STUDIES ON THE DETERMINATION OF PTEROYLGLUTAMIC ACID

A Thesis

Submitted to the Faculty of Graduate Studies and Research in Partial Fulfilment of the Requirements for the Degree of Master of Science

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Section 1.0 GENERAL INTRODUCTION

The most widely used colorimetric method for the determination of pteroylglutamic acid (PGA) in pharmaceutical preparations is that developed by Hutchings et al. (1947). This method is based on the colour reaction for free amines as reported by Bratton & Marshall (1939). Various modifications of this method have been suggested but all of them may be criticized on similar grounds. In order to apply the procedure, a sample must contain at least 2 per cent PGA. Interference due to ferrous sulphate and liver concentrates must be removed. Since polarographic and fluorometric methods are subject to the same difficulties, it was obvious that a new approach to the assay of PGA was needed.

Early work on the isolation and synthesis of PGA has indicated that its absorbance characteristics varied greatly with variations of pH. It was felt that such changes with pH might offer a new approach to the problem and possibly provide the basis for a simpler procedure than the existing colorimetric assay.

Since PGA is known to be but very slightly soluble at pH 2.0 to 3.0 it seemed possible that it might also be feasible to remove interfering substances which are soluble at this pH. A technique of this kind would be useful for application to either the existing colorimetric assay or to any new procedure which might be developed.

It is the purpose of this thesis to report studies on the development and application of a method for the estimation of PGA based on its spectral characteristics at different pH levels. Techniques for the removal of interfering substances along with their limitations are described and the merit of various standards investigated. Certain limitations of the new method are also subjected to critical review.

Section 2.0 HISTORICAL

2.1 The Anti-anemia Factor and Pteroylglutamic Acid

The presence in yeast and liver of an anti-anemia and growth factor that would prevent tropical macrocytic anemia and that was necessary for monkeys and chicks, was demonstrated by Wills (1931), by Day et al. (1935) and by Hogan & Parrott (1939). Snell et al. (1940) and Hutchings et al. (1941) found this factor to be necessary for the growth of microorganisms such as Streptococcus faecalis and Lactobacillus casei.

Numerous names were given to the factor by several workers. These are summarized according to source and test subject or organism in Table I.

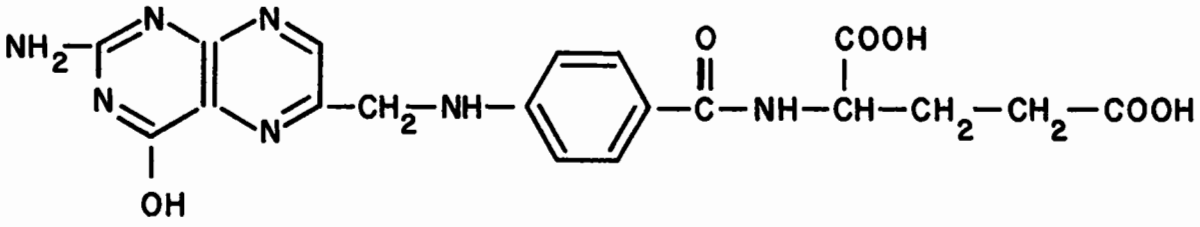
Each of these active factors was later shown to be pteroylglutamic acid (PGA), except for the spinach and bacterial growth filtrates, where pteroyltriglutamic acid (PTGA) was found to be the active substance.

A pure crystalline substance was isolated from liver by Pfiffner et al. (1943). Shortly thereafter Stokstad (1943) isolated a similar substance from both yeast and liver. The ultraviolet absorbance spectra of these substances were identical.

Angier et al. (1945) synthesized a compound whose near ultraviolet spectrum, infrared spectrum, crystalline structure and biological properties were the same as those of a substance isolated from liver. Later these workers demonstrated that the synthetic substance was N- 4- (2-amino-4-hydroxy-6-pteridyl) methyl - amino -benzoyl - glutamic acid.

Table I - Various Names Given to the Anti-anemia Factor and Their Source

Subject	Source			
	Yeast	Liver	Spinach	Bacterial Growth Filtrate
Human	Wills' factor, (1931)	--	--	--
Monkey	Vitamin M, (1935)	Vitamin M, (1935)	--	--
Chicks	--	Vitamin B_c, (1939)	--	--
L. casei	Norit eluate, (1940) L. casei factor, (1943)	L. casei factor, (1943)	Folic Acid, (1941)	--
S. lactis R.	--	--	Folic Acid, (1941)	S.L.R. Factor, (1943)



Pteroylglutamic Acid (PGA)

The compound was given the name pterylglutamic acid (PGA) and was identical with the factor isolated from yeast or liver. Subsequent observations made by Wittle et al. (1947) verified the structure proposed by Angier et al. (1946).

Pfiffner et al. (1943) had originally observed that the factor crystallized from water as orange coloured spherulites, which, after repeated recrystallization, separated into thin, yellow spear-shaped platelets. The crystals did not melt below 360°C but darkened and charred about 250°C. Stokstad (1943) noted that when attempts were made to crystallize the substance from alcohol, it separated from solution as a yellow, gelatinous mass. These properties were used by Angier et al. (1945;1946) to show that the synthetic and natural compounds were identical.

2.2 Stability

Various groups, including Snell et al. (1940), Hutchings et al. (1941), and O'Dell & Hogan (1943), found that crude preparations were labile to acid, light, heat and oxidizing agents, but were stable in moderate concentrations of alkali. These observations were verified by Angier et al. (1945;1946) and Pfiffner et al. (1947) for the synthetic preparation. Biamonte & Schneller (1950) found that PGA was less stable at an acid pH in the presence of riboflavin, thiamine, niacin and panthenol.

2.3 Solubility

Hutchings et al. (1941) and O'Dell & Hogan (1943) found the growth factor to be insoluble in ether, chloroform, pyridine, ethanol, butanol and acetone. On the other hand, Pfiffner et al. (1947) observed that the crystalline compound was slightly soluble in methanol, less so in ethanol and butanol, but relatively soluble in pyridine. All groups confirmed its solubility in acetic acid and phenol. Pfiffner et al. (1947) found that the solubility in aqueous solution at pH 3.0 and 25°C was 16 mcg. per ml. and that at 100°C it was 1000 mcg. per ml. Stokstad et al. (1948) reported a solubility of 10 mcg. per ml. at 2°C, which increased to 500 mcg. per ml. at 100°C. Beamonte and Schneller (1950) were unable to dissolve more than 25 mcg. per ml. of water at 25°C after 48 hours equilibration; and a sodium phosphate citric acid buffer at pH 5.0 was found to dissolve not more than 80 mcg. per ml. When the temperature was increased to 95°C, the solubility in water increased to 200 mcg. per ml.

2.4 Precipitants

Complete precipitation of the chick growth factor was accomplished with phosphotungstic acid by O'Dell & Hogan (1943), the completeness of precipitation being judged on the basis of microbiological assays. Various other groups, e.g. Snell et al. (1940), Hutchings et al. (1941), Mitchell et al. (1941) and O'Dell & Hogan (1943), used the insolubility of the lead, mercury, copper, zinc, silver, cadmium, barium, calcium and nickel salts to concentrate the crude factor. Hutchings et al. (1941) stated that there was a measure of selectivity when zinc salts were used as precipitant.

2.5 Absorbants*

Absorption of the factor (later shown to be PGA) occurred at an acid pH while elution was accomplished by washing with a more alkaline solution. Norite and fuller's earth were used extensively by Hogan & Parrott (1939), Snell & Peterson (1940) and Hutchings et al. (1941). Other absorbants, such as superfiltrol, aluminium hydroxide, aluminium oxide, anthranilic acid, charcoal and amberlite IR 4 were used to a limited extent by Hutchings et al. (1941), O'Dell & Parrott (1943) and Pfiffner et al. (1943). The separation of aminopterin from PGA using the anion exchanger, Dowex 1, was reported by Heinrick et al. (1953). Considerable difficulty was experienced by all groups in obtaining high yields because of the instability of PGA.

2.6 Absorbance Spectra

The characteristic changes in absorbance in the near ultraviolet region that occurred with changes in pH of the solvent medium was an important factor in solving the structure of PGA. Stokstad (1943), Bloom et al. (1944) and Mitchell (1944) pointed out the similarity of the spectrum to that of flavins, alloxazines and pterins. Stokstad (1943) observed changes of maximal absorbance with changes in the pH of the solvent. For example, in 0.1 N hydrochloric acid one maximum occurred at 290 mμ. At pH 7.0 the maximum shifted to 280 mμ with an increased absorbance, while a shoulder appeared at 365 mμ. When the solvent was 0.1 N sodium hydroxide, three maxima occurred, one at 250 mμ, a second at 285 mμ and a third at

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* The more general term absorbant has been used instead of adsorbant because some of the agents presumably acted by ion exchange rather than by adsorption.

365 mμ. The $E^{1\%}_{1\ cm}$ values for the maxima occurring at an alkaline pH for both the synthetic and isolated substance as reported by various other groups are given in Table II.

2.7 Biological Methods

The first procedure for determining the relative concentration of various sources of the anti-anemia and growth factor was described by O'Dell & Hogan (1943). This was a chick bioassay in which the recovery of red cell volume of anemic birds was used as the response.

A microbiological procedure in which the pure crystalline compound was used as a reference standard was described by Telply & Elvehjem (1945). Collaborative assays under the chairmanship of Flynn (1948;1949;1950) led to the recommendation that Streptococcus faecalis R rather than Lactobacillus casei be used as the test organism. The reference standard used by the collaborators was "Folvite", (Lederle), a brand of PGA.

2.8 Chemical Method

A rational chemical determination was not possible until Angier et al. (1946) had described the chemical structure of the factor. They used the Bratton & Marshall reaction (1939) for free amines qualitatively in order to follow the intermediate steps during synthesis. Hutchings et al. (1947) described a quantitative procedure using the same reaction. Essentially the procedure was as follows: PGA in the presence of acid and zinc dust was split to give a free amine (para-aminobenzoyl glutamic acid) and a pterin. The PABG was then coupled with N-(1-naphthyl)-ethylenediamine dihydrochloride to give a pink coloured complex with a maximal absorbance at 550 mμ. The difference in absorbance for the coloured complex of a

Table II - A Summary of the $E^{1\%}_{1\ cm}$ Values for PGA as Given by Several Groups

Source	Solvent	Conc.	, mμ	$E^{1\%}_{1\ cm}$	Reference
Liver	0.1 N NaOH	-	256 283 365	565 550 195	Angier et al. (1946)
Synthetic	0.005N NaOH	-	256 283 365	542 531 194	Doube and Bombas (1950)
Synthetic	NaOH, pH 11.1	0.001%	256 283 365	578 559 194	Flynn (1950)
Synthetic	0.1 N NaOH	0.0015% w/v	256 283 368	550 530 190	British Pharmacopoeia (1953)
Synthetic	0.1 N NaOH	0.0015% w/v	256 283 369	- - -	U.S.P. XV (1955)

reduced and unreduced portion of the sample was proportional to the PGA content over the range of the concentration range used. PABA was chosen as a reference standard because it was readily available and gave the same molal colour. The PGA content of a sample as calculated from PABA was based on the molecular factor 3.22 together with the appropriate dilution. Correlation between the chemical value and the biological value was obtained only with the active isomer. An excess of gelatin was added before the cleavage reaction of PGA to protect the free amine (PABG) from further cleavage. The accuracy of the procedure was limited to that of the Bratton & Marshall (1939) reaction, about $\pm$ 2 per cent. It was not recommended for samples that contained less than 5 per cent PGA.

The procedure was adopted by United States Pharmacopeia (1950) and British Pharmacopoeia (1953), although the latter used zinc amalgam in preference to zinc dust for the cleavage and omitted the gelatin. It was recommended for application only to concentrates of folic acid powders and tablets. The United States Pharmacopeia (1955) recommended the reaction for decavitamin preparations.

Various groups have studied the relative merits of using zinc dust or zinc amalgam as a reductant and also the effectiveness of gelatin in providing a protective action on PABG during the reduction step. Hutchings et al. (1947) commented that zinc amalgam was satisfactory as a reductant, although the reducing time had to be extended to thirty minutes. They quoted Stokstad et al. (1947) as the original reference with respect to the use of zinc amalgam but no mention of this has been found by the writer in this paper of Stokstad et al. (1947). Zinc dust was used by

Wollisch (1948) and Ganguly (1950) but zinc amalgam was preferred by Jones et al. (1950), Biamonte & Schneller (1950) and Ware & Cronheim (1950). According to Kaselis et al. (1951), recovery of PGA at high concentrations was not influenced to a significant extent either by the use of zinc dust or amalgam. However, recovery was lower with the dust as reductant at low concentrations of PGA. Ilver (1953) agreed with Kaselis et al. (1951) but preferred the zinc dust as a reductant because of the time factor. There appears to have been a general agreement that zinc dust gave somewhat lower recoveries at low concentrations of PGA because of over-reduction. Gelatin had no protective action in this respect, according to Kaselis et al. (1951) and Ilver (1953).

