THE USE OF SEPHADEX FOR THE CONCENTRATION OF PTERIDINES*

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INTRODUCTION

The possibility of using Sephadex as a means of isolation and purification of pteridines was first suggested by ZIEGLER[1]. Working with extracts of the eyes of insects she showed that ultraviolet-absorbing substances and ninhydrin positive substances pass rapidly through columns of Sephadex G-25 (fine or medium) and that pteridines appear only in later fractions.

This technique seemed an obvious one to try in an attempt to isolate the unconjugated pteridine(s) produced by the ciliate, *Tetrahymena pyriformis*. In this case, however, the material is much less satisfactory than extracts from insects, in which the pteridine concentration, in the eyes especially, is high. On the other hand, in cultures of the ciliate, the bulk of the pteridine(s) produced is in the culture medium in relatively low concentration. While it would have been possible to concentrate cultures by immediate lyophilization it was found that preliminary passage through Sephadex G-25 (fine) not only eliminated a large part of the solids in the culture but gave some degree of concentration of the pteridine(s).

This technique has also been applied to extracts of cells of *Rhodospirillum rubrum* and of whole fruit flies (*Drosophila melanogaster*, sepia/vestigial mutant).

MATERIALS AND METHODS

Sephadex G-25 (fine) and G-10 (fine) were obtained from Pharmacia. The gels were allowed to swell in water according to the manufacturers' directions and poured into columns of various sizes. Preliminary experiments were carried out using a column 2.5 × 27 cm; total bed volume 130 ml, void volume (V_0), 50 ml. For larger cultures columns 7 × 27 cm (V_0 = 400 ml) or 4 × 65 cm (V_0 = 300 ml) were used. Elution was carried out with distilled water or with 0.1 % 2-mercaptoethanol. With the smallest column 3 ml fractions were collected using an automatic fraction collector; with the larger columns fractions were 20 ml. Void volumes were determined using Blue Dextran.

Tetrahymena pyriformis W was grown in medium A of KIDDER, DEWEY AND HEINRICH[2], in which glucose and vitamin concentrations were doubled. Low form growth flasks containing 400 ml of medium or 1 l Erlenmeyer flasks containing 60 ml were inoculated (1:200) and the culture allowed to grow in the dark. After five days

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2-mercaptoethanol was added to a concentration of 0.1% and ammonia to pH 9–10. Celite Analytical Filter Aid (about 1 g) was then added and the mixture stirred. After 15 min, this was filtered with the aid of suction through a pad of Celite on a Buchner funnel and washed with a small volume of water. In some experiments the culture was neutralized before filtration. This step appeared to make no difference in chromatographic behavior.

Essentially the same procedure was used with light-grown *Rhodospirillum rubrum* (yeast extract–acetate medium) and with *Drosophila*. In either case sufficient dilute ammonia (pH 9–10) was added to make a convenient volume with which to work. The final volume for 15–16 g wet weight of *R. rubrum* cells was 50–60 ml. For 1–2 g wet weight of flies, 15–20 ml is sufficient. To increase the efficiency of extraction of pteridines from the flies, they were ground in a mortar in dilute ammonia.

Emergence of the peaks from the column was followed by reading the fluorescence of the fractions in a Farrand Fluorometer A-2 equipped with a primary filter of 365 mμ and a secondary of 450 mμ. In some cases ultraviolet spectrophotometry at 272 mμ or 420 mμ was employed, as was biological testing, using growth responses of *Crithidia fasciculata* and *Tetrahymena pyriformis*.

All work was carried out in subdued light or under red light to minimize destruction of pteridines.

RESULTS

Preliminary trials showed that volumes up to and even slightly exceeding the void volume of a Sephadex G-25 column could be used successfully. A volume much in excess of the void volume led to smearing of the peaks. For this reason cultures of 400 ml were used for chromatography on the 7 × 27 cm column.

Chromatography of folic acid and biopterin in aqueous solution on Sephadex G-25 indicated that the two could be separated (Fig. 1). It was found, however, when folic acid (radioactive) was added to a culture filtrate just before chromatography, that it was eluted in the same fractions as the fluorescent pteridines, although folic activity (not radioactive) was detected in earlier fractions (Fig. 2). This material could consist of metabolically produced pteroylpolyglutamic acids.

In spite of this drawback, the procedure had two valuable aspects, *viz.* (1) a large amount of undesirable material could be eliminated at the same time as, (2) the pteridines were concentrated at least two fold. As Fig. 3 shows, from the original volume of 400 ml put on the column, the peak tubes for growth and fluorescence had a volume of 160 ml when combined. At the same time a large amount of ultraviolet-absorbing material was eliminated. A second chromatography on the same column (Fig. 4), or the 4 × 65 cm column, while it further decreased the combined volume of the peak fractions to 60–120 ml, eliminated less contaminating material.

On the other hand when, after lyophilization, the peak material from Sephadex G-25 was chromatographed on Sephadex G-10 (2.5 × 27 cm), partial separation of folic acid activity from the fluorescent pteridines was obtained along with elimination of ultraviolet-absorbing material (Fig. 5).

