THE EFFECT OF 3—4 BENZPYRENE IN RESPECT TO THE NON-DISJUNCTION FREQUENCY IN DROSOPHILA MELANOGASTER.

By: GYULA FÁBIÁN and A. GEDEON MATOLTSY.

(From the Hungarian Biological Research Institute, Tihany, Lake Balaton.)

With 4 Figures and 1 Table in the text.

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In considering the well-known theory that cancer originates from mutation, it is important to know what special effects cancerogenic substances may have on mutation. On the Drosophila, the best known genetical experimental material so far tried, cancer-causing hydrocarbons have been used to good purpose in this respect. *Auerbach* and *Friedrich-Freksa* (1940) have dealt with such Drosophila experiments in certain of their publications. They did not find that cancerogenic substances changed the gene mutation in Drosophila, which they explained as due either to the local concentration being scanty, or to the time during which the substance could act being insufficient.

In the following pages we deal with experiments in which we examined the dispersal of a cancerogenic hydrocarbon, benzpyrene, in the Drosophila organism under different life cycles, and investigated how its presence in the Drosophila egg may have affected the non-disjunction of the X-chromosomes.

TECHNIC.

Our investigations were carried out on 5-year, pure inbred, white-eyed stock, the animals constantly protected from rays of light during the experiment, at a temperature of 25°. We found the best means of introducing the substance was to mix it, in the form of minute crystals, in the standard Drosophila food. To the food of the imagos 30 mgr. benzpyrene was mixed with 4.5 gr. thin, yeasty food. This mixture

was smeared on pieces of filter paper the size of a postage stamp. By this method of feeding, benzpyrene crystals were constantly in the intestinal canals of the larvae or the imagos, and so there was constant absorption of benzpyrene into the tissues.

The presence of benzpyrene in the tissues and organs was established by flourescence microscope. The absorbed benzpyrene showed a pale blue fluorescence in the cells and tissues. Whole larvae and dissected organs in Ringer solution were also examined.

BENZPYRENE IN THE DROSOPHILA ORGANISM DURING THE DIFFERENT LIFE CYCLES.

We used a strain of specimens taken from white stock for the experiment, raised on a constant benzpyrene diet, in the dark. The Drosophila, raised in the dark on the constant benzpyrene diet showed no toxic effects. We followed this stock through 6 generations — each generation receiving the constant benzpyrene diet — and examined about 2000 individuals without finding any sort of abnormality which would have indicated a disturbance in the development.

It could be established in one cell, in relation to benzpyrene absorption, that the vacuoles and granules of the plasma showed lively, strong fluorescence, the nucleus none whatever. In the Drosophila the large, flat, 6-sided fat -cells and the malpighi tube cells are particularly suitable for such observations. (Fig. 1.) Our observations here coincide with those of GRAFFI (1939.), who, with another experimental object in view, also got benzpyrene dispersed in the plasma but not in the nucleus. It is possible that some special chemical substance in the cell nucleus "defends" it from the benzpyrene fluorescence, or that the benzpyrene molecules do not permeate the membrane of the nucleus; or again that such a small amount of benzpyrene is present that the fluorescence cannot be seen. In general our studies of the Drosophila so far show that the benzpyrene accumulates in the fat-containing granules and vacuoles of the cells, in the plasma dispersing uniformly, and its appearance in the organs also conforms to this fat-attracting phenomenon.

In the intestinal canals of larvae of the I or II stadium deposited on benzpyrene medium, solid benzpyrene crystals could be seen from the first day. 24 hours later a blue fluorescence diffused the whole organism, which shows that the absorption of benzpyrene began in the organs of the larvae. The systems of the control larvae, without benz-

pyrene, also showed a slight fluorescence in the ultra violet light of the fluorescence microscope, but this fluorescence was very light and greenish, in contrast to the benzpyrene-treated specimens, which were alight with a strong blue colour. We undertook partial examinations of the III. stadium larvae before pupating. (Fig. 2.) Decidedly strong fluorescence could be seen in their fat-bodies. Equally strongly fluorescent were the walls of the intestinal canal and malpighi tubes. Less strong, yet well differentiated from the controls, was the benzpyrene fluorescence in the imaginal buds and the cerebral ganglions. No trace of benzpyrene could be established in the salivary glands or in the sexual organs of either sex. While, for example, the fat-lobes adhering to the salivary glands were lighted by a bright blue fluorescence, the tissues and cells of the salivary glands themselves showed absolutely no fluorescence. In exactly the same way, the fat lobes surrounding the sexual organs were remarkably fluorescent, while the sexual organs themselves were entirely unilluminated.

