

IDENTIFICATION OF *DROSOPHILA* v^+ HORMONE OF BACTERIAL ORIGIN*

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(Received for publication, March 24, 1941)

The production of the brown pigment component of *Drosophila* and certain other insect eyes is controlled by diffusible substances, termed eye color hormones. These are sequentially related in *Drosophila* as follows:

Precursor $\rightarrow v^+$ hormone $\rightarrow cn^+$ hormone \rightarrow brown pigment

The properties of these eye color hormones and methods of testing them in *Drosophila* and other insects have been described by several workers (1-4). All the available evidence agrees in indicating their amino acid-like nature. It has been found (5) that certain bacteria can synthesize a substance which is active in *Drosophila* in replacing the first hormone in the series, the v^+ hormone. This substance is formed from tryptophane and has been isolated in pure crystalline form (6). During the time our investigation of the structure of this active substance was in progress, a note by Butenandt, Weidel, and Becker (7) appeared. These authors, following the lead offered by the bacterial synthesis from tryptophane, had systematically tested all known intermediates in tryptophane metabolism and found that *l*-kynurenine had v^+ hormone activity in *Drosophila* and in *Ephesia*. We have confirmed activity of *l*-kynurenine¹ in *Drosophila*. Kynurenine had

* This work was supported in part by funds granted by the Rockefeller Foundation.

¹ The authors wish to express their thanks to Dr. Clarence P. Berg, State University of Iowa, Iowa City, for his kindness in supplying the samples of *l*-kynurenine, *d*-kynurenine, *d*-tryptophane, and kynurenic acid used in this investigation.

previously been isolated and characterized by Kotake (8) who showed that it was a product of tryptophane metabolism by *Bacillus subtilis* and by mammals under certain conditions.

Our investigation of the structure of the bacterially produced substance has established that a sucrose derivative of *l*-kynurenine is formed, apparently an ester. The relation of the naturally occurring *Drosophila v⁺* hormone to kynurenine has not as yet been demonstrated, but the evidence indicates that these substances are at least very closely related. The activity of *l*-kynurenine and its production only from *l*-tryptophane make it probable that *l*-tryptophane is likewise the precursor of the *Drosophila v⁺* hormone.

Isolation of Crystalline Material—The preparation and isolation of the pure hormone of bacterial origin have been previously described (6). They involve the production of the substance by the *Bacillus* sp. grown on an agar medium containing *l*-tryptophane, sucrose, and dead yeast. The active substance was extracted from the medium with water-alcohol mixtures, and finally crystallized from hot 90 per cent ethyl alcohol. The yield is about 25 per cent of the theoretical from the *l*-tryptophane added.

Properties of Material—The bacterially produced hormone crystallizes in fine needles from alcohol, often arranged in rosettes. It has no definite melting point, but softens and decomposes, beginning at around 140°. It is optically active. The specific rotation of a 0.74 per cent solution in water is +13.5°. The substance is easily soluble in water, and in hot 90 per cent ethyl alcohol, and can be extracted from water by normal butyl alcohol, but is insoluble in absolute alcohols and in other organic solvents. Its activity is not lost on treatment with acid but is easily destroyed with alkali. A water solution gives a strong ninhydrin reaction. These properties agree with those reported for the naturally occurring fly hormones (4), indicating the close relationship.

Elementary Composition—The elementary analyses had been provisionally interpreted (6) as indicating an empirical formula of $C_{21}H_{34}N_2O_{14}$. However, the isolation of *l*-kynurenine and sucrose after hydrolysis of the material indicates that the alternative empirical formula $C_{23}H_{32}N_2O_{14} \cdot 2H_2O$ is correct. The material was dried in a high vacuum at 56° and analyzed.