Inasmuch as the amount of pteridine to be isolated comprises only a few micrograms, this preliminary and quite rapid concentration and purification makes possible the use of the technique of REMBOLD AND BUSCHMANN[3,4] for final purification

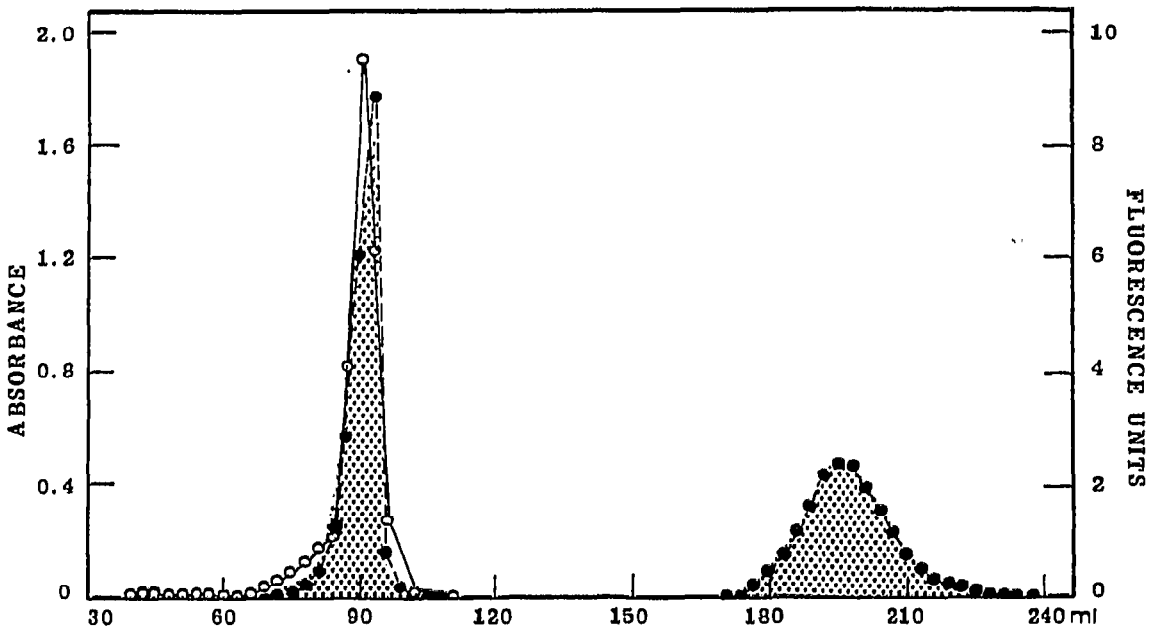

Fig. 1. Chromatography of folic acid and biopterin on Sephadex G-25 (27 × 2.5 cm) 3 ml fractions eluted with 0.1 % mercaptoethanol. Shaded peaks = fluorescence at 450 mμ. Open circles = absorbance at 272 mμ. Note the lack of correspondence of the absorbing peak at 90 ml and the fluorescent peak at 93 ml. The latter is due to a fluorescent impurity in folic acid, which is itself nonfluorescent. The second fluorescent peak is biopterin.

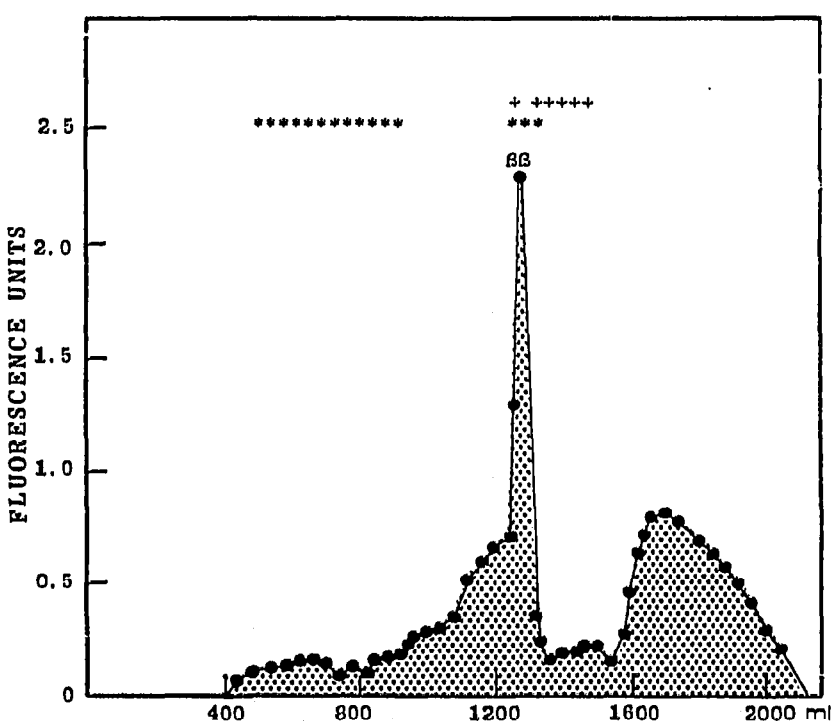

Fig. 2. 250 ml culture of *Crithidia fasciculata* chromatographed on Sephadex G-25 (27 × 7 cm), to which had been added 3 μC of folic acid 2-^{14}C immediately before it was put on the column. Elution with 0.1 % mercaptoethanol; 20 ml fractions. Solid circles = fluorescence at 450 mμ. ** = growth of *T. pyriformis* ("folic") activity. + + + = growth of *C. fasciculata* ("biopterin") activity. ββ = radioactivity.

of the material. The lyophilized fractions are brought to a neutral pH (if necessary) and applied to a small column (0.8 × 9 cm) of Serva phosphocellulose (H^+) (Fig. 6) or Dowex 1 X8 (formate) and eluted with water or water containing 0.1 % mercaptoethanol.

