

- I. CHEMICAL STUDY OF NECROTIC CORN MUTANTS
- II. METABOLISM OF A KININ
- III. THE CHEMICAL NATURE OF AN INSECT GALL GROWTH FACTOR

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ABSTRACT

Part I.

The leaves of nec rd (a single gene necrotic maize mutant) become necrotic when they are exposed to light for a few days. Extensive analysis has failed to reveal any difference in chemical composition between leaves of normal plants and of homozygous nec rd plants before any necrotic symptoms are visible (prenecrotic leaves). Treatment of nec rd seedlings with various metabolites (B vitamins, purines, pyrimidines, amino acids, etc.) did not prevent the appearance of necrosis. The rate of photosynthesis of preneecrotic leaves is normal at low light intensities but only 20 to 50% of normal at saturating light intensity. $C^{14}O_2$ feeding experiments indicate that the carbon fixing reactions function normally in the mutant. Hill reaction rates are also similar in mutant and normal plants, as is the metabolism of labelled inorganic phosphate. CMU, which specifically inhibits photosynthesis to the extent of about 90%, delays the onset of visible necrotic damage and reduces the severity of subsequent necrotic symptoms. It is suggested that the nec rd lesion is in some reaction associated with photosynthesis and that it causes the accumulation of one or more toxic substances. These lower rate of photosynthesis and damage cell membranes. The necrotic phenotype would appear to be the result of the breakdown of cell compartmentalization.

Part II.

The kinin, 6-benzylaminopurine (benzyladenine), is converted to a number of low molecular weight materials by senescing leaves of Xanthium pensylvanicum Wall. (cocklebur). A major product is the riboside, benzyladenosine, which has been identified by

comparison of its properties with those of benzyladenosine synthesized enzymatically using E. coli nucleoside phosphorylase and by degradative studies. The ribotide, benzyladenylic acid, also appears to be present. Labelled adenylic, guanylic and inosinic acids are produced, as are small amounts of adenine and guanine. Substantial amounts of label are also found in urea and in a ureide. Small amounts of labelled adenylic and guanylic acids are found in the RNA of the leaf, but benzyladenylic acid itself does not appear to be incorporated into RNA in measurable amounts.

Part III.

Low molecular weight material obtained from excised accessory glands of Pontania pacifica (gall-wasp) promotes the growth of Pontania galls on Salix alba (willow). Paper chromatographic analysis has indicated that uridine, uric acid and two unidentified adenine containing compounds are prominent constituents of this mixture. Uric acid and the two adenine containing compounds substantially increase the growth rate of small galls from which the larva has been removed while uridine has slight growth promoting activity. Various related compounds (e.g. adenosine, adenine and guanosine) also have growth-promoting activity. It appears likely that such compounds play an important role in the growth and development of Pontania galls.

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PART I. CHEMICAL STUDY OF A NECROTIC CORN MUTANT

INTRODUCTION

Among the many mutant forms which have been observed in higher plants, one abundant class is that of lethals. Of these, the lethal chlorophyll mutants have received the most attention. Some mutants of the alga, Chlorella, are known which are unable to make the normal photosynthetic pigments. However, all the chlorophyll mutants of higher plants thus far examined are able to make pigment but owing to some, as yet unknown, defect, destroy this pigment at an abnormally rapid rate so that the net result is an albino plant. Another class of lethals is that studied by Langridge (1958) who has shown that some mutants of the crucifer Arabidopsis thaliana are unable to make one or more essential metabolites, for example thiamine, biotin or choline. The seeds which give rise to these plants apparently obtain sufficient of these substances from the maternal plant to carry them through germination and some seedling development. Later growth is slow or ceases altogether. The so called necrotics form still another class of lethal mutants. These are not so conspicuous as the chlorophyll mutants and, indeed, in the field may be missed altogether or dismissed as the result of cutworm or smog damage. Typical necrotic symptoms include yellowing and browning of the leaf tissue. In many mutants the tissue becomes waterlogged and exudation may occur. Necrosis may first appear at any time from the seedling stage to maturity depending on the mutant. Most necrotic mutants are recessive, that is, only plants having two doses of the necrotic gene show necrotic symptoms. The heterozygotes present a normal appearance. The physiology of necrotic mutants has

been little studied and to the author's knowledge the basic causes of this phenotype are completely unknown.

The present study was aimed at determining the nature of the abnormality which leads to the necrotic phenotype in a maize mutant, nec rd.

LITERATURE SURVEY

Several examples of necrotic mutants of higher plants are recorded in the biological literature and undoubtedly many more such mutants have been found but have gone unrecorded.

Wide crosses within the genus Triticum (wheat) have given rise to necrotic progenies (Morrison, 1957). The phenotypes of the necrotic plants produced by such crosses vary from rapid, progressive and lethal necrosis of the primary leaves, to late "firing" of the leaves at the flowering stage (Caldwell and Compton, 1943). Hermsen (1960) has analyzed in considerable detail the genetics of semi-lethal necrosis in crosses of several varieties of Triticum aestivum (common wheat). Three complementary genes account for the observed results. The severity with which the necrotic symptoms appear is related to light intensity.

Tomato plants (Lycopersicon esculentum) which are homozygous for a recessive gene, ne, and which in addition carry the unlinked factor Cfp₁ (Cladosporium resistance introduced from L. Pimpinelli-folium) develop necrotic lesions on the leaves and pedicels (Langford, 1948). The severity of these symptoms is increased by high temperatures and light intensity.

Anderson (unpublished) has found a large series of necrotic mutants in maize. Some of these mutants are described in the

material and methods section.

Harrison (1960) has described the appearance of excrudescent necrotic lesions on the stems, leaves, and calyces of hybrid progeny from the cross Antirrhinum orontium x A. meoanthum. In this case the hybrid progeny are sterile so the genetic basis of the condition cannot be studied.

Chandler and Barton (1955) have reported exudate formation and necrosis in tetraploid Plantago ovata (plantain). Since the diploid plants from which the tetraploids were derived (by the use of colchicine) are perfectly normal, the factors leading to exudate formation and necrosis seem likely to be of a very subtle nature. The authors studied the physiology of the condition in some detail and suggest that it may be associated with water relations.

While no attempt will be made to review the vast amount of literature on pathogen induced necrosis, the following cases are pertinent. Gäumann (summary, 1950) has shown that rapid necrosis following pathogen invasion may be the basis of resistance to systemic infection. Potato varieties which are resistant to Synchytrium endobioticum undergo a rapid local necrosis which destroys the infected cells thereby isolating or killing the pathogen while the remainder of the tuber remains healthy. Since no such necrotic breakdown occurs in infected cells of susceptible varieties, widespread infection results. Similar necrogenous reactions are involved in varieties of red clover (Trifolium pratense) which are resistant to powdery mildew (Erysiphe martii). In the case of late blight of potatoes (caused by Phytophthora infestans) resistance or susceptibility of a variety is determined by the rate at which the necrotic defense reactions occur. In resistant varieties necrosis

occurs rapidly while in susceptible varieties the necrogenic reactions take place too slowly to keep the pathogen from infecting large areas. A similar basis has been found for the resistance of Malakoff wheat to biotype 11 of Puccinia triticina (brown rust of wheat). The variety Little Club does not respond by such a rapid necrogenic reaction and is therefore highly susceptible to this pathogen.

The difference between these resistant varieties which possess the ability to isolate and kill invading pathogens by means of rapid local necrosis and the corresponding susceptible varieties may be due either to a single gene pair or to two or more different gene pairs (Müller, 1959).

