oxytocin secretion. Thus (a) hypothalamic lesions in the rat inhibit oxytocin release and prevent litters from obtaining milk, although milk production and hence prolactin secretion continue in a normal fashion(8); (b) single pituitaries transplanted to the kidney of rats secrete greater than normal amounts of prolactin and initiate mammary secretion, despite absence of obvious oxytocin stimulation(9); (c) whereas suckling evokes an immediate decrease in pituitary prolactin content, indicating discharge of this hormone, injections of oxytocin are ineffective in this respect(4). Benson and Folley's(1) demonstration that oxytocin inhibits mammary involution in postpartum rats can be explained by its direct action on the mammary gland(5,10).

Summary. Oxytocin failed to initiate lactation when injected 4 times daily into 45 mature female New Zealand White rabbits initially primed with estradiol or made pseudopregnant to develop their mammary glands. Prolactin injections initiated copious milk production in pseudopregnant rabbits. 2. Neither oxytocin nor vasopressin inhibited mammary involution or induced secretion in estrogen-primed rats, whereas injections of prolactin alone retarded mammary involution and addition of ACTH initiated mammary secretion. 3. It is concluded that oxytocin does not induce prolactin release in rabbits or rats.

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Effect of Diazoalkane-yielding Compounds on Strains of *Escherichia coli* Resistant to 1-Methyl-3-nitro-1-nitrosoguanidine.* (26144)

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1-Methyl-3-nitro-1-nitrosoguanidine (NG) has anticancer activity in mice(1,2,3) and antibacterial activity *in vitro*(4,5). Two mutants of *Escherichia coli* strain S which are resistant to NG have been selected. The first-step resistant mutant S/Ng 1 was selected from the wild type, strain S. The second-step mutant S/Ng 2 was selected from S/Ng 1(6). It was believed that some insight into the mechanism of action of NG could be gained by testing the strains of *E. coli* resistant to NG against structurally similar compounds.

Skinner *et al.*(3) have synthesized a series of nitrosoguanidines differing from NG in the substituent on N-1. In addition, 2 commercially available compounds, like NG, yield diazomethane under alkaline conditions. These are N-methyl-N-nitrosourea (MNU) and N - methyl - N - nitroso - *p* - toluenesulfonamide (MNTS). Another compound, S - methyl-N-nitropseudothiourea (MNPT) (7) will, like NG(8,9) nitroguanisate amino acids.

Methods. Strain S (obtained from Dr. A.

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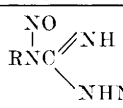
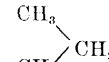
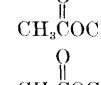
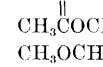
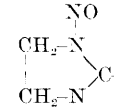
TABLE I. Effect of Various Nitrosoguanidines against *E. coli* Mutants Resistant to 1-Methyl-3-nitro-1-nitrosoguanidine. (Disc assay)



R	μg/disc	Diameter of zone of inhibition (mm)* *E. coli* strain S (parent)	S/Ng 1	S/Ng 2
CH_3 (NG)	25	23	10	0
CH_3CH_2	50	30	22	0
$CH_3CH_2CH_2$	50	30	27	10
$CH_3CH_2CH_2CH_2$	50	24	13	9
	20	18	7	2
	50	18	0	0
$CH_3CH_2CH_2CH_2CH_2$	50	12	0	0
Benzyl	20	14	3	0
	50	31	27	20
	50	28	13	±
$CH_3OCH_2CH_2$	50	29	14	11
$ClCH_2CH_2$	50	11	8	7
$ClCH_2CH_2CH_2$	50	31	27	20
	100	10	±	4

* In excess of disc diameter.

± = Barely visible zone of inhibition.

D. Hershey, Cold Spring Harbor, N. Y.) of *E. coli*, the parent, NG-sensitive strain, and the 2 mutants, S/Ng 1 and S/Ng 2, were used. The organisms were grown in the synthetic medium of Davis and Mingioli(10), pH 6.8, containing 1.5% agar, or in tryptone-agar, pH 5.5 (T-agar). T-agar is made as follows: 10 g of tryptone, 1 g glucose, 5 g sodium chloride, 12 g agar made up to one liter with distilled water and adjusted to pH 5.5 with hydrochloric acid.

