between germfree and conventional animals (figure 1). Experiment 3. Vaginal graft histology revealed cyclic changes in the appearance of cells which had been shed from the vaginal epithelial surface at various stages of the ovarian cycle. Massive penetration of leucocytes was observed in 4 grafts of 2 animals autopsied at metoestrus-2 (figure 2). Early signs of leucocytic influx were visible in the epithelium of two vaginal grafts in 1 animal sacrified at metoestrus-1. Signs of leucocytic remnants were present in the shedded cell layers of all vaginal transplants obtained during other stages of the ovarian cycle.

Discussion. The present experiments confirm cyclic changes in bacterial numbers during the ovarian cycle in the vagina of female mice[7-9]. In accordance with data in rats, bacterial numbers are low during the dioestrous period, and, generally, high when the vaginal epithelium shows maximal development around the time of ovulation. It was not clear whether the bacteria had already disappeared at metoestrus-1 when leucocytes were present in the upper layers of the vaginal epithelium but not yet in the vaginal lumen, or whether disappearance of bacteria coincided with the appearance of leucocytes in the vagina lumen at metoestrus-2.

Daily vaginal smears from germfree mice revealed cyclic changes in the vaginal smear contents. Leucocytic smears occurred regularly in all 3 strains of mice. In the germfree animals, leucocytes appeared in the vaginal epithelium at the same stage of the cycle (metoestrus-1; figure 1) in the upper epithelial layers as in conventional animals[6,10]; the crowding of leucocytes in the epithelium at that time did not seem to be different from that in conventional animals. Leucocytes were present in large numbers in the vaginal epithelium, lumen, and smear at metoestrus-2. It thus seems clear that influx of leucocytes in the postovulatory period in mice occurs in the absence of leucotactic stimuli from bacterial origin. It is difficult to conclude from the lower number of leucocytes in vaginal smears from germfree animals that the leucocytic response in germfree mice is quantitatively different from that in conventional mice. The technique of making smears from germfree animals in their special homecages is different from that of making smears from conventional animals in open cages.

The morphology of sterile transplanted vaginal tissue in cyclic mice revealed large numbers of leucocytes penetrating into the epithelium at metoestrus-1 and metoestrus-2 but not during the period with epithelial keratinization. Such leucocytic response was never seen in similar studies with rats[3]. This finding confirms the above conclusion of leucocytic influx into the vaginal epithelium in the absence of leucotactic stimuli of bacterial origin. It is concluded from the occurrence of leucocytes in the transplants that leucotactic stimuli arise in the epithelium itself. This conclusion is further supported by comparison of the histology of leucocytic influx in rats and mice. In rats leucocytes only penetrate the vaginal epithelium after it has lost its covering layers of cornified cells about 12 h after ovulation[4]. In mice, however, leucocytes start to penetrate in large numbers into the vaginal epithelium when still covered by a thick densely packed layer of cornified cells[6,10] (figure 1). Since cornified cells by themselves are not leucotactic[11] and, indeed, aggregation of leucocytes onto cornified cells is never observed, one would assume that the layers of disintegrating nucleated cells beneath the cornified cells start the formation of leucotactic material in the period after ovulation. It is wellknown that dying cells can produce leucotactic substances during autolysis[12].

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On the importance of thiols and disulphides and the antiviral action of dichloropyrimidines[1]

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Summary. Free SH or SS groups of infected cells are not involved in the antiviral action of dichloropyrimidines.

Dichloropyrimidines inhibit the growth of Polio 1, Vaccinia and Herpes simplex virus in cell cultures[2,3]. Considering that the inhibitory effect is antagonized by the combined addition of cysteine (or cystine) plus glutamine to the culture medium[4] and, in addition, that several thiols and disulphides enhance virus growth[5], the hypothesis might be advanced according to which dichloropyrimidines react with either SH or SS groups needed for virus growth, thus impairing production of infectious particles. To shed some light on that question, it has been deemed useful to establish whether sulphidryl reagents, known to react strongly with thiols, have an antiviral effect or potentiate that of dichloropyrimidines and, on the other hand, to determine if and which thiols and disulphides antagonize dichloropyrimidine inhibition of virus growth.

