the lack of correspondence of the patterns of the overstory and smaller size-classes of a species on a two-dimensional environmental gradient. One alternative is that overstory composition would change in time. This change could be directional with one species gaining prominence at the expense of another, or the overstory composition in any portion of the forest could fluctuate through time. The other alternative is that there might be differential mortality in the understory layer that would cause the canopy pattern to remain relatively unchanged.

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18. This is a contribution of the Hubbard Brook Ecosystem Study, funded by the National Science Foundation. Support during manuscript preparation was from the Montana Forest and Conservation Experiment Station.

21 March 1975

Occurrence of *S*-Methyl Thioesters in Urines of Humans After They Have Eaten Asparagus

Abstract. *Gas chromatography–mass spectrometry was used to determine the odor-causing agent (or agents) present in the urines of humans after they have eaten asparagus.* S-*Methyl thioacrylate and* S-*methyl 3-(methylthio)thiopropionate were identified from methylene chloride extracts of such urines and appear to be the odor-causing compounds. Methanethiol, the previously reported odor-causing agent, was not detected in these methylene chloride extracts.*

It has been recognized for many years that, after eating asparagus, some humans excrete a substance or substances which produce a characteristic odor in the urine (*1*). This strong odor appears within an hour after only a few spears of asparagus have been consumed (*2*). Allison and McWhirter found evidence that the ability to produce the odor is controlled by a single autosomal dominate gene (*3*). In a random sample of 115 individuals, 46 were found to produce the odor after eating asparagus and 63 did not. The odor-bearing compound was tentatively identified as methanethiol in 1891 by Nencki who distilled the urines of four individuals who had eaten a total of 7 kg of asparagus (*1*). Identification was based on the odor of the distillate and precipitation of mercury and lead mercaptides. Allison and McWhirter also reported the compound as methanethiol on the basis of the isolation of the silver methyl mercaptide (*3*).

Recently, dimethyl disulfide was identified as a normal trace volatile of human urine (*4*). The presence of the free mercaptan methanethiol would not be expected, however, because the compound is rapidly oxidized to the disulfide by oxygen. In addition, small doses of methanethiol injected into rats are reported to be metabolized rapidly to inorganic sulfate and carbon dioxide (*5*).

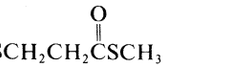
Efforts to identify methanethiol in odorous urine (kept at 80°C) by passing its vapors through a mercuric chloride solution in an attempt to isolate the mercaptide were unsuccessful. An attempt to trap the CH_3SH by reaction of the urine or its methylene chloride extract with 1-fluoro-2,4-dinitrobenzene (DNFB) was also unsuccessful. A water sample containing ~ 1.0 μmole of CH_3SH gave the DNFB derivative that was easily assayed by gas chromatography–mass spectrometry (GC-MS). Attempts to find by the use of GC-MS abnormal amounts of dimethyl disulfide in ethyl ether and methylene chloride extracts of the urines of humans who had eaten asparagus were also unsuccessful.

Analyses were carried out on urine samples of approximately 500 ml, which were collected over a period of 12 hours on several occasions after the subject had eaten a standard diet. When included in the diet, ~ 100 g of asparagus was prepared by cooking for 10 minutes in boiling water. The urine samples were collected in a flask containing 50 ml of methylene chloride (distilled in glass) and cooled to 3°C. The flask and contents were shaken for 1 hour and the resulting emulsion was separated by centrifugation; the organic layer was evaporated at 40°C to a small volume in a stream of nitrogen. The resulting concentrated methylene chloride solution was examined with an LKB 9000 GC-MS (*6*).

Closer examination of the GC-MS data of these extracts, however, revealed the presence of compounds with molecular ions and fragmentation patterns corresponding to *S*-methyl thioacrylate (M^+, $m/e = 102$)

$$CH_2 = CHCSCH_3$$

and its methanethiol addition product *S*-methyl 3-(methylthio)thiopropionate (M^+, $m/e = 150$)



$$CH_3SCH_2CH_2CSCH_3$$

These two compounds were synthesized (*7*), and were found to have the same gas chromatographic retention times and mass spectra as the two compounds found in the urine (*8*). The addition of these synthesized compounds to nonodorous urine produced the characteristic odor found in the urine after the subjects had eaten asparagus. In addition to these compounds smaller amounts of other sulfur compounds were also detected on occasion. These included dimethyl trisulfide CH_3SSSCH_3, tetrahydrothiophene $CH_2CH_2CH_2CH_2S$, and an unidentified compound (M^+, $m/e = 126$) containing two sulfurs (*9*). None of these compounds could be detected in the urine unless asparagus had been consumed. The expected ease of hydrolysis of these compounds to produce methanethiol under the distillation or reaction conditions (or both) used by previous investigators would account for their identifications of methanethiol as the odoriferous compound.

