EFFECTS OF DNA BASE ANALOGUES ON THE SCALES OF THE WING OF EPHESTIA¹

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OUR knowledge concerning genic structure and function has been mostly derived from the study of microorganisms. The question, to what extent the conclusions from this work can be applied to multicellular organisms, has been repeatedly posed. In attempts to answer these questions, it appears desirable to apply to cells of higher organisms methods analogous to those which have been used so successfully with microorganisms. Consequently, much of the work on gene structure and function in higher organisms has been carried out on cultures of mammalian cells. Unfortunately, these cultured cells are in some respects atypical, having frequently aneuploid chromosome numbers, and showing abnormal nutritional requirements (DAVIDSON 1964). It appeared, therefore, desirable to attack the problem of gene structure and gene action in a normal developing system for which methods analogous to those used in microbial genetics are available.

Such a system was found in the scales of the hind wing of the moth *Anagasta (Ephestia) kühniella*. Every scale is a single highly differentiated cell. While the scales are somewhat variable in different parts of the wing, the normal variations are easily recognized so that scales of aberrant shape can be scored. On the surface of the hind wing, the scales stand singly, except on the veins and near the margin of the wing where they are denser and partly overlap. The hind wing shows no color pattern. The total number of scales on the hind wing has been estimated by MÜLLER, LÖBBECKE, and OLTMANN (1962) to be 6,250 so that in ten animals more than 10^5 cells can be scored. This system is therefore suitable for the study of somatic mutations, and has been used for this purpose by POHLEY (1955) and by LÖBBECKE and MÜLLER (1960). These authors irradiated *Ephestia* larvae during the last larval instar and obtained aberrant scales which they interpret as radiation-induced mutations. Very exact dosage/mutation-rate curves could be established in this system.

The development of the wing imaginal disc of *Ephestia* has been thoroughly investigated from a morphological point of view (KÖHLER 1932; POHLEY 1959; MUTH 1961). At the time of the last larval molt, the future wing surface consists only of about 300 to 400 cells, while the remainder of the wing imaginal disc will give rise to the joint and to the distal part of the wing which degenerates in the pupa. The wing grows considerably during the last larval instar by mitotic

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divisions which are oriented predominantly in a proximo-distal direction (POHLEY 1959). At the ninth day after the last larval molt, the wing surface reaches about 60,000 cells; at this time the processes preparatory to pupation start, and the number of mitoses drops to zero. Shortly after pupation, further mitoses appear which are assumed to represent differential divisions, separating scale stem-cells from epithelial cells; at all events, these two types of cells can be distinguished shortly after pupation by their size. The scale cell arises from the scale stem-cell by two differential mitotic divisions which give rise to an abortive cell, the socket cell and the scale cell (STROSSBERG 1937). After these differential divisions, no further cell divisions occur on the wing surface.

Since the wing scales of *Ephestia* have proved to be a convenient system for the study of X-ray induced somatic mutations (POHLEY 1955; LÖBBECKE and MÜLLER 1960), it was attempted to apply chemical mutagens of reasonably well investigated action mechanisms to this system. Among the chemical mutagens, some substituted purines and pyrimidines and their nucleosides have been thoroughly investigated in microbial systems and were chosen, therefore, for the present experiments.

It has been shown that 5-bromouracil (5-BU) and 2-aminopurine (2-AP) are mutagenic in phages (FREESE 1959) and in bacteria (RUDNER 1961; STRELZOFF 1962). Their mutagenicity is ascribed to the fact that both of these substances are incorporated into DNA. The mechanism of mutagenic action of these two substances has been thoroughly investigated by FREESE and collaborators (review 1963). FREESE distinguishes two types of errors, "errors of replication" and "errors of incorporation". In the former, 5-BU is incorporated into DNA instead of thymine and has a tendency to pair with guanine in the course of further replications. In "errors of incorporation", 5-BU is substituted for cytosine, in which case it would be expected to pair with adenine in the next replication cycle. The mutational events consist, therefore, in transition mutations from adenine-thymine to guanine-cytosine and from guanine-cytosine to adenine-thymine. It would be concluded from this theory that for the appearance of a transition mutation after an error of incorporation, two cycles of DNA replication are necessary. After an "error of replication", at least three cycles of DNA replication would be needed. Evidence that in *E. coli* at least two divisional steps are needed for the appearance of BU-induced mutations has been obtained by RUDNER (1961) and STRELZOFF (1962). This consequence is important for the present purpose, because in the *Ephestia* wing the number of future mitotic divisions for scale-forming cells at any time of treatment can be estimated.

If it is assumed that these substances act in *Ephestia* in a way similar to their action in microorganisms, it would be expected that 5-BU, 5-BDU (5-bromo-deoxyuridine), and 2-AP might induce somatic mutations. Their effect would therefore be similar to that of X-irradiation investigated by POHLEY and by LÖBBECKE and MÜLLER.

MATERIALS AND METHODS

Most of the experiments were carried out with the highly inbred strain NCR, a wild-type strain homozygous for the gene *rt*. The animals were reared in plastic refrigerator jars, 13 × 7 × 6.5 cm, on yellow granulated cornmeal in a temperature-constant cabinet at 25°C.

In order to obtain larvae of known ages, larvae were removed from the food shortly before the last larval molt, about 30 days after the eggs had been laid. In such a culture, larvae belonging to the last larval instar and to the next to the last instar can be distinguished by the size of their head capsules. Larvae belonging to the next to last instar were collected in Petri dishes with little cornmeal, so that the individual animals could be observed. Every morning the Petri dishes were checked and all larvae which had molted during the last day were removed to another Petri dish containing cornmeal, where they were left until the time of the experiment. Most experiments were carried out on the 8th day after the last larval molt, but in Experiments III and IV larvae on the 4th and 6th day after the last molt were used in addition.

The injections were carried out under ether anesthesia. For the injection of larvae, a small cut was made in the 8th abdominal segment dorsally or at the side, and the injection needle was introduced through this opening farther anteriorly into the body cavity. In the preliminary Experiment I, the injection apparatus consisted of a Spemann pipette drawn from glass tubing and operated by the finger. In this experiment, the amounts injected were not determined. In all other experiments, a micropipette similar to that used with the Chambers micromanipulator was employed: a 1 ml syringe was mounted on a holder and the plunger moved by means of a micrometer screw, so that the amount injected could be controlled by the movement of the micrometer screw. Extended preliminary experiments were undertaken to determine a suitable medium for injection: deionized and singly distilled water proved to be highly toxic, but doubly glass-distilled water was harmless and permitted good survival. The results were not improved if, instead of doubly distilled water, insect Ringer solution, 0.7% NaCl or $M/15$ phosphate buffer, pH 6.8 was used. Consequently, all drugs were dissolved in doubly distilled water and injected in this form. The operated animals were permitted to finish development at 25°C.

When they had hatched from the pupa, the fore and hind wings were preserved. The adults were etherized and the wings cut off with iridectomy scissors. They were then transferred with a watchmaker forceps onto the surface of a drop of "Caedax" (Merck A. G. Darmstadt) which had been put on a glass slide; the wings spread out neatly on the surface of the oil so that they were ready, after drying, to be observed under the microscope. The wings were scored for aberrant scales under the microscope, using a Zeiss planachromat objective 0.22 (10 $\times$), or for closer study, a planachromat objective 0.45 (25 $\times$). The position of the aberrant scales found was indicated on a sketch of the wing, and drawings of all aberrant scales were made on the margin.

