THE RELATIONSHIP OF FOLIC ACID COMPOUNDS

TO ONE-CARBON METABOLISM IN THE

TOBACCO PLANT

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A THESIS

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VITA

The writer was born in Detroit, Michigan in 1934 and received her secondary education at Busch High School in Center Line, Michigan. In 1955 she received a Bachelor of Science degree from Michigan College of Mining and Technology with a major in medical technology.

Since that time she has worked in the clinical chemistry department of E.W. Sparrow Hospital in Lansing, Michigan and spent one year as a Graduate Teaching Assistant at Michigan State University. During summer and fall terms of 1957 she was a Special Graduate Research Assistant under the auspices of the National Institutes of Health.

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AN ABSTRACT

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ABSTRACT

The present study was undertaken in an attempt to elucidate the role of "folic acid" compounds in one-carbon metabolism of higher plants.

Formaldehyde-C^{14} was administered to tobacco plants both hydroponically and directly through the stem. The plants were allowed to grow for a period of one or four days. Fluorescent materials which possessed radioactivity and resembled "folic acid" compounds in ultraviolet absorption spectra and paper chromatographic characteristics were isolated from the plants.

In a further study a mixture of tetrahydropteroyl-glutamic acid and formaldehyde-C^{14} was administered to isolated leaves of a tobacco plant by vacuum infiltration. After allowing the leaves to metabolize in the presence of light for 10.5 hours a single fluorescent material which possessed radioactivity was recovered from the leaves. It was found to be identical in ultraviolet absorption spectrum and paper chromatographic characteristics with a fluorescent material which was a component of the administered mixture.

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INTRODUCTION

INTRODUCTION

The nature of the "active one-carbon unit" which is
utilized in many biosynthetic pathways in plants and
animals has been under intensive investigation in the last
ten years. Workers have concluded that the compound or com-
pounds in question are derivatives of the "folic acid"
group of bacterial growth factors. These derivatives are
found in many tissues, and are especially abundant in green
leafy plants, liver, and yeast.

"Active C_1" has been implicated as a cofactor in the
biosynthesis of purines (1,2), in serine-glycine intercon-
version (3), in the metabolism of glutamic acid, glycine,
and methionine (4,5,6) and in synthesis of the thymine
methyl group (7,8).

The exact chemical identity of the active cofactor is
still unknown. Research has been complicated by the fact
that a multiplicity of related compounds can function as
growth factors for Lactobacillus casei, Streptococcus faecalis,
and Leuconostoc citrovorum (Pediococcus cerevisiae). All
of the "folic acid"compounds contain the pteroylglutamic
acid nucleus. Differences lie in the state of oxidation
of the pyrazine ring, the number of glutamic acid residues
present, and the one-carbon constituent at the N^5 or N^{10}
position. Further complication arises as the compounds can
be metabolically interconverted through a series of ATP and
DPN dependent reactions (3,8,15,17) and can also be altered

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in isolation procedures (9,10,11).

Most recent investigations have demonstrated that derivatives of 5,6,7,8-tetrahydropteroylglutamic acid (tetrahydrofolic acid, FH_4) are carriers of the one-carbon unit. These are the 5-formyl derivative (citrovorum factor, leucovorin, f^5FH_4)(12), the 10-formyl derivative ($f^{10}FH_4$) and the 5- or 10-hydroxymethyl compounds (h^5FH_4 and $h^{10}FH_4$ respectively)(3,6,9,14-16). The formyl derivatives which are at the oxidation level of formate are required for the biosynthesis of the purine ureide carbon, whereas the hydroxymethyl derivatives which are at the oxidation level of formaldehyde seem to be active in serine-glycine interconversion and the formation of methionine from homocysteine.

The above metabolic pathways have been elucidated largely through work with animals and microorganisms. The role of "folic acid" derivatives in plant metabolism has not been extensively studied. Since many leafy plants contain appreciable amounts of these compounds, it can be postulated that reactions similar to those in animals can take place.

Studies in this laboratory concerned with transmethylation in higher plants have shown that formate-C^{14} can be incorporated into the methyl group of methionine and the methoxy group of lignin (18) and into the N-methyl group of nicotine in tobacco plants (19,20). Formaldehyde-C^{14} has been shown

to be incorporated to a greater extent into the N-methyl group of nicotine than formate (21). Similar results have been observed in animal tissue, that is, greater incorporation of formaldehyde than formate in certain reactions (3,6,22). This seems consistant with the concept that formate must undergo reduction to be utilized at the hydroxymethyl oxidation level and subsequently in the formation of methyl groups.

Zakrzewski and Nichol, using formate-C^{14}, demonstrated its invorporation into the 5-formyl group of citrovorum factor by cells of S. faecalis (23). Since formaldehyde is apparently a more direct precursor of methyl groups in tobacco plants than formate, it seemed of interest to attempt to demonstrate its incorporation into "folic acid" compound(s) in the intact plant. The formaldehyde would presumably enter the compound or compounds as an hydroxymethyl group if metabolic pathways in higher plants are analogous to those in animals.

Formaldehyde-C^{14} was administered to growing tobacco plants and an attempt was made to isolate compounds of the "folic acid" type which had incorporated the radioactivity of the formaldehyde. Because of difficulties encountered in isolation and identification of these compounds, no absolute proof of the incorporation of formaldehyde-C^{14} can be

put forth at this time. However, results of paper chromat-
ographic separation seem to indicate that the radioactivity
was taken up by fluorescent compounds with R_f values in the
same range as known "folic acid" derivatives. Ultraviolet
absorption spectra of the plant compounds also reveal certain
similarities to "folic acid" derivatives.

EXPERIMENTATION AND RESULTS

EXPERIMENTATION AND RESULTS

Preparation of tobacco plants

It has been demonstrated previously in this laboratory that tobacco plants can absorb organic compounds from a nutrient solution through their root systems (19,21,24). Tobacco plants used in this study were of a high nicotine strain, Nicotiana rustica L., var. humilis. They were chosen for their availability and to extend one-carbon studies which have been done on this particular species.

Seeds were planted in flats containing vermiculite (commercial heat expanded mica) and transplanted when seedlings were two to three weeks old. Plants were watered daily with tap water and twice weekly with a nutrient solution containing 1.0 gm. $MgSo_4 \cdot 7H_2O$, 1.0 gm. K_2HPO_4, and 5.8 gm. $Ca(NO_3)_2 \cdot 4H_2O$ in four liters of tap water.

Plants were removed from the flats and vermiculite was washed from the roots with tap water. For hydroponic administration of formaldehyde-C^{14} the plants were placed in 125 ml. Erlenmeyer flasks containing 50 ml. of a 1:3 dilution of nutrient solution with the following composition.

Water	1 l.	Magnesium sulfate	250 mg.
Calcium nitrate	1 gm.	Ammonium sulfate	250 mg.
Potassium chloride	250 mg.	Potassium dihydrogen phosphate	350 mg.
Ferric chloride	2 mg.		

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One ml. of an aqueous solution containing 0.5 mg. of aureo-mycin was added to each flask to reduce the microbial population.

Five ml. of a solution of formaldehyde-C14 containing approximately 2 mg. of formaldehyde was added to each flask. A cotton plug was placed around the plant stem at the neck of the flask to prevent loss of formaldehyde by volatilization. Plants were allowed to grow for periods varying from one to four days in a special fume hood to remove any radioactive carbon dioxide which may have formed. During the growing period water was added to the flasks as needed to maintain the 50 ml. volume of solution.

The light source consisted of two 36 inch 30 watt fluorescent tubes and one 100 watt incandescent bulb which were lighted for 12 hours during each 24 hour period. Light intensity was approximately 200-250 foot-candles at the top of the plants.

Administration of formaldehyde-C^{14} directly through the stem of the plant and by vacuum infiltration of leaves will be described later.

Preparation of formaldehyde-C^{14} solution

Formaldehyde-C^{14} solution was prepared by adding radio-active formaldehyde stock to a solution of formaldehyde containing approximately 2 mg. in 5 ml. Formaldehyde-C^{14} was

obtained from Volk Radiochemical Company, Chicago. This
solution was standardized in triplicate as to radioactivity
and formaldehyde content by precipitation with dimedon
(5,5-dimethylcyclohexanedione-1,3) according to the method
of Yoe and Reid (25).

A 10 ml. aliquot of formaldehyde solution was added to
a solution containing 50 ml. of acetate buffer (pH 4.6) and
10 ml. of 0.8 per cent dimedon in 95 per cent ethanol. The
mixture was allowed to stand at room temperature overnight.
The fluffy white dimedon derivative was removed by filtration
through a tared sintered glass crucible and washed with water
slightly acidified with acetic acid. The precipitate was
dried at 75°C. for one hour and weighed. Multiplication of
the weight of the derivative by a factor of 0.1027 gave the
weight of formaldehyde in the sample.

A sample of the precipitate from each determination
was transferred to a tared aluminum planchet and plated for
radioactivity measurement. Counting was done on a Nuclear-
Chicago scaler, model 192X, with a Tracerlab SC-16 internal
gas-flow Geiger-Müller tube. Efficiency of the instrument
was 40 per cent. Counts were corrected to "infinite thinness"
by use of a self-absorption curve.

Three formaldehyde solutions were prepared and standard-
ized in this way. Five ml. of solution contained 1.99 mg. of
formaldehyde with radioactivity of 1.47×10^5 cpm., 1.88 mg.

of formaldehyde with radioactivity of 3.00×10^5 cpm. and
2.12 mg. of formaldehyde with radioactivity of 1.05×10^6 cpm.