Possibly because bacterial folic acid is usually present in the form of polyglutamate, it is separable almost completely from the unconjugated pteridines of *Rhodospirillum rubrum* (Fig. 7). On phosphocellulose the peak fraction from Sephadex can be separated from a great deal of contaminating material, coming in the early fractions, into at least two pteridines having growth promoting activity for *Crithidia* (Fig. 8).

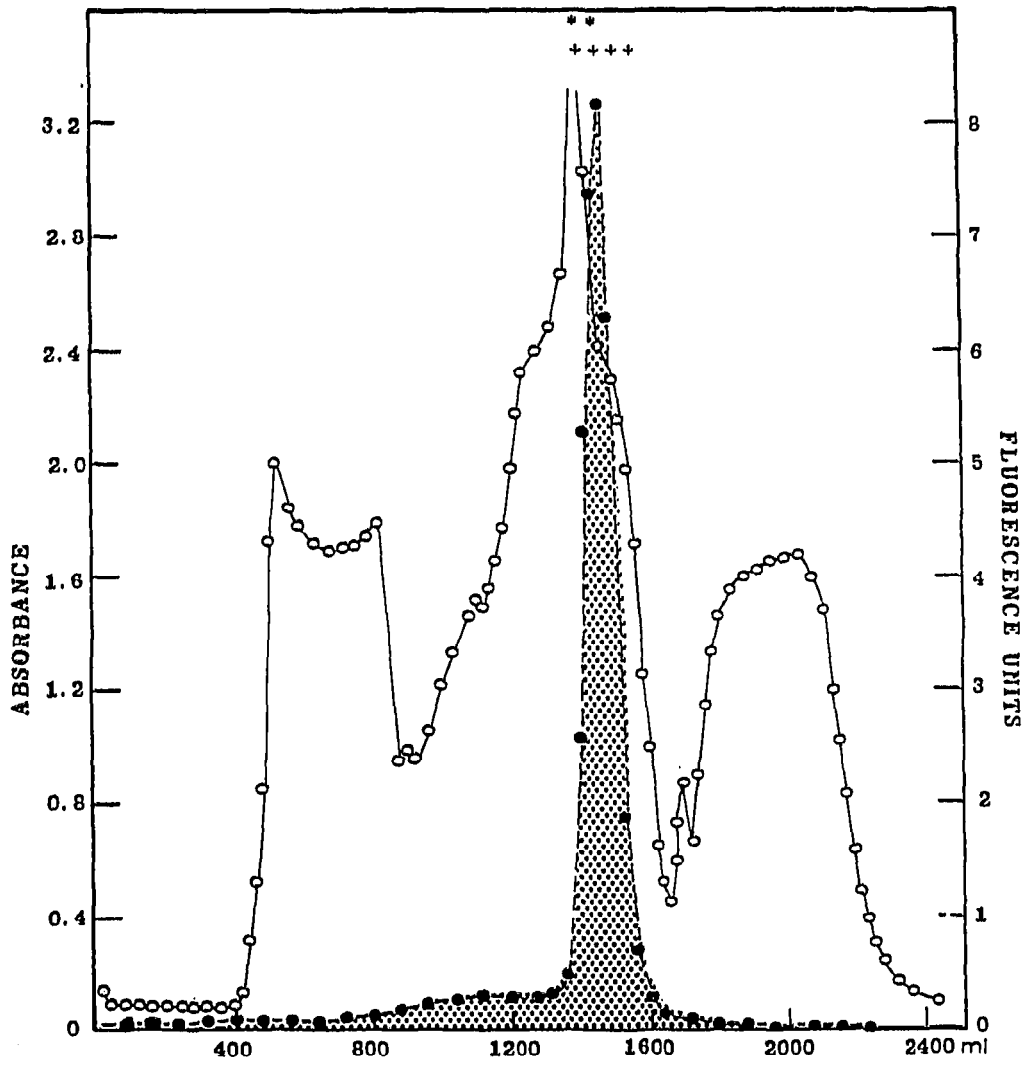

Fig. 3. 400 ml culture filtrate of *Tetrahymena pyriformis* W chromatographed on Sephadex G-25 (27 × 7 cm). 20 ml fractions eluted with 0.1 % mercaptoethanol. Shaded areas = fluorescence at 450 mμ. Open circles = absorbance at 272 mμ. ** = "folic" activity. ++++ = "biopterin" activity.

