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Extra-Mural Research - Dr. J.M. Robson

Experiments on the action of toxic gases on Drosophila melanogaster. by

C. Auerbach, M.Y. Ansari and J.M. Robson

(from the Department of Animal Genetics and of Pharmacology, University of Edinburgh).

SUMMARY

1. Tests for an effect on genes and chromosome structure were carried out with the following substances: lewisite, HN2, HN3, T.724, osmic acid and ammonia.

2. HN2 and HN3 were shown to produce both mutations and chromosome rearrangements. T.724 produced mutations; it is probable that it can also break chromosomes, though it is not definitely proven.

3. No effects on chromosomes and genes were obtained wi h L and osmic acid. The data on ammonia do not exclude a very slight effect on mutation rate, but do not make it probable.

Methods.

HN2, Lewisite, HN3, T.724.

In all but the early experiments with lewisite, the spray method described by Koller et al. (1943) was used. HN2 was used in solution in cyclohexane, lewisite in solution in alcohol, while HN3 and T.724 were sprayed as the pure liquids. The weights of the substances delivered at each compression were as follows;

HN2: 1 in 1 in cyclohexane: 3.75 mg.
Lewisite: 1 in 6 in alcohol: 0.74 mg.
HN3 (pure): 0.77 mg.
T.724(pure): 0.36 mg.

In early experiments with lewisite, the substance was vaporised in a large chamber (about 1 cubic metre). The spray method was, however, much more convenient and was used in the later experiments.

The methods used with ammonia and osmic acid are described in the text.

As has been discussed in some detail in the second report on the action of mustard gas on Drosophila (Auerbach and Robson, 1942), it was found that the genetical effect of a given dose depends not only on environmental conditions, notably temperature, which were not controlled, but also to a high degree on the constitution and behaviour of the flies themselves. These factors exercise their influence presumably by determining how much of the gas present in the environment of the fly reaches the germ cells, or in other words by determining the effective dose to which the germ cells are actually exposed. Hence one has to distinguish between the dose as measured by the investigator and the effective dose acting on the germ cells. For a comparison, therefore, between different experiments, a biological assay of the effective dose is required. The ClB test (see report I, Auerbach and Robson, 1942) was chosen for the purpose, and it was carried out as a routine check in all experiments in which it did not already form part of the main design.

/Three

Three breeding methods have been used for determining whether or not a substance produces an effect on the chromosomes.

Method 1. (see report II). Treated ♂♂ were mated to untreated virgin ♀♀ and the hatchability of the eggs laid by these ♀♀ was determined. Decreased hatchability as compared with controls was taken as suggesting that chromosomal disturbances had been produced by the substance. Normal hatchability makes it unlikely that chromosome breaks are produced, but does not exclude the possibility of induced genic mutations or small deficiencies. This method was used for every substance as a preliminary test, and as a means of determining the most suitable dose for further experiments.

Method 2. Treated ♂♂ were subjected to the standard ClB test (see report 1), and the frequency of newly arisen sex-linked recessive lethals was determined by inspection of the F_2 cultures, each culture representing one treated X-chromosome. This method too, was used with every substance. A positive result was taken to indicate that the substance had an effect on the genes. The stocks used for treatment were (a) Florida 4(Fo4), a closely inbred wild-type stock, and (b) Oregon-K(OrK), another wild-type stock kept in mass cultures. The spontaneous mutation rates were determined once for each stock. Separate controls for each experiment were not considered necessary in the search for substances which, by analogy with the results obtained with H, were expected to produce effects significantly exceeding the range of spontaneous mutation rates.

Method 3. Treated ♂♂ were mated to attached-X ♀♀ homozygous for the recessive sex-linked marker genes yellow (y), vermilion (v), and forked (f). The ♂♂ were left with the ♀♀ for about a week. This method furnishes a test for the ability of the substance to produce chromosome breaks. It gives a quantitative measure for the occurrence of two types of re-arrangement, namely: (a) deletion, i.e. loss of a large fragment out of the X-chromosome, and (b) translocation, occurring before the reduction division, by which a large piece of the X becomes transferred to the Y or to an autosome; subsequent separation of the two chromosomes involved in the translocation leads to the formation of spermatozoa with a deleted X only, as in (a).

♀♀ which receive a deleted X from their father in addition to their attached X-s will have one or more of the markers covered up by their normal allelomorphs. They will also usually show more or less pronounced signs of hyperploidy, though not to the same degree as the small percentage of regularly occurring triple-X ♀♀ in which all 3 markers are covered up by the presence of a complete paternal X. This test was carried out only when the results of at least the first method indicated that a positive effect might be expected. The ♂♂ used for it belonged to stock Fo4.