The fluorescence continued after the larvae treated with benzpyrene had pupated. During the entire pupal life, with its complete metamorphosis, the blue fluorescence did not disappear. The accumulation of benzpyrene could be so marked that its blue light shone through the chitinous surface of the purparium under ultra violet irradiation, while only a greenish light could be seen in the untreated pupae. Just before emerging too, dissection of the pupae showed that it was primarily the fat cells and the intestinal canal that were lighted up.

The newly hatched imagos carried the benzpyrene on from the larval and pupal life and kept it for a time. If the imagos which had been fed on benzpyrene in their larval life were changed to a normal diet on hatching out (as imagos) the benzpyrene could still nevertheless be seen in their organisms for 72 hours. The benzpyrene could be seen in the fat cells, the intestinal canals and malpighi tubes of females 1—2 hours old, and in the developing oöcytes in females 12 hours old. After 24 hours the fluorescence in the fully developed ovaries could scarcely be distinguished from that of the controls, but the malpighi tubes and intestinal canals were still fluorescent. After 72 hours only the malpighi tubes showed traces of the benzpyrene.

Even if the larvae had not been fed on benzpyrene the imagos' organisms absorbed it to a high degree if they were given a mixture of benzpyrene and yeast. Here too the organs above mentioned took up the benzpyrene; only in the muscular system no trace of fluores-

cence could be seen. The most remarkable is the strong, bright fluorescence of the ovaries of the females specimens under this treatment. (Fig. 3.) The male gonads do not take up as much. The eggs deposited by females fed each day on fresh benzpyrene medium were always alight with a bright blue fluorescence, (Fig. 4.) while the control eggs gave a greenish light under the ultra violet rays. In the older eggs (about 12 hours) where the developed embryonic tissues also began to differentiate, the embryonic tissues took a dark blue fluorescence calour. The penetration of the benzpyrene into the eggs takes place in the ovarioles.

The first stage larvae emerge from the fluorescent egg completely viable and carry the benzpyrene fluorescence on with them, without further treatment. This lasts for 6—12 hours. After that time the benzpyrene disappears unless the treatment is repeated. But if they again get on to a benzpyrene culture medium the cycle begins again, as described above.

In summarizing the foregoing we can assert that:

1. Benzpyrene can be taken up by the Drosophila in any stage of the animal's development with the food.

2. During metamorphosis the substance is carried over from one phase to the other.

3. The benzpyrene taken by the mother arrives through the eggs in the I. stadium F/1 generation larvae.

GENETICAL INVESTIGATION.

As we mentioned in our introduction, the aim of our genetical experiments was to inquire into the significance of the presence of benzpyrene in spontaneous non-disjunction frequency. In the present communication we need not enlarge on the phenomenon of non-disjunction itself, so well known from the publications of MORGAN and many others, only touching on the details which seem necessary.

In the organisms of the specimens used in our experiments the quantity of cancerogenic substance taken was apportioned so that it must still give fluorescent light in the oöcytes of the newly hatched females which had been given benzpyrene in the larval state, this fluorescence being due to 13 gr.: 10 cgr. in the food. When it was the imagos which were treated, we wanted the laid eggs to have the same fluorescent colour. That this did take place we were convinced by tests carried out every 2 or 3 days under fluorescence microscope. (4—5 gr. food: 30 mg. benzpyrene).

For crossing we used w/w genotype females and B/y genotype males (Progeny to be expected: red-Bar phenotype females, white-normal males; the exceptions: white normal females, red-Bar males.).

We carried out 2 experiments. In the first (Table I.) we treated the females only in the larval state, immediately after hatching transferring them to a culture without benzpyrene. The males were not treated at all. Each female laid eggs for 8 days. There were 12 cultures with 2—2 females. In the other experiment (Table II.) both the males and females were kept on a benzpyrene culture medium, renewed every 24 hours. Here each female laid eggs during 2 to 4 weeks. We kept the control flies, receiving no benzpyrene, under similar conditions throughout the experiment. (Table III.)

The results of the experiment are shown in the following Table:

Treatment	Number of treated females	Offspring				Total ex. %
		Reg.	Ex.	Reg.	Ex.	
in larval life (I)	24	1190	2	1096	7	0·39±0·13
in adult life (II)	10	1235	—	998	—	
	15	1290	2	1167	—	
	25	2525	2	2165	—	0·04±0·03
Controls (III)	24	1075	2	1049	3	
	10	1466	5	1188	1	
	15	1470	1	1200	3	
	49	4011	8	3427	7	0·2±0·05

According to the above Table, in the control stock the exceptional individuals are present in the frequency of 0.2±0,5%, i. e., the proportion between the exceptions and the regular types is about 1:500. That this high percentage is not a chance effect can be assured by the fact that the total (7463) control progeny were created in three successive breeds and showed equally high percentages in all three steps. (1st control: 2124 regular, 5 exceptions, 2nd control: 2654 regular, 6 exceptions; 3d control: 2670 regular, 4 exceptions.) The 0.2±0,5% can thus be regarded as standard, by which to compare the results in the treated cultures.

