PHYSIOLOGICAL GENETICS OF MELANOTIC TUMORS IN *DROSOPHILA MELANOGASTER*. III. PHENOCRITICAL PERIOD IN RELATION TO TUMOR FORMATION IN THE *tu bw; st su-tu* STRAIN

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IN a series of publications, PLAINE and GLASS (1952, 1955) show that melanotic tumors can be induced in the *tu bw; st su-tu* strain of *Drosophila melanogaster* by feeding larvae a high concentration of L-tryptophan, or by exposing embryos or early larval stadia to X-irradiation. PLAINE (1955a) also shows that both treatments can be counteracted by including L-cysteine in the larval diet. According to PLAINE and GLASS (1955) and GLASS (1957), the similarity in the action of X-ray and tryptophan treatments lies in their influence on the reaction converting tryptophan to formylkynurenine (Figure 1). The enzyme controlling this reaction, tryptophan pyrrolase, acts in the presence of catalytic amounts of hydrogen peroxide. It has recently been assayed and shown to be inducible in *Drosophila* (KAUFMAN 1962; RIZKI and RIZKI 1963). PLAINE and GLASS suggest that an increase in the availability of substrate, brought about by feeding excess tryptophan, accelerates the rate of the reaction. By producing hydrogen peroxide in the larval tissues X-irradiation also catalyses the production of formylkynurenine, presumably from the pool of haemolymph tryptophan. L-cysteine, it is suggested, counteracts the effects of tryptophan and X rays by acting as a reducing agent which removes peroxides formed in the tissues by the ionising radiations, or reduces the peroxide already present in the tissues so that excess tryptophan provided in the diet cannot be used. Direct immersion of dechorionated eggs in solutions of hydrogen peroxide caused a small but significant increase in tumor penetrance (PLAINE 1955b). The *tu bw; st su-tu* strain is homozygous for a second chromosome melanotic tumor gene, with a specific tumor suppressor locus on chromosome 3, and PLAINE and GLASS (1955) and GLASS (1957) conclude that formylkynurenine interferes with the action of the tumor suppressor, thus permitting the phenotypic expression of the tumor gene.

Certain difficulties for this theory arise out of the observations of BURNET and SANG (1964) that dietary sterol deficiency and suboptimal balances of nucleotides in the diet also cause melanotic tumor formation in this strain, since these treatments do not fit into the scheme outlined above in any obvious way. In addition, melanotic tumors can be induced by the same dietary treatments in the *tu^K* strain, which carries a nonallelic tumor gene on the second chromosome (BURNET and

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FIGURE 1.—Enzymic synthesis of kynurenine from L-tryptophan.

SANG 1964). This strain is not readily affected by X-irradiation, suggesting that dietary and radiation treatments may operate through different mechanisms. Since genetic analysis also failed to show the presence of a specific suppressor system in the *tu^k* strain, BURNET and SANG (1964) suggest that, with the possible exception of X-irradiation, tumorigenic treatments may have a direct influence on the reaction controlled by the *tu bw* gene rather than on the activity of the suppressor.

The scheme outlined by PLAINE and GLASS involves two separate propositions, which are: (1) the tumorigenic treatments directly influence the same specified enzyme mediated reaction; (2) the tumorigenic treatments interfere with the action of the tumor suppressor gene. If the tumorigenic effect of the tryptophan and X-ray treatments depends upon their common influence on the tryptophan pyrrolase reaction in the way GLASS (1957) suggests, these treatments should be effective in modifying the phenotype during identical periods in development, and determination of the phenocritical period affords a method for testing this prediction. Also, treatments which interfere with the expression of the suppressor might not be expected to influence tumor penetrance in genotypes carrying the normal allele of the suppressor locus. On live yeasted media, penetrance is normally 95 to 100 percent for such genotypes (BURNET and SANG 1964) so that an increase would not be detected under these conditions, but tumor penetrance is lower on the corresponding aseptic diet, giving an opportunity for testing the second prediction.