MATERIALS AND METHODS

Plant Material

The necrotic mutant with which this study is mainly concerned arose in the maize stocks of Dr. E. G. Anderson. Genetic tests have shown that the necrotic condition appears in plants homozygous for a recessive gene (designated nec rd) which is located on the fourth chromosome. Highkin, Anderson and McNutt (unpublished) carried out preliminary physiological studies on this mutant which seems particularly suitable for such work since the first leaf appears normal for a period after it has unfolded. This makes it possible to work with tissue which is homozygous for the nec rd gene before the many secondary changes inherent in the necrotic process are initiated. They found that etiolated plants do not become necrotic and when heterozygous nec rd plants are crossed to plants heterozygous for an albino gene and the F₁ selfed, no

necrotic plants are found among the albino progeny. Paper chromatographic studies (for details see Appendix I) of the exudate produced by nec rd/nec rd plants showed the presence of glucose, a pentose and many unidentified phenolic and ninhydrin positive compounds (Anderson, unpublished). Two other single gene necrotic mutants were examined briefly. One, necrotic 6697, in which the condition appears before the first leaf has fully expanded, is located on chromosome 8. The other, necrotic 8376, is due to a recessive gene on chromosome 1. The latter mutant has pale green leaves which become necrotic several days after emergence.

In the following the term necrotic, when used without further explanation will refer to nec rd.

Cultivation of Plants

Seedlings were grown at 26°C in a controlled temperature room in the Earhart Plant Research Laboratory (Went, 1957). Artificial illumination provided light intensity of about 700 foot candles and a day length of 16 hours. Seeds were planted in 4" x 8" plastic pans in a mixture of quartz and vermiculite and supplied twice daily with nutrient solution.

In order to obtain known pre-necrotic tissue, plants were generally left until the first visible sign of necrosis appeared; namely, until the tips of the first leaves of the necrotic segregants were visibly waterlogged. The plants were then harvested, the visibly damaged areas removed, and the remaining tissue used. Normal segregants were used for comparison.

Where indicated, experiments were performed on leaf tissue taken from plants before any visible necrosis appeared. The rest of each plant was allowed to develop for several days and then

examined for necrotic symptoms. In this way it was possible to relate the experimental results obtained to the phenotype of the leaf tissue used.

Quantitative Analytical Methods

Total soluble carbohydrate was determined by use of the anthrone reagent described by Morris (1946); reducing sugar by the Somogi (1945) procedure using the colorimetric reagent of Nelson (1944). Nitrogen was determined by Nesslerization of the ammonia formed by a micro-Kjehldahl digestion as outlined by Miller and Miller (1948). Total phenols were determined by the colorimetric procedure of Folin and Ciocalteau (1921).

Extraction of the Tissue

Leaf or root tissue was dropped into 10-50 volumes of boiling 80% ethanol. After about 15 minutes the ethanolic extract was decanted, fresh 80% ethanol added, and gentle boiling continued for a further period of 15 minutes. This process was repeated once more with leaves and twice more with roots. The combined extracts were evaporated to dryness in a rotary evaporator.

In experiments where ion exchange fractionation was used or where, for other reasons, it was advisable to remove lipids, the following procedure was used: Filter aid (Hyflo-Supercell) was added to dried extract followed by sufficient hot water to make a thick paste. Several volumes of hot water were then added, the mixture filtered and the residue washed thoroughly. In some experiments the filter aid residue was then washed successively with hot ethanol, ether and benzene:ether (1:1) to recover the crude "lipid" fraction.

Fractionation of Extracts

In order to facilitate the identification of carbon-14 containing compounds, the crude extract was separated into acidic, basic (amino acid), and neutral fractions according to the following procedure described by Romberger (1960). The solution (30 ml) containing water soluble compounds was run slowly through a 0.9 cm diameter column containing 10 ml of purified Amberlite IR 120 cation exchange resin which had been converted to the hydrogen form with HCl. The eluate from this column was then passed through a similar column containing Amberlite IR 45 anion exchange resin in the OH form. Each column was washed with 10 ml water. Material which was not absorbed on either column made up the neutral fraction. The basic fraction was recovered from the IR 120 with 30 ml of 4N ammonium hydroxide while the acidic fraction was eluted from the IR 45 with 150 ml of ammonium carbonate solution prepared by adjusting the pH of 16% ammonium bicarbonate to pH 8.5 with ammonium hydroxide. Each solution was then evaporated to dryness in a rotary evaporator. In order to remove all the ammonium carbonate, water was added to the residue from the acid fraction and the solution re-evaporated. This process was repeated several times. Aliquots of each redissolved residue were plated for determination of carbon-14.

Determination of Carbon-14

Aliquots of extracts and aqueous solutions were plated on glass planchets at infinite thinness and counted to $\pm 3\%$ accuracy in a Nuclear Chicago D-47 gas flow counter fitted with a "Micromil" window. Absolute counting efficiency was approximately 25%.

Carbon-14 content of the insoluble material was determined after wet combustion of the sample with Van Slyke (Van Slyke et al.,

1961) fluid. The carbon dioxide produced was trapped in potassium hydroxide and precipitated as barium carbonate after addition of barium chloride (Aronoff, 1956a). The barium carbonate was plated on filter paper discs in a sintered glass funnel and counted as described above (Aronoff, 1956b). This method was calibrated using solutions containing sodium carbonate at various concentrations and specific activities. In later experiments the carbon dioxide from wet combustion was trapped in IN hyamine and counted in a Packard "Tri-Carb" scintillation counter after addition of scintillation fluid (Hours and Kaufman, 1959). The amount and distribution of carbon-14 on paper chromatograms was determined by means of a Nuclear-Chicago "Actigraph I" chromatogram scanner.

Determination of the Rate of Photosynthesis of Leaf Discs

Small pieces of leaf tissue (10 to 20 mg) were rapidly weighed and placed in an erlenmeyer flask of appropriate volume (50 ml for 2 discs-500 ml for 12) fitted with a stopper carrying a side tube with a small bulb. A small volume of solution containing carbon-14 labelled sodium bicarbonate was placed in the bulb, the tube sealed with a rubber cap, and about 0.1 ml of N sulfuric acid added by means of a hypodermic syringe. Ten ml of air was forced in to sweep the carbon dioxide into the flask. The flask was then illuminated from the bottom with light from a 150 watt reflector lamp 4½ inches from the flask and shielded with 2½ inches of water. A variac was used to control the intensity of illumination which was measured with a Weston photometer calibrated in foot candles. After 5 or 10 minutes of illumination the discs were removed from the flask and dropped into boiling 80% ethanol in a 15 ml graduated centrifuge tube and exhaustively extracted. The volume of the

extract was made up to 10 ml and aliquots taken for determination of carbon-14.

Determinations carried out simultaneously on paired pieces of tissue from the same leaf agreed to within 10%. That the incorporation of carbon-14 from carbon dioxide into the 80% ethanol soluble material gives a meaningful comparison of normal and necrotic tissue is indicated by the fact that the fraction of the total carbon-14 incorporated into such material was found to be the same in the two tissues. The rate of dark fixation of carbon dioxide into ethanol soluble compounds was never more than 1% of the rate at 1000 foot candles.

Preparation of Isolated Chloroplasts

The method described by Jagendorf and Avron (1957) was used. All procedures were carried out at 3°C. Leaves were ground in a mortar with about 1.5 volumes of a medium containing 0.4M sucrose, 0.01 N NaCl and 0.05 M tris-(hydroxymethyl)-amino methane adjusted to pH 7.8 with HCl. The resulting mixture was filtered through "Miracloth" and centrifuged at 200 x g for 90 sec. The supernatant was decanted into a clean tube and centrifuged at 1000 x g for 7 minutes. The supernatant was discarded and the pellet resuspended in fresh grinding medium with the aid of a small glass homogenizer.