Results

(a) Lewisite. With the doses used, about 50% of the exposed ♂♂ died immediately or shortly after exposure. Eggs laid by survivors (of which the majority died during the next few days) had normal hatchability.

Two independent ClB tests were carried out on OrK ♂♂, the first of them with controls. The results were as follows:

/Series

Series	No. of tested X-chromosomes (F_2 cultures)	No. of lethals	% of lethals
L 5, treated	1271	6 (+ 1 semileth)	0.5
L 5, control	691	8	0.9
L 11, treated	774	1	0.1

Doses used: L 5 was carried out in the gas chamber, the flies being exposed for 30 min. at 18°C., 1.5 c.c. of lewisite was vaporised in the chamber. L 11 was carried out with the spray apparatus, the flies being exposed to a solution of 1 part lewisite in 6 parts absolute alcohol, sprayed at 1 min. intervals for 6 min. with 3 litre air supplied per min.

The percentages of lethals observed in both the treated and control series of experiment L 5 are near the upper limit of the range of spontaneous mutation rates. Since, however, it was even higher in the controls than in the treated group, the data do not indicate that an effect was produced by the gas. The mutation rate in experiment L 11 was well within the normal range. The two tests show that with the doses used - which are lethal to the majority of the flies - lewisite produces no effect on genes or on chromosomes. It was therefore considered unnecessary to try the third method.

(b) HN2. Sublethal doses of HN2 applied to adult ♂♂ markedly reduced their fertility. ♀♀, too, became partly or completely sterilized by appropriate sublethal doses. Dissection of some of these ♀♀ revealed abnormalities of ovarian development similar to those previously described for H (see report 1).

Several CLB tests on ♂♂ F♭4 aimed in proving that HN2 produces a considerable effect on the mutation rate. The data are as follows:

Expt.	No. of tested X-chromosomes	No. of lethals	% of lethals
HN2 1	69	9	13
HN2 2	609	31 (+ 7 semileth)	5
HN2 6	629	34 (semilethals not scored)	5.4

The records of the doses in HN2 1 and HN2 2 were unfortunately lost. In HN2 6 the dose was: 7 min. exposure to a concentration of 1 in 1 in cyclohexane, sprayed at intervals of 20 sec. with an air supply of 2 litre per min.

For the deletion test (third method) F♭4 ♂♂ were used. They were exposed simultaneously and in the same container as the ♂♂ for the CLB test HN2 6. Their ♀ progeny consisted of 2006 regular diploid ♀♀ showing all three markers, 196 triplo-X ♀♀, and 8 exceptional ♀♀ in which 1 or 2 of the markers were covered by a deleted X. Only one of the exceptions occurred among 1044 ♀♀ which had hatched from eggs laid within 10 days after treatment of the ♂♂. The remaining 7 exceptions were found among the 1166 ♀♀ which had hatched from eggs laid 11 or more days after treatment. In detail the exceptional ♀♀ consisted of 2f, 3v, 2vf, and 1 yv ♀. Two of the 3 v ♀♀ were found in the same culture bottle, and the possibility cannot be excluded that they were produced by

/the same

the same initial re-arrangement occurring at some time during spermatogenesis. This cannot have occurred with any of the other exceptions; hence at least 7 independent re-arrangements arose in the course of the experiment. The spontaneous occurrence of chromosome breaks is so extremely rare that this result can be taken as proof of the ability of HN2 to produce chromosome breaks and re-arrangements.

Other genetical abnormalities discovered in the experiments with HN2 include a number of total and fractional Minutes, one mutation to garnet, one to lozenge, as well as certain other unidentified mutations. Two ♀♀ heterozygous for a treated chromosome, did not hand it on to any of their progeny of either sex, presumably owing to a re-arrangement which caused the chromosome to be eliminated into the polar body.

The most interesting mutation was observed in a ♂, son of a treated father by an attached-X ♀, who both somatically and genetically was a mosaic for two different allelomorphs of white, neither of which was white proper. In the following generation, each allelomorph bred true, without tendency to further mutations.[x]

(c) HN3. ♂♂ treated with sublethal doses showed a marked decrease in fertility. The ClB test was carried out on ♂♂ Fo4, with a simultaneous determination of the spontaneous mutation rate of the stock. The results were as follows:

Series	No. of tested X-chromosomes	No. of lethals		% of lethals
Treated	741	46	semilethals not scored	6.2
Controls	1274	3		0.25

Dose: pure HN3, sprayed at 10 sec. interval, for 5 min. with an air supply of 2 litres per min.

