A STUDY OF TRYPTOPHANE IN EYE COLOR MUTANTS OF DROSOPHILA[1]

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Received December 21, 1948

EARLIER studies have yielded evidence which points to the amino acid tryptophane as a precursor of the brown eye pigment in adult Drosophilae as well as in other insects. The precise mechanism whereby tryptophane is converted to brown pigment has been in part clarified as a result of a number of investigations. In the case of *Drosophila melanogaster* these investigations have been primarily concerned with the mutants vermilion and cinnabar, in which the phenotype is characterized by a lack of brown eye pigment. The transplantation studies of BEADLE and EPHRUSSI (cf. EPHRUSSI 1942 for a summary) confirmed earlier observations of STURTEVANT (1932) that genetically vermilion eye discs develop non-autonomously in a wild type host, that is, they form brown pigment. They suggested that the failure of brown pigment formation in vermilion flies was related to the absence of a substance designated the v^+ hormone. Further study demonstrated the non-autonomous behavior of cinnabar eye discs when transplanted into wild type hosts and led to the suggestion that a second substance, designated the cn^+ hormone, was necessary for the development of brown pigment.

The nature of the v^+ hormone was ultimately determined from the results of a number of investigations. In the course of nutritional studies on the vermilion gene of *D. melanogaster*, TATUM (1939) observed that when vermilion flies were raised on a tryptophane-containing medium which had been contaminated by an unknown bacillus, brown pigment was laid down in the adult eyes. BUTENANDT, WEIDEL, and BECKER (1940) then showed that the tryptophane metabolite kynurenin derived from rabbit urine, when injected into or fed to developing vermilion flies, resulted in the deposition of brown pigment in the adult eyes. The bacterial substance was then demonstrated to be kynurenin (TATUM and HAAGEN-SMIT 1941). Final proof of the identity of the v^+ hormone with kynurenin was offered by KIKKAWA (1941), who isolated the kynurenin from cinnabar pupae of *D. melanogaster*.

It has been proposed by a number of investigators that in the formation of brown eye pigment, tryptophane is metabolized in the following manner:

tryptophane→α oxytryptophane→kynurenin→cn^+ hormone—brown pigment

KIKKAWA has suggested that the cn^+ substance is the chromogen of the brown pigment. However, at the present time the nature of the cn^+ hormone, and the intervening steps to brown pigment formation, are unknown.

[1] Aided by a grant from the RESEARCH COUNCIL, UNIVERSITY OF MISSOURI.

The present investigation was undertaken in an attempt to determine the fate of tryptophane in various eye color mutants of *D. melanogaster* and *D. virilis*. In the case of the vermilion mutant of Drosophila, where the lack of brown pigment is associated with a failure in kynurenin formation, it is of interest to learn the reason for this failure in the oxidation of tryptophane. One explanation offered is that an insufficient quantity of non-protein tryptophane is available to developing vermilion flies with a resultant absence of kynurenin. CASPARI (1946), studying the recessive *a* mutation in Ephestia which is similarly characterized by a failure in the formation of kynurenin, observed a significant increase in the protein tryptophane in the mutant as compared with the normal (a^+) moths. He suggested that the increased protein tryptophane might in part account for the failure in kynurenin formation by limiting the non-protein tryptophane available for oxidation. A second possibility is that in vermilion, the tryptophane is metabolized along an entirely different pathway which excludes the formation of kynurenin. Such an explanation has been suggested by BEADLE and TATUM (1941), who pointed out that the tryptophane might be oxidized to kynurenic acid in place of kynurenin. A third possible explanation is that the vermilion flies possess sufficient non-protein tryptophane for conversion to kynurenin but lack the enzyme(s) which are capable of catalyzing this oxidation. If this were the case, one would expect an accumulation of non-protein tryptophane.

In the case of the other eye color mutants, where it is known that tryptophane is oxidized to kynurenin, it is of interest to know how much of the tryptophane is converted to kynurenin; and especially in the case of the *cn* mutant, what the fate of kynurenin is in these flies.

To select the most satisfactory explanation, a number of tryptophane analyses as well as estimates of kynurenin have been made on certain eye color mutants of *D. melanogaster* and *D. virilis*. A preliminary report of these results has been made (GREEN 1948).