An aliquot of the chloroplast preparation was pipetted into 4 volumes of acetone. The solution was then filtered, and the optical density at 652 m μ determined in a Beckman Model B spectrophotometer. The concentration of chlorophyll was then calculated using the formula (Arnon, 1949):

$$C_{(mg/l)} = \frac{O.D. \times 1000}{34.5}$$

Determination of Hill Reaction Rates

Hill reaction rates were determined using the method of Krogman and Jagendorf (1957). Chloroplast preparation (equivalent to 0.1 to 0.2 mg of chlorophyll) plus sufficient grinding medium to make a total of 2.9 ml was mixed with 0.1 ml of 0.1 M potassium ferricyanide in a Beckman cuvette. The cuvette was then illuminated for four minutes with light from a 150 watt reflector flood lamp placed 4½ inches away and screened by 2½ inches of cold water. After illumination, 0.1 ml of 2N sodium citrate, 0.1 ml of 0.1M ferric chloride, and 0.1 ml of 0.1M o-phenanthroline were added successively with mixing. The optical density at 570 mμ was then determined. A cuvette containing the same materials but not illuminated served as the control.

RESULTS AND DISCUSSION

Symptomatology

The first visible necrotic symptoms appear about two days after the first leaf has broken through the coleoptile. The tip of the first leaf becomes waterlogged and a characteristic red-brown exudate is produced in substantial quantities (0.05 ml from an affected area weighing 100 mg). The affected area enlarges progressively until the entire first leaf is necrotic. The second and third leaves are similarly afflicted. The fourth leaf rarely develops fully since the endosperm reserves are exhausted by this time. In the early stages of this process there is a very sharp line of demarkation between the waterlogged and the unaffected areas of the leaf. Over the course of about two days the waterlogged tissue turns yellow, then brown and finally dries out.

Normal and pre-necrotic leaf tissue appear identical when examined under the microscope. Mesophyll cells of waterlogged tissue are somewhat shrunken and their contents appear very granular. No vacuole or nucleus can be seen in these cells after they are stained with neutral red. Sections cut from yellowed leaves show almost complete collapse of the mesophyll cells but the vascular bundles are relatively little affected except for the presence of abnormally granular cytoplasm in the bundle sheath. Thus it appears that necrosis in this mutant involves the breakdown of mesophyll cells.

The residue after exhaustive 80% ethanol extraction of intact normal leaves is white while corresponding material from waterlogged necrotic leaves is a dirty grey color. The black pigment is insoluble in all organic solvents tested and in aqueous alkali or acid. Visibly damaged coleoptile and root tissue have never been observed, even when the leaves of the same plant are completely necrotic.

For convenience, the successive stages of necrosis will be referred to as follows: (see also section on symptomatology)

1. Pre-necrotic: tissue appears normal.
2. Waterlogged: tissue waterlogged, exudate on leaf but leaf still green.
3. Yellowing.
4. Brown and desiccated.

The Light Requirement

The fact that necrotic plants do not develop in etiolated plants was first observed by Anderson et al. and readily confirmed in the present study. Furthermore, it was found that necrotic damage

appeared within 36 hours after 8 day old etiolated plants were placed in light of 700 foot candles. The same result was obtained when excised etiolated leaves were maintained in light with their bases in water. Necrotic mutants could be distinguished from normal plants in populations which were grown under red, green or blue light so the quality of the light is not very critical for the development of necrotic symptoms. If populations of light grown plants were placed in the dark soon after expansion of the first leaf, all plants remained healthy.

Illuminated pre-necrotic areas of leaf tissue became necrotic even after they had been plasmolyzed in 0.5 M mannitol.

Two further necrotic corn mutants were examined to determine whether light is necessary in the development of their necrotic phenotypes. Etiolated plants of both necrotic 8376 and necrotic 6697 exhibited normal appearance but developed necrotic leaf tissue shortly after being placed in the light.

It is well known that aminotriazole prevents the development of normal chloroplast pigments (Woodford et al., 1958). When corn seedlings are treated with aminotriazole at an early stage, the first leaf is entirely white. Necrosis has never been observed in such leaves. If, on the other hand, application of aminotriazole is delayed until the first leaf has partially expanded it is possible to obtain plants in which the first leaf has a green tip and white base. Necrosis develops rapidly in the green areas of such plants but does not spread into the unpigmented portion.

Thus it appears that both chlorophyll and light are essential for the expression of the necrotic phenotype.

Chemical Changes

The most dramatic feature of the necrotic phenotype is the appearance of droplets of red-brown exudate on the surface of the leaves. The composition of this exudate was studied in some detail as was that of normal, pre-necrotic, and waterlogged tissue. When four volumes of absolute ethanol were added to concentrated exudate, only a very small precipitate formed. Aqueous extracts of normal corn leaves yield a much larger precipitate when treated in the same way. Hydrolysis of the precipitate from the exudate yielded only amino acids, indicating that a little protein is present but polysaccharides of any size are absent.

Paper chromatographic methods were used to study the low molecular weight compounds present in the exudate and tissue. Phenolic compounds with R_f values of 0.27 and 0.31 were identified as caffeic acid derivatives by comparison of their U.V. spectra with that of the pure acid and by hydrolysis to free caffeic acid which was then identified by chromatography in several solvents. The chromatographic properties and U.V. absorption spectrum of the R_f 0.81 phenol were found to be identical with those of a sample of 1,3-dihydroxy-2-keto-6-methoxy-1,4-benzoxazine which was kindly provided by R. H. Hamilton through R. S. Bandurski. The R_f 0.52 phenol gave the same spectrum as the benzoxazine derivative and probably represents its N-glucoside. Both these compounds have been reported to occur in corn (R. H. Hamilton, 1960). The R_f 0.85 phenol which occurs only in the necrotic tissue remains unidentified. It has an absorption spectrum characterized by a sharp peak at 283 m μ and gives a yellow color with diazotized sulfanilic acid. The sugars were identified by their paper chromatographic behavior

in a number of solvents and by electrophoresis of their borate complexes.

Table I shows that considerable changes in the sugars and phenols take place when the tissue becomes necrotic. It will be noted, however, that normal and preneurotic tissue cannot be distinguished on the basis of these compounds.

Table I. Composition of Tissue, Extracts and Autolyzate

Compound	Rf in BAW	Normal Tissue	Relative Amount in:			
			Pre-necrotic Tissue	Water-logged Tissue	Exudate	Autolyzate
Phenols						
Caffeic acid derivative	0.27	++++	++++	++	++	+
Caffeic acid derivative	0.31	++++	++++	++	++	+
Glucoside of DHB ^a	0.52	++++	++++	+	trace	trace
DHB	0.81 ^b	+	+++	++++
Unknown phenol	0.85	+	+++	++++
Sugars						
Sucrose		+	+	trace
Glucose		+++	+++	+	++++	++
Fructose		trace	trace
Ribose		+ ^c	+	++
Other Pentose		trace	+
Uronic Acid		trace	+

^a1,3-Dihydroxy-2-Keto-6 methoxy-1,4-benzoxazine.

^bUndetectable.

^cRibose present to the extent of 100 µg/gm F.W.