5-BDU and thymidine were purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio, and 5-BU, 2-AP and aminopterin from Mann Research Laboratories, New York, N.Y.

In order to compare the effect of base analogues with that of X rays in the strain used in these experiments, larvae of the 8th day after the last molt were exposed to X rays. The irradiations were carried out at the Atomic Energy Project at the University of Rochester, and we thank Mrs. FLORENCE VAN SLYKE for her aid and advice in these experiments. The animals were irradiated at 250 kv, 15 ma, with aluminum parabolic and 5 mm copper filters at a distance of 50 cm.

EXPERIMENTAL RESULTS

The experiments to be discussed in this section deal with the effects of injection of drugs into *Ephestia* larvae. Most experiments are concerned with the effects of 5-BDU, but in addition 5-BU and 2-AP were tested. 5-BDU was furthermore used in combination with thymidine and aminopterin. Since 5-BDU is assumed to be incorporated into DNA instead of thymidine, simultaneous injections of 5-BDU and thymidine would be expected to lead to a reduction of the mutagenic effect of 5-BDU. Aminopterin is a folic acid inhibitor and, since folic acid is used as a cofactor in the synthesis of purines and pyrimidines, aminopterin would be expected to increase the incorporation of 5-BDU into the DNA, and therefore increase its mutagenic effect.

The material was collected in four different experiments. Experiment I was a pilot experiment intended to find out whether any changes can be obtained by

means of base analogues. Experiments II and III are mainly intended to obtain information on the effects of different dosages of BDU. In Experiment II, different amounts of a single 5-BDU solution (5 mg BDU/ml) were used, while in Experiment III, 4 μ l of different concentrations of BDU were injected. Experiment IV deals with injection of BDU at different times during the last larval instar.

(a) *The effect of treatments on viability, time of development and the production of aberrant scales:* The survival of treated larvae and the time necessary for their development are summarized in Tables 1, 2 and 3 for Experiments I, II and III. Experiment I is not very instructive since as much solution as possible was injected with a Spemann pipette, and it is certain that in many instances some of the material injected was lost by flowing out back through the wound. The data merely indicate that survival, though lower than in the controls, is still quite good, and that the time of development is increased consistently in all treatments, even though the difference for the control is significant for AP only ($t = 3.2$). For the material presented in Table 2, survival is high when low amounts of BDU are injected, but it drops with the injection of larger amounts. The fact that injection of 12 μ l H₂O is as lethal as BDU suggests that the impor-

TABLE 1

*Survival and hatching time after injections on the 8th day after the last larval molt.
Experiment I*

Treatment	Number of animals treated	Percent survival	Time to hatching (days) $\pm$ se
Control	40	92	19.78 $\pm$ 0.83
Aminopterin (0.5 mg/ml)	40	60	22.17 $\pm$ 1.01
2-Aminopurine (10 mg/ml)	80	47	23.79 $\pm$ 0.95
5-Bromodeoxyuridine (5 mg/ml)	80	47	21.11 $\pm$ 1.01
Aminopterin (0.5 mg/ml) + 5-BDU (5 mg/ml)	40	20	21.75 $\pm$ 1.28
Aminopterin (0.5 mg/ml) + 2-AP (10 mg/ml)	40	10	21.50 $\pm$ 1.27

TABLE 2

*Survival and hatching time after treatments on the 8th day after the last larval molt.
Experiment II*

Treatment	Number of animals treated	Percent survival	Time to hatching (days) $\pm$ se
Control	30	96.7	17.48 $\pm$.247
H ₂ O (12 μ l)	74	29.7	22.27 $\pm$.567
5-BDU (1 μ l, 5 mg/ml)	15	73.3	20.09 $\pm$.625
5-BDU (2 μ l, 5 mg/ml)	20	60.0	19.17 $\pm$.613
5-BDU (4 μ l, 5 mg/ml)	20	65.0	20.00 $\pm$.519
5-BDU (6 μ l, 5 mg/ml)	20	35.0	23.00 $\pm$ 1.512
5-BDU (12 μ l, 5 mg/ml)	160	25.0	26.05 $\pm$.575
5-BDU + aminopterin (12 μ l, 2.5 mg/ml + 500 μ g/ml)	48	2	18
X rays (900r)	50	98.0	18.51 $\pm$.313

TABLE 3

*Survival and hatching time after injections on the 8th day after the last larval molt.
Experiment III*

Treatment	Number of animals treated	Percent survival	Time to hatching (days) $\pm$ se
Control	35	100	22.20 $\pm$ 0.71
H ₂ O, 2 μ l	25	92	23.26 $\pm$ 0.74
H ₂ O, 4 μ l	25	68	20.77 $\pm$ 0.74
5-BDU, 4 μ l, 0.25 mg/ml (= 1 μ g)	25	76	20.79 $\pm$ 0.89
5-BDU, 4 μ l, 0.5 mg/ml (= 2 μ g)	25	92	22.00 $\pm$ 0.36
5-BDU, 4 μ l, 1.25 mg/ml (= 5 μ g)	25	84	20.62 $\pm$ 1.91
5-BDU, 4 μ l, 2.5 mg/ml (= 10 μ g)	25	76	21.79 $\pm$ 0.91
5-BDU, 4 μ l, 5 mg/ml (= 20 μ g)	25	92	24.26 $\pm$ 0.93
5-BDU, 4 μ l, 7.5 mg/ml (= 30 μ g)	25	80	22.55 $\pm$ 0.77
5-BDU, 4 μ l, 10 mg/ml (= 40 μ g)	25	84	21.00 $\pm$ 0.67
5-BU, 4 μ l, 0.5 mg/ml (= 2 μ g)	25	88	24.50 $\pm$ 0.74
2-AP, 4 μ l, 2.5 mg/ml (= 10 μ g)	25	32	25.50 $\pm$ 1.73
Aminopterin, 4 μ l, 0.125 mg/ml (= 0.5 μ g)	25	0	
5-BDU + aminopterin, 4 μ l	25	0	
2.5 mg/ml + 0.125 mg/ml (= 10 μ g + 0.5 μ g)			
5-BDU + thymidine, 4 μ l	25	88	24.18 $\pm$ 1.01
2.5 mg/ml + 2.0 mg/ml (= 10 μ g + 8 μ g)			
X rays, 1300r	25	100	21.43 $\pm$ 0.32

tant factor in killing the animals is the amount of fluid injected, rather than the amount of BDU. This is borne out by the data presented in Table 3 in which uniformly 4 μ l of fluid containing different concentrations of BDU were injected. In this experiment, no difference in survival for different amounts of BDU is evident; the highest concentration, 40 μ g BDU per animal, permitted slightly higher survival (84%) than its water control (68%). Of the other treatments, X rays and 5-BU have little effect on survival while 2-AP and aminopterin reduce survival significantly. It appears, therefore, that 5-BDU and 5-BU do not affect viability even at the highest dosages used while 2-AP and aminopterin appear to be toxic.

