The Structure of the Yellow Pigment from Drosophila

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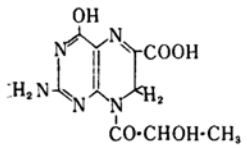
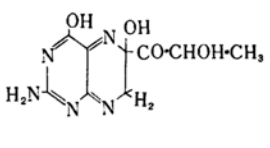
It has been known that the wild type eye-color of *Drosophila melanogaster* is due to the presence of two pigments, the red and the brown. It is of particular interest to study the chemistry of the eye pigments in order to clarify the relationship between genes and biochemical processes, since the various genetic interrelationships are well established in the group of eye-color mutants of *Drosophila*. Hadorn and Mitchell[1] have mentioned for the first time the presence of a yellow pigment, which was later named Sepiapterin by Ziegler-Günder and Hadorn[2], in *Drosophila* by a simple chromatographic technique and that the mutant *sepia* contained much greater amounts of Sepiapterin in comparison with the wild type. In 1954 Nawa and Taira[3] have reported that the yellow pigment, Xanthopterin-B[4], found in the epidermis of the mutant *lemon* of silkworm is a derivative of 2-amino-4-hydroxypteridine and is indistinguishable from Sepiapterin. In the same year Forrest and Mitchell[5] have isolated Sepiapterin as a crystalline form and proposed the structure I for it, although I was later modified to structure II by Forrest et al.[6] In the light of some new experiments on Sepiapterin, however, its structure will be discussed in this paper. And we now wish to propose the structure III for Sepiapterin on the basis of the following findings.



I



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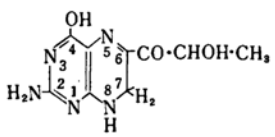

III

Experimental

Isolation of Sepiapterin. — One handred grams (wet) of flies (*D. melanogaster*, mutant *sepia*) were homogenized in a Waring Blender in 30% ethanol (500 ml.). The suspension was heated at 80°C for 5 min. and allowed to stand overnight in an ice box. The reddish yellow supernatant obtained by centrifugation was concentrated in vacuo to a small volume (about 50 ml.) and then spread on 8 sheets (40×40 cm.) of heavy filter paper (Toyo Roshi Co. Ltd., No. 26). These were then chromatographed with water. The yellow band was cut off and eluted with water. The eluate was then concentrated and chromatographed again on the heavy filter paper (4 sheets) with a mixture of *n*-butanol, acetic acid and water (4 : 1 : 1). After allowing the chromatogram to dry, the appropriate band corresponding to Sepiapterin was eluted with water. The concentrated solution was applied to a column (6×40 cm.) of cellulose powder and developed with water. The fraction of yellow pigment was collected and evaporated to dryness in vacuo. The residue was dissolved in hot water (8 ml.) and allowed to stand overnight in an ice box, when the product (13 mg.) crystallized as orange needles. All the above procedure was carried out in the dark to avoid photolysis. Eight handred gram of flies gave 105 mg. of crystals in repetition of this procedure.

Found: C, 45.9; H, 4.5; N, 29.2. Calcd. for $C_9H_{11}N_5O_3$: C, 45.6; H, 4.7; N, 29.5%.

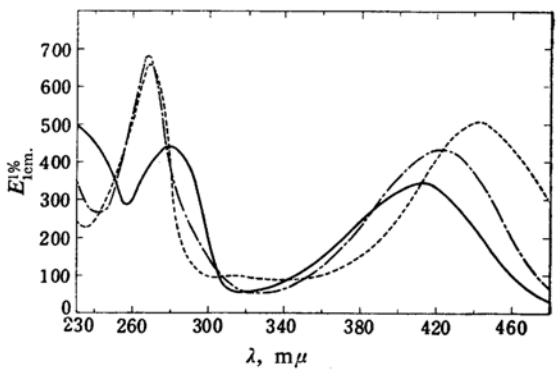

Fig. 1. The ultraviolet absorption of Sepiapterin: ——, at pH 1.0; — — —, at pH 5.0; and – – –, at pH 11.0.

