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THE CHARACTERIZATION OF MULTIPLE FORMS OF KYNURENINE FORMIDASE IN *DROSOPHILA MELANOGASTER*

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Summary

Two enzymic forms of kynurenine formamidase (EC 3.5.1.9) from *Drosophila melanogaster* were separated and partially purified by pH fractionation, $(NH_4)_2SO_4$ fractionation and Sephadex G-75 gel filtration. The enzymes were also separated by DEAE-cellulose ion-exchange chromatography and distinguished by their different rates of thermal inactivation.

The multiple forms are termed formamidase I and formamidase II. The molecular weight of formamidase I as measured by Sephadex G-75 chromatography is 60 000 and that of formamidase II is 31 000. The pH optima are broad, ranging between 6.7 and 7.8 for formamidase I and 6.5 and 8.0 for formamidase II. The apparent K_m values are $5 \cdot 10^{-3}$ and $0.83 \cdot 10^{-3}$ M, respectively. The possibility that formamidase II is an active subunit of formamidase I is discussed, although neither enzyme will convert to the other when separated and rechromatographed. Eight organisms were tested for the presence or absence of multiple forms of formamidase. *Drosophila melanogaster* and *Drosophila virilis* have both enzymes; cow, chicken, yeast and housefly have formamidase I only, and mouse and frog have formamidase II only.

Introduction

The enzyme kynurenine formamidase (aryl-formylamine amido hydrolase, EC 3.5.1.9) is the second enzyme in the pathway of tryptophan degradation. It catalyzes the hydrolysis of *N*-formyl-L-kynurenine, yielding kynurenine and formate as products.

Kynurenine formamidase (formamidase) has been found in the liver of rat, guinea pig, horse, rabbit, pig and chicken as well as in whole extracts of *Neurospora crassa*, *Anagasta kuhnilla* and *Drosophila melanogaster*. The enzyme has been purified from rat liver by Shinohara and Ishiguro [1] and from chicken liver by Bailey and Wagner [2]. The latter authors also demonstrated the

existence of a second formamidase in pre-hatching chick embryos. This enzyme was not extensively studied but was shown to be immunologically unrelated to the adult chicken liver enzyme.

In *Drosophila*, kynurenine is converted to 3-hydroxykynurenine which is a precursor of the ommochromes, the brown eye pigments. Although the pathway of pigment production in *Drosophila* had been extensively studied, little is known about formamidase in this organism. It has never been purified from *Drosophila* nor has its structural gene been identified. No mutant deficient in its production has been found, although a relatively large number of eye color mutants have been tested for its activity [3]. Because of our continuing interest in the biochemical nature and genetic regulation of the eye pigment production pathway we have undertaken a study of formamidase in *Drosophila*.

In this report we demonstrate the existence of two forms of formamidase, as shown by their separation by gel filtration and ion-exchange chromatography as well as their different rates of thermal inactivation. The partial purification and characterization of the two enzymes is described. Although one of the enzymes could be an active subunit of the other, they are shown not to interconvert under the conditions used in this study. A number of diverse species are described with regard to the presence or absence of multiple forms of formamidase and a model is presented to account for the results.

Materials and Methods

Animal procedures

D. melanogaster used in this study are an Oregon R strain maintained in our laboratory. The flies were grown at 25° C on a standard medium of corn meal, dextrose, sucrose, agar and yeast and maintained in plexiglass cages. Adult flies of 9–11 days from oviposition were used for all enzyme preparations except where the use of 4- and 5-day-old larvae is specifically indicated. The mice were randomly bred CF-1 Swiss albinos, both males and females. The *Musca domestica* (housefly) were kindly supplied by Dr T. Nakatsugawa of the SUNY College of Environmental Science and Forestry. The yeast was a wild type strain kindly provided by Dr R. MacIntyre of Cornell University. It was grown in liquid culture medium composed of dextrose, peptone and yeast and swirled at 30° C for 24 h.

Formamidase assay

Formamidase activity was assayed by a method similar to that of Glassman [3]. The assay was carried out at 25° C with the temperature held constant with a circulating water bath attachment for the Gilson model 240 spectrophotometer. To a 1 ml glass cuvette was added 0.8 ml of 0.1 M potassium phosphate (pH 7.4), 0.1 ml of a 15 mM solution of *N*-formyl-L-kynurenine (Calbiochem) and 0.1 ml enzyme preparation. The increase of absorption at 360 nm (due to kynurenine formation) was continuously recorded. The rate of kynurenine formation was proportional to the amount of enzyme added and boiling caused complete loss of activity.

