A FLUOROMETRIC METHOD FOR THE DETERMINATION OF PTEROYLGLUTAMIC ACID*

By V. ALLFREY,† L. J. TEPLY, C. GEFFEN, AND C. G. KING

(From the Department of Chemistry, Columbia University, New York)

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The characteristic fluorescence of the antipernicious anemia factor was suggested as a measure of its concentration in natural materials by Jacobson and Simpson (1) and a quantitative study of the fluorescence of synthetic pteroylglutamic acid (PGA) was made by Villela (2).

Attempts to apply such direct fluorescence measurements to yeast extracts prepared in this laboratory indicated that a variety of interfering substances could greatly augment the intensity of the fluorescent beam. Riboflavin and many of the pterins, for example, are strongly fluorescent under the conditions recommended by Villela. Consequently, a study has been made of the fluorescence of pteroylglutamic acid under controlled conditions and a method has been developed for its quantitative estimation in a variety of synthetic and natural mixtures.

Permanganate oxidation of PGA yields a strongly fluorescent substance, identified as 2-amino-4-hydroxypteridine-6-carboxylic acid (3). In the absence of fluorescent pigments (*e.g.*, xanthopterin) which are attacked by permanganate, the increment in the intensity of fluorescence, on oxidation, is directly proportional to PGA concentration. When interfering pigments are present, the PGA oxidation product can be isolated chromatographically and its fluorescence determined directly.

Method

Apparatus—The intensity of fluorescence was measured in a Farrand microfluorophotometer, with filters recommended by Dr. O. H. Lowry for the microdetermination of thiochrome, xanthopterin, and related substances (4).

Primary filter. Corning No. 5860, transmitting in the ultraviolet at 365 mμ.

Secondary filter. Corning No. 4308 + Wratten gelatin No. 2A + Corning No. 3389, in that order, with Filter 3389 facing the phototube.

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The secondary filter combination transmits in the blue region at 470 mμ, blocks out stray ultraviolet radiations, and reduces filter fluorescence to a minimum.

Adsorption columns. Pyrex glass tubes, 5 mm. inside diameter and 120 mm. in length, with a bell-shaped top of 22 mm. inside diameter and 50 mm. in length, and a capillary constriction at the bottom, 1 mm. inside diameter and 15 mm. in length, over-all length 180 mm. When the tubes are charged, the rate of flow should be 15 to 20 drops per minute.

Reagents—Pteroylglutamic acid, crystalline reference standard (generously supplied by Dr. T. H. Jukes of the Lederle Laboratories Division, American Cyanamid Company), 4 per cent potassium permanganate solution, 3 per cent hydrogen peroxide solution, prepared as needed by 1:10 dilution of 30 per cent H_2O_2, 2 N hydrochloric acid, 4 per cent sodium tetraborate solution ($Na_2B_4O_7 \cdot 10H_2O$), 40 per cent sodium hydroxide solution, and 1 mg. per cent of quinine sulfate in 0.1 N sulfuric acid. Dilutions of this quinine sulfate standard serve as reference points in the selection and adjustment of instrument sensitivity.

2.5 M acetate buffer, pH 3.95, prepared as follows: To 500 ml. of 5 M acetic acid add 105 ml. of 5 N NaOH and dilute to 1 liter.

Florisil, 60 to 100 mesh, prepared as follows: 500 gm. of Florisil are suspended in 2 liters of 4 per cent sodium tetraborate solution and boiled for 30 minutes. After decantation, the process is repeated. The Florisil is washed with distilled water and resuspended in 2 liters of 0.25 M acetate buffer (prepared by 1:10 dilution of the acetate buffer described above). This suspension is boiled for 30 minutes and filtered. The Florisil is washed on the funnel with 10 liters of 0.25 M acetate buffer and air-dried at room temperature.

Procedure

Two procedures are available, depending upon the nature and complexity of the material to be analyzed.

Method I—In the absence of fluorescent pigments which are altered by permanganate oxidation, the following direct method may be applied: To 10.0 ml. of a neutral, unbuffered solution containing 0.01 to 10.0 γ of pteroylglutamic acid per ml. is added 0.1 ml. of 2.5 M acetate buffer. The pH at this point should lie between 3.9 and 4.1. The intensity of fluorescence of the solution is determined, with the appropriate sensitivity setting of the instrument. To the 10.1 ml. of buffered solution is then added 0.05 ml. of 4 per cent potassium permanganate solution, followed after 5 minutes by 0.10 ml. of 3 per cent hydrogen peroxide. The final volume is 10.25 ml. The final pH lies between 4.1 and 4.4. After gentle stirring for 2 to 3 minutes the intensity of fluorescence is again determined.