MATERIALS AND METHODS

Melanotic tumors are usually localised in the abdomen and become melanised just prior to the close of the third larval stadium. They generally number less than four per individual. The proportion of individuals with tumors (tumor penetrance) was scored from adult flies and from unhatched pupae present in the cultures, no account being taken of expressivity. The strains used are: (1) *tu bw*; *st su-tu* (2) *tu bw*; *Sb +su-tu/TM3, ri P^b bx^{34c} e^s Ser su-tu* and (3) *tu bw*; *+su-tu*. All three are isogenic for the same second chromosome carrying brown with *tu* at locus 83.9 (GLASS 1954). Strain 1 is homozygous for scarlet and the suppressor. Strain 2 carries a balanced lethal third-chromosome combination heterozygous for an allele of the suppressor locus. The chromosome carrying the normal allele of the suppressor is marked with Stubble and is balanced by the TM3 chromosome carrying the suppressor and marked with Serrate (BURNET and SANG 1964). TM3 carries a complex system of inversions which suppress crossing over in chromosome 3. The third strain is homozygous for a wild-type chromosome carrying the normal allele of the suppressor, and with Strain 1 was kindly supplied by PROFESSOR BENTLEY GLASS. The strains were maintained as brother-sister mated lines. The *er* and *su-er* system also carried in these strains is not considered below.

Eggs were collected during a 4-hour period, and the larvae hatching 20 to 24 hours later

were inoculated into defined media under aseptic conditions, of which full details are given by SANG (1956) and SANG and BURNET (1963a). Forty larvae were placed in each culture, with 8 to 10 replicate cultures for each treatment.

Addition of substances to the germ-free cultures at different times during their development presents some technical difficulties. Where the substances are stable and soluble, like the pentose nucleotides, they were autoclaved in bulb pipettes set in the tubes prior to inoculation, in the manner described by SANG (1962). The sterile bulbs were then broken at the required times, when the solution rapidly diffused into the gel medium. When the substances were insoluble at the required concentrations, like L-tryptophan or cholesterol, a sterile suspension was added by pipette under aseptic conditions. This was a far from satisfactory procedure since larvae could avoid the high surface concentrations by feeding at the sides of the tubes. An alternative method, used for adding cholesterol, was to grind the cholesterol into a mixture of autoclaved bakers' and brewers' yeast in the required amount, and to drop this on the surface of the medium where the larvae then fed. When the substance was unstable, like L-cysteine, it was Seitz-filtered in acid solution (pH 2) and added to the autoclaved medium immediately before gelation.

Embryos and newly hatched larvae were irradiated on plain agar plates (kvp 140, 5 ma, distance 20 cm, 347 r/min) and subsequently grown on yeasted cornmeal-molasses media. Older larvae were irradiated on the yeasted medium in which they were growing. Since the effects of protective agents had to be assessed using defined, aseptic media, the larvae had to be irradiated *in situ*, the dose on the axis of the tube at the surface of the gel medium having been previously calibrated by direct measurement. Control and supplemented cultures were irradiated together (kvp 140, 5 ma, Fsd 25 cm from tube axis, filter 1 mm aluminium, 114 r/min measured at gel surface). A preliminary test showed that twice the maximum dose reported here produced no radiochemical changes in the medium which affected survival, development rate or tumor frequency among untreated larvae inoculated into irradiated cultures.

The rate of larval development is not the same on different treatment media, and this complicates any comparisons between different experiments. In the present instance we have standardised development times by simple, proportional adjustment of the larval period to a standard 96-hour period (BODENSTEIN 1950). Since there was no significant heterogeneity amongst replicates, significance of differences is tested by the usual 2 by 2 chi-square.

RESULTS

1. *Phenocritical periods*: The phenocritical period (NEEDHAM 1942) is a period in the development of the organism during which the effect of a gene can readily be influenced by externally applied factors. It has usually been measured as the temperature effective period, and there are few comparisons of phenocritical periods determined using different treatments (for an example see SANG and McDONALD 1954). The evidence suggests that agencies with the same effect act during the same, or overlapping, periods. It is therefore of special interest in the present case to find if the physical and chemical treatments appear to act at the same time, or whether the different chemical treatments are distinguishable from one another. The technical difficulties in the way of doing this have already been listed; in addition, we must also note that we are dealing with populations which vary in physiological age as they grow. Consequently, even a brief, all-or-none response phase in the individual will appear as spread over a period corresponding to the physiological stages within the population (CHILD 1935). This sampling effect cannot be distinguished from graded responses which depend on the amount of treatment accumulated within the critical period.

