tained which decomposed slightly at 85° and melted at 111–113.5°, and changed to a brown amorphous solid in two hours.

Anal. Calcd. for $C_{20}H_{16}O_4N_2$: N, 8.05. Found: N, 7.78.

Determination of Ferric Ion Consumed.—In each of two erlenmeyer flasks was pipetted 5.00 ml. of approximately 0.2 *N* ferric chloride in 10% perchloric acid solution. To one of these was added 0.0138 g. (0.079 millimole) of 3-indoleacetic acid. After 45 minutes, the solution was extracted with isobutyl alcohol to remove the colored product. The blank was treated in a similar manner. The blank and the reaction mixture were each titrated with standard (0.02 *N*) potassium dichromate solution. A difference of 0.083 milliequivalent of dichromate was found between the two solutions, corresponding to one milliequivalent of dichromate being required per millimole of 3-indoleacetic acid oxidized.

Biological Properties

The biological activity of N-hydroxy-3-indoleacetic acid and the N,N′-diindolyl-3,3′-diacetic acid in stimulating growth responses in higher plants was ascertained using 3-indoleacetic acid as a control comparison.[15] In both the induction of parthenocarpy in young tomato fruits and negative curvatures of bean seedling hypocotyls, N,N′-diindolyl-3,3′-diacetic acid was equally as active as 3-indoleacetic acid. In contrast, N-hydroxy-3-indoleacetic acid showed less than 1% of the activity characteristic of 3-indoleacetic acid.

Acknowledgment.—The authors wish to thank Dr. M. E. Speeter of the Upjohn Company, Kalamazoo, Michigan, for the infrared and ultraviolet spectral analysis of N-hydroxy-3-indoleacetic acid.

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[CONTRIBUTION FROM THE KERCKHOFF LABORATORIES OF BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

Pteridines from *Drosophila*. I. Isolation of a Yellow Pigment[1]

BY H. S. FORREST[2] AND H. K. MITCHELL

RECEIVED APRIL 7, 1954

A method is described for the isolation in pure crystalline form, of a yellow pigment occurring chiefly in the eyes of *Drosophila melanogaster* and in many times larger amounts in a mutant, *sepia*.

During the last 10–12 years, various attempts have been made to study the chemistry of the eye pigments of *Drosophila melanogaster*, not only because of their interest *per se* but also because of the possibility of providing further insight into the relationship between genes and biochemical processes. Provided some knowledge of the underlying chemistry is available, the group of eye color mutants in *Drosophila* would be very useful for such a study because the various genetic interrelationships are well established. This has been recognized, of course, in the pioneering work of Beadle, Tatum and Ephrussi and their co-workers[3] in which it was shown that kynurenine (v^+ substance) and a second compound (cn^+ substance) later shown by Butenandt[4] to be hydroxykynurenine are implicated in the synthesis of the so-called brown pigment of the eyes. Practically nothing is known, however, of the chemistry of the red pigments beyond an intimation of their possible pteridine nature[5] and a denial of even this allegation.[6]

Of the various groups which have made sporadic attempts to investigate these compounds[6-8] all have concentrated on the red pigments themselves, but these have been found to be extraordinarily difficult to handle from the chemical standpoint for three main reasons, firstly the difficulty of isolation in quantity, secondly that of separating efficiently several closely related compounds and thirdly that of their inherent instability. Thus the main outcome of these efforts has been the publication[9] of empirical formulas for various fractions of the red pigment complex, but no attempts have been made to relate these formulas to any chemical structure.

A new approach to this problem was made apparent by the discovery[10] of a simple chromatographic technique for the identification and approximate estimation of the fluorescent pigments in *Drosophila* and in various mutants thereof. By this method it was shown that a yellow pigment occurred in about 5 times greater amount in a mutant, *sepia*, than in *wild type* flies. This mutant does not produce red pigment and it seemed probable that the yellow substance is an intermediate or is related to an intermediate in the biosynthesis of the red pigments. It appeared that this compound would be easier to obtain in quantity, the difficulty of separating a number of very similar compounds would be eliminated and furthermore, a determination of its structure would be of considerable value in elucidating those of the red pigments if indeed it lay in the biochemical pathway toward them. Another point of interest lay in the early discovery that this compound was highly photosensitive, in contrast to the red pigments, and its possible importance in the visual processes of the flies was therefore appreciated.