Uptake studies

Ringler (21) had shown previously that formaldehyde is
taken up from nutrient solution by tobacco plants under the
conditions which were employed in this experiment, and further,
that formaldehyde in the concentration used was not toxic to
the plants. He also demonstrated that the uptake of formal-
dehyde was true absorption rather than adsorption on the plant
roots. Eighty-nine per cent of a 2 mg. dose was taken up by
the plants in two days in his experiments.

To confirm the uptake results of Ringler two groups of
two plants each were given 5 ml. of formaldehyde-C^{14} solution.
One group was allowed to grow for 24 hours and the other for
four days. The remaining nutrient solution was filtered and
the flasks washed with distilled water to bring the total
volume up to about 30 ml. Three ml. of dimedon solution was
added and the pH was adjusted to 4.6 with acetic acid. The
mixture was then allowed to stand overnight and the precipitate
treated as before. The average uptake of formaldehyde by the
two plants was 88 per cent in 24 hours and 96 per cent in four
days.

The fact that a sample of formaldehyde was quantitatively
recovered from a mixture of formaldehyde and nutrient solution
indicates that the nutrient solution did not interfere with
the analytical procedure.

Isolation and identification of "folic acid" compounds

Isolation. In 1950 Keresztesy and Silverman first reported the isolation and partial purification of a growth factor for L. citrovorum from liver extract (26). The isolation was effected by making a water extract of the liver, adjusting the pH to 5.5, and adsorbing the active compound on charcoal. The charcoal was eluted with an ethanol-water-sodium hydroxide mixture and the eluate was extracted with water-saturated butanol. The butanol fraction then was washed with water, the aqueous extract acidified and the growth promoting activity precipitated as the silver salt. The factor was further purified by precipitating it as the barium salt and by chromatography of it on an aluminum oxide column. Modifications of this procedure have been used by other investigators to isolate citrovorum factor (27, 28), polyglutamyl-folic acids (27,29), N^{10}-formylfolic acid (30), and some unidentified compounds with activity for L. citrovorum and S. faecalis (31).

Sauberlich in 1951 (28) reported the isolation of citrovorum factor from fresh spinach by allowing a water extract of the plant to autolyze for 15 hours, precipitating the proteins with ethanol, and adsorbing the compound on charcoal. This was followed by elution, extraction with butanol, and precipitation of the activity as the barium salt.

The procedure used for isolation of "folic acid" compounds in this study was a modification of the methods of Silverman et al. (30) and Sauberlich. Plants which had been fed formaldehyde-C^{14} were removed from the nutrient solution and rinsed with distilled water. Roots were removed and discarded; the leaves and stems of the plants (usually two plants) were cut into small pieces with scissors. The plant material was then placed in a Waring blendor containing 100 ml. of an 8 per cent solution of $Na_2HPO_4 \cdot 12H_2O$. The air in the Waring blendor had been displaced with nitrogen to minimize air oxidation of reduced "folic acid" compounds. The plant material then was homogenized for 15 seconds, the solid material removed by filtration, and the filtrate transferred to an Erlenmeyer flask. Nitrogen was bubbled through the mixture for five minutes. Ten ml. of toluene was added to reduce microbial growth and the mixture was incubated overnight at $37^{\circ}C$. It was assumed that the incubation would allow autolysis of any polyglutamyl compounds to take place.

Fifty ml. of 95 per cent ethanol was added to precipitate proteins and the mixture heated to $83-85^{\circ}C$. for five minutes while stirring with a stream of nitrogen. The proteins were removed by filtration.

About 2 gm. of Norite A was added to the filtrate to absorb the aromatic organic compounds. The solution was

adjusted to pH 6-7 with hydrochloric acid and heated to 35-40°C. for 30 minutes. Charcoal was removed by filtration and the filtrate treated again with charcoal. The charcoal cake was eluted two times with a mixture of ethanol-ammonium hydroxide-water (4:1:3) for 30 minutes at 35-40°C. The total volume of eluate was about 200 ml. The tan colored eluate was evaporated to dryness under reduced pressure at room temperature and the resulting tan solid material stored at 6°C.

Paper chromatography of reference compounds. Paper chromtographic characteristics of "folic acid" compounds were determined in the following manner. A 45 X 15 cm. glass cylinder was used as a chromatographic chamber for ascending development of papers. Approximately 50 ml. of solvent was placed in the bottom of the cylinder and allowed to saturate the air in the cylinder by standing at least 24 hours.

Sheets of Whatman No. 1 filter paper were cut into 16 X 14 inch pieces and samples of plant material and reference compounds were placed two inches apart one-and-one-half inches from the narrower end of the paper. Samples were applied with a micro pipet in spots about one cm. in diameter and dried between applications by a stream of warm air.

The paper was stapled together to form a cylinder in such a manner that the edges of the paper were not in contact with each other. The self-supporting cylinder was placed in

the chromatographic chamber and allowed to develop at room temperature for about four hours in the case of the aqueous solvent or 18-20 hours for the organic solvent.

Reference compounds used in chromatography were pteroylglutamic acid, leucovorin, acid-treated leucovorin, tetrahydropteroylglutamic acid, and tetrahydropteroylglutamic acid treated with formaldehyde-C^{14}.

Pteroylglutamic acid was obtained from General Biochemicals, Incorporated, Chagrin Falls, Ohio. Leucovorin was the gift of Dr. Harry P. Broquist, Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

Acid transformation products of leucovorin were prepared by making a solution of leucovorin in 0.1 M hydrochloric acid and allowing the mixture to stand overnight at room temperature. Treatment of leucovorin with mineral acid has been reported to result in the formation of transformation products which are N^5-N^{10}-imidazolinium derivatives(32,33).

Tetrahydropteroylglutamic acid was prepared by hydrogenation of pteroylglutamic acid in glacial acetic acid over platinum. The reduction was carried out in a flask attached to a Warburg manometer in a constant temperature water bath at 25°C. The procedure was a modification of that of O'Dell, et al. (35) which was also used by Blakley (16). Pteroylglutamic acid takes up two moles of hydrogen to form the tetrahydro derivative under these conditions.

Twenty-five mg. of pteroylglutamic acid was placed in the side arm of a 125 ml. Warburg flask; 5 ml. of glacial acetic acid and 13 mg. of platinum oxide were placed in the bottom of the flask. Hydrogen gas was introduced into the system to reduce the catalyst and saturate the acetic acid until the gas pressure as recorded on the manometer remained constant. The pteroylglutamic acid was tipped into the mixture and the reaction was allowed to progress until 80 per cent of the theoretical amount of hydrogen had been taken up.

The catalyst and unchanged pteroylglutamic acid were removed by filtration and the filtrate was poured into 50 ml. of ethyl ether. The tan precipitate which resulted was recovered by centrifugation and washed three times with 30 ml. volumes of ethyl ether. The product was dried in a desiccator under reduced pressure over potassium hydroxide and stored in the dark.

Tetrahydropteroylglutamic acid prepared as described above was added to an equimolar solution of formaldehyde-C^{14}, assuming that the tetrahydro compound was pure. Blakley (16) reported the equimolar condensation of tetrahydropteroylglutamic acid with radioactive formaldehyde _in vitro_. The results of his studies indicated that the compound formed was the 5-hydroxymethyl derivative.

Since the "folic acid" group of compounds have characteristic absorption of ultraviolet light or distinct fluorescence, completed chromatograms were examined under an ultraviolet lamp

and the fluorescent or absorbant zones were marked with a pencil.

Following are the R_f values obtained using the reference compounds mentioned above.

Compound	Solvent	UV Character	$R_f \times 10^2$
Pteroylglutamic acid	EBAW*	A[t]	27
		F[tt]	50
	K$_2$HPO$_4$**	A	20
		F	31
Leucovorin	EBAW	F	21
		F	32
	K$_2$HPO$_4$	F	44
		A	71
Acid-treated leucovorin	EBAW	yellow F	1
		blue F	16
		F	20
	K$_2$HPO$_4$	F	0
		F	24
		blue F	56
		F	73
Tetrahydropteroyl-glutamic acid	EBAW	F	0
		green-blue F	15
		blue F	21
		blue F	35
	K$_2$HPO$_4$	F	0
		yellow F	21
		blue F	29
		blue F	44
		F	74
Tetrahydropteroyl-glutamic acid and formaldehyde mixture	EBAW	F	1
		green-blue F	14
		blue F	22
		blue F	35
	K$_2$HPO$_4$	F	1
		yellow F	21
		blue F	32
		blue F	46
		F	76

*Ethanol-n-butanol-ammomia-water (50:15:10:25) (29)
**0.1 M dipotassium hydrogen phosphate (34)
[t]Absorbant
[tt] Fluorescent

The R_f values reported above are the average of two or more chromatographic runs. A tabulation of R_f values of these and other "folic acid" compounds in similar solvents which have been reported in the literature by other workers is shown in the appendix.

