ditional series of control tests were carried out: 1) 3 mice at the height of reaction to the induced encephalomyelitis were killed and suspensions of the brain and cord of each, in dilution of 10^{-1}, were injected intracerebrally into 10 mice. None of the 30 W-Swiss mice exhibited neurological signs. 2) The material used for inoculation was tested for the presence of an infective element transmissible to mice; none was found. It would thus appear that the induced encephalomyelitis is not caused by a transmissible agent present in the inocula or in the stock mice.

To conclude, disseminated encephalomyelitis was readily induced in W-Swiss mice by means of intramuscular injections of brain tissue combined with liquid petrolatum and killed tubercle bacilli—the latter being the constitutents of a modified "adjuvant" technic of Freund and McDermott.[3]

[3] Freund, J., and McDermott, K., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 548.

17009

Tests for Chemical Mutagens in *Drosophila* Using the Vaginal Douche Technic.*†

IRWIN H. HERSKOWITZ. (Introduced by F. G. Brazda.)

From the Department of Surgery, Louisiana State University School of Medicine, New Orleans.

In a previous note[1] data were presented demonstrating that the rate of mutation is increased in spermatozoa when the latter are exposed to methyl bis (β-chloroethyl) amine hydrochloride introduced into females of *Drosophila melanogaster* by vaginal douche. In addition, there seemed to be an increase in the number of mutations occurring in the egg chromosomes of females that were treated with the N mustard in this manner. It is the purpose of this paper to offer additional data which confirms these observations and to present data on the mutagenic activity of copper sulfate, formaldehyde, 2,4-dinitrophenol, hydrazine hydrate and trypsin, employing this technic. These results will be discussed together with those of other workers testing these chemicals in *Drosophila*.

Material and method. The Oregon-R wild type and the Muller-5 (sc^{81} B In-S w^a sc^8) stocks of *Drosophila melanogaster* were used in these experiments. Virgin females from either one of these stocks were aged for 2 to 5 days, injected with a chemical substance, and then individually placed in vials with several males from the other stock. The males had been isolated soon after hatching. Injections were made as described previously,[1] but in the later experiments a set-up of ring-stand clamps, arranged to steady the hands during injection, was substituted for the micromanipulator. Females were observed for copulation for 2 hours after the injection. Only a single mating was permitted, after which the males were killed and the females placed individually in "creamers" or vials containing culture medium. Transfers to fresh creamers or vials were made every 2 to 3 days until no further offspring were produced. As in the earlier experiments, all the first generation females except those lost through accident were tested for lethal mutations involving the X chromosome from the Ore-R stock. Except for a period in the N mustard experiments reported here, in which the food was not enriched with brewer's yeast, all the first generation females were also tested for lethals involving the X chromosome from the Muller-5 stock. The plan of the crosses made for

* Part of this investigation was done at Columbia University, New York, and the Long Island Biological Laboratory at Cold Spring Harbor, N. Y.

† This work has been supported by a grant from the U. S. Public Health Service.

[1] Herskowitz, I. H., *Evolution*, 1947, **1**, 111.

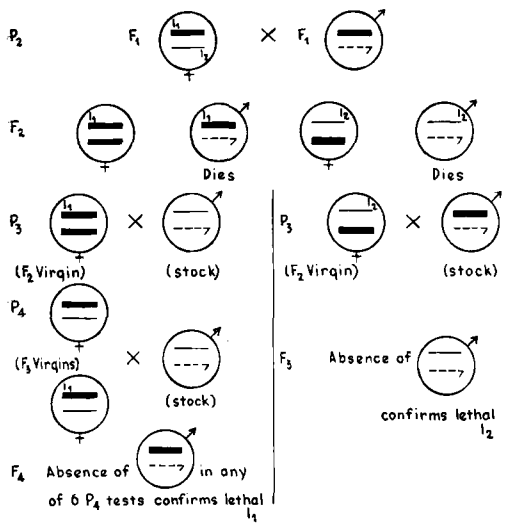

FIG. 1.