In the first experiment, where the females were treated in the larval stage, there is no significant difference between the control and the treated stocks as to proportion of exceptional animals. (0,39±0,13.)

Under the second treatment, among the progeny from the 4692 eggs

of benzpyrene-treated parents, there was not a single exceptional male and we found 2 exceptional females brought about by the non-dividing of the X-chromosome. The proportion here is 1:2300. Expressed in percentage, the progeny hatched from the benzpyrene eggs was $0.04 \pm 0.03\%$ of exceptions. The difference between the control and the 2nd experiment was $0.16 \pm 0.05\%$ in respect to presence of exceptional animals. This difference (0.16) is 2.7 times greater than their standard error (0.059). In any case such a value makes it extremely likely that there is really a difference between the control and the 2nd experiment.

DISCUSSION.

The non-disjunction of the X chromosomes usually occurs with uniform frequency in groups taken from the same stocks in the manner of the gene mutation. This spontaneous proportion depends primarily on the genes of the stock, but after all in certain cases the physical and chemical state reigning in the plasma gives the last impulse when the reduction division of the cell takes place. This has been proved by well-known experiments in which heat, X-rays, as well as colchicine, (Gelei-Csik, 1940) have brought about changes in frequency of non-disjunction. From these studies we might expect that in our investigations of a chemical substance, benzpyrene, in the plasma the number of non-disjunctions might also be affected. In judging the results of our experiments from that standpoint, however, we must keep in mind the difference between the conditions of the lst and 2nd experiments; for in the lst experiment fluorescence microscopic observation disclosed that the mothers treated in the larval state had benzpyrene only in the young oöcytes. The eggs when laid were not fluorescent. Thus this type of pre-treatment could not be effective, nor do we find a significant difference from the standard values.

But in the 2nd experiment benzpyrene was constantly present, not only in the eggs developing in the ovarioles, but also in the eggs, when laid. Thus the benzpyrene was also there in the plasma during the egg's reduction division. This might lead us to expect that the chemical substance would have some effect on the standard number of non-disjunctions. And in reality it turned out that we got fewer exceptional progeny from the benzpyrene-treated eggs than were to be expected even of the control specimens. The statistical valuation showed the difference very strikingly; and further, if we consider the apparent proportion of exceptions in the 3 control cultures and the lst experiment

(1:500), and the exceptions among the progeny hatched from benzpyrene-treated eggs (1:2300), then we must conclude that the presence of benzpyrene actually diminishes the X-chromosome's non-disjunction frequency.

In a negative direction it is more difficult to determine changes in the usual proportions of spontaneous mutation, as we know of few similar cases which could be used for comparison. BERG and SEVERTZOFF (1941) observed in intraspecific hybridization the degree of descent in spontaneous mutation: IGNATIEV and SHAPIRO (1943) experienced the same on the effect of modifying factors; and according to ZAMENHOF (1943): "a decrease in the mutation rate of an unstable Drosophila gene has been effected by adding copper sulphate, sodium hydroxide, or ammonium hydroxyde to the food." We consider it imaginable that the stocks we used in our investigations may also be standard for non-disjunction, which after all depends on the genotype of the stock, the benzpyrene diminishing the gene mutation in the way ZAMENHOF describes in the case of other chemicals.

Another possible explanation is that the benzpyrene so affected the plasmatic condition of the eggs by way of physico-chemial, e. g. viscosity changes, that it "helped" the separation of the X-chromosome's from the synapsis.

Our experiments warrant the conclusion that benzpyrene, a photo-active cancerogenic substance, causes a change in the mechanism of heredity.

Whether this change, the decrease in the frequency of the non-disjunction, has an influence on the development of cancer or not should be investigated with further experiments.

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SUMMARY.

1.) A cancerogenic substance, benzpyrene, can be taken up by the Drosophila in any stage of the animal's development with its food, and its presence demonstrated by fluorescence microscope.

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2.) During metamorphosis the substance is carried over from one phase to the other.

3.) The cancerogenic substance is absorbed from the body of the mother through the ovarium into the eggs, and so can be seen in the eggs when laid and in the Ist stadium larvae.