In chromatography of the tetrahydro compound and formaldehyde mixture an area of high radioactivity corresponding to formaldehyde-C^{14} (R_f 0.60 in EBAW) was detected by use of a chromatographic strip counter (Nuclear-Chicago windowless rate meter coupled with an Esterline-Angus recorder, efficiency approximately 4 per cent). Counting with this instrument indicated that there was a considerable excess of formaldehyde and hence the tetrahydropteroylglutamate preparation was not pure. The detection of four or five fluorescent spots on the chromatograph of the preparation also indicates impurity or reoxidation to the more stable pteroylglutamic acid. Greenberg (1), Blakley (16) and Kisliuk (36) also reported the presence of a number of products upon hydrogenation of pteroylglutamic acid.

Tetrahydropteroylglutamic acid and the tetrahydropteroylglutamic acid and formaldehyde mixture seem to have identical R_f values by the chromatographic methods employed. It will be shown later by ultraviolet absorption spectra that at least one of the fluorescent materials of the same R_f value in the two preparations is not the same.

Chromatography and counting of plant extracts. The plant
preparations were chromatographed under the same conditions
as the reference compounds. A portion of the dry plant extract
was resuspended in 200to 500 μl of distilled water and spotted
on the papers. Fluorescent material was detectable on com-
pleted chromatograms but resolution was poor in both solvents.

In the first experiment two plants were used and were
allowed to metabolize the radioactive formaldehyde for 24 hours.
The radioactivity of the hydroponically administered formalde-
hyde was 1.47 X 10^5 cpm. per plant. Chromatography of the
plant extract in EBAW revealed the presence of fluorescing
material in a streak extending approximately 6 cm. No radio-
activity in this area could be detected on the chromatographic
strip counter.

In order to increase the possible radioactivity of the
fluorescent compound(s), a new formaldehyde-C^{14} solution was
prepared and standardized. This solution contained 3.00 X 10^5
cpm. in 5 ml.

The new formaldehyde solution was administered to two
groups of two plants each. One group was allowed to grow in
the presence of formaldehyde for 24 hours and the other was
allowed to grow for four days. Chromatographic results were
similar to those of the first experiment. Again no radioactiv-
ity was detected in the fluorescing material.

In a third experiment two plants were given formaldehyde
containing 1.05 X 10^6 cpm. in 5 ml. and were allowed to grow
for four days. The plants were worked up in the usual

manner. The plant extract was applied to the paper in a one inch strip at the origin, rather than in a single spot, to increase the concentration of material on the paper.

A number of fluorescent areas were noted on the completed chromatograms which gave reproducible peaks of radioactivity when measured on the chromatographic strip counter. These spots were cut out of the chromatograms and eluted with water directly into tared aluminum planchets for counting with the internal gas-flow Geiger-Müller tube.

Elution was performed by stapling a rectangular filter paper wick to one end of the paper containing the material and a pointed wick to the other end. The rectangular wick was dipped into a water reservoir and the water allowed to elute the paper by capillarity. The pointed end of the paper was placed in the aluminum planchet. When approximately 0.3 ml. had been eluted the paper was removed and the liquid in the planchet was evaporated at 30-40°C. under a stream of air.

Weight of eluted material in each case was less than one mg. Counts were corrected for background. Since the weight of material was very small no correction for self-absorption was necessary.

The following data were obtained in this experiment.

Solvent	UV Character	R_f X 10^2	Cpm./mg.
EBAW	fluorescent	27	29
	fluorescent	37	60
	fluorescent	53	115
	absorbant	82	8
K_2HPO_4	fluorescent	50 (streaked)	5
	fluorescent	80 "	12

In order to get formaldehyde into the plants in a
shorter period of time a fourth experiment was performed.
The roots of two plants were removed by cutting diagonally
across the stem one-half inch above the roots. The cut end
of the stem was placed in a mixture of 20 ml. of diluted
nutrient solution and 5 ml. of formaldehyde solution contain-
ing 1.05 X 10^6 cpm. After 22 hours the plants both had taken
up approximately 22 ml. of the mixture. The plants were worked
up in the usual manner and the extract was separated by paper
chromatography as was done in the third experiment. Elution
and counting with the internal gas-flow tube gave the following
results.

Solvent	UV Character	R_f X 10^2	Cpm./mg.
EBAW	fluorescent	30	29
	fluorescent	37	37
	fluorescent	45	81
	absorbant	81	11
K_2HPO_4	fluorescent	50 (streaked)	-
	fluorescent	80 "	8

Ultraviolet absorption spectra. Absorption spectra of refer-
ence compounds and materials isolated from the plants were
determined using a Beckman DK-2 recording spectrophotometer.

These are shown in the appendix. Since the compounds in
question were ultraviolet absorbing or fluorescent the wave
length range of 220 to 340 mμ was chosen.

Absorption spectra of leucovorin, pteroylglutamic acid,
and tetrahydropteroylglutamic acid were run on diluted stock
solutions. For materials which had been eluted into planchets
and dried for counting it was found convenient to wash the
planchets with approximately two to three ml. of distilled
water and determine the spectra of the resulting solutions.

In some cases when the material had not been counted
(for example, the products of acid-treated leucovorin) the
spots from the chromatograms were cut out, placed in two to
three ml. of distilled water and allowed to stand at least
one hour with occasional agitation to form a solution for the
determination of spectral absorption.

Because of the limited amount of material eluted from
the chromatograms the ultraviolet absorption of the water
solutions were determined first. Then the solutions were
divided into two equal portions and one volume of approximately
0.2 M sodium hydroxide or hydrochloric acid was added and the
ultraviolet absorption spectra were determined again. This
method gave spectra in acid or base at one-half the concen-
tration of those in water.

Leaf infiltration experiments

In order to determine how the plants metabolize pre-formed "folic acid" compounds a fifth experiment was under-taken. This experiment also served to increase the concen-tration of these compounds in the plants. Since nothing was known about the absorption of such materials through the roots of the plants another method of administration seemed desir-able. Therefore the vacuum infiltration procedure of Kursanov (37) was employed.

Three isolated leaves of a tobacco plant which had been weighed previously were immersed in a mixture of 4.0 mg. of tetrahydropteroylglutamic acid preparation and 10 ml. of form-aldehyde solution containing 4.24 mg. of formaldehyde and 2.10×10^6 cpm.

Air was removed from the leaves by reducing the pressure to 25 mm. of mercury. As air was readmitted to the system the air spaces of the leaves were filled with the surrounding liquid. The leaves were removed from the liquid, blotted dry, and weighed again. Average absorption of the liquid mixture was 0.3 gm. per leaf. The total absorption was 0.90 gm. or approximately 1.56×10^5 cpm.

The leaves were allowed to metabolize in the presence of light for 10.5 hours. They then were worked up in the manner described previously and chromatographed in EBAW and K_2HPO_4.

The following results were obtained.

Solvent	UV Character	R_f X 10^2	Cpm./mg.
EBAW	green-blue fluorescent	21	---
	blue fluorescent	31	---
K_2HPO_4	fluorescent	1 (faint)	---
	fluorescent	75	34

The fluorescent material at R_f 0.75 in K_2HPO_4 was eluted into an aluminum planchet and counted with the internal gas-flow tube. Since chromatography of the tetrahydropteroylglutamic acid and formaldehyde-C^{14} mixture revealed a fluorescent area at R_f 0.76 in K_2HPO_4, this material was also eluted and counted. Radioactivity was 17 cpm./mg. above background.

In order to further identify the material isolated from the plant tne ultraviolet absorption spectra of all materials in the reference compounds which had R_f values near 0.75 in K_2HPO_4 were determined. These were 1) R_f 0.76 material from the tetrahydropteroylglutamic acid and formaldehyde mixture, 2) R_f 0.74 material from the tetrahydropteroylglutamic acid preparation, 3) R_f 0.56 material from acid-treated leucovorin, and 4) R_f 0.73 material from acid treated leucovorin. Results of these determinations are shown in the appendix.

DISCUSSION

DISCUSSION

The identity of the radioactive fluorescent materials isolated from the plants after administration of formaldehyde-C^{14} is difficult to establish from the data presented in this study. Examination of the paper chromatographic data on "folic acid" compounds that have been presented in the literature reveals certain disparities in the behavior of individual compounds. This is especially evident in the case of the reduced compounds. Zakrzewski and Nichol (38) have shown that the mobility of certain pteroylglutamic acid derivatives changes with the pH of the developing solvent, and also that their mobility increases with decreasing concentration of salts in the solvent. This may account for some of the apparent discrepancies, since the solvent was not the same in all cases. The R_f values reported for dihydro and tetrahydropteroylglutamic acids seem to indicate some disagreement as to the chromatographic identity of these compounds, however.

Since the conditions of isolation and chromatography under which the present study was conducted were not rigorously anaerobic, it seems reasonable to expect the more oxygensensitive "folic acid" compounds to be oxidized to more stable forms during these procedures. May, et al. (41) have shown that the N^{10} substituted derivatives are more oxygen-labile than those that are N^{5} substituted. The work of Cosulich, et al. (32) indicates that the N^{5}-N^{10}-imidazolinium derivative

is especially stable to oxygen because of the possibility of resonance of the quaternary nitrogen. This reasoning cannot be strictly applied to the N^5-N^{10}-methylene (or hydroxymethyl, as it is sometimes named) bridge compounds since no quaternary nitrogen is involved.

Cosulich and Smith (42) synthesized N^{10}-methyl pteroic and N^{10}-methyl pteroylglutamic acids and showed that N^{10}-methyl pteroic anid is stable to aerobic alkaline oxidation.

A comparison and correlation of the ultraviolet absorption spectra and R_f values of the compounds isolated from the plants and those of reference compounds in the light of the above considerations reveals similarities which are as follows.

