PHYSIOLOGICAL GENETICS OF MELANOTIC TUMORS IN DROSOPHILA MELANOGASTER. I. THE EFFECTS OF NUTRIENT BALANCE ON TUMOR PENETRANCE IN THE tu^K STRAIN

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GENETICALLY determined melanotic tumors have been described in a number of strains of *Drosophila melanogaster*, usually involving major loci on the second chromosome (see GHÉLÉLOVITCH 1958 and 1959). Nearly all have the common feature that in isogenic strains only a proportion of individuals actually exhibit the melanotic tumor phenotype; that is to say, they belong to the troublesome class of genes of variable penetrance. This variability suggests that the position of the developing system with respect to the threshold between normal and atypical development is set by particular components of the genotype-environment interaction, and directly poses the problem of what special environmental variables are involved, and the nature of their physiological effect. An approach to this problem has recently become possible through development of a technique for culturing Drosophila on chemically defined media under completely germ-free conditions during the entire life cycle, permitting environmental manipulations impossible on the yeasted media commonly in use. As mutant genes are thought to act by causing quantitative as well as qualitative metabolic changes during development, it seems reasonable to expect that environmental influences which modify the phenotypic expression of mutant genes will afford some clue as to the nature of these changes. This is the primary concern of this series of studies.

This first paper describes results obtained with a new melanotic tumor strain which normally shows very low penetrance of the tumor phenotype. By altering the balance of nutrients available to larvae grown in defined media, it is possible to produce changes in the pattern of pentose nucleotide and sterol utilization, which cause melanotic tumors to appear in the majority of the larvae. A preliminary report of this work was given by BURNET and SANG (1962).

MATERIALS AND METHODS

The strain used in this investigation is an inbred Oregon-K line carrying the fourth chromosome marker recessive eyeless (ey^K) which has been kept for over 300 generations under a system of full sib mating. It will be referred to below as the tu^K strain. The pure line is propagated on the usual maize-meal molasses medium fortified with dried yeast and seeded with live baker's yeast. The tech-

nique for collecting and sterilizing eggs from mature females is as previously described by SANG (1956). The sterilized eggs are incubated at 25°C on sterile agar plates and germ-free newly hatched larvae transferred to chemically defined, aseptic media by sterile paper spoons. Unless mentioned otherwise, each culture contains 40 larvae.

Five ml of the chemically defined medium shown in Table 1 is delivered to 6×1 inch boiling tubes and autoclaved for a standard time (20 minutes at 15 lbs pressure and 120°C). The medium differs from that previously described (SANG 1956), in that sucrose is substituted for fructose in order to minimize the Malliard reaction between sugar and amino acids during autoclaving. Ribonucleic acid (RNA) is omitted from the medium for all experiments involving addition of pentose or deoxypentose nucleotides.

Compounds to be added to the medium at different times during the larval period are included in solution in bulb pipettes which are autoclaved with the medium. The procedure is described in detail by SANG (1962). Routine subcultures in nutrient broth, and nutrient agar slants incubated at 37°C, are taken to ensure sterility of the cultures. Any infected culture is rejected.

Larval development time is taken as the number of days to eclosion of the adult, minus the pupal period, and expressed as the logarithm averaged for replicate cultures. Melanotic tumors are scored in adult flies, and such unhatched pupae as may be present in the culture. Tumor frequency estimates based on counts of larvae are unreliable for reasons which will become evident below. Tumor penetrance is expressed as the percentage of tumor-bearing individuals for a given treatment averaged for replicate cultures. In Figure 9, results are plotted on a probit scale in order to correct the inaccuracy of percentage estimates at the upper and lower limits (FINNEY 1947).

TABLE 1

Composition of the larval food medium

	grams
Agar (Oxoid, Kobe No. 1)	3.00
Casein (Genatosan, low vitamin)	5.50
Sucrose	0.75
Cholesterol	0.03
Lecithin	0.40
Ribonucleic acid (California Biochem.)	0.40
Thiamine, HCl	0.0002
Riboflavin	0.0001
Niacin	0.0012
Ca pantothenate	0.0016
Pyridoxine, HCl	0.00025
Biotin	0.000016
Folic acid	0.0003
$NaHCO_3$	0.140
KH_2PO_4	0.183
Na_2HPO_4	0.189
Water to	100 ml

The histology of melanotic tumors: Melanotic tumors first become evident in the tu^K strain towards the end of the third instar, as small black nodules, varying in number, but seldom exceeding three or four. They lie freely in the abdominal hemocele but may occasionally be associated with the fat body. Tumors in this and in other strains examined involve aggregations of one or more of the hemocytes of the larval hemolymph (GHÉLÉLOVITCH 1959; LEWIS 1954; and RIZKI 1957a, 1962.) A detailed description of the blood cells of Drosophila larvae has been given by RIZKI (1957b) who distinguishes four types of hemocyte: small spherical plasmatocytes, crystal cells which are much larger and contain crystaline inclusions, podocytes, and disc-shaped lamellocytes. Podocytes are considered to be a transitional stage in the transformation of plasmatocytes to lamellocytes. RIZKI finds that larvae of tumorous strains show a precocious transformation of plasmatocytes to lamellocytes at the beginning of the third instar, a process normally occurring only at pupation in non-tumorous larvae.

In the tu^K strain, small cell groups consisting of plasmatocytes and crystal cells can be found in the hemocele at the beginning of the third instar. These tend to congregate in the posterior segments and occur both in tumorous and non-tumorous larvae (Figure 1A). Unpigmented tumors consist of similar aggregations of small spherical cells resembling plasmatocytes encapsulated by lamellocytes which form a dense stroma (Figure 1B). At the onset of pupation the tumor becomes melanised. Deposition of black pigment begins in the stroma and spreads centripetally, producing an inert black mass in which the cellular structure is obscured (Figure 1C). In brief, the tumors arise in the same way and are similar in form to those described by the authors noted above.

Dietary-environmental treatments. The effect of larval density and temperature on tumor penetrance: Melanotic tumors occur at very low frequency in larvae grown on fully supplemented live yeast or on sterile synthetic media, at the optimum density of 40 larvae per culture. Progressive degrees of inanition can be achieved by crowding larvae and, in this way, increasing the degree of competition for available nutrients (SANG 1950, 1959). Table 2 shows the effect of varying larval density on live-yeast medium, and on sterile synthetic medium, at two temperatures, 25°C which is the optimum for growth, and 18°C which more than doubles the total larval growth period. None of the four series shows a systematic change in tumor frequency with larval density, suggesting that generalized food shortage has no effect on tumor penetrance for the strain. There is complete agreement between the 18°C and 25°C series for each type of medium, showing that the rate of development as such has no effect on penetrance. These results suggest that tumor penetrance in the tu^K strain is influenced by the balance of nutrients rather than by their overall availability.