Nucleotides: Tumor penetrance in the *tu bw*; *st su-tu* strain is quantitatively

determined by the relative concentrations of purine and pyrimidine ribonucleotides in the larval food medium (BURNET and SANG 1964). In nucleic acid-free media, increasing amounts of adenylic acid lower tumor penetrance, whereas increasing amounts of cytidylic acid increase tumor penetrance. The sensitive period for nucleotide balance can therefore be tested by increasing tumor penetrance by adding 0.093 percent cytidylic acid to RNA-free media or, conversely, by lowering tumor frequency by adding adenylic acid to a cytidylic acid-containing medium. In both series (Figure 2 a and b) addition of the nucleotide is fully effective up to about 40 hours of larval life, after which it becomes less effective. The two responses intersect at about 52 hours, which is near the midpoint of both curves, and this is the closest estimate we can make of the mean sensitive period for the populations. It is of considerable interest that the sensitive period for a strain (*tu^K*) carrying a nonallelic tumor gene is similar to that determined above (SANG and BURNET 1963 a).

Cholesterol deficiency: Figure 2 c shows the effect of adding a yeast + cholesterol mixture to cholesterol deficient medium. The tumorigenic effect of the deficiency becomes irreversible after about 40 hours and is completely so by 65 hours of the larval period, giving an estimate of 50 to 55 hours for the midpoint of the cholesterol sensitive period.

Tryptophan: Addition of a suspension of L-tryptophan to the synthetic medium

FIGURE 2.—Determination of the sensitive periods for X-ray and dietary environmental treatments in the *tu bw*; *st su-tu* strain. Nucleotides: (a) 0.093 percent cytidylic acid added to RNA free medium, (b) 0.2 percent adenylic acid added to medium containing cytidylic acid. Cholesterol: (c) yeast cholesterol mixture added to cholesterol deficient (0.00156 percent) diet. Tryptophan: (d) addition of 0.8 percent L-tryptophan to the synthetic diet, (e) addition of yeast mixture to a diet containing 0.8 percent tryptophan. X rays: (f) 1000r X rays given to embryos or larvae *in situ* on the culture medium.

(Figure 2, series d) proved to be toxic to first instar larvae owing to the high concentration of the amino acid remaining at the surface of the gel. Maximum tumor penetrance attained in this series is also lower than expected because of the uneven distribution of the suspension to older larvae which feed below the surface of the medium. For the converse manipulation (Figure 2, series e) larvae were inoculated onto a medium containing 0.8 percent tryptophan, and an autoclaved yeast mixture added after different intervals. The two series give an estimate of 48 to 51 hours for the midpoint of the tryptophan sensitive period.

X-irradiation: A significant increase in tumor penetrance can be detected when embryos are exposed to 1000r X rays as early as 3 hours from the time of laying. The response to the same dose reaches a maximum around the time of eclosion of first instar larvae from the egg, but falls rapidly after the close of the first larval stadium. The duration of the X-ray sensitive period appears to cover the whole of the embryonic period and approximately the first 36 hours of the larval period (Figure 2, series f).

The phenocritical period for the melanotic tumor gene *tu bw* lasts from early in the embryonic period to the beginning of the third larval instar, but consists of two distinct periods of sensitivity to different types of environmental influence, the X-ray and dietary sensitive periods. Since dietary treatments are reversible during the first and most of the second instar, they are effective only during a short period which includes the ecdysis between the second and third larval stadia, whereas the X-ray sensitive period begins early in embryonic development, covers the first larval instar, but terminates before the beginning of the dietary sensitive period. Thus, the different environmental treatments influence the expression of the tumor gene through temporally different processes. The possibility cannot be discounted that X rays may nevertheless influence the phenotype at the same time as the tryptophan treatment by a delayed effect through some intermediate reaction. If this is indeed the case, the action of X rays on tumor penetrance cannot be through the simple direct mechanism postulated by PLAINE and GLASS (1955).