Since the injections are carried out in the larval stage, it must be concluded that BDU does not interfere, to any large degree, with the developmental processes leading to pupation and with the differentiation of the imaginal organs in the pupa. Out of 350 animals treated in Experiment III, 91 died as larvae. This includes 5 out of 50 treated with H₂O, 20 out of 250 animals treated with different concentrations of 5-BDU, all 50 animals injected with aminopterin, and 16 out of 25 moths treated with 2-AP. This result seems to indicate that in the case of injections with water and 5-BDU the treated larva dies as a result of injury, while in the case of aminopterin and 2-AP the toxic action of the substances seems to be responsible. In the same experiment, 12 animals died in the process of pupation and four more during the pupal stage. Three animals were alive in their pupal cases when the experiment was terminated.

Similar results are obtained with respect to the time necessary for development.

In Experiment II (Table 2), the time necessary for development is longer than that of the uninjected controls in every case; the differences between the uninjected controls and treated animals are significant at the 5% level for X rays ($t = 2.6$) and 2 μ l BDU ($t = 2.6$), and at the 1% level for all the others. In this experiment, there is also evidence that 5-BDU itself delays development even farther, since the hatching time for 12 μ l BDU is significantly higher than that for 12 μ l H₂O ($t = 4.7$). The hatching time at lower amounts of BDU is significantly lower than that for 12 μ l H₂O, indicating an effect of the volume of fluid injected (1 μ l BDU: $t = 2.6$; 2 μ l BDU: $t = 3.7$; 4 μ l BDU: $t = 2.95$).

In Experiment III (Table 3), on the other hand, there is no evidence for an influence by the treatment with BDU on time of development. Only the injection of 4 μ l BDU, 5 mg per ml, leads to a significantly longer time to hatching than for the H₂O control ($t = 2.9$), but this is due to some degree to the very short time to hatching for 4 μ l H₂O; the value for the same concentration of BDU is not significantly different from that for the uninjected control ($t = 1.8$) and 2 μ l H₂O ($t = 0.7$). For the same reason, the difference between 2-AP and 5-BU and the controls is not convincing. It must therefore be concluded that injection of large amounts of fluid (over 4 μ l) results in a delay in development. But there is no evidence that 5-BDU, even in high concentrations, delays hatching time, nor is there good evidence to assume a delay for 2-AP and 5-BU.

It was observed that after injection of water and 2-AP, particularly in large amounts, the scales of the wing may show disturbances. In large areas of the wing the scales appear twisted, bent or folded. It was originally thought that this might be an effect of osmotic shock; but injection of 0.7% and 0.9% NaCl, does not improve the shape of the scales. The effect did not disturb the scoring of aberrant scales, however, since the effect is relieved by 5-BDU, particularly in high concentrations, and also by 5-BU. Furthermore, the aberrant scales occur singly or in small clusters, and have a different shape from those induced by injection of water. It was therefore possible to score for aberrant scales even on wings from animals injected with low concentrations of the drug in which occasionally relatively large areas carried contorted scales.

Already Experiment I (Table 4, Column 2) indicated that aberrant scales occur with considerable frequency on the wings of animals treated with BDU. They occur very rarely in control animals. Combined treatment with BDU and aminopterin may appear to lead to a further increase in aberrant scales, as expected; but the difference is not significant. Contrary to BDU, AP does not appear to induce aberrant scales. This may be due either to the fact that AP is not mutagenic in our system, or that it does not penetrate into the cells. The fact that BU induces a low number of aberrant scales, similar to AP and different from equimolar amounts of BDU, favors the second alternative (Table 6). The facts are best explained by assuming that BDU is taken up by the cells more easily than BU and AP. These results have in general been confirmed in Experiments II and III (Tables 5 and 6). BDU induced, in all concentrations used, a recognizable increase in the number of aberrant scales, while aberrant scales occurred only occasionally in uninjected controls. The effect of BU and AP is certainly lower

TABLE 4

Number of aberrant scales on the hind wing of animals treated on the 8th day after the last larval molt: Experiment I

Treatment	Sex	No. of wings scored	No. of aberrant scales	No. of scales type "leaf"	No. of homogeneous clusters	No. of heterogeneous clusters	Mean mutational events per wing $\pm$ SE
Control	♂	20	0	0	.	.	
	♀	20	1	0	.	.	.05
Aminopterin (0.5 mg/ml)	♂	14	0	0	.	.	
	♀	20	1	0	.	.	.05
2-AP (10 mg/ml)	♂	20	0	0	.	.	
	♀	20	1	0	.	.	.05
5-BDU (5 mg/ml)	♂	20	38	22	2	2	1.70 $\pm$.450
	♀	20	66	25	5	3	2.90 $\pm$.500
Aminopterin + 5-BDU	♂	4	9	5	0	1	2.00 $\pm$.913
	♀	8	40	23	1	1	4.50 $\pm$ 1.500
Aminopterin + 2-AP	♂	6	0	0	.	.	
	♀	.	.	.	.	.	

than that of BDU. It is doubtful whether the increase in aberrant scales after treatment with these two drugs over the controls is real.

Finally, it should be pointed out that combined treatment with BDU and thymidine in equimolar amounts ($4 \mu\text{l } 8 \times 10^{-3} \text{ M}$) drastically reduces the number of aberrant scales induced (Table 6, line 9). This would be predicted under FREESE's (1959) theory of the action of BDU, since it is assumed to be incorporated instead of thymidine into DNA, and the two pyrimidines compete with each other for incorporation (STRELZOFF 1962). A similar suppression of BU-induced mutations by thymidine has been described in phages by LITMAN and PARDEE (1960).

(b) *The types of scale aberrations:* It was already noticed in Experiment I that amongst the aberrant scales one type is prevalent and easily recognizable. Contrary to the normal scale, it is pointed at its free end and has been designated as "leaf". It is shown in Figure 1c, and in Figure 2a. This type of scale which is easily distinguished from the normal scale, and constitutes a well defined phenotype, made up about 50% of the abnormal scales in Experiment I. It has never been found in our irradiated animals, nor in the massive materials obtained from irradiated animals by POHLEY and by LÖBBECKE (personal communication). It has regularly appeared after treatment with BDU, and, as shown in Tables 7 and 8, it constitutes a high proportion of all aberrant scales at low amounts of BDU. At higher amounts of BDU, it becomes relatively less frequent.

Two different types of scales can be distinguished among the scales described under the term "leaf". The more frequent type has bent or curved trabecles and usually ends with a pointed tip at the free end. This type is well illustrated in

TABLE 5

Number of aberrant scales on the hind wings of animals treated on the 8th day after the last larval molt. Experiment II

Treatment	Sex	No. of wings scored	No. of aberrant scales	No. of homogeneous clusters	No. of heterogeneous clusters	Mean mutational events per wing $\pm$ SE
Control	♂	4	0	.	.	0
	♀	4	0	.	.	0
H ₂ O 12 μ l	♂	4	0	.	.	0
	♀	4	0	.	.	0
5-BDU 12 μ l (5 mg/ml; 60 μ g)	♂	12	89	3	5*	6.58 $\pm$ 0.90
	♀	12	69	3	4	5.75 $\pm$ 1.09
5-BDU 6 μ l (5 mg/ml; 30 μ g)	♂	6	45	1	3	6.83 $\pm$ 1.82
	♀	4	20	2	0	4.50 $\pm$ 1.55
5-BDU 4 μ l (5 mg/ml; 20 μ g)	♂	6	59	1	9†	8.00 $\pm$ 1.13
	♀	4	57	5	4	12.00 $\pm$ 0.71
5-BDU 2 μ l (5 mg/mg; 10 μ g)	♂	2	20	0	1	9.50
	♀	8	74	1	6‡	8.25 $\pm$ 0.73
5-BDU 1 μ l (5 mg/ml; 5 μ g)	♂	4	38	2	2	8.25 $\pm$ 2.97
	♀	6	17	0	1	2.67 $\pm$ 1.51
5-BDU + aminopterin (12 μ l; 2.5 mg/ml + 500 μ g/ml)	♂	0	.	.	.	...
	♀	2	15	0	0	7.50
X rays 900r	♂	20	7	0	0	0.35 $\pm$ 0.30
	♀	19	66	2‡	0	2.95 $\pm$ 0.43

* One cluster of four scales.