Specific nutritional deficiencies: The effect of specific nutritional deficiencies on the penetrance and expressivity of melanotic tumors has not hitherto been examined using chemically defined aseptic media. Owing to the limitations inherent in yeasted media, the closest approach to specific deficiencies has been the inclusion of antimetabolites in the food medium by FRIEDMAN, HARNLEY, and KOPAC (1955). While this is a useful technique in some respects, the in-

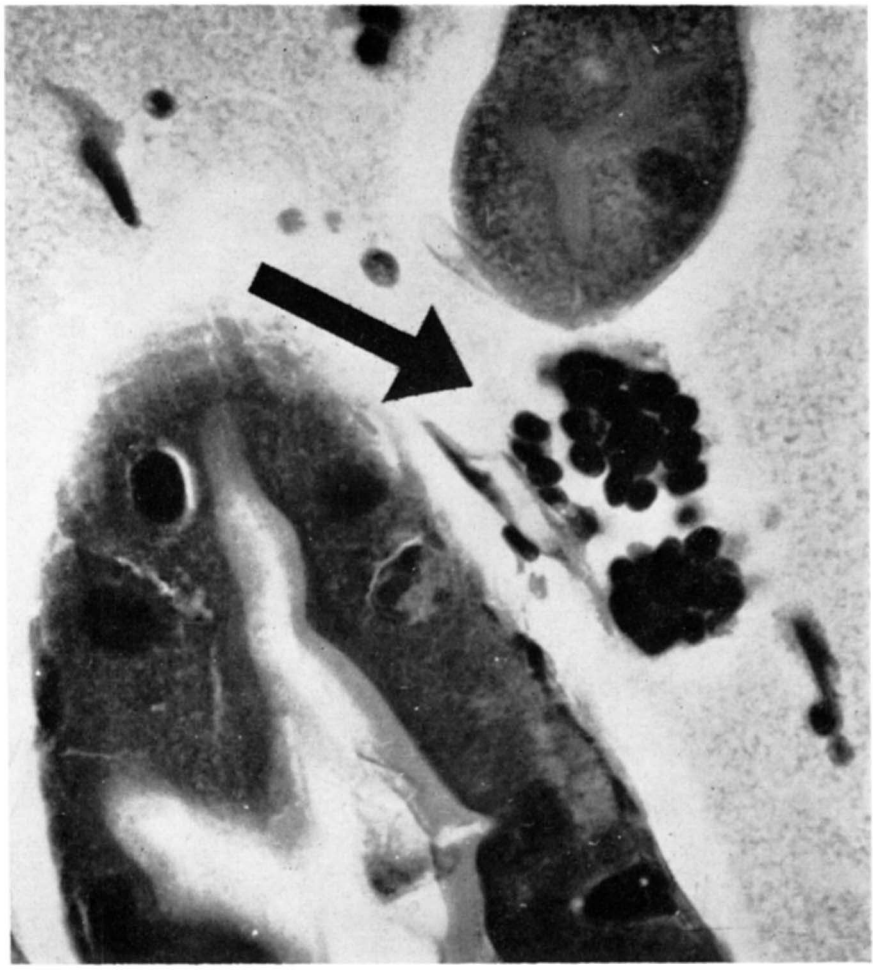

FIGURE 1A.—Aggregation of hemocytes consisting of plasmatocytes and crystal cells in the larval hemocele. 900×.

terpretation of results obtained with growth factor analogues must always be contingent upon comparison with the effects of an actual deficiency of the metabolite which is thought to be antagonized.

A complete analysis of the effects of specific deficiencies on larval growth rate and body size is available, and need not be repeated here (SANG 1956). In the following experiments, the contents of the medium are maintained at the control level shown in Table 1, with exception of the nutrient to be examined, which is reduced to the concentration shown in Table 3. Omission of ribonucleic acid from the medium induces melanotic tumors in a high proportion of individuals. In addition, reduction of the concentration of cholesterol and biotin, which are obligate requirements for survival of larvae, also causes a highly significant increase in penetrance above the control level. Biotin deficiency produces a sharp

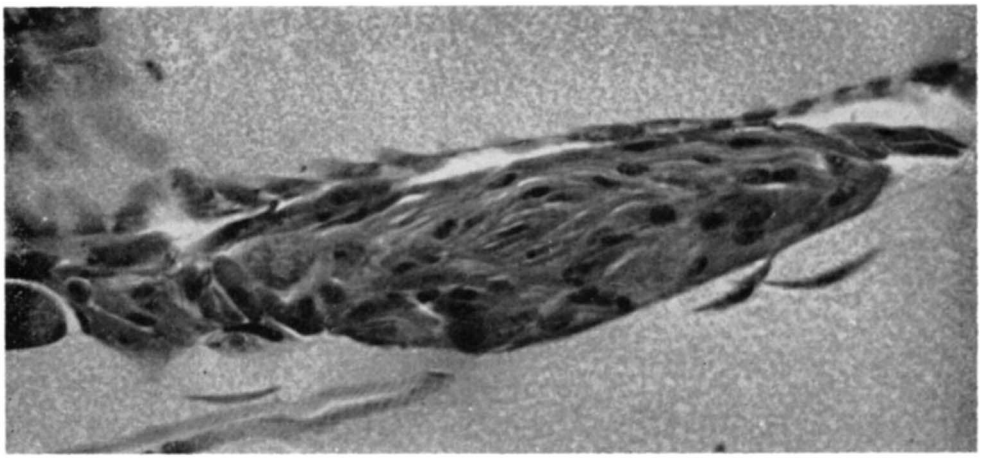

FIGURE 1B.—Unpigmented tumor showing formation of the stroma by encapsulating lamellocytes. Notice the two crescent-shaped lamellocytes to the right of the tumor. 650× magnification.

decline in the proportion of pupae which yield viable adults, in addition to its effect on tumor frequency. None of the other nutrients causes a significant increase in the proportion of affected individuals at concentrations permitting survival of 50 percent or more of the larvae.