Unfortunately these hoped for simplifications

(1) These investigations were supported by funds from the Rockefeller Foundation, the Williams-Waterman Fund for the Combat of Dietary Diseases and by funds from the Atomic Energy Commission administered through contract with the Office of Naval Research, contract No. N-6-onr-244, Task Order 5.

(2) U. S. Public Health Service Postdoctoral Fellow during part of the work described.

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were not altogether realized and fresh complications arose in the course of the work, but it is the purpose of this paper to describe various attempted methods and one successful one for purifying this yellow compound which resulted finally in its being obtained in crystalline form. In a later paper we shall describe the degradative experiments carried out in the investigation of its structure and present a tentative formula for it based on these.

The starting material for all isolation procedures was the *sepia* mutant of *Drosophila*. This was grown in relatively large quantities in $^1/_2$ pint milk bottles in the normal manner[11] and the yield per bottle from two collections on the 18th and 28th day after inoculation was about 1000 flies (1 g.). The flies were stored at −30° until required for use. The first method used on a fairly large scale to isolate the pigment was based on the solubility of the yellow pigment in aqueous methanol[6] and its precipitation from this as a silver salt. This preliminary concentration and purification was followed by chromatography on columns of powdered cellulose and "crystallization" of the purified fractions from this.

Six hundred grams of *se* flies were macerated in a Waring Blender in methanol (1.2 l.). The suspension was allowed to stand for 12 hr. and was then filtered through muslin in a wine press, and the residue was allowed to stand again for 12 hr. with 60% methanol (800 ml.). After filtration, this process was repeated two further times, thus removing most of the yellow pigment from the flies. The combined filtrates were refiltered through a bed of Hyflo Supercel and the resulting clear yellow solution was treated with silver nitrate (6–10 g.) in water (60 ml.). The red precipitate was allowed to settle and was then collected by centrifugation. It was resuspended in 50% aqueous methanol (200 ml.) and this was treated with hydrogen sulfide. The silver sulfide was removed by filtration and thoroughly washed with further quantities of 50% methanol. The combined filtrates were evaporated to dryness *in vacuo* and the residue was suspended in 5% acetic acid (300 ml.), the suspension was filtered and the filtrate, in 100-ml. lots, was passed through columns (8 × 44 cm.) of acid-washed, powdered cellulose prepared according to the method of Campbell, Work and Mellanby[12] and equilibrated with the same solvent. The crude concentrate separated into four main fluorescent bands during the development of the column, the first two being almost colorless but with intense blue and purple fluorescences, respectively, the third and fourth being yellow in color and yellow fluorescent. The main yellow band (3rd in order of elution) was collected and the solution evaporated to dryness *in vacuo*. The residue was "crystallized" from 80% ethanol (hot filtration) and the resulting micro-crystalline, yellow solid was collected by centrifugation, washed with alcohol and ether, and dried. The yield from several separate preparations was between 200 and 300 mg.

As determined by physical measurements on pure crystalline substance (see later) this material was only 50–60% pure, yet by the methods available for testing its purity (analysis, recrystallization and the effect of this on absorption spectrum, and paper chromatography) no impurities could be detected and it was therefore used in a number of the earlier degradative experiments. Several disadvantages, however, were apparent in the purification procedure. The silver salt, for instance, was very sensitive to light and rapidly turned black even in artificial light. By working through this stage very quickly and in red light this trouble was minimized but never completely eliminated. Another disadvantage lay in the adsorptive property of the silver sulfide for the yellow pigment, resulting in a constant loss at this stage. Numerous attempts were therefore made to eliminate this step in the purification completely and these were chiefly concerned with a search for suitable adsorbents for the pigment. An obvious possibility was charcoal, which has been used for the red pigments[8] but although this adsorbs the substance completely from methanol extracts, no satisfactory eluting agents could be found, *e.g.*, aqueous phenol or pyridine–acetic acid mixtures, were only very slightly effective, and even preliminary deactivation of the charcoal by boiling it with pyridine, aniline or ammonia, or by impregnation with stearic acid[13] was of no value in this process.