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The increment in fluorescence intensity due to oxidation is directly proportional to the concentration of pteroylglutamic acid. The concentration of PGA may be read directly from a standard linear plot of fluorescence increment (Δ) *versus* concentration.

Standardization—Three such plots have been established. The first (Fig. 1, Curve C) covers the concentration range of 0.01 to 0.1 γ of PGA per ml. The reference point of instrument sensitivity for this range is obtained by setting the galvanometer deflection due to a 0.0024 mg. per cent quinine sulfate solution in 0.1 N sulfuric acid at 70 on the 100 unit galvanometer scale. Points on the curve are obtained as follows: 10.0 ml. of a neutral standard solution containing the indicated concen-

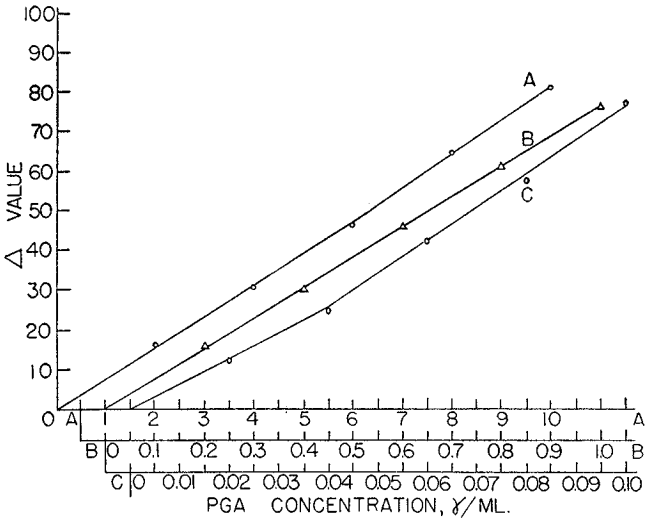

FIG. 1. Fluorescence increment as a function of PGA concentration

trations of PGA are adjusted to pH 4.02 by the addition of 0.1 ml. of 2.5 M acetate buffer. The initial intensity of fluorescence is determined and recorded. 0.05 ml. of 4 per cent $KMnO_4$ solution is then added, followed after 5.0 minutes by 0.10 ml. of 3 per cent H_2O_2. After gentle stirring for 2 to 3 minutes the final intensity of fluorescence is determined. The difference, Δ, between initial and final readings is plotted against the concentration of PGA in the original 10.0 ml. of solution.

As indicated in Fig. 1, Curve C, a slight deviation from linearity occurs at the lower PGA concentrations.

The second standard plot (Fig. 1, Curve B) covers the range from 0.1 to 1.0 γ of PGA per ml. The reference point of instrument sensitivity for this range is obtained by setting the galvanometer deflection due to a 0.024 mg. per cent quinine sulfate solution in 0.1 N H_2SO_4 at 75 on the

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galvanometer scale. The oxidation technique is the same as that used above.

In this range the increment, Δ, is directly proportional to PGA concentration.

The third curve (Fig. 1, Curve A) applies for concentrations between 1 and 10 γ of PGA per ml. The reference point here is a galvanometer deflection of 75 for a 0.2 mg. per cent solution of quinine sulfate in 0.1 N H_2SO_4.

There is a slight deviation from linearity at the higher PGA concentrations. However, concentrations of this magnitude are not often encountered in the analysis of natural products.

It is of primary importance that the standards used be freshly prepared and shielded from direct sunlight. Exposure to light or long standing results in a partial cleavage of PGA to the corresponding pteridyl aldehyde which fluoresces. The initial intensity of fluorescence of pure PGA standards is no higher than the "blank" fluorescence of the reagents employed.

Method II—When interfering pigments are present, the PGA oxidation product is isolated by adsorption on Florisil at pH 4 and elution in 4 per cent sodium tetraborate solution. The fluorescence of the eluate at pH 4 is determined and recorded. After the addition of 40 per cent NaOH solution the fluorescence is again determined. The difference between initial and final readings (corrected for dilution and the slight fluorescence of the oxidation product in alkaline solution) corresponds to the Δ values of the standard curves in Fig. 1.

