The effects of inhibition of protein synthesis on the phenotype of *Abnormal-abdomen*

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SUMMARY

Penetrance and expressivity of the *Drosophila* mutant *Abnormal-abdomen* \(A^{53g}\) may be reduced by adding inhibitors of protein synthesis, of RNA synthesis, and of oxidative phosphorylation to the defined, sterile medium on which larvae are developing. When grown on regular diet, the mutant flies contain a higher concentration of total protein than do wild-type flies. The metabolic inhibitors which reduce the expressivity of the mutation also lower protein concentration in these mutant flies. The reduction of protein synthesis is directly correlated with the reduction of expression of the mutant genotype. The regulation of the morphological abdominal abnormalities by the complex \(A^{53g}\) genotype is discussed in relation to the control, by this same genetic system, of the mechanisms of protein synthesis.

1. INTRODUCTION

The mutation *Abnormal-abdomen* \(A^{53g}\) of *Drosophila melanogaster* results in a phenotype which ranges from a ragged appearance of the lateral edges of the tergites to the complete absence of tergite on the dorsal abdominal surface (Hillman, 1973). In addition to its effect on the development of imaginal hypoderm, the mutant genotype also is responsible for an increase in the incorporation of labelled amino acids into a cell-free system prepared from *Drosophila* and for an increase in the aminoacylation of tRNA (Rose & Hillman, 1969). These latter experiments, utilizing the aminoacylating enzyme systems from both \(A^{53g}\) and wild-type flies, show that the post-microsomal supernatant fraction from homogenates of \(A^{53g}\) adult flies is more effective in aminoacylating *Drosophila*, yeast, calf liver and *E. coli* tRNA’s than is the corresponding fraction from wild-type flies.

Evidence to be presented elsewhere (Rose & Hillman, 1973) indicates clearly that the aminoacylating enzyme systems of both mutant and wild-type flies charge the same tRNA’s, but that the efficiency of the mutant enzyme system is greater than that of the wild-type. On the basis of these data it has been hypothesized
first, that the mutant genotype is responsible for an increase in protein synthesis in the fly and second, that this increase is related to the morphological expression of the mutant phenotype. In order to test these hypotheses, it is necessary to measure the protein concentration in both mutant and wild-type flies and to interfere with protein synthesis in the $A^{s3g}$ organism in order to determine if a reduction in protein synthetic activity will result in a more normal phenotype.

2. MATERIAL AND METHOD

Oregon-R wild-type strain of Drosophila melanogaster and a highly selected line of $A^{s3g}$ were used in these experiments. The Oregon-R stock had been maintained in unselected small mass matings, while the $A^{s3g}$ stock had been selected each generation for the most extreme expression of the abdominal abnormalities and maintained as small mass matings of extremely abnormal flies. These same stocks had been used for both incorporation and aminoacylation studies (Rose & Hillman, 1969).

Parental flies were maintained on a standard cornmeal, molasses (or Karo) medium, seeded with live yeast. The experiments were carried out at 25 °C, using germ-free larvae cultured on a sterile, chemically defined medium, as described by Sang (1956). Culture tubes, containing approximately 5 ml of medium with or without the additives described, were inoculated with between 40 and 50 first-instar larvae. The tubes were incubated until all adults emerged. Contaminated tubes were discarded.

Flies carrying the $A^{s3g}$ genotype were graded from 1 to 4; 1 being normal, two slightly abnormal, three moderate, and four extremely abnormal. For any single experiment a measure of expressivity was arrived at by summing the products of the number of flies in each grade times the grade and dividing by the total number of flies in the experiment. Penetrance was calculated by the usual method, the number of flies showing the mutant phenotype in any experiment divided by the number of flies in that experiment carrying the mutant genotype. Protein determinations were done by the method of Lowry et al. (1951) on the postmitochondrial fractions of whole-fly homogenates.