The effect of ribonucleic and deoxyribonucleic acid: Ribonucleic acid (RNA) is not an essential dietary requirement for most inbred strains of *D. melanogaster*, but its inclusion is necessary for optimum rate of development. An exogenous source of deoxyribonucleic acid (DNA) is unnecessary, the requirement presumably being satisfied by *de novo* synthesis, or by utilization of ribonucleotides as precursors. Therefore, although the results presented in Table 3 suggest that realization of the normal phenotype depends upon an adequate supply of dietary RNA, the question arises as to whether the effect may not, in reality, be a concealed requirement for DNA.

Figure 2 shows the relationship between the frequency of individuals with tumors and the concentration of RNA provided in the medium. Decrease in concentration from the control level of 0.4 percent to 0.1 percent has no effect, but below this threshold there is a linear rise in the proportion affected. The final frequency does not reach 100 percent on medium without nucleic acid, presumably reflecting the known *de novo* synthesis of RNA by the larvae.

The effect of two preparations of DNA, from herring sperm and a highly polymerized preparation from calf thymus, were examined on RNA-free media. Increasing the concentration of DNA in the medium causes a reduction in the proportion of individuals with tumors, but in both cases the distribution flattens out at 16 to 24 percent affected. DNA is less effective than RNA in reducing tumor penetrance, and eight to 16 times the equivalent concentration is required to achieve the maximum effect.

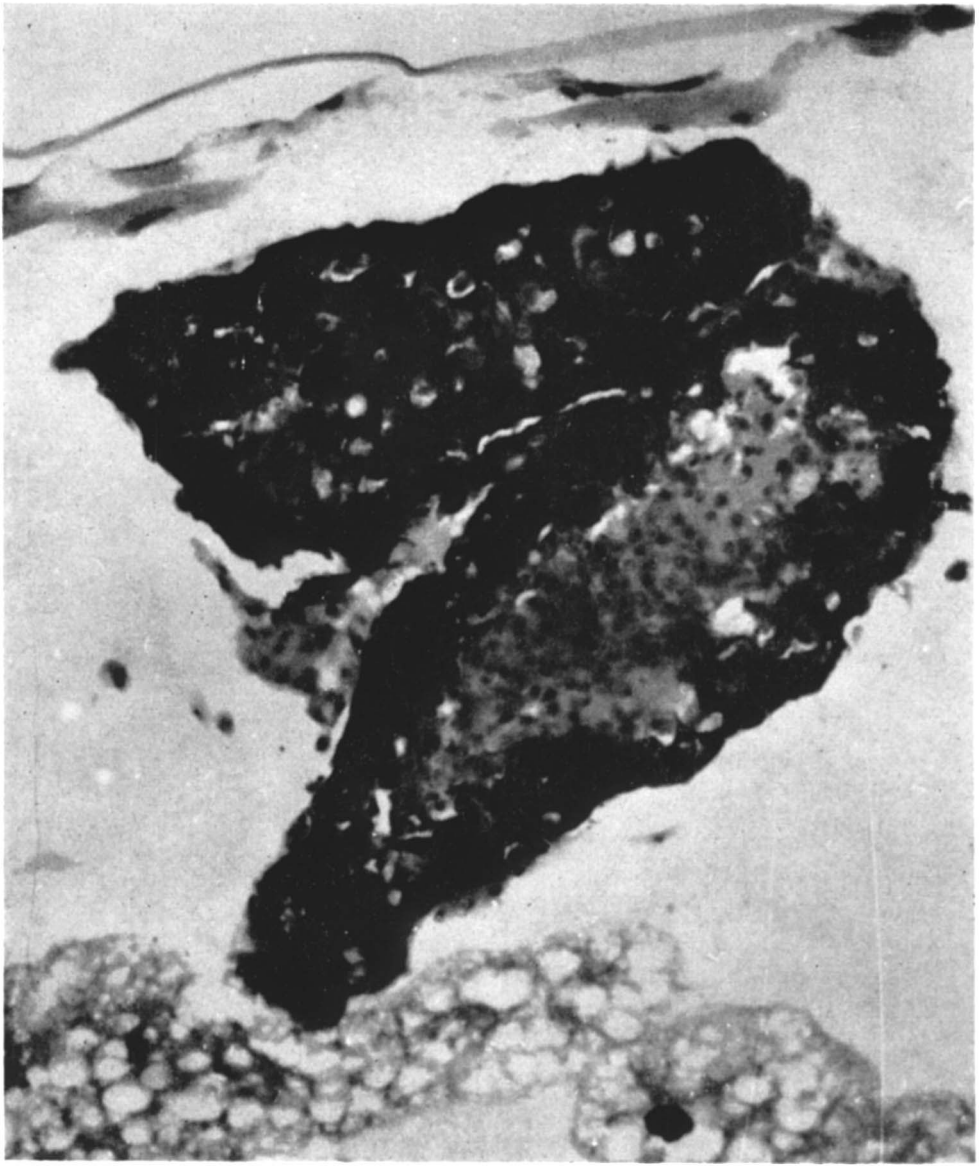

FIGURE 1C.—Melanization of a pair of tumors. Melanin is confined to the stroma in the lower tumor. 350× magnification.

The three nucleic acid preparations used are all of comparable purity (80 percent of air dry weight, as judged by total phosphorus content), so that the difference in effectiveness is not to be sought in differences in quality of the preparations. As we shall see below, the degree of polymerization is not of any consequence either. The results indicate that the requirement is for RNA or its constituent nucleotides, and does not represent a concealed DNA requirement.

TABLE 2

Tumor penetrance in larvae raised on live yeast and on germ-free media at 18°C and 25°C, and at different densities of larvae per culture. Control cultures each contain 40 larvae

Larval density	Percent with tumors			
	Live yeast		Medium D	
	18°C	25°C	18°C	25°C
5	Zero	2.15	Zero	Zero
10	4.5	1.7	8.5	0.65
20	1.3	Zero	Zero	Zero
40	Zero	Zero	Zero	Zero
80	Zero	Zero	Zero	Zero
160	0.3	Zero	4.8	0.3
320	Zero	Zero	0.25	0.2

TABLE 3

Series A, effect of individual deficiencies on tumor penetrance. Series B, effect of the same deficiencies as in Series A, but on media containing only 0.1 percent RNA. The control medium is shown in Table 1

	Percent with tumors	
	Series A	Series B
Control	1.6	1.8
Casein 2.5%	1.24	0.34
Sucrose zero %	1.5	Zero
Cholesterol 0.00156%	75.0*	90.0*
Lecithin 0.0125%	Zero	Zero
Thiamine 0.4 μg	Zero	59.7*
Pyridoxine 0.25 μg	Zero	43.8*
Niacin 2.5 μg	4.2	0.83
Pantothenate 3.0 μg	Zero	0.54
Riboflavin 1.5 μg	2.8	1.5
Folic acid zero	1.1	74.2*
Biotin 0.0025 μg	45.8*	91.5*
RNA zero %	70.4*	. . .