† One cluster of three scales.

‡ One cluster of ten scales, one of two.

Figure 1c, Figure 2a, and Figure 3b; in the latter photograph the trabecle structure is particularly well expressed. The second type possesses trabecles which run parallel to each other and to the edge of the scale; these scales frequently have a blunter tip. This type of "leaf" scale is illustrated in Figure 3a. Since these two types of scales intergrade with each other, they are lumped together under the term "leaf".

A second frequent phenotype of abnormal scales are "dwarf" scales. These do not constitute a homogeneous type like "leaf" but occur in a large variety of different shapes, as shown in Figure 1a and Figure 2d. As shown in Table 8, "dwarf" scales did not occur at low concentrations of BDU (1 and 2 μ g) but make up a considerable proportion of the aberrant scales at medium and high concentrations. They occur rarely after irradiation with X rays.

The type of scale most frequently induced by radiation has been called "ES1" by POHLEY (1955). It is a clear-cut homogeneous type of scale, narrower than the normal scale, more darkly pigmented and with reduced teeth at the free

TABLE 6

Number of aberrant scales on the hind wings of animals treated on the 8th day after the last larval molt. In all cases, 4 μ l injected. Experiment III

Treatment	Sex	No. of wings scored	No. of aberrant scales	No. of homogeneous clusters	No. of heterogeneous clusters	Mean mutational events per wing $\pm$ se
H ₂ O	♂	8	1	0	0	0.125
	♀	12	1	0	0	0.08
5-BDU, 1 μ g	♂	10	16	1	0	1.5 $\pm$ 0.601
	♀	10	4	0	0	0.4 $\pm$ 0.163
5-BDU, 2 μ g	♂	10	22	1	0	2.1 $\pm$ 0.277
	♀	10	14	0	0	1.4 $\pm$ 0.108
5-BDU, 5 μ g	♂	10	62	2	2	5.8 $\pm$ 1.548
	♀	10	129	4	5	12.0 $\pm$ 1.932
5-BDU, 10 μ g	♂	10	90	1	6	8.3 $\pm$ 1.461
	♀	10	124	3	5	11.6 $\pm$ 1.707
5-BDU, 20 μ g	♂	10	92	1	3	8.8 $\pm$ 0.814
	♀	10	142	3	14*	12.4 $\pm$ 1.301
5-BDU, 30 μ g	♂	10	113	1	9	10.3 $\pm$ 2.595
	♀	10	119	2	5	11.2 $\pm$ 1.153
5-BDU, 40 μ g	♂	10	136	1*	10	12.4 $\pm$ 1.319
	♀	10	209	2*	17	18.9 $\pm$ 3.716
5-BDU, 10 μ g + thymidine 8 μ g	♂	10	2	0	0	0.2 $\pm$ 0.133
	♀	10	6	0	0	0.6 $\pm$ 0.163
5-BU 2 μ g	♂	10	2	0	0	0.2 $\pm$ 0.133
	♀	10	4	0	0	0.4 $\pm$ 0.221
2-AP, 10 μ g	♂	6	0	0	0	...
	♀	10	4	0	0	0.4 $\pm$ 0.221
X rays, 1300r	♂	10	5	0	0	0.5 $\pm$ 0.167
	♀	10	77	14†	0	4.8 $\pm$ 0.448

* One cluster of three scales.

† One cluster of 7 scales, two clusters of 5, four clusters of 3, and seven clusters of 2.

margin. As shown in Tables 7 and 8, it is frequently found in our irradiated material, but only rarely in material treated with BDU, and usually at high concentrations. It is found considerably more frequently in females than in males (Figure 1d).

Another type of aberrant scale found both in BDU treated and irradiated material has been called "fan". It is illustrated in Figure 1b, and is similar to POHLEY's types ES2 and ES10. As shown by the fact that POHLEY divided them into two groups, the "fan" type is variable, but we have been unable to divide these scales into two or more well defined classes. Like ES1, they occur frequently

FIGURE 1.—Different types of aberrant scales on hind wings of animals treated with BDU. a. dwarf; b. fan; c. leaf; d. ES1.

after irradiation, but relatively rarely after BDU treatment and mostly at high concentrations. They also occur more frequently in the female sex than in the male in Experiment III, though not in Experiment II.

Other types of aberrant scales occur more rarely or do not constitute a homogeneous group. The unpigmented scales listed in Tables 7 and 8 occur in a variety of different shapes. Giant scales (Figure 2c) occur more rarely. Double scales, such as the one pictured in Figure 2b, are very rare but their occurrence is of interest from a developmental point of view.

FIGURE 2.—Types of aberrant scales on hind wings of animals treated with BDU. a. homogeneous cluster consisting of two aberrant scales of type “leaf”; b. double scale; c. giant; d. dwarf.

The recognition of cell lethals, which would be represented by lack of a scale, presents difficulties. The place where a scale is missing can easily be detected. Since the moths may lose scales in the process of fluttering, lack of a scale does not necessarily indicate a lethal event. When a scale is lost, its position can still be recognized by the remaining socket. The criterion for a lethal event, therefore, was absence of both scale and socket. As shown in Tables 7 and 8, lethals defined in this way occur rarely after treatment with BDU.

It is seen from Tables 7 and 8 that at the lower concentrations, up to 10 to 20

TABLE 7

*Types of abnormal scales found on wings treated on the 8th day after the last larval molt.
Experiment II*

Treatment	Sex	No. of abnormal scales	ES1	Leaf	Fan	Dwarf	Unpig- mented	Lethal	Giant
5-BDU, 60 μ g	♂	89	.	5	8	3	2	.	.
	♀	69	1	3	8	3	.	.	.
5-BDU, 30 μ g	♂	45	.	5	2	9	5	4	1
	♀	20	.	.	.	3	1	.	.
5-BDU, 20 μ g	♂	59	.	20	1	12	3	3	.
	♀	57	.	10	1	21	4	.	.
5-BDU, 10 μ g	♂	20	.	5	1	6	.	.	.
	♀	74	.	18	3	33	2	1	1
5-BDU, 5 μ g	♂	38	.	11	.	16	2	.	.
	♀	17	.	7	.	5	.	.	.
5-BDU + amino- pterin (30 μ g + 3 μ g)	♀	15	.	4	.	7	.	.	.
X rays 900r	♂	7	.	.	1	1	.	.	.
	♀	66	14	.	9	.	2	.	.

μ g BDU, a high proportion of the aberrant scales (50% or more) is constituted by the relatively well defined types described above. At higher concentrations, their proportion declines.