1) E. Hadorn and H. K. Mitchell, *Proc. Nat. Acad. Sci. U. S.*, 37, 650 (1951).
2) Ziegler-Günder and E. Hadorn, *Z. Vererbungslehre*, 89, 235 (1958).
3) S. Nawa and T. Taira, *Proc. Japan Acad.*, 30, 632 (1954).
4) Y. Hirata, K. Nakanishi and H. Kikkawa, This Bulletin, 23, 76 (1950).
5) H. S. Forrest and H. K. Mitchell, *J. Am. Chem. Soc.*, 76, 5656, 5658 (1954).
6) H. S. Forrest, D. Hatfield and C. V. Baalen, *Nature*, 183, 1269 (1959).

The ultraviolet absorption spectra are shown in Fig. 1. (At pH 1, λ_{max} 281, 410 mμ, $E^{1\%}_{1cm.}$ 440, 340. At pH 11, λ_{max} 268, 440 mμ, $E^{1\%}_{1cm.}$ 665. 510.)

Air Oxidation in Borax Solution. — Sepiapterin (25 mg.) was dissolved in 5% borax (50 ml.) and shaken at 35°C for 5 hr. in the dark to avoid photodecomposition, when the yellow color of the solution gradually disappeared and the yellow fluorescence changed to green.

a) Estimation of 7,8-Dihydroxanthopterin (IV). —When this reaction mixture was allowed to stand overnight in an ice box, the product (14.6 mg.) precipitated. The compound was recrystallized from 0.25 N sulfuric acid as the sulfate (10.7 mg.).

Found: C, 30.0; H, 3.9; N, 29.2. Calcd. for $C_6H_7N_5O_2 \cdot 1/2H_2SO_4 \cdot H_2O$: C, 29.0; H, 4.0; N, 28.2%.

The ultraviolet absorption curves as shown in Fig. 2a. (At pH 1, λ_{max} 276, 305 mμ, $E^{1\%}_{1cm.}$, 535, 310. At pH 11, λ_{max} 276 mμ, $E^{1\%}_{1cm.}$ 515.) This compound was readily oxidized to xanthopterin (V) with manganese dioxide in alkaline solution. In neutral solution, moreover, the compound was oxidized to V accompanied by consumption of one mole of iodine. These properties, absorption spectra and R_f values of the compound are perfectly identical with those of the authentic 7,8-dihydroxanthopterin (IV)[7-9]. Some R_f values compared with those for authentic IV (in parentheses) are: *n*-propanol, 1% ammonia (2:1), 0.16 (0.16); 2% K_2HPO_4, 0.32 (0.32), 3% sodium citrate, 0.35 (0.35). The absorption curves of IV obtained from the catalytic hydrogenation of xanthopterin (Nutritional Biochemicals Co., U. S. A.) are shown in Fig. 2b.

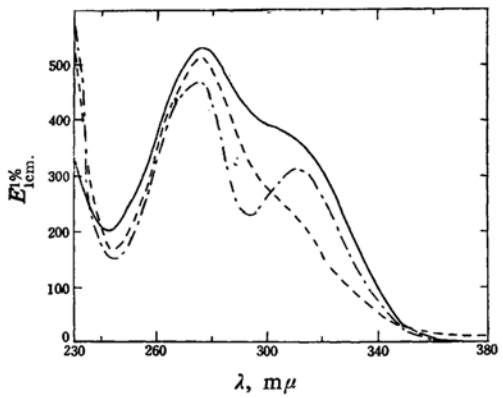

Fig. 2a. The ultraviolet absorption of the product from decomposition of Sepiapterin: ——, at pH 1.0; ——·——, at pH 5.0; and - - -, at pH 11.0.

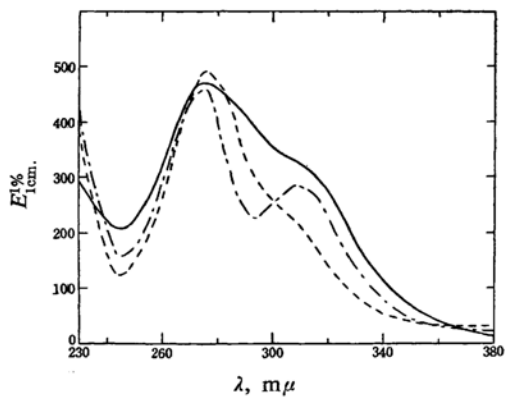

Fig. 2b. The ultraviolet absorption of 7,8-dihydroxanthopterin (IV) obtained from catalytic reduction of xanthopterin: ——, at pH 1.0; ——·——, at pH 5.0; and - - -, at pH 11.0.