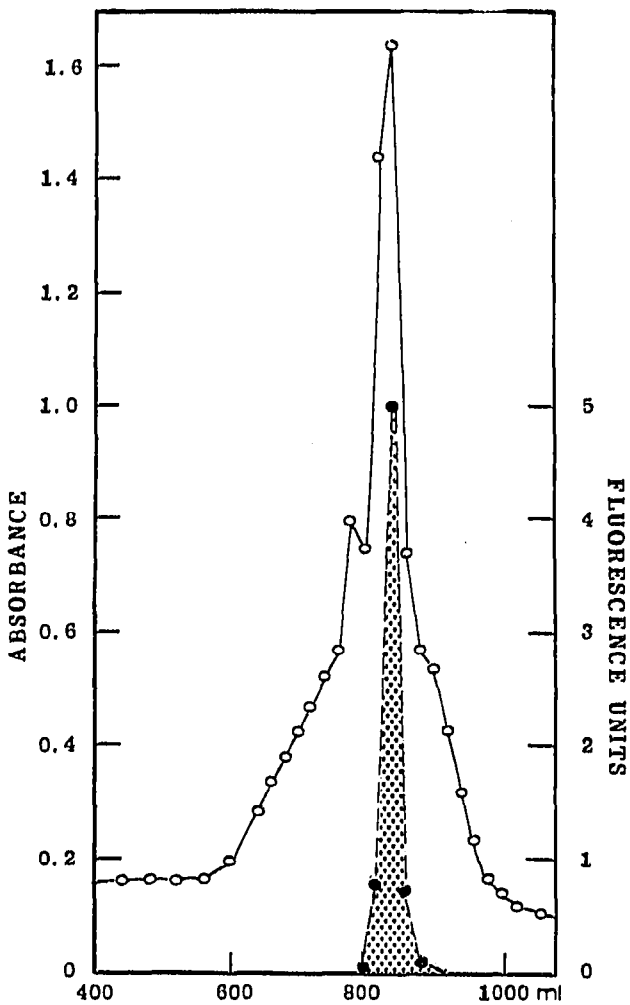

Fig. 4. Peak fluorescent fractions from column illustrated in Fig. 3 rechromatographed in exactly the same way, except on a column 65 × 4 cm.

Data on the chromatography of the *Drosophila* extract are included because it was found that sepiapterin was separated almost entirely from blue-fluorescent pteridines occurring in the flies (Fig. 9). There are three peaks showing absorption at 420 mμ, the latest and largest containing mainly sepiapterin. After lyophilization of the peak fractions, this material was chromatographed on phosphocellulose (Fig. 10) which separated the sepiapterin from 2 or 3 blue-fluorescent substances and from ultraviolet-absorbing material. While the ultraviolet absorption spectrum of the peak fraction corresponded exactly to those published by FORREST AND MITCHELL[5] and by VISCONTINI[6], paper chromatography indicated the presence of several minor contaminants. Further purification has been achieved by rechromatography on ECTEOLA (formate).

