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Physiological genetics of melanotic tumours in *Drosophila melanogaster*

VIII. The role of choline in the expression of the tumour gene *tu bw* and of its suppressor, *su-tu*

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SUMMARY

The melanotic tumour gene *tu bw* of *Drosophila melanogaster* has a specific suppressor *su-tu*. The genotypes *tu bw*; $+^{su-tu}$ and *tu bw*; *su-tu* show opposite responses, as measured by tumour penetrance, to increasing choline levels in the defined axenic medium. The three major metabolic functions of choline have been examined using additions to the axenic medium to determine which biochemical pathway(s) are different in the two genotypes. It is concluded that the opposite strain responses are due to changes in the pattern of phospholipid synthesis, and that the gene product of the *su-tu* gene probably functions in this area of metabolism.

1. INTRODUCTION

Burnet & Sang (1964) were able to alter the penetrance of melanotic tumours in the *tu bw*; $+^{su-tu}$ strain by nutritional manipulation of the defined axenic culture medium. They showed deficiencies of protein, cholesterol, thiamine pyridoxine and magnesium ions raise tumour incidence, as do excesses of tryptophan and phenylalanine. Since such treatments are known to modify many unrelated metabolic pathways, the problem is: how do they all influence the precocious transformation of the plasmatocyes, the cellular basis of tumorigenesis (Rizki, 1957, 1960, 1962)?

Sang (1968) argued that the interrelationships of metabolism are so intimate that imbalances of other pathways may also modulate the functioning of the 'mutant pathways', and attempted to draw the current data into the framework of a hypothesis. He considered that at least two pathways were involved; one regulating hormone balance and the other influencing the ability of the target cells to respond to that balance.

In a further attempt to localize the lesion caused by the second chromosome *tu bw* mutant, Sang & Burnet (1967) studied tumorigenesis in two strains, the unsuppressed strain, *tu bw*; $+^{su-tu}$ and the suppressed strain, *tu bw*; *st su-tu*. This latter strain carries the tumour-suppressor gene, *su-tu* (Glass, 1957) on the third chromosome. The rationale behind these experiments was to exploit the

apparent specificity of the suppressor gene (Burnet, 1966). Treatments affecting tumorigenesis in the two strains in different ways, increasing or decreasing tumour incidence, could then be further investigated in the belief that the different responses were due to both genes affecting a small area of metabolism. Such responses would then make it easier to localize the pathway affected by *tu bw*. Of all the excesses and deficiencies of the normal constituents of the defined medium which were tested, only two nutritional treatments affected tumour penetrance of the suppressed and unsuppressed strains in an opposite manner. These were deficiences of choline, or of nicotinic acid. This paper is a further investigation of the effects of dietary choline on the expression of the *tu bw* and *su-tu* genes.

Choline is considered to have three general functions in insects (Dadd, 1970): as a metabolic donor of methyl groups, as acetylcholine in neural transmission, and as a constituent of lecithins (phosphatidyl-cholines) in structural and transport lipids (Thomas & Gilbert, 1967). Each of these choline functions has been investigated with regard to their possible role in tumorigenesis and, specifically, for their involvement in the expression of the *su-tu* gene. The metabolic pathways concerned are illustrated in Fig. 1.

2. MATERIALS AND METHODS

The melanotic tumour strains used in this study are both homozygous for the melanotic tumour gene *tu bw* which maps at 80·5 on the second chromosome (Lindsley & Grell, 1968). The *tu bw; +$^{su-tu}$* strain, described as the unsuppressed strain, carries a wild-type third chromosome whilst the suppressed strain, *tu bw; st su-tu*, differs from it in having a third chromosome which carries the eye colour mutant scarlet and a specific suppressor of *tu bw* designated *su-tu* (Glass, 1954; Burnet, 1966). These strains have been described previously (Sang & Burnet, 1967) and are maintained as sib-mated lines.