Two methods of assay of drug activity were used: (1) Filter paper disc-assay. In this method, 10^7 log phase bacteria were spread on solid agar. Filter paper discs 0.67 mm in diameter were placed on the agar, and 20 μl of a properly diluted solution, usually methanolic, of test drug were added to the disc by micropipette. (2) The gradient plate technic of Szybalski and Bryson(11).

All cultures were incubated at 37°C for 18 hours, after which the zone of inhibition (disc-assay) or length of the streak showing confluent growth (gradient plates) was measured. Compounds were always tested concurrently on all 3 strains of *E. coli*.

Results. Table I shows results obtained with a series of nitrosoguanidines, varying in the substituent on N-1, against *E. coli* strain S, S/Ng 1, and S/Ng 2, grown on DM medium at pH 6.8. The last compound in the table (1-nitroso-5-nitraminoimidazoline) is included because it can be considered as a condensed nitrosoguanidine. Every compound in the table was less effective against S/Ng 1 than against S. With 4 of the compounds (R = propyl, acetoxyethyl, chloroethyl, and chloropropyl), the difference in zone size between S and S/Ng 1 was small and possibly within the error of the method. On the other hand, every compound was unequivocally less effective against S/Ng 2 than against S. Furthermore, wherever such a comparison is possible (zone size against S/Ng 1>0), and with only 2 exceptions, the compounds were less effective against S/Ng 2 than against S/Ng 1. The two exceptions were: R = chloroethyl and the nitrosoimidazoline. This last compound appeared to be less effective against S/Ng 1 than against S/Ng 2.

MNU and MNTS were inactive at concentrations up to 250 μg/disc when tested against *E. coli* strain S, DM medium, at pH 6.8. We had observed earlier that antibacterial activity of NG increased as pH of the medium at start of the experiment decreased. As measured by the effect on colony-forming ability of *E. coli* strain S, NG was over 1000 times as active in tryptone-agar at pH 5.5 (T-5.5 agar) as in DM medium at 6.8. When an amount of tryptone equivalent to that in T-5.5 agar was added to DM medium at pH 6.8, the activity of NG was significantly inhibited. It is possible that NG might be even more effective at pH 5.5 in absence of tryptone, but we have not yet been able to test this possibility, since *E. coli* did not grow at all in a salts-glucose medium at pH 5.5.

It was conceivable that MNO and MNTS

TABLE II. Effect of N-methyl-N-nitrosourea (MNU) and N-methyl-N-nitroso-*p*-toluenesulfonamide (MNTS) against *E. coli* in Tryptone-agar pH 5.5.

Compound	μg/disc	Diameter of zone of inhibition (mm)* Strain of *E. coli* S	S/Ng 1	S/Ng 2	S/Mnu 1
MNU	200	15	±	±	2
	100	21	±	0	2
	50	16			
	20	11			
	10	7			
MNTS	250	13	5	14	
NG	50		2	4	21
	25	23	10	0	15

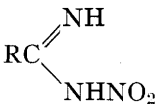
* In excess of disc diameter.

± = Barely visible zone of inhibition.

might inhibit *E. coli* on T-5.5 agar, even though inactive at pH 6.8. Both compounds produced zones of inhibition, even though at very high concentrations, under these conditions (Table II). Furthermore, S/Ng 1 was more resistant to both compounds than was S. However, S/Ng 2 was no more resistant to MNTS than was S. It was not possible to determine by this method whether S/Ng 2 was more or less resistant to MNU than was S/Ng 1. For this reason, we determined minimum inhibitory concentration (mic) of MNU on gradient plates (T-5.5 agar). The mic of MNU against *E. coli* strain S was 10 μg/ml; against S/Ng 1, 250 μg/ml; and against S/Ng 2, 165 μg/ml. As with MNTS and 1-nitroso-5-nitraminoimidazoline, S/Ng 2 was more sensitive to MNU than was S/Ng 1.