(c) *The occurrence of clusters of aberrant scales:* In the radiation experiments of POHLEY (1955), of LÖBBECKE and MÜLLER (1960) and of MÜLLER *et al.* (1962) it was found that aberrant scales do not always occur as single scales surrounded by normal scales, but that frequently a number of aberrant scales stand close together on the wing, forming clusters. It has been found that the size of these clusters depends on the time of irradiation. After irradiation on the 8th day after the last larval molt, the average cluster size is about 2, while after treatment on the 1st day after the last molt it is somewhat above 50 (POHLEY 1959). This finding suggests that the mutational event induced by the radiation is transmitted in successive cell divisions to the offspring of the mutated cell. The fact that the clusters tend to appear as longitudinal stripes agrees with the observation that the mitotic spindles stand predominantly in a proximo-distal direction. The behavior of clusters agrees with the hypothesis that the mutational events induced by radiation are transmitted to the progeny cells at mitosis. A whole cluster of mutant cells is therefore regarded as a single mutational event.

Already in Experiment I, it was observed that clusters occur also in animals treated on the 8th day after the last larval molt with BDU. Some of these clusters consist of two scales of identical morphology, as shown in Figure 2a for two "leaves". Clusters of this type are designated in the tables as "homogeneous"

TABLE 8

*Types of abnormal scales found on wings treated on the 8th day after the last larval molt.
Experiment III*

Treatment	Sex	No. of abnormal scales	ES1	Leaf	Fan	Dwarf	Unpig- mented	Lethal	Giant
H ₂ O	♂	1	.	.	.	.	.	.	.
	♀	1	.	.	.	.	.	.	1
5-BDU, 1 µg	♂	16	.	5	.	.	.	.	2
	♀	4	1	.	.	.	.	.	2
5-BDU, 2 µg	♂	22	.	15	.	.	.	.	1
	♀	14	.	7	.	.	.	.	.
5-BDU, 5 µg	♂	62	.	20	.	13	1	1	1
	♀	129	.	45	1	24	8	.	2
5-BDU, 10 µg	♂	90	.	19	.	20	7	.	.
	♀	124	1	40	2	23	.	.	.
5-BDU, 20 µg	♂	92	.	17	.	18	.	.	.
	♀	142	.	17	.	30	9	1	2
5-BDU, 30 µg	♂	113	.	18	.	21	8	1	1
	♀	119	.	22	2	23	8	.	3
5-BDU, 40 µg	♂	136	.	8	.	2	8	.	1
	♀	209	3	24	2	51	6	.	3
5-BDU, 10 µg + thymidine 8 µg	♂	2	.	.	.	.	1	.	1
	♀	6	.	.	.	.	1	.	.
5-BU, 2 µg	♂	2	.	.	.	1	.	.	.
	♀	4	.	.	.	1	.	.	1
2 AP, 10 µg	♂	0	.	.	.	.	.	.	.
	♀	4	2	.	.	1	.	.	.
X rays, 1300r	♂	5	1	.	.	1	.	.	.
	♀	77	26	.	19	2	3	.	1

clusters. In addition, there occur in material treated with BDU, clusters of aberrant scales in which the scales have different morphology. Examples of this phenomenon are shown in Figure 3a (1 leaf + 1 narrow scale attenuated at the base), and 3b (1 leaf + 1 triangle). These clusters are called "heterogeneous" clusters. While some of them may be assumed to represent two aberrant scales which stand close to each other by chance, the phenomenon is too frequent to be accounted for in this manner, and it is therefore assumed that a heterogeneous cluster represents a single mutational event.

A certain difficulty arises from the fact that in many cases the differentiation between homogeneous and heterogeneous clusters is arbitrary, if the cells representing a cluster are similar, but not identical. It occurs frequently, for example, that two cells close to each other have the same shape, but different sizes (ex-

FIGURE 3.—Heterogeneous clusters of aberrant scales on hind wings of animals treated with BDU. a. heterogeneous cluster consisting of a blunt “leaf,” and a slender scale with drawn-out base anterior and to the left of it. b. heterogeneous cluster consisting of a triangular scale and a “leaf” scale anterior to it.

ample: 1 large and 1 small leaf). These were counted as homogeneous clusters while clearly different scales, e.g. a leaf and a fan, or a colorless and a yellow scale, are regarded as heterogeneous clusters.

The numbers of homogeneous and heterogeneous clusters in Experiments I, II and III are given in Tables 4, 5, and 6. It is seen that most clusters induced by BDU consist of two scales. Only two homogeneous clusters of 3, three heterogeneous clusters of 3, and one heterogeneous cluster of 4 were found. In contrast, X rays induce only homogeneous clusters. The clusters induced by irradiation are always clearly homogeneous; even the difference in size is lacking. Many of the clusters are of three or more scales, the largest cluster consisting of ten scales.

It must therefore be concluded that the heterogeneous cluster is a phenomenon which is characteristic for BDU-treated animals. It would be in agreement with the theory of FREESE which predicts that cells which have incorporated BDU may themselves be mutated, and give rise in successive cell division to differently mutated offspring, depending on the locations in the genome of the copying errors leading to transition mutations. The smaller size of clusters induced by BDU would also be predicted from FREESE's theory, since two or three cell divisions would be needed for a mutation to be induced. Experiments to test this possibility further will be described in a later section. It should be mentioned here that they do not seem to support the hypothesis.

(d) *The relation of “mutational events” and dosage of 5-BDU:* Since clusters are assumed to be expressions of one event affecting several scales, the effectiveness of treatments has been measured as “mean mutational events per wing”.

This means the average number of aberrant scales per wing corrected for the occurrence of clusters of different sizes. It is not intended to imply by this term that the event is a *gene* mutation; the nature of these events will be discussed later.

Data on the frequency of mutational events per wing are presented in Tables 4, 5, and 6. It should be mentioned that considerable differences have been found for different animals even in the same experiment, while the correlation between the two wings of the same animal is always high.

In Experiment II (Table 5) the number of mutational events per wing rises from 5 μg to 20 μg BDU per animal. The differences from 5 μg to 10 μg and from 10 μg to 20 μg are significant in the females ($t = 3.3$ and $t = 3.7$); in the males, there is, however, no evidence for an increase in mutational events. At higher amounts of BDU there appears to be a drop in the number of mutational events per wing which is significant in the females (20 μg –30 μg , $t = 4.4$). Since in this experiment the different dosages were produced by injecting different amounts of fluid, it is possible that after injection of 6 μl and 12 μl a certain amount of the injected fluid flowed out through the wound.

For this reason, the data from Experiment III (Table 6) appear more meaningful. In the females, we have a significant rise in mutational events per wing from 1 μg to 5 μg BDU per animal ($t = 5.1$ and $t = 5.5$). At higher dosages, however, there is no evidence for a further increase; a plateau is reached at 5 μg , and the value stays constant at about 12 mutational events per wing up to 30 μg BDU. This value corresponds to a rate of about 1.9×10^{-3} , an increase of about 100 times over the control rate. The rise at 40 μg is not significant ($t = 2.0$). In the males, a similar increase is observed between 1 μg and 5 μg which is significant at the 5% level for the difference between 2 μg and 5 μg ($t = 2.4$). It appears, therefore, that at lower levels of BDU, an increase in mutational events per wing takes place which reaches a plateau between 5 and 10 μg BDU per animal.