The technique of axenic culture of *Drosophila melanogaster* has been well described (Sang, 1956; Sang & Burnet, 1963; Bryant & Sang, 1969). Eggs were washed and sterilized in a new egg-washing apparatus (Sparrow, 1971*a*) which permits quicker handling, and gives lower rates of culture infection than the earlier reversing pump (Sang, 1956). The constitution of the defined medium was that of Sang's medium C with the exceptions that (i) sucrose was used instead of fructose to minimize the browning reaction between the sugar and the amino acids of the diet, (ii) the medium contained magnesium (as sulphate), a requirement that is not satisfied by the magnesium content of the Oxoid No. 3 agar used (Sang & Burnet, 1967), (iii) KH_2PO_4 replaced Na_2HPO_4 to give a more optimal sodium/potassium ratio (Bryant, 1967), (iv) the lecithin (a phosphatidyl-choline) was replaced completely by choline so that the exact dietary concentration of choline was known. The low choline medium contained 1×10^{-4} M choline. This is the dietary choline level which Geer & Vovis (1965) found was the minimal quantity for normal development rate and survival of wild-type *D. melanogaster*. The high choline medium contained 8×10^{-4} M choline.

Adult flies were cleared using Sang's fructose method (1966), after which melanotic tumours are easily scored without the need to dissect the flies. The tumour penetrance was expressed in terms of percentage tumourous flies, and a chi-square test used to determine the significance of effective treatments.

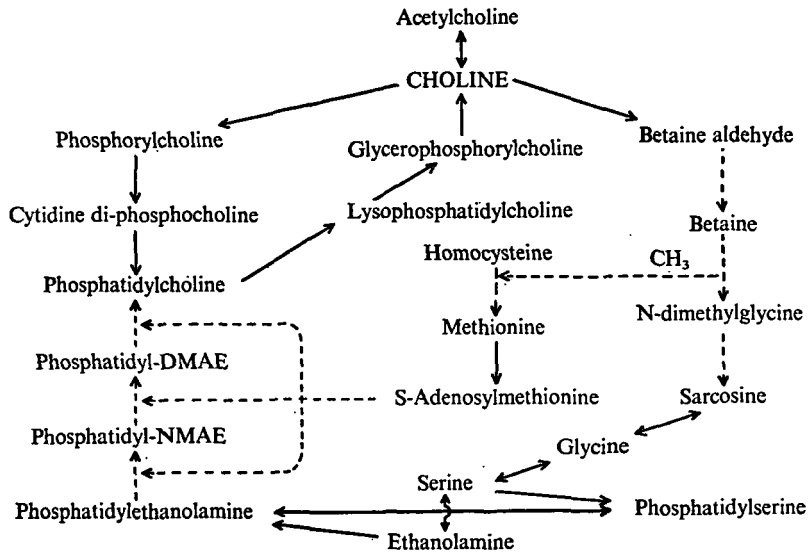

Fig. 1. Metabolism of choline. The solid lines show the pathways known to be functional in Diptera (Bridges, 1972). The broken lines indicate the pathways found in vertebrates but apparently absent in Diptera. Phosphatidylethanolamine (cephalin) is synthesized by a cytidinediphosphoryl pathway as illustrated for choline, and the other analogues, including carnitine (as β-methylcholine) similarly. For simplicity the synthesis of sphingomyelin from CDP-choline is omitted from the diagram. This synthesis does not occur in Diptera.

3. RESULTS

The results of growing the suppressed (*tu bw; st su-tu*) and unsuppressed (*tu bw;* $+^{su-tu}$) strains on a low choline medium and of making choline additions are shown in Table 1. At the lower dietary choline levels tumour penetrance of the unsuppressed strain is reduced whilst the converse is true for the suppressed strain. These results confirm those obtained by Sang & Burnet (1967).