3. RESULTS

Protein in $A^{s3g}$ and wild-type flies was determined, and the results may be seen in Table 1. The data show clearly that there is an increased amount of protein in mutant flies when compared with wild-type flies of the same age. Further, the evidence shows that, allowing for the smaller size of the extremely abnormal flies, the concentration of protein on a wet weight basis is proportional to the degree of expression of the mutant phenotype. There is a direct correlation, therefore, between synthesis of total protein in the mutant fly and the abnormal development of the imaginal hypoderm.

The relationship between protein synthesis and penetrance and expressivity of the mutant genotype was next investigated in two series of experiments which
Table 1. Protein concentrations in Oregon-R and A^53g adult flies

| Age (h) | Genotype/phenotype | mg protein/fly | mg protein/mg wet weight |
|---------|-------------------|---------------|--------------------------|
|         |                   | ♀             | ♂             | ♀             | ♂             |
| 12      | Oregon-R/wild type| 0.031         | 0.026         | —             | —             |
|         | A^53g/mutant      | 0.041         | 0.034         | —             | —             |
| 66–80   | Oregon-R/wild-type| 0.038         | 0.017         | 0.039         | 0.023         |
|         | A^53g/slight      | 0.047         | 0.023         | 0.046         | 0.029         |
|         | moderate          | 0.064         | 0.031         | 0.065         | 0.039         |
|         | extreme           | 0.063         | 0.031         | 0.070         | 0.044         |

Table 2. Effect of the addition of cycloheximide to Sang’s medium on the penetrance and expressivity of A^53g

| mg/ml | Penetrance | Expressivity | Viability |
|-------|------------|--------------|-----------|
|       | ♀          | ♂            | ♀         | ♂         | ♀         | ♂         | |
| 0.00  | 152        | 159          | 1.00      | 0.89      | 2.34      | 1.97      | 0.48     |
| 0.15  | 52         | 45           | 0.98      | 0.89      | 2.08*     | 1.96      | 0.48     |
| 0.20  | 56         | 38           | 0.93      | 0.89      | 2.04*     | 1.92      | 0.52     |
| 0.30  | 75         | 68           | 0.79      | 0.73      | 1.80*     | 1.71*     | 0.41     |
| 0.40  | 31         | 33           | 0.81      | 0.61      | 1.84*     | 1.61*     | 0.30     |

* Effect significant at the 5% level.

were designed to study the effects of a reduction of protein synthesis on the A^53g phenotype. The first involved the addition of cycloheximide or chloramphenicol to the bacteria-free defined culture medium in order to test the effect of these inhibitors on the development and differentiation of the A^53g abdominal hypoderm. Chloramphenicol was found to have no effect on the penetrance, expressivity, or viability of mutant flies at concentrations as high as 3 mg/ml of medium. Cycloheximide, which has been shown to inhibit protein synthesis through its effect on the binding, transfer enzyme II-dependent movement and release of tRNA from ribosomes (Obrig et al. 1971), does change the phenotype of A^53g adults. When fed to developing A^53g larvae at sublethal dosages, cycloheximide normalizes the phenotype of the adult (Table 2).

The reduction of the penetrance and expressivity of the A^53g genotype by an inhibitor of protein synthesis led to a second series of experiments designed to interfere with protein synthesis in the mutant flies. Amino acid analogues were added to Sang’s medium, and A^53g larvae were placed in these cultures. Analogues which had no significant effect on the penetrance and expressivity of A^53g under these conditions included 5-methyl tryptophan and d-leucine. It is possible that the lack of phenotypic effect in these cases might be related to the competitive effect of the normal amino acids present in the casein used in the medium, or to the fact that the analogue is not used in protein synthesis (Geer, 1966). There were, however, two amino acid analogues tested which did reduce the penetrance and