Plan of crosses to detect and confirm lethals in either X chromosome of F_1 ♀♀ from crosses between Ore-R and Muller-5 stocks. Heavy line = X chromosome from one of these stocks; light line = X chromosome from the other; broken line = Y chromosome. Single pair matings throughout.

the detection and confirmation of lethals involving either of the X chromosomes of F_1 females is presented in Fig. I where it is assumed for clarity that both X chromosomes have different lethal mutations (l_1 and l_2). Lethal mutations originating in the sperm X chromosome (l_2) can be detected in the F_2 and retested in the F_3 generation. Lethals originating in the egg X chromosome (l_1) also can be detected in the F_2 but require an F_4 generation for confirmation.

In the N mustard experiments females failing to copulate within the 2 hour period after injection were isolated individually with one male for an unlimited period of time. The rest of the procedure was the same for these females as for those that had copulated within the time allotted, with one exception. Instead of using all the first generation females, only random samples from the first vial and from the transfer vials yielding the last female progeny were taken for tests of lethals.

F_2 cultures containing potential lethals were counted every 2 to 3 days for a month after the cross was made or until no further offspring emerged. If few or no males appeared of either the Ore-R or Muller-5 type the appropriate X chromosome was retested in a subsequent generation. Retests for mutations were performed under the same experimental conditions used for obtaining the F_2 generation. A lethal mutation was considered confirmed if the total retest population contained 1% or less of males with the tested X chromosome, and a semilethal confirmed if 1-10% of the individuals in the retest generation were males with the X chromosome tested.

Saline, prepared according to Buck and Melland,[2] was the solvent for all the chemical substances except formaldehyde, which was prepared with distilled water, and hydrazine hydrate which was used undiluted. The solutions were made just prior to use. Cultures were kept at room temperature.

Results. The fate of 414 females treated with chemicals by vaginal douche is presented in Table I. Of these, 294 females (71%) copulated within 2 hours after injection. However, this figure is composed of only 20 of the 66 Ore-R females treated (30%) as compared with 274 of the 348 Muller-5 females (79%). In a total of 359 copulations observed with Muller-5 females in these and similar experiments, 47 (13%) occurred while the females were anesthetized; among the 20 copulations observed with Ore-R females none occurred while the females were still under the effects of ether. Copulation with etherized females occurred in a variety of experiments involving the injection of 7 different chemical substances and resulted in apparently normal deliveries of sperm. The concentration of the chemical substance injected seems to affect the number of females that oviposit and, of these, the number that produce adult offspring. When the highest concentrations of copper sulfate and formaldehyde were used only 10 of 54 females that copulated (19%) deposited eggs. When intermediate concentrations of these chemicals were injected 20 of 34 females oviposited (60%), and with the lowest doses 28 of 42 (67%). With the highest concentrations of copper sulfate and formaldehyde none of the 10 ovipositing females produced adult pro-

[2] Buck, J. B., and Melland, A. M., *J. Hered.*, 1942, **33**, 173.

TABLE I.
Fate of ♀ ♀ Treated with Chemicals by Vaginal Douche.

Chemical	Conc., %	Date	Stock	No. ♀ ♀ Injected	Copulating within 2 hr	Ovipositing sterile eggs	Producing adult offspring
N mustard	4	9/48	M-5	71	55	9	26
			Ore-R	50	12	—	8
Formaldehyde	10	9/47	M-5	19	18	2	0
	5		M-5	28	22	3	13
	1		M-5	19	14	1	12
Copper sulfate	saturated sol. and 5%	9/47	M-5	46	36	7	0 (1 larvae)
	1.0		M-5	15	12	3	1
	0.2		M-5	34	28	5	9 "
2,4-Dinitrophenol	saturated	5/47	M-5	78	53	2	32
	sol.	7/47	Ore-R	16	8	1	6
Hydrazine hydrate	85	7/47	M-5	25	23	1	16
Trypsin	conc. sol.	7/47	M-5	13	13	0	8

