Morphogenic Effects of Halogenated Thymidine Analogues on Drosophila

VI. Causal Analysis of Bromodeoxyuridine Induced Growth Lesions

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Variations in the treatment conditions which affect the frequency of BUdR-induced morphogenic lesions in *Drosophila melanogaster* have been studied. By varying the concentrations of BUdR and FU, or by changing the duration of treatment, it has been demonstrated that increased incorporation of BUdR into DNA results in a concomitant increase in morphogenic lesions.

A quantitative analysis of the data on BUdR-induced lesions as a function of the age of the larvae at the time of treatment indicates that an analog pulse during early larval life induces fewer, but larger lesions than a similar treatment given during later stages of development. These observations support the hypothesis that the developmental modifications are the result of genetic changes which in subsequent replication cycles of DNA are transmitted to descendant cells, and are not necessarily due to the presence of BUdR in DNA *per se* of cells in the developing organism.

Introduction

Pulse treatment of *Drosophila* larvae with 5-bromouracil deoxyriboside (BUdR) induces growth lesions in the adult flies. The thymidine analogue is substituted in *Drosophila* DNA under these conditions, but its incorporation can be markedly increased by giving 5-fluorouracil (FU) together with BUdR [1]. In the latter instance, the frequency and intensity of morphogenic disruptions is also enhanced [2]. Previous studies suggested that FU ingested by *Drosophila* larvae is converted to an inhibitor of thymidylate synthetase and the consequent deficiency of thymidine monophosphate (dTMP) facilitates incorporation of BUdR into *Drosophila* DNA [3, 4]. Depression of dTMP synthesis by other inhibitors including methotrexate (MTX) and trifluoromethyl deoxyriboside (F_3TdR) will also increase incorporation of BUdR into DNA of *Drosophila* larvae, and morphological modifications similar to those found in BUdR + FU treated individuals are present in the adults [5]. 5-Iodouracil deoxyriboside can be substituted in place of BUdR as the thymidine analogue for such studies [6], and F_3TdR, which is a thymidine analogue capable of being incorporated in DNA as well as an inhibitor of thymidylate synthetase, is an effective lesion inductor for *Drosophila* in the absence of any other inhibitor [5]. These series of studies suggest that the primary cause of the growth lesions in the imaginal tissues of *Drosophila* is the incorporation of a thymidine analogue in DNA.

Extensive sets of data from morphological examinations and DNA samples were acculuated in our earlier studies, but the experiments utilised only a limited set of treatment conditions so a number of questions remain in evaluating the molecular events which lead to the modifications in growth and development of the organism. The present investigations were therefore undertaken to obtain additional information on the variables which could affect the concordance or discordance of BUdR incorporation into DNA at the molecular level and the morphological effects at the organismic level. These variables include the duration of the pulse treatment with the analogues, and the developmental age of the larvae during these treatments.

Methods and Materials

The methods of larval collection, administration of analogues, tabulation of morphological lesions, and isolation of *Drosophila* DNA have been enumerated previously [2]. It is important to note that the incorporation of BUdR into *Drosophila* DNA as well as the induction of

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morphogenic lesions were obtained by pulse treatment with aqueous solutions of base analogues in the absence of other nourishment. At the conclusion of the treatments, the larvae were returned to regular *Drosophila* culture medium to complete their development since the morphological effects are scored in the adult stage. The period of treatment is therefore indicated by larval age at the beginning and termination of analogue feeding. Larval ages were timed from egg eclosion: at 23–24° C the first larval molt occurs at 24 h, the second molt occurs at 48 h, and puparium formation begins at approximately 96 h. On this developmental schedule, *Oregon-R* (*Ore-R*) wild type larvae of *D. melanogaster* will continue development if they are deprived of food after 65 h, and the hatching adults will be morphologically normal. For the extended feeding intervals with nucleic acid base analogues, treatment was begun after this critical age of larval development. Control groups maintained on H_2O therefore pupated on schedule in these experiments, whereas larvae given analogues for extended intervals were returned to food containers, and pupation proceeded after a feeding interval with yeast. Unless indicated otherwise in Table or Figure Legends, the following concentrations of analogues have been used throughout these studies: BUdR and thymidine (Tdr), 6.5×10^{-4} M; FU, 1.15×10^{-3} M.

Results

In order to correlate morphogenic effectiveness of analogue treatment with BUdR incorporation into DNA, conditions of treatment with BUdR were varied, and data on the frequency of morphologic deviants and BUdR incorporation into DNA were obtained for each of the specified conditions. BUdR incorporated DNA samples were subjected to CsCl density-gradient centrifugation for analysis, and morphological effects were assessed by examination of the wings of the hatching adult flies.