Other possible methods of purification were explored at this time. Ion-exchange columns (Amberlite 1R-100; Amberlite 1R-50; Dowex 50) were ineffective; chromatopiles using some of the solvents known to be effective for small scale separations, were not successful; variations in the powdered cellulose (*e.g.*, with small additions of talc, calcium carbonate, etc.) or in the solvents used in the cellulose columns gave no improvement over the method described above. However, identification of the pigment as a pteridine derivative (see following paper) suggested fresh possibilities as regards its purification. Thus Florisil (a synthetic magnesium silicate) has been used successfully in the purification of pteroyltriglutamic acid,[14] and for the preliminary isolation of flavins (benzpteridine derivatives)[15]; it was observed that it could be used as an adsorbent for the yellow pigment from 80% methanol or 6% trichloroacetic acid extracts of flies. In the latter case (*i.e.*, the 6% trichloroacetic acid extract) adsorption was quite strong and elution could be effected with water or dilute pyridine solution; but the separations achieved on chromatographic columns of Florisil were not clear cut and two additional difficulties were encountered. The first was due to the accumulation in the final product of silica washed off the column in small amounts during elution, and the second to the decomposition of the compound by the adsorbent. Silica or silicates have of course been used by previous workers in this field,[6–8] and difficulties of this type have been commented on. However, further exploration of this group of adsorbents led to attempts to use the "Filtrol" adsorbents which are essentially fuller's earths[16] and one of these "Filtrol Grade 58" has proved to be by far the most useful adsorbent yet found and has been used to obtain the pigment in crystalline form.

The "Filtrol Grade 58" was thoroughly washed by decantation (to remove most of the very fine material contained in the commercial product) with dilute acetic acid and water, and for column chromatography, an additional washing was given with dilute ammonia (1%) followed by further washings with dilute acetic acid (5%) and water. The acid washed "Filtrol" (10 g.) was added to a 6% trichloroacetic acid extract of blenderized flies (100 g.; total volume of extract after filtration and washing, 500 ml.) and after shaking for 30 min., the Filtrol was collected, washed thoroughly with water, then with alcohol and dried. Most of the yellow pigment was adsorbed from the extract by the Filtrol. The dried adsorbent was packed into a chromatographic column (3.5 cm.) on top of a further quantity (10 g.) of fresh Filtrol, and elution was immediately commenced with 50% aqueous acetone containing ammonia (0.05%). The yellow pigment was slowly washed from the column and the eluates containing it were evaporated to dryness and the residue was crystallized from dilute acetic acid to give the compound (40–50 mg.) as an orange micro-crystalline solid.

This was further purified in the following way. The partially pure material (20 mg.) was dissolved in 50% aqueous acetone (4 ml.) and this solution was applied to a column of acid and ammonia washed Filtrol (2.5 × 29 cm.) packed in the column in the same solvent. The yellow material in this solvent passed almost straight through the column and the impurities were left on it. The yellow eluate was collected, evaporated to dryness and the residue crystallized from a small volume (1.5 ml.) of water to give the compound (14 mg.) crystallizing in long tiny yellow needles. The total yield obtained by similar purification of mother liquors and less pure eluates from the first column was between 30 and 40 mg. in repetitions of this procedure. These columns must be run as quickly as is consistent with efficient separation since, again, the pigment is decomposed by contact with the adsorbent.

Physical Properties of the Pigment.—The absorption spectrum is shown in Fig. 1. In alkaline solution (0.1 *N* sodium hydroxide) there are two

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(16) Kindly supplied by the Filtrol Corporation, Los Angeles 14, California.

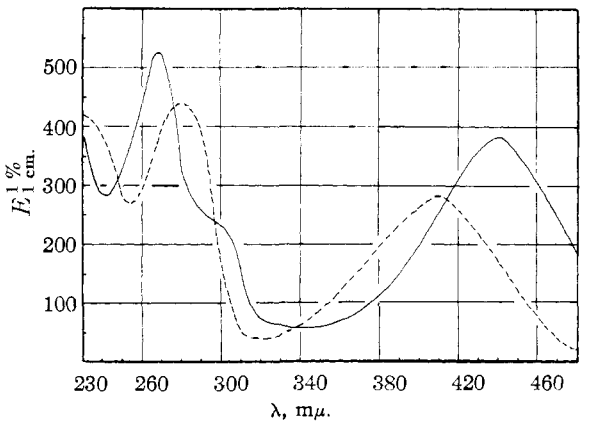

Fig. 1.—Ultraviolet absorption spectrum of the yellow pigment: —, in 0.1 *N* sodium hydroxide; ---, in 0.1 *N* hydrochloric acid.