When the procedure was applied to tissue homogenates by Glazko & Wolf (1949) it was found necessary to use titanous chloride as the reductant in order to eliminate fallaciously high values due to the presence of adenine.

The use of PAB as a reference standard has been criticized by Kaselis et al. (1951) on the basis that the absorbance maximum at the concentrations useful for analysis for PGA is situated at a somewhat higher wavelength than for an equivalent concentration of PAB. Further, the peak absorbance of the PAB coloured complex shifted towards a lower wavelength as its concentration was increased. Over the concentration range 0.7 mcg. per ml. to 1.5 mcg. per ml., the wavelength shift was not significant. PABG had a 5 per cent greater molal absorbancy than did PAB. On this basis PABG was preferred as a reference standard. Other groups that used PABG as a reference standard included Jones et al. (1950), Biamonte & Schneller (1950) and Ware & Cronheim (1950).

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Several groups were unable to apply the Hutchings et al. (1947) procedure directly to complex multivitamin preparations. Wollisch (1950) made the following observations regarding factors that interfered:

1. The background colour changed on reduction.
2. The presence of ferrous iron inhibited the colour formation.
3. Large amounts of ascorbic acid and of B complex liver fraction gave inconsistent values.

It has been stated that the effect of background colour may be eliminated by subtracting the absorbance of the reduced and unreduced solutions from the absorbance when the coloured complex was present and then using their difference as being equivalent to the PGA content. A similar procedure was used by Jones et al. (1950), Ware & Cronheim (1950) and Kaselis et al. (1951).

Interference by ferrous iron was removed by precipitation of the iron in the presence of potassium ferricyanide. Particular care was required with this step to ensure that not more than 5 per cent of the ferricyanide is added, since under these conditions an excess will react with the coupling reagent to give a white, colloidal precipitate. Ware & Cronheim (1950) used sodium gluconate to remove the interference by ferrous salts. A soluble complex was formed but at the same time the combined action was such that it contributed significantly to the reduction of PGA. Consequently, it was necessary to carry out all determinations as quickly as possible. Jones et al. (1950) were able to remove ferrous iron, as well as oils and waxes, from the sample by extraction of the acid solution with several portions of ethyl ether.

Interference by ascorbic acid was removed by Jones et al. (1950) and Wollisch (1950) by increasing the proportions of sodium nitrite and ammonium

sulphamate. Care was required to ensure that the excess sodium nitrite was destroyed, otherwise the formation of a brown colour interfered with the determination.

When powdered liver was present, Wollisch could not determine PGA with any of his modified procedures, whereas parenteral and oral liver extracts could be readily assayed. Jones et al. (1950), by using an extraction medium of 20 per cent hydrochloric acid, observed that most of the desiccated liver in their preparations was insoluble and could be removed by filtration. Ware & Cronheim (1950), providing the PGA content was sufficiently high, were able to dilute their preparations sufficiently to overcome the liver interference by subtracting its absorbance from the absorbance of the reduced and unreduced colour complex solutions.

2.9 Fluorimetric and Polarographic Methods

Similar fluorimetric methods were described independently by Andreeva (1949) and Alfrey et al. (1949). The strong fluorescence of the permanganate oxidation product of PGA, 2-amino-4-hydroxypteridine-6-carboxylic acid, was used as the basis for a procedure to estimate PGA concentration. The method was sensitive for concentrations of PGA down to 0.01 mcg. per ml. Rigid control over the concentration of salts, and particularly of acetate and phosphate, was required as they exerted an extreme quenching effect.

A polarographic method was used by Mader & Frediani (1948) but could only be applied to fairly pure samples in the absence of iron salts.

The direct spectrophotometric estimation of PGA concentration was investigated by Ilver (1953). However, he could not obtain agreement between concentration and extinction in either alkaline or acid solutions.

2.10 Summary

Bioassays and microbiological procedures were of fundamental importance in the researches that led to the recognition and eventual separation of the vitamin, and they were of great value in assaying the biological activity of crude concentrates. However, such methods are undesirably time consuming and expensive for routine control of commercial preparations and they also call for rather skilled manipulation. There was an urgent need for some simplified method based on readily measured physical or chemical properties of the vitamin.

The procedure of Hutchings et al. (1947) was sufficiently reliable for the estimation of PGA in mixtures of relatively high concentration. The modifications described by other groups represent so many attempts to apply the procedure to complex mixtures containing less than 5 per cent of the active isomer. These modifications were successful for mixtures containing low ratios of ferrous salts, of ascorbic acid and of some liver concentrates to PGA but they were not satisfactory when higher proportions of these interfering substances were present.

The fluorimetric procedure reported by two independent groups had received a limited application and its use by other groups has not been reported. A polarographic procedure is of limited application in so far as it requires special apparatus and an exacting technique, and presents special difficulties due to the unknown interfering substances that may be present in complex mixtures.

The work described in the present thesis was undertaken with the following objectives:-

(a) to examine the possibility of estimating PGA concentrations in samples containing relatively high concentrations of PGA by means of absorbance

measurements in the near ultraviolet region of the spectrum;

(b) to devise procedures which would permit of the removal of interfering substances and the concentration of the vitamin from relatively complex pharmaceutical preparations; and

(c) to re-investigate the relative merits of PAB and PABG as standards in the colorimetric procedure of Hutchings *et al.* (1947).

Section 3.0 EXPERIMENTAL RESULTS

3.1 Selection and Preparation of PGA Reference Standard

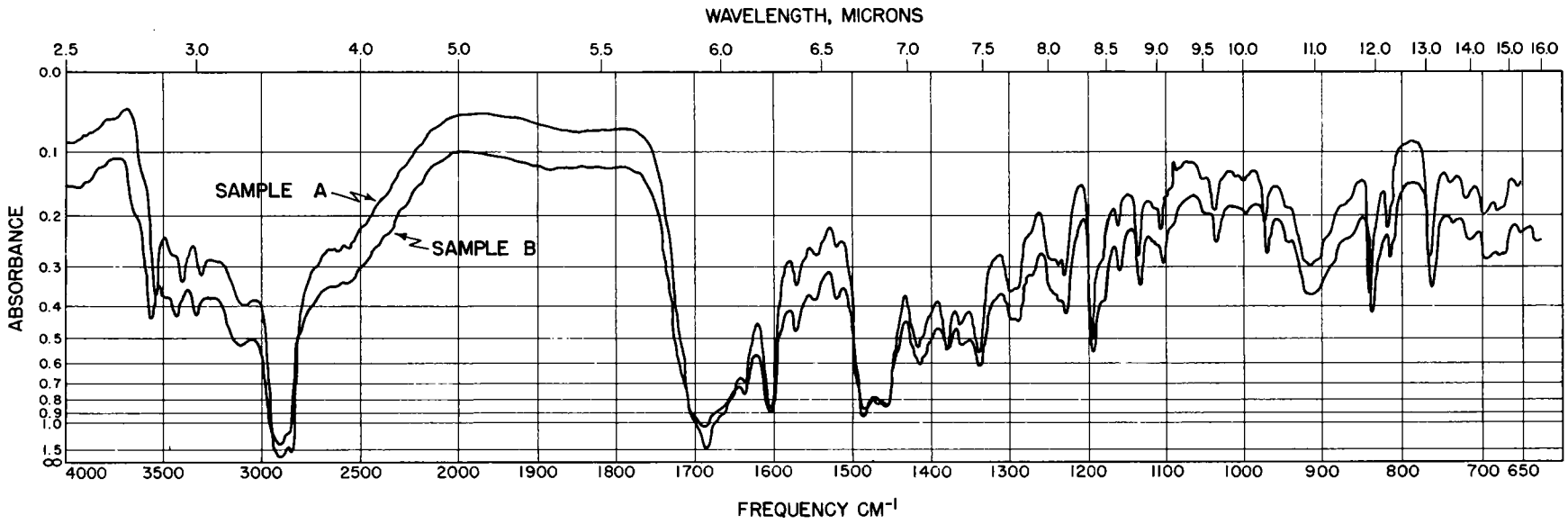
A suitable reference standard was required for the investigational work. Since there is no official source of the vitamin one had to be selected from available material. Sample A was the product of the Calco Division, American Cyanamid Company; Sample B of the British Drug Houses, (Canada). They had been packed in an actinic glass and clear glass bottle respectively and each had a metal, screw type cover.

The Pharmacopeia of the United States XIV and XV and the British Pharmacopoeia (1950) contain similar monographs on PGA. These monographs specify acceptable levels of purity as well as the assay procedures to be used in estimating such purity. These procedures were used to determine which of the two samples available was the most suitable as a reference standard.

The infra red spectrum of each was obtained on a Perkin-Elmer recording spectrophotometer, model 21, as a Nujol mull. The spectra are given in Figure 1. It will be seen that no apparent differences exist between the two samples for maximal wavelengths. Differences exist for the maximal absorbance values. They are, however, related to PGA concentration and are not indicative of difference in structure. The two samples were essentially the same on a chemical basis.

The moisture content was determined by dissolving 50.0 mg. of each sample in separate 25.0 ml. portions of a 1.0 per cent, w/v, potassium hydroxide, absolute methanol solution, then titrating with Karl Fischer reagent. A Fisher titrimeter was used to determine the end point. The insolubility of PGA in non-aqueous solvents caused some difficulty in

Figure 1 - The Infra Red Spectra of Two Sources of PGA Concentrate


WAVELENGTH, MICRONS
2.5
3.0
4.0
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
11.0
12.0
13.0
14.0
15.0
16.0
ABSORBANCE
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.5
SAMPLE A
SAMPLE B
4000
3500
3000
2500
2000
1900
1800
1700
1600
1500
1400
1300
1200
1100
1000
900
800
700
650
FREQUENCY CM-1

obtaining a solution. Glacial acetic acid was not satisfactory since it gave erratic values for its own water content. Reagent grade potassium hydroxide pellets and absolute methanol were found to be satisfactory as a solvent medium.

The $E^{1\%}_{1\ cm}$ values were calculated for 365 mμ, with 0.1N sodium hydroxide as the solvent, from absorbance values determined on a Beckman spectrophotometer, model DU.

Each sample was assayed for PGA content by the procedure outlined in Pharmacopeia of the United States XIV.

All values relating to the previous determinations are listed in Table III. The results indicate that the samples were essentially of the same purity. Sample A was selected as the reference standard for all subsequent spectrophotometric determinations on synthetic and commercial preparations. Calculations were adjusted to the 100 per cent purity level on the basis that the standard was 94.7 per cent pure.

3.2 Development of the Ultra Violet Method

3.2.1. The Effect of pH on PGA Absorbance

Solution A was prepared by dissolving 100 mg. of PGA reference standard in approximately thirty ml. of 0.1 N sodium hydroxide then diluting to 100 ml. with distilled water. Solution B was prepared by transferring 1.0 ml. of solution A to a beaker containing forty ml. of 0.1 N sodium hydroxide. The pH was accurately adjusted with five per cent, w/v, sodium hydroxide or five per cent, v/v, hydrochloric acid to 11.0 using a Beckman pH meter, model G. This solution was quantitatively transferred to a fifty ml. volumetric flask and diluted to volume with distilled water. One half of solution B was adjusted to pH 1.0 using a few drops of concentrated

Table III - A Summary of Some of the Chemical and Physical Characteristics of Two Sources of PGA Concentrate

Sample	Per Cent Purity	Per Cent Free Amine	Per Cent Water	$E^{1\%}_{1\ cm}$	Colour
A	94.7	2.4	1.9	193.2	Lemon-yellow
B	93.4	3.1	2.9	191.0	Orange-yellow

hydrochloric acid and was labelled solution C.

The absorbance spectra of solutions B and C for the spectral region 210 mμ to 400 mμ were obtained using a Cary recording spectrophotometer, model 11M, with distilled water as a reference solution. The spectra are given in Figure 2. Three maxima occurred at pH 11.0, namely, at 255 mμ, 285 mμ and 365 mμ. At pH 1.0, only two maxima occurred, one at 215 mμ the other at 300 mμ. The difference in absorbance at the two pH levels at 260 mμ and 365 mμ constitute a means of estimating PGA concentration in the presence of irrelevant substances whose absorbance does not change under these conditions. As the majority of the vitamins have maxima below 315 mμ, the spectral region 365 mμ was selected for further study.

3.2.2 Characteristic of PGA Absorbance Increment for the Region 340 mμ to 400 mμ Over the Range pH 1.0 to pH 11.0

PGA solutions of 20.0 mcg. per ml. at pH 11.0 were prepared as for solution B in subsection 3.2.1. A Beckman spectrophotometer, model Du, was balanced with the solution of pH 1.0 and the absorbance of the solution at pH 11.0 was estimated at the following wavelengths: 340 mμ, 350 mμ, 360 mμ to 370 in steps of one mμ, 380 mμ, 390 mμ, 400 mμ and 410 mμ.