2. *The protective effect of cysteine:* The protective effect of cysteine against both tryptophan and X-ray treatments argues in favor of their having a common mechanism of action in tumorigenesis. PLAINE (1955a) and GLASS (1957) visualised the role of cysteine essentially as that of a radical scavenger, removing peroxide necessary for the activity of tryptophan pyrrolase, and so blocking formation of the effective tumorigenic agent. But, to the extent that tryptophan and X-irradiation operate through different mechanisms, for the reasons shown above, so the protective effect of cysteine might also be different in each case. Cysteine is one of a group of aminothiols which afford protection against radiation damage for certain mammals, although the evidence is equivocal for insects. Additionally, cysteine may act as a sulfur donor in the detoxication and excretion of abnormal quantities of aromatic amines resulting from high concentrations of dietary tryptophan, a possibility suggested by the data of WHITE and WHITE (1938) who were able to reverse the growth-inhibiting effects of certain carcinogenic hydrocarbons on rats, by dietary supplements of sulfur amino acids. This mechanism

finds no obvious application in the case of cholesterol deficiency or nucleotide balance effects, and the following experiments were designed to test whether cysteine has any protective effect against these treatments.

Larvae were irradiated *in situ*, in germ-free cultures, as described in MATERIALS and METHODS, with a dose of 1000r, 20 hours after inoculation onto the medium, and the tumorigenic dietary treatments employed were 0.8 percent tryptophan, 0.00156 percent cholesterol, and 0.0465 percent cytidylic acid, respectively.

Dietary administration of L-cysteine in germ-free synthetic diets presents certain technical problems which require description here. The amino acid is unstable in solution at neutral pH and rapidly oxidises to cystine, which is insoluble. Accordingly, L-cysteine HCl was Seitz-filtered in aqueous solution at pH2 and delivered to autoclaved cultures immediately before gelation, to give a concentration of 0.1 percent cysteine in the medium finally.

The results summarised in Table 1 show that cysteine reduced the proportion of X-ray induced tumors but had no significant effect on the dietary treatments. The effect of cysteine with respect to X-ray treatment confirms the observations of PLAINE (1955a), whilst failure to obtain any reduction in tumor penetrance with this compound in the case of the dietary treatments could be due to the oxidation of the amino acid, during the interval between its inclusion in the medium and the end of the second larval instar. Since the gel medium is buffered at pH 6.8, oxidation of cysteine to cystine would be expected to occur at a fairly rapid rate, and although larvae accumulate sufficient cysteine during the 20-hour feeding period before X-irradiation, an effective dietary concentration may no longer be available at the beginning of the dietary sensitive period of the *tu* gene. However, repeat tests for the cysteine protective effect do not yield consistent results and this avenue has not been pursued further owing to this technical difficulty.

PLAINE and GLASS (1955) seek to account for the action of X rays and of tryptophan by an influence through the activity of tryptophan pyrrolase on the formation of formylkynurenene which, they suggest, may be the effective tumorigenic agent. Now tryptophan pyrrolase in *Drosophila* is controlled by the vermilion locus, and in the presence of the *v* mutant the activity of this enzyme is only 1 to 2 percent of the wild type, which effectively blocks the synthesis of formylkynurenine. Hence we would expect tryptophan treatment to have no effect on

TABLE 1

Effect of L-cysteine (0.1 percent) on the tumorigenic effect of X-rays (1000r) and dietary treatments (excess L-tryptophan 0.8 percent, cholesterol deficiency 0.00156 percent, and cytidylic acid 0.0465 percent) in the tu bw; st su-tu strain.
Tumor penetrance is expressed in percent

	Treatment			
	X rays	Tryptophan	Cholesterol	Cytidylic acid
Control	71.5	89.2	83.0	26.2
+ Cysteine	49.5**	91.5	88.4	22.6

** Significantly different from control at the 1 percent level.

tumor penetrance when the *v* mutant is present, but this expectation is contradicted by the data of KANEHISA (1956), who reports an increase in tumor penetrance in a *v tu* strain when tryptophan is included in the diet. (The tumor gene in the *v tu* strain synthesised by KANEHISA was derived from the *tu bw* strain studied by HARTUNG [1950], which is reported to be an allele of the *tu* gene in the *tu bw*; *st su-tu* strain [GLASS 1954]).