The data show conclusively that HN3 produces mutations.

For the deletion test (method 3) ♂♂ Fo4 were exposed simultaneously and in the same container as the ♂♂ for the ClB test. Their ♀ progeny consisted of 980 regular diploid yvf ♀♀, 50 triplo-X ♀♀ and 1 exceptional hyperploid vf ♀. This ♀ was tested by breeding and was found to carry a translocation of the left end of the X to the Y. The presence of 1 translocation in 980 flies might perhaps be considered a chance occurrence, not due to the action of the gas. A second translocation, between X and III, was, however, discovered genetically in one of the F_2 ♀♀ of the ClB test; the presence of the translocation was subsequently confirmed cytologically by Dr. Slizynski. The occurrence of two translocations strongly suggests that HN3 can produce chromosome breaks and re-arrangements.

Other genetical abnormalities obtained after treatment with HN3 included two mutations to lozenge (identified by phenotype only, not by breeding), several total and fractional minutes and a sterile ♀ which was both Minute and aristapedia.

(d) T.724. ♂♂ exposed to sublethal doses showed a marked decrease in fertility. The ClB test, carried out on ♂♂ Fo4, gave the following result:

No. of tested X-chromosomes	No. of lethals	% of lethals
586	50 (+ 4 probable lethals and 5 semilethals)	8.5

/The

x This finding, together with others which suggest that these substances may have an after effect on the treated chromosomes, will be discussed at a later date.

The dose was 5 min, of pure T.724, applied at a spray interval of 10 sec. with an air supply of 2 litres per min. The result proves that T.724 produces mutations.

For the deletion test ♂♂ Fo4 were exposed simultaneously and in the same container as those used for the ClB test. Their ♀ progeny consisted of 834 regular diploid yvf ♀♀, 4 triplo-X ♀♀, and 1 exceptional ♀ vf. This result, though it does not prove that T.724 produces chromosome breakage by T.724, at least points in this direction, especially as the small percentage of triplo-X ♀♀ indicates that the culture conditions were not favourable for the survival of unbalanced types.

Other abnormal flies in the experiments with T.724 included a number of total and fractional Minutes.

(e) Osmic acid. It seemed possible that the action of H on chromosomes and genes was dependant not so much on specific chemical properties of the H, as on certain more general characteristics, viz. the power to penetrate readily into the cell, and the capacity to fix proteins (see Foll, 1942). Consequently, another chemical substance possessing these properties was tested. The substance chosen was osmic acid. When flies are exposed to osmic acid vapour, a certain percentage, depending on the dosage, die within the next few days. Surviving ♂♂ are often unwilling or unable to mate, as seen from the fact that the receptacles of virgin ♀♀ placed with such ♂♂ frequently do not contain any spermatozoa. ♂♂ which copulate are perfectly fertile. The absence of zygotic lethality indicates that no gross chromosomal changes are produced in treated sperm.

The ClB test gave 3 lethals and 1 semilethal in 940 tested X-chromosomes, corresponding to 0.3% lethals, i.e. about the normal mutation rate for this stock (compare controls to HN3, ClB test).

(f) Ammonia. Ammonia vapour was tested for several reasons: firstly, it has a high power of penetration; secondly, it is among the few substances for which a slight effect on mutation rate has been claimed (Lobashow, 1937); and thirdly, there exists a certain relationship between the simple chemical structure of NH_3 and the more complicated ones of some active substances, like HN3. Flies were exposed at room temperature to the vapour of a 10% solution of ammonia in water (i.e. 1 in 10 of ammonia S.G. 0.88 in water). If kept constantly in this atmosphere, the animals die rapidly, But when small quantities of air are frequently introduced into the chamber containing the flies, exposures up to two hours are tolerated by a minority. Survivors, like the survivors from osmic acid treatment, often fail to mate, but matings which occur are perfectly fertile.

For the ClB test, ♂♂ Fo4 were exposed to a treatment extending over a total of one and a half hours, given in two parts separated by a 30 min. recovery period. About 25% of the flies died within the next 2 days, and the majority of the remainder during the following week. The test was carried out on two broods produced by the same parents. Brood I was produced during the first 4 days following treatment when presumably sperm was used which had already been mature at the time of exposure. Brood II was produced 11 or more days after treatment so that the sperm used for fertilization had presumably been exposed in earlier developmental stages. The following result was obtained:

Brood	No. of tested X-chromosomes	No. of lethals	% of lethals
I	597	4 (+ 1 possible)	0.7
II	485	1	0.2
Total	1082	5	0.5

/The data

The data - especially for mature sperm - are near the upper limit for the range of spontaneous mutation rates, but do not exceed it (compare e.g. the controls in the lewisite experiment which was, however, carried out on a different wild-type stock). These results show that ammonia certainly does not affect chromosomes and genes in the same drastic manner as mustard gas and related substances. The possibility of a very much slighter action is not excluded by our data.