Morphological Studies

Growth modifications following analogue treatment are not limited to the wings, but this structure was selected for these studies since it can be easily removed from treated specimens and permanent mounts of large numbers of wings on microscope slides can be examined and scored in a given experiment. The growth lesions on the wings were classified as belonging to one of the following categories: (1) a single bristle event is the appearance of a bristle (chaeta) on the wing in a site usually occupied by a wing hair (trichome); (2) a bristle cluster includes a group of similar bristles induced in a region normally occupied by hairs or a different kind of bristle; (3) the supernumerary category includes all other aberrant tissue formations whether these remain small, undifferentiated nodules or show differentiation of bristles, veins, and sense organs. It should be stressed that absence of wing structures is occasionally encountered in treated specimens, but these are excluded from the tabulation since inhibitory effects of similar types can be induced by feeding a variety of antimetabolites to *Drosophila* larvae. Only events that represent extra growth or new differentiations with growth in otherwise normal wings have been included in these studies. Photographs representing lesion varieties induced by thymidine analogue treatment have been published in earlier papers [2, 6].

A. Effects of Duration of the Analogue Pulse: Larvae from a single collection period were divided into five groups at 68 h of age and transferred to the following solutions: (a) BUdR, (b) BUdR + FU, (c) FU, (d) FU + TdR, (e) H_2O. A sample of larvae was removed from each of the feeding chambers after 6, 12, 24, and 48 h and returned to regular *Drosophila* medium to permit pupation. Since larvae transferred to H_2O after the mid third instar pupate at the normal age of 96 h, this treatment can be tested only through a 24-h feeding interval.

Table 1 presents the data on growth lesions scored under the three categories: single bristles, clusters of bristles, and supernumeraries. The number of wings examined for each treatment is given in the first column together with the fraction of wings in the group which showed growth lesions. Entries in the succeeding columns represent the coefficient of the total number of specific lesions divided by the total wings examined. The last column represents the sum of the lesions in the three categories divided by the total wings examined in the group, and this factor is designated as lesion frequency per wing.

Lesion frequency increased as exposure to BUdR was lengthened and reached a maximum of 0.17 lesions/wing in the 48-h sample. Flies treated with BUdR for 48 h contained melanised mesenchymal cells [3] in the wings and one-fourth of the specimens remaining on BUdR for 48 h did not hatch. With a six-hour treatment of BUdR + FU, lesion frequency was 0.37/wing and an increase to 0.89/wing was found following 12 h of treatment. Melanised mesenchymal cells were apparent in some of the wings of the 12-h specimens with no apparent effect on viability but extension of the treatment with BUdR + FU to 24 and 48 h resulted in lethality. Many of the flies in the 24-h sample were badly mutilated, legs were deformed, wings remained folded. These flies were visibly weak and many died during emergence or shortly thereafter. The 24-h sample also contained several dead pupae, and continuation of treatment with BUdR + FU to 48 h resulted in the death of most of the specimens during the pupal stage. Among the control groups of flies resulting from larvae treated with FU, FU + TdR, and H_2O none showed bristle inductions or supernumerary structures. Therefore the number of wings examined at each age interval for these groups are presented as pooled counts. High concentra-

Table 1. Developmental lesions induced by BUdR and FU

Drosophila larvae were transferred to analogue solutions when they were 68 h old and returned to culture medium following the treatment interval indicated in hours. The fraction of wings affected represents the number of wings with lesions per total wings examined. Lesion frequency is based on the total number of wings in the sample.

Treatment	(hours)	Wings		Lesions per wing			
		Total	Fraction affected	Single	Cluster	Super-numerary	Total
BUdR	6	174	0	0	0	0	0
	12	203	0.039	0.039	0	0	0.039
	24	184	0.120*	0.114	0	0.006	0.120
	48	110	0.173*	0.164	0	0.009	0.173
BUdR+FU	6	332	0.304	0.265	0.015	0.090	0.370
	12	177	0.554	0.723	0.034	0.130	0.887
	24	**					
	48	**					
FU	6–48	564	0***	0	0	0	0
FU+TdR	6–48	637	0***	0	0	0	0
Water	6–24	432	0	0	0	0	0

* Melanised mesenchymal cells similar to those found following treatment with BUdR + FU appear in these wing samples.

** Many of the treated specimens died in the pupal stage; those flies that hatched were weak and most of the wings remained folded, so classification of lesions could not be completed for these samples.

*** Suppression of vein development, particularly the fifth vein, was found in approximately 25% of the flies remaining on FU or FU + TdR for 24 h or longer. Dead pupae and flies with unopened wings were also found in the 24- and 48-h treatments with FU (28 specimens) and FU + TdR (14 specimens).

tions of FU inhibit *Drosophila* development and these inhibitory effects are particularly noticeable in eye and wing formation [7]. In the present study, suppression of wing vein development, especially the fifth vein, occurred in approximately one-fourth of the flies that remained on FU or FU + TdR for 24 and 48 h. Dead pupae and flies with unopened wings were also found in the 24- and 48-h samples of FU and FU + TdR.