$C_{23}H_{32}N_2O_{14} \cdot 2H_2O$.	Calculated.	C 46.31, H 6.08, N 4.70
	Found.	" 46.77, " 6.40, " 5.28 (Dumas)
		" 46.49, " 6.39, " 5.11
		" 4.78 (ter Meulen) ²

That this formula and not a multiple thereof is correct was shown by determining the rate of diffusion through agar blocks, as previously described (4), the concentration in the different blocks being measured by the biological test method.

Hydrolysis with Alkali—Kynurenine is decomposed by hydrolysis with weak alkali with the production of NH_3 . The bacterial hormone was inactivated on treatment with hot alkali. The theoretical yield of NH_4OH was obtained on hydrolysis of the material with 0.01 N NaOH.

4.81 mg. gave 0.625 cc. 0.0136 N NH_4OH
 4.81 " " 0.622 " 0.0136 " "

$C_{23}H_{32}N_2O_{14} \cdot 2H_2O$. Calculated, N 2.35; found, N 2.48, N 2.47

Hydrolysis of kynurenine with stronger alkali causes a more complete decomposition in which *o*-aminoacetophenone is produced. This is also true of the bacterial hormone on treatment with hot 1 N NaOH. The *o*-aminoacetophenone, which is easily distinguished by its jasmine-like odor, was extracted from the hydrolyzed product with ether, dried in a vacuum, and the acetyl derivative made by treatment of the residue with acetic anhydride. The derivative was crystallized from alcohol. The acetyl derivative melted at 73° (uncorrected). The reported melting point is 75°.

Acid Hydrolysis—Kynurenine is stable towards acid hydrolysis, and forms an easily crystallizable sulfate. The bacterial product retains its activity on acid hydrolysis, but the sugar residue is removed. The kynurenine may be easily isolated as follows: A hot saturated solution of the bacterial compound in 90 per cent alcohol is made acid with 1 N H_2SO_4 and the solution kept hot for a few minutes. On cooling, kynurenine sulfate crystallizes out. This material darkened at 165° and blackened completely at 180° without melting. The recrystallized material was dried at 80° for 12 hours and analyzed.

² The authors are indebted to Dr. C. B. van Niel for carrying out this determination as well as for many helpful suggestions and criticisms.

$C_{11}H_{10}N_2O_3 \cdot H_2SO_4 \cdot 2H_2O$.	Calculated.	C 37.39,	H 4.56,	N 7.93
	Found.	" 37.78,	" 5.25,	" 8.02
		" 37.80,	" 5.17	

The bromine addition product was made by adding dilute bromine water to a water solution of the sulfate. The product was recrystallized from 50 per cent alcohol. It melted at 206–207° (decomposition). The melting point of the bromine compound made from authentic *l*-kynurenine was the same, as was that of a mixture of the two preparations. The recrystallized bromine derivative was dried at 100° for 24 hours and analyzed.

$C_{11}H_{10}N_2O_3Br_2 \cdot 2H_2O$.	Calculated.	C 31.90,	H 3.41,	N 6.77
	Found.	" 31.69,	" 2.97,	" 7.51, 7.68

Identification of Sucrose—A solution of the bacterial hormone reduces Fehling's solution only after acid hydrolysis. Estimation of the sugar as sucrose by determining the reducing power after hydrolysis gave the correct theoretical amount.

$C_{23}H_{32}N_2O_{14} \cdot 2H_2O$.	Calculated.	Sucrose 57.3,
	Found.	" 58.7, 58.4

On hydrolysis with 0.01 N NaOH the sucrose is split from the molecule without further hydrolysis. The kynurenine is decomposed by the alkali and its decomposition products were removed from acid solution with butyl alcohol. The sugar concentration in the remaining solution was estimated to be 0.278 per cent by determining the reducing power on an aliquot after acid hydrolysis. The specific rotation of the sugar solution before, +67.2°, and after inversion, -21.5°, proved that it contained sucrose. The values for pure sucrose are +66.5° and -19.84°, respectively.