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Table 3. The effect of the addition of two amino acid analogues to Sang’s medium on the penetrance and expressivity of A53°

|                  | Number | Penetrance | Expressivity | Viability |
|------------------|--------|------------|--------------|-----------|
|                  |        |            |              |           |
|                  | 90     | 0.98       | 2.20         | 0.52      |
| Azetidine-2-carboxylic Acid | 100    | 0.84       | 1.84         | 0.65      |
|                  | 200    | 0.95       | 2.09*        | 0.52      |
|                  | 400    | 0.91       | 1.91*        | 0.28      |
| Ethionine        | 200    | 0.99       | 2.17         | 0.60      |
|                  | 100    | 0.91       | 1.98*        | 0.62      |
|                  | 200    | 0.94       | 1.94*        | 0.48      |
|                  | 400    | 0.72       | 1.72*        | 0.33      |

* Effect significant at the 5% level.

expressivity of A53°. One of these was azetidine-2-carboxylic acid, a proline analogue, and the second was ethionine, a methionine analogue (Table 3). In both of these cases, the analogues were effective in reducing expressivity before their lethal dosages were reached.

Azetidine-2-carboxylic acid replaces proline in protein synthesis in both Escherichia coli and Phaseolus aureus. The replacement is in the primary protein structure and is assumed to change the tertiary structure, and thus the function, of the protein molecules (Fowden & Richmond, 1963). Ethionine is also incorporated into proteins (Levine & Tarver, 1951) and has an inhibitory effect on protein synthesis. This latter effect is considered, however, to be a secondary manifestation of either the acceleration of RNA breakdown (Stewart & Farber, 1968) or of the inhibition of ATP synthesis (Farber et al. 1964; Villa-Trevino, Shell & Farber, 1963, 1966; Gordon & Farber, 1965) by this analogue. On the basis of these latter considerations, it was decided to investigate the effect of an interference with nucleic acid synthesis and oxidative phosphorylation during A53° development.

Data from experiments designed to test the effect of the interference with RNA and DNA synthesis in A53° flies may be found in Table 4. 5-Bromouridine was chosen as an RNA analogue because while it results in a reduction of from 20 to 50% in RNA synthesis (Kahan & Hurwitz, 1962; Shapiro & August, 1965; Kapular et al. 1969), it also reduces the incorporation of labelled amino acids in a cell-free system, where it replaces UTP, to approximately 30% of normal (Furth, Kahan & Hurwitz, 1962). The evidence indicates clearly that a reduction in protein synthesis which is caused by a change in RNA structure results in a significant loss in penetrance and expressivity of the mutation.

The interference with DNA synthesis, on the other hand, has no effect. 5-Bromodeoxyuridine (B UdR) is lethal to the organism at very low concentrations, but at sublethal dosages does not change the phenotypic expression of A53°. The
addition of hydroxyurea to the growth medium does cause a reduction in the mutant phenotypic effect, but this normalization occurs at a dosage which is at the level of lethality. Since BUdR is without effect; and since hydroxyurea, in addition to inducing a reversible inhibition of DNA synthesis by interference with ribonucleotide reductase activity (Rosenkranz et al. 1966; Elford, 1968), has been shown to inhibit oxidative phosphorylation (Fishbein & Carbone, 1963); a study of the phenotypic effect of a reduction in ATP was initiated. The results may be seen in Tables 5 and 6.
Table 6. The effect of the addition of adenosine to Sang’s medium, less RNA, on the penetrance and expressivity of A53g (cytidine added, 0·375 mg/ml)

| mg/ml | Number | Penetrance | Expressivity | Viability |
|-------|--------|------------|--------------|-----------|
|       | ♂      | ♀          | ♂            | ♀         |
| 0·00  | 56     | 64         | 0·78          | 0·56      | 1·78      | 1·56      | 0·48      |
| 0·10  | 49     | 54         | 0·71          | 0·57      | 1·73      | 1·57      | 0·52      |
| 0·20  | 41     | 47         | 0·88          | 0·70      | 1·88      | 1·70      | 0·59      |
| 0·40  | 37     | 41         | 0·95          | 0·90      | 2·08*     | 1·95*     | 0·52      |

* Effect significant at the 5% level.