(e) *The effect of 5-BDU on males and females:* In *Ephestia*, as in all *Lepidoptera*, the female is the heterogametic sex. If the induced scale aberrations are gene mutations, it would be expected that more mutations appear in the female than in the male sex, if recessive sex-linked mutations constitute part of the mutational events. It is in agreement with this assumption that after X-ray treatment, the difference in mutational events between males and females is highly significant (Tables 5 and 6), in agreement with the radiation results of POHLEY (1955).

The situation is more complex in BDU-treated animals. In Experiment I (Table 4) more mutational events are found in females than in males, but the difference is not significant ($t = 1.8$). In Experiment II (Table 5), a significant difference between males and females is found at 20 μg BDU ($t = 3.0$) but not with larger or smaller amounts. In Experiment III (Table 6), no difference between the sexes is found for 1 μg . At 2 μg more mutational events occur in males than in females, and this difference is significant at the 2% level ($t = 2.8$). At all amounts of BDU higher than 5 μg per animal, the females show a slightly

higher number of mutational events than the males, and this difference is significant at the 5% level for 5 μg BDU ($t = 2.5$) and 20 μg BDU ($t = 2.3$).

POHLEY (1955) has pointed out that somatic mutations should occur in two forms; sex-linked recessives which would be restricted to the females, and autosomal dominants which would occur in both sexes with equal frequency. In agreement with this expectation, it was found that certain phenotypic types (ES1, ES2, ES3, ES4 and others) occur exclusively or almost exclusively in females, while others (ES5, ES6, ES7 and others) occur in both males and females. It appears possible, therefore, that in the BDU-induced aberrations, we are dealing with a mixture of sex-linked recessives and autosomal dominants which have different sensitivities to BDU. The distribution of different types of BDU-induced aberrations was therefore investigated (Tables 7 and 8). ES1, in agreement with POHLEY's data, occurs almost exclusively in females. In Experiment II, fan occurred with equal frequencies in males and females (12:12), but in Experiment III, all seven fan scales were found in females. The largest material is available for leaf. In Experiment I, 22 leaf mutations were found in males, and 26 in females. In Experiment II, 46 leaves occurred in males and 38 in females, an insignificant difference. In Experiment III, however, 155 leaf scales were found in the females and 102 in the males, a difference significant at the 1% level ($\chi^2 = 10.9$ for 1 df). The data, furthermore, indicate that at low levels of BDU (1 μg and 2 μg), the excess is in favor of the males, and only at 5 μg BDU and higher does the larger number of leaf scales occur in the females. Still, the number of leaves in males is sufficiently high that this aberration cannot be regarded as a sex-linked recessive.

The same phenomenon appears even more clearly for the dwarf scales. In Experiment II, 46 dwarf scales were found in males and 65 in females, an insignificant difference ($\chi^2 = 3.3$). But in Experiment III, 74 dwarf scales were found in males and 151 in females, a highly significant difference ($\chi^2 = 26.4$ for 1 df). As in the case of leaf, the sex difference is most pronounced at the highest concentration of BDU, 40 μg .

(f) *The effect of time of treatment on the formation of aberrant scales:* As was pointed out earlier, aberrant scales induced by X rays and BDU occur frequently in clusters. It was pointed out that the clusters induced by BDU are smaller than those induced on the 8th day after the last larval molt by X rays. Further, after X-ray treatment, the size of clusters becomes larger, if the treatment is applied earlier during the last larval instar, indicating that the clusters represent clones of mutated cells. In order to test this possibility for BDU, larvae were injected 4, 6, and 8 days after the last larval molt. The results are represented in Table 9.

It appears from the table that the time of injection does not have a strong effect on the production of aberrant scales. The number of mutational events per wing is about the same for the same concentration at all times. Also, cluster size does not appear to be strongly influenced; except for one homogeneous cluster of seven scales, four scales per cluster was the largest size observed in both homogeneous and heterogeneous clusters.

TABLE 9

Occurrence of clusters after treatment of larvae at different ages

Experiment	Treatment	Sex	Time of treatment (days after last molt)	No. of mutational events	Mutational events per wing	Homogeneous clusters of 2	Homogeneous clusters of 3	Homogeneous clusters of 4+	Heterogeneous clusters of 2	Heterogeneous clusters of 3	Heterogeneous clusters of 4+
III	H ₂ O	♂	8	1	0.125	0	0	0	0	0	0
		♀	8	1	0.08	0	0	0	0	0	0
	5-BDU, 10 µg	♂	8	83	8.30	1	0	0	7	0	0
		♀	8	116	11.60	3	0	0	5	0	0
	5-BDU, 10 µg	♂	6	78	8.00	1	0	1(7)	13	0	0
		♀	6	96	9.75	1	1	0	9	0	0
IV	5-BDU, 2 µg	♂	8	25	2.08	1	1	0	1	1	0
		♀	8	30	2.14	3	0	0	1	1	0
	5-BDU, 2 µg	♂	6	25	2.27	0	0	1(4)	0	0	0
		♀	6	15	2.50	0	1	0	0	0	1(4)
	5-BDU, 2 µg	♂	4	25	2.08	0	0	0	0	0	0
		♀	4	36	3.00	0	0	0	2	0	0

This conclusion is borne out by a preliminary experiment. On the 8th day after the last larval molt, the left hind wing bud was extirpated. This operation leads to regeneration of the wing, a process which takes 8 days and goes on under considerable cell division (POHLEY 1961). Three days after the operation, an unspecified amount of BDU was injected with a Spemann pipette. Twenty-nine treated animals developed into adults, 12 males and 17 females. A total number of 12 clusters was found on these 58 wings, eight on the operated side and four on the control side. Nine of these clusters consisted of two cells, and one cluster each on the operated and on the control side consisted of three cells. One cluster of four cells was found on the operated side.

The two experiments agree in indicating that the process induced by BDU which leads to the formation of abnormal cells does not result in the production of large mutant clones. One possibility to account for this fact would be that BDU after incorporation interferes with further cell division. This appears likely because an inhibition of cell division by BDU has been described for *E. coli* (RUNNER 1961; STRELZOFF 1962). Since in *Ephestia*, the cell divisions in the wing bud proceed predominantly in a proximo-distal direction, it would be expected that BDU treatment might lead to a shorter, plumper wing.

Accordingly, the width and length of wings spread on Caedax was measured with an eyepiece micrometer. Since there was a high correlation between the two wings of the same animal, only the right wing was measured, except in cases where it had been injured in the process of transfer to Caedax; in this case the left wing was substituted for the right wing. The length was measured as the distance from the point where the root of the veins radius + media approaches closely the root of the cubitus vein, to the tip of the wing between radius 2 and

media 1. The width was measured from the end point of the vein axillaris α to the anterior margin, passing through the point of closest approach of media 3 and cubitus 1. The results, in millimeters are given in Table 10.

In Table 10, males and females have been pooled, since there was no evidence for a difference between the sexes. Amongst the 30 comparisons possible, in only one case (width at 30 $\mu\text{g}/\text{ml}$) was the difference between the sexes significant ($t = 2.9$). In 15 of the comparisons, the difference was smaller than its standard error. The sex differences seem therefore to be normally distributed around the zero point.