The detailed procedure is as follows: To 1 to 10 ml. of sample containing 0.25 to 250 γ of PGA are added 0.1 ml. of 2.5 M acetate buffer and sufficient water to bring the volume to 10.1 ml. The pH at this point should be approximately 4. 0.05 ml. of 4 per cent $KMnO_4$ is then added. (In the presence of high concentrations of citrate, oxalate, or other oxidizable substances, more permanganate may be necessary. Enough permanganate should be present to impart a definite red color to the solution throughout the oxidation period.) After 5 minutes, 0.10 ml. of 3 per cent H_2O_2 is added and the clear solution is passed through about 10 cm. of Florisil in an adsorption column. (Adsorption columns are prepared by inserting a small plug of glass wool above the capillary constriction at the base of the tube and filling to a height of 10 to 11 cm. with dry Florisil. Immediately before using the column is washed with 10 ml. of 0.25 M acetate buffer.) If permanganate causes precipitation in the solution being examined, it is advisable to centrifuge before adsorption. When the sample has passed through the Florisil, the column is washed with five 10 ml. portions of 0.25 M acetate buffer. (Mild suction may be applied

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to hasten the washing of the column.) To elute, four 5 ml. portions of boiling 4 per cent sodium tetraborate solution are passed through the column. With mild suction an elution rate of 15 to 20 drops per minute can be obtained. The eluate is adjusted to pH 4.0 to 4.5 by the addition of about 1.8 ml. of 2 N HCl, and then diluted to 25 ml. in a volumetric flask.

Duplicate 10 ml. aliquots are withdrawn and 0.1 ml. of 2.5 M acetate buffer is added to each. The intensity of fluorescence is determined on 1 ml. of these solutions before and after the addition of 0.1 ml. of 40 per cent NaOH. Instrument sensitivity is selected by using one of the three quinine sulfate calibration points used for the standard curves. The choice depends on the initial fluorescence of the eluate and the magnitude of the decrease on addition of the alkali.

The difference between the initial reading and the final reading multiplied by the dilution factor 1.1 gives a Δ value which must be further increased by 4 per cent to allow for the fluorescence of the oxidation product in alkaline solution. The corresponding concentration can then be read from the standard curves of Fig. 1.

It is advisable to run "recovery" samples and to correct for losses due to coprecipitation, incomplete elution, or to other causes, depending on the material being analyzed.

In many natural products (*e.g.*, yeast and bacterial extracts) a more complete isolation of the PGA oxidation product is required. The recommended procedure is described in the section covering the application of the method to natural products.

EXPERIMENTAL

Oxidation—As indicated in Table I, the increment, Δ, in fluorescence intensity is essentially constant over a wide range of permanganate concentrations.

10 ml. aliquots of a standard solution containing 2 γ of PGA per ml. were oxidized for 5.0 minutes with 0.05 ml. of the indicated concentrations of potassium permanganate. The solutions were buffered with 0.2 ml. of 2.5 M acetate buffer. Permanganate remaining at the end of the oxidation period was removed with 0.2 ml. of 3 per cent H_2O_2. The difference, Δ, between final and initial intensities of fluorescence was determined at an instrument sensitivity adjustment corresponding to a galvanometer deflection of 34 scale divisions for a 0.024 mg. per cent solution of quinine sulfate in 0.1 N H_2SO_4.

Oxidation Time—The variation of fluorescence increment with time of oxidation is shown in Fig. 2. The increment is constant between 5 and 11 minutes. Data were obtained as follows: 10 ml. aliquots of a standard

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PGA solution (1.2 γ per ml.) were buffered with 0.1 ml. of 2.5 M acetate buffer. 0.05 ml. of 4 per cent $KMnO_4$ was then added. After the times indicated by points on the curve, 0.1 ml. of 3 per cent H_2O_2 was added to reduce the remaining permanganate. The increment in intensity of fluorescence due to oxidation was determined at an instrument sensitivity

TABLE I

Effect of Permanganate Concentration on Fluorescence Increment

$KMnO_4$ concentration	Fluorescence increment*
per cent	
1	80
2	80
4	80
6	81
8	83
10	83
20	82

* Observed at an instrument sensitivity corresponding to a galvanometer deflection of 34 scale divisions for a 0.024 mg. per cent quinine sulfate standard.

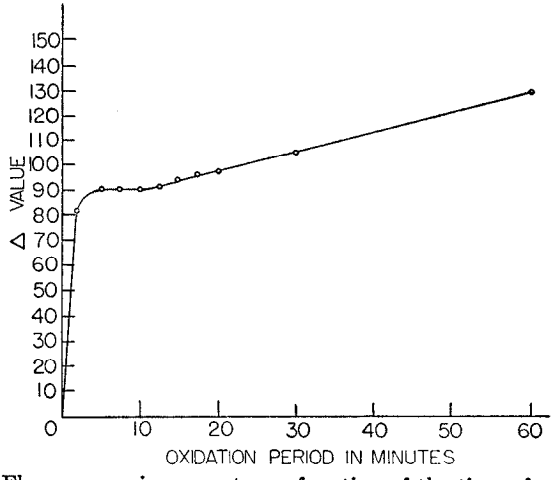

FIG. 2. Fluorescence increment as a function of the time of oxidation

corresponding to a galvanometer deflection of 75 for a 0.024 mg. per cent solution of quinine sulfate in 0.1 N H_2SO_4.