TABLE II.
Summary of Tests for Mutagenic Action of Several Chemical Substances.*

Chemical	Conc., %	X chromosome lethals†				
		Muller-5 ♀ ♀ injected		Oregon-R ♀ ♀ injected		
		No. (Ore-R) lethals/ No. sperm tested	No. (M-5) lethals/ No. sperm tested	No. (M-5) lethals/ No. sperm tested	No. (Ore-R) lethals/ No. eggs tested	Total lethals/ total gametes tested
N mustard	4	16/1043	6/710	7/373	3/373	32/2499
Formaldehyde	1 and 5	1/549	0/547			1/1096
Copper sulfate	0.2 and 1.0	0/183	1/150			1/333
2,4-Dinitrophenol	saturated sol.	1/751	2/739	0/562	1/562	4/2614
Hydrazine hydrate	85	1/623	2/593			3/1216
Trypsin	conc. sol.	2/506	2/506			4/1012

* Only includes tests from ♀ ♀ copulating within 2 hr after injection.
† All lethals were confirmed.

geny; however, when lower concentrations of these chemicals were used 35 of the 48 females that oviposited (73%) produced adult offspring.

The results of the tests for X chromosome lethals, in the daughters of females that copulated within 2 hours after injection with different chemical substances, are summarized in Table II. Only 13 lethals were observed in the 6271 sperm or egg X chromosomes tested in the formaldehyde, copper sulfate, 2,4-dinitrophenol, hydrazine hydrate and trypsin experiments. However, the N mustard treatments produced 32 lethals in 2499 sperm or egg X chromosomes tested, of which 23 lethals from 11 origins occurred in 1416 sperm chromosomes tested and 9 lethals from 8 origins occurred in 1083 egg chromosomes tested.

In the N mustard experiments females failing to copulate within the time allotted were not discarded. Instead, these females were utilized to obtain offspring that were the issue of the single pair matings taking place sometime later than 2 hours after injection. The results of egg and sperm X chromosome lethal

TABLE III.
The X Chromosome Lethal Mutation Rate in ♀♀ Copulating Later than 2 Hours After Injection with 4% N Mustard.*

Stock ♀♀ injected	No. fecund/ No. treated	No. (Ore-R) lethals/ No. sperm tested	No. (M-5) lethals/ No. eggs tested	No. (M-5) lethals/ No. sperm tested	No. (Ore-R) lethals/ No. eggs tested
Muller-5	7/16	1/444	4/363	—	—
Oregon-R	23/38	—	—	6/1091	10/1091

* All lethals were confirmed except 2 lost.

TABLE IV.
The Distribution, in the 4% N Mustard Experiments, of F_1 ♀♀ Bearing Lethals in Relation to the Total Number Tested.

Transfer vial No.	0	1	2	3	4	5	6 and 7
No. ♀♀ with sperm lethals/ No. ♀♀ tested	9/649	6/601	3/366	3/382	2/309	5/511	2/133
No. ♀♀ with egg lethals/ No. ♀♀ tested	18/649	1/564	1/188	1/268	1/246	1/489	0/133

tests with such female progeny are presented in Table III. Twenty-one lethals were obtained in a total of 2989 egg and sperm X chromosomes tested. However, only 7 of these lethals (from 6 origins) occurred in the 1535 sperm tested, whereas 14 lethals (from 13 origins) arose in the 1454 eggs tested.

Table IV shows the distribution in the various transfer vials of lethal bearing F_1 females in relation to the total number of females tested. Transfers were made every 2 days. The data come from parents copulating before as well as after the 2 hour period following injection with 4% N mustard. The 30 sperm lethals in the 2951 sperm chromosomes tested seem to be distributed at random in the transfer vials. This is not the case for the 23 egg lethals in the 2537 egg chromosomes tested. The frequency of egg lethals in F_1 females oviposited during the first two days (18/649) is significantly larger ($P < .01$) than the frequency of egg lethals in females coming from eggs deposited after that time (5/1888).

Besides the lethals, 2 visible mutants (rough and white eyes) and 2 semi-lethals occurred in the 4% N mustard experiments. These mutants occurred in the sperm X chromosomes of progeny from 3 females which had copulated within the two hours allotted. In addition, each of these parents had produced sperm lethals.