B. Effects of Analogue Concentration: An increase in BUdR concentration from 0.5 mg/ml to 4.0 mg/ml produced a seven-fold increase in bristle and supernumerary lesions in an earlier study [2]. In this same study, it was demonstrated that bristle and supernumerary lesions are not induced by treatment with FU alone even at high doses. If the role of FU in lesion induction is the inhibition of thymidylare synthetase, thereby permitting increased BUdR substitution in DNA, then concentrations of FU that more effectively inhibit this enzyme in feeding larvae will be correlated with higher lesion frequencies. Carpenter [4] has already demonstrated that cell-free extracts from groups of *Drosophila* larvae fed inceasing concentrations of FU (0.075, 0.15, 0.030 mg/ml) show a parallel decrease in thymidylate synthetase activity. It should be noted that only a limited amount of liquid-soaked cellulose powder is ingested by the larvae during the period of treatment, and it is difficult to determine the exact concentration of these analogues in the intra- and extracellular spaces of the body of the larva. However, by varying the concentration of FU and keeping the BUdR concentration constant, information on the effects of FU concentration on BUdR induced lesions can be obtained. For these studies, the concentration of BUdR was maintained at 6.5×10^{-4} M and the concentration of FU was tested over a four-fold range. Table 2 presents the data from these experiments. A two-fold dilution of FU decreased lesion frequency fifty per cent (from 0.578 to 0.280), and a four-fold dilution of FU (2.875×10^{-4} M) yielded a lesion frequency of 0.063 per wing.

Table 2. Effect of FU concentration on the induction of morphogenic lesions

Drosophila larvae were fed 6.5×10^{-4} BUdR with concentrations of FU indicated during the age interval 52–58 h. Lesion frequencies were calculated on the basis of the total wing samples. No lesions were obtained by brief treatments with FU, so the data for these control groups have been summarised by including the number of wings examined for these samples.

Treatment		Wings		Lesions per wing			
		Total	Fraction affected	Single	Cluster	Super-numerary	Total
BUdR + FU	1.15×10^{-3} M	296	0.382	0.311	0.041	0.226	0.578
BUdR + FU	0.58×10^{-4} M	386	0.218	0.158	0.003	0.119	0.280
BUdR + FU	0.29×10^{-4} M	315	0.060	0.041	0	0.022	0.063

Control groups	Wings examined	Lesion frequency
FU 1.15×10^{-3} M	333	0
FU 0.58×10^{-4} M	247	0
FU 0.29×10^{-4} M	335	0

Incorporation of BUdR into DNA

A. Effects of Duration of the Analogue Pulse: The incorporation of ^{3}H-BUdR into larval DNA in the presence and in the absence of FU was examined from groups of larvae after 6, 12, and 24 h of analogue exposure. An additional feeding series utilised ^{14}C-BUdR with and without FU for these same intervals, and in a third experiment DNA samples were isolated from larvae given ^{3}H-BUdR with or without FU for 10 and 22 h. The DNA samples from the three experimental series were centrifuged to equilibrium in CsCl and the results of isopycnic centrifugation in CsCl of one series of B-DNA (DNA from larvae fed BUdR) and FB-DNA (DNA from larvae given BUdR + FU) are presented in Fig. 1. The sequence illustrates the results obtained in the other series as well.

Dispersion of the optical density (A_{260}) of normal DNA as well as BUdR substituted DNA in CsCl gradients shows a major peak at 1.700 g cm^{-3}. Radioactivity in the form of ^{3}H-BUdR-labelled DNA, however, is detected in the denser regions of the gradient as well as dispersed to the lighter side of the A_{260} peak, and both B-DNA and FB-DNA samples show three maxima with approximate densities of 1.696, 1.714, and 1.731 g cm^{-3}. In B-DNA samples less radioactivity is found in the heaviest region of the gradient than in FB-DNA samples. There is variability in the proportion of radioactivity in this region of the gradient among DNA samples isolated at different times, but in comparing B-DNA and FB-DNA samples within any given set of experimental conditions, the amount of material in this region of the FB-DNA sample always exceeds that in the B-DNA sample. Furthermore, as illustrated in the sequence of gradients in Fig. 1, radioactivity in the region corresponding to densities of 1.72 - 1.73 g cm^{-3} increases considerably as larvae continue to feed on BUdR + FU while the accumulation of this material in B-DNA samples remains limited.

It was previously demonstrated that ^{3}H-BUdR incorporated in *Drosophila* nuclei by pulse treatment of the larvae could be detected in nuclei of the pupal stage by autoradiography [1]. In view of this retention of BUdR in nuclei, and the consistent difference in the B-DNA and FB-DNA samples resulting from prolonged treatment of the larvae, the question now arises whether the isopycnic patterns of B-DNA and FB-DNA as displayed in CsCl density gradients are retained during subsequent development. To answer this question, larvae were fed ^{3}H-BUdR or ^{3}H-BUdR + FU for 12 h, and DNA was isolated from half of the batch of larvae in each group while the remaining larvae were returned to regular *Drosophila* medium to feed for 24 h prior to DNA isolation. The results of isopycnic centrifugation of the four DNA samples presented in Fig. 2 clearly indicate maintenance of pattern differences between B-DNA and FB-DNA samples. There is, however, some indication of an increase in the dense material in both samples and a concomitant decrease in the lighter radioactive regions of the gradients following a recovery period of 24 h. These relative changes may represent a lag in incorporation of ingested analogue that was in the lumen of the gut at the time the larvae were transferred to food.

B. Effects of Analogue Concentration: BUdR substituted DNA was isolated from larvae in experimental series paralleling those used for evaluating the effects of analog concentration on lesion induction. The DNA samples were centrifuged to equilibrium in CsCl and Fig. 3 presents CsCl pycnographic comparisons of these DNA samples.