The protein determinations were made by the method of Lowry et al. [4] using crystalline bovine serum albumin as a standard.

Preparation of formamidase multiple forms

The enzymes were prepared for study as follows with all steps done at 4° C or in an ice bath.

(1) 30 g of adult flies were ground in a mortar and pestle in 0.1 M potassium phosphate buffer (pH 7.4) with 2% phenylthiourea and 2 mM dithiothreitol. The final dilution was to 100 ml. The homogenate was centrifuged at 12 000 × *g* for 20 min in a Sorval RC2B centrifuge.

(2) The supernatant was filtered through nylon mesh and centrifuged at 100 000 × *g* for 90 min in a Beckman L3-40 ultracentrifuge in the type 30 rotor.

(3) The supernatant was brought to pH 5.0 with 1 M acetic acid, incubated 1 h then centrifuged at 12 000 × *g* for 20 min.

(4) Enough of a saturated $(NH_4)_2SO_4$ solution to reach 40% was added to the supernatant which was incubated 2 h then centrifuged at 12 000 × *g* for 20 min. Enough saturated $(NH_4)_2SO_4$ solution was then added to the supernatant to reach 65% saturation. It was incubated 2 h and centrifuged as before.

(5) The $(NH_4)_2SO_4$ was resuspended in (a) 10 ml of 0.1 M phosphate buffer (pH 7.4) for Sephadex G-75 gel filtration or (b) 0.05 M Tris · HCl (pH 8.0) for DEAE-cellulose ion-exchange chromatography. Prior to application to the DEAE-cellulose column the enzyme preparation was dialyzed overnight against four large volume changes of 0.05 M Tris · HCl (pH 8.0), $5 \cdot 10^{-4}$ M dithiothreitol and 10^{-4} M EDTA.

(6) The resuspended pellet, treated as in (5a) above, was applied to a Sephadex G-75 column (70 × 2.6 cm) equilibrated in phosphate buffer (pH 7.4). The column was developed with the same buffer at a flow rate of 23 ml/h. Fraction size was 3 ml. Flow was descending with a hydrostatic pressure head of 30 cm. Fractions which contained formamidase activity were pooled for further study.

Results and Discussion

Demonstration of multiple forms of formamidase

During the course of our preparation of formamidase, two well separated activity peaks were observed when extracts were run on Sephadex G-75. This elution profile is shown in Fig. 1. The faster moving, larger molecule we have termed formamidase I and the more retarded, smaller molecule we have named formamidase II. The two forms can be partially separated by $(NH_4)_2SO_4$ or pH fractionation and Fig. 2 shows that they can also be separated by ion-exchange chromatography using DEAE-cellulose. Chromatography of the separated DEAE-cellulose peaks on Sephadex G-75 showed that the peak more tightly bound on DEAE-cellulose is formamidase I, eluting at 0.15 M NaCl, and the less tightly bound peak eluting at 0.1 M NaCl, is formamidase II.

The existence of multiple forms of formamidase is further substantiated by the biphasic nature of the thermal inactivation profile shown in Fig. 3. Thermal inactivation studies on preparations of the separated enzymes showed that formamidase I is the more heat stable form (see below). Extrapolation of the inactivation rate of formamidase I to the ordinate (Fig. 3) gives an estimate of the amount of formamidase I relative to formamidase II. An estimate of 52%

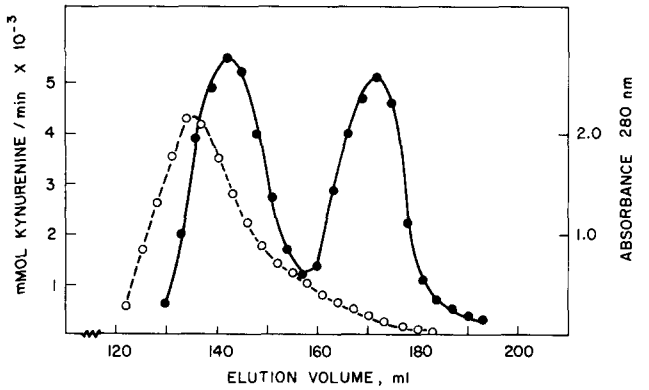

Fig. 1. Gel filtration of kynurenine formamidase activity on Sephadex G-75. The sample applied was the resuspended pellet obtained in the 40—65% $(NH_4)_2SO_4$ cut of an extract of adult flies. The left ordinate and closed circles are formamidase activity. The right ordinate and open circles are absorbance at 280 nm.