The constancy of the increment between 5 and 11 minutes and its independence, within wide limits, of the permanganate concentration permit a precision in duplicate determinations to within 1 scale division on the galvanometer.

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Properties of Oxidation Product—The fluorescence of the oxidation product, as prepared by the adsorption-elution technique outlined above, does not change appreciably within 240 hours. It varies considerably with pH, however, and with the concentration of borate and acetate ions in the solution.

The variation of the fluorescence intensity of the PGA oxidation product with pH is illustrated in Fig. 3. The data were obtained as follows: 12.5 ml. of a PGA standard solution containing 100 γ per ml. were oxidized by the standard procedure and the solution passed through about 10 cm. of Florisil in an adsorption column. The oxidation product was eluted with four 5 ml. portions of boiling 4 per cent sodium tetraborate

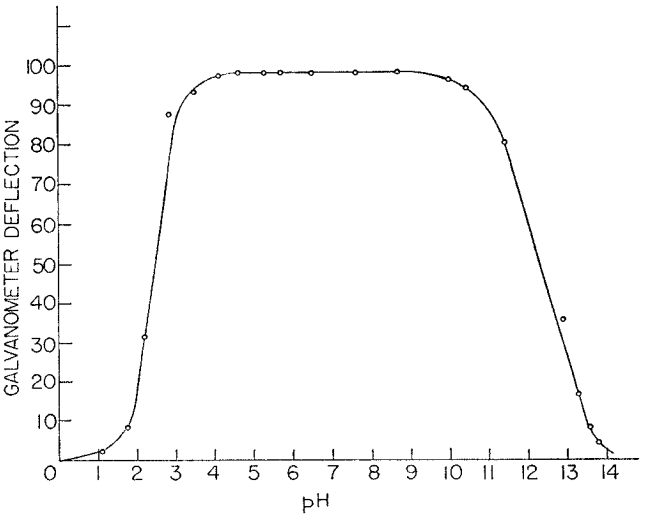

FIG. 3. Fluorescence of the PGA oxidation product as a function of pH

solution. The eluate was made up to 500 ml. with distilled water. 10 ml. aliquots of this solution were brought to the indicated pH values in a final volume of 25 ml. (pH values below 10 were measured on a Beckman pH meter. Values above pH 10 were calculated from the dilutions of 1.0 N NaOH used in the titrations.) Fluorescence intensities were determined at an instrument sensitivity corresponding to a galvanometer deflection of 75 for a 0.024 mg. per cent quinine sulfate standard.

The fluorescence under these conditions is constant between pH 4 and 9.5 and falls off rapidly in more strongly acid and alkaline solutions. The fluorescence intensity of the oxidation product in 1 N NaOH is about 4 per cent of the value determined at pH 4.

In the direct method, in which the oxidation product is not isolated,

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the constancy of increment holds only between pH 3.9 and 4.4. Above pH 4.4 the formation of MnO_2 causes a significant decrease in the measured increment. The 2.5 M acetate buffer recommended serves to keep the pH within these limits of optimum fluorescence intensity.

Salt Effects—The intensity of fluorescence of the oxidation product varies considerably with its ionic environment. The data in Table II indicate the magnitude of the variation in different concentrations of salts. The final concentration of PGA oxidation product is 1 γ per ml.

TABLE II

Variation of Fluorescence Intensity of Oxidation Product with Salt Concentration

Solution (containing 1 γ oxidation product per ml.)	Concentration	pH	Fluorescence intensity*
	M		
H_2O		7.1	90
NaCl	0.1	5.1	86
	0.5	5.1	84
	1.0	5.1	80
NaH_2PO_4	0.1	4.45	60
	0.5	4.15	32
	1.0	4.0	22
$(NH_4)_2SO_4$	0.1	5.1	90
	0.5	5.0	97
	1.0	5.0	102
$NaC_2H_3O_2$	0.01	6.85	77
	0.05	7.4	67
	0.10	7.6	65
	0.50	7.7	70
	1.0	7.8	75
$Na_2B_4O_7$	0.01	9.15	83
	0.05	9.15	88
	0.10	9.2	91
	0.15	9.2	94

* Observed at an instrument sensitivity corresponding to a galvanometer deflection of 42 scale divisions for a 0.024 mg. per cent quinine sulfate standard.

in all cases. Since the fluorescence of the oxidation product is constant between pH 4 and 9.5, the observed differences probably represent specific ion effects. Fluorescence measurements were made at an instrument sensitivity corresponding to a galvanometer deflection of 42 for a 0.024 mg. per cent quinine sulfate standard. The salt solutions themselves do not give a measurable fluorescence at this sensitivity.