The absorbance increments were plotted against wavelength and the data are presented in diagrammatic form in Figure 3. The portion of the spectrum covered exhibits features characteristic of PGA under the specified conditions. The absorbance increment increases sharply from a value of nil at 340 mμ to the region of 360 mμ, at which region the curve flattens out to give a plateau extending from approximately 360 mμ to 370 mμ. Thereafter the curve falls off smoothly to about 400 mμ, where there are indications of a slight inflection. Any deviations from the foregoing

Figure 2 - The Spectra of PGA Solutions at pH 1.0 and at pH 11.0 for the 200 mμ to 410 mμ Region

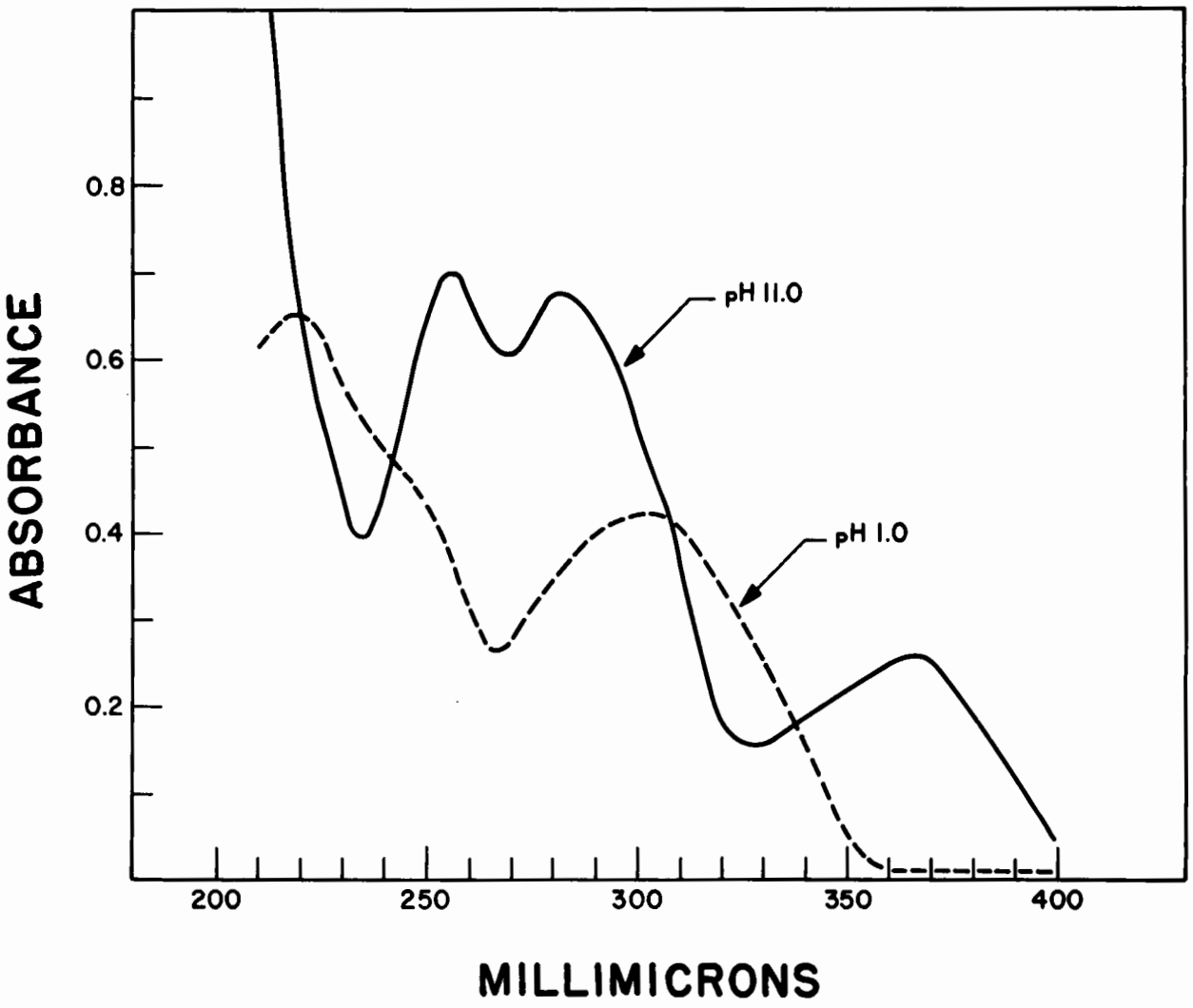
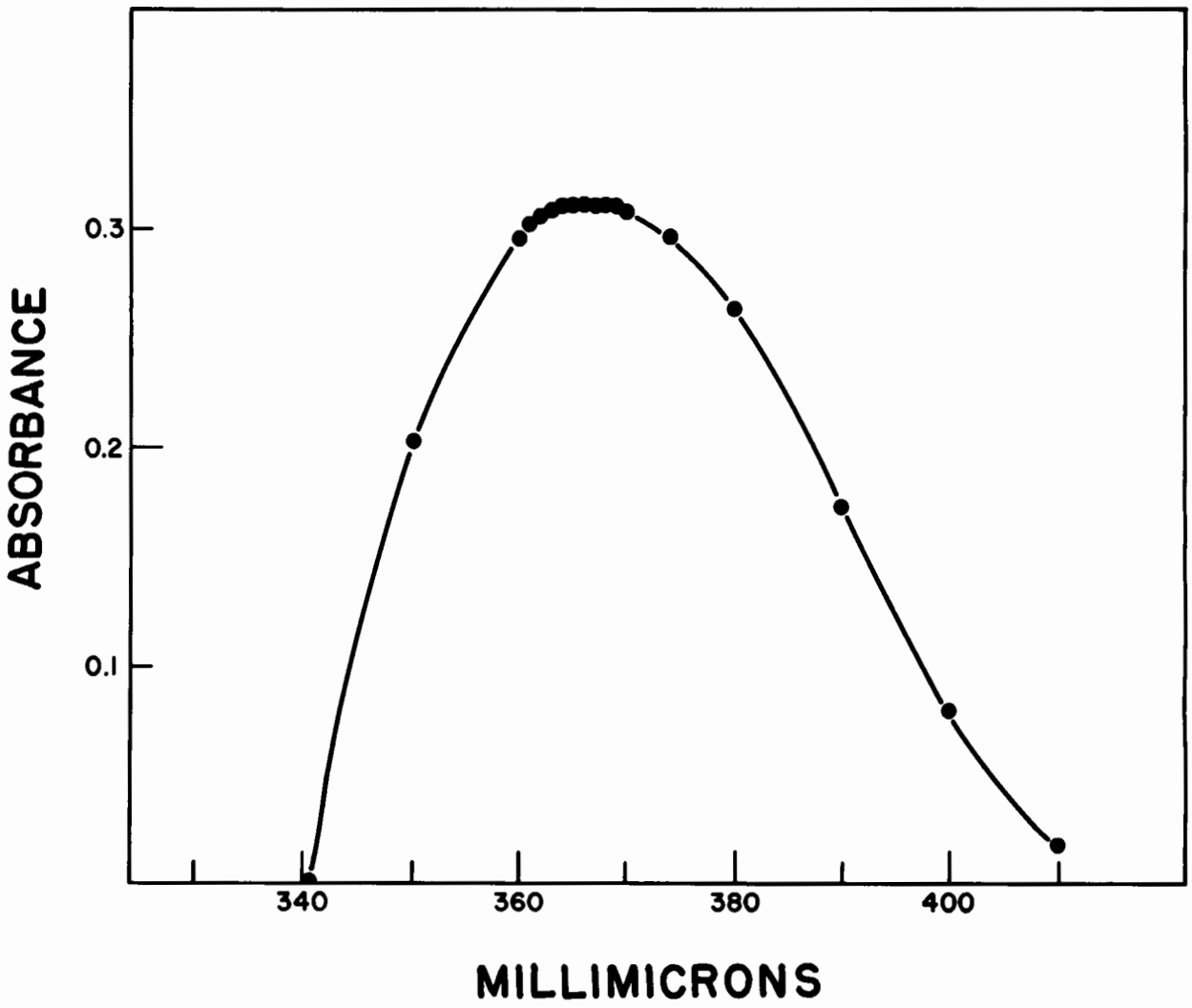

ABSORBANCE
0.8
0.6
0.4
0.2
pH 11.0
pH 1.0
200
250
300
350
400
MILLIMICRONS

Figure 3 - The Absorbance Difference Spectrum of Solutions of PGA at pH 1.0 and pH 11.0 for the 340 mμ to 410 mμ Region


ABSORBANCE
0.3
0.2
0.1
340
360
380
400
MILLIMICRONS

characteristic features were taken as indicative of the presence of irrelevant absorbance, and hence as providing a means of evaluating the reliability of any particular procedure for estimating the PGA content of either known or unknown materials.

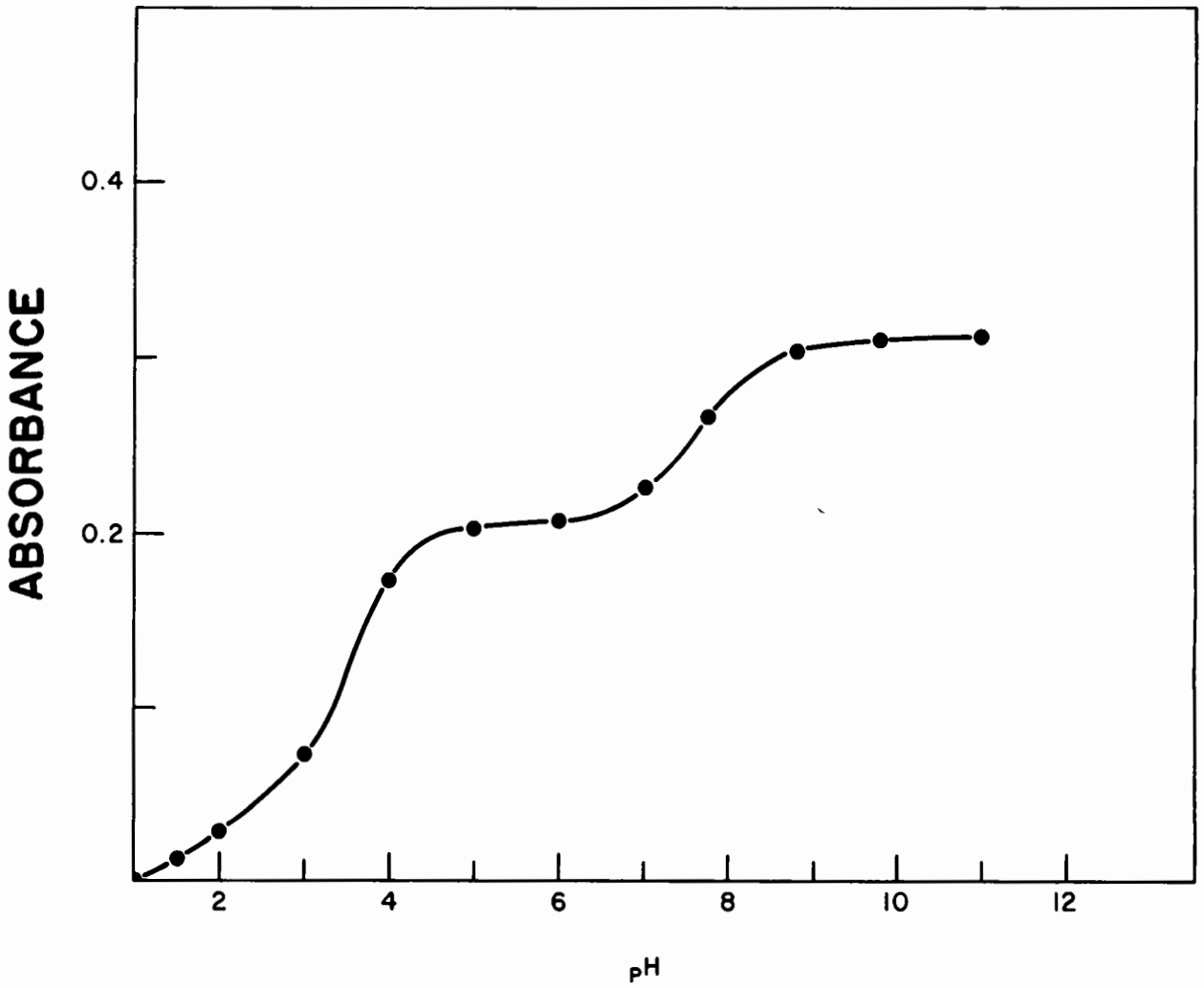
3.2.3 The Effect of pH of PGA Absorbance Increment at 365 mμ

It was necessary to select the pH at which the absorbance increment would be most sensitive in estimating PGA concentration. Twelve separate solutions, whose PGA concentration was 20.0 mcg. per ml., were prepared from solution A, subsection 3.2.1, covering the range pH 1.0 to pH 11.0. The absorbance increment for each solution was determined at 365 mμ by the procedure outlined in subsection 3.2.2. All values obtained were plotted against their corresponding pH and a smooth curve was drawn through the points. The resultant curve is shown as Figure 4. With increased pH there was an increase in absorbance. Plateaus occur between pH 4.0 to pH 6.0 between pH 9.0 and pH 11.0. The sensitivity is greatest between pH one and eleven. The pH must be carefully controlled in the region of pH 1.0 in order to ensure reproducibility of the results. Furthermore, while the increment in absorbance from pH 1.0 to pH 5.0 and from pH 5.0 to pH 11.0 is less sensitive, it offers the greater possibility that the absorbance of irrelevant substances will not change for these more restricted regions.