Experiment two. No definite R_f data was obtained, but the absorption spectra in acid, alkali, and distilled water (Appendix II, Figure 6), show an absorption maximum at approximately 267 mμ with little change in acid or alkali. None of the reference compounds which were investigated show this type of behavior in ultraviolet light.

May, et al. (41) reported the absorption spectrum of N^{10}-formylpteroylglutamic acid in 0.1 M sodium hydroxide solution in which a characteristic absorption maximum at 260 mμ was observed. Since it is not known whether the material that was isolated from the plants possessed any radioactivity or whether the compound was chromatographically pure, it seems inadvisable to attempt to draw any conclusions as to its

identity.

Experiment three. It is unfortunate that absorption spectra in acid and alkali were not determined on all of the fluorescent materials isolated from the plants in this experiment. It is difficult to make any comparisons without knowledge of their behavior in these solvents. The fact that four fluorescent materials were separated by EBAW solvent whereas only two were obtained in K_2HPO_4 may be rationalized when one considers that EBAW is a basic solvent containing 10 per cent ammonium hydroxide. May, et al.(41) showed that interconversions of leucovorin and its derivatives may be brought about under the influence of alkali. The N^5-N^{10}-imidazolinium tetrahydro compound can be converted to N^{10}-formyltetrahydropteroylglutamic acid or leucovorin under appropriate conditions of high pH. Further aerobic oxidation of the N^{10}-formyltetrahydro compound gives N^{10}-formylpteroylglutamic acid. The N^{10}-formyl group may also be hydrolyzed by dilute alkali.

If an analogy can be drawn in the case of the N^5-N^{10}-methylene compounds it may be postulated that such transformations could have occurred in EBAW solvent during chromatography.

The absorption spectra of the material which exhibited an R_f of 0.80 in K_2HPO_4 (Appendix II, Figure 11) shows a distinct absorption maximum at 305 mμ in 0.1 M sodium hydroxide. A compound which was separated from acid treated leucovorin (Appendix II, Figure 14) shows an absorption maximum at this

wave length, although the curve is not as sharp, and it also has maxima at 287 and 234 $m\mu$. Cosulich, et al. (32) have reported the absorption spectra in 0.1 M hydrochloric acid of N^5-N^{10}- imidazolinium tetrahydropteroylglutamic acid, anhydroleucovorin A, and anhydroleucovorin B, all of which are N^5-N^{10}-methenyl derivatives of tetrahydropteroylglutamic acid. They may be interconverted at various pH values from 1.3 to 4.0. All three compounds exhibit absorption minima near 300 $m\mu$ in 0.1 M hydrochloric acid. Anhydroleucovorin B shows a maximum at 304 $m\mu$. The spectrum of 5-formyl-10-methyltetrahydropteroylglutamic acid also shows absorption at 310 $m\mu$(32). It is tempting to postulate that the compound isolated from the plants is an N^5-N^{10}-methylene bridge compound, which would be identical to neither the N^5-N^{10}-imidazolinium or the methyl derivatives, but resemble them in ultra-violet absorption.

The R_f of the N^5-N^{10}-methylene bridge compound as reported by Osborn is 0.25 in 0.1 M phosphate buffer, pH 8.0. The absorption spectra of the compounds isolated from acid treated leucovorin at R_f 0.56 and 0.76 in K_2HPO_4 (Appendix II, Figures 14 and 15) appear to be very similar, although their R_f values are quite different. This variation in R_f of apparently similar compounds may lend some support to thehypothesis that the compound isolated from the plants may be an N^5-N^{10}-methylene derivative, even though its R_f is 0.80.

Experiment four. The results of experiment four are for the most part similar to those of experiment three. It may be noted however that the radioactivity of the fluorescent compounds isolated from the plants is lower than that in experiment three. This is not without explanation, since the plants in experiment four were only allowed to metabolize the formaldehyde-C^{14} for one day whereas those in experiment three were grown for four days in the presence of formaldehyde-C^{14}.

Experiment five. The fluorescent material isolated from the leaves which had been infiltrated with a formaldehyde-C^{14} and tetrahydropteroylglutamic acid mixture (R_f 0.75 in K_2HPO_4) is not the same as that isolated from the formaldehyde-C^{14} fed plants, although the R_f value is very nearly the same. The absorption spectra in acid, alkali, and distilled water (Appendix II, Figure 17) are distinctly different than those of experiments three and four. It is also different than the fluorescent material (R_f 0.74 in K_2HPO_4) which was a component of the tetrahydropteroylglutamic acid preparation (Appendix II, Figure 13).

Comparison of the spectra of this plant material and that of the fluorescent material (R_f 0.76 in K_2HPO_4) which was a component of the formaldehyde-C^{14} and tetrahydropteroylglutamic acid mixture reveals very close similarity.

A comparison may also be made with the component of acid-treated leucovorin (R_f 0.73 in K_2HPO_4, Appendix II, Figure 15).

The absorption spectrum in 0.1 M hydrochloric acid and distilled water of both of the compounds appear similar, although that in 0.1M sodium hydroxide is quite different. If the material from acid-treated leucovorin is assumed to be an N^5-N^{10}-imidazolinium derivative with a quaternary nitrogen, its behavior in alkali would be expected to be different than that of an N^5-N^{10}- methylene compound, but perhaps it would be similar in acid.

It is interesting to note that this was essentially the only fluorescent material recovered from the leaves that had been fed the mixture, and that the radioactivity of the plant material was greater than that of the administered component.

Radioactivity of the fluorescent materials has been recorded as cpm./mg. The material which was eluted into planchets for counting and weighed probably contained a certain amount of K_2HPO_4 from the solvent, so that the weight observed was not a true weight of the fluorescent materials. The small amount of material isolated precluded any elaborate purification procedures. The higher radioactivity of the plant material therefore may be more apparent than real.

Further study of this problem should include 1) an unequivocal synthesis of N^5-N^{10}-methylene pteroylglutamic acid derivatives to be used as reference compounds for comparison with plant materials, 2) better control of the tetrahydropteroylglutamic acid synthesis and purification of the product, 3) determination of the ultraviolet absorption spectra in acid

and alkali of those plant materials which can be separated by EBAW, 4) larger scale separation of fluorescent plant materials so that they may be further purified and identified, and 5) hydrolysis of the N^5-and/or N^{10} substituent and examination of that carbon for radioactivity.

SUMMARY

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1. Formaldehyde-C^{14} was administered to tobacco plants both hydroponically and directly through the stem. An attempt was made to isolate and identify "folic acid" compounds which may have incorporated the radioactivity of the formaldehyde. Fluorescent materials recovered from the plants resembled "folic acid" compounds in paper chromatographic characteristics and ultraviolet absorption spectra, but could not be positively identified.

2. A mixture of tetrahydropteroylglutamic acid and formaldehyde-C^{14} was administered to isolated leaves of a tobacco plant by vacuum infiltration. A single fluorescent material was recovered from the leaves which was shown by paper chromatography and ultraviolet absorption spectra to be identical with a component of the administered mixture.

3. A discussion of the possible identity of the fluorescent materials derived from the plants and suggestions for further study were presented.

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REFERENCES

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APPENDIX

APPENDIX I

R_f VALUES OF "FOLIC ACID" COMPOUNDS

REPORTED IN THE LITERATURE

Compound	$R_f \times 10^2$	Solvent*	Reference
Pteroylglutamic acid	37	A	Zakrzewski (38)
	36	B	Zakrzewski (23)
	24	C	Jaenicke (39)
	29	D	Greenberg (1)
	28	E	Wieland (27)
Leucovorin	74	B	Zakrzewski (38)
	74,86	B	Zakrzewski (23)
	62	C	Jaenicke (39)
	65	D	Greenberg (1)
	71	E	Wieland (27)
N^5-N^{10}-methylene-tetrahydropteroyl-glutamic acid	25	G	Osborn (40)
N^{10}-formyltetra-hydropteroylglutamic acid	54	C	Jaenicke (39)
N^{10}-formyldihydro-pteroylglutamic acid	58	C	Greenberg (11)
N^{10}-formylpteroyl-glutamic acid	76	A	Greenberg (11)
	78	C	Jaenicke (39)
	55	D	Greenberg (1)
	56	F	Silverman (30)
N^{10}-methylpteroyl-glutamic acid	75	A	Zakrzewski (38)
Tetrahydropteroyl-glutamic acid	12,25,48	A	Blakley (16)
	48	C	Jaenicke (39)
	13	G	Osborn (40)
Dihydropteroyl-glutamic acid	10,40,73	A	Blakley (16)
	"several below 40"	D	Greenberg (1)

*Solvents are designated as follows: A, 0.1 M K2HPO4; B, 0.1 M phosphate buffer, pH 7.0; C, 12% Na2HPO4 with 0.2% ethylene-diaminetetraacetate; D, 5% K2HPO4 with isoamyl alcohol; E, 5% Na2HPO4 with isoamyl alcohol; F, 15% Na2HPO4·12H2O; G, 0.1 M phosphate buffer, pH 8.0