(i) *Choline as a methyl group donor*

Supplementary methionine has been shown to reduce tumour incidence in the presence of excess dietary tryptophan or phenylalanine, both of which raise tumour penetrance of the suppressed and unsuppressed strains (Plaine & Glass, 1955). However, methionine added to the complete medium in the absence of either of these amino acids has no measurable effect on tumorigenesis (Burnet & Sang, 1968). Addition of methionine to the low choline medium lowers the tumour penetrance of both the suppressed and unsuppressed strains (Fig. 2). A qualitative

difference in the responses of the two strains would be predicted if methionine were acting in the same manner as choline. The fact that both strains give the same response to this methionine addition suggests that choline is not important in tumorigenesis because of its potential as a labile methyl-group donor.

Table 1. *The effects of dietary choline concentration on the tumour penetrance of the* tu bw; $+^{su-tu}$ *and* tu bw; st su-tu *strains*

Choline concn. ($\times 10^{-4}$ M)	Tumour penetrance	
	tu bw; +su-tu	*tu bw; st su-tu*
1	40·0	68·4
2	34·2	59·3
4	72·9**	33·3**
8	66·6**	39·2**

** Significance at the 1 % level of penetrance from the values obtained on the low-choline medium (1×10^{-4} M).

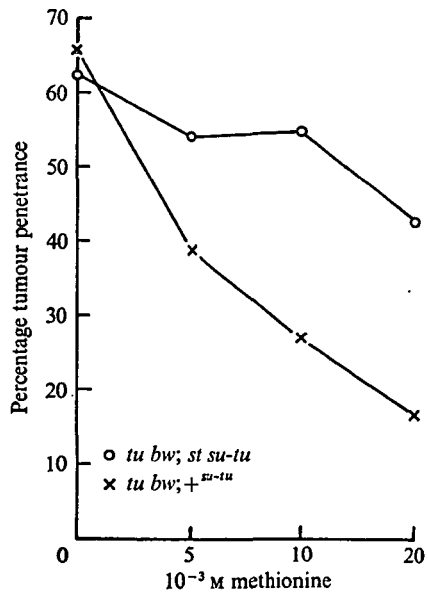

Fig. 2. The effects of methionine additions to the low-choline medium on the penetrance of tumours in the suppressed (*tu bw; st su-tu*) and unsuppressed (*tu bw; +su-tu*) tumour strains.

When high levels of choline are added to the defined medium containing excess tryptophan there is no decrease in the tumour penetrance of either strain (Table 2). Under similar conditions methionine additions reduce the high levels of tumour incidence caused by the excess tryptophan in both tumour strains. Since choline apparently cannot 'spare' for methionine it seems likely that choline is not a methyl group donor in *D. melanogaster* metabolism. This interpretation, however, assumes both that sufficient homocysteine is available for the *in vivo* synthesis of

adequate methionine to lower tumour penetrance (Fig. 1), and that the product of choline demethylation which affects tumour formation is methionine. Addition of excess homocystine to a medium containing high levels of choline and tryptophan does not result in a reduction of tumour penetrance (Table 3), supporting the conclusion that any synthesis of methionine by demethylation of choline, if it occurs, is trivial with respect to tumorigenesis under these conditions.

Table 2. *The effects on tumorigenesis in the suppressed and unsuppressed strains of choline and methionine additions to the high-choline medium (10 mM) containing excess tryptophan*

	Tumour penetrance	
	tu bw; +$^{su\text{-}tu}_{i}$	*tu bw; st su-tu*
Control	45·6 (197)	18·3 (284)
+ 20 mM tryptophan	97·5 (215)	73·7 (366)
+ 20 mM tryptophan + 20 mM methionine	36·6** (191)	31·9** (256)
+ 20 mM tryptophan + 20 mM choline	98·9 (193)	72·2 (229)

Figures in parentheses denote number of flies in each experimental class. Significance of difference from the control + tryptophan class indicated by ** at 1 % level.