Figure 1 shows that formylkynurenine is the immediate precursor of kynurenine, synthesised by the enzyme kynurenine formamidase, present in *Drosophila* in amounts that are not likely to be limiting on kynurenine formation (GLASSMAN 1956; KAUFMAN 1962). Consequently we might expect an increased rate of formylkynurenine synthesis, induced by feeding tryptophan, to be reflected by an increase in rate of kynurenine synthesis, as is amply confirmed by the elegant studies of RIZKI and RIZKI (1963). Using autofluorescence techniques on the larval fat body these authors find that, by feeding tryptophan, kynurenine synthesis can be induced in certain cells which normally synthesise only isoxanthopterin. The larvae must be kept on the tryptophan diet until late in third instar when kynurenine fluorescence normally begins. These observations show that tryptophan induced kynurenine synthesis occurs late in larval development after the close of the phenocritical period of the tumor gene. Thus the tryptophan and X-ray sensitive periods occupy different phases of development, and neither coincides with the time when tryptophan induced kynurenine synthesis can be demonstrated in the larvae, showing that the action of these treatments is unlikely to be mediated through formylkynurenine synthesis in the way that PLAINE and GLASS (1955) suggest.

Such evidence is, however, open to the objection that the autofluorescence technique probably indicates rather the time at which kynurenine is accumulated than when it is synthesised, and synthesis along this pathway may nevertheless begin at a much earlier stage. More definitive data are necessary on this point.

3. *Penetrance in unsuppressed strains:* Although X-ray treatment appears to influence tumor penetrance by a mechanism which, at least in its timing, differs from that of dietary treatments, the responses of the *tu bw*; *st su-tu* strain alone do not allow us to distinguish which treatments operate directly on the expression of the tumor gene, and which indirectly through the expression of the suppressor. To decide this point it is necessary to examine the effect of treatments on penetrance in unsuppressed *tu* strains, since those of the latter group should not be effective in the presence of the normal allele at the suppressor locus.

Table 2 shows tumor penetrance for three genotypes isogenic for the *tu bw* second chromosome but with different allelic combinations at the suppressor locus. On the live yeast medium, which provides optimal growth conditions for these strains, the *tu* gene is almost fully penetrant in the presence of the wild-type allele of the suppressor ($+^{su-tu}$), but the aseptic medium is in some respects suboptimal for tumor penetrance which is 70.0 and 47.5 percent in the *tu*; *su-tu*/ $+^{su-tu}$ and *tu*; $+^{su-tu}$ strains respectively. Tumor penetrance in the *tu*; $+^{su-tu}$ strain was higher than the control for larvae cultured on cholesterol deficient and excess tryptophan media, or irradiated *in situ* 20 hours after inoculation into asep-

TABLE 2

Tumor penetrance (percent) in strains carrying different allelic combinations at the suppressor locus, on live yeast medium, and on fully supplemented aseptic synthetic medium, with three tumorigenic treatments: cholesterol deficiency (0.00156 percent), excess L-tryptophan (0.8 percent) and X rays (1000r)

	<i>tu bw; st su-tu</i>	<i>tu bw; Ser su-tu/Sb +su-tu</i>	<i>tu bw; +su-tu</i>
Live yeast	7.5	99.5**	95.0**
Aseptic synthetic (Control)	10.6	70.0	47.5
Cholesterol deficiency	88.8**	61.2	72.9**
L-tryptophan excess	90.7**	95.0**	87.0**
X rays	87.4**	96.3**	95.2**

** Significantly different from control at the 1 percent level.

tic cultures. With the exception of cholesterol deficiency, the response to treatment is in the same direction for *tu; su-tu/+su-tu*.

These observations show unequivocally that the response to tryptophan and X-ray treatments is in the same direction, namely increased tumor penetrance, irrespective of which allelic combination is present at the suppressor locus. Consequently the treatments must have some direct influence on the expression of the *tu* gene as BURNET and SANG (1964) have suggested, rather than an indirect influence by interfering only with the expression of the suppressor.

The difference between the level of penetrance on synthetic medium for the two unsuppressed strains illustrates the complexity of the situation, which does not yield to a simplified explanation in terms of a single gene-environment interaction involving either the tumor gene, or the suppressor, alone. The strain difference is possibly a consequence of residual variation between the different third chromosomes, or the remainder of the genome excluding the second chromosome; or it may derive directly from the particular alleles at the suppressor locus, since these are of independent origin in each of three stocks used here. A possibility suggested by GLASS's (1957) analysis of the erupt suppressor system is that wild-type alleles of different origin at the tumor suppressor locus may turn out to be a group of isoalleles of varying potency, that is, differing in their threshold of action, which can only be distinguished when in specific genetic combinations or under special environmental conditions. On the other hand, the live yeast and synthetic media may differ with respect to environmental agencies which influence the expression of the tumor gene in *tu; +su-tu* combinations by biochemical mechanism quite different to that through which the suppressor operates. The effect would be subject to modulation by such differences in genetic background as are present in the three strains studied here. Analysis of these possibilities is difficult and calls for information on the nature of the underlying metabolic effects of both the tumor gene and the suppressor. Due to the low level of penetrance in the *tu; su-tu* strain attention has necessarily been focussed on dietary manipulations which increase tumor penetrance, but current investigations on the *tu; +su-tu* (SANG and BURNET, unpublished) reveal a complex array of interactions by which tumor penetrance can be varied in both directions, and will be reported in a further publication of this series.