b) Characterization of Lactic Acid. — The supernatant from the reaction mixture mentioned above showed the color reaction[10] characteristic of lactic acid. The supernatant (0.01 ml.) was withdrawn and heated at 100°C for 5 min. with 3 ml. of sulfuric acid and 0.05 ml. of 2% $CuSO_4$. After cooling,, 0.05 ml. of 1.5% *p*-hydroxydiphenyl reagent was added and allowed to stand at 30°C for 30 min. And then the color developed was estimated at 570 mμ in a Beckman spectrophotometer. From a standard curve obtained from known amounts of lithium lactate, 5 μg. of Sepiapterin (0.01 ml. of the reaction mixture) was estimated to give 1.5 μg. of lactic acid (with a molecular weight of 237, the theory for one mole of lactic acid per mole of Sepiapterin is 1.57 μg.). The remained supernatant was concentrated to a small volume and paper chromatographed in various solvent systems. Some R_f values compared with those for lactic acid (in parentheses) are: *n*-butanol, acetic acid, water (4:1:1), 0.75 (0.75); ethanol,10% ammonia (4:1), 0.77 (0.77); *n*-butanol, pyridine, water (1:1:1), 0.39 (0.39).

c) Relationship of Oxygen Uptake, and IV and Lactic Acid Production. — Sepiapterin (4.7 mg.) was dissolved in 5% borax solution (20 ml.) and each aliquots (2 ml., 0.47 mg.) were shaken in Warburg flasks at 35°C in the dark. At various

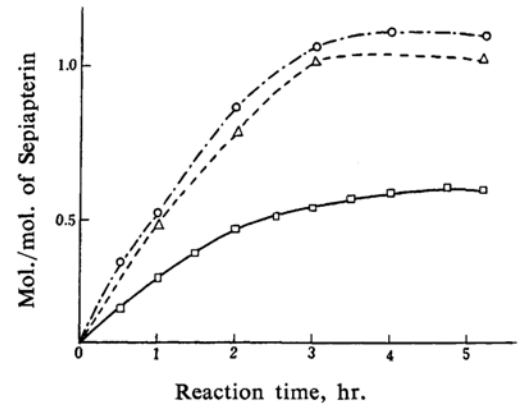

Fig. 3. Air oxidation of Sepiapterin in the presenc of borax: -□-, oxygen consumed; -○-, lactic acid produced; and -△-, 7,8-dihydroxanthopterin produced.

7) B. L. O'Dell, J. M. Vandenbelt, E. S. Bloom and J. J. Pfiffner, *J. Am. Chem. Soc.*, 69, 250 (1947).
8) W. R. Boon and T. Leigh, *J. Chem. Soc.*, 1951 1497.
9) G. B. Elion and G. H. Hitchings, *J. Am. Chem. Soc.*, 74, 3877 (1952).
10) S. B. Barker and W. H. Summerson, *J. Biol. Chem.*, 138, 535 (1941).

time intervals, oxygen uptake was measured, the amounts of IV produced were calculated using the known extinction constant at 310 mμ at pH 7 and the amounts of lactic acid were determined by the procedure described previously. As shown in Fig. 3, the molar ratio of oxygen consumed, and IV and lactic acid produced was 0.5 : 1 : 1 at all reaction intervals. This reaction did not occur in the absence of oxygen. It should be noted that the rate of reaction is variable. Since the reaction is accerelated by addition of small amounts of heavy metal ions and inhibited by chelating agents, it is concluded that the variable rate is due to the presence of metal-ion impurities.