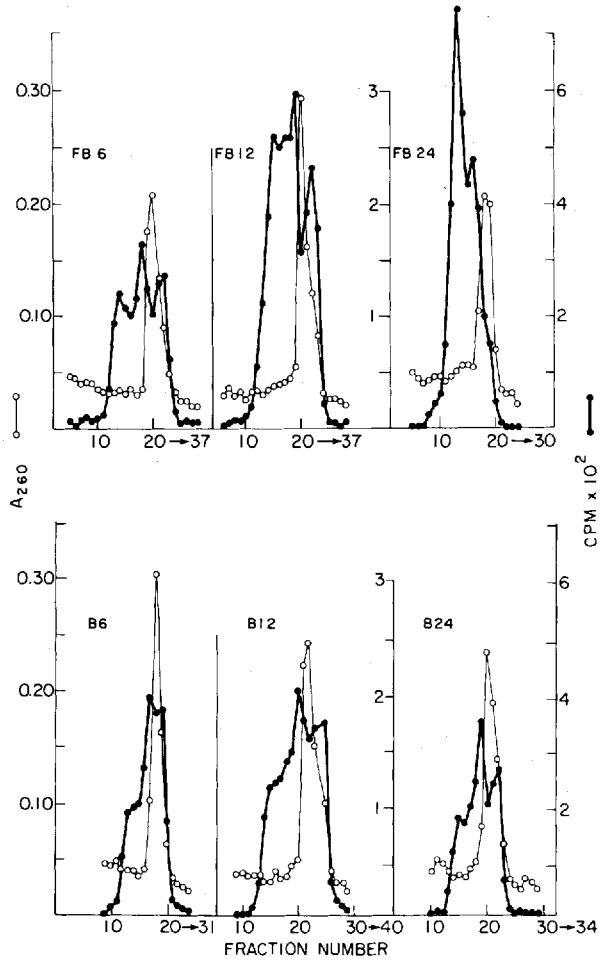

Fig. 1. CsCl density gradients of DNA isolated from *Drosophila* larvae given ^{3}H-BUdR with or without FU. Larvae (67 h) from a single collection period were divided into two groups and transferred to the following solutions: B series–7 μCi/0.65 μmole BUdR/ml and FB series–7 μCi/0.65 μmole BUdR/1.15 μmole FU/ml. DNA was isolated from larvae removed from the feeding chambers after 6, 12, and 24 h. Approximately 70 μg of each DNA sample in 3 ml CsCl was centrifuged to equilibrium at 33,000 rpm for 60 h (SW 39 rotor, Spinco Model L). Fractions were collected dropwise from the bottom of the tubes and diluted with 1 ml of distilled H_2O; the arrow indicates the total number of fractions for each gradient. Optical density readings at 260 nm were taken with a Zeiss spectrophotometer and 10 ml of scintillation fluid was then added for counting radioactivity. The efficiency of the counting system for ^{3}H was 10%; counts were corrected for background. The density of selected fractions of one gradient analysis for each DNA sample was determined by refractometry

It is apparent that increased BUdR incorporation in DNA obtained by higher doses of both BUdR or FU ($B_{\times 20}$ and $F_{\times 4}B$) produces a higher proportion of DNA molecules with densities of approximately 1.72 to 1.73 g cm^{-3}. The occurrence of this BUdR-DNA in both cases corresponds with the more effective morphogenic treatment.

Finally, we may inquire whether the effects of FU on the pattern of BUdR incorporation in DNA can be overcome by simultaneous administration of TdR along with FU + BUdR, since treatment of larvae with FU + BUdR +