* Significantly different from control at the one percent level of probability.

DNA forms about 10 percent of the total nucleic acid in Drosophila larvae (LESLIE 1955), so that the addition of DNA to an RNA-free medium may have some sparing effect on the RNA requirement, and so explain the reduction in frequency of affected individuals. A second possibility is that there may be some reutilization of the nitrogenous bases from DNA as RNA precursors, after splitting off the deoxyribose-phosphate moiety. It may be noted here that these observations show for the first time that *D. melanogaster* is able to utilize dietary sources of DNA.

The role of individual nucleotides: In the results presented above we cannot distinguish whether the requirement for the normal phenotype is for the whole RNA molecule or for one or more of its constituent nucleotides. The effects of

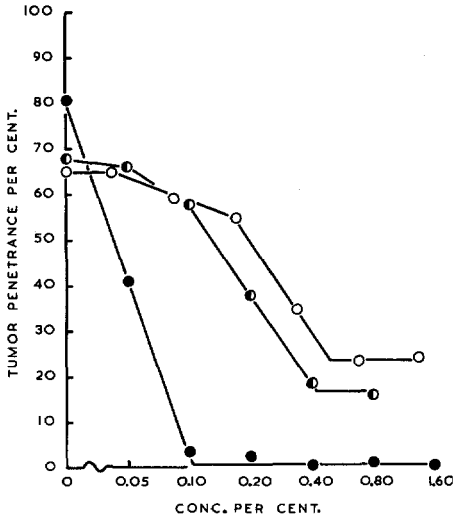

FIGURE 2.—Relation between tumor penetrance and the concentration of nucleic acid in the larval food medium. RNA (solid circles); DNA from herring sperm (half solid circles); DNA from calf thymus (hollow circles).

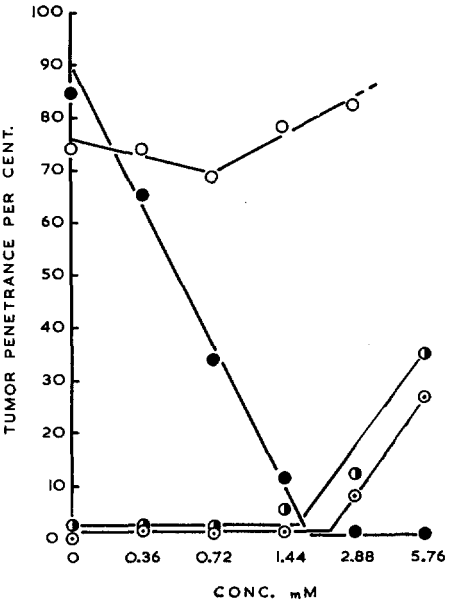

FIGURE 3.—Effect of individual purines and pyrimidines on tumor penetrance. Inosine response on 0.72 mM (0.0465 percent) cytidylic acid (solid circles); guanylic acid response on 0.36 mM (0.0233 percent) cytidylic acid (hollow circles); thymidylic acid (half solid circles) and orotic acid (circle and dot) responses on 0.72 mM (0.025 percent) adenylic acid.

individual purines and pyrimidines on larval growth rate are described in detail by SANG (1957), who has shown that the nucleic acid requirements of Drosophila larvae are satisfied by providing the nucleotides or nucleosides of adenine and cytosine in the diet, both being required.

The effects of purines were tested on a medium without nucleic acid, which contained 0.0465 percent cytidylic acid as a pyrimidine source supplemented with the purines to be tested at equimolar concentrations. A similar medium containing 0.025 percent adenylic acid as a purine source was supplemented with the pyrimidine nucleotides to be tested. Unsupplemented media act as controls in each case, as shown in Table 4. A high proportion of flies raised on the control diet containing cytidylic acid alone develop melanotic tumors: addition of deoxyadenylic acid to the medium causes some improvement, but deoxyguanylic proved to be toxic to the larvae, all of which died during the second instar. Adenylic acid, and its precursor inosine, produced a reduction in tumor frequency comparable to that of whole RNA, whereas only a slight effect of guanylic acid is apparent, owing possibly to a sparing of the requirement for adenylic acid (Figure 3).

TABLE 4

Effect of pentose and deoxypentose nucleotides on tumor penetrance. Series A, addition of purine nucleotides to media containing 0.0465 percent cytidylic acid. All purine additions are molar equivalents of 0.1 percent adenylic acid. Series B, addition of pyrimidine nucleotides to media containing 0.025 percent adenylic acid. All pyrimidine additions are molar equivalents of 0.093 percent cytidylic acid

Series A	Penetrance percent	Series B	Penetrance percent
Cytidylic acid (0.0465%)	77.2	Adenylic acid (0.025%)	8.0
+ Deoxyadenylic acid	42.4*	+ Deoxycytidylic acid	3.8
+ Adenylic acid	2.92*	+ Cytidylic acid	64.2*
+ Deoxyguanylic acid	Toxic	+ Thymidylic acid	25.7*
+ Guanylic acid	67.4	+ Uridylic acid	64.0*
+ Inosine	4.16*	+ Orotidylic acid	13.0

* Significantly different from control at the one percent level of probability.

Flies raised on the control diet containing adenylic acid alone show a low frequency of tumors. Addition of deoxycytidylic acid has no effect, whereas thymidylic acid causes a significant rise above the control level in the proportion with tumors (Table 4). Both cytidylic and uridylic acid give a large increase, but their precursors orotidylic and orotic acid have only limited effects. The effects of orotic and thymidylic acid become apparent only at eight to 16 times the equivalent molecular concentration of cytidylic acid, implying that their action is rather indirect (Figure 3).

The results show that adenylic acid, or its precursor inosine, is required for *prevention* of melanotic tumor formation; but, unexpectedly, they also reveal that cytidylic and uridylic acid act antagonistically by *inducing* tumor formation. In view of the rapid interconversion of uridylic and cytidylic acid it is not possible to say whether the effect is a property of one or both of them, but the low activity of orotidylic acid (orotidine-5-phosphate), which is thought to be the immediate precursor of uridylic acid (REICHARD 1959), is interesting in this connection. In what follows we shall refer to cytidylic acid only, fully aware that its effect may be mediated through uridylic acid.