Periodate Oxidation.—A solution of Sepiapterin (2.0 mg.) in 2% sodium bicarbonate (1.2 ml.) was treated with 1% sodium metaperiodate (0.8 ml.) in an ice bath. At various intervals the undestroyed periodate was estimated. Aliquots (0.1 ml.) were withdrawn and quenched in 0.001 N sodium arsenite (1.0 ml.) containing potassium iodide (10 mg.). After 15 min. the samples were titrated with standard potassium iodide-iodine (0.002 N) to a starch end-point. Acetaldehyde was determined by the color development[11] with *p*-hydroxydiphenyl in sulfuric acid. To aliquots (0.01 ml.) was added 0.1% sodium arsenite (0.5 ml.). After 15 min. the solution was treated with sulfuric acid (3 ml.), 2% $CuSO_4$ (0.05 ml.) and 1.5% *p*-hydroxydiphenyl reagent (0.05 ml.) at 30°C for 30 min. The color developed was estimated at 570 mμ. As shown in Fig. 4, the

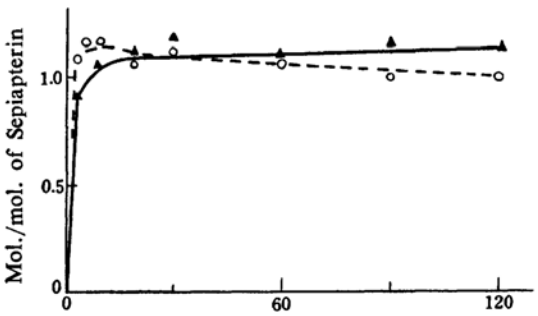

Fig. 4. Periodate oxidation of Sepiapterin: -▲-, periodate consumed; --○--, acetaldehyde produced.

curves of the acetaldehyde production and periodate consumption reached to plateaus as soon as periodate was added to the solution of Sepiapterin. In the reaction the yellow color of the solution rapidly disappeared and the yellow fluorescence changed to green.

2-Amino-4-hydroxy-7, 8-dihydropteridine-6-carboxylic Acid (VI).—To a solution of Sepiapterin (20 mg.) in 2% sodium bicarbonate (12 ml.), 1% sodium metaperiodate (8 ml.) was added. After standing 30 min. at 0°C, the reaction mixture was diluted with cold water (20 ml.), applied immediately to a column (5×40 cm.) of cellulose powder and developed with 0.002% ammonia. During the development of the column, the fluorescent products were separated into two bands, major greenish yellow and minor blue fluorescent. The minor blue fluorescent compound (1.6 mg.) was 2-amino-4-hydroxypteridine-6-carboxylic acid (VII) with identity of ultraviolet absorption and its paper chromatographic behavior. The main greenish yellow fluorescent band was collected and a pale greyish residue (7.4 mg.) was obtained by freeze-drying. For convenience, this compound is henceforth referred to as Compound-A.

Found: C, 40.78; H, 3.21; N, 32.75. Calcd. for $C_7H_7N_5O_3$: C, 40.19; H, 3.37; N, 33.48%.

The ultraviolet absorption spectra are shown in Fig. 5. (At pH 1, λ_{max} 280, 380 mμ. At pH 11,

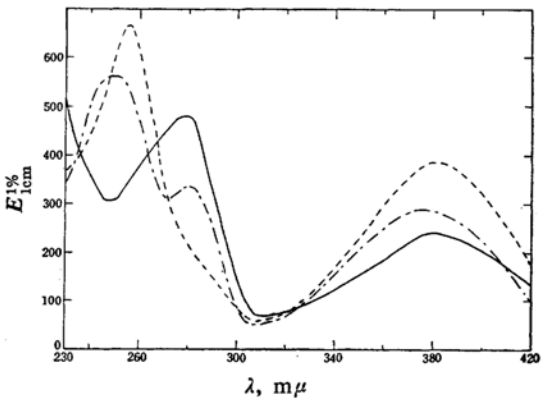

Fig. 5. The ultraviolet absorption of pteridine (VI) obtained from periodate oxidation of Sepiapterin: ——, at pH 1.0; — — —, at pH 5.0; and - - -, at pH 11.0.