Several ninhydrin positive compounds, all but one of which could be matched with a known amino acid, were found on two dimensional paper chromatograms. However, hydrolysis of the material from each individual spot in 6 N HCl at 105°C for 20 hours produced

a mixture of amino acids. In each case the predominant amino acid found after hydrolysis appeared to be that inferred from the chromatographic behavior of the unhydrolyzed material. It seems probable that each zone consists of some free amino acid and one or more peptides with similar chromatographic properties. Since the ninhydrin positive material of the exudate and tissues appeared to be very similar this aspect was not pursued further.

The red-brown "pigment" found in the exudate appears to be a complex mixture of polyphenolic materials. When chromatographed in butanol, acetic acid, water, it gives a phenol positive streak extending from the origin to about Rf 0.10-0.15. Some of the colored material passed through a dialysis membrane, but a considerable portion of it is non-dialyzable. This pigment is probably formed by the action of polyphenol oxidase on endogenous phenolic compounds. This enzyme has previously been reported to occur in corn leaves (Haskins, 1955) and in the present study was found to be equally active in normal and preneurotic tissue. Table II shows that dialyzed extracts of normal corn convert caffeic acid to a colored non-dialyzable product. The benzoxazine derivative is also known to form colored polymers (R. S. Bandurski, private communication).

The data of table I have already indicated that the chemical composition of preneurotic leaves is very similar to that of normal leaves. This conclusion is further supported by the data of table III which showed that these two tissues cannot be distinguished on the basis of percent dry weight, soluble or insoluble nitrogen, phosphorous, reducing sugar, total carbohydrate or total phenolic

material. Yellowed tissue, however, was found to contain somewhat less of each of these substances.

Table II. "Pigment" Production from Caffeic Acid by Dialyzed Extracts of Corn

Enzyme ^a	Substrate ^b	ΔOD_{425}	OD	Caffeic Acid Remaining ^c
Active	0.24	++++
Boiled	+	0.29	0.05	++++
Active	+	0.73	0.49	trace

^a Leaf tissue (15 gm.) ground in 10 ml. 0.05 M, pH 5.6 acetate buffer. Supernatant dialyzed against buffer for 20 hours in the cold.

^b Saturated solution of caffeic acid. 0.1 ml. incubated for 6 hours with 3 ml. enzyme preparation.

^c Estimated from paper chromatograms.

Table III. Quantitative Analysis of Normal and Prenecrotic Tissue

Phenotype	Dry Weight ^a	Insol. N ^a	Sol. N ^a	Total Soluble Carbo- hydrate ^a	Reducing Sugar ^a	Total Phenols ^a	Total P ^b
Normal	107 ± 4	6.4 ± 0.4	2.3 ± 0.1	15.0 ± 1.0	8.0 ± 2.1	2.1 ± 0.5	1.03 ± 0.07
Necrotic	111 ± 2	6.6 ± 0.4	2.3 ± 0.2	16.7 ± 4.2	9.4 ± 2.7	2.3 ± 0.3	1.04 ± 0.01

^a Mg. per g. F W.

^b µg per g. F W.

Plastid pigments were extracted from normal and prenecrotic leaves and separated into carotene, xanthophyll and chlorophyll fractions by chromatography on icing sugar columns (Tswett, 1906). The absorption spectrum of each fraction from prenecrotic tissue was found to be identical with that from the corresponding normal

fraction.

Toxicity of the Exudate

Highkin and McNutt found that exudate from waterlogged tissue caused small necrotic spots when applied to the leaves of unrelated normal seedlings. This observation was easily confirmed. However, in view of the known toxicity of many metabolites to plant tissue (Curtis, 1943; Audus and Quastel, 1947; and Schreiner and Reed, 1908), it seems likely that this is a very non-specific process which is unrelated to the basic cause of necrosis in the mutant. Further support for this view derives from the finding that the same type of necrotic leaf spot can be produced on normal leaves by an autolyzate of normal corn leaves (prepared by grinding surface sterilized leaves in a mortar, allowing the paste to stand about four hours, straining the liquid through "Miracloth," heat coagulating the protein, and centrifuging off the debris). Paper chromatographic studies (table I, last column) of such an autolyzate indicate that the degradative changes involved in the formation of exudate in the necrotic mutant are similar to autolytic processes in ground normal corn.

Supplementation Experiments

In order to test the possibility that the lesion in necrotic plants is lack of ability to make an essential metabolite, seeds were grown aseptically on mineral agar supplemented with 2% sucrose and other organic compounds as shown in table IV. Each plant was grown in an 18 x 150 mm test tube containing 10 ml of media and covered with heavy aluminum foil. Temperature was maintained at 26°C with light intensity of about 700 foot candles for a 16 hour

day. Under these conditions normal plants grew well, but at a somewhat slower rate than those grown in vermiculite.

Table IV. Organic Supplements Supplied to, but without Effect on, the Phenotype of nec rd Mutant

Exp	Compound Added	Amount per Plant	Exp	Compound Added	Amount per Plant
1	Casein		5	Adenine	0.3 mg
	hydrolysate	20 mg		Guanine	0.1
	Tryptophan	0.3 mg		Hypoxathine	0.1
2	Thiamine	3.3 µg	6	Xanthine	0.1
	Riboflavin	3.3		Thymine	0.1
	Pyridoxine	17		Uracil	0.1
	Niacin	17			Low High
	Pantothenate	3.3		Adenosine	0.1 mg 0.5 mg
	Folic Acid	0.3		Guanosine	0.1 0.5
	Biotin	3.3		Uridine	0.1 0.5
3	Yeast Extract	1 mg	Thymidine	0.1 0.5	
			Cytidine	0.1 0.5	
4	Yeast Extract	8 mg	7	Choline	0.3 3.0
				8	Ethanolamine

None of the supplements used prevented the appearance of necrotic plants in a ratio of about one necrotic to three normal. It thus appears that the basic lesion in the mutant is not due to inability to make one of these simple metabolites.

Respiration

The rate of respiration of pre-necrotic leaf tissue was not significantly different from that found in normal tissue. The respiratory quotients of the two tissues were also very similar. Waterlogged tissue respired at about 30% of the normal rate while in yellow tissue respiration has almost ceased (3% of normal). Sections cut from roots of normal and necrotic plants respired at the same rate.

Photosynthesis

Preliminary measurements of carbon dioxide exchange rates in the light and dark, using a Beckman infrared carbon dioxide analyzer, indicated that the rate of photosynthesis of preneecrotic tissue in light of 1000 to 2000 foot candles was only 20-50% of the rate observed when normal leaf tissue was used. This finding was confirmed in feeding experiments with carbon-14 labelled carbon dioxide. It was also found, however, that when light of lower intensity was used, the rate of photosynthesis of the preneecrotic leaves was equal to that of normal leaves. The data of figure 1 and table V indicate that the decrease in the rate of photosynthesis in the mutant at high light intensity is caused by a partial inhibition of the process in all chloroplasts and not due to a complete cessation of photosynthesis in a portion of the chloroplasts (or cells). If preneecrotic tissue did consist of such a mixture of normal and non-functional photosynthetic units, the rate vs. intensity curve for preneecrotic tissue would be lower than the normal curve at all intensities and would level off at the same intensity as the normal curve.

It is perhaps worthy of mention that only two classes of plants can be distinguished on the basis of rate of carbon dioxide fixation. The heterozygous nec rd plants do not differ from the normal homozygotes in this respect.

Table V also shows that the degree of inhibition of photosynthesis in preneecrotic tissue becomes progressively higher as the onset of visible necrotic symptoms approaches.