Because of the increased sensitivity, the extreme range from pH 1.0 to pH 11.0 was selected for further study.

3.2.4 Effect of PGA Concentration and the Pteridine and p-amino Benzoyl Glutamic Acid Functions of PGA on the Absorbance Increment at

Figure 4 - The Effect of pH on the Absorbance of PGA at 365 mμ


ABSORBANCE
0.4
0.2
2
4
6
8
10
12
pH

365 mμ

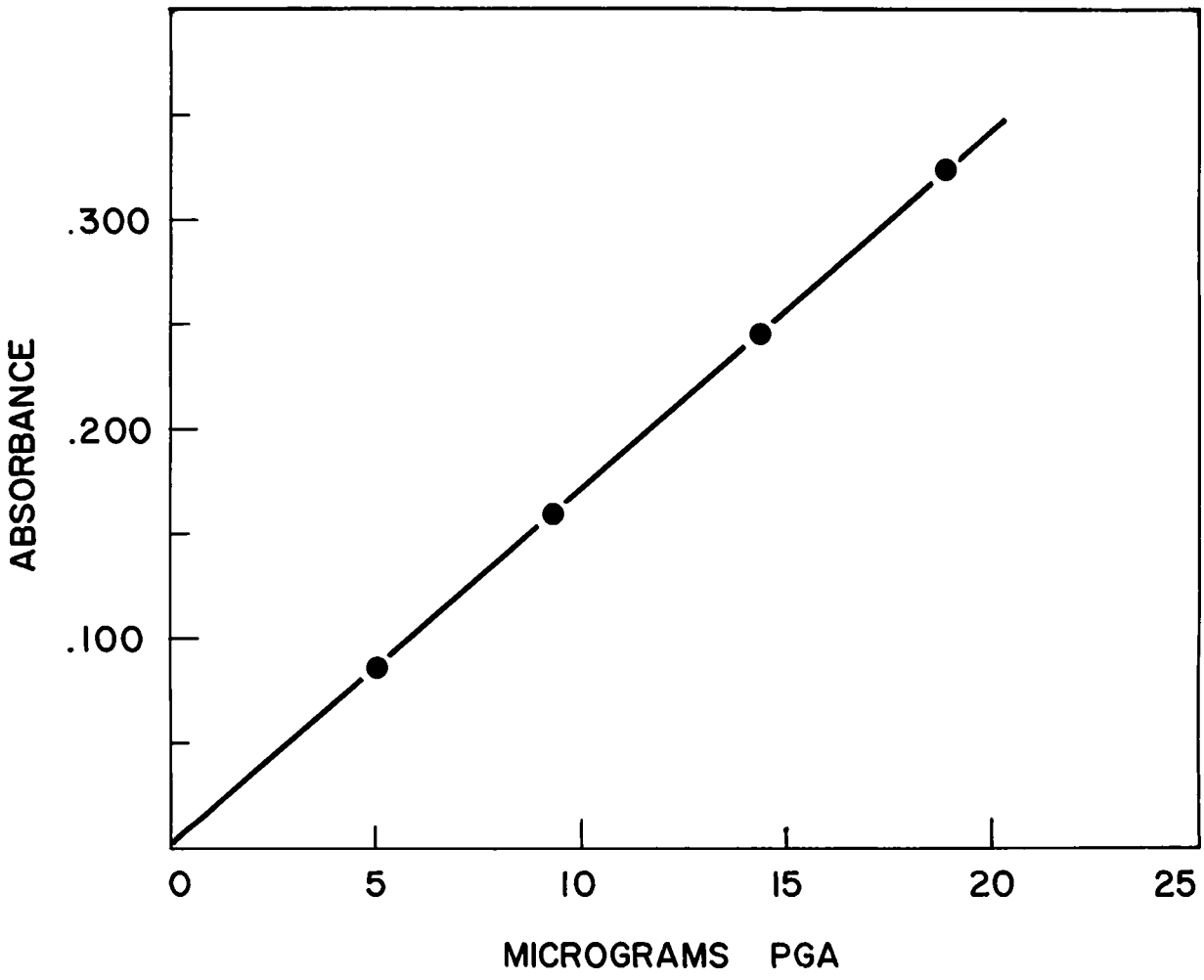
In order to verify the validity of an absorbance increment reading at one concentration level of a PGA standard as the basis for calculating the concentration of an unknown, observations were carried out at several levels. On the assumption that the response of the spectrophotometer was linear, information regarding Beer's law was obtained by the following procedure.

Appropriate aliquots of solution A, subsection 3.2.1, were diluted to four concentration levels of 5.0, 10.0, 15.0 and 20.0 mcg. per ml., and all at pH 11.0. Portions of each were then adjusted to pH 1.0 with a few drops of concentrated hydrochloric acid and the absorbance increments of the alkaline solutions determined as in subsection 3.2.2. The resulting values were plotted against concentration in Figure 5.

A linear response is obtained for the concentrations observed and can be extrapolated to pass through the origin. It is therefore valid to use, as the basis for calculating the concentration of an unknown from its absorbance increment, its relationship to the absorbance increment of a reference standard of known concentration; providing, that is, that irrelevant absorbance is not present. Under these conditions, the absorbance increment of a twenty mcg. per ml. solution of PGA was 0.338 and is within the most sensitive portion of the spectrophotometer absorbance scale. For this reason the following relationship was used as the basis for calculating the concentration of unknowns from their absorbance increments:

$$\frac{\text{absorbance increment of unknown}}{0.338} \times 20$$

Figure 5 - The Effect of PGA Concentration on the Absorbance Increment at 365 mμ for Solutions at pH 1.0 and pH 11.0


ABSORBANCE
.300
.200
.100
0
5
10
15
20
25
MICROGRAMS PGA

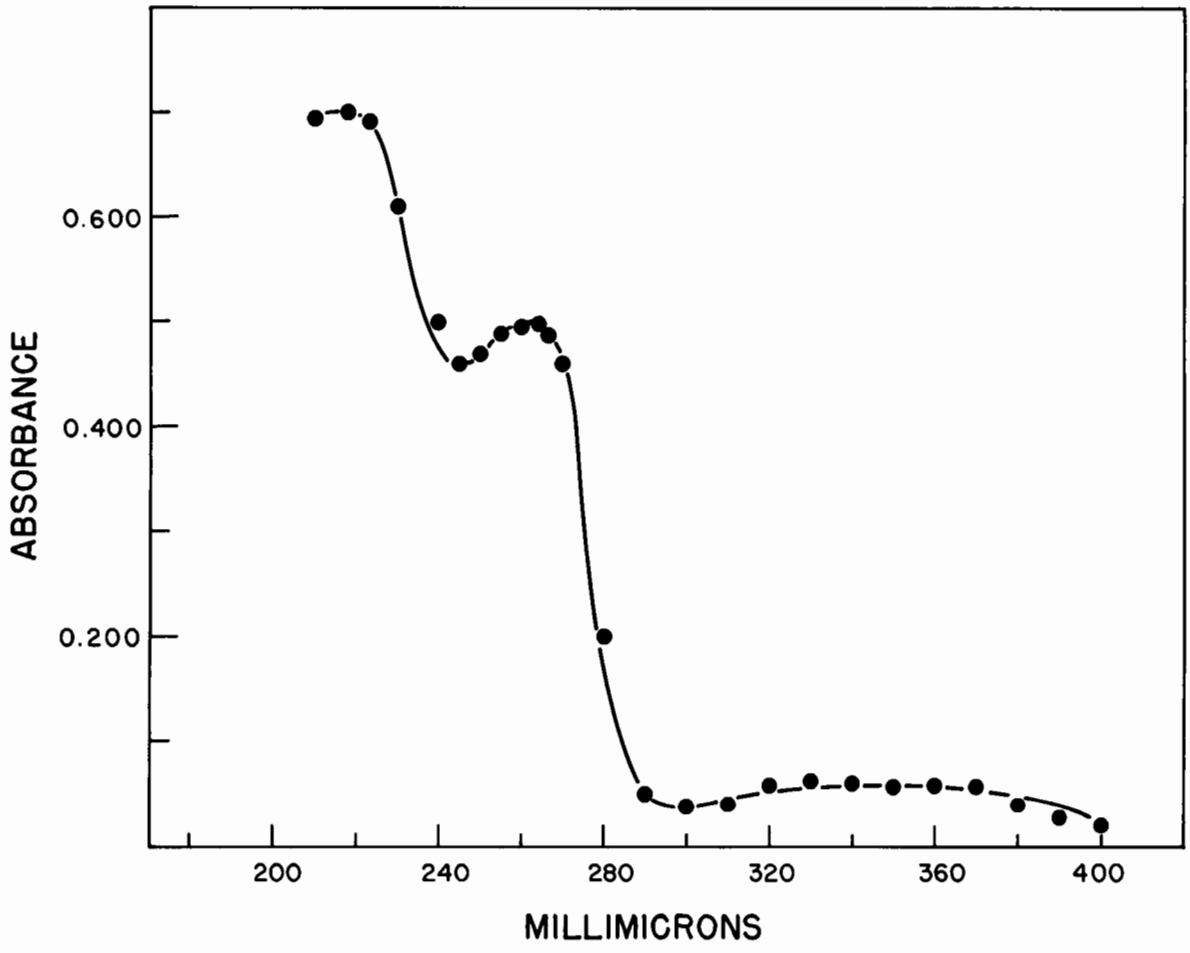
Since the decomposition products of PGA are not biologically active, it was necessary to ascertain the degree to which they might interfere with the determination of PGA by the absorption increment. An aliquot of solution A, subsection 3.2.1, was subjected to reduction by zinc dust and hydrochloric acid as outlined in U.S.P. XIV. An aliquot of the resultant solution was diluted so that its equivalent PGA concentration was 20 mcg. per ml. The absorbance increment was then determined at 365 mμ and was found to be 0.005. However, it should be remarked here that above pH 3.0 there is a heavy precipitation of zinc hydroxide. In addition the spectral characteristics of the solution at pH 1.0 were obtained on a Cary Recording Spectrophotometer, model 11 for the range 210 mμ to 400 mμ and using a reagent blank as the reference solution. The data recorded are presented in Figure 6. The characteristics of this curve are discussed in Section 4.0

3.2.5 The Estimation of PGA by the Absorbance Increment Method in Some Commercial Preparations

A few commercial preparations containing PGA in mg. quantities were available. They consisted of two tablet preparations, two injectable preparations and one elixir preparation. Mineral salt and vitamins that could be expected to interfere were not present. The liver concentrate contained in one of the injectable preparations was an ether extract of whole fresh liver. Each sample was assayed by the spectrophotometric absorbance increment procedure and by the procedure described in the U.S.P. XIV for assay of folic acid. All manipulations were carried out under subdued light.

For the spectrophotometric procedure five tablets were finely ground, then extracted with approximately 20 ml. of 0.1 N sodium hydroxide by

Figure 6 - The Spectrum of the Decomposition Product at pH 1.0, Obtained by Hydrogen Cleavage of PGA


ABSORBANCE
0.600
0.400
0.200
200
240
280
320
360
400
MILLIMICRONS

shaking intermittently, over a period of ten minutes. The residue and extractant were diluted to a PGA concentration of approximately 100 mcg. per ml.

Ten ml. aliquots were taken and the pH adjusted as for solution B, subsection 3.2.1, then diluted to 50.0 ml. A portion of each was adjusted to pH 1.0 and the absorbance increments determined at 340 mμ, 350 mμ, 360 mμ to 370 mμ in steps of one mμ at 380 mμ. Appropriate aliquots of the injectable preparations and elixir were diluted and then assayed in the same manner.

The PGA content was calculated by the following formula:

$$\text{mg. PGA/tablet or cc.} = \frac{\text{absorbance increment}}{0.338} \times \frac{20}{1000} \times \text{dilution factor}$$

The results of both procedures are given in Table IV. Good agreement was found between methods except in the case of liver extract injectable. It assayed below label claim by the U.S.P. XIV procedure but met label claim by the absorbance increment method. This difference could be explained by any one or a combination of the following reasons:

1. PGA has cleaved during storage and handling to give products that have an absorbance increment similar to that of the parent compound. This finds some support from the high free amine correction which was observed with the U.S.P. XIV method.