APPENDIX II

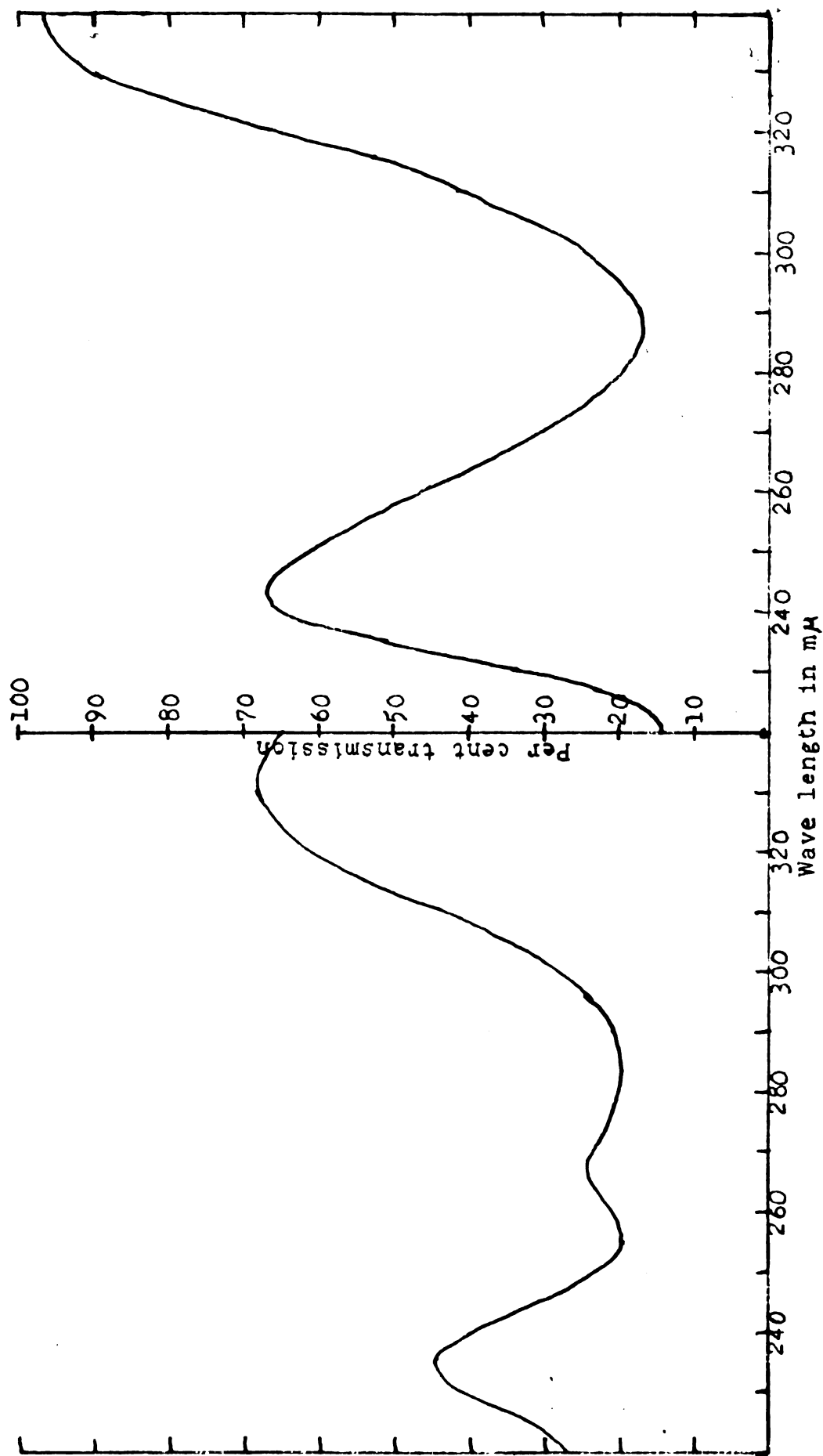ULTRAVIOLET ABSORPTION SPECTRA OF REFERENCE COMPOUNDS AND PLANT MATERIALS

Figure 1. Pteroylglutamic acid in distilled water.
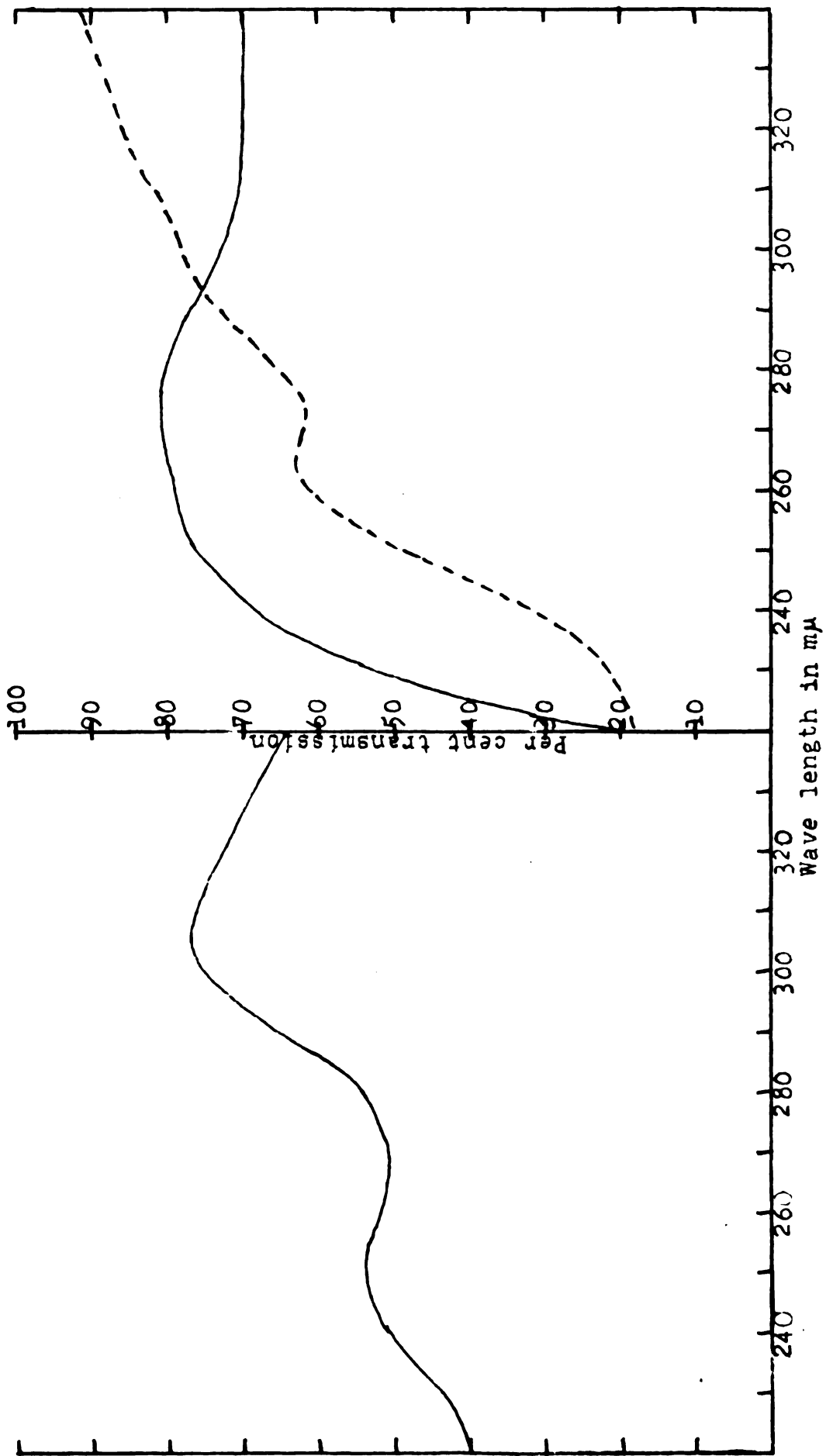
Figure 2. Leucovorin in distilled water.