An interesting effect is observed in the concentration series for sodium acetate, in which a definite minimum is observed at about 0.1 M. A similar effect is evident in sodium tetraborate solutions in which the initial loss due

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to "quenching" in 0.01 M solution is restored at 0.1 M (about 4 per cent), and at higher concentrations the fluorescence may exceed that of the water solution.

The extreme "quenching" effects of acetate and phosphate ions emphasize the importance of rigid control in the analytical procedure. Such observations point to the necessity of comparing fluorescence intensities under identical or reasonably identical conditions. It is for this reason that the Florisil eluate is titrated to pH 4 and buffered in acetate before comparison with the standards of Fig. 1.

Adsorption-Elution

General—Barium and calcium carbonates do not adsorb PGA from neutral or weakly alkaline solutions. Both PGA and its oxidation product are partially adsorbed on Decalso and on tricalcium phosphate from neutral or weakly acid solutions. The adsorption of PGA on Magnasol[1] at pH 4 to 5 appears to be quantitative. A number of ion exchange resins were tested for adsorptive capacity, but in all cases these were sufficiently soluble to give excessive and variable "blank" fluorescence readings.

Florisil—Both PGA and its oxidation product are quantitatively adsorbed on Florisil at pH 4. Both are recovered in greater than 90 per cent yield with the recommended elution procedure. Under these conditions riboflavin is adsorbed but not recovered from the column. Xanthopterin is adsorbed and is recovered in good yield. Xanthopterin, however, does not interfere with the method, since permanganate oxidation, prior to adsorption, converts it to leucopterin which does not fluoresce appreciably under these conditions.

The quantitative nature of the adsorption of the PGA oxidation product is illustrated in Columns 2 and 3 of Table III, where the fluorescence intensities before and after adsorption are recorded for different PGA concentration levels. Columns 4 and 6 of Table III list the decrement in eluate fluorescence on addition of 40 per cent NaOH and the corresponding Δ values for PGA standards at the same final concentrations. The data are listed for triplicate determinations. The extent of recovery on elution is recorded in Column 7.

The data were obtained as follows: 10.0 ml. of solution containing the indicated total concentrations of PGA were buffered at pH 4 by the addition of 0.1 ml. of 2.5 M acetate buffer and oxidized by the standard technique previously described. The intensity of fluorescence following oxidation was recorded and the solution was passed through about 12 cm. of Florisil in an adsorption column. The columns were washed with two 10 ml. portions of 0.25 M acetate buffer. The fluorescence intensities of

[1] Supplied by the Westvaco Chemical Corporation, New York.

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the first filtrates and of the washings were determined and recorded. The columns were then eluted with four 5 ml. portions of boiling 4 per cent sodium tetraborate solution. The eluate was titrated to pH 4 to 4.5 by the addition of 1.7 ml. of 2 N HCl, and diluted to 25 ml. Then 0.25 ml.

TABLE III

Adsorption-Elution Recovery Data

PGA oxidized and adsorbed	Fluorescence before adsorption	Intensity* after adsorption	Fluorescence decrement of eluate†		Standard Δ value	Recovery
				Average		
(1)	(2)	(3)	(4)	(5)	(6)	(7)
γ						*per cent*
25	86	0	78			
	87	1.5	77	76.3	76.5	100
	86	0	74			
20	68	1	58			
	67	0	58	58	59.5	97
	67	0	58			
15	54	0	45			
	55	0	45	45.7	47.5	96
	56	1.5	47			
10	39	0	36			
	39	0	36	36	35.5	101
	39	0	36			
5	19	0	18			
	19	0	17	17.7	17	104
	19	0	18			
1	39	0	30			
	39	0	31	30	32	94
	39	0	29			
0.5	18	0	10			
	18	0	10	10.7	11	97
	19	0	12			

Fluorescence intensities for 0.5 and 1 γ of PGA oxidized and adsorbed were determined at an instrument sensitivity corresponding to a deflection of 75 scale divisions for a 0.0024 mg. per cent quinine sulfate standard. All other observations were made at a sensitivity of 75 units deflection for a 0.024 mg. per cent quinine sulfate standard.