With respect to length, it is possible to conclude that injection of H_2O by itself affects the value. The controls are significantly longer than wings of animals injected with 2 μl H_2O ($t = 3.8$) but not with 4 μl H_2O ($t = 1.4$). The wings treated with BDU are best compared to those injected with 4 μl H_2O since the experimental animals were injected with BDU dissolved in the same amount of fluid. No shortening of the wing is found at low concentrations of BDU, except for 2 μg ($t = 2.9$). At 20 μg BDU, however, and at all higher concentrations, the wings were significantly shortened ($t = 3.5$, $t = 4.1$, $t = 5.2$). An inhibiting effect of high dosages of BDU appears, therefore, well established.

A similar effect is found concerning width. It appears to be reduced by 2 μl H_2O ($t = 2.7$), but not by 4 μl H_2O . It is not reduced significantly at low levels of BDU—only at 1 μg is the width significantly reduced ($t = 2.95$)—but it is consistently and significantly lowered at dosages of BDU above 20 μg ($t = 2.0$, 3.1 and 3.1).

It appears then, that the pattern in the reduction of length and width is similar,

TABLE 10

Effect of treatment with 5-bromodeoxyuridine and X rays on the 8th day after the last larval molt on wing size of Ephestia (males and females combined)

Treatment	Wing length $\pm$ SE	n	Wing width $\pm$ SE	n
Control	9.73 $\pm$ 0.067	29	4.52 $\pm$ 0.035	29
H_2O , 2 μl	9.31 $\pm$ 0.088	20	4.34 $\pm$ 0.057	20
H_2O , 4 μl	9.56 $\pm$ 0.104	16	4.44 $\pm$ 0.052	17
5-BDU, 1 μg	9.14 $\pm$ 0.310	17	4.24 $\pm$ 0.044	18
5-BDU, 2 μg	9.21 $\pm$ 0.063	22	4.32 $\pm$ 0.038	21
5-BDU, 5 μg	9.40 $\pm$ 0.104	21	4.37 $\pm$ 0.066	21
5-BDU, 10 μg	9.43 $\pm$ 0.121	20	4.41 $\pm$ 0.058	19
5-BDU, 20 μg	9.07 $\pm$ 0.098	20	4.30 $\pm$ 0.054	20
5-BDU, 30 μg	8.97 $\pm$ 0.101	20	4.23 $\pm$ 0.050	20
5-BDU, 40 μg	8.93 $\pm$ 0.073	20	4.23 $\pm$ 0.045	21
5-BU, 2 μg	9.42 $\pm$ 0.090	18	4.38 $\pm$ 0.046	18
2-AP, 10 μg	8.80 $\pm$ 0.227	8	4.08 $\pm$ 0.109	8
5-BDU + thymidine (10 μg + 8 μg)	9.36 $\pm$ 0.074	19	4.43 $\pm$ 0.039	20
X rays, 900r	9.51 $\pm$ 0.052	29	4.47 $\pm$ 0.019	29
X rays, 1300r	9.91 $\pm$ 0.069	28	4.61 $\pm$ 0.039	29

significant reductions in both values being found at 20 μ g, 30 μ g and 40 μ g BDU. There is, in addition, some evidence that length is more strongly affected than width. The average ratio length/width is remarkably constant in the uninjected animals: 2.15 for untreated controls, both water controls and 1300r X rays. At low dosages of BDU, the value becomes more variable, 2.13 to 2.16. But the wings of animals injected with 20 μ g, 30 μ g and 40 μ g BDU are consistently shorter: 2.11, 2.12 and 2.11.

At all events, these data show clearly that BDU reduces the size of the wing, probably by interfering with the growth processes. There is some evidence that growth in length is more strongly affected than growth in width. It may, therefore, be possible to ascribe the lack of large clusters of aberrant scales to an inhibition of growth. This interpretation is, however, contradicted by the fact that it may be assumed that the scale-forming cells undergo the two differential mitoses giving rise to the scale in a normal way.

WITKIN and SICURELLA (1964) investigated the appearance of lactose negative mutants in *E. coli* treated with 5-BU, by studying the size of the mutant clones induced. They found that mutant clones appear either as pure clones or as large sectors, indicating that the DNA of all mutated daughter cells is derived from the same mutated DNA strand, or that several DNA strands of the same organism have mutated in the same way. It is apparent that this result is quite different from that obtained with *Ephestia* scales, in view of the fact that the two systems of investigation are sufficiently similar to allow comparison. It appears likely, therefore, that differences exist between the two types of cells in their reaction to base analogues.

DISCUSSION

The mutagenic effect of BDU is well established for bacteria and phages. The theory of FREESE (1959), that the molecular basis of BDU-induced mutation is the transition AT $\rightarrow$ GC, and GC $\rightarrow$ AT is supported by the extensive investigations by FREESE and collaborators (review 1963) on the reversion pattern of BU-induced mutations by treatment with base analogues, and by the demonstration of RUDNER (1961) that for the appearance of BU-induced mutations two mitotic cycles are necessary. It is astonishing that little evidence is available for a mutagenic action of BDU in multicellular organisms. KAUFMANN *et al.* (1962) obtained an increase in sex-linked lethals after injection of BDU into *Drosophila* males. BROWN and SMITH (1964) obtained from the seeds of *Arabidopsis* treated with BDU and IDU phenotypes which are called "laggards". Some of them were lethal while others survived and could be bred. It appeared possible, in successive generations, to select for the incidence of "laggards", indicating that the condition has a genetic basis. It is not clear, however, what the genetic basis of this character is, and whether it is dependent on analogue-induced mutations. NAKAJIMA (1962) induced somatic mutations from + to *od* by injection of BDU and BU into early *Bombyx* larvae.

The lack of direct evidence for BDU-induced mutations in multicellular organisms is astonishing because incorporation of BDU and IDU into the chromo-

somes of animals and plants has been repeatedly demonstrated [DJORDJEVIC and SZYBALSKI (1960) for human cell lines; SMITH *et al.* (1963) for *Vicia*; BROWN and SMITH (1964) for *Arabidopsis*]. It has been observed that BDU may induce chromosome breaks in cells of higher organisms by HSU and SOMERS (1961) for mammalian cell cultures, and by KOO (1963) for three species of plants. The chromosome breaks are located in the heterochromatin and particularly in the centromere region. Other authors [DJORDJEVIC and SZYBALSKI (1960) for human cells; TAYLOR, HAUT and TUNG (1962) and KIHLMAN (1962) for plant cells] did not observe any chromosome damage after exposure to BDU. KAUFMANN *et al.* (1962) did not observe any chromosome aberrations after treatment of *Drosophila* males. The explanation for these contradictory results seems to be based on the concentrations and lengths of exposure used by various authors. CHU (1963) states that at low dosages (10^{-5} M) of BDU and IDU he obtained no chromosome breakage except for a slight effect after prolonged exposure; but at higher concentrations, "severe damage" was observed.

In bacteria, other effects of BDU have been noted: killing of cells, reduction of growth rate and increased sensitivity to radiation. The latter has also been noted in animals and plants. Furthermore, HSU and SOMERS (1962) observed extensive cellular damage and death in mouse fibroblasts treated with 5-BDU; BDU-resistant strains could be obtained by selection. Morphogenetic effects of BDU and IDU have been obtained by BROWN (1962) and BROWN and SMITH (1964) in *Arabidopsis*; some of these may be explained by an effect on the growth rate of the apical meristems.