Material and methods. Cysteine and cystine, cysteamine and cystamine, mercaptopropionylglycine, glutathione SH and SS, N-ethylmaleimide, parachloromercuribenzoic acid (CMB), and $CuCl_2$ were furnished by Fluka, Buchs SG, Switzerland, as well as 2-amino-4,6-dichloropyrimidine (ADCP), which was the only dichloropyrimidine tested. Ethacrynic acid was obtained as aqueous extract from tablets of Reomax (Bioindustria, Italy). Virus strains (kindly provided by the National Institutes of Health, Bethesda, Md., USA) were Polio 1, Vesicular stomatitis, Encephalomyocarditis, Newcastle disease, Vaccinia and Herpes simplex 1 virus. Experiments were

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Table 1. Effect of sulphidryl reagents on virus growth and on antiviral effect of 2-amino-4,6-dichloropyrimidine (ADCP)

Virus growth (in infectious units) after 24 h at 37 °C Drugs in Eagle's MEM (μg/ml)*		Polio 1 virus	Vaccinia virus	Herpes simplex 1 virus	Encephalomyocarditis virus	Vesicular stomatitis virus	Newcastle disease virus
–		6.5×10^7	1.1×10^7	3.4×10^7	5.9×10^7	8.2×10^6	6.9×10^6
ADCP	100	3.6×10^5	3.2×10^5	8.4×10^5	6.2×10^7	7.1×10^6	5.5×10^6
N-ethylmaleimide	1.5	5.8×10^7	9.9×10^6	1.8×10^7	3.8×10^7	9.2×10^6	6.6×10^6
CMB	1.5	6.3×10^7	1.2×10^7	1.9×10^7	4.1×10^7	1.2×10^7	5.3×10^6
$CuCl_2$	1.2	3.9×10^7	8.4×10^6	3.6×10^7	4.5×10^7	5.9×10^6	7.2×10^6
Ethacrynic acid	15	4.4×10^7	4.5×10^5	2.2×10^7	5.6×10^7	7.1×10^6	4.8×10^6
ADCP + N-ethylmaleimide	100 + 1.5	3.5×10^5	4.1×10^5	9.1×10^5	6.2×10^7	6.5×10^6	6.6×10^6
ADCP + CMB	100 + 1.5	1.8×10^5	3.2×10^5	7.5×10^5	5.1×10^7	6.9×10^6	5.4×10^6
ADCP + $CuCl_2$	100 + 1.2	5.1×10^5	3.3×10^5	1.1×10^6	1.1×10^7	8.4×10^6	3.7×10^6
ADCP + ethacrynic acid	100 + 15	2.2×10^5	2.0×10^4	9.5×10^4	6.3×10^7	8.3×10^6	4.4×10^6

* ADCP was used at $^1/_2$ of one MNCTD; the other drugs at $^2/_3$ of one MNCTD.

carried out on human aneuploid HEp 2 cells (American type culture collection, Rockville, Md., USA) which were grown in Eagle's MEM (Hank's base, pH 7.3)[6] supplemented with 7% calf serum. 16-h-old cell monolayers (2×10^6 cells/sample) were infected with 10 infectious units of each virus per cell and incubated at 20 °C for 1 h. Cells were then washed 3 times in Hank's BSS and incubated at 37 °C either in Eagle's MEM (2% calf serum, pH 7.5) or in the same medium, deprived of the amino acid supplement. Drugs were added at time zero after infection, at $^2/_3$ of the maximum non-cytotoxic dose (MNCTD) previously determined. Production of infectious virus was measured 24 h later, starting with the whole cultures. More details of technique have been given previously[2].