* Fluorescence intensities corrected for "blank" fluorescence of H_2O controls.

† Decrements corrected for "blank" fluorescence of reagents and fluorescence of the oxidation product in alkaline solution.

of 2.5 M acetate buffer was added and the intensity of fluorescence determined on 1 ml. of this solution before and after the addition of 0.1 ml. of 40 per cent NaOH.

The decrease in fluorescence intensity was compared with the Δ values of standard PGA solutions at the same final concentrations and at the same instrument sensitivities.

The data in Table III indicate the essential completeness of the adsorp-

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tion-elution step. Similar results have been obtained for the isolation of PGA.

The PGA oxidation product may be adsorbed from volumes far in excess of 10 ml., complete recoveries having been obtained for 10 γ of total oxidation product in as much as 200 ml. of solution passing the column. Similarly, washing in acetate buffer at pH 4 can be extended to remove contaminants without a corresponding loss of the oxidation product.

The volume of eluting agent cannot be reduced below 20 ml. without a corresponding decrease in the recovery of oxidation product from the column. Elution at 30° and 60° is likewise incomplete. Two 10 ml. portions may be used in place of four 5 ml. portions of eluting agent without appreciable difference in recoveries. The use of successive small portions at 100°, however, minimizes cooling during elution. Steam-jacketed adsorption columns could be used to advantage at this step.

Interfering Compounds—The direct method (measuring fluorescence increment after oxidation) may be applied without error in the presence of many of the B vitamins, purines, pyrimidines, and amino acids. Riboflavin, for example, in concentrations as high as 10 γ per ml. is unaffected by the oxidation, and PGA is readily determined in admixture with it.

In Table IV are listed many of the compounds which have been examined for their behavior on permanganate oxidation. In Column 3 are recorded the observed fluorescence increments at the indicated concentration levels. For purposes of comparison, the Δ values given by a PGA standard (1 γ per ml.) at the same instrument sensitivity are recorded in Column 4.

From Table IV it can be seen that xanthopterin, isoxanthopterin, and pteroic acid interfere significantly with the determination of PGA with the direct method. When the PGA oxidation product is isolated by adsorption and elution, xanthopterin is converted to leucopterin which does not fluoresce. Isoxanthopterin is not widely distributed and is not a likely source of error. Pteroic acid, however, interferes in both methods, since it yields the same dibasic acid on oxidation as does PGA (and PGA conjugates). Similarly, high concentrations of tyrosine or tryptophan give erroneously high results in both methods. The products resulting from the oxidation of these amino acids are not completely removed in the adsorption-elution step. (They do not interfere, however, in the modified procedure described in the section covering the application of the method to natural products.) Histidine does not interfere.[2]

Pteroylglutamic acid can be determined in admixture with xanthopterin by a modification of the direct oxidation technique. The method depends on the preliminary conversion of xanthopterin to leucopterin by incubation with the enzyme, xanthine oxidase. Subsequent permanganate oxida-

[2] Vitamin B_{12} does not interfere in either procedure.

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tion then gives the fluorescence increment characteristic of the PGA component of the mixture.

Several applications of this method are listed in Table V, where the fluorescence intensities after incubation and the increments on subsequent oxidation are listed for mixtures of PGA and xanthopterin. The data

TABLE IV

Effect of Permanganate Oxidation on Fluorescence

Substance (1)	Concentration (2)	Increment on oxidation (3)	Δ, 1 γ PGA per ml. (at this sensitivity) (4)
	γ per ml.		
Riboflavin	1	0	78
	10	0	78
Thiamine	2	0	78
	10	2	78
Nicotinic acid	2	0	78
	10	0	78
Thymine	2	0	78
	10	0	78
Uracil	10	0	78
Xanthine	10	1	78
Adenine	200	1	78
Guanine	250	1	78
DL-Phenylalanine	1000	0	78
L-Tyrosine	50	8	78
	1000	36	78
DL-Tryptophan	1000	65	78
Pyridoxine	1	1	78
	10	15	78
N^1-Methylnicotinamide	1	0	78
	10	2	78
Xanthopterin	1	−76	78
Isoxanthopterin	1	−15	11
Rhizopterin*	3.4	0	35
Pteroic acid	0.8	36	80

* Supplied by the Research Laboratories of Merck and Company, Rahway, New Jersey.