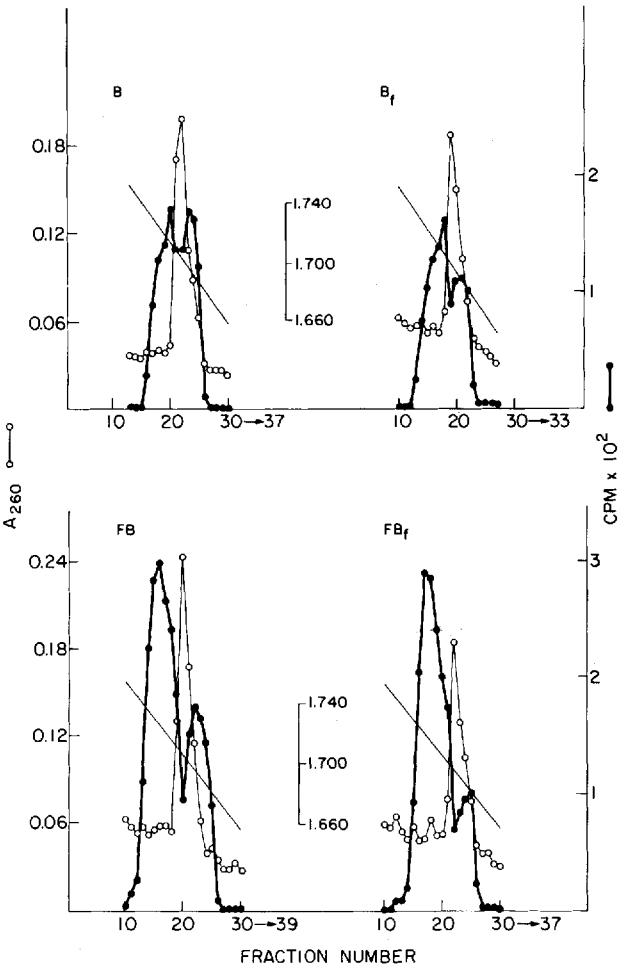

Fig. 2. CsCl density gradient analysis of *Drosophila* DNA from larvae (60 h) given ^{3}H-BUdR (B) and ^{3}H-BUdR + FU (FB) for 12 h. A group of larvae from each feeding chamber was returned to regular *Drosophila* medium for an additional 24 h before DNA was isolated and these samples are designated B_f and FB_f. The methods used were the same as described in Fig. 1. ^{3}H-BUdR (specific activity 26.1 Ci/mmole) was added to aqueous solutions to give final concentrations of 12.5 μCi/0.65 μmole BUdR/1.15 μmole FU/ml. The density gradient is represented by the diagonal line in each plot and the center scale indicates the density

TdR is morphogenically ineffective. Comparison of gradients labelled FB+T and FB in Fig. 4 demonstrates that equimolar TdR and BUdR given along with FU limits the incorporation of BUdR into DNA molecules corresponding to the density for major peak *Drosophila* DNA.

Lesion Frequency and Expressivity as a Function of Larval Age

The question whether lavae of all ages are equally susceptible to morphogenic effects of BUdR + FU treatment was examined by feeding the analogues to larvae of different ages and scoring the wings of the resulting adults for lesions. Beginning at six hours after eclosion, larvae were pulse treated with BUdR + FU for a six-hour period and then returned to normal feeding conditions. Table 3 summarises these data.

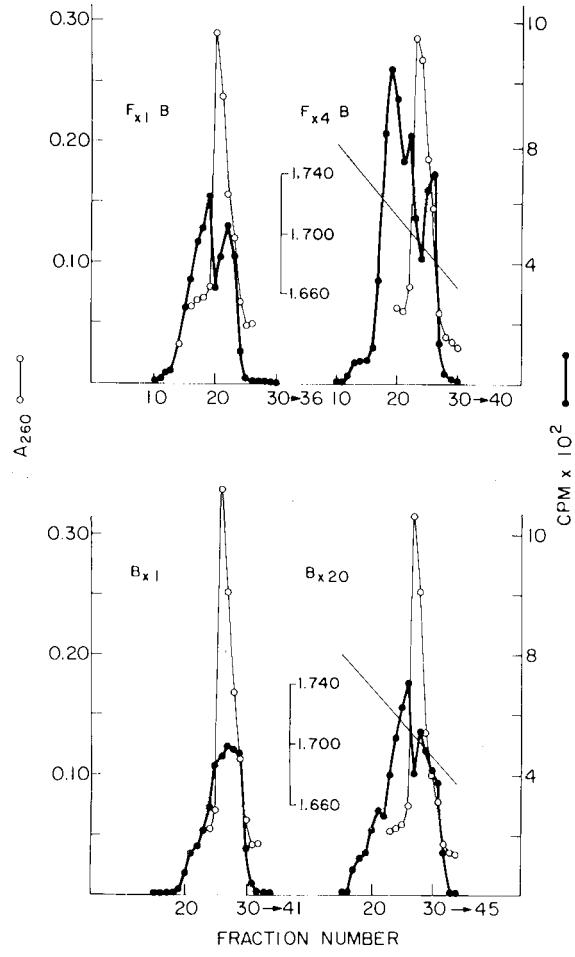

Fig. 3. A comparison of the density gradient profiles of BUdR substituted DNA from *Drosophila* larvae given two different concentrations of FU together with the same concentration of BUdR (upper), and from *Drosophila* larvae given a low and a high concentration of BUdR without FU (lower). For the upper gradient pair: $F_{\times 1}B$–20 μCi/0.65 μmole BUdR/0.29 μmole FU/ml; $F_{\times 4}B$–20 μCi/0.65 μmole BUdR/1.15 μmole FU/ml. For the lower two gradients ^{3}H-BUdR (26.1 specific activity) was diluted with an aqueous solution of BUdR to yield the following solutions for feeding: $B_{\times 1}$–20 μCi/0.65 μmole BUdR/ml; $B_{\times 20}$–20 μCi/13.0 μmole BUdR/ml

All treated groups showed morphogenic effects except the 6–12 h group. The incidence of lesions in the first and second instars was low, so lesion absence in the 6–12 h group may be due to limited sample size since the number of cells in the wing forming region of the disk at this age is small [8, 9]. Maximum response was achieved by analogue treatment during the first half of the third instar when a lesion frequency of 0.58 was recorded for ages 52–58 and 64–70 h. The frequency of bristle and supernumerary lesions, however, differed between these two groups; an increase in the frequency of bristle lesions was found in the 64–70 h group, while the induction of supernumeraries decreased in the latter half of the third larval instar. The low incidence of lesions in the 76–82 h group may represent changes in the endogenous pools of thymidylic acid. It may, on the other hand, be due to the fact that larvae at this stage tend to leave cellulose powder whether it is soaked with the analogue or other non-attractant medium such as water. It should be recalled that larvae after 60–65 h of regular feeding no longer require nutrients for pupation and metamorphosis, and low-lesion frequency therefore could be caused by a lower intake of analogue.