Table 3. *The effects of homocystine on tumorigenesis in the unsuppressed and suppressed strains grown on a high-choline medium (10 mM) containing excess tryptophan*

	Tumour penetrance	
	tu bw; +$^{su\text{-}tu}$	*tu bw; st su-tu*
Control	57·4 (190)	34·3 (105)
+ 20 mM tryptophan	91·9 (184)	82·1 (106)
+ 20 mM tryptophan + 20 mM homocystine	91·5 (199)	74·0 (142)
+ 20 mM homocystine	59·0 (166)	22·9 (107)

The question as to whether other products of choline demethylation are important was tested by adding the intermediates of the betaine pathway to the low choline medium. It is well known from studies on both micro-organisms and vertebrates that choline is first oxidized to betaine before donating methyl groups to the one-carbon pool. The addition of either betaine, dimethylglycine or sarcosine to the low choline medium was without effect on the tumour penetrance of either strain (Table 4), even at concentrations in excess of those required for choline to affect tumorigenesis.

(ii) *Choline as acetylcholine*

Acetylcholine is synthesized *in vivo* by choline acetyltransferase which acetylates choline by condensation with acetyl coenzyme A. This latter compound is central to intermediary metabolism, and it is not possible to alter its level without affecting other pathways already documented as influencing tumorigenesis. Inhibitors of

Table 4. *The effects of betaine, dimethylglycine and sarcosine additions to the low-choline medium*

(Results expressed as differences in tumour penetrance from the control grown on unsupplemented low choline medium.)

	Tumour penetrance difference from control	
	tu bw; +$^{su\text{-}tu}$	*tu bw; st su-tu*
$+8 \times 10^{-4}$ M betaine	−5·7 (142)	+2·3 (38)
$+8 \times 10^{-4}$ M dimethylglycine	−3·2 (143)	+4·6 (271)
$+8 \times 10^{-4}$ M sarcosine	−2·1 (99)	−1·4 (167)

All differences from control are insignificant.

Table 5. *The effects of eserine sulphate on tumour formation, pupal and adult survival (expressed as per cent of surviving individuals to the particular stage) of the suppressed and unsuppressed strains grown on the low-choline medium*

	tu bw; +$^{su\text{-}tu}$			*tu bw; st su-tu*		
Eserine concn.	Pupal viability	Adult viability	tu P	Pupal viability	Adult viability	tu P
Control	88·7	83·3	46·6	64·0	58·5	91·4
$1{\cdot}9 \times 10^{-5}$ M	62·5**	58·5**	45·6	74·5**	56·0	88·6
$3{\cdot}8 \times 10^{-5}$ M	21·5**	20·0**	48·8	60·0	45·0**	90·1
$7{\cdot}6 \times 10^{-5}$ M	14·0**	12·5**	46·4	45·5**	37·0**	92·5
$15{\cdot}2 \times 10^{-5}$ M	Lethal during second instar for both strains					

** Values differing significantly from control levels at $P = 0{\cdot}01$.

Pupal and adult viability are expressed as percent of larvae reaching the pupal and adult stages respectively. tu P, Tumour penetrance.

Table 6. *The effects of hemicholinium-3 on tumorigenesis, and on pupal and adult survival of the suppressed and unsuppressed strains grown on the low-choline medium*

	tu bw; +$^{su\text{-}tu}$			*tu bw; st su-tu*		
Hemicholinium-3 concn.	Pupal viability	Adult viability	tu P	Pupal viability	Adult viability	tu P
0	94·5	91·5	55·0	64·5	52·0	59·0
2×10^{-4} M	82·4**	69·6**	61·2	36·4**	15·6**	60·5
8×10^{-4} M	37·6**	0·0	55·3	Died as second-third instar larvae		

tu P, Tumour penetrance.

** Values differing significantly from control levels at $P = 0{\cdot}01$.

acetylcholine metabolism have therefore been used to investigate the possible role of this metabolite in tumorigenesis. Addition of eserine sulphate, an inhibitor of acetylcholinesterase (Lord & Potter, 1951), to the low choline medium is without effect (Table 5) even at levels which cause reduced viability of the larvae of both strains. Hemicholinium-3, an inhibitor which interferes with choline transport, particularly into the mitochondria, hence depriving choline acetyltransferase of

its substrate (MacIntosh, Birks & Sastry, 1956), is also without effect on tumour penetrance in either strain (Table 6). This inhibitor also affects the viability of both strains. These results imply, though do not prove, that acetylcholine is not the choline metabolite which is effecting the changes in tumour penetrance of the unsuppressed and suppressed strains on diets containing different levels of choline.