It was then found that a short treatment with 0.1 N acid in 90 per cent alcohol solution caused a splitting of the molecule into sucrose and kynurenine without leading to further hydrolysis of the sucrose. After the alcoholic solution was cooled and the crystallized kynurenine sulfate removed, the addition of a little absolute alcohol and ether cause the crystallization of sucrose from the solution. It was dried and analyzed.

$C_{12}H_{22}O_{11}$.	Calculated,	C 42.1,	H 6.5;	found,	C 42.0,	H 6.6
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Constitution of Intact Molecule—After the isolation and identification of both hydrolytic products of the bacterial hormone, *l*-

kynurenine and sucrose, there is left only the question of how the two components are combined. No definitive information is available regarding the point of attachment on the sucrose molecule. Neither carboxyl group can be titrated in water solution. This eliminates a possible linkage of the sugar through an amino group. Only 1 equivalent of aqueous alkali is required for titration in 95 per cent alcohol solution with phenolphthalein. This shows that only one carboxyl group is in equilibrium with a charged basic group, and indicates that the second carboxyl group is not free. It therefore seems probable that the sucrose is esterified with one of the carboxyl groups of kynurenine.

Biological Aspects of Kynurenine Production and Specificity—The production of the active substance by the bacteria is strictly limited to its formation from *l*-tryptophane. A number of related substances have been tested for their ability to replace *l*-tryptophane but all these were ineffective. They include indole, skatole, indoleacetic acid, indolepropionic acid, tryptamine, and *d*-tryptophane. The results showed that only *l*-tryptophane can be used for the bacterial synthesis.

As pointed out previously (5) this synthesis takes place from *l*-tryptophane only under aerobic conditions. It has also been found that it takes place only in the presence of an excess of carbohydrate, glucose or, preferably, sucrose. Presumably in the absence of carbohydrate, the kynurenine formed is further oxidized by the bacteria.

The biological activity in *Drosophila* is also quite specific. *d*-Kynurenine is inactive. Kynurenic acid is also inactive as either the v^+ or the cn^+ hormone.

The absolute activity of the various compounds of *l*-kynurenine has been determined by injection of solutions into vermilion-brown larvae.³ The results were as follows:

Substance tested	Activity* units per mM $\times 10^6$
Known kynurenine.....	9
Kynurenine-sucrose derivative.....	12
Kynurenine sulfate from bacterial derivative.....	13

* See Tatum and Beadle (4).

³ For details of testing and for definition of the unit of activity see Tatum and Beadle (4).

It should be pointed out that the unavoidable experimental errors in the biological tests make this agreement quite acceptable.

SUMMARY

The substance produced by certain bacteria from *l*-tryptophane and which possesses *v⁺* hormone activity in *Drosophila* has been found to be a sucrose ester of *l*-kynurenine. The *l*-kynurenine is the essential active portion of the molecule. *l*-Kynurenine, *l*-kynurenine sulfate, and the *l*-kynurenine-sucrose derivative have the same molar activity when tested in *Drosophila* larvae. This value approximates 12×10^6 units per mm.

BIBLIOGRAPHY

1. Beadle, G. W., and Ephrussi, B., *J. Genetics*, **22**, 76 (1937).
2. Ephrussi, B., *Am. Naturalist*, **72**, 5 (1938).
3. Plagge, E., and Becker, E., *Biol. Zentr.*, **58**, 231 (1938).
4. Tatum, E. L., and Beadle, G. W., *J. Gen. Physiol.*, **22**, 239 (1938).
5. Tatum, E. L., *Proc. Nat. Acad. Sc.*, **25**, 486 (1939).
6. Tatum, E. L., and Beadle, G. W., *Science*, **91**, 458 (1940).
7. Butenandt, A., Weidel, W., and Becker, E., *Naturwissenschaften*, **28**, 63 (1940).
8. Kotake, Y., *Ergebn. Physiol.*, **37**, 245 (1935).