MATERIALS AND METHODS

The following homozygous mutants of *Drosophila melanogaster* were investigated: vermilion (*v*); cinnabar (*cn*); scarlet (*st*); brown (*bw*); suppressor of vermilion in combination with vermilion (su^2-*s*, *v*); and white (*w*). A Stephenville wild type strain served as the wild type control. In *Drosophila virilis* the following homozygous mutants were investigated: vermilion (*v*); cinnabar (*cn*); scarlet (*st*); eosinoid (*es*); and white (*w*). A standard wild type strain derived from a collection at Pasadena, California, served as the control. Each stock was cultured en masse on the usual corn meal, yeast and agar medium at a room temperature of 22–23°C. The flies were raised in quarter pint milk bottles and care was taken to prevent overcrowding by allowing the parental females to oviposit for a maximum of 24 hours. The adult flies were collected at intervals of 0–2 hours after emergence in order to limit the food intake to a minimum. Immediately after collection, the adults were frozen by immersion in liquid air. The flies were kept in the frozen state for at least 30 minutes after which they were transferred to a vacuum desiccator and dried

in vacuo over Al_2O_3. It was found that desiccation for 24 hours was sufficient to dry the flies to a constant dry weight. After complete drying, the flies were pulverized with an agate mortar and pestle and then stored *in vacuo* over Al_2O_3 until used.

In making the tryptophane determinations two methods were used. The initial analyzes were made according to the method of ECKERT (1943). Subsequent tests to determine the specificity of this method showed that it is relatively non-specific. Other compounds, especially those involved in tryptophane metabolism, such as indole and kynurenin, react in a manner similar to tryptophane (CASPARI and GREEN, unpublished) and their presence may lead to excessively high results. While this non-specificity is disadvantageous, it will be pointed out that certain results obtained with this method aid in the clarification of the action of certain of the mutant genes studied. The second method used was the modification of the May-Rose p-dimethylaminobenzaldehyde method developed by HORN and JONES (1945). This method has been shown to be most specific in its reaction with tryptophane. Results obtained by performing duplicate analyses with this method and a bioassay method based on the growth of a tryptophane-requiring strain of *Escherichia coli* were identical (CASPARI and GREEN, unpublished).

Non-protein tryptophane was determined for each mutant by first precipitating the fly proteins with 0.3M trichloracetic acid followed by analysis of the filtrates for tryptophane content.

Total tryptophane was determined after first hydrolyzing the fly proteins. The first attempts at determining the total tryptophane depended upon the alkaline hydrolysis of the proteins. This method, in which the dried flies were autoclaved with 5N NaOH, gave erratic results and was discarded. The procedure finally adopted was a slight modification of the method of GREENHUT, SCHWEIGERT and ELVEHJEM (1946). This method is based on the enzymatic hydrolysis of the proteins. The enzymes used here were a combination of the proteolytic enzymes of dried pancreas (pancreatin) and of dried small intestinal mucosa (duodenin). To fly samples of approximately 50 mgm were added 10 mgm of pancreatin and 5 mgm of duodenin as a solution in phosphate buffer of Ph 8.2. The samples were layered with toluene to prevent bacterial growth and were then shaken in an incubator at 37°C. After 20-24 hours of incubation, the added enzymes were precipitated with trichloracetic acid and the tryptophane content of the filtrates determined by the method of HORN and JONES. In agreement with the report of GREENHUT *et al.*, shaking was necessary for complete hydrolysis. Shaking for less than 18 hours resulted in low tryptophane results, while no increase was found after shaking for longer than 24 hours.

In addition to the tryptophane content, determinations of the non-protein α amino nitrogen and total α amino nitrogen were made on the appropriate filtrates using the method of POPE and STEVENS (1939).

All results presented represent the means of three or more duplicate analyses. Calculations have been made as milligrams of tryptophane or α amino

nitrogen per gram of dried flies. Protein tryptophane and protein α amino nitrogen have been calculated as the difference between the total and non-protein values.