Discussion. The data in Table I offer several points of interest concerning the use and effects of vaginal douches in relation to studies of chemical mutagens in *Drosophila*. Much stronger concentrations of chemical substances may be used in vaginal douche treatments than in most other technics designed to treat gametes with chemicals for the purpose of inducing mutations. This seems to be due to the fact that only a portion of the individual is treated with the chemical substance in this procedure. Low concentrations of mutagens may be effective because of the direct contact of the chemical with the sperm.[1] However, very strong concentrations of chemical substances can produce definite harmful effects on the females treated. Failure of injected females to oviposit is due in part to the fact that many females are weakened by the chemical and die in the first few days after the treatment. This is reflected in the decrease in the percent of females which oviposit after copulation as the concentrations of copper sulfate and formaldehyde are increased. The evidence that the percent of ovipositing females pro-

ducing adult progeny is lower with the highest concentrations of copper sulfate and formaldehyde than with more moderate concentrations has several possible interpretations. High concentrations of these chemicals may cause many gametes to become non-functional and/or may produce genetic or physiological changes subsequent to fertilization that result in death before maturity. Such effects should also result in a decrease in the number of adult offspring produced when the concentration of the chemical substance injected is increased. The vaginal douche experiments that employed 0.2% and 10.0% N mustard[1] were performed under comparable experimental conditions and furnish information on this point. Unfortunately, data from the experiments with 4% N mustard are not suitable for comparison since the food used had been enriched with brewer's yeast. The average number of males and females in the F_1 progeny per fecund female was 74 in the 0.2% treatments (from 24 parents) but was only 29 (from 9 parents) in the 10.0% treatments.

In the formaldehyde, copper sulfate, 2,4-dinitrophenol, hydrazine hydrate and trypsin experiments (Table II) there were no lethals in 562 Muller-5 sperm X chromosomes tested and 7 lethals in 2535 Muller-5 egg X chromosomes tested. In the same experiments there were 5 lethals in 2612 Ore-R sperm X chromosomes tested and one lethal in 562 Ore-R egg X chromosomes tested. This suggests that the spontaneous lethal mutation rate in the Muller-5 X chromosome is much the same as in the Ore-R.[3-5] Under comparable experimental conditions we may also note the mutational response of these two chromosomes when exposed to the action of a mutagenic agent (Table II). In treatments with 4% N mustard 16 lethals occurred in 1043 Ore-R sperm tested and 7 in 373 Muller-5 sperm tested; 3 lethals occurred in 373 Ore-R eggs tested and 6 in 710 Muller-5 eggs tested. These data suggest that both the Oregon-R and the Muller-5 X chromosomes mutate with similar frequencies when exposed to N mustard. Accordingly, no distinction will be made between Ore-R and Muller-5 X chromosomes in the discussion which follows.

Vaginal douches with India ink suspensions showed that particles of the ink remained in the vagina of some virgins when these were dissected 12 hours after injection.[1] It seemed desirable, nevertheless, to test the egg and sperm lethal mutation rate in females copulating later than 2 hours after vaginal douche with 4% N mustard solution. In these experiments (Table III), there were 7 lethals in 1535 sperm chromosomes tested while in a similar number of egg chromosomes tested, 1454, there were 14 lethals. While the egg mutation rate in these experiments (14/1454) and the mutation rate in eggs from females copulating within 2 hours after injection (9/1083, Table II) seem to be comparable, the sperm mutation rate (7/1535) is considerably lower than the one in sperm deposited soon after injection (23/1416, Table II). This effect may be attributed to the removal of the mutagenic agent from the vagina sometime after the two hours allotted for copulation. The removal of the mutagenic agent may have been the result of any one or more of the following: diffusion through the body, expulsion from the vagina, chemical decay of the agent, and chemical combination with the body substances.