were obtained as follows: 10.0 ml. aliquots of standard solutions containing the indicated concentrations of xanthopterin and PGA were buffered to pH 6.3 by the addition of 1.0 ml. of 0.1 M phosphate buffer. The solution was then incubated for 60 minutes at 37° with 0.04 ml. of a xanthine oxidase preparation. (The enzyme was prepared according to the method of Ball (5) and was suspended in 4 M $(NH_4)_2SO_4$. 0.04 ml. of this suspen-

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sion completed the enzymatic oxidation of 10.0 ml. of a 2 γ per ml. xanthopterin standard in 45 minutes at 37°.) Following incubation, 0.1 ml. of 2.5 M acetate buffer was added and the intensity of fluorescence was determined before and after permanganate oxidation. In Column 5 are recorded the Δ values obtained by the above procedure for the same concentrations of PGA at the same instrument sensitivity.

The full fluorescence increment characteristic of the PGA oxidation product is recovered in all cases. Enzyme-free controls treated in the same way show a fluorescence decrement on permanganate oxidation.

By determining the fluorescence decrement due to enzymatic oxidation in standard xanthopterin solutions, the method can be extended to give both the xanthopterin and PGA concentrations in a mixture. By comparing the loss of fluorescence on incubation with that of xanthopterin standards, one determines the xanthopterin concentration of the solution. The

TABLE V

Determination of PGA on Admixture with Xanthopterin

Solution (1)	Fluorescence after incubation (2)	Intensity after oxidation (3)	Increment (4)	Δ values of PGA control (5)
0.33 γ PGA + 0.33 γ xanthopterin per ml.	21	48	27	27
0.5 " " + 1.0 " " " "	25	58	33	33
1.0 " " + 1.0 " " " "	14	43	28	28
1.5 " " + 1.0 " " " "	12	47	35	35
2.0 " " + 1.0 " " " "	15	58	43	43

subsequent increment on permanganate oxidation is proportional to the PGA concentration.

Application of Method—Application of Method II to water extracts of some natural products gave the results indicated in Table VI. The procedure was as follows: The weighed sample was ground with water for 5 minutes in a Waring blendor. The resultant suspension was brought to the indicated concentration and heated in a 100° water bath for 15 minutes. After filtration, 10 ml. aliquots of the clear filtrate were oxidized according to the standard procedure and passed through Florisil columns. Columns were washed with 50 ml. of 0.25 M acetate buffer and eluted with four 5 ml. portions of boiling 4 per cent sodium tetraborate solution. Eluates were titrated to pH 4 to 4.5, diluted to 25 ml., and buffered at pH 4 by the addition of 0.25 ml. of 2.5 M acetate buffer. The fluorescence intensities were determined on 1 ml. of these solutions before and after the addition of 0.1 ml. of 40 per cent NaOH.

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The decrement in fluorescence intensity was then calculated. The corresponding PGA concentrations were read from the standard curves of Fig. 1.

In Column 4 of Table VI are recorded the PGA concentrations in the filtrates as determined by microbiological assay with *Lactobacillus casei*. (The method used was essentially that of Roberts and Snell (6) as modified by Olson, Fager, Burris, and Elvehjem (7).) Since both methods of analysis were applied to the filtrates obtained after heating the suspensions, the analytical data are expressed as γ per ml. rather than as γ per gm. of sample. This permits a comparison of the methods without the additional assumption that the extraction procedure was completely effective in releasing bound PGA from the tissues examined.

The results obtained by the chemical method are listed in Column 3 of Table VI. They are higher than the microbiological results in all cases, although the agreement is fair for the four samples listed. The

TABLE VI

PGA Content of Natural Extracts

Sample (1)	Concentration of suspension (2)	PGA content of filtrate, γ per ml.	
		Chemical (3)	Microbiological (4)
	gm. per ml.		
Lettuce	0.5	0.29	0.23
Carrots	0.5	0.27	0.20
Potato	0.5	0.29	0.20
Beef muscle	0.4	0.13	0.10

discrepancy may be due, in part, to the presence of PGA conjugates, 6-pteridyl aldehyde, pteroic acid, or other PGA hydrolysis products in the extracts examined. (Such compounds would not produce a growth response in PGA-deficient lactobacilli.) High concentrations of tyrosine and tryptophan also interfere with the method, since their oxidation products are fluorescent and follow the recommended adsorption-elution procedure.