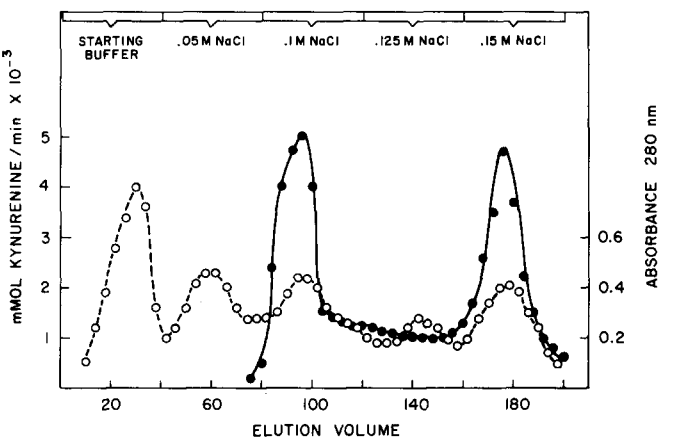

Fig. 2. Elution profile of formamidase from DEAE-cellulose using a stepwise increase in NaCl concentration. The sample applied was the resuspended pellet obtained in the 40—65% $(NH_4)_2SO_4$ cut of an extract of adult flies. Left ordinate and closed circles refer to formamidase activity. Right ordinate and open circles to absorbance at 280 nm.

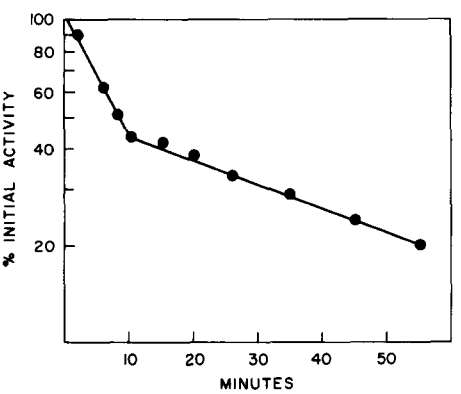

Fig. 3. Thermal inactivation profile of formamidase activity. 0.5-ml aliquots of extracts of third instar larvae were placed in closed Durham tubes and simultaneously immersed in a 47°C water bath. Tubes were removed at times indicated, rapidly chilled, and assayed for formamidase activity.

formamidase I and 48% formamidase II is obtained. This estimate is only approximate but indicates that one form is not present in large excess relative to the other. This conclusion is confirmed by the gel filtration and ion-exchange chromatography profiles of Figs 1 and 2. The relative amounts of the two forms do not appear to vary greatly from the larval to the adult stages of development.

Although rate of thermal inactivation has not been as widely used to detect multiple forms of enzymes as electrophoretic techniques, it offers the advantage of speed and ease of execution. Theoretically it is, in fact, a more sensitive means of detecting different forms since a single amino acid substitution is more likely to affect the rate of thermal inactivation than the rate of electrophoretic mobility [5,6]. One disadvantage of using thermal inactivation procedures in non-homogeneous preparations is the possibility of interactions between the denaturing protein and unknown substances in the mixture. Such interactions may change the rate of thermal inactivation. In fact, we have observed that the rates of thermal inactivation of both formamidase I and II change during the development of the organism. Both forms become more heat stable in the adult animal but the shift is more marked in formamidase II, so that by the adult stage only a slight difference can be detected between the two (although gel filtration verifies that both forms are still present).

General characteristics of formamidase I and II

Table I summarizes the procedure used to prepare formamidase I and II. The yield from Sephadex G-75 could be greatly improved except that only the fractions with highest activity were retained. A further 4—5-fold purification could be obtained on DEAE-cellulose but this step was not employed in the preparations used in this study. Stored at 4°C in 0.1 M phosphate buffer (pH 7.4) with no stabilizing agents, formamidase I retained 58% of its activity after 1 week and formamidase II retained 72% of its activity. 10% glycerol reduced activity loss to about 10% for both forms over the same time period. Increasing salt concentration as well as the presence of reducing agents also had some stabilizing affect, especially on formamidase II.

TABLE I

PARTIAL PURIFICATION OF KYNURENINE FORMIDASE I AND II

A unit of formidase is defined as the change of absorbance of 0.1 per min, which is equivalent to 0.23 μmol of kyurenine.