NON-PROTEIN TRYPTOPHANE COMPOSITION

In table 1 are presented the results of non-protein tryptophane determinations by the two methods used as well as the non-protein α amino nitrogen in wild type and mutant *D. melanogaster.* It may be observed that the results obtained for the various mutants by the method of HORN and JONES are essentially in agreement with one another and with wild type, with the exception of *v* and of *su*^{2}*-s, v.* In these two mutants there appears to be a significant excess of non-protein tryptophane. The six-fold increase in the non-protein tryptophane content of *v* in comparison with wild type flies appears to be an increase in this amino acid alone. Examination of the results obtained for α amino nitrogen indicate no general increase in the non-protein amino acids as compared with wild type. A similar increase in the non-protein tryptophane is observed in the case of *su*^{2}*-s, v*, but in this case the increase is not as marked as in *v*.

TABLE 1

Non-protein tryptophane composition of D. melanogaster.
(All results expressed as mgm/gm dried flies.)

GENOTYPE	HORN AND JONES METHOD	ECKERT METHOD	NON-PROTEIN α AMINO NITROGEN
wild type	0.215	0.329	4.82
v	1.389	1.151	4.93
su^2*-s, v*	0.512	0.539	5.13
bw	0.236	0.574	5.36
cn	0.235	1.253	5.39
st	0.250	0.565	5.13
w	0.171	0.306	4.27

The results of non-protein tryptophane determinations as made by the method of ECKERT are essentially in agreement with those found by the method of HORN and JONES. One exception is evident. It will be noted that the results obtained for *cn* by the two methods are in sharp disagreement. As pointed out previously, the ECKERT method is relatively non-specific for tryptophane and such compounds as indole and kynurenin when present could lead to excessively high results. The discrepancy between the values obtained by the two methods undoubtedly results from the presence of kynurenin in the *cn* flies. Kynurenin has been isolated from *cn* pupae (KIKKAWA 1941). Moreover, trichloracetic acid filtrates of *cn* adults when tested for the presence of kynurenin by the OTANI-NISHINO reaction gave a strongly positive test, whereas similar tests performed on wild type and mutants other than *cn* were negative. It will be noted that in the case of all determinations except that for *v*, the results obtained by the ECKERT method were somewhat higher than

those obtained by that of HORN and JONES. It is suspected that in all these cases the higher results may be related to the presence of small amounts of kynurenin in the adult flies.

Non-protein tryptophane results for the wild type and mutants of *D. virilis* are listed in table 2. As in *D. melanogaster*, the results obtained by the HORN and JONES method indicate a significant excess of non-protein tryptophane in *v* as compared with the other mutants. In addition there is a strong suggestion that the non-protein tryptophane content of *w* of *D. virilis* is also significantly greater. This finding differs from the results obtained for the *w* mutant of *D. melanogaster*, where the non-protein tryptophane content was slightly lower than in wild type. It has been suggested that the *w* mutants in the two species are homologous. The finding of this difference may be an indication of the non-homology of the two mutants. However, additional information must be obtained before a conclusive answer can be given to this problem. Similar to the findings for *D. melanogaster*, the results obtained in *D. virilis* using the

TABLE 2

Non-protein tryptophane composition of D. virilis.
(All results expressed as mgm/gm dried flies.)

GENOTYPE	HORN AND JONES METHOD	ECKERT METHOD	NON-PROTEIN α AMINO NITROGEN
wild type	0.201	0.303	4.36
v	0.892	0.842	5.28
es	0.224	0.343	5.57
st	0.285	1.116	4.89
cn	0.223	0.432	4.98
w	0.343	0.598	5.63

method of ECKERT are essentially in agreement with the results obtained by the HORN and JONES method, except for the mutant *st*. This discrepancy can be accounted for by the presence of kynurenin in *st* flies as indicated by a strongly positive OTANI-NISHINO test. These findings support the conclusion of STURTEVANT and NOVITSKI (1941) that *cn* of *D. melanogaster* and *st* of *D. virilis* represent homologous mutations.

PROTEIN TRYPTOPHANE COMPOSITION

Table 3 lists the results obtained for the total tryptophane of wild type and mutant adult flies of *D. melanogaster*. Superficial examination of these values indicates that there is a wide variation in the total tryptophane of *D. melanogaster*. Evaluation of these results will show that the differences found reflect variations in the non-protein tryptophane and in the total protein content of the flies. However, no significant difference in the protein tryptophane composition among the various mutants was found.

The protein tryptophane in the various mutants has been calculated as the difference between the mean total tryptophane and the mean non-protein tryptophane as obtained by the HORN and JONES method (table 3). From the values obtained for the protein tryptophane, the protein tryptophane α amino

nitrogen has been calculated on the basis of tryptophane containing 6.86 percent α amino nitrogen (table 3).