Figure 4. Tetrahydropteroylglutamic acid
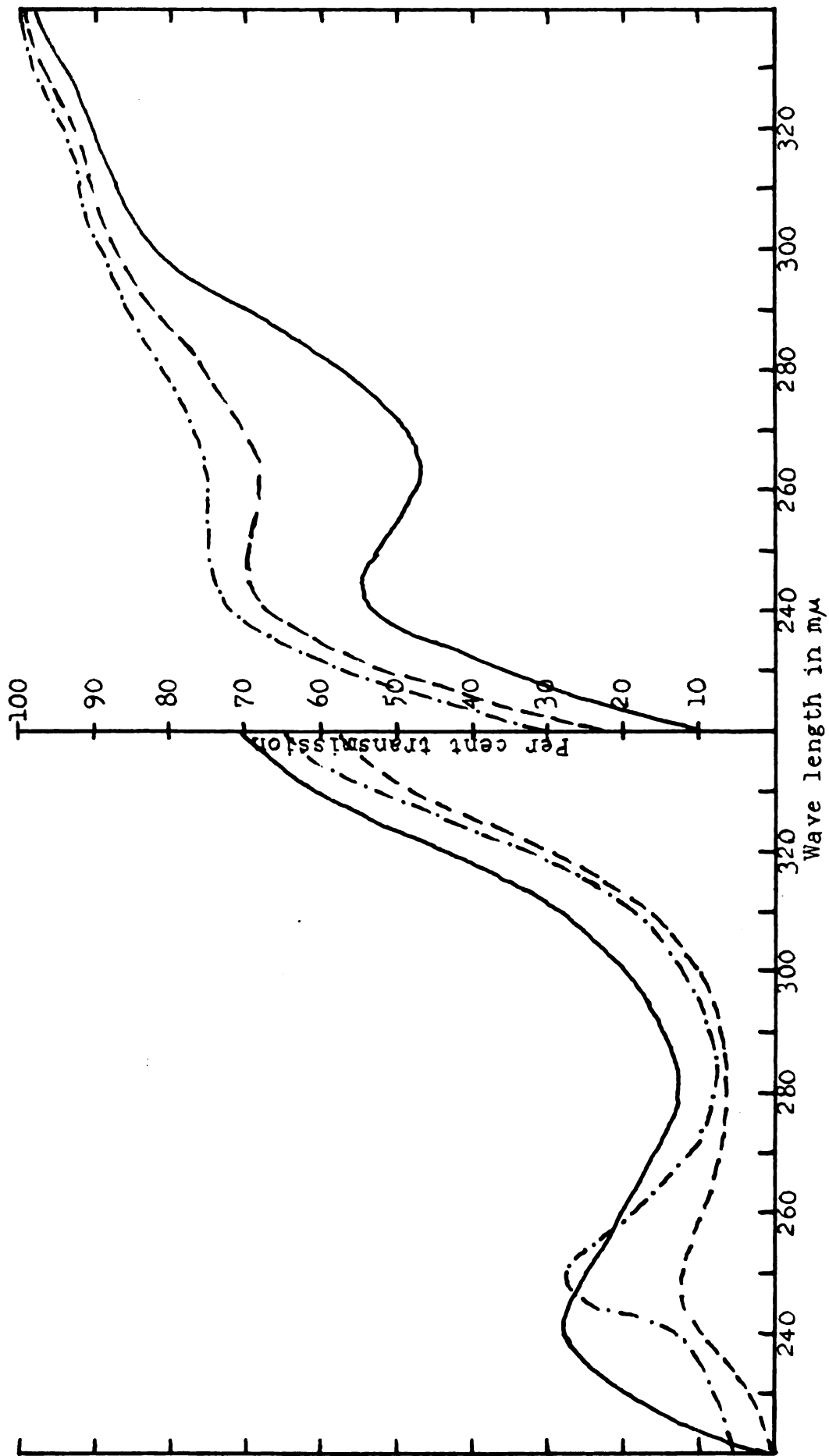in 0.1 M sodium hydroxide ————, and 0.1 M
hydrochloric acid ———.

Per cent transmission

Wave length in mμ

Figure 3. Leucovorin in 0.1 M hydrochloric
acid.

Figure 5. Tetrahydropteroylglutamic acid +
formaldehyde in 0.1 M sodium hydroxide ————,
0.1 M hydrochloric acid _ _ _, and distilled
water ─•─•─•─.

Figure 6. Fluorescent plant material from
experiment two in 0.1 M sodium hydroxide ————,
0.1 M hydrochloric acid _ _ _, and distil-
led water ─•─•─•─.

Figure 8. Fluorescent plant material, experiment three, EBAW Rf .53 in distilled water ⸺ Absorbant plant material, experiment three, EBAW Rf .82 in distilled water ⸺ ⸺
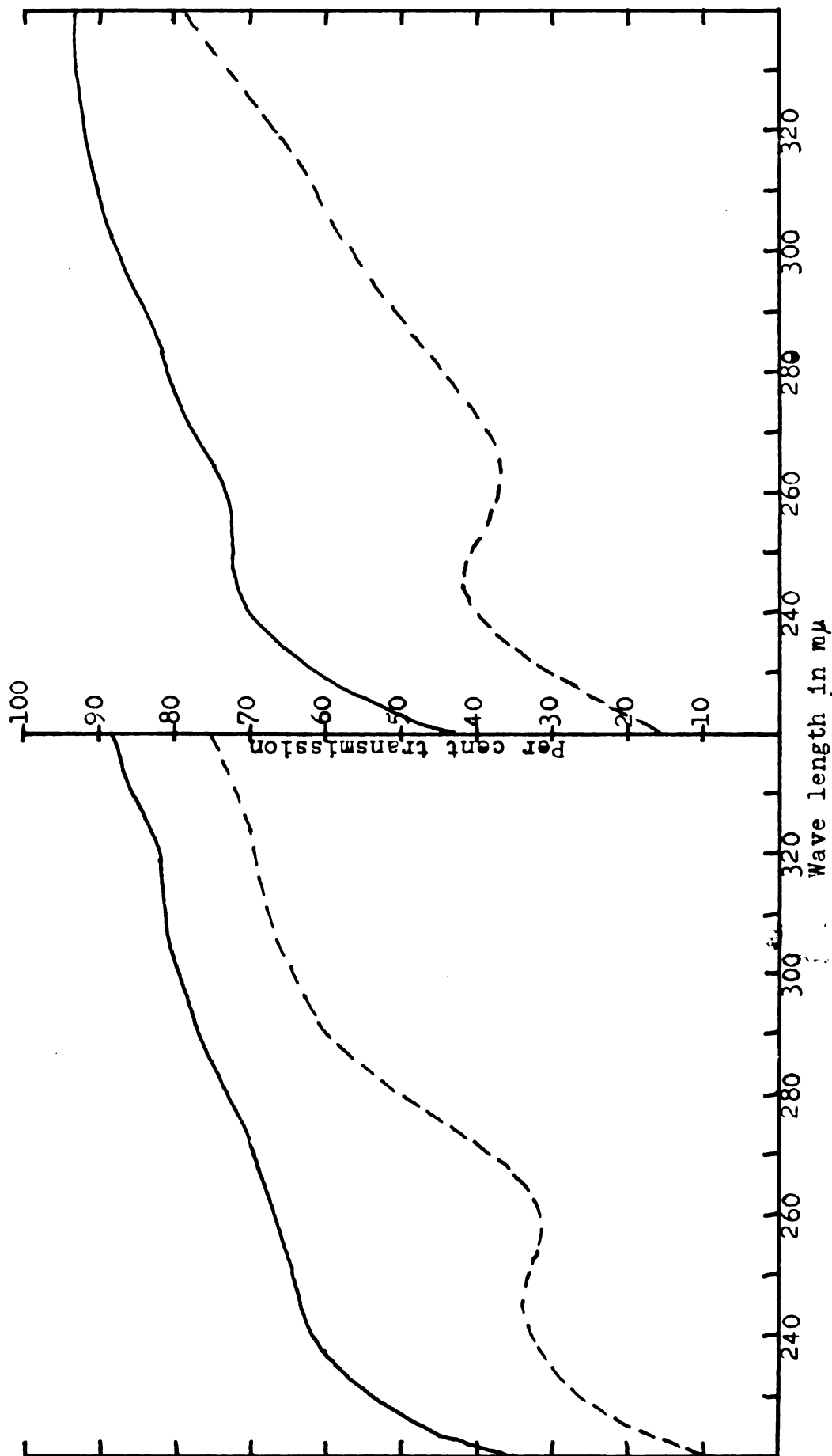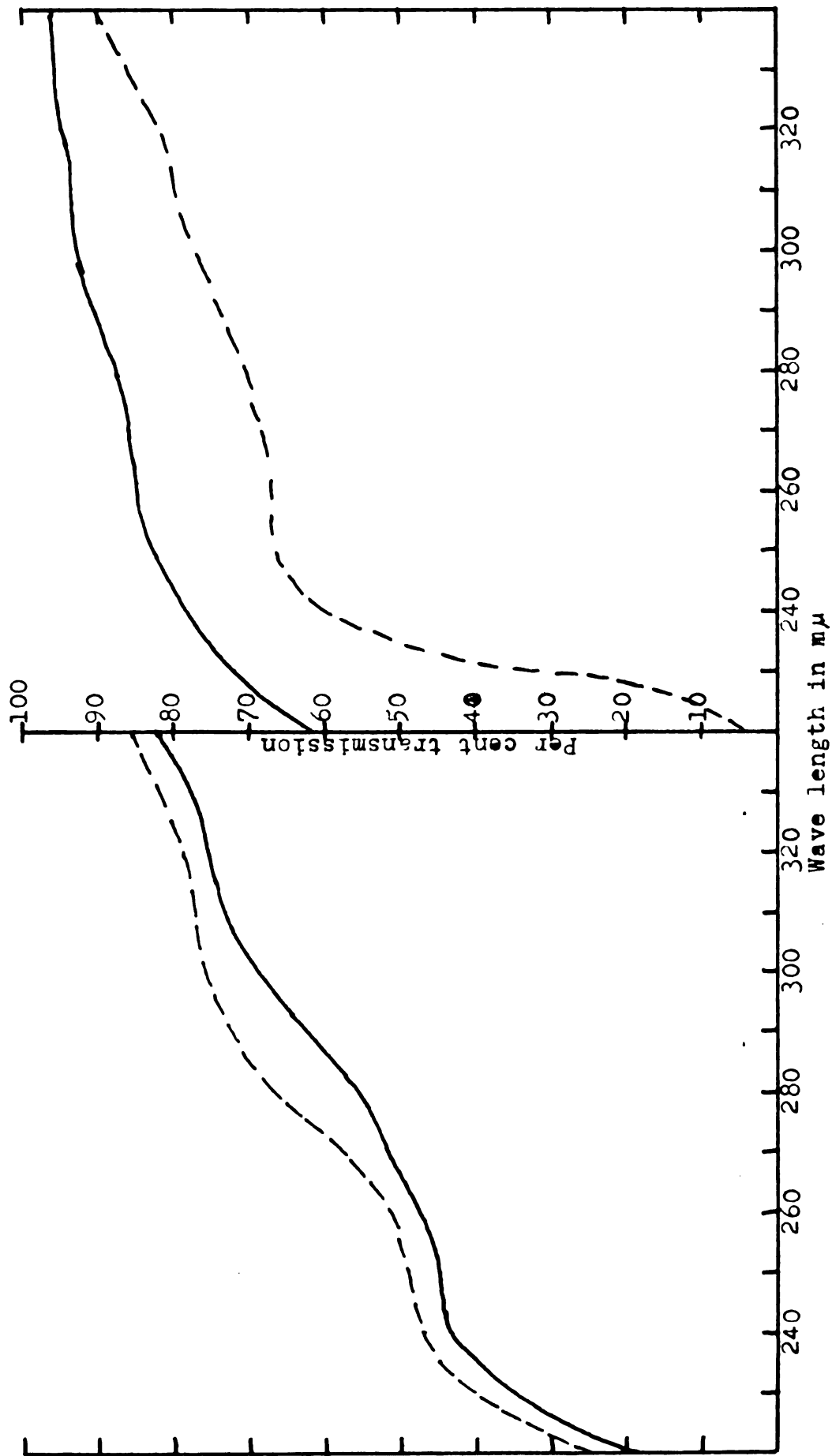
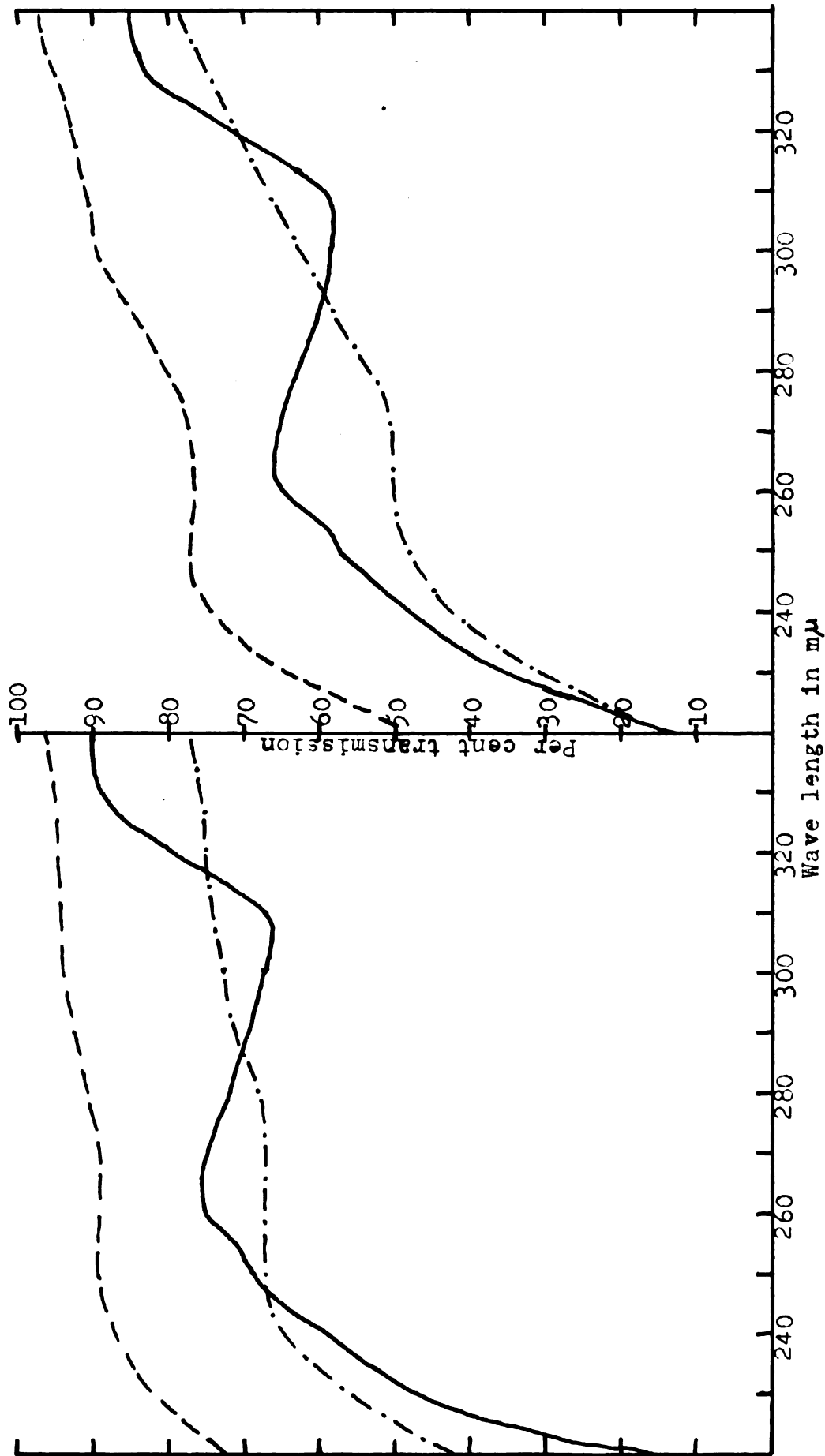Figure 7. Fluorescent plant material, experiment three, EBAW Rf .27 in distilled water ⸺ Fluorescent plant material, experiment three, EBAW Rf .37 in distilled water ⸺ ⸺