The reciprocal effect of adenylic and cytidylic acid implies an interaction when both are supplied as the whole RNA tetranucleotide. The form of this interaction was investigated by examining the response to adenylic acid on various levels of cytidylic acid, and vice versa (Figures 4 and 5). When cytidylic acid is added to a purine-free medium, the tumor penetrance rises from about 70 percent to over 90 percent (Figure 4). When adenylic acid is included in the medium, there is a sharp decline in the initial proportion affected and the response to cytidylic acid is shifted along the abscissa, progressively higher concentrations being required to give the same effect. There is a marked loss of activity above 0.025 percent adenylic acid, and at 0.1 percent, no tumorigenic activity of cytidylic acid is apparent within the range tested.

There is a steep decline in tumor penetrance when adenylic acid is added to a

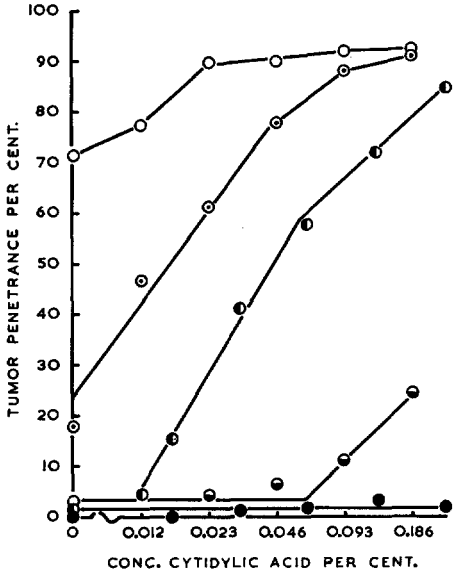

FIGURE 4.—Cytidylic acid response curves on five different levels of dietary adenylic acid: 0.1 percent (solid circles); 0.05 percent (solid bottom circles); 0.031 percent (solid side circles); 0.0125 percent (circle and dot); zero percent (hollow circles).

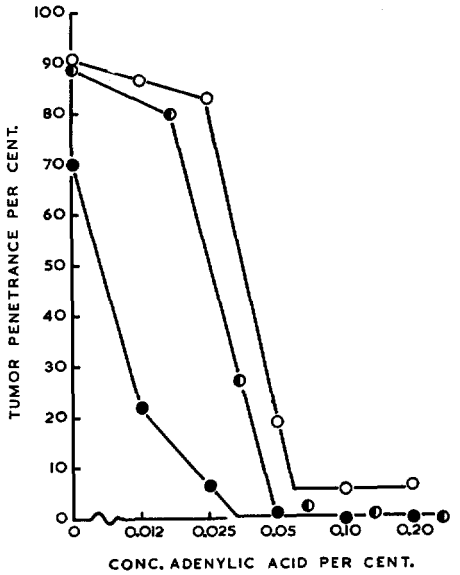

FIGURE 5.—Adenylic acid response curves on three different levels of dietary cytidylic acid. Zero percent (solid circles); 0.05 percent (half solid circles); 0.18 percent (hollow circles).

pyrimidine-free medium (Figure 5). When cytidylic acid is included in the medium, more adenylic acid is required to bring about the same reduction in the tumor penetrance, but the effect is not directly proportional to the increase in cytidylic acid concentration.

The results from these two families of responses, involving nearly 15,000 individuals, are more readily appreciated, with respect to developmental implications, in the form of the three dimensional construction (Figure 9), which shows the relationship of tumor incidence to adenylic-cytidylic balances.

An adequate provision of dietary adenylic acid is required for development of the normal phenotype, but supplementary cytidylic acid is also necessary for the optimal rate of development, which takes place at 2.88 mM (0.10 percent) adenylic acid + 2.88 mM (0.046 percent) cytidylic acid, shown by the arrow in Figure 9. The mean larval development time 0.773 log days compares favorably with the optimum, 0.744 log days, when 0.4 percent whole RNA is fed to the larvae.

The point of optimum growth rate is surrounded by an area in which the penetrance of melanotic tumors is very low; that is to say, there is a considerable safety margin between the conditions which are most favorable for growth and development, and conditions under which the wild-type phenotype can no longer be realized. The dimensions and shape of this area give a measure of the developmental buffering of the strain, to use WADDINGTON's (1957) terminology.

The particular point at which this breaks down depends on at least two factors: principally the concentration of adenylic acid in the medium, and secondly, the molecular ratio of adenylic and cytidylic acid. This point is seen best in Figure 4. For example, with 0.031 percent adenylic acid in the medium, tumor penetrance rises if the molecular ratio of cytidylic and adenylic acid exceeds 1:2, whereas with 0.1 percent adenylic acid in the medium a ratio of 2:1 is tolerated. However, as the strain is highly inbred these relationships are likely to be particular to it.

Since Drosophila is capable of synthesizing part of its purine and pyrimidine requirements, it is difficult to determine from Figure 9 if the normal phenotype breaks down at a particular adenylic:cytidylic ratio and if the tumor level depends on such a balance, or whether there is a more complex relationship between adenylic and cytidylic levels. It can be calculated from the data of Figures 4 and 5 that the ratio of adenylic to cytidylic acids formed *de novo* is of the order of three to one, but the data are too variable to permit an assessment of the amounts synthesized. This explains why a high percentage of tumors is found when RNA is omitted from the diet and indicates the danger of interpreting the results in too great detail at the present time. The data do show, however, that increased dietary cytidylic acid influences the pattern of purine nucleotide utilization so as to raise the requirement for dietary adenylic acid, or, lacking this latter supply, to increase the penetrance of melanotic tumors.

Nucleic acid interactions: The normal diet contains 0.4 percent RNA, so that the original test of individual deficiencies (Table 3) was carried out at an insensitive part of the nucleic acid interaction system. This might explain why no effect of folic acid was apparent, for instance, when such a result might have been predicted from the established role of this vitamin for nucleic acid synthesis and in view of the considerable amounts apparently synthesized by the larvae. Individual deficiencies were therefore retested on 0.1 percent RNA, a level which is at the point of inflexion of the RNA response curve but not low enough to produce tumors alone. This test would expose only interactions involving purine synthesis. Table 3 shows that in addition to cholesterol deficiency, low biotin causes a rise in the proportion with tumors, and three new effective deficiencies show up: thiamine, pyridoxine, and as predicted, folic acid. The four vitamin deficiencies are examined in more detail in Figure 6, which shows the effect of each vitamin deficiency on the response to dietary RNA.