Step	Volume (ml)	Protein (mg/ml)	Total protein	Activity units	Specificity activity (units/mg)	Purification	Yield
1 Homogenate	100	34.3	3430	98.6	0.029	1	100
2 100000 × *g* supernatant	94	19.2	1805	98.0	0.054	1.9	99
3 pH 5 supernatant	90	7.4	667	90.2	0.135	4.7	91.5
4 40—65% $(NH_4)_2SO_4$	10	30.3	303	76.4	0.252	8.9	77.5
5 Sephadex G-75							
(a) formamidase I	10	1.8	18	20.4	1.13	38.3	20.7
(b) formamidase II	10	1.2	12	26.0	2.1	72.9	26.5

The pH optimum of formamidase I is broad, ranging between 6.7 and 7.8. The optimum of formamidase II is also broad between 6.5 and 8.0 with a slight maximum at 7.7. Broad pH optima of this nature have been reported in rat liver formamidase [1], *Neurospora* [7] and chicken liver [2]. Kimmel [8] reports two activity peaks in *Drosophila* formamidase, one at pH 6.7 and one at pH 7.4.

Both formamidases are inhibited at substrate concentrations greater than 2 mM. This apparent substrate inhibition might result from impurities in the commercial preparation of *N*-formyl-L-kynurenine. One likely contaminant is kynurenine itself since the conversion of *N*-formyl kynurenine to kynurenine can take place spontaneously. However, Kimmel [8] has reported, and we have confirmed, that kynurenine (0.2 mM) does not inhibit formamidase activity. The apparent K_m of formamidase I and II are $5 \cdot 10^{-3}$ and $0.83 \cdot 10^{-3}$ M respectively, based on the double reciprocal plots of Lineweaver and Burke [9]. It is worth noting that strong substrate inhibition may have the effect of distorting the double reciprocal plot and may lead to inaccuracy in the calculation of K_m values.

The molecular weights of formamidase I and II were determined by Sephadex G-75 chromatography as shown in Fig. 4. The estimated molecular weights are 60 000 and 31 000, respectively. These values raise the possibility that formamidase II is an active subunit of formamidase I. It is relevant to note that when Baily and Wagner [2] sized their purified chicken formamidase by Sephadex gel filtration they obtained a value of 59 000. When they performed the sizing experiment on sodium dodecyl sulfate electrophoretic gels, however, they found a value of 30 000. This suggests the existence of subunits. However, under the conditions we have used for separation of formamidase I and II the enzymes do not spontaneously interconvert. If free association or disassociation can take place, then the separated peaks from DEAE-cellulose, when run individually on Sephadex G-75, should show two peaks. One should correspond to the monomer and one to the dimer. Fig. 5 shows chromatography of the two forms after separation on DEAE-cellulose. Separated formamidase I shows no disassociation to formamidase II, nor does formamidase II combine to give a

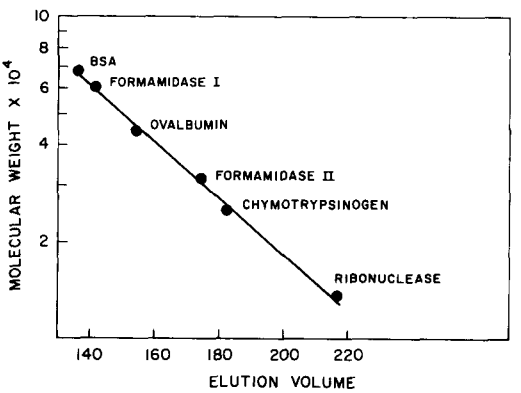

Fig. 4. Molecular weight estimations of formamidase I and II using gel filtration on a standardized Sephadex G-75 column. Standards were obtained from Pharmacia. Separated formamidases were run individually.

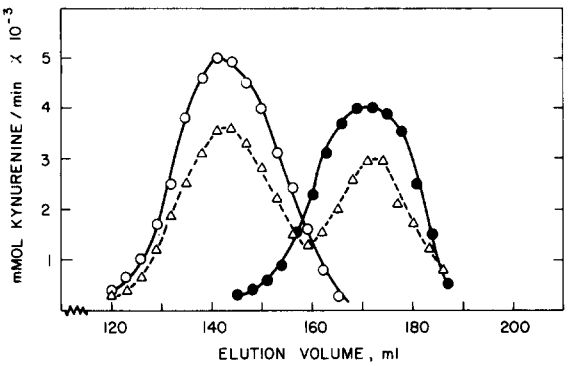

Fig. 5. Sephadex G-75 chromatography of formamidase I and II following separation by DEAE-cellulose ion-exchange chromatography. Open circles are formamidase I. Closed circles are formamidase II. Triangles are separated formamidase I and II recombined and then subjected to gel filtration.

peak corresponding to formamidase I. Similarly, when the enzymes were separated on Sephadex G-75, then rechromatographed on Sephadex G-75, no interconversion was observed. This indicates that the two forms of formamidase are not freely interconvertible.