(iii) *Choline as a phospholipid constituent*

Geer & Vovis (1965) used a number of choline analogues to study the metabolism of choline in *D. melanogaster*. They found that carnitine and its metabolite beta-methylcholine could replace choline in the larval diet to give more or less normal growth and development. *N*,*N*-dimethylaminoethanol (DMAE) and *N*-methylaminoethanol (NMAE) would support larvae to pupation in the absence of dietary

Table 7. *The effect of carnitine additions to a choline-free diet on tumour levels, and on the survival of the suppressed and unsuppressed tumour strains*

	tu bw : $+^{su\text{-}tu}$			*tu bw*; *st su-tu*		
Carnitine concn.	Pupal viability	Adult viability	tu P	Pupal viability	Adult viability	tu P
1×10^{-4} M	50·0	2·8	32·0	7·6	0·0	63·1
2×10^{-4} M	59·5	21·5**	44·5	39·1**	0·8	59·0
4×10^{-4} M	61·3	39·1**	55·1**	45·1**	3·1	47·7**
8×10^{-4} M	58·5	50·0**	51·3**	36·9**	11·2	31·4**

tu P, tumour penetrance.
** A significant difference from the values obtained on 1×10^{-4} M carnitine at $P = 0{\cdot}01$.

choline, but ethanolamine was ineffective. Geer *et al.* (1971) have shown that carnitine, as its product beta-methylcholine, is incorporated into the phospholipids of *D. melanogaster* when fed in the diet in the absence of choline. Similar studies have shown that beta-methylcholine, DMAE and NMAE are also incorporated into the phospholipids of *Musca domestica* when fed in the absence of dietary choline (Bridges, Ricketts & Cox, 1965). Ethanolamine is a normal constituent of insect phospholipids (Gilmour, 1961).

When increasing carnitine concentrations are included in a choline-free diet the suppressed and unsuppressed strains are affected in opposite ways (Table 7). These changes are similar to those previously observed when the dietary choline concentration is varied. An increase in dietary carnitine increases the tumour penetrance of the unsuppressed strain, but causes a decrease in that of the suppressed strain. This suggests that carnitine is acting in the same manner as choline and is substituting for it. This effect is not due to the absence of dietary choline since carnitine additions to the low choline medium cause the same effects.

The addition of either DMAE or NMAE to the low choline diet has effects on tumorigenesis (Table 8). DMAE causes a decrease in tumour penetrance of both strains. This is somewhat surprising considering the close structural relationship between DMAE and choline. NMAE has a different effect on the two strains. It

raises the level of tumours in the unsuppressed strain but causes no change in that of the suppressed strain. There are striking similarities between this last result and that obtained when ethanolamine is added to the low choline diet (Table 9). Ethanolamine addition raises tumour penetrance in the unsuppressed strain but

Table 8. *The effects of addition of DMAE and NMAE to the low-choline medium on the tumour penetrance of the unsuppressed and suppressed strains*

	tu bw; +su-tu			*tu bw; st su-tu*		
	tu P	pv	av	tu P	pv	av
Control	44·1 (134)	53·7	49·7	49·6 (127)	50·8	30·8
$+8 \times 10^{-4}$ M DMAE	24·3** (111)	56·0	47·3	10·9** (110)	37·6**	23·6
$+8 \times 10^{-4}$ M NMAE	63·6** (140)	55·5	48·0	43·6 (94)	46·0	31·2

tu P, Tumour penetrance; pv, pupal viability; av, adult viability.
** Values differing significantly from control levels at $P = 0{\cdot}01$.