In certain natural extracts, therefore, a more specific isolation of the PGA oxidation product is required. This can be achieved as follows: The Florisil eluate is titrated to pH 5, brought to 25 ml., and buffered with 0.25 ml. of 2.5 M acetate buffer (pH 5.0). The intensity of fluorescence of the solution is determined before and after the addition of about 0.5 gm. of Magnasol (prewashed with acetate buffer of pH 5). The decrement in fluorescence intensity represents the specific adsorption of the PGA oxidation product. If a further check is desired, the oxidation product may be eluted from the Magnasol with four 5 ml. portions of boiling 4 per cent sodium tetraborate solution, and the fluorescence decrement between pH 4 and 14 determined as before.

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Application of this modified procedure to yeast extracts gives analytical results in satisfactory agreement with the PGA concentrations as determined by microbiological assay.

The recovery of added amounts of PGA from the filtrates, according to Method II, is essentially complete in all cases. In Table VII are recorded the fluorescence decrements ($-\Delta$) of the filtrates and of "recovery" samples containing the indicated added amounts of PGA. The difference between the decrement observed in the "recovery" sample and that obtained in the original filtrate is given in Column 4. In Column 5 are

TABLE VII

Recovery of Added PGA in Extracts of Natural Products

Sample (1)	PGA added per 10 ml. extract (2)	Fluorescence observed (3)	Decrement difference* (4)	$-\Delta$ of PGA control (5)	Recovery (6)
	γ				*per cent*
Lettuce	0	11.5			
	4	21.0	10.5	10.5	100
	8	30.5	19.0	20.5	93
	10	37.0	25.5	27.0	95
	20	56.0	44.5	46.5	96
Beef muscle	0	3.5			
	2.5	10.3	6.8	7.0	97
	5.0	18.0	14.5	14.5	100
	10	29.0	25.5	27.0	94
	20	50.5	47.0	46.5	102
Salmon	0	35.0			
	12	65.5	30.5	31.5	97
Carrots	0	8.5			
	3	17.0	8.5	8.5	100
Onion	0	27.0			
	4	37.0	10.0	10.5	95
Milk	0	38.0			
	3	46.0	8.0	8.5	94

* Average of three determinations.

recorded the corresponding $-\Delta$ values of PGA standards at the same final concentrations and at the same instrument sensitivity. The percentage recovery of the added PGA is given in Column 6. The recoveries of added amounts of PGA from the filtrates are greater than 90 per cent in all cases.

DISCUSSION

The methods described do not include an efficient procedure for extracting bound PGA from tissues. Preliminary experiments indicate that PGA is stable to autoclaving (15 minutes at 15 pounds pressure) in neu-

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tral or faintly acid solutions (pH $\geqslant$ 4.5) and in 0.005 to 0.01 N NaOH. It is partially hydrolyzed by autoclaving in 0.1 N alkali and almost completely hydrolyzed in 0.1 N acid. The extraction procedures recommended for the microbiological method by Olson *et al.* (7) (heating in a boiling water bath for 3 minutes at pH 4.5 or 7; autoclaving at pH 4.5 for 5 minutes) do not result in hydrolytic destruction of the vitamin as followed fluorometrically. However, it cannot be concluded that such methods make possible the extraction of the total PGA content of natural materials. The effects of hydrolytic enzymes and the conjugases of hog kidney (8) and chicken pancreas (9) are currently being investigated.

Solutions of PGA are not stable to prolonged standing or to exposure to direct sunlight. Since pure PGA does not fluoresce, it is likely that the observations of Villela (2) represent partial hydrolysis of his synthetic PGA standards.

Inspection of the recovery data and internal standardization curves for lettuce and beef muscle extracts indicates that the method is accurate within about ± 6 per cent. The precision in duplicate determinations by both procedures is about ± 3 per cent.

SUMMARY

1. By permanganate oxidation pteroylglutamic acid (PGA) is converted to 2-amino-4-hydroxypteridine-6-carboxylic acid which fluoresces strongly at 470 mμ when irradiated with light of wave-length 365 mμ. The increment in intensity of fluorescence is directly proportional to PGA concentration over a wide range. (*a*) In the absence of fluorescent pigments which are altered by permanganate oxidation, the reaction may be standardized to permit the determination of PGA in concentrations between 0.01 and 10 γ per ml. (*b*) When interfering pigments are present, the oxidation product may be isolated chromatographically. Its concentration is then determined by the decrease in intensity of fluorescence between pH 4 and 14.

2. The variation of fluorescence intensity of the PGA oxidation product with pH and salt concentration is described.

3. Results obtained by application of the fluorometric method to several natural extracts are compared with the analytical values obtained by microbiological assay with *Lactobacillus casei*.

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V. Allfrey, L. J. Teply, C. Geffen and C. G. King

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