In addition to scoring each lesion as a single event, the magnitude of response to analogue treatment was estimated by counting the number of bristles in each cluster, and by measuring the length and width of each supernumerary structure in ocular micrometer units at standard magnification. This information is summarised in Table 4. Bristle clusters have been grouped into four classes based on the number of bristles in each cluster, those containing 2–4, 5–8, 9–16, and more than 17 bristles per cluster. The supernumeraries have also been grouped into four classes designated in arbitrary units obtained by multiplying the length and width of each supernumerary. The 30–36-h sample had 3 clusters and the mean number of bristles per cluster in this group was 9.3; treatment during 52–58 h gave 17 clusters and the average number of bristles per cluster in this group was 5.5; in the 65–70-h sample, the average bristle number for the 16 clusters was 3.3. It is clear that there is a systematic trend in the mean number of bristles per cluster, and the number is higher among the larvae treated at an earlier stage of development. A similar trend is apparent by examining the mean size of supernumeraries; treatment during early larval life gives rise to large supernumerary lesions whereas treatment during later stages results in lesions that are small.

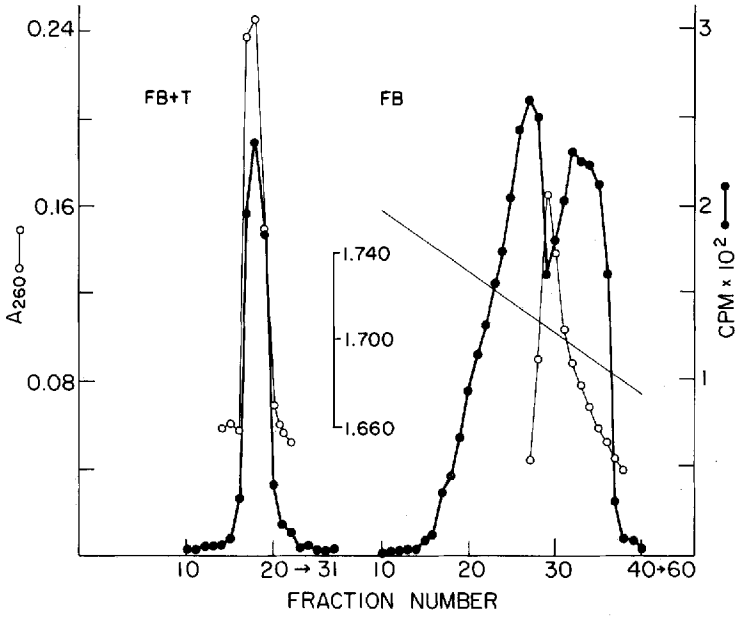

Fig. 4. Comparison of ^{3}H-BUdR incorporation in *Drosophila* DNA in the presence of equimolar TdR and in its absence. ^{3}H-BUdR (specific activity 25.9 Ci/mmole) was diluted with an aqueous solution of BUdR and FU to give a final concentration of 25 μCi/0.65 μmole BUdR/1.15 μmole FU/ml

Table 3. Morphogenetic lesions induced by BUdR + FU treatment at different larval ages

Treatment age (in hours)	Wings		Lesions per wing			
	Total	Fraction affected	Single	Cluster	Super-numerary	Total
6–12	178	0	0	0	0	0
16–22	203	0.030	0.005	0	0.025	0.030
30–36	308	0.032	0	0.010	0.023	0.033
40–46	263	0.019	0	0	0.023	0.023
52–58	373	0.354	0.284	0.048	0.244	0.576
64–70	261	0.364	0.398	0.069	0.111	0.578
76–82	270	0.141	0.189	0.004	0.015	0.208

Table 4. Intensity of BUdR + FU effect as a function of larval age

Treatment age (in hours)	Size of bristle cluster					Size of supernumerary growth				
	>17	16–9	8–5	4–2	Mean	6600–501	500–51	50–6	<5	Mean
6–12	–	–	–	–	–	–	–	–	–	–
16–22	–	–	–	–	–	4	1	–	–	5078
30–36	–	1	1	1	9,3	2	3	–	1	2679
40–46	–	–	–	–	–	1	1	3	1	611
52–58	1	2	1	13	5,5	–	7	27	34	24
64–70	–	–	1	15	3,3	–	2	10	16	15
76–82	–	–	–	1	–	–	–	2	2	5

Discussion

In this study the effects of BUdR on developmental processes were observed in the tissues arising from the growth and differentiation of the imaginal disks and the analysis of analogue incorporation in DNA was based on total DNA content of third instar larvae. Since autoradiographs of *Drosophila* larvae fed ^{3}H-BUdR show label in both larval and imaginal disk nuclei [1], the studies on total-body DNA assume that the patterns of BUdR incorporation in DNA represent an overall average of all nuclei in the developing organism. This assumption may require modification if differential incorporation of BUdR among various body tissues does in fact occur. At any rate, the present study not only confirms that BUdR incorporated DNA in *Drosophila* larvae is retained when the larvae are returned to medium without BUdR, but it further demonstrates that the pattern characteristics for B-DNA and FB-DNA generated by isopycnic centrifugation in CsCl immediately following the pulse treatment are also retained after larvae are removed from BUdR.