4.) The presence of the cancerogenic substance in the egg diminishes the X-chromosome's non-disjunction frequency.

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EXPLANATION OF THE FIGURES.

Figure 1. The intestinal canal of an adult female Drosophila treated constantly with benzpyrene. "A" intestinal canal with brilliant benzpyrene crystals. "B" fat cell. "C" malpighian tube. Photograph under ultra violet light.

Figure 2. Larvae of Drosophila treated with benzpyrene under ultra violet light. The control is slightly illuminated by its neighbour.

Figure 3. The ovaries of an adult female Drosophila treated constantly with benzpyrene (the two luminous parts left and right), the dark ones are the two controls. Photograph under ultra violet light.

Figure 4. Eggs of Drosophila maintained on food containing benzpyrene, under ultra violet light. In the middle are three dark controls.

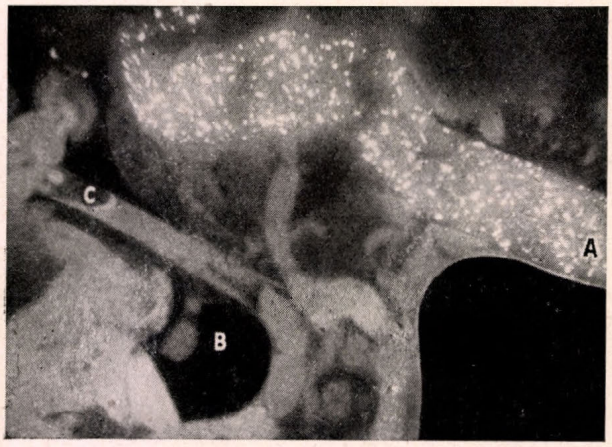

Fig. 1.

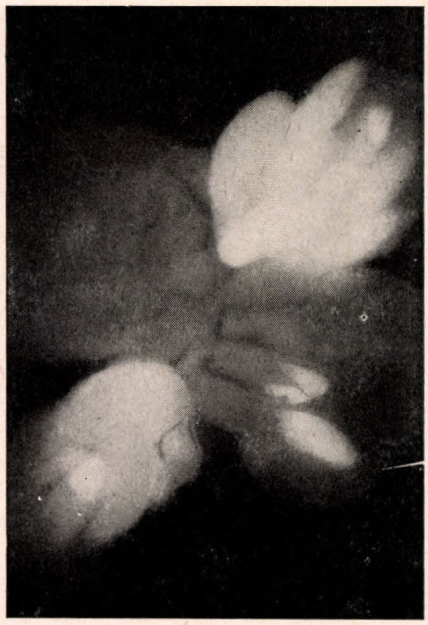

Fig. 3.

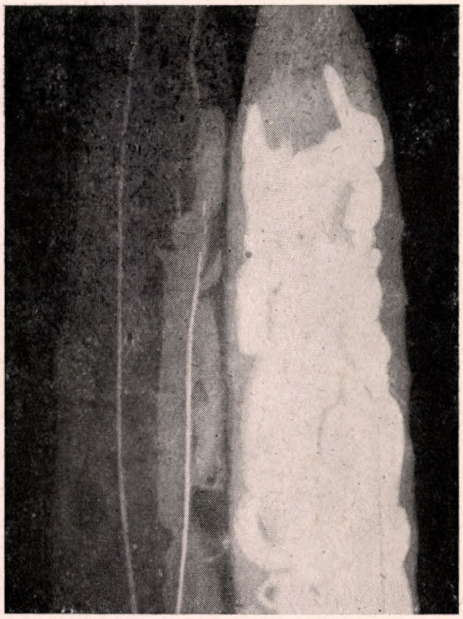

Fig. 2.

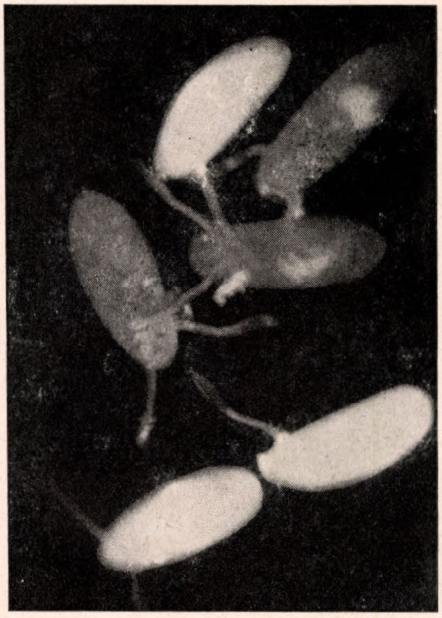

Fig. 4.