Interference with ATP production and energy metabolism in developing A53g flies normalizes the phenotype. The addition to the diet of diethylstilbestrol (Tenenhouse & Quastel, 1960) or of 2,4-dinitrophenol (Loomis & Lipmann, 1948) lowers the penetrance and expressivity of the mutation (Table 5). Conversely, the addition of adenosine to a cytidine-rich, adenosine-deficient diet increases both penetrance and expressivity to a normal level (Table 6). These experiments clearly show that a reduction in energy metabolism as well as an interference in RNA and protein synthesis normalizes the A53g phenotype.

The final series of experiments was designed to determine if the loss of penetrance and expressivity caused by metabolic inhibitors was correlated with a reduction in the amount of protein in A53g flies. Bacteria-free larvae were collected and placed on sterile medium to which had been added either metabolic inhibitors or analogues. Adults emerging from these cultures were graded for penetrance and expressivity, homogenized and assayed for total protein. The results of these experiments may be seen in Table 7.

Three of the experimental procedures reported in Table 7 have been previously discussed. The fourth involved the use of DL-p-fluorophenylalanine as a limited inhibitor of normal protein synthesis (Zeuthen & Rasmussen, 1966; Leick, 1969). The action of DL-p-fluorophenylalanine was highly variable in the system studied here, possibly due to the high concentration of phenylalanine in the medium. Consequently, this amino acid analogue was not reported among those which affected the penetrance and expressivity of A53g. In the experiment reported in Table 7, however, its addition resulted in a significant decrease in expressivity among A53g males at the dosage used. For this reason, it is included among the results.

The evidence presented in Table 7 supports the hypothesis of a direct relationship between the penetrance and expressivity of A53g and the protein content of the fly. In each of the experiments reported, the loss of penetrance, and secondarily that of expressivity, is accompanied by a parallel reduction in protein. The increased protein, resulting from an increase in the reactions associated with protein synthesis, is clearly a part of the physiological phenotype of the A53g genotype. This increase in protein synthesis in turn bears a special relationship to the penetrance and expressivity of the morphological abnormality.
Table 7. The relationship between penetrance, expressivity, and protein concentration in 2-day-old A530 adults after development on sterile medium containing inhibitors of protein synthesis

| Treatment-concentration | Penetrance | Expressivity | mg protein/fly |
|-------------------------|------------|--------------|----------------|
| 5-Bromouridine          |            |              |                |
| ♀♂ Control              | 1-00       | 2-42 (47)*   | 0-058 (40)     |
| 0-15 mg/ml              | 0-98       | 2-31 (61)    | 0-054 (40)     |
| 0-30 mg/ml              | 1-00       | 2-20 (46)    | 0-047 (40)     |
| ♀♂ Control              | 0-97       | 2-00 (60)    | 0-049 (60)     |
| 0-15 mg/ml              | 0-93       | 2-00 (59)    | 0-047 (40)     |
| 0-30 mg/ml              | 0-75       | 1-77 (53)    | 0-042 (40)     |
| Ethionine               |            |              |                |
| ♀♂ Control              | 1-00       | 2-12 (69)    | 0-070 (60)     |
| 2 mg/ml                 | 0-94       | 1-94 (48)    | 0-050 (40)     |
| 4 mg/ml                 | 0-72       | 1-73 (32)    | 0-036 (28)     |
| ♀♂ Control              | 0-89       | 1-92 (64)    | 0-041 (60)     |
| 2 mg/ml                 | 0-56       | 1-56 (73)    | 0-037 (60)     |
| 4 mg/ml                 | 0-41       | 1-41 (51)    | 0-030 (40)     |
| Cycloheximide           |            |              |                |
| ♀♂ Control              | 1-00       | 2-48 (25)    | 0-065 (24)     |
| 0-15 mg/ml              | 0-98       | 2-08 (52)    | 0-036 (18)     |
| 0-30 mg/ml              | 0-77       | 1-78 (60)    | 0-023 (40)     |
| ♀♂ Control              | 0-94       | 2-06 (34)    | 0-036 (32)     |
| 0-15 mg/ml              | 0-89       | 1-96 (45)    | 0-025 (27)     |
| 0-30 mg/ml              | 0-70       | 1-70 (44)    | 0-019 (37)     |
| dl-p-fluorophenylalanine|            |              |                |
| ♀♂ Control              | 1-00       | 2-45 (74)    | 0-058 (60)     |
| 1-5 mg/ml               | 0-97       | 2-28 (39)    | 0-038 (28)     |
| ♀♂ Control              | 0-91       | 2-06 (53)    | 0-035 (50)     |
| 1-5 mg/ml               | 0-80       | 1-82 (40)    | 0-028 (24)     |