DISCUSSION

Phenocritical period, used here in the sense defined by NEEDHAM (1942), must be distinguished from phenocritical phase used by HADORN (1961) to denote the stage at which the first difference between the mutant and wild-type phenotype can be recognised. Sensitive period is used here to define the period during which the phenotypic expression of a gene can be influenced by a *specified* environmental treatment. HADORN (1961) uses the term sensitive phase in a more general sense. The temperature and dextrose sensitive periods for the antennaless mutant studied by BEGG and SANG (1945) coincide with each other and with the first detectable differences in growth between normal and antennaless imaginal discs. In this case phenocritical and sensitive phases of HADORN's (1961) terminology coincide, but in other cases the sensitive period may precede the earliest detected differences in phenotype by a considerable interval.

Comparison of the sensitive periods for different tumor genes is complicated by differences in the accuracy with which material has been timed for different strains, and by strain and treatment differences of development rate. This difficulty may be overcome by comparing the observations of different authors on the standard scale mentioned above (MATERIALS and METHODS), bearing in mind the underlying assumption that factors influencing development rate operate uniformly over the whole larval period. Tumor penetrance in the *tu-e* strain is influenced by dietary variations and, using a killed yeast medium, FRIEDMAN, HARNLEY and KOPAC (1955) find the sensitive period to be at 72 hours of larval life, when larvae are transferred from yeast deficient to complete medium. Larvae kept on the deficient medium for the whole of their development required 10.3 days to emergence, so that subtracting the pupal period (approximately 4.3 days at 25°C) this represents a larval period of 144 hours, placing the sensitive period at around 48 hours on the standard 96-hour scale, which coincides with the dietary sensitive period for *tu bw*. There is also a close agreement between the sensi-

TABLE 3

The phenocritical periods determined by X-ray and dietary environmental treatments for different melanotic tumor genes

Tumor strain	Treatment	Sensitive period*	Source
<i>tu-e</i>	Yeast deficiency	Circa 48 hr	FRIEDMAN et al. (1955)
<i>tu 48 a</i>	X-irradiation	Embryonic period to 42-58 hr	GHELÉLOVITCH (1961)
	Temperature	After 48 hr	GHELÉLOVITCH (1958)
	Yeast deficiency	Third instar	GHELÉLOVITCH (1958)
<i>tu^K</i>	Nucleotide balance	Circa 48 hr	SANG and BURNET (1963a)
<i>tu bw; st su-tu</i>	X-irradiation	Embryonic period to circa 36 hr	PLAINE (1955)
	Nucleotide balance Cholesterol deficiency L-tryptophan excess	45-55 hr	This study

* Larval age is expressed in hours after eclosion from the egg, corrected in each case to a 96-hour larval period.

tive period for dietary nucleotide changes for *tu bw* and *tu^K* determined in both cases on defined diets. GHÉLÉLOVITCH (1958) examined both the temperature and nutritional sensitive periods for *tu48a* (2–29.5), both of which start at the beginning of the third instar, but differ in duration with different genetic backgrounds. The sensitive period for yeast deficiency found by this author extends well into the third instar. The period of X-ray sensitivity for *tu48a* began in the embryo and ended between 75 and 95 hours after oviposition (GHÉLÉLOVITCH 1961), and the unirradiated control larvae pupated towards the end of the sixth day after oviposition (GHÉLÉLOVITCH, personal communication). Thus, allowing 22 hours for the embryonic period, the phase of X-ray sensitivity ends between 42 and 58 hours of larval development on the standard scale.