Results. Data from experiments with sulphidryl reagents (reported in table 1) show that potent thiol inactivating agents, such as N-ethylmaleimide[7], CMB[8] and $CuCl_2$[9] are practically deprived of both antiviral action and potentiating effect on that of 2-amino-4,6-dichloropyrimidine, even if added at $^2/_3$ of MNCTD. The sole active component of this group is ethacrynic acid[10] which, however, inhibits vaccinia virus only and potentiates the effect of 2-amino-4,6-dichloropyrimidine on Vaccinia and Herpes simplex virus. Since cystine and glutamine, which together counteract the antiviral effect of dichloropyrimidines[3], are both included in the amino acid supplement of Eagle's MEM, experiments on the antagonistic effect of thiols and disulphides were carried out in amino-acid-free Eagle's MEM and therefore restricted to Polio virus 1 which, among dichloropyrimidine-sensitive viruses, is the only one able to grow in such poor medium. Data from these experiments, reported in table 2, show that, among all thiols and disulphides tested, only mercaptopropionylglycine antagonizes the antipolio effect of 2-amino-4,6-dichlopyrimidine, while cysteine and cystine, cysteamine and cystamine, glutathione SH and SS are deprived of this antagonistic effect. As shown in the second column of table 2, simultaneous addition of glutamine to the medium makes, as expected[4], cysteine and cystine able to antagonize antiviral action of the dichloropyrimine, but has no such effect on the other thiols and disulphides. In conclusion, data reported here indicate that the typical, wide-range antiviral effect of dichloropyrimidines is neither shared nor potentiated by strong sulphidryl reagents, nor is it antagonized by several thiols and disulphides, all found able to enhance virus growth[5] and including cysteine and cystine as well as glutathione SH and SS which amount to about 100% of the intracellular SH and SS groups. Taking into account these data, the possibility that dichloropyrimidines inhibit virus growth by merely impairing functions of intracellular thiols and disulphides is to be ruled out. In previous papers, a hypothesis was advanced tentatively to explain the mechanism of antiviral action of dichloropyrimidines. Based on the ability of these drugs to impair assembly of Polio virus and on the antagonism produced by cysteine (or cystine) plus glutamine on that effect, it was proposed that dichloropyrimidines interact with cysteine and glutamine radicals located in critical points of structural polypeptides of Polio virus, thus preventing organization of virus particles[3]. In the light of the data referred to here, that hypothesis seems even more tenable. The presence of the above radicals in mercaptopropionylglycine would make this thiol a potent antagonist of the antiviral effect of dichloropyrimidines.

Table 2. Antagonism produced by thiols and disulphides on the antipolio effect of 2-amino-4,6-dichloropyrimidine (ADCP)

Drugs in amino acid-free medium (AFE)	(ng/ml)*	Virus yield at 24 h in AFE medium containing no glutamine	Virus yield at 24 h in AFE medium containing glutamine (20 μg/ml)
–		3.5×10^7	6.4×10^7
ADCP	100	4.1×10^5	4.3×10^5
Cysteine	40	3.9×10^7	6.9×10^7
Cystine	40	5.1×10^7	5.4×10^7
Cysteamine	20	4.1×10^7	4.8×10^7
Cystamine	20	5.3×10^7	4.2×10^7
Glutathione SH	800	6.9×10^7	6.4×10^7
Glutathione SS	800	7.1×10^7	7.7×10^7
Mercaptopropionylglycine	600	7.0×10^7	6.4×10^7
ADCP + cysteine	100 + 40	9.4×10^5	2.2×10^7
ADCP + cystine	100 + 40	8.5×10^5	3.1×10^7
ADCP + cysteamine	100 + 20	6.6×10^5	7.5×10^5
ADCP + cystamine	100 + 20	5.4×10^5	4.1×10^5
ADCP + glutathione SH	100 + 800	6.1×10^5	6.4×10^5
ADCP + glutathione SS	100 + 800	7.1×10^5	7.4×10^5
ADCP + mercaptopropionylglycine	100 + 600	2.8×10^7	1.9×10^7

*ADCP was used at $^1/_2$ of one MNCTD; the other drugs at $^2/_3$ of one MNCTD.

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