Per cent transmission

Wave length in mμ

Figure 9. Fluorescent plant material, experiment four, EBAW Rf .30 in distilled water ———, Fluorescent plant material, experiment four, EBAW R$_f$ .37 in distilled water – – –°

Figure 10. Fluorescent plant material, experiment four, EBAW Rf .45 in distilled water ———, Absorbant plant material, experiment four, EBAW Rf .81 in distilled water – – –°

Figure 11. Fluorescent plant material, experiment three, K2HPO4 Rf .80 in 0.1 M sodium hydroxide ——— , 0.1 M hydrochloric acid ——— , and distilled water o—o—o.

Figure 12. Fluorescent plant material, experiment four, K2HPO4 Rf .80 in 0.1 M sodium hydroxide ——— , 0.1 M hydrochloric acid —·—·—, and distilled water o—o—o.
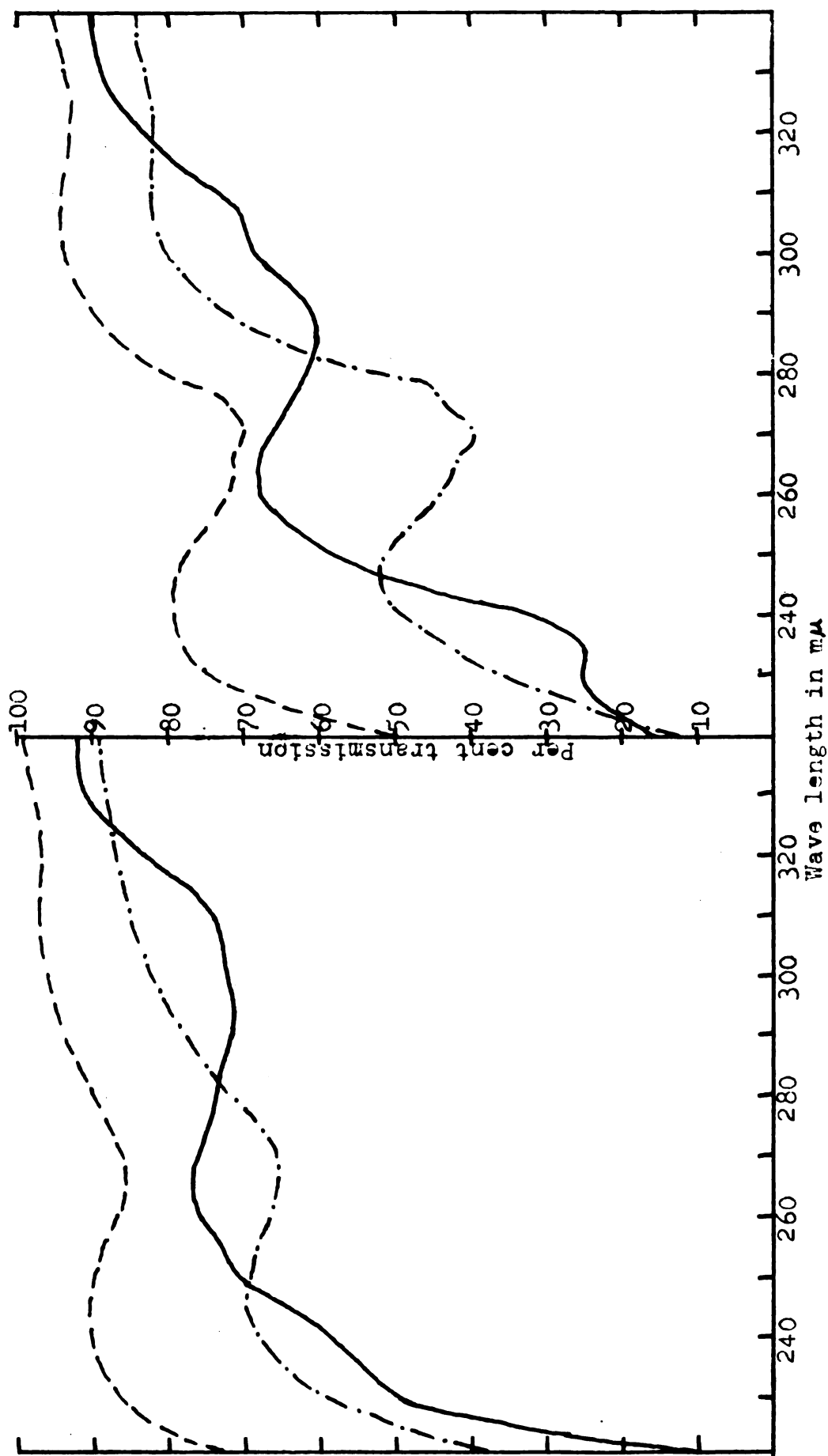
Per cent transmission