Table 9. *The effects of supplementary ethanolamine in the low-choline medium on tumour levels in the suppressed and unsuppressed tumour strains*

	tu bw: +su-tu			*tu bw; st su-tu*		
	tu P	pv	av	tu P	pv	av
Control	59·8 (101)	67·1	64·7	52·5 (112)	49·6	40·4
$+8 \times 10^{-4}$ M ethanolamine	71·4* (133)	52·1**	47·2**	51·5 (96)	40·0	30·0*

*, ** Values differing significantly from control levels at $P = 0{\cdot}05$ and $P = 0{\cdot}01$ respectively.

Table 10. *The effects of adding ethanolamine or NMAE to a high-choline medium (10 mM choline) on tumorigenesis in the unsuppressed and suppressed tumour strains*

(Results from separate experiments.)

	Tumour penetrance	
	tu bw; +su-tu	*tu bw; st su-tu*
Control	35·5 (185)	24·4 (94)
+5 mM ethanolamine	70·2** (191)	18·4 (87)
Control	54·4 (209)	30·3 (175)
+5 mM NMAE	47·0 (168)	24·2 (161)

** Values differing significantly from control levels at $P = 0{\cdot}01$.

is without effect on the suppressed strain. The similarity of effects between NMAE and ethanolamine could possibly be accounted for by the fact that they differ by only one methyl group. However, addition of ethanolamine or NMAE to a diet containing 10 mM choline does not give similar results (Table 10). Under these conditions, ethanolamine addition still raises tumour levels in the unsuppressed

strain and leaves the suppressed strain unchanged, demonstrating the independence of the ethanolamine effect on tumour formation from the dietary choline concentration. Addition of NMAE to the high choline diet does not affect tumorigenesis in either strain, suggesting that ethanolamine and NMAE are not influencing tumour levels by precisely the same mechanism. Not surprisingly, additions of these substances to the high carnitine containing medium have the same effects (Table 11), which will be discussed in the next section.

Table 11. *Effects on tumour penetrance of additions of ethanolamine, NMAE and DMAE to the high-carnitine medium*

	Tumour penetrance			
	tu bu; +$^{su\text{-}tu}$		*tu bw; st su-tu*	
Carnitine	75·0	(197)	42·3	(66)
+Ethanolamine	97·1*	(177)	40·5	(79)
+NMAE	82·5	(132)	54·8	(31)
+DMAE	60·6*	(165)	13·6*	(88)

All substances provided at 8×10^{-4} M.
* Values differing from control levels at $P = 0{\cdot}05$.

4. DISCUSSION

Burnet & Sang (1964) concluded from the fact that they could alter tumour penetrance of the *tu bw* gene by nutritional manipulation of the defined medium that the gene produces a defective protein rather than no protein at all. A similar argument may be applied to the case of tumour suppression by the *su-tu* gene: the results presented here, and elsewhere (Sang & Burnet, 1967), demonstrate that the *su-tu* gene affects the expression of the *tu bw* gene in a quantitative manner. These facts suggest the suppressor acts not to increase the wild-type activity of the *tu bw* gene but causes some metabolic, or physiological, reversal of the lesion caused by *tu bw*.

Of all the nutritional treatments that have been examined previously only deficiences of choline, or of nicotinic acid in the defined medium cause differences in the tumorigenic response of the *tu bw; +*$^{su\text{-}tu}$ and *tu bw; st su-tu* strains (Sang & Burnet, 1967). The many roles that nicotinic acid derivatives play in intermediary metabolism makes further analysis of this response exceedingly difficult, especially since manipulation of most of these pathways is already known to affect tumorigenesis (Sang, 1968). The choline response is more accessible to experimentation.

Choline is apparently not synthesized by *D. melanogaster*. In the absence of dietary choline larvae cannot survive (Sang, 1956; Geer & Vovis, 1965). The feeding of possible choline precursors, e.g. methionine, ethanolamine and betaine, or the two substituted aminoalcohols, NMAE and DMAE, does not relieve the lethal effects of a choline-free diet (Geer & Vovis, 1965). The same results have been found over a wide range of insects (listed by Hodgson, Smith & Snyder, 1972)

and it seems fair to conclude that choline is an essential nutritional requirement for most, probably all, insects.