2. Substances present in liver have an absorbance increment similar to that of PGA. Any absorbance increment observed at 340 mμ would indicate the presence of such substances. Of the five samples assayed, the liver extract injectable had an appreciable absorbance at 340 mμ, thus indicating irrelevant absorbance adjacent to the maximum for PGA.

3. Substances present in liver react preferentially with the reagents of

Table IV - A Comparison of the Estimated PGA Content of Some Commercial Concentrates by the Absorbance Increment and United States Pharmacopoeia XIV Procedures

Sample	Labelled Content Mg./cc. or Tablet	Estimated Content Mg./cc. or per Tablet		
		U.S.P. XIV Net	Gross	Absorbance Increment
Tablet # 1	5.0	6.5		6.4
Tablet # 2	5.0	6.1		5.8
Elixir	5.0/4 cc.	5.8		5.8
Liver Ext. Injt.	5.0	3.2	5.2	5.0
Injectable	15.0	16.7		16.6

the colorimetric reaction or are destroyed by the reduction step. This would mean a lower total amine value, a true free amine value and consequently a low value for the PGA content.

3.2.6 Effect of Other Vitamins on the Absorbance Increment

The effect of thiamine, riboflavin, niacin, niacinamide, vitamin C, pyridoxine, calcium d-pantothenate and vitamin B_{12} on the absorbance increment was studied for each vitamin alone, with PGA, and together. The ratio of PGA to other vitamins was similar to that usually found in commercial preparations.

Separate solutions of each vitamin in mg. per ml. were prepared as follows: PGA 1.0; thiamine 3.0; riboflavin 2.5; niacin 30.0; niacinamide 30.0; pyridoxine 5.0; Ca d-pantothenate 5.0; vitamin C 100.0; vitamin B_{12} 0.005. Using one ml. aliquots of these solutions a seconal series was made consisting of each vitamin alone, each with PGA and all together. The pH and final volume were the same as solution B, subsection 3.2.1. The absorbance increments were determined and expressed as a percentage of the absorbance increment for the PGA solution alone. By this method of calculation the effect, if any, on the recovery of PGA estimated by the absorbance increment method was readily demonstrated.

Table V lists the apparent PGA content contributed by other vitamins and their ratio of concentration to that of PGA. Riboflavin and vitamin C interfere seriously; fallaciously high recoveries of PGA were experienced in their presence. Ca d-pantothenate gave some irrelevant absorbance, while the other vitamins had no effect. It is obvious that if the absorbance increment is to be measured between the pH limits 1.0 to 11.0 then interference by vitamin C and riboflavin must be removed or, if possible,

Table V - The Apparent PGA Concentration Contributed by Other Vitamins in Synthetic Mixtures

Vitamin	PGA to Vitamin Ratio	Contribution in Per Cent	
		Alone	with PGA
Thiamine	1:3	0	0
Riboflavin	1:2.5	16	29
Niacin	1:30	0	0
Niacinamide	1:30	0	0
Pyridoxine	1:5	0	0
Ca Pantothenate	1:5	1	2
Vitamin C	1:100	5	15
Vitamin B	1:0.005	0	0
All Vitamins	--	-	33

a correction must be made for their presence.

3.2.7 Effect of Ferrous Sulphate on the Absorbance Increment

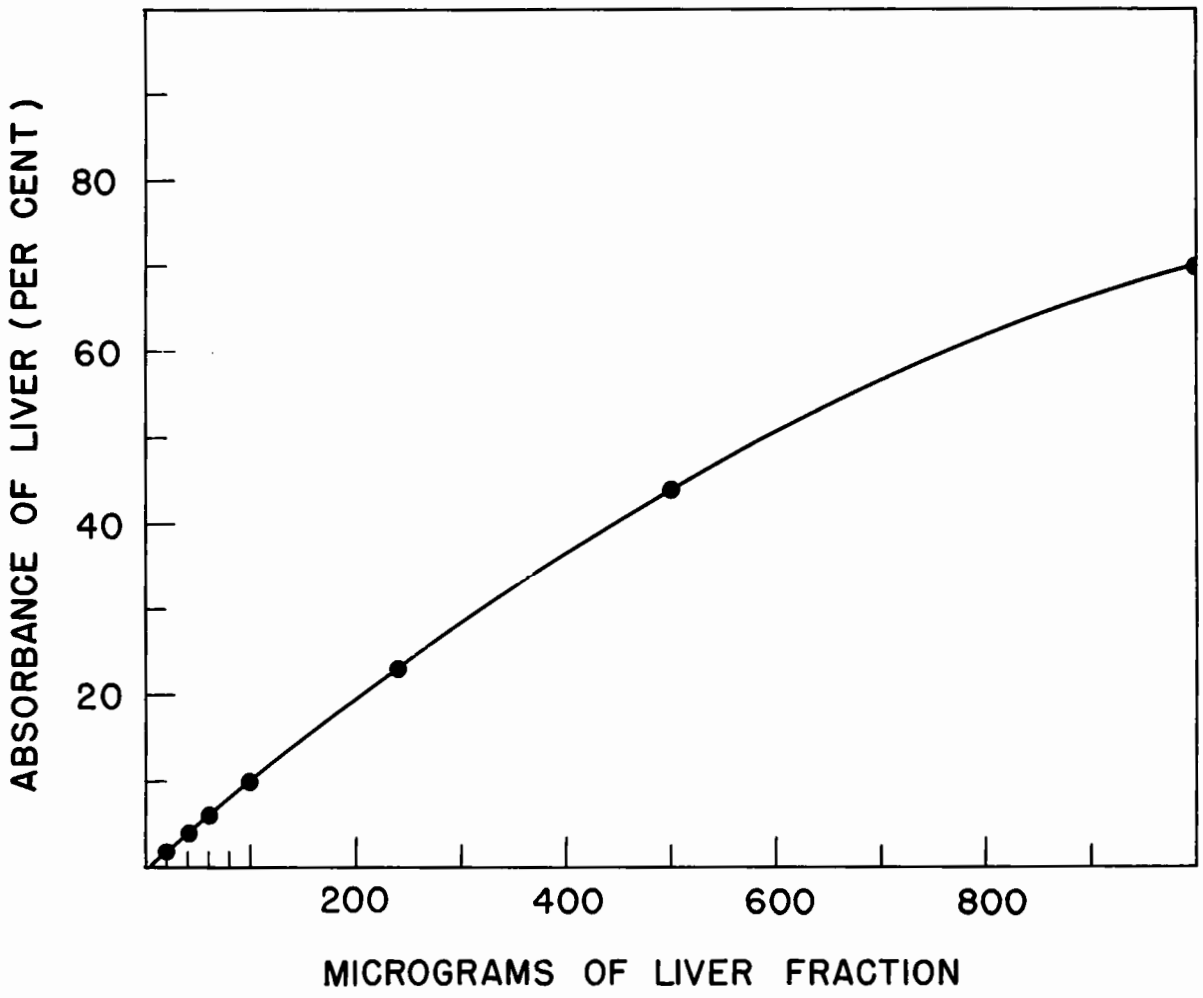
Many multi-vitamin preparations contain a high proportion of ferrous sulphate, usually in the ratios of one to twelve or one to three hundred fifty. Attempts to recover PGA from solutions containing such concentrations of reduced iron were not succesful. Recoveries were very low, probably due to the vitamin being absorbed or occluded by the flocculent precipitate above pH 5.0 or destroyed at some point in the manipulation steps. Repeated washing of the flocculent precipitate of ferrous hydroxide did not increase the yield to any appreciable extent. On the basis of these observations it was clearly necessary to remove ferrous sulphate before attempting to apply the spectrometric method.

3.2.8 Effect of Liver Concentrate on PGA Absorbance Increment

In this study the dried liver powder used was "Liver Fraction NF". A solution containing 50 mg. per ml. in 0.1 N sodium hydroxide was prepared. Aliquots of this solution were diluted with distilled water to give five concentration levels of 1.0, 2.0, 3.0, 12.5 and 25.0 mg. per ml. One ml. portion was then added to solution B, as outlined in Section 3.2.1, before the pH adjustment, to obtain the required proportion of liver fraction to PGA. The absorbance increment values were expressed as a percentage of that for a PGA reference standard taken through the same procedure at the same concentration and were illustrated graphically as Figure 7.

It is apparent that liver concentrate seriously interferes with the proposed method. As with riboflavin, vitamin C and ferrous sulphate, liver concentrate must be removed from solutions containing PGA before the absorbance increment is determined for the purpose of estimating PGA, except

Figure 7 - The Per Cent Contribution to the Absorbance Increment of PGA of Increasing Amounts of Liver Fraction NF2 at 365 mμ


ABSORBANCE OF LIVER (PER CENT)
80
60
40
20
200
400
600
800
MICROGRAMS OF LIVER FRACTION

where the ratio is 1:1 or less.

3.2.9 Recovery of PGA at pH 2.0

Since PGA is least soluble in the range pH 2.0 to pH 4.0, the extraction of interfering substances a pH 2.0, but leaving PGA as an insoluble residue, seemed to be feasible. Consequently the recovery of PGA standard subjected to such an extraction procedure was investigated.

A buffer at pH 2.0 was prepared by mixing 59.5 ml. of M/5 hydrochloric acid with 441.0 ml. of M/5 potassium chloride, then diluting to 1000 mls. with distilled water, (Morgan & Ceprini, 1952). Six accurately weighed portions of about 10 mg. of the PGA reference standard, (sample A), were transferred to separate 40 ml. glass stoppered Maizel-Gerson reaction flasks and numbered one to six respectively. Approximately 500 mg. of powdered cellulose was added to each to act as a clarifying agent and to occlude to some extent the finely suspended standard. To the mixtures were added 5.0 ml. portions of the buffer, the whole was then vigorously shaken for two minutes followed by 2 minutes centrifuging. The supernatant was decanted into a Whatman 30, 12.0 cm. filter paper. The number of extractions to which each sample was subjected were as follows:

1. Sample number one; four extractions.
2. Samples number two, three and four; six extractions.
3. Sample number five; eight extractions.
4. Sample number six; ten extractions.

The residue from each, together with their respective filter papers, were transferred to separate 400 ml. beakers. To each was added approximately 300 mls. of 0.1 N sodium hydroxide, the contents were then thoroughly mixed and the pH adjusted to 11.0. These solutions were transferred to

separate 500 ml. volumetrics and diluted to volume with distilled water. The absorbance increment of each was determined and used to calculate the per cent recoveries by the following formula:

$$\text{Per cent recovery} = \frac{\text{Observed absorbance}}{0.338} \times 100$$

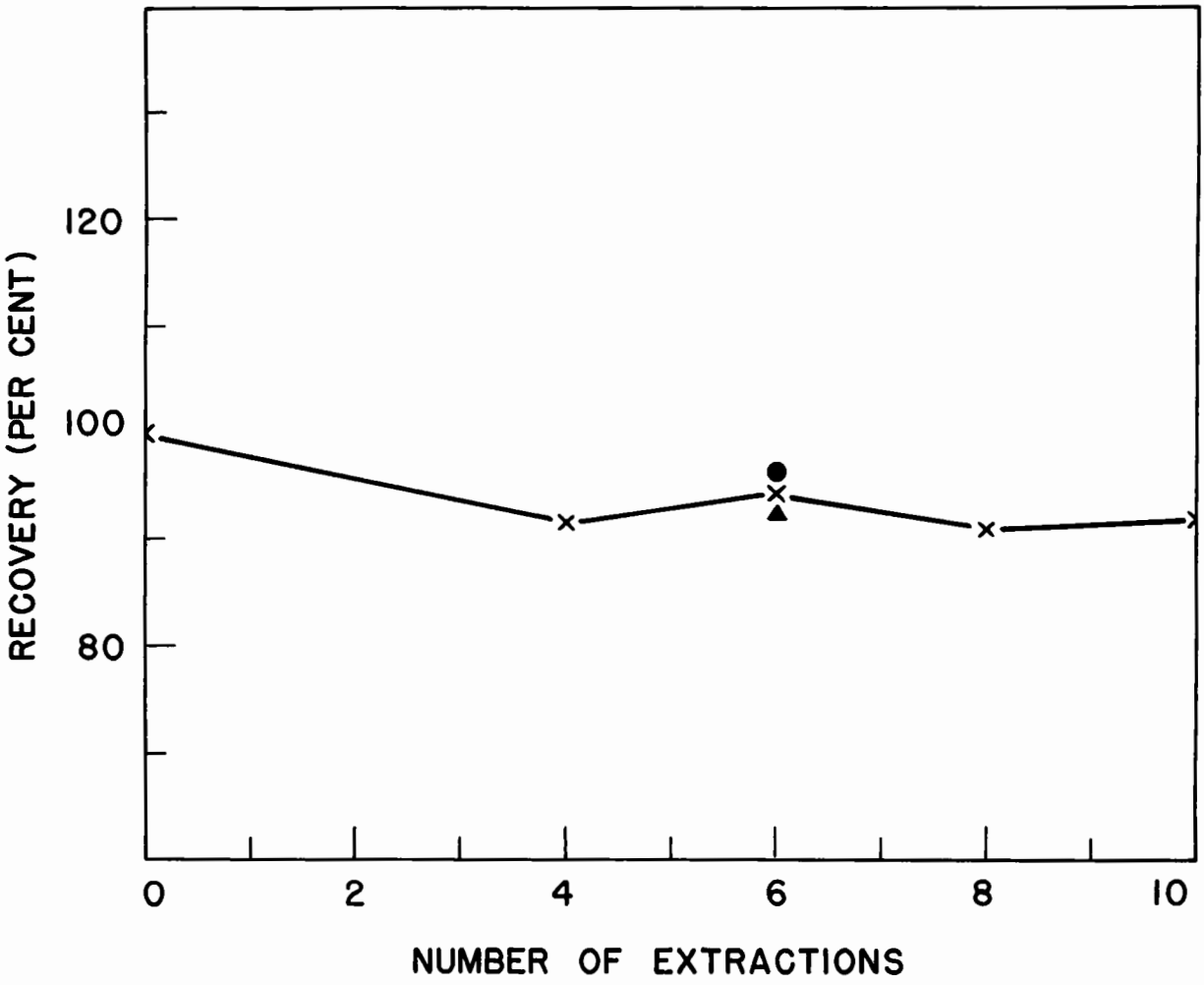
The results were expressed graphically in Figure 8. It is surprising to note that the per cent recovery remains consistent between 90 to 95 per cent with repeated extractions of the same residue. One would expect that it should decrease in a linear manner with repeated extractions. However, it is evident that by carrying a reference standard along with the unknown samples it would be possible to correct for a 5 to 10 per cent loss of PGA during the extraction procedure.