Parallel examination of BUdR substituted DNA and BUdR effects on development for a variety of analogue treatment conditions suggest that the probability that growth lesions will be induced in the imaginal tissues of *Drosophila* is correlated with an increase in the heavier (1.73 g cm^{-3}) BUdR substituted DNA molecules in CsCl gradients. This will be apparent by comparing the total lesion frequency per wing in Table 1 obtained by the treatments labelled BUdR and BUdR + FU for 12 h with the results of the CsCl density gradients of DNA samples labelled B-12 and FB-12 in Fig. 2. It appears that effective incorporation entailing specific conditions must take place before lesions can be induced. Furthermore, the endogenous TdR or dTMP seems to compete with the conversion and incorporation of the ingested BUdR since higher levels of FU ingested along with BUdR not only increase the lesion frequency per wing (Table 2), but they also increase the fraction of heavy DNA (Fig. 3: $F_{\times 1}B$ and $F_{\times 4}B$). An alternate way for the ingested BUdR to compete successfully with endogenous TdR and dTMP is to give the larvae massive doses of BUdR which increases the frequency of lesions [2] and, as tested in the present study, increases the proportion of heavy DNA (Fig. 3: $B_{\times 1}$ and $B_{\times 20}$). Further support for the statement that BUdR competes with TdR is apparent from the DNA samples in Fig. 4 where the presence of TdR along with ingested BUdR + FU totally eliminates the heavy component. It is evident from the earlier reports [1, 2] and the study of the various parameters examined in this communication that incorporation of BUdR in DNA is a prerequisite for the induction of neoplastic lesions in *Drosophila*.

Exposure to BUdR induces a variety of developmental disturbances in embryonic systems [10–12]. Metabolic upsets during critical periods of embryogenesis would be expected to produce altered patterns of differentiation as a result of inhibition or enhancement of the growth of specific groups of differentiating cells. Schubert and Jacob [13] have demonstrated that BUdR hastens the incipient differentiation of cultured neuroblastoma cells primarily by an interaction between BUdR, the cell surface, and the substrate on which the cells are growing. This enhancement of differentiation occurred in the absence of DNA synthesis and the metabolic effects did not result in new paths of cell differentiation. Some of the events induced by BUdR in *Drosophila* development may be due to metabolic disturbances caused by the analogue. This possibility was examined by combining BUdR treatment with different inhibitors of thymidylic acid synthesis which have distinct overall metabolic effects [5]. For example, MTX affects folic acid-requiring metabolic steps in addition to inhibiting dTMP synthesis, and FU is incorporated in RNA and affects RNA-dependent processes. If metabolic effects play a significant role in generating BUdR induced growth lesions, then the outcome of treatment with MTX + BUdR and treatment with FU + BUdR would differ. The data did not support this expectation, but were consistent with the primary role of FU and MTX as inhibitors of endogenous dTMP synthesis [5].

Incorporation of BUdR in DNA would also be expected to disrupt normal differentiation, and the underlying mechanism in this case could relate to either aspect of DNA template activity, replication or transcription. Distortion of the transcriptive function of BUdR substituted DNA in 3T6 mouse fibroblasts *in vitro* has been reported recently by Hill *et al.* [14]. In their study, abnormal ratios of adenine to guanine were maximal in RNA transcribed from BUdR-substituted chromatin, and decreased with removal of protein from the chromatin. Production of altered mRNAs by a BUdR containing cell would affect the overall metabolic balance of the translated pool of cell proteins, and perhaps change the fate of the cell in differentiation. Such events, however, would be restricted to the cells retaining BUdR in their DNA since these properties would not be transmitted to descendant cells replicating in the absence of BUdR. The lack of fidelity of BUdR-incorporated DNA in RNA transcription raises a question regarding the fidelity of replication of BUdR-substituted DNA. It is well known that BUdR is mutagenic for microbial cells by virtue of its incorporation in DNA [15], and Chu *et al.* [16] have demonstrated that under the proper conditions for detection of mutations, BUdR is also an effective mutagen for mammalian cells in culture. It therefore becomes necessary to consider the possibility that the

modified patterns of cell differentiation found in the imaginal disks of *Drosophila* may be the result of somatic cell mutational events. The detection of mutated somatic cells in a diploid organism will be limited to dominant mutants, recessive sex-linked mutants in the heterogametic sex, and "operator constitutive" type mutations which by definition will be dominant. Nix [17] has demonstrated that the lethality of $sc^{sl}/0$ *Drosophila* larvae is due primrily to suppression of ribosomal RNA genes. In these long inversion-carrying nullo-Y males where the nucleolus organiser has been positioned near the left tip of the X chomosome, there has been no loss of ribosomal DNA but there is a 15% reduction in ribosomal RNA. This work was based on the elegant genetic analysis of Baker [18] who showed that deficiency of a region proximal to the centromere can restore the viability of X/O males carrying the inversion. It follows from these studies that in *Drosophila* at least, the activities of some genes are under negative control and must consist of "operator-like" elements in their organisation.