A mutant of *E. coli*, S/Mnu 1, was selected which was 25-fold resistant to MNU as determined on gradient plates. This strain was as resistant to NG as was S/Ng 1 (Table II).

Saroff and Evans(7) showed that MNPT nitroguanisated amino acids. This is a reaction characteristic of NG, and the two compounds resemble each other in having the structure



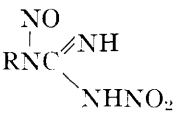
Since resistance to NG, at least in S/Ng 2, seemed to be a function of the nitroguanidine portion of the molecule, it seemed possible that NG and MNPT might be cross-resistant. However, MNPT was inactive against *E. coli* strain S at concentration of 100 μg/ml at pH's from 5.0 to 8.0.

Discussion. It is apparent from these results that resistance to NG is accompanied by resistance to all nitrosoguanidines, regardless of the alkyl substituent on N-1. At least this is true with the particular mutants we selected for resistance to NG. The demonstration of cross-resistance among nitrosoguanidines is unequivocal in S/Ng 2, and is questionable in only 4 of 11 compounds in S/Ng 1. In most instances it is clear that S/Ng 2 is more resistant to nitrosoguanidines than is S/Ng 1. It seems likely that if a more precise method for estimating degree of resistance were used, there would be no question about any of the compounds. Resistance of S/Ng 2 to NG and related compounds has previously been demonstrated clearly by conventional turbidimetric measurements(5). However, resistance to S/Ng 1 could not be demonstrated turbidimetrically; but where strain S responded to NG and related nitrosoguanidines by producing filaments, S/Ng 1 over an identical range of drug concentrations did not. Resistance of S/Ng 1 could be demonstrated only by examining the morphology of the organisms grown in liquid culture or by colony-forming ability on solid medium. The gradient plate technic might yield more precise quantitative data on relative degree of resistance among nitrosoguanidines. This method has been used by us with 3 different nitrosoguanidines, and the results, which substantiate the general conclusion regarding cross-resistance of any nitrosoguanidines, will be published later.

With the imidazoline derivative the results were different than with the nitrosoguanidines. Both S/Ng 1 and S/Ng 2 were resistant, but S/Ng 2 appeared to be less resistant than S/Ng 1. This was true also of MNU and MNTS: S/Ng 1 was resistant to both, and S/Ng 2 was no more resistant and was probably less resistant than was S/Ng 1.

How is this information related to what we already know? We know that S/Ng 1 is

resistant to a variety of radiomimetic compounds (azaserine, Mitomycin C, nitrogen mustard) and to ultraviolet radiation (unpublished information). S/Ng 2 is no more resistant to these agents, and may be less resistant, than S/Ng 1. From this we conclude that the first step mutation, S/Ng 1, probably involves some general physiological change which makes the organism resistant to radiation and radiomimetic compounds. This change is associated with resistance to filament formation, or with some mechanism which permits the cell to divide properly or, on agar plates, to form colonies. In S/Ng 2, the second step mutation involves some mechanism which is concerned with the precise chemical configuration of NG. The problem is: how precise? It is obvious from the present data that S/Ng 2 shows increased resistance to compounds of the configuration



Therefore, the nature of the alkyl substituent on N-1 is of little or no importance in this stage of resistance. On the other hand, S/Ng 2 does discriminate between NG and MNU, or between a urea and a nitroguanidine, and between NG and MNTS. Resistance of S/Ng 2 is not against compounds which can yield diazomethane.

Since S/Ng 1 was resistant to MNU and MNTS, these compounds belong, in this respect, to a class of agents which include nitrogen mustard, azaserine, Mitomycin C, and which are radiomimetic. It is pertinent, therefore, that a mutant resistant to MNU was resistant to NG. This, again, we have observed with other compounds of this class: that first stage resistant mutants of each are cross-resistant with all other compounds of this class.

