been described. These bacteria would be conducting a novel reaction at an incredible pace to make excess nitrate possible.

2) Intestinal synthesis of nitrite or nitrate could account for the *N*-nitroso compounds reported in feces (*5*), since intestinal bacteria can nitrosate amines in the presence of these ions (*6*). Such *N*-nitroso compounds could explain the etiology of colorectal cancer.

3) Concerns over the carcinogenicity of exogenous nitrite (*7*), such as that added to processed foods, would seem to be unfounded, since it has been suggested that thousands of times more nitrite is synthesized in the intestinal tract than could be ingested with food (*8*).

4) Intestinal synthesis of nitrite or nitrate could play a role in the etiology of other types of human cancer, not only by increasing the amount of *N*-nitroso compounds in the body but also through direct carcinogenic action (*7, 9*).

5) Various *N*-oxy and *N*-hydroxy compounds, which could be intermediates of microbial HIN, have antimicrobial activity (*10*) and might play an important role in controlling the microbial ecology of the intestinal tract.

Earlier (*11*), we offered alternative explanations for the presence of nitrite and nitrate in the intestinal tract, argued (*12*) that HIN is unlikely, and suggested other sources of urinary nitrate. This report provides further data relating to the nondietary origin of nitrate in rats.

If intestinal bacteria or fungi are involved in nitrogen oxidation in the intestinal tract and cause an excess of urinary nitrate, the urine of conventional rats should contain more nitrate than the urine of germfree rats, assuming that dietary nitrate intakes are comparable and that nitrite synthesized in the intestine can be absorbed and stoichiometrically converted to nitrate in the bloodstream (*13*). In our first study we determined the nitrate balance of rats before and after they were removed from a germfree isolator and allowed to acquire a complex microbial flora. These rats were fed Rat Chow (Ralston Purina diet

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Table 1. Daily dietary intake and urinary output of nitrate by germfree and conventional Sprague-Dawley rats. All rats were analyzed daily for 14 days. The daily intake and output of nitrate were summed for each rat over the experimental period and expressed as means ± standard errors. In the difference column, means ± standard errors were calculated by subtracting the intake from the output each day. The Rat Chow was powdered and thoroughly mixed to ensure homogeneity. Weighed portions offered to the rats were made to a pastelike consistency with double-distilled water (DDW). The chow offered to the germfree rats was autoclaved. This feeding system resulted in minimal (approximately 0.1 g) loss of food. At the end of each 24-hour period the remaining food (if any) was removed, dried, and corrected for water loss to estimate the amount of food consumed. Before, during, and after the experiment, water extracts of the food were analyzed for nitrate (*14*). Before additional weighed food samples were given to the rats, the plastic metabolism cages (Nalge) were thoroughly washed with DDW and the urinary nitrate output was analyzed (*14*) on that same day. Urine samples from the conventional rats were preserved with toluene, or the urine collector was placed in a Dewar flask containing dry ice and absolute ethanol to immediately freeze the urine. Rats maintained on Rat Chow were given DDW to drink. Rats on a nitrate-free diet were fed 5 percent glucose in DDW.

Microbial status	Diet	Rat	Nitrate (μmole/day)		
			Intake	Ouput	Difference
Germfree	Chow	1	10.0 ± 0.99	11.1 ± 0.93	+1.1 ± 0.30
	Chow	2	8.9 ± 0.86	10.5 ± 0.92	+1.6 ± 0.47
	Chow	3	9.7 ± 0.97	11.3 ± 1.0	+1.6 ± 0.19
Conventional	Chow	1	10.2 ± 1.03	6.1 ± 0.64	−4.1 ± 0.82
	Chow	2	9.2 ± 0.90	6.1 ± 0.51	−3.2 ± 0.67
	Chow	3	9.7 ± 0.97	4.1 ± 0.33	−5.6 ± 0.81
Germfree	Glucose	4	0.0	3.8 ± 0.24	+3.8 ± 0.24
	Glucose	5	0.0	3.4 ± 0.23	+3.4 ± 0.23
	Glucose	6	0.0	3.6 ± 0.29	+3.6 ± 0.29
Convention	Clucose	7	0.0	1.2 ± 0.17	+1.2 ± 0.17
	Glucose	8	0.0	0.9 ± 0.15	+0.9 ± 0.15
	Glucose	9	0.0	0.7 ± 0.15	+0.7 ± 0.15

5010C) throughout the experiment; the food was autoclaved while the rats were in the germfree state. Dietary and urinary nitrate were assayed by high-performance liquid chromatography (*14*). As shown in Table 1, while the rats were germfree they had an excess of nitrate in their urine compared to the amount of nitrate they ingested. After the rats were removed from their isolator less nitrate appeared in their urine than they ingested. This decrease in urinary nitrate indicates that intestinal flora is involved in the degradation or utilization of dietary nitrate rather than in its synthesis (*15*). When these rats were fed antibiotics (neomycin and tetracycline), their urinary nitrate excretion increased, in one case matching the output maintained in the germfree state. The antibiotics themselves were not *N*-oxidized because they did not increase the urinary nitrate output of germfree rats.

In a second experiment, we fed other germfree and conventional rats a nitrate-free diet (5 percent glucose in distilled deionized water). The rats excreted nitrate in their urine for 14 days despite the fact no dietary nitrate was available to them. The germfree rats excreted an average of 3.6 μmole of nitrate per day and the conventional rats excreted 0.9 μmole per day. The continued urinary excretion of nitrate by both germfree and conventional rats when no known nitrate was being ingested indicates that endogenous synthesis of nitrate does occur but is a function of the host rather than its complex microbial flora. Even when conventional rats were maintained on a nitrate-free diet, the amount of urinary nitrate excreted was decreased by the intestinal flora.

These data and our data on nitrate pharmacokinetics (*16*) clearly do not support the HIN hypothesis of Tannenbaum and colleagues (*17*). It appears that the intestinal flora does not produce appreciable amounts of nitrate or nitrite in vivo. On the contrary, our data demonstrate that the intestinal flora can decrease urinary nitrate output in rats. The intermediates or end products of this metabolism are unknown, but nitrite is certainly one possibility (*16*). Nitrate metabolism, which appears to depend on dynamic interactions between the host and the microbial flora, can be altered by factors that eliminate or change the intestinal flora. Whether such changes are important in the endogenous formation of *N*-nitroso compounds remains to be determined.

Finally, it is evident that the output of nitrate in the urine can exceed dietary intake of nitrate. This is illustrated by our data on conventional rats, for which excess urinary nitrate was noted in the absence of dietary nitrate. A similar situation has also been reported in humans (*18*). Apparently a metabolic process occurs whereby the host's tissues oxidize nitrogenous compounds to either nitrite or nitrate. This suggests that nitrate is not xenobiotic. We do not know where this process occurs or which nitrogenous compounds might serve as substrates for nitrate synthesis. These and other data (*19*) are in agreement, however, that nitrate can be synthesized by the mammal.

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15. The microbial degradation of nitrate was attributed to an in vivo metabolism (rather than to an in vitro metabolism that could have occurred between the time the urine was voided and the time it was assayed) because neither viable bacteria nor nitrite, which would represent an active bacterial nitrate reductase, was detected in urine samples.
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