The distribution of carbon-14 in various fractions of the 80% ethanol soluble extract of preneecrotic leaf tissue after

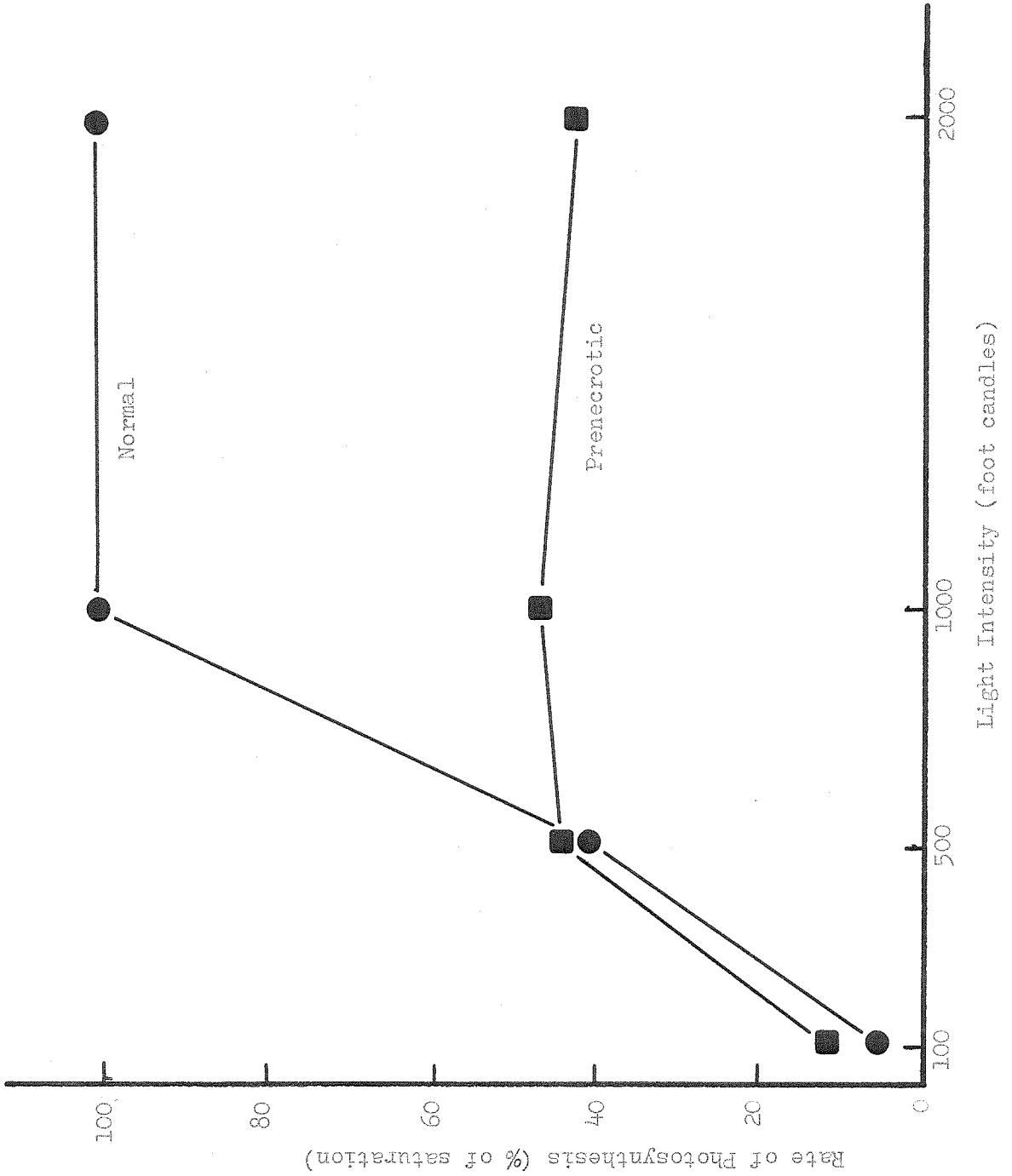


Fig. 1. Rate of photosynthesis of normal and preneurotic leaf discs at various light intensities.

Table V. Rate of Photosynthesis of Leaf Discs from Normal and Preneurotic Corn Seedlings at Various Light Intensities

Exp	Phenotype	Leaf	Time to visible Necrosis	C ¹⁴ O ₂ Incorporation (% of saturation value) at Light Intensity (Foot Candles) of:			
				100	500	1000	2000
1	Normal	1	14	100
	Necrotic	1	few hours	12	22
2	Normal	2	5	41	100	100
	Necrotic	2	several hours	12	44	46	41
3	Normal	2	18	37	100
	Necrotic	2	one day	15	36	68
4	Normal	1	7	28	100
	Necrotic	1	few hours	7	28	21

exposure to labelled carbon dioxide was found to be very similar to that obtained from normal tissue (Table VI). Furthermore, the same compounds became labelled to about the same degree in the two tissues (Table VII). The 80% ethanol insoluble fraction contains the same proportion of the total carbon-14 in preneurotic and normal leaves ($21 \pm 4\%$ and $24 \pm 3\%$ respectively).

It must be concluded from these experiments that the photosynthetic carbon fixing reactions all function normally in the mutant.

In order to gain further information concerning the integrity of biosynthetic processes in the necrotic mutant, the metabolism of glucose and of acetate was investigated.

Glucose (uniformly C-14 labelled) was fed to seedlings through the cut stem. After two hours in light of about 100 foot candles, the tissue was extracted and fractionated in the usual way.

Table VI. Distribution of 80% Ethanol Soluble Carbon-14 in Leaves after Exposure to Labelled Carbon Dioxide^a

Phenotype	Exp IV Intact Plants		Exp V Leaf Discs ^c		
	Normal	Necrotic ^b	Normal	Normal	Necrotic
Total Soluble C-14 (CPM $\times 10^4$ /100 mg F.W.)	204	99	11.3	10.7	3.9
Relative Soluble C-14	100 ^d	48	100 ^d	96	35
Percent of Total Soluble C-14 in:					
a) Neutral Fraction	35	28	36	38	39
b) Basic Fraction	28	27	35	34	33
c) Acidic Fraction	9	15	17	13	20
Water Insoluble Fraction	9	16	6	6	6
Recovery (%)	81	86	94	91	98

^aC₁₄O₂ fed for 15 minutes at 1000 foot candles. Plants then killed by plunging into boiling 80% EtOH.

^bSmall waterlogged area at tip of first leaf removed as was corresponding area from normal leaf.

^cRemoved from plant before any visible sign of necrosis.

^dArbitrarily set at 100%.

The results obtained with pre-necrotic tissue were identical to those obtained with normal leaves. The principal labelled compounds were: sucrose, fructose, raffinose, succinic, malic and low R_f acids, aspartic acid, asparagine, glutamic acid, glutamine, alanine, tyrosine, phenylalanine and tryptophan. The glucose and fructose moieties of sucrose were equally labelled.