Discussion

The data show that a group of vesicant substances can cause pronounced disturbances in the nuclear mechanism. Not only do they produce mutations, i.e. abnormalities which, by their very nature, are transmitted from a cell in which they arise to all its descendants, but they are also capable of breaking the continuity of the chromosome thread, thus giving rise to rearrangements which may interfere with the normal process of cell division. The capacity to produce mutations has been proved for H, HN2, HN3 and T.724 and these substances are indeed potent in this respect. In addition H and HN2 can definitely produce chromosome breaks, and it seems probable that HN3 and T.724 can also do so. Corroborative cytological evidence for the action of H, HN2, and HN3 on the chromosomes (of Tradescantia pollen grain) has been obtained by Koller et al. (1943 unpublished data).

The negative results with lewisite require some discussion, especially as they are in agreement with the results of Koller et al. (unpublished data) that lewisite produces no nuclear effects on the pollen grain of Tradescantia. In the case of Drosophila, it seems possible that lewisite kills the flies by an action on some essential organ or process at doses which are below the threshold necessary to produce an effect on the chromosomes. Even in the case of Tradescantia it is possible that the cytoplasm is more susceptible than the nucleus to the action of L, and that the cell rapidly dies from cytoplasmic poisoning before the effective dose for the nucleus is attained, or before any action which may have been exerted on the nucleus can become evident. This may, of course, also explain the effects observed in Drosophila. Hence it seems probable that the immediate pharmacological action of lewisite is not due to a primary effect on the nucleus. It would thus appear that the development of vesication is not necessarily linked up with visible chromosome disturbances and, in view of the negative result of the CIB test with lewisite, that vesication does not depend on an effect on the genes. It must be added, however, that this conclusion only holds on the assumption that the relative susceptibility of nucleus and cytoplasm to lewisite is similar in mammalian tissues and in the lower forms of life studied in this experiments.

The negative results with boric acid show that something more than mere power of penetration and ability of fixing proteins is required to produce a derangement of the genic material. Chemical constitution must be an important factor, and the difference between the action of H, HN2, HN3 and T.724 on the one hand and lewisite on the other, is probably dependent on differences in the chemical constitution. The chemical composition of ammonia shows certain similarities to HN2 and HN3. This is of interest in view of the contention of Lobashow (1937) that ammonia can increase the mutation rate in Drosophila. His results, obtained after injecting imagines with a solution of ammonia in Ringer or after exposing larvae to ammonia vapour, show a significant increase in mutation rate over that on the controls; the mutation rates in his controls were exceptionally low (0.05% as compared with 0.5% in the treated flies). Since the same controls obviously were used for several experiments it seems unlikely that treated and control flies were taken from the same culture bottle and tested at identical times, a condition which should be fulfilled when the significance of slight differences in mutation

/rate is

rate is estimated (Auerbach, 1941). In the present experiment with ammonia the mutation rate obtained in the treated flies was almost the same as in Lobashow's treated flies. No controls were taken, and for the reason just stated comparison with controls taken for other experiments are not reliable. However, all mutation rates taken over a long period of years in this laboratory agree in giving values of at least 0.1% with an average of about 0.3%; a rate of 0.7% as observed by us for mature spermatozoa after NH_3 treatment is not outside the range of the observed normal rates. Without further large scale and carefully controlled experiments it is thus impossible to decide whether NH_3 is able to exercise a slight action on the genic substance. It seems hardly worth while to carry out such laborious tests before substances which are intermediate in chemical character between the HN2 type and NH_3 have been tried, e.g. the ethylenimes. Koller, in extensive cytological tests with Tradescantia, obtained no evidence that ammonia can produce visible chromosome disturbances (personal communication).

The possible mode of action of H on the nucleus has been previously discussed (Koller et al, 1943) and similar consideration

probably applies to the action of HN2, HN3, and T.724. Further work on substances capable of producing mutations and chromosome breaks may throw more light on the actual chemical mechanisms involved. As has already been pointed out, it is not, at present, possible to say to what extent these nuclear changes are responsible for the effects which follow within a short time of contamination, but it seems likely that they play a part in retarding the healing of wounds, and they may also give an explanation for the tendency of these lesions to break down after healing is apparently complete.

References

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