Similarly the protein α amino nitrogen has been calculated as the difference between the mean total α amino nitrogen and the mean non-protein α amino nitrogen (table 3). The protein α amino nitrogen may serve as a measure of the protein content of the flies. On this basis, it would appear that the protein content is somewhat variable. However, the only significant differences are those of approximately ten percent between wild type and *bw* and *v*. Since it happens that the flies of these three genotypes were not raised concurrently, further tests would be necessary to show whether these differences are intrinsic or the result of variations in the culture media.

The percentage tryptophane composition of the fly proteins has been calculated as 100× the ratio of protein tryptophane α amino nitrogen to protein

TABLE 3

Total and protein tryptophane composition of D. melanogaster.
(All results expressed as mgm/gm dried flies except as noted in final column.)

GENOTYPE	TOTAL TRYPTOPHANE	PROTEIN TRYPTOPHANE	TOTAL α AMINO NITROGEN	PROTEIN α AMINO NITROGEN	PROTEIN TRYPTOPHANE α AMINO NITROGEN	PERCENT PROTEIN TRYPTOPHANE COMPOSITION
wild type	5.63	5.42	48.16	43.34	0.37	0.85
v	7.33	5.94	54.02	49.09	0.41	0.83
su^2-*s*, *v*	6.32	5.81	51.02	46.89	0.40	0.85
bw	6.23	5.99	55.15	49.79	0.41	0.82
cn	6.05	5.82	53.71	48.32	0.40	0.83
st	6.03	5.78	52.35	47.22	0.40	0.84
w	5.85	5.68	50.31	46.04	0.39	0.85

α amino nitrogen. These results may be found in the last column in table 3. It may be seen that the protein tryptophane composition among the various mutants studied varied from 0.82–0.85 percent. In view of the errors inherent in the analytical procedures used, such a variation is not unexpected and the differences, where they occur, cannot be considered as significant. Therefore it can be concluded that in *D. melanogaster* no significant difference in the protein tryptophane composition in the various mutants studied occurs. Where there are differences, they may be resolved as being the result either of increased non-protein tryptophane, as in the case of *v*, or of an overall increase in the fly proteins.

In table 4, determinations of the total tryptophane content of the mutants in *D. virilis* have been presented. Calculations of the protein tryptophane and the percentage protein tryptophane composition in these mutants have been made exactly as they were made for the mutants of *D. melanogaster*. In the case of *D. virilis*, the protein tryptophane composition varied between 0.95–0.98 percent, indicating that here too there is no significant difference among the mutants studied.

TABLE 4

Total and protein tryptophane composition of D. virilis.
(All results expressed as mgm/gm dried flies except as noted in final column.)

GENOTYPE	TOTAL TRYPTOPHANE	PROTEIN TRYPTOPHANE	TOTAL α AMINO NITROGEN	PROTEIN α AMINO NITROGEN	PROTEIN TRYPTOPHANE α AMINO NITROGEN	PERCENT PROTEIN TRYPTOPHANE COMPOSITION
wild type	6.24	6.04	47.47	43.11	0.41	0.96
v	7.96	7.07	55.87	49.59	0.48	0.96
es	6.80	6.58	53.08	47.51	0.45	0.95
st	7.13	6.85	52.93	48.04	0.47	0.98
cn	7.06	6.84	54.19	49.21	0.47	0.95
w	7.54	7.20	56.84	51.21	0.49	0.96

It may be pointed out that while within each species the protein tryptophane composition is constant, there does appear to be a real difference between *D. melanogaster* and *D. virilis* with respect to protein tryptophane.

DISCUSSION

The results presented here support the conclusion that the eye color mutants of *D. melanogaster* and *D. virilis* do not influence the protein tryptophane composition of the flies. This observation is at variance with the findings reported for *a* and a^+ in Ephestia by CASPARI (1946). It has been suggested that the *v* mutation of Drosophila and the *a* mutation of Ephestia are homologous; it seems justifiable to conclude here that in so far as their effects on the protein tryptophane composition are concerned, the two mutations are not homologous.