Acetate-1-C-14 was fed in the same way. Again, no difference was found in the distribution of the carbon-14 in extracts of normal and pre-necrotic tissue. In a separate experiment acetate-1-carbon-14 fed tissue was extracted in chloroform:methanol (2:1;v/v)

Table VII. Principal Soluble C-14 Containing Compounds after Feeding Labelled CO₂ to Intact Plants for 15 Minutes

Fraction and Compound	Percent Distribution of Carbon-14 with each Fraction:	
	Normal	Necrotic
Neutral:		
Sucrose	99	99
Glucose	trace	trace
Fructose	trace	trace
Raffinose	trace	trace
Basic:		
Aspartic Acid	50	52
Alanine	39	35
Glutamic Acid	11	13
Acidic:		
Low Rf Acids ^a	55	51
Malic Acid	39	43
Succinic Acid	6	6
Fumaric Acid	trace	trace

^aIncluding sugar acids, phosphates and glycosides.

at 0°C with subsequent extractions at room temperature and 50°C. The combined extracts were evaporated under a stream of pre-purified nitrogen. The residue was taken up in a few drops of benzene-isoamylalcohol and applied to silicic acid impregnated filter paper. The chromatograms were then developed in the diisobutylketone, acetic acid water solvent (8:5:1;v/v). Again the distribution of carbon-14 on the chromatograms from normal and pre-necrotic tissue were identical.

These results provide further evidence that the major carbon pathways appear to be operating normally in the mutant.

Effect of Inhibitors of Photosynthesis

Since both light and chlorophyll are required for expression

of the necrotic phenotype, and since the photosynthetic apparatus becomes damaged before any other detectable changes occur, it seemed crucial to try to establish whether an "error" in the process of photosynthesis leads to the development of necrosis. The effect of inhibitors of photosynthesis was therefore examined.

3-p-(Chlorophenyl)-1,1-dimethylurea (CMU) is known to be a very selective inhibitor of photosynthesis which acts by blocking the electron transfer pathway (Wessels and Van der Veen, 1956, Jagendorf and Margulies, 1960) in the chloroplast. CMU treatment (4×10^{-4} M CMU solution poured into the pan in which the plants were growing just before the first leaf unfolds from the coleoptile) was found to inhibit 85 to 90% of photosynthetic carbon fixation but did not affect linear growth or dry weight accumulation of the leaves over a five day period. Increasing the CMU concentration ten fold did not appreciably increase the extent of inhibition. CMU treatment delayed appearance of necrotic symptoms by about two days and greatly reduced the rate at which necrosis later spread to the remaining leaf tissue. It seems reasonable to conclude that if photosynthesis could be 100% inhibited by CMU no necrotic symptoms would appear. As previously noted by Minshall (1960) CMU treated corn seedlings begin to degenerate after about one week so these experiments could be continued for only a few days.

Attempts to extend this type of study met with failure because the two other inhibitors tried, phenylurethane and iodoacetamide, were rapidly toxic even at concentrations where photosynthesis was only slightly inhibited.

Hill Reaction and Photophosphorylation

From the data presented in previous sections the process of

photosynthesis appears to be involved in the development of necrosis in the mutant nec rd but the pathways by which carbon compounds are formed appear normal. In an attempt to further localize the lesion, the Hill reaction and the process of photosynthetic phosphorylation were studied. Levine (1960) has recently found a mutant of the alga, Chlamydomonas which is unable to carry out the latter reaction.

Table VIII gives data comparing the rates of reduction of ferricyanide of illuminated (1000 foot candles) chloroplasts from normal and preneurotic leaves. These data show that chloroplasts from preneurotic leaves are able to carry out the Hill reaction and that although the rate is lower than normal in two of the four experiments it is unlikely that the primary lesion in this mutant is inability to carry out that part of photosynthetic electron transfer which makes up the Hill reaction.

Table VIII. Reduction of Ferricyanide (Hill Reaction)
by Illuminated Chloroplasts

Chloroplasts from	uM Ferricyanide Reduced per mg Chlorophyll per Hour			
	Exp 1 ^a	Exp 2	Exp 3	Exp 4
Normal Leaf	35	29	16	21
Preneurotic Leaf ^b	43	14	17	11
Waterlogged Leaf	0.08

^a Each experiment is the average of two determinations on the same chloroplast preparation. The individual experiments were done on different lots of seedlings.

^b Rate of photosynthesis of leaves from which the preneurotic chloroplasts were obtained was 20-40% of normal.

Extensive attempts were made to compare the rates of photosynthetic phosphorylation by chloroplasts prepared from normal and preneurotic tissue. However, using the standard technique developed

for spinach and applied successfully to a number of other plants with soft leaves, no esterification of inorganic phosphate could be detected even with preparations from normal corn. (Rates similar to those reported in the literature were obtained with spinach chloroplasts.) So far as the author is aware, there are no published reports of photophosphorylation by chloroplasts from a monocotyledon. It seems that special techniques will be necessary for such material.

Because it proved impossible to study photosynthetic phosphorylation in this mutant by use of a cell free system, experiments comparing the metabolism of inorganic phosphate containing P^{32} by normal and preneurotic leaves were carried out. The labelled phosphate was fed to intact plants through their roots and to excised leaves through their bases. With either of these procedures it was necessary to allow considerable time for uptake of the tracer. Short term (10 min.) experiments were therefore carried out by floating leaf discs on a solution of radioactive phosphate which contained 0.05% "Tween 80" to reduce the surface tension. After the period allowed for feeding and metabolism, the tissue was killed and extracted in the usual way. In early experiments, the extracts were chromatographed in the solvent used by Laughman and Martin to separate phosphate containing compounds in barley (t-butanol, water picric acid). This solvent gave fairly good separation of the material into nucleotide, sugar phosphate and inorganic phosphate zones but was very slow running and the presence of picric acid on the chromatogram interfered with the use of spray reagents. For these reasons the n-butanol, acetic acid, water (74:19:50;v/v) solvent devised by Schwink was used. The ATP zone was eluted from the paper and electrophoresed in pH 4.6 buffer in order to confirm the

identity of ATP. The results of these experiments show no consistent differences in the proportion of P^{32} incorporated into ATP, other nucleotide phosphates or sugar phosphates by the pre-necrotic and normal leaf tissue either in the light or dark.

CONCLUSION

The intimate relation of the primary lesion to photosynthesis in the necrotic mutant is suggested by the complete absence of necrotic symptoms from illuminated, chlorophyll free leaf tissue and from etiolated leaves. Mitigation of the necrotic phenotype following inhibition of 85 to 90% of normal photosynthetic carbon fixation with CMU provides further convincing evidence for this view.

The best hypothesis as to the nature of the primary biochemical lesion seems to be the following: Because of the lesion a compound which is ordinarily present in very small amounts accumulates to toxic concentrations during photosynthesis and gradually damages the photosynthetic apparatus. This compound could be a minor carbon-containing product of photosynthesis or a compound allowed to accumulate because of a defect in a reaction involving either the reducing or oxidizing product of water photolysis. That it cannot be one of the major products of photosynthetic carbon fixation is indicated by the results of labelled CO_2 feeding experiments. This same toxic compound must cause, directly or indirectly, the breakdown of the membranes of the mesophyll cells. The presence of an abnormal dark pigment on the residue of pre-necrotic leaves before any other visible damage has occurred suggests that the tonoplast becomes leaky and allows mixing of cytoplasmic and vacuolar material before the plasma membrane breaks down.

With the breakdown of cell compartmentalization, mixing of normally separated cellular components takes place. It seems likely that here, as with other damaged tissues, normally masked degradative enzymes become activated. It is little wonder then that extensive chemical changes are taking place by the time the tissue becomes visibly necrotic.

In some of the other necrotic corn mutants and much pathogen-induced necrosis no exudate is produced and the tissue has a dry appearance. The extensive exudate production by homozygous nec rd plants probably results from continued water movement due to root pressure through the relatively undamaged xylem elements at the time the mesophyll cells have become leaky. Eventually, of course, the xylem is damaged and the tissue becomes dried out.