There are, therefore, at least two different types of events in the course of developing increased resistance to NG. One is a change in the physiology of the organism which involves a response to a variety of chemical compounds, all of which are radiomimetic, and to radiation itself. The other rejects the inhibitor *per se* or structurally related chemicals. With NG we have localized the portion of the molecule which seems to be concerned in the second type of resistance. We do not know, but may assume, that it is this portion of the molecule which is important to the inhibitory properties of the compound.

These experiments suggest many questions: Is the sequence of mutations—chemically nonspecific prior to chemically specific—inevitable? Or is the one more readily induced than the other? Or does the nonspecific mutation prime the organism for subsequent mutations which are chemically specific? Or does the specific mutation require a larger amount of inducer which is possible only in strains able to tolerate these amounts because of the nonspecific mutation? Do similar sequences occur in other radiomimetic agents? And, finally, do these events occur in vertebrate cells and could they account for resistance to radiomimetic agents in tumors?

Summary. A series of analogues of 1-methyl-3-nitro-1-nitrosoguanidines (NG), differing in the alkyl substituent, was tested against 2 mutants of *E. coli* strain S, selected for successively increasing resistance to NG. In general, increasing resistance to NG was observed to be accompanied by increasing resistance to all the nitrosoguanidines. When N-methyl-N-nitrosourea and N-methyl-N-nitroso - *p* - toluenesulfonamide were tested against NG-resistant strains of *E. coli*, it was found that the first step resistant mutant was cross-resistant. However, the second step mutant, derived from the first, was more sensitive to both of these compounds than was the first step mutant. A first step mutant of *E. coli* strain S, selected for resistance to N-methyl-N-nitrosourea, was resistant to NG. S-methyl-N-nitropseudothiourea did not inhibit *E. coli* strain S.

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Erythrocyte Survival in Vitamin E-Deficient Monkeys.* (26145)

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Anemia as the earliest sign of Vit. E deficiency in the monkey was first reported by Day and Dinning(1). The anemia was slightly macrocytic, and was accompanied by a granulocytosis as evidence of marrow activity. These findings suggested that the anemia occurring might be the result of an increased rate of erythrocyte destruction. Results of an experiment designed to test this hypothesis are the subject of this report.

Methods. Monkeys of the *rhesus* type which had been on a diet deficient in Vit. E for varying periods of time were selected for this study. The composition and preparation of the diet have been described(2).

Erythrocytes were tagged with radio-active chromium according to the principles set forth by Gray and Sterling(3) and Ebaugh *et al.*(4) and modified for application to small animals by Marvin and Lucy(5). The one departure from the technic as described was use of the brachial vein rather than cardiac puncture for obtaining blood for tagging, and use of the ear for obtaining repeated samples. Complete blood counts were made at intervals, also from the ear veins. Radioactive chromium (Cr^{51}) was determined in a scintillation well counter using a thallium-activated sodium iodide crystal.

Results and discussion. Survival curves of 6 studies on 4 monkeys are shown in Fig. 1. Monkey #211 served as a replete control, having never been given an E-deficient diet. Its erythrocyte count was 5.8 million per cu mm and hemoglobin and hematocrits were normal. Maximal survival time was 98 days; the curve was symmetrical with no apparent changes in rate of chromium loss. This normal value agrees well with that of 100 ± 20 days reported by Harne, *et al.*(6) using the consecutive reticulocyte peak method.

Monkey #204 had been on the deficient diet only 35 days when its cells were tagged and injected. On the 137th day of the dietary regimen, no activity could be detected; a maximal survival time of 102 days. During this time, erythrocyte count was 5.7-5.9 millions per cu mm. Monkey #205 had an 88 day survival time when measured between the 188th and the 276th day on the deficient diet. During this time red cell count decreased from 5.55 to 4.24 millions per cu mm. This is possibly not a significant, but certainly a suggestive, shortening of the life span.

On the other hand, after 349 or more days on the diet, survival was shortened to 49, 45, and 35 days in monkeys 207, 205, and 204, respectively. Erythrocyte counts were between 3.02 and 3.98 millions during the time these determinations were being made.

Five months after addition of adequate daily supplements of tocopherol were given monkey #205, red cell count had risen from 3.07 to 5.85 millions. Life span of the eryth-

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