Fig. 6 shows the thermal inactivation profile of the separated enzymes after preparation similar to that outlined in Table I. Note that each separated and partially purified enzyme inactivates in a linear fashion as opposed to the broken line seen in Fig. 3. This again indicates lack of interconvertibility since if the enzymes could interconvert a broken line would be expected after separation and thermal inactivation. The rate of inactivation of the separated enzymes is somewhat more rapid than that of the crude preparation seen before (this is especially true of formamidase II). This may be due to removal of some stabilizing agent during purification, or the two forms of formamidase may stabilize each other. Alternatively the lower protein concentration of the partially purified preparation may have an effect on the rate of thermal inactivation.

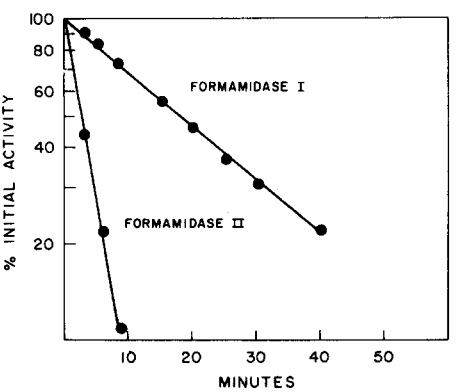

Fig. 6. Thermal inactivation profiles of formamidase I and II after separation by gel filtration.

Formamidase content of other organisms

A number of organisms were examined to determine whether the existence of multiple forms of formamidase is restricted to *D. melanogaster* or is of general occurrence. Chromatography on a standardized Sephadex G-75 column was used to determine the molecular weight of formamidase from each organism. The results presented in Table II show that every organism tested, with the exception of the *Drosophila* species, has only one molecular weight form of formamidase. Bovine and adult chicken liver extracts, and whole extracts of yeast and housefly each have one formamidase of molecular weight close to 60 000. Frog and mouse liver extracts each have one formamidase of molecular weight 30 000 and 36 000, respectively. *D. virilis* is similar to *D. melanogaster* in having two forms of formamidase, one of molecular weight 36 000 and one of molecular weight 60 000. Thermal inactivation profiles of mouse and frog extracts were linear, confirming the absence of multiple forms. In all the organisms tested, the peak from Sephadex G-75 was symmetrical, did not show any shoulder or give any evidence for heterogeneity.

The regular variation in molecular weight of this enzyme from species to species is quite striking. There is no obvious evolutionary basis for the observed differences in formamidase molecular weight since such diverse organisms as cow, chicken, yeast and housefly all have formamidase close to 60 000, while the phylogenetically similar mouse and cow have formamidase of very different molecular weight. Also the housefly is different from the two *Drosophila* species (all members of class Diptera) in that the housefly has the 60 000 molecular weight enzyme only, while the *Drosophila* species have both the large and the small enzymes.

One model which can explain the pattern of formamidase molecular weight variation from species to species is as follows: A 30 000 molecular weight molecule contains an amino acid sequence involved in the subunit interaction necessary for non-reversible dimerization to a 60 000 molecular weight enzyme. In mouse and frog this sequence is not present so that dimerization does not occur. This implies that the sequence involved with subunit interac-

TABLE II

MOLECULAR WEIGHTS OF THE KYNURENINE FORMAMIDASE

Molecular weights were determined by analysing a 100000 × *g* supernatant of each organism on a Sephadex G-75 column. The elution volume was determined and compared to a standard curve of the log of the molecular weight plotted against elution volume (see Fig. 4).