In the 4% N mustard treatments the lethal mutation rate in egg X chromosomes (23/2537, Tables II and III) appears to be lower than the rate in sperm X chromosomes from females copulating soon after injection (23/1416, Table II). An examination of the distribution of the egg and sperm lethals from the 4% N mustard experiments in relation to the time of hatching of the F_1 females may lead to a better understanding of the basis for the apparent difference in mutability of egg and sperm X chromosomes (Table IV). The distribution of sperm lethals in the various transfer vials seems to be at random. Accepting the hypothesis that the mutagenic agent disappears from the vagina soon after injection, this distribution is consistent with the

[3] Demerec, M., *Carnegie Inst. Wash. Yearb.*, 1946, **45**, 156.

[4] ———, *Nature*, 1947, **159**, 604.

[5] ———, *Genetics*, 1948, **33**, 337.

evidence that the genes in mature sperm are not functional.[6,7] The preponderant occurrence of egg lethals in the individuals oviposited during the first 2 days may have several explanations. Since, in all the experiments with N mustard there were no F_1 females with lethals on both X chromosomes, and in view of the data indicating that egg chromosomes mutate when sperm chromosomes do not (Table III), it is suggested that the production of egg mutations is independent of the treatment of sperm and that eggs are treated with the mutagenic agent directly. However, the means whereby eggs are exposed to the mutagenic agent still remains obscure and the apparent difference in the mutability of eggs and sperm may be the result of differences in the concentration of mutagen to which each is exposed or to a difference in the mutability of mature and immature ova.

We shall consider briefly the results of lethal tests from the N mustard, copper sulfate, formaldehyde, and 2,4-dinitrophenol experiments (Table II) with reference to the studies of other workers employing these and related chemicals in *Drosophila*. The pioneer work of Auerbach and Robson[8-10] clearly demonstrated that mustard gas and certain N and S mustards are chemical mutagens. Using the vapor and spray technics to treat mature sperm, they obtained up to 24% lethals with mustard gas and up to 13% with N mustard. Demerec[3,4] found the N mustard, methyl bis (β-chloroethyl) amine hydrochloride, to be mutagenic in treatments of spermatocytes and mature sperm using the aerosol technic. The author, in this and a previous paper,[1] has presented evidence that mutations in eggs and mature sperm are produced when this N mustard is used in vaginal douches. Such factors as the solvent for, and the concentration of, the chemical substance used should be taken into consideration in evaluating different technics for the chemical induction of mutations.

Magrizhikovskaja,[11] by bathing whole eggs, Law,[12] by injecting larvae and bathing dechorionated eggs, and Zamehof,[13] by feeding larvae, all obtained data indicating that copper sulfate is mutagenic. However, Demerec,[3] using aerosols to treat mature sperm and spermatocytes, and the author, using vaginal douches to treat eggs and mature sperm, obtained negative results with this chemical. Rapoport[14] and Kaplan[15] found positive mutagenic effects after feeding early developmental stages with formaldehyde but the author, using vaginal douches, obtained negative results. Thornton[16] has shown that 2,4-dinitrophenol decreases the growth rate and delays the moulting and pupation of *Drosophila* larvae. This drug is known to speed up the intracellular oxidative metabolism, principally of fat, in rats[17] and to inhibit phosphate uptake in staphylococci and yeast.[18] Assuming the effects of this chemical are similar in *Drosophila* it seemed desirable to test whether this chemical could influence the mutation rate via some metabolic effect. Vaginal douches with solutions of this chemical have not produced a detectable increase in the lethal mutation rate of sperm or egg X chromosomes.

Although negative results, in general, may be due to a variety of factors, it may be of interest to suggest some reasons for the failure of the aerosol and vaginal douche technics to produce mutations after treatments with copper sulfate and formaldehyde. If these

6 Muller, H. J., and Settles, F., *Z. f. ind. Abst.-u. Verer.*, 1927, **43**, 285.

7 Dobzhansky, Th., *Genetics*, 1930, **15**, 347.

8 Auerbach, C., and Robson, J. M., *Nature*, 1946, **157**, 302.

9 ———, *Proc. Roy. Soc. Edin.*, Sec. B, 1947, **62**, 271.

10 ———, *Proc. Roy. Soc. Edin.*, Sec. B, 1947, **62**, 284.