λ_{max} 255, 380 mμ.) Some R_f values compared with those for VII (in brackets) are: *n*-propanol, 1% ammonia (2 : 1), 0.11 (0.14); *n*-butanol, acetic acid, water (4 : 1 : 1), 0.09 (0.14); 3% sodium citrate. 0.37 (0.53); 5% acetic acid, 0.30 (0.47); 2% K_2HPO_4, 0.35 (0.44). Compound-A was comparatively stable to auto-oxidation in neutral solution when stored at 0°C in the dark, but it was readily converted to VII on exposure to light and at that time consumed 0.5 mol. of oxgen. In alkaline solution Compound-A was oxidized into VII with manganese dioxide. In neutral or acidic solution, furthermore, Compound-A consumed 1 mol. of iodine and gave VII. From these results, it is concluded that Compound-A is a dihydro-form of VII, i. e. 2-amino-4-hydroxy-7, 8-dihydropteridine-6-carboxylic acid (VI). This is also confirmed by the following findings.

a) Reduction of Compound-A with Sodium Borohydride.—A solution of Compound-A (0.1 mg.) in 0.01 N acetic acid (10 ml.) was treated with sodium borohydride (1 mg.), when the greenish yellow fluorescence of the solution immediately dissappeared. Aliquots of the reaction mixture were adjusted to various pH and the absorption spectra were recorded as shown in Fig. 6. The curves are characteristic of tetrahydropteridies[7,12,13].

11) R. J. Block and D. Bolling, *J. Biol. Chem.*, **130**, 365 (1939).

12) M. Viscontini and H. R. Weilenmann, *Helv. Chim. Acta.*, **41**, 2170 (1958).

13) S. Kaufman, *J. Biol. Chem.*, **234**, 2677 (1959).

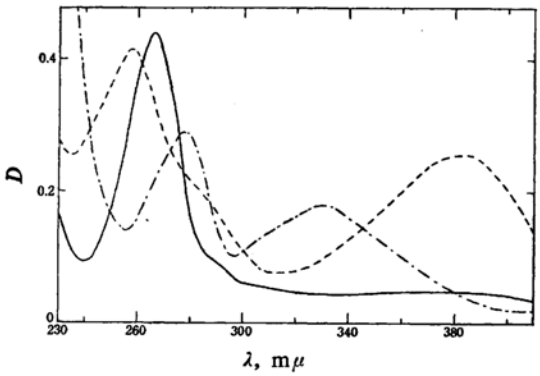

Fig. 6. The absorption of the product resulting from $NaBH_4$ reduction of VI: ——, at pH 1.0; — — — —, at pH 5; and – – –, at pH 11.0.

Upon shaking the reaction mixture in air, its fluorescence became blue. Paper chromatography of this solution showed the presence of two blue fluorescent compounds. The major spot was VII and the weaker was 2-amino-4-hydroxypteridine (VIII). Neither VII nor VIII was attacked by $NaBH_4$.

b) Conversion of Compound-A to 2, 6-Diamino-4-hydroxypteridine (IX).—A solution of compound-A (0.5 mg.) in 1% ammonia (1 ml.) was heated at 80°C for 30 min. The reaction mixture was applied to a column of cellulose powder and developed with 0.002% ammonia. The main bright green fluorescent band was eluted. The absorption spectra of this eluate in various pH (Fig. 7) correspond to these of 2,6-diamino-4-hydroxypteridine (IX)[14], which were obtained from the catalytic reduction of VIII in dilute ammonia, and the identity of this compound was confirmed by paper chromatographic comparison with the authentic sample. When the compound was incubated with milk xanthine oxidase at pH 8, it was oxidized to a purplish blue

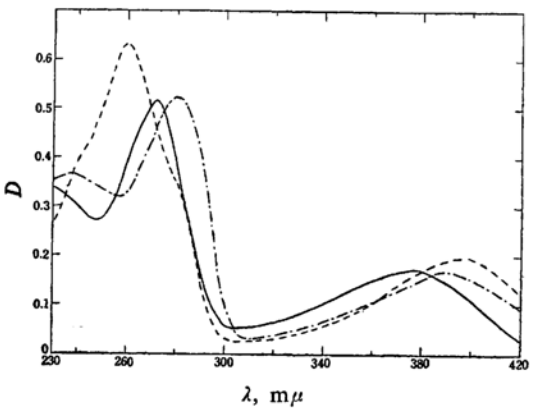

Fig. 7. The absorption of pteridine (IX) obtained from VI by treating with ammonia: ——, at pH 1.0; — — — —, at pH 5.0; and – – –, at pH 11.0.

14) C. V. Baalen and H. S. Forrest, *J. Am. Chem. Soc.*, **81**, 1770 (1959).