Source	Formamidase I	Formamidase II
D. melanogaster	60000	31000
D. virilis	60000	36500
Bovine liver	59000	—
Yeast	58500	—
Chicken liver	59000	—
M. domestica (housefly)	60000	—
Rana pipiens liver	—	30500
Mouse liver	—	36000

tion is not associated with the active site so that the monomer is enzymologically active. During the evolution of *Drosophilids*, gene duplication and subsequent divergence of one gene has resulted in the occurrence of both the non-dimerizing monomer (formamidase II) and the non-disassociating dimer (formamidase I). Purification and amino acid analysis of formamidase I and II may help clarify the biochemical relationship between the two. This work, as well as genetic analysis to determine the map position of the genes for each of the forms is now in progress.

This model implies that the two forms of formamidase in *Drosophila* are not biochemical derivatives of one another, that is, subunits or otherwise epigenetically modified. This is supported by our failure to observe interconvertability of the enzymes. In addition, if epigenetic modification were responsible for the biochemical difference between formamidase I and II we have reasoned that some compartmentalization with respect to formamidase should exist in order to account for the presence of substantial quantities of both forms. It seems reasonable to expect that if all of the enzyme content of a cell were exposed to the same modifying factors then one form should exist in substantial excess over the other. We have previously shown that all formamidase activity is in the soluble fraction in differential centrifugation experiments [10]. Therefore, no subcellular compartmentalization is evident. We investigated the possibility of differential tissue localization by examining the formamidase content of isolated fat body which is the principle tissue of kynurenine synthesis [11]. Larvae were minced and fat body floated by centrifugation in 0.162 M sucrose containing 0.02 M phosphate buffer (pH 6.8). The resultant preparation was examined microscopically and appeared to contain only pieces of fat body. The isolated fat body was homogenized, centrifuged and the extract chromatographed on DEAE-cellulose with stepwise NaCl elution as in Fig. 2. 70% of the activity eluted at 0.1 M, corresponding to formamidase II and 30% eluted at 0.15 M NaCl, corresponding to formamidase I. The total yield was close to 100%. The existence of significant amounts of both forms of formamidase in fat body suggests that tissue compartmentalization is not the basis for the existence of two forms of formamidase. We have previously mentioned that the relative amounts of the two forms do not change appreciably during development. The absence of any compartmentalization of formamidase coupled with the existence of about equal amounts of both forms suggests that one form is not a derivative of the other.

Our data do not rule out the possibility that only one of the enzymes is involved with the conversion of *N*-formyl kynurenine to kynurenine in vivo. The other could be an enzyme not normally involved with this conversion in vivo, but able to catalyze the reaction in vitro. We do not favor this interpretation for two reasons. Table II shows that organisms other than *Drosophila* have formamidase of molecular weight either 60 000 or about 30 000. If one of the forms in *Drosophila* were not involved with kynurenine synthesis, it would be a remarkable coincidence if it were to have a molecular weight so similar to one of the two categories of formamidase seen in other organisms.

A second indication that both *Drosophila* enzymes convert *N*-formyl kynurenine to kynurenine in vivo is the lack of a structural gene mutant for formamidase. The phenotype of such a mutant would be the bright red eye

color (due to lack of ommochrome pigment) similar to the familiar cinnabar or vermilion. This type of mutant should have been easy to detect. When one considers the large number of eye color mutants in existence, it is evident that some unusual circumstance has prevented the finding of a mutant deficient in formamidase activity. If both forms of formamidase were separate gene products and each could catalyze the hydrolysis of *N*-formyl kynurenine in vivo, two genetic mutations would be necessary to block kynurenine production at this step. This would be a rare event. The existence of two formamidase enzymes, both of which are active in kynurenine synthesis and coded for by separate genes, may explain the difficulty in finding a formamidase mutant in the existing collection of eye color variants.

Acknowledgements

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References

1 Shinohara, R. and Ishiguro, I. (1970) Biochim. Biophys. Acta 198, 324—331
2 Bailey, C. and Wagner, C. (1974) J. Biol. Chem. 249, 4439—4444
3 Glassman, E. (1956) Genetics 41, 566—574
4 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
5 Paigen, K. (1971) Enzyme Synthesis and Degradation in Mammalian Systems, p. 1—46, Karger, Basel
6 Langridge, J. (1968) J. Bacteriol. 96, 1711—1717
7 Jakoby, W. (1954) J. Biol. Chem. 207, 657—663
8 Kimmel, D. (1970) Genetics 64, s33
9 Lineweaver, H. and Burke, D. (1934) J. Am. Chem. Soc. 56, 658—666
10 Sullivan, D., Grillo, S. and Kitos, R. (1974) J. Exp. Zool 118, 225—234
11 Rizki, M. (1963) J. Cell Biol. 16, 513—520