11 Magrzhikovskaja, K. W., *Biol. Zh.*, 1938, **7**, 635.

12 Law, L. W., *Proc. Nat. Acad. Sci.*, 1938, **24**, 546.

13 Zamenhof, S., *J. Genet.*, 1945, **47**, 69.

14 Rapoport, I. A., (Doklady), *C.R. Acad. Sci. U.R.S.S.*, 1947, **56**, 537.

15 Kaplan, W. D., *Science*, 1948, **108**, 43.

16 Thornton, D., *Growth*, 1947, **11**, 51.

17 Hall, V. E., Field, J., Sahyun, M., Cutting, W. C., and Tainter, M. L., *Am. J. Physiol.*, 1933, **106**, 432.

18 Hotchkiss, R. D., *Advances in Enzymology*, 1944, **4**, 153. Interscience Pub., Inc., New York.

chemicals do reach and affect eggs, spermatocytes and mature sperm, and some support for this has been presented, the negative results obtained with these technics may be due to the relatively short duration of the treatments or to differences in the mutability of the germ plasm at various stages of its differentiation.

Summary. Vaginal douches of 4% methyl bis (β-chloroethyl) amine hydrochloride produced 53 lethals in 5488 egg and sperm X chromosomes tested while only 13 lethals were observed in the 6271 sperm or egg X chromosomes tested after treatments with formaldehyde, copper sulfate, 2,4-dinitrophenol, hydrazine hydrate and trypsin. The practicality of the vaginal douche technic for the induction of mutations with N mustard in both sperm and eggs, suggested from previous experiments, is confirmed in that 30 lethals occurred in the 2951 sperm tested and 23 in the 2537 eggs tested. The number of sperm X chromosome lethals seems to be considerably higher in F_1 females resulting from single copulations within a two hour period from the time of injection (23 lethals in 1416 tests) than it is in females from copulations after this interval (7 lethals in 1535 tests); the mutation rate for the egg X chromosome under these conditions remains apparently unaffected (9/1083 and 14/1454, respectively). Whereas the 30 sperm lethals seem to be distributed at random among the F_1 females oviposited at various times, the frequency of egg lethals in individuals oviposited during the first two days (18/649) is statistically larger than the frequency of lethals in F_1 females coming from eggs deposited after that time (5/1888).

The use and effects of vaginal douche treatments are discussed and results of the experiments evaluated in the light of studies of other workers testing these chemicals in Drosophila.

I wish to express my sincere appreciation to Dr. Walter J. Burdette for many helpful suggestions concerning the manuscript and to Professor Th. Dobzhansky for numerous discussions and suggestions throughout the course of these investigations.

17010

Effect of Alloxan Diabetes on Fertility and Gestation in the Rat.*

JO ANNE SINDEN AND BERNARD B. LONGWELL.

From the Department of Biochemistry, University of Colorado, School of Medicine, Denver.

The discovery of the diabetogenic properties of alloxan[1] gave to the study of metabolism a tool by means of which many vexing problems may be investigated. One of these is the effect of diabetes on reproduction, a problem whose importance has been emphasized by White and Hunt[2] in extensive investigations on human diabetics. Reproduction in animals made diabetic with alloxan has been studied by Miller,[3] Friedgood and Miller,[4] Davis, Fugo and Lawrence[5] and Hultquist.[6] Reported below are some observations on the rat made diabetic with alloxan in which the problem of fertility, gestation and the via-

* From the thesis presented to the Graduate School of the University of Colorado by Jo Anne Sinden in partial fulfillment of the requirements for the M.S. degree.

1 Dunn, J. S., and McLetchie, N. G. B., *Lancet*, 1943, **2**, 384.

2 White, P., and Hunt, H., *J. Clin. Endocrinology*, 1943, **3**, 500.

3 Miller, H. C., *Endocrinology*, 1947, **40**, 251.

4 Friedgood, C. E., and Miller, A. A., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 61.

5 Davis, M. E., Fugo, N. W., and Lawrence, K. G., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 638.

6 Hultquist, G., *Acta Path. et Microbiol. Scand.*, 1948, **25**, 131.