* Number of organisms in sample shown in parentheses.

4. DISCUSSION

The evidence presented in this paper supports the hypothesis that a genetically controlled change in a biochemical synthetic system results in a change in the organism at the level of the morphological phenotype. Interference with both protein synthesis and with oxidative phosphorylation is reflected by a normalization of the phenotypic effect of the A530 genotype. A question which arises from the work reported is whether the decreased protein synthesis or the reduced ATP production is responsible for the changes in development which are recorded as a reduction of penetrance and expressivity of the mutation. The major piece of evidence which bears upon this point is the effect of 5-bromouridine on the penetrance and expressivity of A530.

The addition of 5-bromouridine to a medium already rich in nucleic acids has a normalizing effect on the phenotype. The metabolic effects of 5-bromouridine are first on RNA synthesis, second on protein synthesis, and third on ATP production through a lowering of enzyme concentration. Based upon this evidence, the most
consistent explanation for the normalization of the phenotype is that the treatments used reduced the penetrance and expressivity of $A^{53g}$ through their effect on protein synthetic reactions. In support of the hypothesis that it is the reduction in protein synthesis which is responsible for the change in phenotypic expression is the evidence that total protein per fly is directly correlated with the extent of the abdominal abnormality which that fly exhibits. If the increase in ATP production is the controlling factor in producing the abnormality, the absolute correlation might not have been seen.

Observations reported by Shafer (1971) also bear on the question. Shafer, studying synthetic activity in $A^{53g}$ larvae and pupae, reported an increase in RNA synthesis at approximately 48 h after the prepupal molt, followed by an increase in protein synthesis 2–3 h later. Although a similar increase is found in the wild-type organism, the concentration of protein in mutant pupae 56–60 h old is approximately 20% higher than in wild-type pupae of the same age. Since the increase in protein concentration is found at the time at which the final differentiation of the abdominal hypoderm is taking place (Robertson, 1936), this increase may be assumed to be associated with the abnormal folding of the imaginal hypoderm.

A second question which arises from data reported involves the relationship between the metabolic changes observed and the $A^{53g}$ genotype. If the primary effect of the genotype is the stimulation of RNA and protein synthesis, the experimental reduction of oxidative phosphorylation can reduce protein synthesis by reducing the energy available for synthetic reactions. On the other hand, if the primary effect of the genotype is an increase in the rate of ATP production by an increased rate of oxidative phosphorylation, the experimental reduction in protein synthesis would tend to lower ATP production by reducing oxidative enzyme concentrations.

The experiments reported here cannot distinguish between these two alternative explanations for initial gene activity. The details of the effect of the complex mutant genotype on the developmental system of $A^{53g}$ flies remain unknown. The hypothesis being tested is that the modifier genes, which have been described as controlling the expression of $A^{53g}$ (Hillman, 1973), control protein synthesis through their effect on those enzymes which are responsible for the aminoacylation of tRNA. The major gene then functions in reaction to these changes in protein synthetic activity by producing cellular abnormalities. Thus, one would predict changes in $A^{53g}$ cells and tissues when they are placed under certain metabolic conditions not found in the wild-type cellular environment. Such experiments are now in progress.

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Protein synthesis of Abnormal-abdomen phenotype

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