The tumorigenic effects of folic acid, pyridoxine and thiamine deficiencies are all removed by providing sufficient RNA (Figure 6), but that of biotin is not. In the case of the first three, the implication is that the deficiencies interfere with purine nucleotide synthesis. This can be explained for folic acid owing to its involvement in the transfer of single carbon units to positions 2 and 8 of the purine ring, but it is more difficult to assign such direct roles to pyridoxine and thiamine. It may be that a pyridoxine shortage affects the availability of aspartate, and, therefore, of nitrogen for position 1 of the purine ring, and thiamine may conceivably be involved in the synthesis of ribose-phosphate, but these possibilities have not yet been examined.

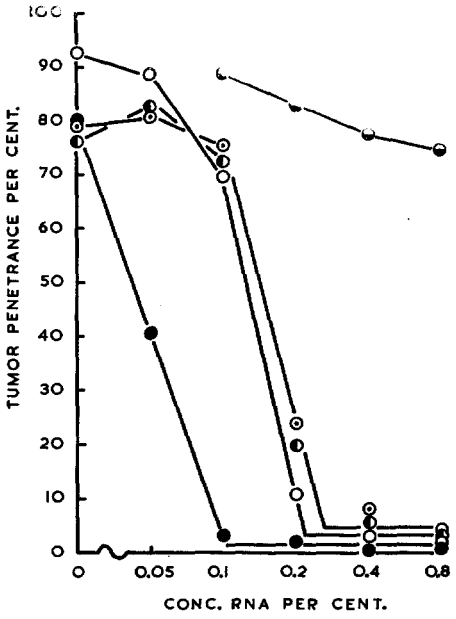

FIGURE 6.—Relation between tumor penetrance and the concentration of dietary ribonucleic acid on vitamin deficient diets. All vitamins at the control level (solid circles); pyridoxine deficiency 0.25 μ.gm per tube (hollow circles); folic acid deficiency 0.2 μ.gm per tube (solid side circles); thiamine deficiency 0.4 μ.gm per tube (circle and dot); biotin deficiency 0.0025 μ.gm per tube (solid bottom circles).

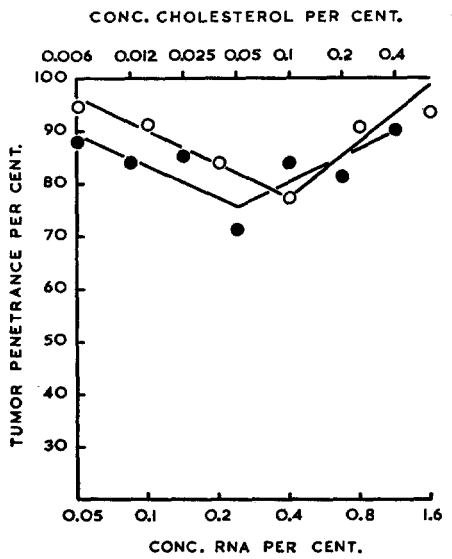

FIGURE 7.—Interaction between ribonucleic acid and cholesterol deficiencies. Response to RNA on 0.0015 percent cholesterol (solid circles); cholesterol response on zero percent RNA (hollow circles). The optimal requirements for growth are 0.4 percent RNA and 0.03 percent cholesterol.

The RNA response on low biotin shows that the former has some biotin sparing effect (Figure 6), and thus presumably reflects some function of biotin in purine synthesis. However, failure of RNA to compensate completely indicates some further role for biotin directly related to tumorigenesis. This is now being studied.

The effect of sterol deficiency: All insects so far examined have an absolute requirement for dietary sterol, and the range of acceptable sterols is rather narrow. According to LEVINSON and BERGMAN (1957) the presence of a hydroxyl group, which may be esterified, at position 3 of the sterol nucleus is necessary for utilization, whereas a hydroxyl or ketone group at position 7 prevents utilization. With Drosophila the sterol requirement is completely satisfied by cholesterol (SANG 1956).

On a cholesterol deficient medium (Table 3) a high proportion of larvae develop melanotic tumors, and the effect is somewhat greater when the RNA concentration is suboptimal. Supplementary RNA in excess of the optimum growth requirement does not reverse the effects of deficient cholesterol, neither

has excess cholesterol any sparing effect on the requirement for RNA. There are indications in Figure 7 of some interaction between the cholesterol and RNA on the proportion of tumor bearing flies, but its significance is not clear. Sterol and RNA deficiencies appear to be biochemically unrelated, but the histopathology of the tumors induced is identical.

The three deficiencies which can be shown to be tumorigenic only on 0.1 percent RNA are noted here to illustrate how the potential effects of some nutritional imbalances may be concealed when the reaction system has a considerable safety margin between normal dietary supplies and restriction of the amounts of the major components (adenylic and cytidylic acids) which lead to the appearance of some tumors. The two deficiencies (biotin and cholesterol) which produce tumors in the presence of normal supplies of dietary RNA are reported to illustrate how intereference with biochemical systems, apparently not directly related to one another, may have the same phenotypic consequences. In this context, we must also note that a further, apparently unrelated, dietary manipulation (provision of excess dietary tryptophan) may also cause the appearance of tumors (PLAINE and GLASS 1955, for references). It does so also in the present strain (BURNET and SANG, unpublished).

Timing of the tumor response. The effective period of the tu^K *gene:* We have seen above that histological changes indicative of tumor formation begin prior to the middle of the third larval instar. This poses questions about the period during which dietary manipulations are effective, and the time of action of the tu^K gene. The sensitivity of the system to dietary nucleotide balance enables us to examine these questions by switching the molecular ratio of adenylic and cytidylic acid at different times during larval development, from tumor inducing to tumor suppressing, and vice versa.

In the first series (Figure 8) larvae are fed on a pyrimidine-free medium containing sufficient adenylic acid to prevent tumor formation. Cytidylic acid is added at successively later stages of larval development. Addition of cytidylic acid at any time before 45 hours of larval life induces tumors in a large proportion of flies, but addition after 55 hours has no effect, the proportion being no greater than in flies raised throughout on a pyrimidine-free diet. Similarly, in the second series, adenylic acid is added after successive time intervals to a purine-free diet containing cytidylic acid. Addition of adenylic acid at any time up to 45 hours prevents the effect of the purine-free diet, but after 55 hours, addition of adenylic acid is without effect, and the proportion of tumor-bearing flies is as great as when no addition is made at all.