3.2.10 The Separation of PGA and Ferrous Sulphate

The separation of PGA and ferrous sulphate by extraction of the ferrous salt with five ml. portions of a buffer at pH 2.0 was investigated on a synthetic and a commercial preparation. A mixture of PGA and ferrous sulphate was prepared by thoroughly mixing small portions of anhydrous ferrous sulphate with 0.7237 g. of PGA until 149 gm. of the salt had been added. This gave a PGA to salt ratio of 1 in 206. Two samples of a commercial preparation formulated as tablets were obtained. They were said to contain 1.7 mg. of PGA and 350 mg. of ferrous sulphate as active ingredients while the remainder consisted of a filler and a red dye as an outer coating.

The PGA content of each was estimated in duplicate by the absorbance increment procedure as outlined in sub-section 3.2.9 using four extractions, and the procedure of Hutchings *et al.* (1947) as modified by Wollisch (1948).

Figure 8 - The Percentage Recovery of Pteroylglutamic Acid after Repeated 5.0 ml. Portion Extractions with Buffer at pH 2.0


RECOVERY (PER CENT)
120
100
80
0
2
4
6
8
10
NUMBER OF EXTRACTIONS

For the prepared mixture a one-gram sample was taken, while for the commercial preparation, five tablets each were used. The tablets were carefully washed free of dye with a few ml. of buffer, dried with the aid of filter paper, then finely ground in a mortar. A concentration of 20 mcg. per ml. at pH 11.0 was used in estimating the absorbance increment spectrum from 340 mμ to 380 mμ.

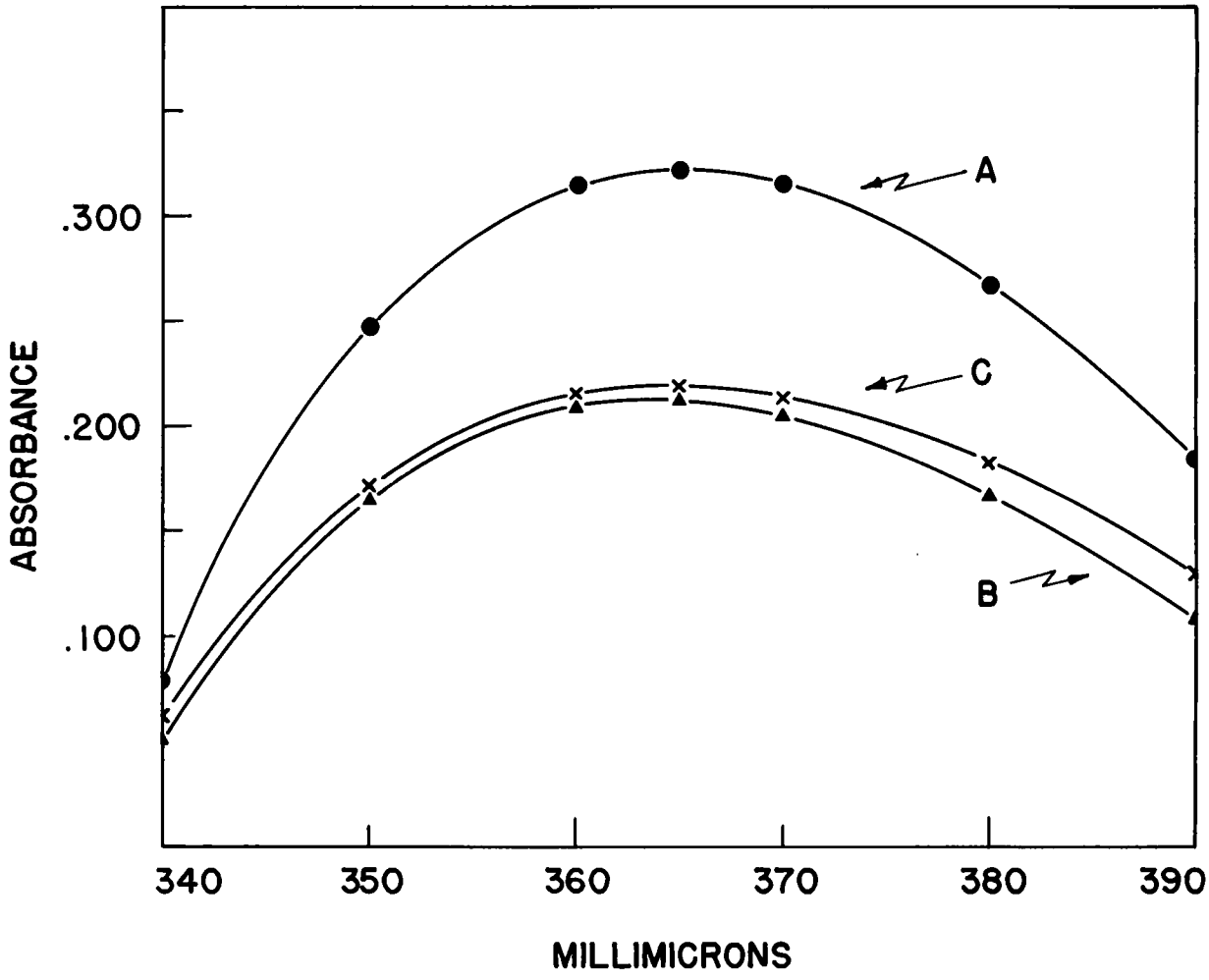
The recoveries by both methods are listed in Table VI while the absorbance increment spectra are given in Figure 9. From the date in Table VI, it can be seen that agreement between methods was good for all samples, although there was a tendency for the absorbance increment procedure to give slightly higher values. The two commercial preparations were below the content claimed on the label. In sample B the high free amine correction observed with the Hutchings et al. (1947) procedure, indicated that the product probably met labelled claim at the time it was prepared but that destruction had occurred during the handling and storage. However, sample C, the same product but a different lot, had little or no free amine connection, so that it was difficult to interpret the available data in terms of whether or not it may have met the labelled claim at the time it was compounded. Further, it is possible that complete destruction of the vitamin content, represented by the difference between claimed content and assayed content, took place so that no free amine would be found.

The spectra as found in Figure 9, indicate predominance of PGA in the extracted residue. With each sample, the maximum occurred at 365 mμ and the overall characteristic shape of the curves was similar to that of a PGA standard under the same conditions. Some irrelevant absorbance was present at 340 mμ but did not seriously interfere with the absorbance at 365 mμ for this particular product.

Table VI - A Comparison of the Estimated PGA Content of a Prepared Mixture and Some Commercial Preparations Containing Ferrous Sulphate by the Modified Absorbance Increment and the Wollisch Procedures

Sample	Ratio PGA to Iron	Labelled Content Mg./gm. or Tablet	Estimated Content		
			U.S.P. XIV		Absorbance Increment
			Net	Gross	
Mixture A	1-206	4.8	3.9	4.9	5.0
Tablet B	1-206	1.7	1.0	1.5	1.1
Tablet C	1-206	1.7	0.9	1.1	1.1

Figure 9 - The Absorbance Increment Spectra of PGA and Ferrous Sulphate Mixtures for a Prepared Mixture A and Two Commercial Tablet Preparations, B and C, Following Extraction with Buffer at pH 2.0


ABSORBANCE
.300
.200
.100
A
C
B
340
350
360
370
380
390
MILLIMICRONS

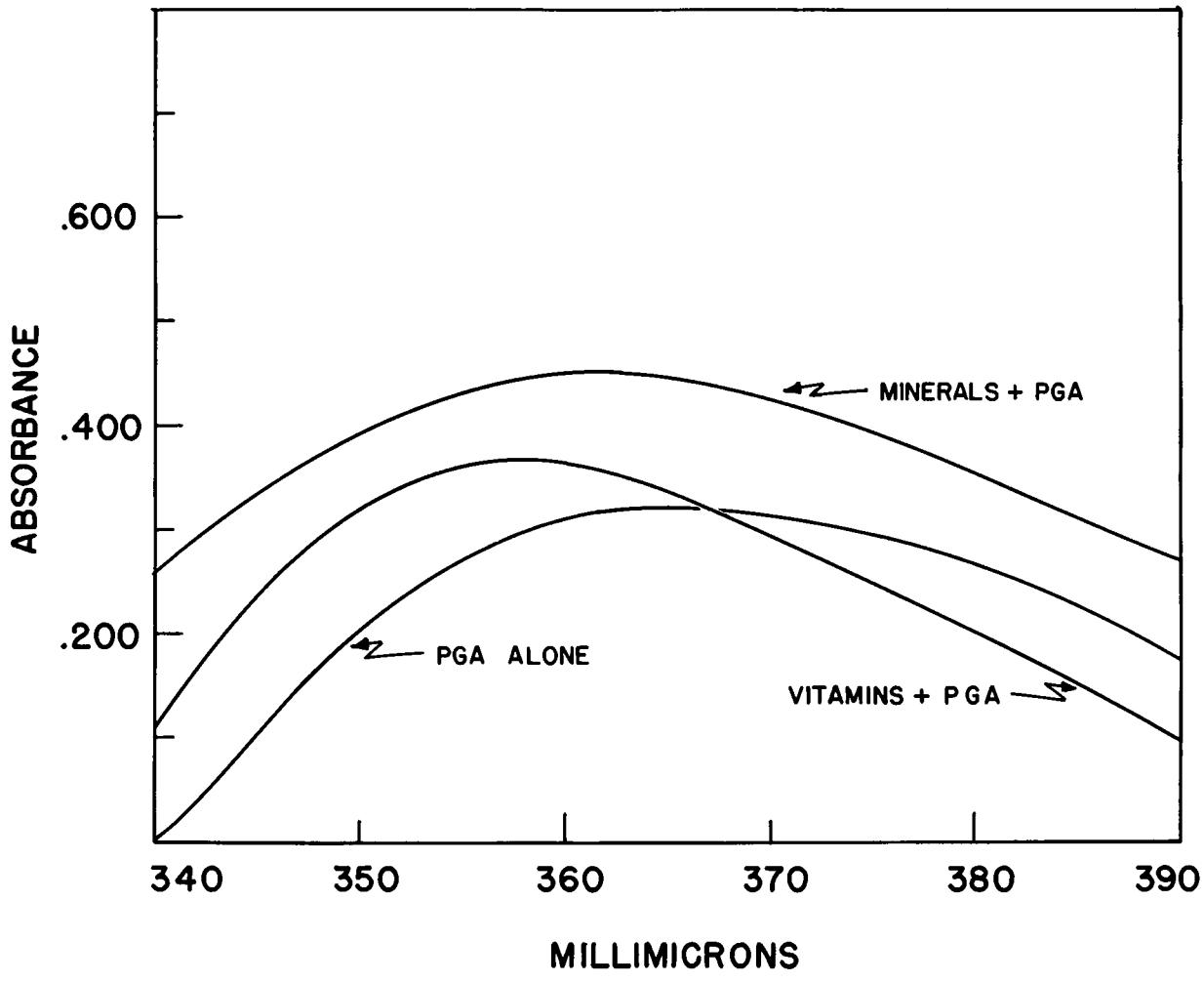
3.2.11 The Separation of PGA from other Interferences