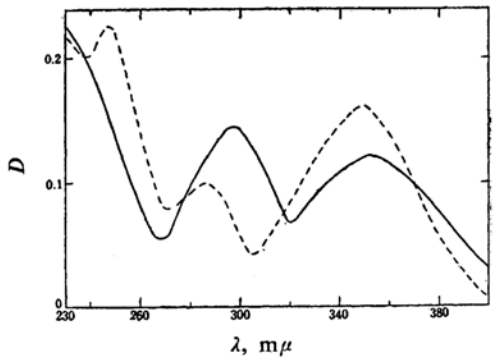

Fig. 8. The absorption of pteridine (X) obtained from IX by treatment with milk xanthine oxidase: ——, at pH 1.0; – – –, at pH 11.0.

fluorescent substance. Its absorption spectra (Fig. 8) resemble those of isoxanthopterin (2-amino-4, 7-dihydroxypteridine)[15]. From the evidence[16,17] that milk xanthine oxidase attacks the 7-position of the pteridine skeleton, it is probable that the substance is 2,6-diamino-4,7-dihydroxypteridine(X).

Photodecomposition.—Sepiapterin (0.47 mg., 2.0 μmol.) dissolved in water (2 ml.) was exposed to sunlight in a Warburg flask at 35°C. An oxygen uptake of 50 μl. was observed, when the yellow color of the solution had disappeared. Acetaldehyde (0.086 mg., 1.95 μmol.) was ascertained by the procedure described above. VII (0.39 mg., 1.88 μmol.) was determined using the known optical density for this acid, after separating by paper chromatography.

Discussion

It has been known that both photodecomposition[3,5] and permanganate oxidation[5] of Sepiapterin gave 2-amino-4-hydroxypteridine-6-carboxylic acid (VII). This shows that Sepiapterin is a 6-substituted pteridine. In connection with the result by Forrest et al.[5], who obtained VII and acetaldehyde from periodate oxidation of Sepiapterin, the present observation that one mole of lactic acid is quantitatively obtained from decomposition of Sepiapterin suggests that the substituent is a lactyl group ($CH_3 \cdot CHOH \cdot CO-$). Since the analytical data indicate that Sepiaterin has three carbon atoms other than its pteridine skeleton, the lactyl group seems to be a sole side chain. The isolation of 7, 8-dihydroxanthopterin (IV) from air oxidation of Sepiapterin suggest whether the 6-hydroxy group of IV is an integral part of the molecule of Sepiapterin or a hydroxylation at the 6-position occurs at

15) A. Albert and H. C. S. Wood, *J. Appl. Chem.* (London), 3, 521 (1953).
16) O. H. Lowry, O. A. Bessey and E. J. Crawford, *J. Biol. Chem.*, **180**, 389 (1949).
17) F. Bergmann and H. Kwietny, *Biochem. et Biophys. Acta*, **26**, 613 (1958).

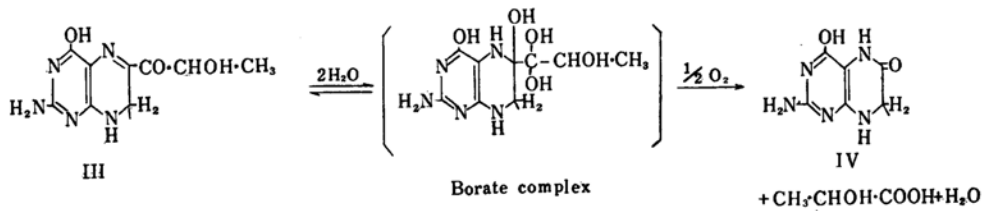

Fig. 9.

the same time as the lactyl side chain is split off. Since this reaction did not occur in the absence of oxygen (0.5 mol. of oxygen were consumed), it is sure that the side chain is split off oxidatively. In this connection, Blakley[18] has reported the production of xanthopterin in aerated solution of dihydro- or tetrahydrofolic acid. It seems reasonable to assume, therefore, that the oxygen atom in the 6-position of IV is oxidatively introduced and then Sepiapterin has no OH group in its 6-position. The finding that the decomposition of Sepiapterin give one mole each of IV and lactic acid accompanied by consumption of 0.5 mol. of oxygen, therefore, suggests that Sepiapterin has the structure 2-amino-4-hydroxy-7, 8-dihydro-6-lactylpteridine (III). From the evidence[19] that polyhydroxy compounds form borate complexes and from the experimental result that 0.5 mol. of oxygen are consumed in the reaction of sepiapterin with borax, the schema shown in Fig. 9 is postulated.