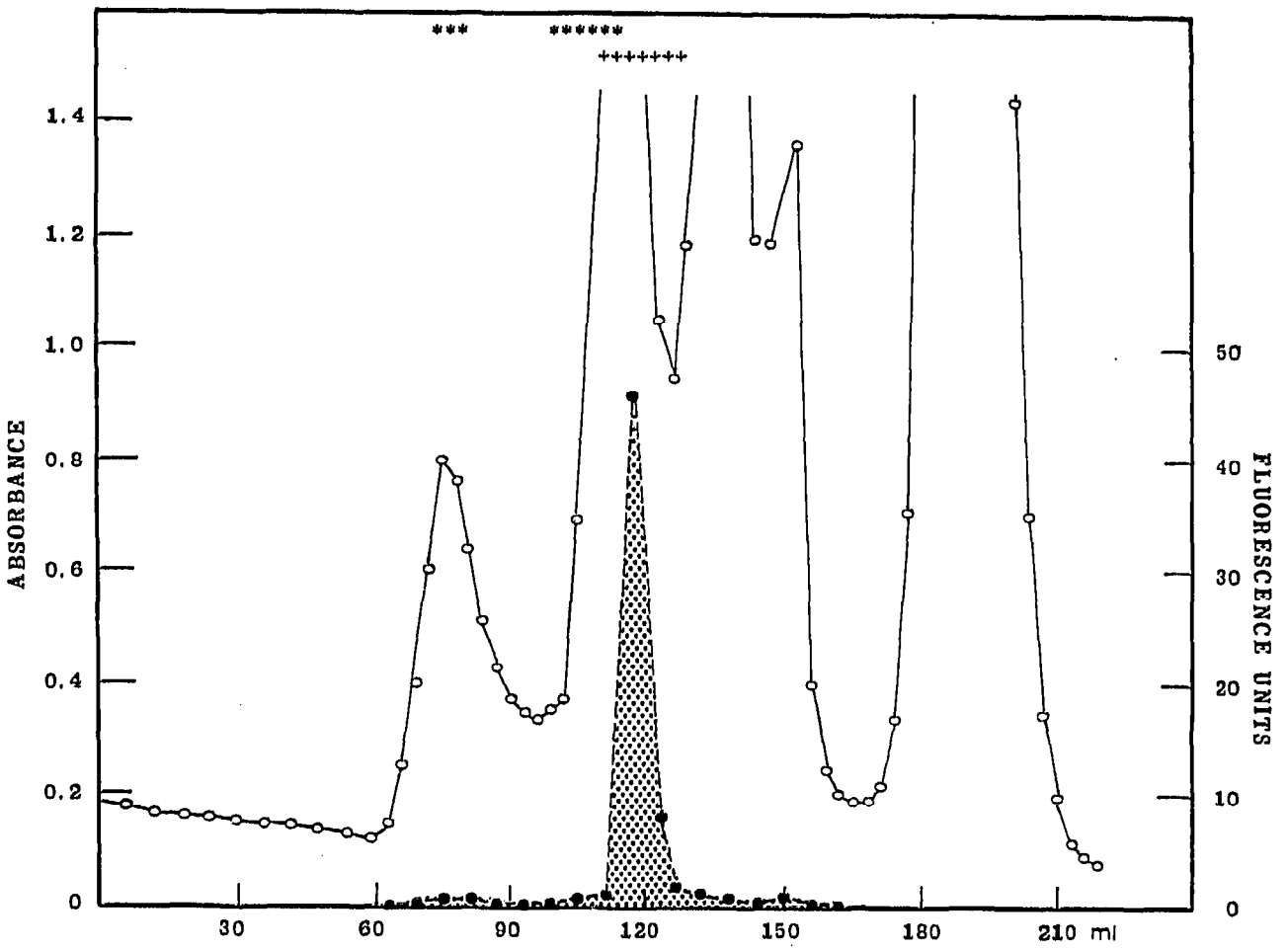

Fig. 5. Peak fluorescent fractions from column illustrated in Fig. 4 chromatographed on Sephadex G-10 (27 × 2.5 cm). 3 ml fractions eluted with 0.1 % mercaptoethanol. Shaded area = fluorescence at 450 mμ. Open circles = absorbance at 272 mμ. ** = "folic" activity. +++ = "biopterin" activity.

DISCUSSION

One of the advantages of the use of Sephadex for isolation of pteridines is the rapidity with which a large volume of material can be handled. With flow rates of from 2 to 10 ml/min even the large columns can be run in several hours. This feature is of particular value when dealing with pteridines and especially with the di- and tetrahydropteridines which are particularly susceptible to degradation by light and by oxygen. While little mention has been made of the reduced compounds here, since the main object was the isolation and identification of the major fluorescent pteridine produced by *Tetrahymena*[7], the reduced pteridines are in fact the biologically active cofactor forms[8]. The fluorescent material in *Tetrahymena pyriformis* cultures is not

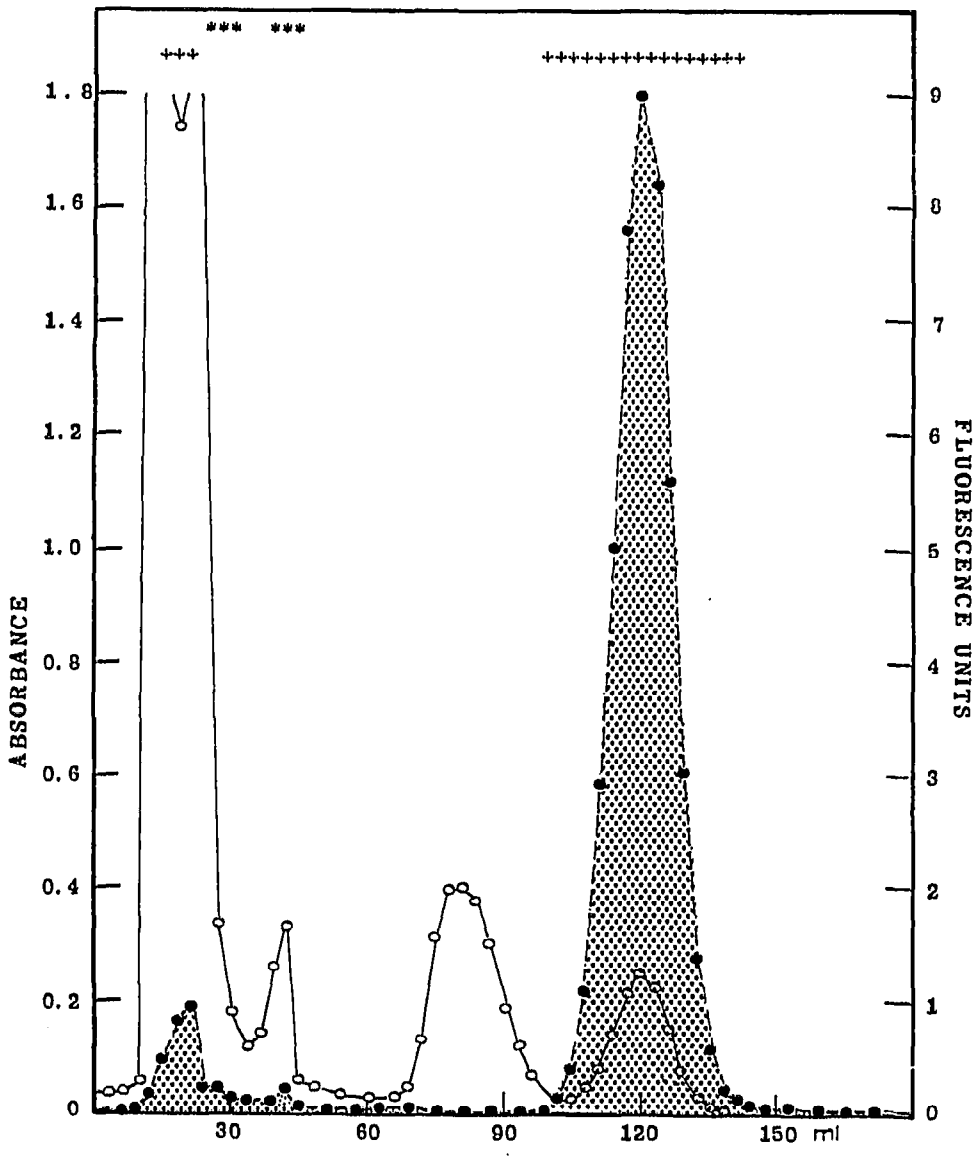

Fig. 6. Peak fluorescent fractions from a column similar to that illustrated in Fig. 4 lyophilized and chromatographed on a column of Serva phosphocellulose (H^+) (25 × 0.8 cm) and eluted with 0.1% mercaptoethanol; 3 ml fractions. Shaded area = fluorescence at 450 mμ. Open circles = absorbance at 272 mμ. ** = "folic" activity. +++ = "biopterin" activity.

the result of artifactual degradation, since it is present whether or not mercaptoethanol is used as the eluant.