The extraction procedure was applied to specially prepared mixtures containing PGA and minerals or other vitamins. In order to evaluate the effectiveness of the extraction technique in removing vitamins and minerals, the absorbance increment was determined on solutions of the residue for the spectral region 340 mμ to 390 mμ, using a recording spectrophotometer. Such a measurement is semi-quantitative only, but does offer a quick and effective means of indicating the efficiency of such a procedure through the overall spectral characteristics. The following samples were prepared of PGA with minerals or with vitamins in mg. quantities:

Sample A:	PGA	10.0
	ferrous sulphate	625
	di-calcium phosphate	1250
	magnesium sulphate	150
	zinc sulphate	2.5
	copper sulphate	1.3
	manganese sulphate	1.3
	potassium iodide	1.3
	cobalt sulphate	1.3
	sodium molybdate	1.3
Sample B:	PGA	10.0
	ascorbic acid	250
	niacinamide	100
	Ca d-pantothenate	25
	thiamine hydrochloride	10
	riboflavin	10
	pyridoxine hydrochloride	5
Sample C:	PGA	10.0

Each sample was subjected to the procedure as recommended for PGA in subsection 3.2.9 using four extractions. The absorbance increment spectrum was determined for the spectral region 340 mμ to 390 mμ using a Beckman recording spectrophotometer, model DK 2. The resulting spectra are given in Figure 10. It is evident that the absorbance increment would be invalid when used as the basis of calculating PGA concentration in the presence of

Figure 10 - The Absorbance Increment Spectra of PGA with Minerals or with Vitamins Following Extraction with Buffer at pH 2.0


.600
.400
.200
ABSORBANCE
MINERALS + PGA
PGA ALONE
VITAMINS + PGA
340
350
360
370
380
390
MILLIMICRONS

the minerals and vitamins used. The spectra are atypical for such mixtures. With minerals or vitamins there is both a hypsochromic and hyperchromic shift of the maximum as compared to that at 365 mμ for PGA alone. Further, the absorbance present at 340 mμ for the mixtures is strongly indicative of a high proportion of irrelevant absorbance. Although the isobestic point present for the vitamin mixture with PGA and PGA alone occurs at 365 mμ for the prepared mixture under study, this cannot be accepted as occurring with unknown mixtures of different proportions. Otherwise, the absorbance increment value observed at 365 mμ for such mixtures, would give essentially complete recovery of the added PGA.

It is doubtful whether an increase in the number of extractions would be beneficial as losses of PGA through a slight solubility would occur. This could be tolerated and corrected for in samples containing approximately five to ten mg. of PGA, but with amounts, less than this, the loss through solubility would become a considerable proportion of the total. Experience has indicated that the concentration of PGA found in multi-vitamin preparations has become on the average approximately 0.75 mg. per tablet or capsule and in some instances is 0.2 mg., while the vitamin and mineral content has increased both in quantity and number of elements involved.

3.2.12 The Separation of PGA from Liver Concentrates

The effectiveness of the extraction procedure in removing liver interference was investigated. An estimation of the PGA content and its spectrum for the region 340 mμ to 380 mμ by the procedure outlined in sub-section 3.2.11 was made on a mixture consisting of 1.5 gm. of liver fraction NF and 6.67 mg. of PGA. A 1.5 gm. sample of liver alone was treated in the same manner. Each determination was carried out in duplicate.

The spectra are given in Figure 11. They are similar except for an overall increased absorbance, particularly at 340 mμ. The values for liver alone or with PGA at 340 mμ are nearly equal. This indicates that the absorbance at this wavelength is for the most part entirely due to liver interference. On this basis, as well as on the basis of linearity exhibited by the absorbance of the liver residue, the subtraction of these values from those corresponding to liver plus PGA should give a more accurate estimation of the PGA recovery. Such spectra, for samples A and B, together with that of a PGA reference standard, are given in Figure 12. The per cent recoveries of samples A and B on this corrected basis were 110 and 101 respectively, while the overall characteristics of the spectra, except for absorbance values, are identical.

The separation of liver concentrate from PGA can be accomplished by a selective extraction of the liver at pH 2.0 on a semi-quantitative basis. Use of the absorbance increment alone as the basis of estimating PGA in samples containing liver fraction NF at the ratio of 1 to 24, is not valid as high recoveries are experienced. The proportion of interference to PGA is greatly reduced and on this basis the extraction procedure would be of value with respect to the Hutchings et al. (1947) procedure and its application to such products.

3.3 A Study of Some of the Factors Effecting the Colorimetric Procedure

3.3.1 The Effect of Liver Concentrate on and its Removal from PGA Mixtures

It had been reported by Wollisch (1950) that the colorimetric procedure could not be applied in the presence of powdered liver. This was substantiated by his experience with vitamin preparations containing a

Figure 11 - The Absorbance Increment Spectra of Duplicate Assays of Prepared Mixtures of PGA with Liver (A & B) and of Liver Alone (A, & B,) Following Extraction with Buffer at pH 2.0

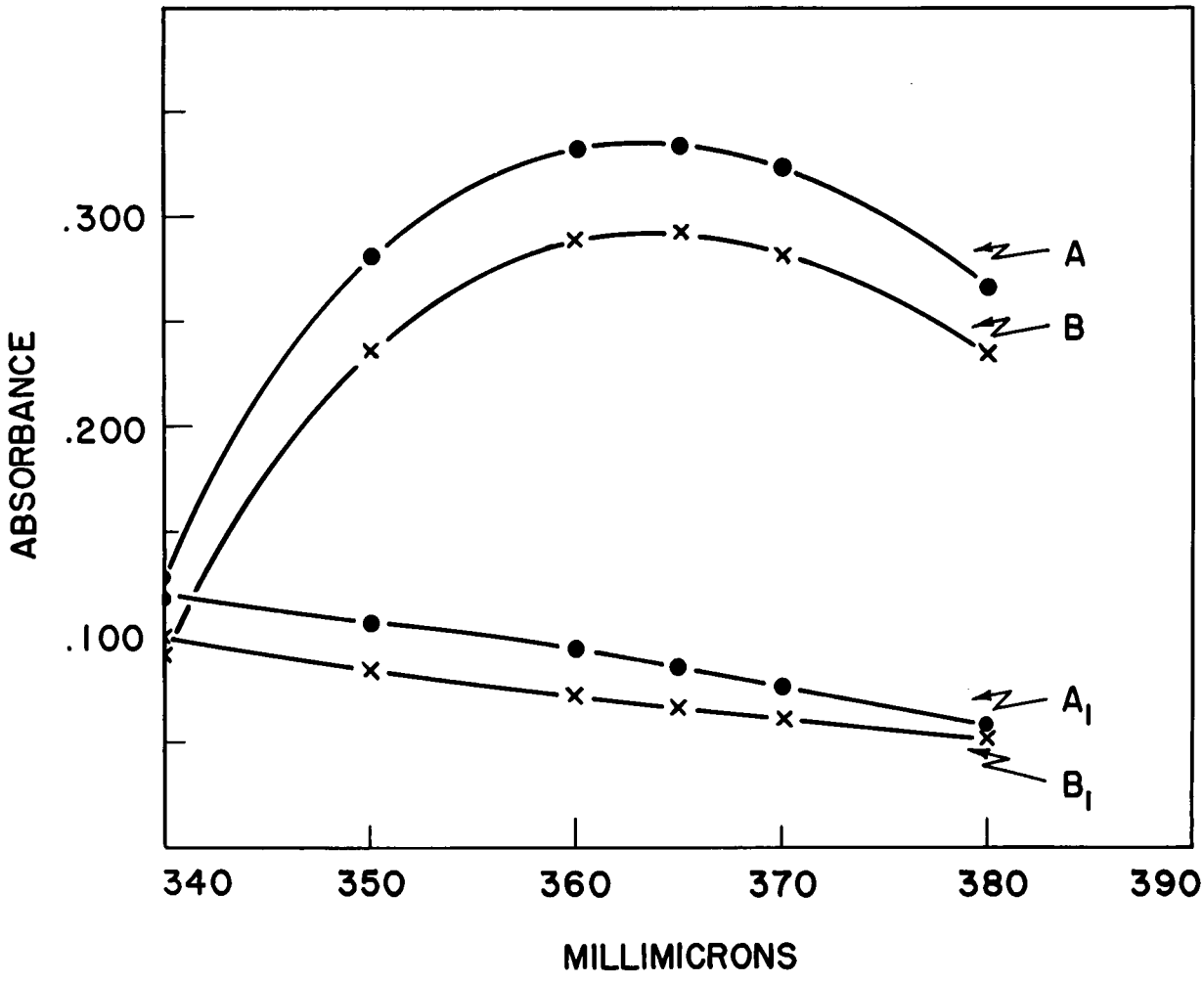
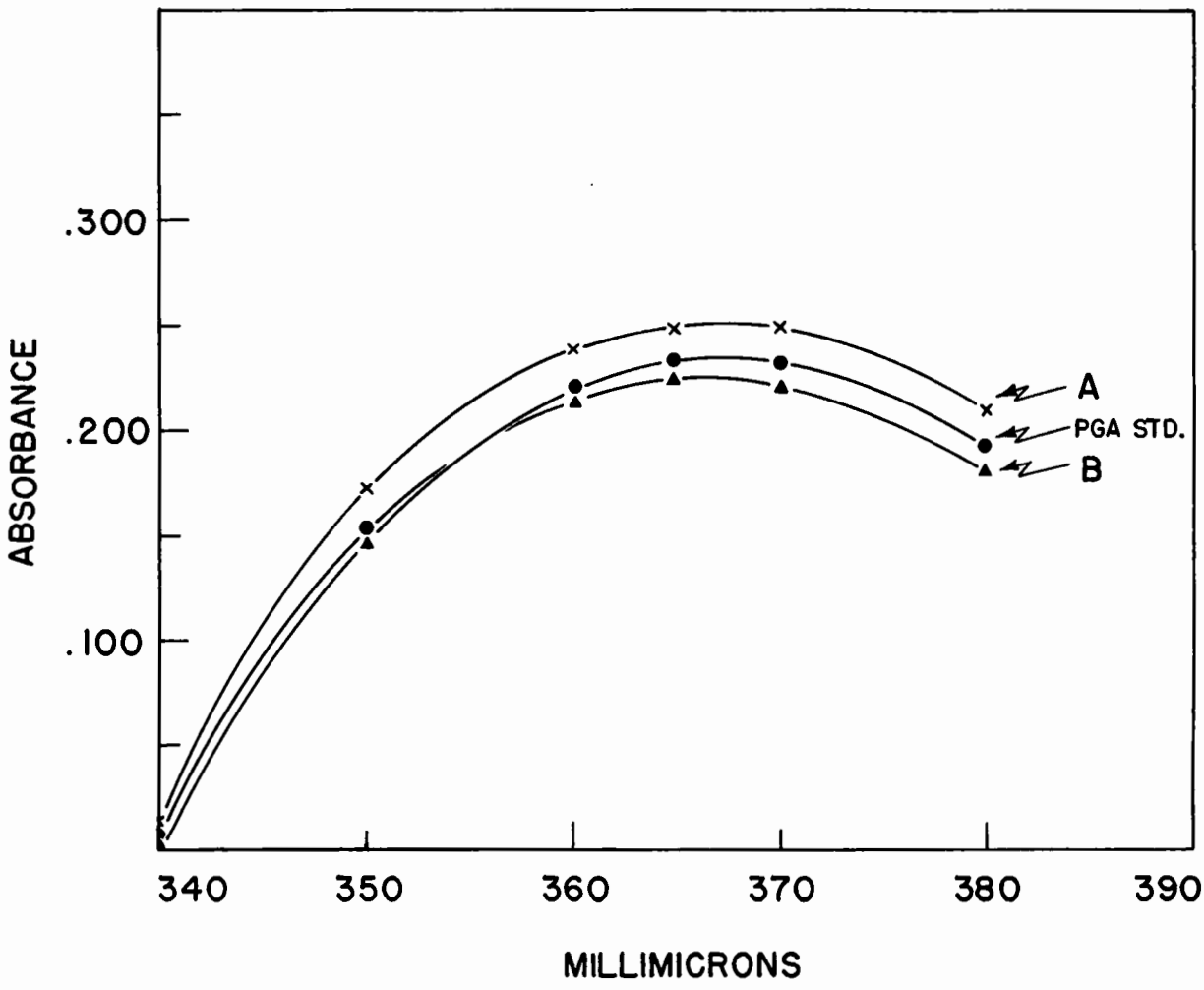

.300
.200
.100
ABSORBANCE
A
B
A1
B1
340
350
360
370
380
390
MILLIMICRONS

Figure 12 - The Absorbance Increment Spectra of Duplicate Assays of Prepared Mixtures of PGA with Liver (A & B), Corrected for the Apparent Liver Contribution to the Absorbance Increment


.300
.200
.100
ABSORBANCE
340
350
360
370
380
390
MILLIMICRONS
A
PGA STD.
B

large proportion of this substance. Consequently, the recovery of PGA from a prepared mixture with liver concentrate NF, was attempted on weighed portions which were extracted as in subsection 3.2.10 or not extracted. Liver concentrate alone was subjected to the same procedure. In each instance the residue was assayed by the procedure outlined in U.S.P. XIV for folic acid. Recoveries were expressed in mg. of PGA except where pre-extraction was not carried out and made it necessary to use per cent transmission values of the total and free amine. All data are given in Table VII, and from the results it can be seen that the liver interference can be removed by its solubility at pH 2.0. Recovery of the added PGA was consistent but five to ten per cent lower than the amount added.