Moreover it is apparent that in the case of the *v* mutants of Drosophila, the failure to make brown pigment is not correlated with excessive storage of tryptophane in the proteins. The findings reported here point to a marked accumulation of non-protein tryptophane in the *v* flies, indicating that while there is sufficient tryptophane available during development, *v* flies are completely incapable of carrying out the oxidation of tryptophane to kynurenin. Further support for this conclusion may be drawn from the observation that when the su^2-*s* mutation is introduced into *v* flies, the biosynthesis of brown pigment is correlated directly with the utilization of tryptophane. Thus in su^2-*s*, *v* flies, in which the eyes approach wild type pigmentation, the non-protein tryptophane is reduced to less than 50 percent of the amount found in *v* flies. Since it has been reported that kynurenin is formed in su^2-*s*, *v* flies, though on a reduced scale in comparison with wild type (BEADLE and EPHRUSSI 1936), it follows that the relative drop in non-protein tryptophane in su^2-*s*, *v* as compared with *v* is the direct result of oxidation of tryptophane to kynurenin. The accumulation of tryptophane in *v* flies appears to be comparable to the findings of TATUM, BONNER and BEADLE (1944) in a tryptophaneless mutant of Neuro-

spora. Here it was observed that the mutant strain, which cannot convert anthranilic acid to indole, accumulates anthranilic acid.

Similar results have been reported here with the mutants *cn* of *D. melanogaster* and *st* of *D. virilis*. In these mutants the conversion of tryptophane to kynurenin is carried out, but further step(s) in the biosynthesis of brown pigment fail at the kynurenin level such that kynurenin accumulates in these two mutants.

The data presented here do not warrant any clear-cut conclusions on the mechanism of action of the genes *st* of *D. melanogaster* and *cn* of *D. virilis* nor of the *w* mutations in both species. If the amounts of non-protein tryptophane present in these mutants represent a measure of kynurenin synthesis, then it appears that the failure in brown pigment synthesis occurs in some stage after kynurenin formation. Comparison of the results obtained for non-protein tryptophane by the two methods used indicates that kynurenin is also metabolized. However the data do not suggest at what point between the metabolism of kynurenin and the formation of brown pigment the failure occurs. While the homology between the *st* mutant of *D. melanogaster* and the *cn* mutant of *D. virilis* seems to be supported by the data presented here, there is some question with respect to the homology between the *w* mutations of the two species. It may be noted that the non-protein findings for *w* of *D. melanogaster* indicate the amount of tryptophane present to be the same as or less than that found in wild type flies. Conversely, non-protein tryptophane of *w* in *D. virilis* appears to be significantly greater than that found for wild type. There is the possibility that these results indicate different modes of gene action for the *w* mutations of the two species.

From the results of investigations in Neurospora, it has been suggested that each gene controls a specific metabolic step (HOROWITZ *et al.* 1945). The findings for the *v* and *cn* genes of *D. melanogaster* and the *v* and *st* genes of *D. virilis* fit into such a scheme of gene action. It has been argued that if genes control the production of enzymes, a block in a particular reaction chain should be manifested in the accumulation of the substrate immediately prior to the point of blockage (BONNER 1946). Such accumulations are to be noted here in the case of the *v* mutants and *cn* of *D. melanogaster* and *st* of *D. virilis*. These results are then in agreement with the point of view that such accumulations of substrates constitute evidence for the failure in the elaboration of specific enzymes in the presence of specific mutant genes. On the other hand it might be argued that the failure of the metabolic step is the result of the elaboration of a specific enzyme inhibitor. Unpublished experiments with the *v* mutation of *D. melanogaster* however appear to support the former idea, although more information is necessary before a definite conclusion can be drawn.

SUMMARY

Analyses of the protein and non-protein tryptophane content of adults with wild type and mutant eye color in *D. melanogaster* and *D. virilis* have been presented.

No difference was found in the protein tryptophane composition between the various eye color mutants and wild type within each species. However, a difference in the protein tryptophane content between *D. melanogaster* and *D. virilis* was found.

Non-protein tryptophane was found to accumulate in the *v* mutants of both species. Similarly, kynurenin was found to accumulate in the *cn* mutant of *D. melanogaster* and the *st* mutant of *D. virilis*.

It is concluded that the accumulation of tryptophane and kynurenin in the specified mutants supports the hypothesis that each gene mutation fails to elaborate a specific enzyme necessary for the metabolism of the accumulated compounds.

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