That the use of Sephadex for the isolation of tetrahydropteridines is feasible has been demonstrated by ZIEGLER[1]. It would appear from her results that the fully reduced pteridines are eluted from Sephadex first (between 2 and 3 void volumes) and from our results the fully oxidized (aromatic) compounds next (3–4 void volumes) and a yellow (dihydro) pteridine last (5–6 void volumes).

While the principle of gel filtration is separation on the basis of molecular size, it is apparent that some other factor(s) is implicated in the behavior of the pteridines. The fact that Sephadex, according to the manufacturer, contains some free carboxyl groups which lend it some ion-exchange properties, may account for the separation

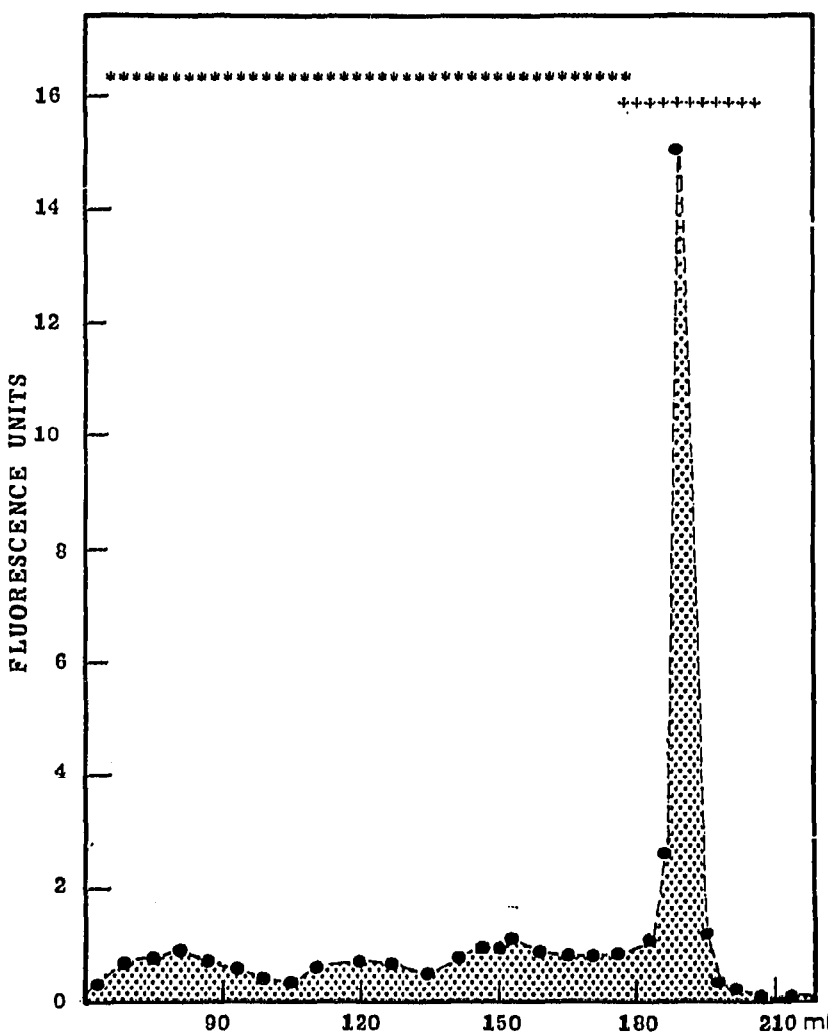

Fig. 7. Extract of *Rhodospirillum rubrum* (*ca.* 50 ml) chromatographed on Sephadex G-25 (27 × 2.5 cm), eluted with 0.1 % mercaptoethanol; 3 ml fractions. Shaded area = fluorescence at 450 mμ. ** = "folic" activity. +++ = "biopterin" activity.