Since the amount of dimethyl sulfone excreted in a 24-hour period is known to be relatively constant (between 4 to 11 mg) (*10*), dimethyl sulfone was used as an internal standard for quantifying *S*-methyl thioacrylate and *S*-methyl 3-(methylthio)thiopropionate in urine. The two thioesters are present in approximately equal amounts after ingestion of asparagus. Furthermore, they occur at levels comparable to that of dimethyl sulfone. Therefore from 2 to 5 mg of each of the thioesters appears to be excreted in the urines of humans within 12 hours of having consumed ~ 100 g of asparagus.

Previous work has demonstrated the presence of two unusual sulfur-containing compounds in asparagus; these include 2,2′-dithiolisobutyric acid (*11*) and a methylsulphonium salt of methionine (α-amino-dimethyl-γ-butyrothetin) (*12*). Ingestion of 10 mg of 2,2′-dithiolisobutyric acid by two individuals produced no odor in the urine (*13*). The methionine analog has also been found in cabbage and parsley, neither of which produce the urine odor observed after the eating of asparagus, which would indicate that this compound is not responsible for the production of the odor (*13*). Although *S*-methyl thioacrylate and *S*-methyl 3-(methylthio)thiopropionate have been identified as the odor-causing compounds, their metabolic origin remains an open question.

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6. Separations were effected with a glass column (1.8

m by 0.32 cm, inside diameter) containing 3 percent OV-17 on Gas Chromosorb Q. After sample injection, the column was maintained isothermal at 60°C for 2 minutes followed by an increase of 10°C per minute until 300°C was reached. The flash heater was maintained at 220°C and the separator at 300°C. Helium flow was 26.4 ml/min.

7. *S*-methyl thioacrylate was prepared by the dicyclohexyl carbodiimide coupling of acrylic acid and methanethiol in methylene chloride. This compound consumed 1 mole of HBr to form *S*-methyl 3-(bromo)thiopropionate (M^+, m/e = 182, 184, 186) and 1 mole of bromine to form *S*-methyl thio-2,3-(dibromo)thiopropionate (M^+, m/e = 260, 262, 264, 266). This compound also reacted with methanethiol to form the β-methanethiol addition compound. The *S*-methyl 3-(methylthio)thiopropionate was also prepared from 3-thiopropionic acid by methylation with methyl iodide in aqueous base followed by formation of the thiol ester by dicyclohexyl carbodiimide coupling in methylene chloride with methanethiol.
8. *S*-Methyl thioacrylate and *S*-methyl 3-(methylthio)thiopropionate gave the following mass spectra m/e (relative intensity) 104 (2.5), 103 (3.0), 102 (45), 75 (6.0), 55 (100), 47 (11), 45 (12); and 152 (3.5), 151 (2.5), 150 (36), 104 (44), 75 (61), 61 (100), 47 (35), 45 (27), respectively. Under the described GC conditions, the former compound had a retention time of 2.6 minutes and the latter 11.6 minutes. Dimethyl sulfone had a retention time of 8.3 minutes.
9. Dimethyl trisulfide was prepared from the corresponding Bunte salt [B. Milligan, B. Saville, J. M. Swan, *J. Chem. Soc. (Lond.)* (1963), p. 3608. Tetrahydrothiophene was prepared by the method of C. S. Marvel and W. W. Williams [*J. Am. Chem. Soc.* **61**, 2714 (1939)].
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14. I thank G. Willson, P. Keogh, and L. White for participating in the experiment. Special thanks go to Prof. S. L. Miller for his critical analysis of this manuscript. Supported by NSF grant MPS 72-05043. GC-MS was obtained in part with NSF grant GP 18245.