Wave length in mμ

Figure 13. Fluorescent material from tetra-
hydropteroylglutamic acid preparation, K_2HPO_4
Rf .74 in 0.1 M sodium hydroxide ____, 0.1 M
hydrochloric acid ___ ___, and distilled water
○─○─○○○

Figure 14. Fluorescent material from leuco-
vorin in acid, K_2HPO_4 Rf .56 in 0.1 M sodium
hydroxide ____, 0.1 M hydrochloric acid ___ ___,
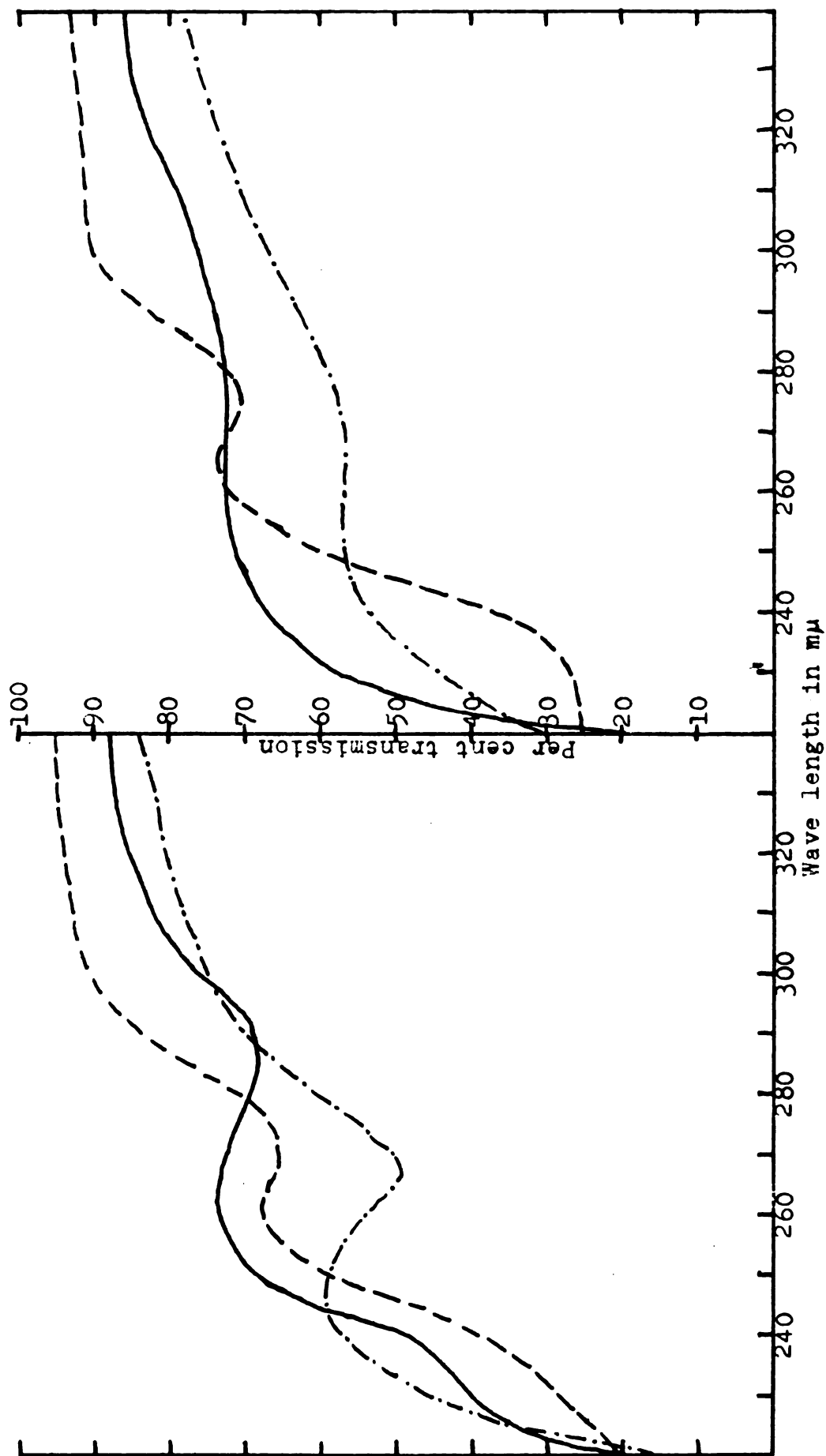and distilled water ○─○─■○○

Wave length in mμ

Per cent transmission

Figure 15. Fluorescent material from leuco-
vorin in acid, K2HPO4 Rf .73 in 0.1 M sodium
hydroxide ———, 0.1 M hydrochloric acid — — —,
and distilled water .o—o..

Figure 16. Fluorescent material from tetra-
hydropteroylglutamic acid + formaldehyde,
K2HPO4 Rf .76 in 0.1 M sodium hydroxide ———,
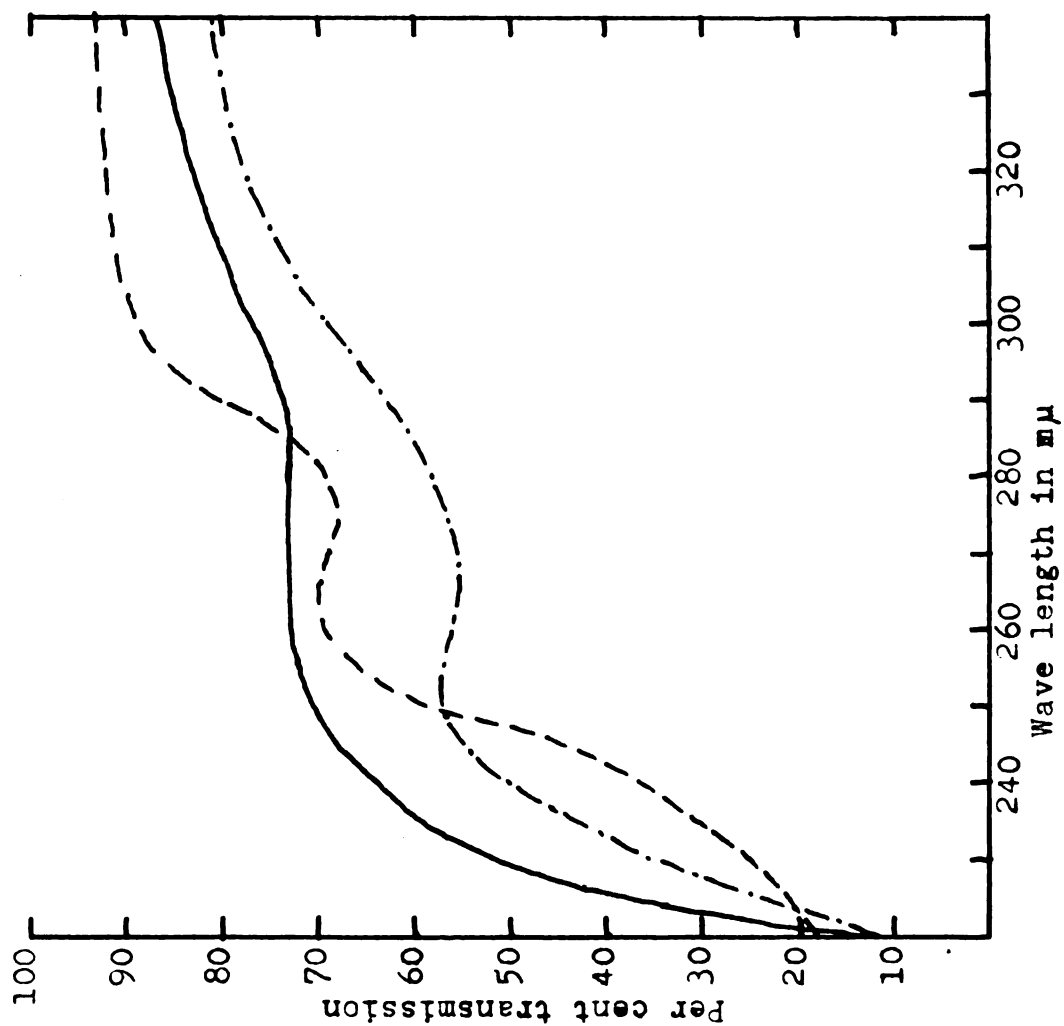0.1 M hydrochloric acid — — —, and distilled
water .o—o..

Per cent transmission

Wave length in mμ

Figure 17. Fluorescent plant material, exper-
iment five, K_2HPO_4 R_f .75 in 0.1 M sodium
hydroxide _____, 0.1 M hydrochloric acid _ _ _ _,
and distilled water °—•—°°