Analysis of the mechanism underlying BUdR-induced growth lesions in *Drosophila* must account for the following: (1) the appearance of lesions at random both among rhe individuals treated as well as among the cells of an affected individual; (2) similarity of types of lesions induced by treatment at any time during the larval growth period; (3) effectiveness of treatment as remote as three molting cycles (two larval and one pupal) prior to the stage of wing differentiation; (4) induction of lesions constituting clones of affected cells. These features are consistent with the hypothesis that BUdR is acting as a mutagen in the somatic cells of *Drosophila*, and in view of the correlation between analog incorporation in DNA and lesion induction, it is reasonable to inquire whether BUdR-DNA *per se* is responsible for the altered differentiation of wing cells, or whether events subsequent to BUdR incorporation into DNA change the prospective fate of the presumptive wing cells and transmission of this new state to progeny cells no longer requires the presence of BUdR in DNA. The morphological consequences of these two alternate interpretations are presented in Fig. 5. If the presence of BUdR in specific sites in DNA is required, then according to the semi-conservative mode of DNA replication only one progeny cell will retain the critically substituted BUdR-DNA or replicon and eventually appear as a single phenotypically altered cell among the clone of normal cells resulting from subsequent multiplication of the parent cell (H_1). If, after BUdR incorporation, the original informational state of the cell is changed, then all subsequent cell progeny will inherit the change and appear as a cluster of cells with the altered phenotype (H_2). Data from the series of larvae treated with BUdR + FU at different ages of

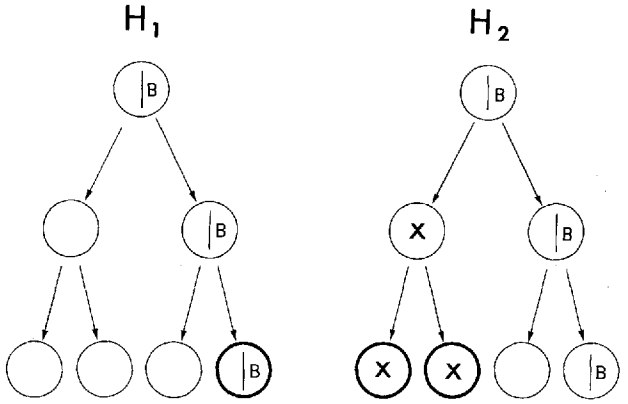

Fig. 5. Diagrammatic representation of two alternate hypotheses explaining the morphogenic effects of BUdR in *Drosophila*. H_1 illustrates the consequence of BUdR incorporation in a morphogenically critical sequence of informational DNA and its transmission to progeny cells according to the semi-conservative model of DNA replication following pulse treatment with BUdR. Under H_1 it is the BUdR incorporation *per se* that brings about a change in differentiation of the cell phenotype and only one of the four cell clones is depicted as an affected cell (heavy circle). H_2 illustrates the transmission of BUdR-DNA similar to H_1, but assumes an informational change (X) in the complementary strand of DNA due to the presence of BUdR in the critical region. H_2 allows formation of clusters of transdetermined cells (heavy circles) and does not require the presence of BUdR in DNA

development can be useful in considering these alternatives. The wing imaginal disk of the young larva consists of fewer presumptive cells than the wing disk from a late larval stage. Overall lesion frequency is lower at the earlier stages as compared to later stages (Table 3), and it is therefore reasonable to conclude that under a standard set of conditions lesion frequency is a function of the number of cells exposed to BUdR. Furthermore, both bristle and supernumerary lesions can be induced during all three larval instars. If the bristle clusters are ordered according to their sizes and examined as a function of time, it becomes obvious that induced clusters of bristles from early larvae given a six-hour pulse of BUdR contain more bristles than clusters induced in late larvae given the same treatment; if the supernumeraries are examined in a similar fashion, it is also apparent that the supernumerary lesions induced in the larvae treated at an early stage are considerably larger than those induced in older larvae. The development of a large supernumerary is indicative of multiplication of cells with a changed fate and the systematic difference in size of bristle clusters from early to late larvae also tends to reject the hypothesis that it is BUdR-DNA *per se* that is responsible for these effects, since according to this hypothesis a clone of phenotypically altered cells will not arise. Recently the data on the distribution of BUdR-induced

lesions was subjected to Poisson statistics (in preparation) by grouping the wings into three categories: wings without lesions, wings with one lesion, and wings with two or more lesions. The analysis is in agreement with Poisson expectations, and supports the hypothesis that these events are rare and randomly distributed rather than a mass response of the BUdR-incorporated cell population as was visualised in autoradiographs of imaginal disks [1]. In conclusion, it should be pointed out that what is hypothesised as changed at the DNA level are the regulatory elements of genes in the cells rather than the prospective potency of the cells since the latter is stored in the cell as the sum total of the structural genes. In terms of differentiation, however, that which is considered here as abnormal represents a change in the prospective fate of some cells since these are viewed as extra bristles or supernumerary structures whose appearance is inconsistent with the normal elements in the final pattern of the differentiated wing.

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