The formation of 2-amino-4-hydroxypteridine-6-carboxylic acid (VII) and acetaldehyde by periodate oxidation of Sepiapterin has been reported by Forrest et al.[5]. However, when in alkaline solution Sepiapterin was treated with periodate, it consumed one mole of periodate to give acetaldehyde and a pteridine different from VII. The pteridine seems to be a dihydro form of VII, i. e. 2-amino-4-hydroxy-7, 8-dihydropteridine-6-carboxylic acid (VI), since it is readily oxidized to VII accompanied by consumption of 0.5 mol. of oxygen or one mole of iodine. Furthermore, the finding that pteridine was converted to 2, 6-diamino-4-hydroxypteridine (IX) by treating with ammonia again supports it to be hydropteridine, in connection with the evidence[14] that the catalytic reduction of 2-amino-4-hydroxypteridine (VIII) in the presence of ammonia gave IX. In progress of our studies, Viscontini and Möhlmann[20] reported that the reduction of Sepiapterin with sodium borohydride give a tetrahydro form of biopterin (2-amino-4-hydroxy-6-(1, 2 - dihydroxypropyl)-pteridine)[21-23]. We observed that the sodium borohydride reduction of VI give a compound with a spectrum characteristic of typical tetrahydropteridine[7,12,13], although neither VII nor VIII is affected. Dihydropteridines, therefore, seems to be reduced to the corresponding tetrahydro form by the action of sodium borohydride. It was also found that Sepiapterin is reduced to a tetrahydro form with the enzyme system from chicken liver, which carries out a reaction of dihydrofolic acid to tetrahydrofolic acid. These observations confirm that Sepiapterin is a dihydropteridine. The present result that photodecomposition of Sepiapterin gave one mole each of acetaldehyde and VII accompanied by consumption of one mole of oxygen, is again in accord with the proposed structure III.

Several different pteridines[3,5,6,20,21,23] have already been isolated from *Drosophila*. It has been assumed[3,5,20] that the red pigment found in the wild type has a structure related closely to the yellow pigment (Sepiapterin) and the latter is an intermediate in the synthesis of the former. It is of particular interest that Sepiapterin is a hydropteridine, in connection with the biological significance[24] of hydrofolic acid. Recently, Kaufman[13] reported the role of hydro-form of simple pteridines in the enzymatic oxidation of phenylalanine to tyrosine. It has been, furthermore, known that the mutant *lemon* of silkworm, which accumulates the yellow pigment identical with Sepiapterin in larval epidermis, shows the inhibition of oxidation of phenolic substances in vivo. From these, it may be expected that in the insect the yellow pigment (Sepiapterin) participates in some oxidation-reduction systems, although its biological significance remains to be determined.

Summary

The yellow pigment (Sepiapterin), occuring in the eyes of *Drosophila melanogaster*,

18) R. L. Blakley, *Biochem. J.*, 65, 331 (1957).
19) J. Böeseken, *Advances in Carbohydrate Chem.*, 4, 189 (1949).
20) M. Viscontini and E. Möhlmann, *Helv. Chim. Acta*, 42, 836. 1679 (1959).
21) H. S. Forrest and H. K. Mitchell, *J. Am. Chem. Soc.*, 77, 4865 (1955).
22) E. L. Patterson, R. Milstrey and E. L. R. Stokstad, ibid., 78, 5868 (1956).
23) M. Viscontini, E. Loeser and P. Karrer, *Helv. Chim. Acta*, 41, 440 (1958).
24) R. L. Blakley, "Current Trends in Heterocyclic Chemistry", A. Albert, editor, Butterworths Scientific Publication, London (1958), p. 140.

decomposed to one mole each of 7, 8-dihydroxanthopterin and lactic acid accompanied by consumption of 0.5 mol. of oxygen. From this and from the results of its photodecomposition and periodate oxidation, it is concluded that the compound has the structure 2-amino-4-hydroxy-7, 8-dihydro-6-lactylpteridine (III).

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