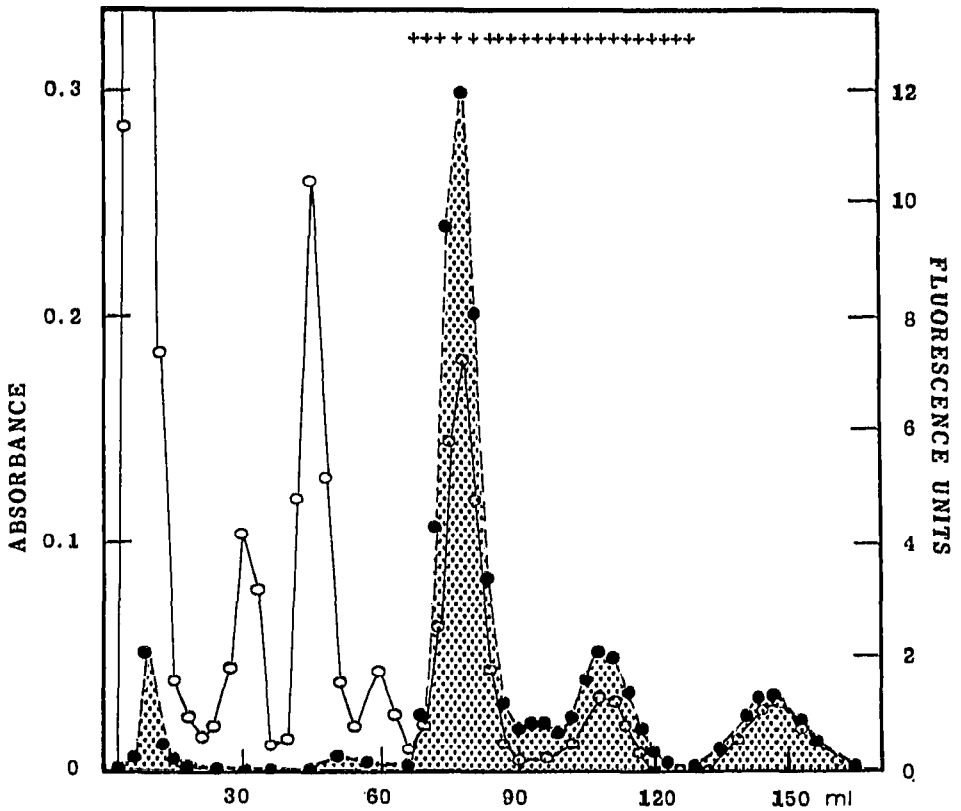

Fig. 8. Peak fluorescent fraction from column illustrated in Fig. 7 chromatographed on Serva phosphocellulose (H^+) (25 × 0.8 cm), eluted with water; 3 ml fractions. Shaded area = fluorescence at 450 mμ. Open circles = absorbance at 272 mμ. +++ = "biopterin" activity.

of the compounds discussed above. This is not completely satisfactory since the reduced pteridines are more basic than the oxidized[9] and should be retained longer on a cation-exchange resin. The manufacturer also states that Sephadex has a certain affinity for aromatic and heterocyclic compounds. This would account for a separation of the fully reduced (nonaromatic) from the fully oxidized (aromatic) compounds. Dihydro compounds such as sepiapterin may occur in a quinonoid form[6] rather than an aromatic form, which might be the basis for its separation from the other types.

There is, however, not even a suggestion of an explanation for the failure of folic acid to separate from unconjugated pteridines when crude culture filtrates are applied to Sephadex while it does separate when no other materials are present. The separation of "folic" activity from "biopterin" activity in the chromatography of *R. rubrum* extract may be due to the fact that the extract from cells is a simpler mixture than the whole culture extract or to the fact that folic acid is present in these cells as polyglutamates of sufficiently large size to be partially excluded from the gel.