maxima, at 268 mμ ($E^{1\%}_{1\ cm.}$ 520) and 440 mμ ($E^{1\%}_{1\ cm.}$ 340); in acid solution, there is a shift toward longer wave lengths of the 1st peak (max. at 279 mμ, $E^{1\%}_{1\ cm.}$ 446) and toward the shorter wave lengths of the visible peak (max. at 409 mμ, $E^{1\%}_{1\ cm.}$ 240). The compound has no melting point, but slowly chars on heating. No attempt was made to measure optical activity because of the difficulty of the intense color of even dilute solutions. Its R_f values in various solvents are given in Table I.

TABLE I

Solvent	R_f
n-Propanol–1% ammonia (2:1)	0.44[9]
n-Butanol–acetic acid–water (4:1:5)	.47
Water–isoamyl alcohol	.46
5% Acetic acid	.56
3% Ammonium chloride	.33

As usual with pteridines, considerable difficulty has been experienced in obtaining consistent and reliable analytical figures for carbon, hydrogen and nitrogen, an added complication in this case being the persistence of a non-combustible residue from material obtained from powdered cellulose columns. Early results therefore have given values between the following figures: C, 39–42; H, 4–6; N, 18.6–24.0. On the crystalline material described above the following results have been obtained: C, 41.7; H, 4.3; N, 24.1. This corresponds roughly to $C_{10}H_{12}N_5O_{5.5}$.

The compound is soluble in water (1 mg./ml.) and in aqueous mixtures of various organic solvents, but is insoluble in most organic solvents. The best illustration of this generalization is in its behavior in acetone in which it is completely insoluble, yet in 50% aqueous acetone it is 4 to 5 times more soluble than it is in water.

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Pteridines from *Drosophila*. II. Structure of the Yellow Pigment[1]

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RECEIVED APRIL 7, 1954

The yellow pigment, occurring principally in the eyes of *Drosophila melanogaster*, which has been isolated in pure crystalline form, has been submitted to a series of degradative experiments and it is concluded from these that the compound has the structure 2-amino-4-hydroxy-7,8-dihydro-8-lactylpteridine-6-carboxylic acid.

In an earlier paper[3] we have described the isolation in crystalline form of a yellow pigment from *Drosophila melanogaster* in which it is concentrated in the eyes and to a lesser extent in the malpighian tubules and testes. A mutant, *sepia*, was used as the source of this compound because it is accumulated in this mutant in much greater amount than in wild-type flies. Its possible significance as an intermediate in the biosynthesis of the red eye pigments of *Drosophila*, and as an important photo-receptor for these flies, has been briefly mentioned. It is the purpose of this paper to present the results of degradative experiments which have led to the tentative assignment of a structure to this compound. Many of these experiments were carried out on material of low purity (as determined by the eventual isolation of crystalline material), but the validity of the conclusions drawn from them has been checked by similar, or, in some cases, simplified experiments on the pure material.

The first clue to the chemical nature of the yellow pigment arose from a study of its photodecomposition products. On exposure to sunlight, it is very rapidly decomposed in dilute alkaline solution, a little more slowly in neutral or acid solution, the color disappearing and an intense blue ultraviolet fluorescence being produced. As revealed by paper chromatography, this fluorescence arises from two components in the solution, but the major one was identified by paper chromatography as 2-amino-4-hydroxypteridine-6-carboxylic acid (I); and this was confirmed by its purification through the disodium salt and comparison of its absorption spectrum in acid and alkaline solution with an authentic specimen of this acid prepared from pteroylglutamic acid[4] and by decarboxylation of, and purification of the products from, both the natural and synthetic acids and com-

(1) These investigations were supported by funds from the Rockefeller Foundation, the Williams-Waterman Fund for the Combat of Dietary Diseases and by funds from the Atomic Energy Commission administered through contract with the Office of Naval Research Contract No. N-6-onr-244, Task Order 5.

(2) U. S. Public Health Service Postdoctoral Fellow during part of the work described.

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