It will be attempted to interpret the results obtained in *Ephesia* on the basis of these data. In view of the cell toxic action of high concentrations of BDU in tissue culture, it was unexpected to find good survival after injection of BDU in *Ephesia*, even when high concentrations were used. Estimates of the total DNA of *Ephesia* by means of the Dische reaction suggested a value of 7.5 to 10 μ g per animal. This means that the amount of BDU injected at higher concentrations is considerably higher than the total DNA of the animal. The lack of toxicity of BDU, even in high concentrations, and its lack of interference with normal development, differ strongly from the effect of 5-fluorouracil which blocks developmental processes at definite stages (MUTH 1965). It should be pointed out that this difference in action between BDU and 5-fluorouracil would be expected, if BDU is primarily a mutagenic agent affecting individual somatic cells, whereas 5-fluorouracil interferes with the transmission of messages initiating developmental processes. The reduction in growth of the wing, in BDU treated animals is in agreement with the expectation from microorganisms and cell cultures.

The most serious problem in the interpretation of the data concerns the nature of the induced scale aberrations. The argument of POHLEY (1955) and of LÖB-BECKE and MÜLLER (1960) for considering the abnormal scales induced by X rays as somatic mutations is based on the more frequent occurrence of the aberrations in the heterogametic sex, with some types of aberrations restricted to the females, and on the clonal distribution of aberrations on the wing. In the case of NAKAJIMA's work with the silkworm, heterozygotes at the *od* locus were treated, and "transparent" skin was scored. In the present case, neither of these argu-

ments is applicable. There is no evidence for the occurrence of large clones of mutated scales, possibly due to an inhibition of the mitotic rate, and the difference between the sexes, though indicated for some mutant types particularly at high levels of BDU, is not as pronounced as it is after X-ray treatment.

Generally speaking, the production of aberrant scales by BDU differs from those induced by X rays by the following characteristics: (1) the morphology of the scales, particularly the occurrence of leaf and dwarf form; (2) the appearance of heterogeneous clusters; (3) the occurrence of types of aberrations, notably leaf and dwarf which are more frequent in the heterogametic sex but not restricted to it, and which therefore do not fit either the expectation for a recessive lethal mutation, nor for an autosomal dominant; and (4) the lack of evidence for clonal transmission.

Theoretically, two types of mutational events might be expected: transition mutations and chromosomal aberrations. It is difficult to decide in our system between these two types of events. Both might be expected to give rise to heterogeneous clusters, the transition mutations because a nucleus which had incorporated BDU might be expected to give rise to mutations in successive cell divisions, and chromosome breaks as a result of breakage-fusion-bridge cycles. The fact that morphologically the pattern of aberrant scales changes with increasing doses suggests that different events are involved at different dosages.

The closest resemblance between X-ray- and BDU-induced mutations is found in the fact that type ES1 is found with both treatments, and that in both cases it behaves like a sex-linked recessive. The same is true for type "fan" in Experiment III; in Experiment II this type does not show a difference between the sexes. Both these types occur mostly at high dosages of BDU. If they are regarded as transition mutations, it would be concluded that mutations of this type would only be likely to appear after incorporation of large amounts of BDU.

If ES1 and possibly "fan" are regarded as transition mutations, it should be concluded that the other types of mutational events, such as leaf, might constitute chromosome aberrations. The fact that leaf and dwarf, contrary to all somatic mutations induced by X rays, appear more frequently but not exclusively in females may be reconciled with this hypothesis since in the chromosome rearrangements the X chromosome may or may not be involved. The difficulty under this hypothesis would be that it would imply that chromosome breakage occurs at low levels of BDU while transition mutations are restricted to higher levels. This is not in agreement with the contradictory result obtained by different authors on chromosome breakage in animal and plant cells discussed earlier, since it appears that in these systems chromosome breakage is only induced after prolonged treatment with high levels of BDU.

Finally, the possibility that the induced aberrations constitute nongenic modifications must be considered. This possibility receives support from the fact that while most of the induced aberrant scales do not resemble those induced by X rays, they appear somewhat similar to the modifications induced by KÜHN (1948) by heat shock at 132 to 144 hours after pupation. Since KÜHN investigated the scales of the fore-wing, the shapes of the scales are not strictly identical; but he found scales with a pointed apical end and disturbances of trabecle structure

reminiscent of the "leaf" type. In addition, he frequently found double scales of the type rarely occurring after BDU treatment, and illustrated in Figure 2b.

One difficulty for the interpretation of the BDU-induced aberrations as non-genetic modifications arises from the fact that theoretically BDU is supposed to be incorporated into DNA instead of thymidine, and that this assumption is supported in our case by the suppression of the BDU effect by thymidine. It could, however, be imagined that incorporation of BDU interferes not only with the correct reproduction of DNA but also with its function. In the differentiation of scales, the synthesis of structural proteins plays a large role, and errors in their structure may lead to deformed scales. It may be mentioned in this connection that the occurrence of nuclear changes which are not genetically transmissible has been observed in a number of cases [RAVIN and NORMAN (1952) in *Aerobacter*, ARWOOD and MUKAI (1954) in *Neurospora*, LÖBBECKE and VON BORSTEL (1964) in *Habrobracon*]. In the latter case, the phenomenon is induced by ultra-violet radiation and nitrogen mustard, but only to a small degree by X rays. If an effect on this kind was induced by BDU in somatic cells, it would appear as a modification.

It must be concluded that the scale aberrations induced by BDU do not fit clearly into any one of the categories discussed above. It appears most likely that they constitute a mixture of several different types of injury. This assumption receives some support from the fact that the effects induced by different amounts of BDU differ from each other not only in quantity, but also qualitatively.

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SUMMARY

Ephestia larvae were treated with 5-bromodeoxyuridine (BDU) by injection of solutions of different concentrations 8 days after the last larval molt. Survival was high and no evidence for delay in development was found. BDU is therefore regarded as nontoxic for *Ephestia*.

On the hind wings of treated animals, individual abnormal scales were found. They occur as single abnormal scales or as homogeneous or heterogeneous clusters. These clusters are regarded as due to the same "mutational event."

The number of abnormal scales per wing increases in the range from 1 to 5 μg BDU per animal. At higher levels it reaches a plateau at an average of 12 "mutational events" on a wing containing about 6,250 scales (about 2×10^{-3}).

The scale abnormalities induced by BDU differ from those induced by X rays in the following respects: (a) The phenotypic appearance of the abnormal scales is different, even though some types of scales are common to both treatments. (b) The occurrence of heterogeneous clusters which have not been found after treatment with X rays. (c) In X ray treated *Ephestia*, most types of aberrant scales are restricted to the heterogametic female sex, while in BDU-treated animals, a slight difference between the sexes appears only at high dosages. (d) X-ray treatment at earlier times in the last larval instar gives rise to large

homogeneous clusters of mutated scales, while after treatment with BDU, 4 and 6 days after the last larval molt, mostly clusters of two cells are observed. No clusters larger than seven scales were found.

The lack of large clusters of aberrant scales induced by BDU may be explained by an inhibition of growth caused by the drug. The wings of animals treated with high concentrations of BDU (20 μ g and over) are significantly smaller than those of controls injected with water.

The induction of abnormal scales by BDU is suppressed by equimolar amounts of thymidine.

The nature of the BDU-induced abnormal scales is discussed. On the basis of the evidence it is impossible to decide whether they represent point mutations, chromosome rearrangements or genetically nontransmissible changes. The evidence seems to indicate, however, that more than one mechanism is involved.

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