3.3.2 A Comparison of PAB, PABG and PGA as Standards for the Colorimetric Procedure

Hutchings *et al.* (1947) had used PAB as a reference standard in the belief that its molal colour development with the Bratton & Marshall reagent (1939) was equal to that for PABG, one of the products of PGA cleavage. Other groups such as Biamonte & Schneller (1950), Jones *et al.* (1955) and Ware & Cronheim (1950) used PABG as their reference standard. It was therefore necessary to compare the colour developed using the Bratton & Marshall reagent with PAB, PABG or PGA that had been cleaved to give PABG plus its pteridine fraction.

For this study PABG reagent was obtained from Brickman and Company, Manufacturing Chemists, Montreal. The PAB used was U.S.P. reference standard and the PGA was the same as used for the previous studies on the ultra violet method. Separate solutions of the substances were prepared in 0.1 N sodium hydroxide. Portions were taken so that the final concentration

Table VII - The Recovery of PGA from Prepared Mixtures Containing Liver Concentrate by the Colorimetric Procedure Alone or Modified to Include a Pre-extraction of the Mixture at pH 2.0

Mixture	Added PGA Mg.	Added Liver Concentrate Mg.	Recovery of PGA		
			Extracted	Not Extracted	
			Mg.	Total Amine % T	Free Amine % T
1	1.0	--	0.95	-	-
2	1.0	1000	0.93	-	-
3	-	1000	0.0	-	-
4	-	1000	-	37.5	43.5
5	1.0	1000	-	37.0	38.5

levels at colour development would be as follows: PAB 0.4, 1.0 and 1.25 mcg. per ml.; PABG 2.0 mcg. per ml., and PGA 1.0, 2.0, 3.0 and 4.0 mcg. per ml. The colour reaction was carried out as outlined in U.S.P. XIV.

Absorbance measurements were made on each solution with a Beckman spectrophotometer, model DU, in 1 cm. cuvettes, for the region 450 mμ to 600 mμ. The spectral characteristics are given in Figure 13.

The data show that with an increased concentration of PAB, a hypsochromic shift occurs for the concentration levels used. It amounts to approximately 4 mμ and does not change the absorbance value to an appreciable extent. However, with PABG reagent or PABG from reduced PGA, no change in the maximum wavelength occurs. Such a difference as was found for PAB would not be observed if the measurements were taken on a spectrophotometer utilizing glass filters.

The factor adopted by Hutchings et al. (1947) for convering PAB to PGA was 3.22 and was derived by dividing the molecular weight of PGA by the molecular weight of PAB (441.42/137.13). If the $E^{1\%}_{1\ cm}$ values for the PAB coloured complex at 550 mμ are calculated from the observed absorbance values and then divided by the $E^{1\%}_{1\ cm}$ value for the PABG coloured complex, the resulting value should be 3.22. These calculations were made and the results given in Table VIII.

Figure 13 - The Spectral Characteristics of the Bratton and Marshall Reaction Products with Varying Amounts of Either PAB, PGA, or PABG

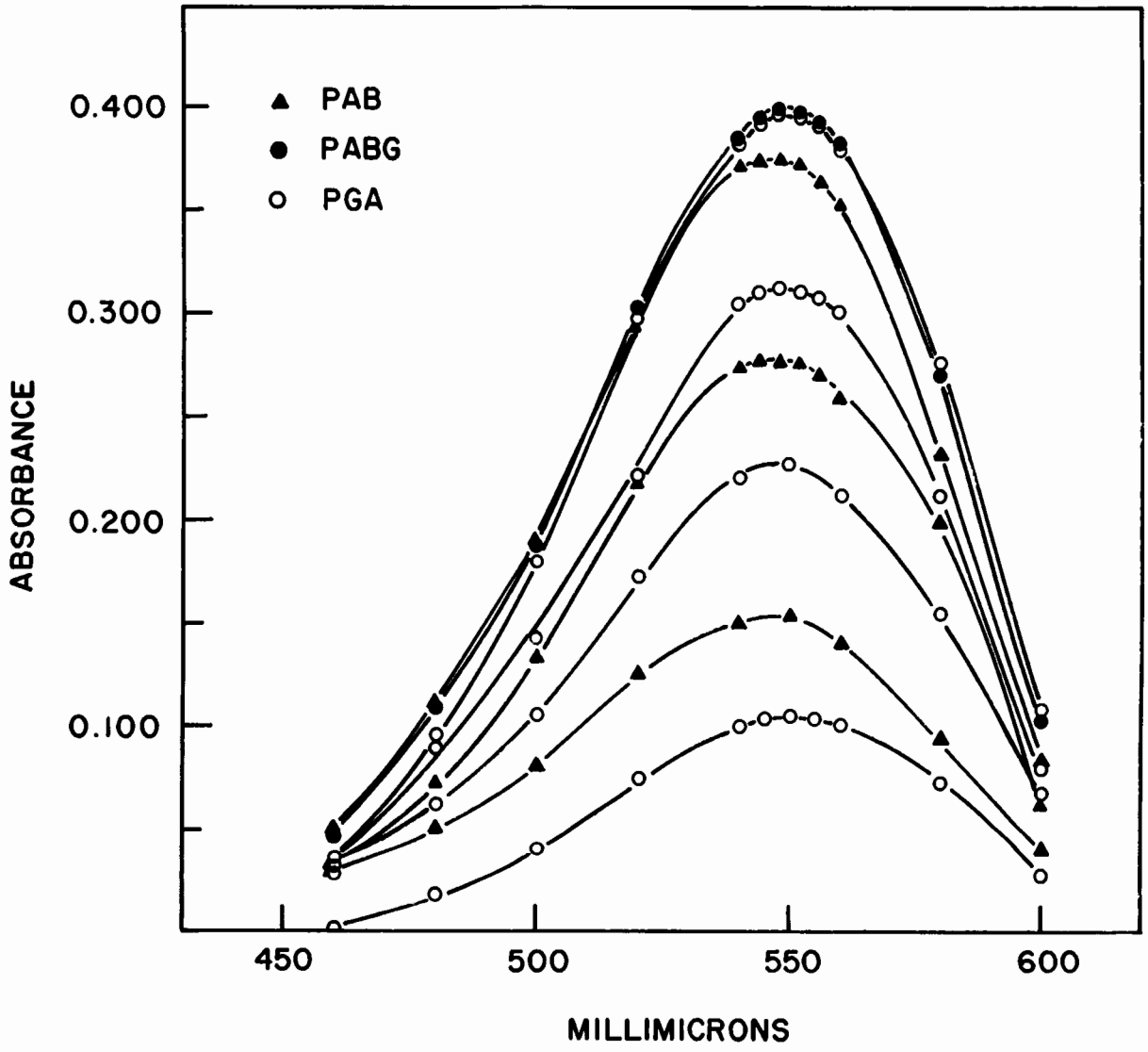

PAB
PABG
PGA
0.400
0.300
0.200
0.100
ABSORBANCE
450
500
550
600
MILLIMICRONS

Table VIII - The Per Cent Deviation of the Conversion Factor for Converting PAB to PGA, Calculated from the $E^{1\%}_{1\ cm}$ Values of PAB at Three Concentration Levels and that of PABG Resulting from PGA Cleavage, from that Based on Molecular Weights

Compound	Concentration Mcg./ml.	$E^{1\%}_{1\ cm}$ x 10^3	$\frac{E^{1\%}_{1\ cm}\ PAB}{E^{1\%}_{1\ cm}\ PGA}$	Per Cent Deviation from 3.22
PAB	0.5	3.46	3.16	-1.8
PAB	1.0	3.55	3.24	+0.8
PAB	1.5	3.47	3.18	-1.2
PABG	3.0	1.09	-	
Average for PAB	-	-	3.19	-0.9

DISCUSSION

The foregoing study of the spectral characteristics of PGA in the ultra violet region at various pH levels has shown that the maxima observed at pH 1.0 and at pH 11.0 were in agreement with the observations of groups such as Angier et al. (1945), Doube & Bombas (1950), Flynn (1950), the British Pharmacopoeia (1953) and the Pharmacopeia of the United States XV, with the following two exceptions. A maximum occuring in the spectral region 360 mμ to 370 mμ, at pH 11.0, was observed at 365 mμ in the present study, whereas British Pharmacopoeia (1953) and U.S.P. XV give this maximum at 368 mμ and 369 mμ respectively. Recently a revision notice for the U.S.P. monograph has changed the 369 mμ figure to 365 mμ.

Although Ilver (1953) had been unsuccessful in applying a direct spectrophotometric measurement as the basis of a procedure for the assay of some PGA concentrates, the large and well defined changes in absorbance at 365 mμ with changes in pH were used here as the basis of an indirect method. The procedure evolved was successfully applied to some synthetic mixtures and commercial preparations where there was predominance of PGA.

However, with other samples, it was apparent that the pteridine fraction from PGA decomposition was remaining intact and contributing to the absorbance increment. This was obvious from the high free amine correction for the samples when assayed by the U.S.P. XIV procedure and the relatively low value for PGA. When an attempt was made to check the extent of pteridine interference by cleavage of PGA through zinc, hydrochloric acid reduction no absorbance increment was present. In addition,

the ultra violet spectrum of the solution at pH 1.0 when compared to the spectra for pteroyl compounds, PGA and PABG as given by Wittle et al. (1943), Mowatt et al. (1948) and O'Dell et al. (1947) indicated predominance of PABG with little or no pteridine fraction. It would thus seem that there is variability in the end products of PGA decomposition resulting from uncontrollable factors.

The removal of interferring substances by extraction with a buffer was successful with ferrous sulphate; Liver concentrates, minerals and vitamins were only partially removed and that portion reamining contributed to the absorbance increment. Because of the low solubility of pteridines in aqueous solutions as reported by Albert (1953) it is doubtful if they would be removed by the extraction procedure. Such difficulties made it apparent that the absorbance increment procedure could not be applied to complex multi-vitamin preparations and retain confidence in its specificity.

The modification of the U.S.P. XIV procedure to include a pre-extraction of interferences with a buffer wash was highly successful in determining PGA in the presence of large amounts of liver concentrate. Furthermore, it would remove ferrous sulphate so that the difficulties experienced with the potassium ferric cyanide precipitation procedure could be avoided.

Although the wavelength for maximum absorbance of the coloured complex for PAB and the Bratton & Marshall reagent varied with PAB concentration it is doubtful whether this is grounds for criticising its use as a standard. The variation was only slight at the concentration levels used in the U.S.P. XIV procedure for PGA and could not be detected

by an instrument using glass filters to select the desired wavelength.

An interesting aspect of this study was the relationship between the absorbance increment at 365 mμ and changes of pH. Approximate dissociation constants expressed as pK_c and pK_a were obtained with successive changes of pH from 1.0 to 11.0. The values were as follows: $pK_c = 3.6$ and $pK_a = 7.8$. Albert (1953) has reported values of $pK_c = 2.31$ and $pK_a = 7.92$ for the 2-amino 4-hydroxy pteridine but does not specify the method used. A similar technique as described here for PGA was used by Stenstrom & Goldsmith (1926) to determine the dissociation constant of phenol. It would seem possible from these observations that the use of such measurements might be of some value in determining the dissociation constants of small amounts of material as well as the means of indicating the end point of non aqueous titrations.

SUMMARY

1. A method for the determination of PGA in pharmaceuticals based on its change in absorbance at 365 mμ with a change in pH was developed. The method was found to be simple and to give reproducible results for preparations containing PGA in the absence of interfering substances.
2. The results obtained by this procedure were affected by the presence of other pteridines produced by decomposition of PGA under certain conditions, and also by ascorbic acid, riboflavin, minerals and liver concentrate.
3. Interference by ferrous sulphate was overcome by extraction with a buffer solution at pH 2.0. This procedure was partially effective in obviating interferences by other factors.
4. It was found possible, by means of the method of the U.S.P. XIV, to determine PGA in the presence of large amounts of liver concentrate provided that the sample had been extracted previously with the buffer at pH 2.0.
5. PAB was found to be a suitable reference standard for the method of the U.S.P. XIV.
6. The absorbance maximum for PGA in 0.1 N sodium hydroxide over the range 360 mμ to 370 mμ was at 365 mμ.
7. Approximate values for the pK_c and pK_a values for PGA were obtained by plotting the values for the absorbance increment at 365 mμ against successive changes in pH.

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