The absence of choline synthesis in *D. melanogaster* means that the effects of the low choline diet on tumour formation in the *tu bw;* $+^{su-tu}$ and *tu bw; st su-tu* strains cannot be ascribed to changes in requirements for choline synthesis. This further implies that it is choline, or one of its metabolic derivatives, which is directly involved in tumorigenesis. As previously mentioned, choline has three distinct general functions in insects (Dadd, 1970): as (i) a source of labile methyl groups, (ii) acetylcholine in neurotransmission, and (iii) a constituent of certain phospholipids.

The possible role of choline as a labile methyl group donor in tumorigenesis was examined by the addition of the intermediates of the choline demethylation pathway known in both micro-organisms and in vertebrates. None of these intermediates produce any effect on tumorigenesis in either of the two tumour strains, at concentrations in excess of those at which choline is effective. These results mean that this pathway is not involved in tumorigenesis. Indeed, the evidence (Hodgson *et al.* 1972) is that transmethylation from choline does not occur in insects, and that betaine is not metabolized.

Earlier evidence implying that methyl groups affect tumour levels comes from the results of Burnet & Sang (1968), which showed that for both tumour strains supplementary methionine reduces the increased tumour incidence caused by the presence of excess dietary tryptophan or phenylalanine. In the absence of either of these aromatic amino acids methionine is without effect. Here we have shown that the inclusion of methionine in the low choline diet lowers the tumour penetrance of both strains. This result, although supporting the contention that methionine is important with respect to tumorigenesis, by the very fact that it shows the same effect on both strains implies that the effects of low dietary choline are not due to a reduction of *in vivo* methionine levels. High levels of dietary choline, even in the presence of homocystine, do not reduce the high tumour levels of either strain caused by the inclusion of excess tryptophan in the defined diet. Since methionine effectively reduces tumours under these conditions this further confirms that there is no donation of the labile methyl groups of choline for the synthesis of methionine. Geer & Vovis (1965) have also shown with *D. melanogaster* that the feeding of homocysteine in a defined diet containing choline does not relieve the lethal effects of a methionine deficiency. It follows that methyl group donation from choline is not the metabolic area relevant either to tumorigenesis or to its suppression.

The role of supplementary methionine in tumour suppression or low choline might be postulated as due to its conversion to *S*-adenosylmethionine, leading to the stepwise methylation of phosphatidylethanolamine (Fig. 1), but the evidence from a number of insects is that this does not occur (Willis & Hodgson, 1970). The evidence for Diptera is that the methyl group of methionine is incorporated into serine, ethanolamine, non-polar lipids and the nucleosides of RNA (Bieber & Rottman, 1973). Adding serine (Sparrow, 1971*b*) or ethanolamine to low-choline

diet raises tumour penetrance in the unsuppressed strain, but has no effect on the suppressed strain, thereby eliminating the first two possibilities. We have not explored the possible effects of non-polar lipid precursors, or of other possible roles of methionine.

Sang (1968) showed that inhibition or enhancement of biogenic amine synthesis raises or lowers tumour penetrance. Juvenile hormone-like substances raise tumours, whilst ecdysone has the opposite effect (Bryant & Sang, 1969; Sang, 1968). Since hormone secretion is under neural control, it seemed likely that choline might be affecting tumorigenesis as its derivative acetylcholine. Unfortunately there are no direct methods to alter the *in vivo* level of this compound except perhaps by altering dietary choline levels. Acetyl-coenzyme A is the only other compound involved in acetylcholine synthesis. This being so central to intermediary metabolism is not easily manipulated. Two inhibitors, hemicholinium-3 for choline synthesis (MacIntosh, Birks & Sastry, 1956) and eserine sulphate for acetylcholinesterase, are without effect on tumour levels even at sublethal doses. Although this type of evidence is not conclusive it does suggest that the cholinergic nervous system is not involved in tumorigenesis. In fact, Dwivedy & Bridges (1973) have shown that choline is preferentially incorporated into the nervous system, so effects on acetylcholine are not to be expected.