The observation of two other mutants in which necrosis develops only when plants are illuminated suggests that there may be a whole class of mutants in which a necrotic phenotype is caused by defects in the photosynthetic system.

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PART II. METABOLISM OF A KININ

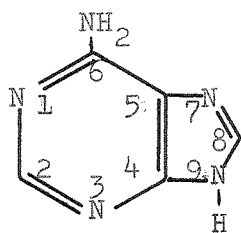
LITERATURE SURVEY

Kinetin and Kinins

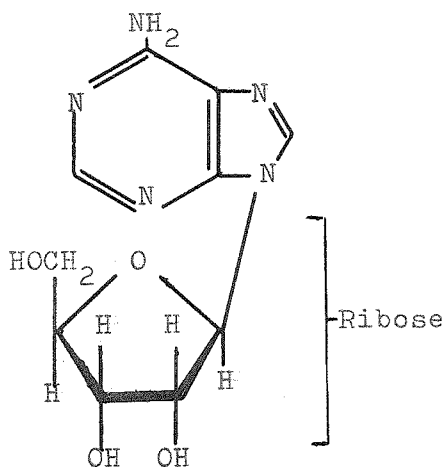
In 1956 Miller et al. reported that 6-furfurylaminopurine isolated from aged deoxyribonucleic acid (DNA) promotes division of tobacco pith cells cultured in vitro provided that auxin was also provided. The trivial name kinetin was given to this compound. It was further proposed by Miller et al. that other compounds, natural as well as synthetic, which have similar physiological properties be known as kinins.

Kinetin was first synthesized by Miller et al. (1956) and other syntheses have since been reported (Baizer et al., 1956, and Bullock et al., 1957).

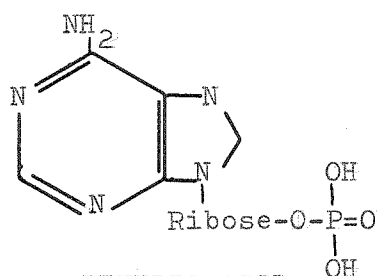
Many kinetin derivatives have been prepared and tested for physiological activity. Hampton et al. (1956) and Kissman and Weiss (1956) have synthesized 6-furfurylamino-9- β -D-ribofuranosyl purine (kinetin riboside) which appears to have the same biological effects as kinetin itself. Kinetin nucleotide, which has been prepared by von Saltza (1960), is also fully active. Many analogues in which the furfuryl group is replaced by some other radical have also been prepared. Thus 6-benzylaminopurine (figure 1g) has full activity, while some derivatives carrying substituents on the benzyl ring have even greater physiological activity than has kinetin itself. In the 6-alkylaminopurines the physiological activity of the methyl and ethyl compounds is small. Physiological activity increases rapidly in the propyl to hexyl members of the series and drops sharply at the heptyl and octyl compounds. Substitution of polar



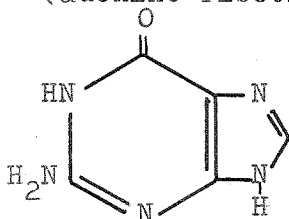
a. ADENINE
(6-aminopurine)



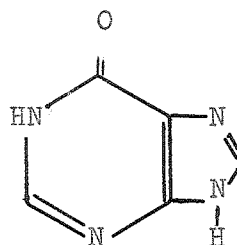
b. ADENOSINE
(adenine riboside)



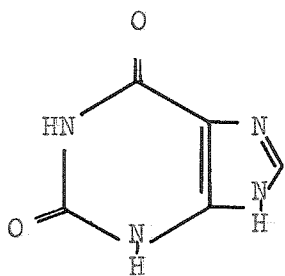
c. ADENYLIC ACID
(adenine ribotide)



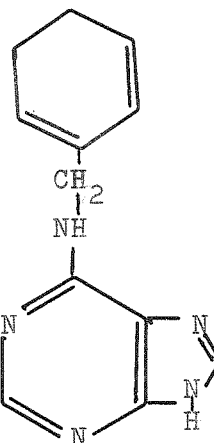
d. GUANINE



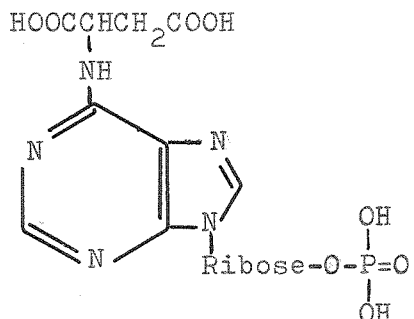
e. HYPOXANTHINE



f. XANTHINE



g. 6-Benzylaminopurine
(Benzyladenine)



h. ADENYLOSUCCINIC ACID

Fig. 1. Purines and Purine Derivatives.

groups into the N-alkyl group leads to inactivation. Substituents on the purine ring of kinetin also lead to loss of physiological activity except for the 2-amino- derivative which retains some activity (Skoog and Miller, 1957).

There can be no doubt that a kinin or kinins occur in the tissues of higher plants and play a role in the guidance of development and growth. Thus Caplin and Steward (1948) have shown that to obtain maximum growth rates of carrot phloem explants in vitro both auxin and a compound present in coconut milk are required. Extensive attempts were made by Strong and his collaborators to isolate the highly polar, water soluble kinin from coconut. Considerable purification (about 4000 fold) was achieved but crystalline material could not be isolated, indeed the final product was not chromatographically pure. Similar results were obtained in attempts to isolate an active factor from yeast (Strong, 1958). Goldacre and Bottomley (1960) have described the partial purification and some of the properties of a kinin from apple fruitlets while Miller (1961) has recently announced the discovery of a purine-like kinin in milk stage corn kernels. The structures of these natural kinins remain to be elucidated.

Since the discovery of the effect of kinetin on the division of pith cells in tissue culture, kinetin has been found to influence a number of other physiological processes. Skoog and Miller (1957) discuss kinetin and auxin interactions in processes involving differentiation of callus tissue in vitro. It is the ratio of kinetin to auxin rather than the absolute amount of either, that is important. High kinetin concentrations promote shoot development while high auxin concentrations promote the development of roots.

Wickson and Thimann (1958) report that kinetin, in suitable concentrations, is able to overcome apical dominance in peas and to free buds cultured in vitro from inhibition by applied auxin.

It is a well known fact that many plant physiological processes are promoted by red light (650-660 mu) but reversibly inhibited by far-red light (730-740 mu). Kinetin appears to mimic the effect of red light in several of these systems. Miller (1956) has shown that kinetin, like red light, increases the germination of lettuce seeds and overcomes the inhibiting effect of a previous exposure to far-red light. However, far-red light applied after kinetin treatment is not inhibitory as it is when applied after red light treatment. Kinetin and red light both promote the expansion of excised leaf discs. However, it appears that the effect of kinetin is to increase the size of the cells while red light increases the number of cells (Powell and Griffith, 1960). Kinetin and red light both promote the extension growth of Avena coleoptiles but to a lesser extent than applied indole-3-acetic acid (IAA) (Shrank, 1957). The dark growth of duckweed (Lemna minor) can be greatly increased by kinetin. A similar increase is obtained when Lemna is exposed to low intensity red light (Hillman, 1957). The effect of kinetin is similar to that of red light in still another system, the inhibition of IAA-induced elongation of pea stem sections (Miller, 1956).

Richmond and Lang (1957) found that kinetin greatly retards the loss of chlorophyll and protein from senescing Xanthium leaves. Other workers have extended this observation to other plants (Mothes et al., 1959).