The results of these two series of experiments show that reversible dietary manipulation of tumor penetrance may be made up to about 50 hours of larval life, after which the course of the reaction system is irreversibly determined. The intersection of the two curves in Figure 8 covers from 45 to 55 hours approximately, implying that this marks the effective period of the tu^K gene, which coincides with the ecdysis separating the second and third larval stadia. Significantly, it precedes the period at which the lamellocyte transformation takes place in other tumorous larvae (RIZKI 1957a, 1962).

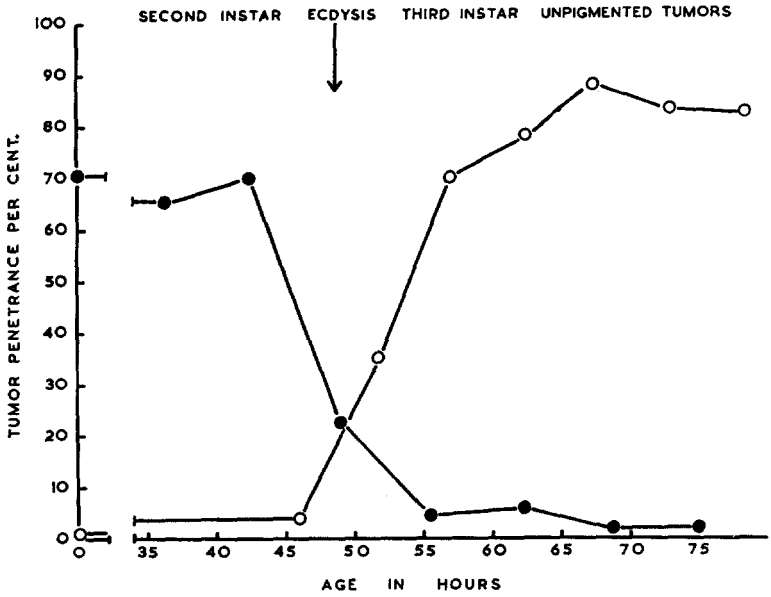

FIGURE 8.—The effect of changing the molecular ratio of adenylic and cytidylic acids at different times during larval development. Solid circles: addition of 0.093 percent cytidylic acid to media containing 0.025 percent adenylic acid. Hollow circles: addition of 0.1 percent adenylic acid to media containing 0.0465 percent cytidylic acid. The age of the larvae is in hours after eclosion from the egg, corrected proportionally to the standard 96-hour larval period.

Humoral control of tumor pigmentation: Unpigmented tumors can be found, in material from larvae fed on appropriate diets, at the middle of the third instar, but the characteristic black pigmentation is not formed until nearer the onset of pupation. It is well known that the activity of hemolymph tyrosinase is inhibited in Drosophila larvae until late in the third instar when the onset of pupation is preceded by a sharp increase in activity of this enzyme (OHNISHI 1953). Observations by DENNELL (1949) on blowflies suggest that the change in redox potential of the hemolymph, which releases tyrosinase inhibition, is under humoral control.

Melanization of abdominal tumors can be prevented by isolating the brain and ring gland by means of a ligature made with a fine strand of silk or nylon. Depending on whether the ligature is placed anterior to or posterior to the brain, the humoral connexion between the ring gland and abdomen may be broken or preserved, provided that the operation is carried out before the critical period when the pupation hormone is released.

Larvae raised on a medium without RNA, and a control group raised on an RNA supplemented medium, were ligatured as shown in Table 5. Histological examination of unligatured larvae of the same age confirmed the presence of unpigmented tumors in the RNA-free series, but not in the controls. The ligature itself sometimes causes damage to the larval tissues which in both normal and tumorous larvae becomes melanized, and may be confused with a tumor. We

TABLE 5

Effect of ligation on the appearance of melanotic tumors in larvae raised on control and zero-RNA media

Position of ligature relative to brain	Control medium non-*tu*	Control medium *tu*	Zero-RNA medium non-*tu*	Zero-RNA medium *tu*
Anterior	25	1	14	12
Posterior	28	2	27	3

can take account of this from the behavior of the control group. Considering the RNA-free group, we find that ligatures posterior to the brain cause a highly significant reduction in the number of melanized tumors ($X_1^2 = 9.26$, $P < 0.01$), associated with failure of the abdomen to pupate. The anterior end of the larva pupated in the normal manner, showing that pupation hormone is prevented from diffusing past the point of constriction into the abdomen. Larvae with ligatures anterior to the brain all pupated in the abdominal portion posterior to the ligature, but not anteriorly.

These results, and those of RIZKI (1960), describe the role of the pupation hormone ecdysone in controlling melanization of tumors through its effect on the activity of tyrosinase. The effect of this hormone is rather indirect and therefore it seems unlikely to have any carcinostatic activity for vertebrate tumors as has been suggested by BURDETTE (1960).

DISCUSSION

A defensive reaction can be provoked in many insects by the implantation of certain parasites or other foreign bodies into them (SALT 1961). This reaction generally takes the form of encapsulation by hemocytes, which may subsequently be melanized, and in Drosophila larvae it is a normal reaction against damaged tissues produced by wounding. Although the process is superficially similar to tumor formation, it differs in one important respect—it does not involve a premature hemocyte transformation (RIZKI 1957a).

Both in normal and tumorous larvae the hemocyte transformation follows important periods of hormonal activity, and suggests that the stimulus for the precocious transformation may derive from defective hormonal balance during the ecdysis between the second and third instar which, as we have seen, coincides with the effective period of tu^K. The recent discovery that the open chain terpene alcohol farnesol has juvenile hormone activity for certain insects (WIGGLESWORTH 1961) offers a new opportunity for experimental investigation of this possibility.

The penetrance of melanotic tumors in the tu^K strain is controlled quantitatively by the concentration of adenylic and cytidylic acids, of cholesterol and of biotin in the larval food medium. This suggests that the tu^K gene causes a metabolic defect which is within the capacity of the developing system to compensate for under certain environmental conditions, but not in others. That is to say, the effect of the gene-controlled defect is near the limit of developmental

buffering of the reaction system, and under certain conditions of stress, this buffering can no longer maintain that developmental pathway which leads to the normal phenotype. The RNA effect conceals a complex interaction involving the availability and relative molecular concentrations of two pentose nucleotides, adenylic and cytidylic acid, and the form of this interaction can be stated in three dimensional terms (Figure 9). The area surrounding the point of optimal nucleotide balance for the larval development rate gives us a measure of the degree to which the normal phenotype is buffered under specified conditions of stress.