12 May 1975; revised 10 June 1975

Immunological Tolerance: Transmission from Mother to Offspring

Abstract. *Mice born and raised by mothers made specifically unresponsive to heterologous erythrocytes by prior treatment with soluble extracts of these cells become themselves tolerant. Tolerance is achieved through nursing since normal neonates fostered by tolerant animals become tolerant, while animals born to tolerant mothers but nursed by normal mice are fully responsive.*

Since theoretical considerations led Burnet and Fenner to postulate that exposure to antigens in prenatal or perinatal life might make an animal unresponsive to the same antigen presented to it later in life, the conceptual framework of immunological tolerance has broadened to include a variety of conditions in which unresponsiveness can be induced not only early in the developmental history of an animal but in adult life as well. While a distinction may be made between transplantation tolerance as originally defined by Billingham, Brent, and Medawar and tolerance to protein or polysaccharide antigens, that distinction has become operationally clouded as the complex interplay among such factors as transplantation antigens, T cells, B cells, and serum antibody and antigen-antibody complexes has been analyzed for the various systems collectively considered as manifestations of immunological unresponsiveness or tolerance (*1*).

Over the past 3 years work from this laboratory has described a system for inducing specific unresponsiveness to heterologous erythrocytes by use of a high-speed supernatant derived from red blood cells lysed in hypotonic solution. Sheep hemolyzate supernatant (SHS) or horse hemolyzate supernatant (HHS) derived, respectively, from sheep red blood cells (SRBC) or horse red blood cells (HRBC), when injected into adult mice, severely reduced the subsequent response of such animals to antigenic challenge by the appropriate intact erythrocyte (*2*). We have reported that the material is nonantigenic as defined by its inability to elicit spleen antibody-forming cells (PFC), its failure to boost a primary response previously induced by intact erythrocytic challenge, and its failure to increase over a 4-week period the serum antibody titer as measured by hemagglutinin or hemolysin titrations. Tolerance was found to be specific and long lasting. Cell transfer studies showed that the tolerance is not expressed by spleen cells from tolerant animals following transfer into normal irradiated hosts, but rather that tolerance is systemic in nature, presumably dependent on serum-mediated blocking of the immune response (*3*). In vitro studies confirmed that cells from tolerant animals respond to erythrocytes in undiminished fashion when placed in cell or organ culture. On the other hand, serum from tolerant animals specifically prevented the in vitro immune reaction of normal spleen cells to the appropriate challenge erythrocyte (*3*).

Our experiments were undertaken to determine the effect on the developing mouse embryo and neonate of treatment of the mother with tolerogenic material obtained from sheep or horse erythrocytes. We used BALB/c mice for all our work. Both SHS and HHS were prepared as described previously and checked for lack of antigenicity prior to use (*2*). Timed pregnancies were obtained by examining daily for presence of vaginal plugs, the day of appearance of a plug being designated day 0. Birth usually occurred 19 days later. To observe the effect of tolerogen administration during pregnancy 0.5 ml of HHS or SHS was administered on days 3, 4, and 5 of development, that is, during the period of embryo implantation and prior to formation of embryonic blood cells or placentation. Other pregnancies were initiated at various intervals after injection of tolerogen. Since we ascertained that there was no significant antigenic competition or cross-reactivity at the PFC level between HRBC and SRBC administered simultaneously, antigenic challenge was achieved by intraperitoneal injection of 0.2 ml of a 1 : 1 mixture of 25 percent SRBC and HRBC 4 days prior to assay of spleen PFC. The normal BALB/c mouse yields about twice as many PFC to SRBC as to HRBC when this regimen is used, and the ratio of PFC response of SRBC/HRBC was used as a useful measure of immunological tolerance. The PFC were enumerated by the method of Cunningham and Szenberg (*4*).

Our initial results indicated that many but not all litters of mice born after treatment of the mother with SHS during pregnancy showed a subsequently reduced response to SRBC, while similar treatment with HHS resulted in a reduction of the response to HRBC. More striking, however, were the results obtained from litters conceived several weeks after administration of tolerogen to the prospective mothers. To date, all litters born to tolerant mothers 5 to 8 weeks after administration of the tolerogenic material and tested between 25 and 45 days of age showed a profound reduction in response to test antigen, while producing a normal level of response to the control erythrocyte (Table 1).

To determine the prenatal versus postnatal influence of the prior induction of maternal tolerance on the immune competence of the litter, rearing experiments were carried out in which newborn mice

Table 1. Effect of tolerance induction of mother on immune response of young. Interval between tolerance induction and conception was 2 to 5 weeks.

Treatment	Litters (No.)	Assays (No.)	PFC/10^6 spleen cells		Ratio SRBC/HRBC
			SRBC	HRBC	
SHS	14	29	67.8*	717.7	0.09
HHS	7	19	523.8	36.1†	14.51
Saline	6	16	697.1	353.3	1.97

*9.7 percent of normal response to SRBC. †10.2 percent of normal response to HRBC.