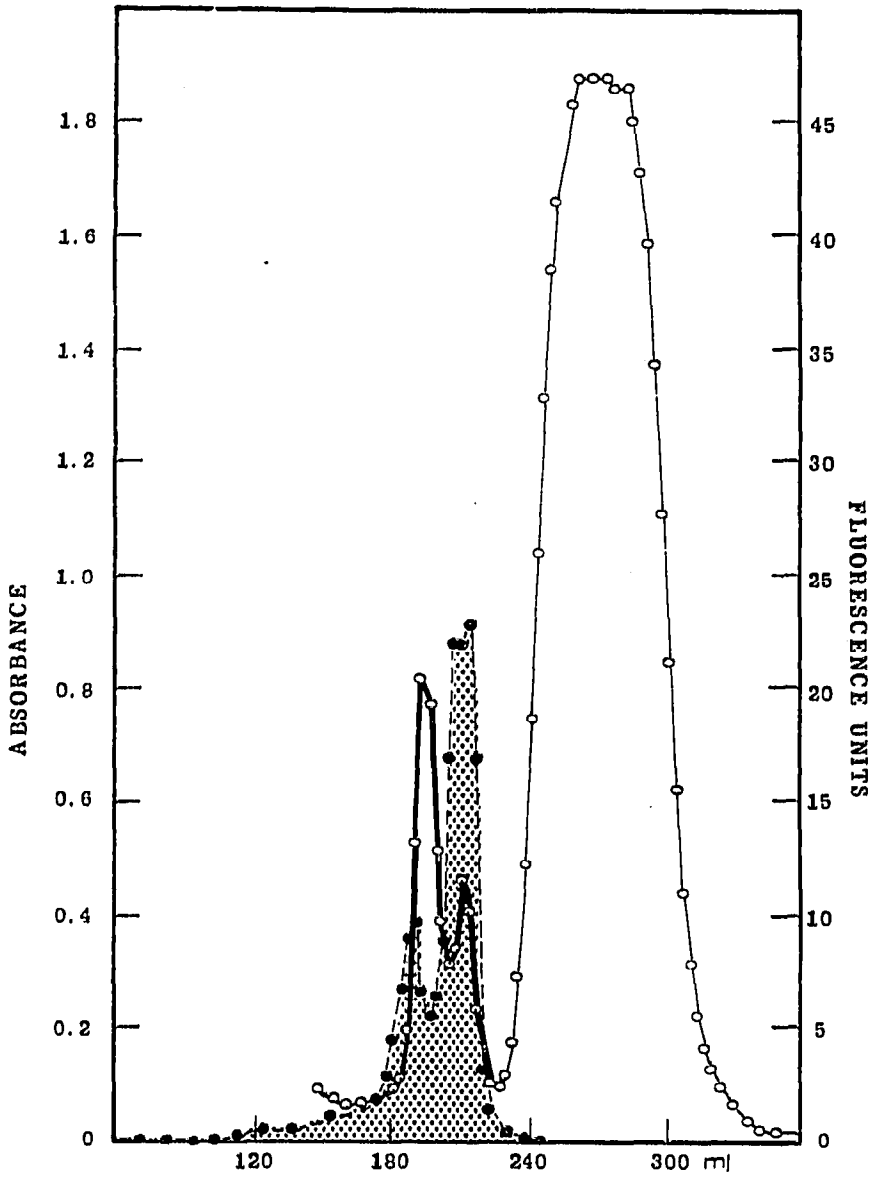

Fig. 9. Extract of *Drosophila melanogaster* se/vg chromatographed on Sephadex G-25 (27 × 2.5 cm); eluted with 0.1% mercaptoethanol; 3 ml fractions. Shaded area = fluorescence at 450 mμ. Open circles = absorbance at 420 mμ.

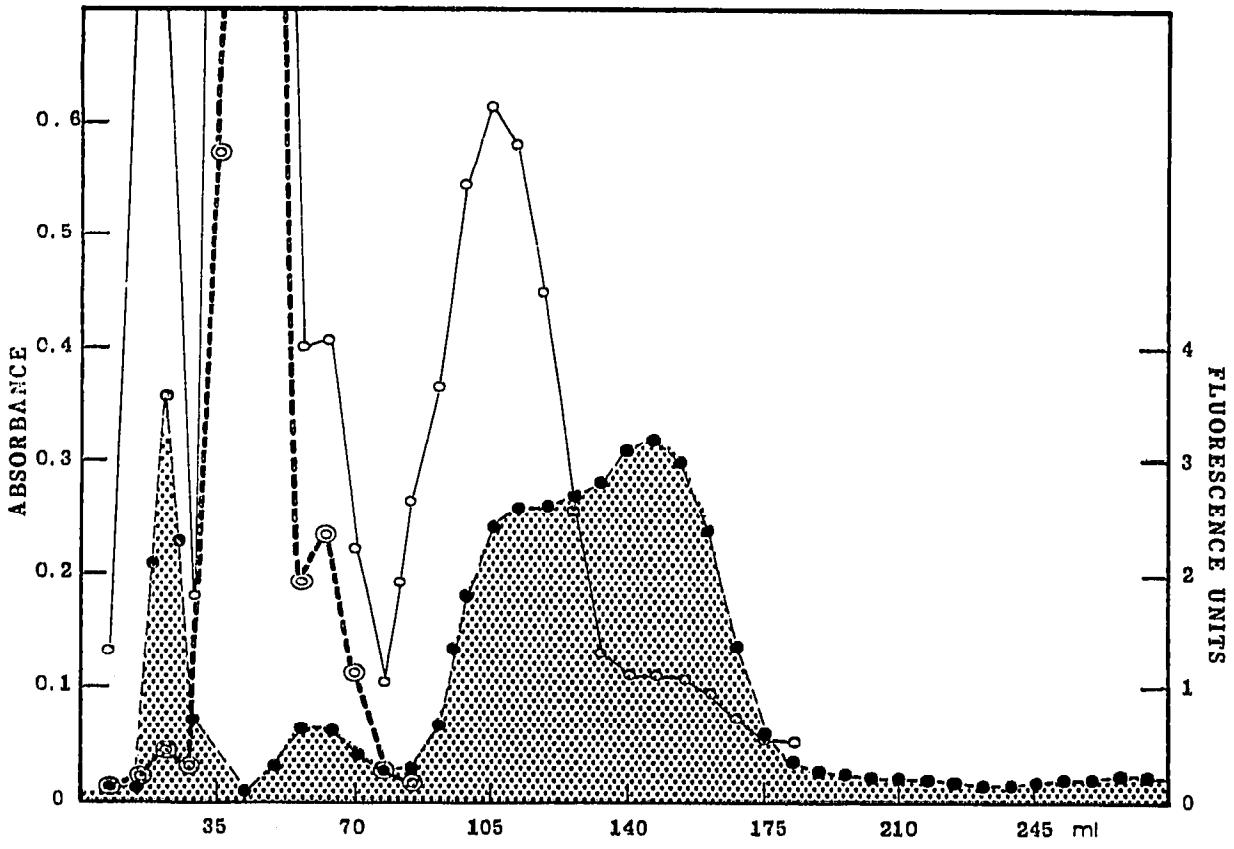

Fig. 10. Combined fractions which absorbed at 420 mμ from column illustrated in Fig. 9, lyophilized and chromatographed on Serva phosphocellulose (H^+) (25 × 0.8 cm); eluted with 0.1% mercaptoethanol; 3 ml fractions. Shaded area = fluorescence at 450 mμ. Open circles = absorbance at 272 mμ. Double circles = absorbance at 420 mμ.

SUMMARY

For the isolation of small quantities of pteridines from dilute solutions, such as the culture filtrates of *Tetrahymena pyriformis*, Sephadex G-25 and G-10 (fine) have proved to be of value. Volumes as large as the void volume of the Sephadex column can be used, resulting in the elimination of large amounts of ultraviolet-absorbing material and in at least a two-fold concentration of the pteridines. Rechromatography on Sephadex results in further concentration. Further purification is achieved using ion-exchange celluloses (cationic and anionic). Ion exchange properties of Sephadex as well as its affinity for aromatic and heterocyclic compounds are postulated as being responsible for these effects.

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