GHÉLÉLOVITCH's observations on *tu48a* agree with those reported here for *tu bw* in that the phenocritical period for the tumor gene consists of more than one sensitive period, a situation which is not peculiar to tumor genes as SANG and BURNET's (1963b) study of the phenocritical period for *ey^K* clearly shows. Genes of this type probably cause disruption of buffering at more than one point in the normal developmental pathway; a fact relevant to the suggestion of HENKE, FINCK and MA (1941), that true and false phenocopies might be distinguished on the basis of a comparison of the time at which a particular environmental stimulus is effective, and the time at which the development of the homologous mutant deviates from the wild type. If we know much too little about the real point of inception of mutant gene action to use it as a criterion for comparisons, as LANDAUER (1959) points out, the additional complications, that sensitive periods may not be identical for different treatments influencing the penetrance of the same mutant gene, places a formidable difficulty in the way of this comparison (GLASS 1957).

The termination of the sensitive periods for *tu bw* fixes the commencement of the third instar as the point beyond which the phenotype is irreversibly determined. But fully pigmented tumors do not make their appearance until 90 to 95 hours, which is just prior to pupation, so that cytological changes indicative of tumor formation might be expected somewhere between 48 and 90 hours of larval life. Tumor strains that have been examined cytologically are alike in the participation of lamellocytes in tumor formation, by encapsulation of other haemocytes or of fat body cells (OFTEDAL 1953; RIZKI 1957; SANG and BURNET 1963a), whereas lamellocytes (spindle cells) do not appear in wild-type larvae until pupation, when they are associated with histolysis of the larval tissues (RIZKI 1957). In the *tu^w* strain, studied in detail by RIZKI (1962), the haemocyte transforma-

TABLE 4

The timing of cytological changes associated with tumor formation in different tumor strains. The timing of each event is expressed in hours after eclosion from the egg, corrected in each case to a 96 hour larval period

Tumor strain	Haemocyte transformation	Encapsulation	Melanized tumors	Source
<i>tu 49^K</i>	52–57.5 hr	61–63 hr	78 hr	OFTEDAL (1953)
<i>tu^w</i>	48–54 hr	70 hr	After 90 hr	RIZKI (1957, 1960)
<i>tu bw; st su-tu</i>	40–60 hr	Circa 72 hr	Circa 90 hr	Unpublished data

tion giving rise to this blood cell variant begins at the ecdysis between the second and third larval stadia, and lamellocytes are present in 54-hour old larvae. OFTEDAL (1953) also noted the appearance of lamellocytes in the *tu49k* strain (at 65 to 72 hours, with 120-hour total larval period) which agrees very closely with the *tu^w* and *tu bw*; *st su-tu* strains on the 96-hour scale, as shown in Table 4. The haemocyte transformation appears to be the earliest cytological change associated with tumor formation so far detected, and gives rise to lamellocytes which form amelanotic tumors by encapsulating other haemocytes, or fat-body cells. The tumors become pigmented toward the close of the third instar to give the characteristic tumor phenotype.

The relationship between the phenocritical period and the timing of the haemocyte transformation implies a critical threshold at the ecdysis between the second and third larval stadia. Possibly, as RIZKI (1957) suggested, the melanotic tumor phenotype is due to a genetic timing error in the reaction to changes in hormone balance at this point in development, a view which gains indirect support from the observations of BURDETTE (1954) and OSTER (1954) on tumor penetrance in genotypes carrying mutant genes thought to cause defects of the humoral system. Further observations involving direct administration of the juvenile and pupation hormones are necessary to test this idea.

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SUMMARY

Attempts to define the phenocritical period for the *tu* gene, in the *tu bw*; *st su-tu* strain, by environmental treatments show that the period in which tumor penetrance can be influenced by X rays differs in time and duration from the periods in which dietary environmental treatments are similarly effective. The X-ray sensitive period begins early in embryonic development and terminates after the beginning of the first larval instar. The sensitive periods for excess dietary tryptophan, cholesterol deficiency, and suboptimal balances of dietary nucleotides, are the same within the limits of resolution of the experimental data, beginning towards the end of the second instar and terminating after the beginning of the third instar. X-ray and dietary environmental treatments consequently influence tumor penetrance through temporally different developmental reactions.

Since X-ray and dietary treatments cause an increase in tumor penetrance in strains homozygous for the *tu bw* second chromosome, irrespective of the allelic combination present at the suppressor locus on chromosome 3, they have a direct influence on the penetrance of the tumor gene, rather than an indirect effect due to interference with the phenotypic expression of the suppressor.

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