It is clear, however, that Figure 9 represents only one possible aspect of what is, in reality, a multidimensional system, the form in this particular case being a function of the availability of two pentose nucleotides. Availability here has two components, the rate of *de novo* synthesis and the concentration of the dietary supplement. Both of these components can be varied experimentally, but probably not independently, because the likelihood is that the rate of *de novo* synthesis is influenced to some extent by the concentration of the dietary supplement. The information presented in Figure 6 suggests that the synthesis of purine nucleotides can be rate limited by deficiencies of at least three essential vitamins involved at different points in the synthetic pathway. It follows that the

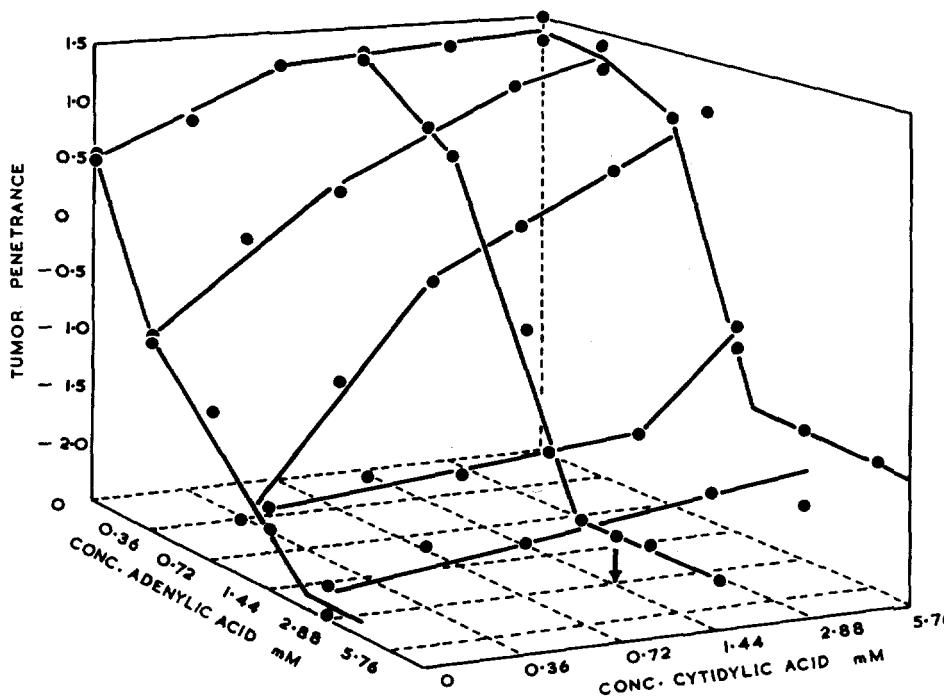

FIGURE 9.—Relation between tumor penetrance expressed in probits (−5) and the concentration of adenylic and cytidylic acid. The concentration scales in mM correspond to the percentage values in Figures 4 and 5. The optimal nucleotide balance for larval growth is indicated by the arrow.

coordinates of the surface in Figure 9 would have different values if the experiments were repeated on media deficient in one or more of three vitamins. The threshold between normal and atypical development would then be closer to the point of "maximal" growth rate and the area over which the normal phenotype could be maintained would be greatly reduced.

An important feature of Figure 9 is that it describes conditions which are relevant, at most, only to the first two larval stadia. It seems that an adequate concentration of adenylic acid in the diet is required to maintain the normal phenotype during these stages. When this is insufficient, the difference between what is supplied and what is required must be synthesized by the larvae, and the threshold concentration must be reached before the time of action of the tu^{K} gene, when the phenotypic response is irreversibly determined at the ecdysis between the second and third instar. Adenosine is involved in a great many biological reactions, so that it is likely that the requirement is really for its participation in some as yet unidentified reaction of a threshold type. Any conditions which tend to limit the rate of adenosine synthesis, or which lead to a restriction of its availability through competing reactions, will mitigate against maintenance of the wild-type phenotype. In this respect, it is interesting to observe that in this system antipurine agents would show carcinogenic rather than carcinostatic activity.

Whatever the nature of the gene-controlled metabolic defect, its consequences can be intensified by three apparently unrelated treatments, which suggests that the system in which it is involved is open to interference in many ways, including alteration of the rest of the genotype. It is therefore not surprising that the different melanotic tumor mutants reported show a variety of penetrance levels when grown on live-yeast media. If the dietary interactions described here are found to be general, these mutants would be expected to show different sensitivities to nutrient deficiencies, and therefore to provide more critical material for the elucidation of the physiological genetics of melanotic tumors.

SUMMARY

The tu^{K} strain of *Drosophila melanogaster* shows a low penetrance of melanotic tumors under standard conditions of culture. The tumors are formed by aggregations of hemocytes in the larval hemocele which become melanized prior to pupation. On germ-free chemically defined media, a deficiency of ribonucleic acid or cholesterol causes a pronounced rise in tumor penetrance, but the two deficiencies have independent effects as an excess of RNA does not counteract the effects of cholesterol deficiency, neither does excess cholesterol counteract the effect of RNA deficiency.

Comparative studies on the effect of pentose and deoxypentose nucleotides show the RNA requirement for tumor suppression to be specifically for the nucleotide or nucleoside of adenine, and in addition disclose an antagonistic interaction between dietary levels of adenylic and cytidylic acid. The form of the interaction, stated as a three dimensional surface, defines the degree of

buffering of the normal phenotype under different conditions of pentose nucleotide imbalance.

The effect of RNA deficiency on tumor penetrance is enhanced by deficiencies of folic acid, pyridoxine, thiamine, and biotin, and it is suggested that deficiencies of these vitamins rate limit *de novo* synthesis of adenosine by the larvae.

Alteration of the molecular concentration of dietary adenylic and cytidylic acid at specified times during development shows that this measure of the time of action of the tu^{K} gene coincides with the ecdysis at the end of the second larval stadium. Melanization of tumors is controlled by the pupation hormone and is thought to coincide with an alteration of the redox potential of the hemolymph which releases an inhibition of the enzyme tyrosinase.

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