Lack of evidence for choline acting as a methyl group donor or as acetylcholine leads to the remaining alternative that it is acting as a phospholipid component. This hypothesis is supported by a number of results. Additions of carnitine to a choline-free diet have the same effects as choline additions, affecting tumour penetrance in both strains in an opposite manner. Under these conditions it is known that carnitine, as beta-methylcholine, is incorporated into the phospholipids of *D. melanogaster* (Geer *et al.* 1971). This effect of carnitine is not due to the absence of choline since the same effect is apparent when carnitine additions are made to a low choline diet. This result itself is a little surprising since Geer *et al.* (1971) have shown that at the dietary choline concentration of the low choline medium (1×10^{-4} M) carnitine is not incorporated to any appreciable degree into phospholipids. It seems more likely that the more normal development rate of larvae in the experiments here reported, compared with that in Geer's work, leads to a higher choline requirement and that carnitine is used even in the presence of low levels of choline.

The only known function of DMAE and NMAE is to be incorporated into phospholipids at low dietary choline levels (Bridges *et al.* 1965), and ethanolamine is a normal constituent of insect phospholipids (Gilmour, 1961). The results obtained when DMAE, NMAE or ethanolamine are included in the low-choline diet are not easy to interpret, due largely to the absence of detailed information on phospholipid metabolism in *D. melanogaster*. DMAE, which is structurally very similar to choline unexpectedly lowers tumours in both the *tu bw*; $+^{su-tu}$ and *tu bw*; *st su-tu* strains. NMAE and ethanolamine, which differ from each other by one terminal methyl group, have the same effect when included in the low-choline diet, increasing the tumour penetrance of the unsuppressed strain but being without effect on the

suppressed strain. This latter result is inexplicable in terms of what is known of the *in vivo* interactions of choline and ethanolamine in phospholipid metabolism. Studies on *Musca domestica* (Bridges *et al.* 1965) showed that larvae grown on a choline-free diet had less phosphatidylcholine but the total phospholipid was the same, the deficit being made up by an increase in the phosphatidylethanolamine fraction. If this result were applied to the tumour system, then the expectation would be that on a low-choline diet additional ethanolamine should increase the tumour levels in the suppressed strain and lower them in the unsuppressed strain, i.e. having the same effect as low choline. This is quite obviously not the case. In fact, in the unsuppressed strain the effect of ethanolamine is similar to that of increased dietary choline. This perhaps indicates that in the unsuppressed strain, but not in the suppressed strain, choline and ethanolamine may be interchangeable in a particular class of phospholipids involved in tumorigenesis. This difference in response to ethanolamine of the two strains coupled with that found with regard to dietary choline levels (Sang & Burnet, 1967; this paper) strongly implicates phospholipid metabolism as the area in which the *su-tu* gene acts to alter tumour formation.

How a change in particular phospholipid patterns in these two strains affects tumorigenesis is not apparent from these experiments. There are a large number of known phospholipid functions in cells and organisms. The cell membranes, of which phospholipids are a constituent, have already been implicated in tumorigenesis by observations that cell aggregation is involved (Sang & Burnet, 1963) and that feeding glucosamine, a known component of cell surfaces (Spiro, 1970), causes changes in tumour formation (Rizki, 1962). Phospholipids as constituents of cell membranes are also involved in all processes that require the integrity of the cell and its organelles. They have been implicated too in the transport of a number of compounds in the body fluids of insects, including ecdysones (Thomas & Gilbert, 1967). It is likely that further examination of the nutritional interactions between choline and other tumorigenic compounds, and the metabolic consequences of growth on a low-choline diet will reveal the role of phosphatidylcholine in the expression of the *tu bw* and *su-tu* genes.

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