Recently, kinetin has been shown to restore the viability of

seeds which have been heat treated so that they would otherwise be unable to germinate (Porto and Siegel, 1960).

The physiological effects of kinetin are not restricted to higher plants:

Kennell (1960) has found that kinetin increases the growth rate of Amoeba proteus and of E. coli. Adenine was also found to promote growth but competition experiments indicated that these two purines are not involved in the same process. In the presence of IAA, kinetin slows the growth rate of yeast for a few generations, after which the control rate is approached and eventually exceeded. When kinetin-treated yeast cells are washed and resuspended in kinetin-free media their growth rate exceeds that of the controls for a few generations. This effect is caused by premature initiation of daughter cell growth; the "daughter cells" begin to grow before the completion of cytokinesis. Adenineless strains of yeast cannot utilize kinetin as a source of adenine.

Ham et al. found that regeneration of Hydra tentacles is inhibited by kinetin but that some other adenine derivatives are many times more active. Kinetin riboside shows a great differential toxicity to adult human fibroblasts in tissue culture. Thus Hampton et al. (1956) found that over 99% of fibroblast cells are killed while other cell types remain unaffected. However, Orr and McSwain (1957) have found that kinetin significantly increases the growth of epithelial cells in human skin cultures.

The following results, while contributing little to our understanding of the problem of kinetin action are extremely intriguing and indicate the fundamental importance of kinetin and kinins:

Braun and his co-workers observed that non-virulent cells of Brucella and Pneumonococcus can be changed into virulent or S-type cells by treatment with DNAase digests of DNA. Kinetin is also able to convert non-S to S type cells although the factor in DNA digests is not kinetin itself (Braun, et al., 1957).

Jaroslawa and Taliaferro (1958) report that enzymatic digests of DNA will restore the production of hemolysin in irradiated rabbits. Kinetin, but not nucleotides or related compounds, is also effective in restoring hemolysin synthesis.

Gale (Summary, 1959) has found that the ability of preparations from Staphylococcus cells to incorporate adenine into RNA and glycine into protein can be greatly increased by the addition of an "incorporation factor" to the incubation mixture. The "incorporation factor" is obtained from enzymatic digests of DNA. Once again kinetin riboside was found to be an effective replacement for the material from DNA digests.

Mode of Action of Kinetin

The mode of action of kinetin remains obscure. Skoog and his collaborators (Das et al., 1956; Patau et al., 1957) have published extensive studies on the effect of kinetin and IAA on cell division in tobacco pith. Kinetin was found to induce DNA synthesis by some unknown mechanism. Cytochemical studies by Guttman (1956, 1957), demonstrated that exposure of onion root-tips to kinetin greatly increases the RNA content of the nuclei of the meristomatic cells. The non-nucleolar fraction in particular was greatly increased. The DNA content of the nuclei, however, was not significantly increased. Olszewska fed adenine-8-C-14 to kinetin-treated and to control onion roots and found that the presence of kinetin

increased incorporation into the nucleolar RNA about three fold while that in the rest of the nucleus and in the cytoplasm was roughly doubled. Osborne (1960, unpublished) found that incorporation of labelled orotic acid into the RNA of senescing Xanthium leaves is increased by kinetin, as is the incorporation of leucine into protein.

To the author's knowledge the sole paper concerning the metabolism of kinetin is that of Bergman and Kweitny (1958). These workers found that mammalian xanthine oxidase converts kinetin into 2-hydroxy- and 2,8-dihydroxy-6-furfurylamino-purine. As this enzyme has not been reported to occur in plants, the significance of the above finding is uncertain.

Conversion of Adenine to Other Purine Containing Compounds

It is well known that adenine is converted to other purines and incorporated into both RNA and DNA when fed to tissues. Böttger (1958) has reviewed the extensive literature on this subject (see figure 2). Published biochemical work with higher plants seems to be lacking. However, cytological studies employing microradiography have shown that adenine is rapidly converted into nucleic acids in Allium (Olszewska, 1959). McNutt (1959) working an adenineless mutant of Neurospora found that the carbon skeleton of uniformly labelled adenosine was incorporated intact into adenylic and guanylic acids of RNA and into the corresponding deoxyribotides of DNA. McNutt (1956) also showed that the isoxalloxazine ring of riboflavin is derived from the pyrimidine ring of adenine.

Metabolism of Purine Analogues

Much experimental work has been carried out on the metabolism of purine analogues in animal cells and in micro-organisms.

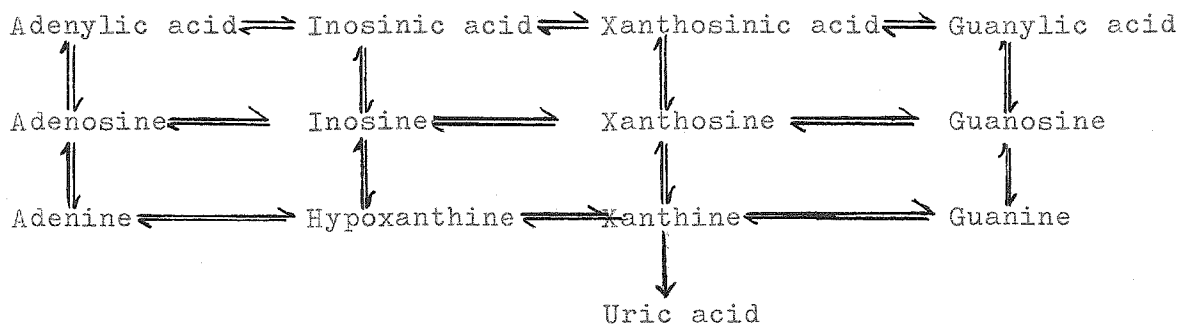


Fig. 2. Interconversion of purines, their ribosides and ribotides (from Böttiger, 1958).

The literature on this subject has recently been reviewed by Anderson and Law (1960).

Both animal and microbial systems convert several purine analogues to the corresponding ribotide. Thus 6-mercaptopurine-treated *E. coli* (Carter, 1959), mouse fibroblasts, and ascites tumor cells (Brockman, 1960) form 6-mercaptopurine ribotide but do not incorporate this compound into nucleic acid. Ascites cells convert 6-mercaptopurine into 6-thioxanthine and 6-thiouric acid as well. 2,6-Diaminopurine and 8-azaguanine (Matthews, 1958) are likewise converted to the ribotide but are also incorporated into nucleic acid. Strains of analogue-resistant cells have arisen by mutation both in microbial and animal cell populations. In a number of cases the biochemical basis of the resistance appears to be lack of ability to convert the purine analogue into its riboside. For example, Brockman (1960) found that nucleotide pyrophosphorylase which catalyses the conversion of 6-mercaptopurine, 8-azaguanine, hypoxanthine and xanthine to the corresponding ribotide is not detectable in resistant tumor cells. The enzymes concerned with the biosynthesis of adenylic and guanylic acids are apparently unaffected in these strains.

Normal purines are known to be involved in feedback control

of purine biosynthesis. Gots and Gollub (1959) have shown that several purine analogues can also inhibit early reactions in the purine nucleotide biosynthesis pathway in vivo and suggest that these analogues interfere with the biosynthesis of purine nucleotides as well as with their subsequent utilization.

Degradation of Purines

The metabolic pathway by which animal tissues degrade adenine has been well worked out (figure 2). While some details of metabolism of adenine in higher plants remain to be studied, it appears that a similar pathway is operative. The field is thoroughly covered in the reviews by Tracey (1955) and Böttger (1958).

Barnes (1959) fed adenine-8-C-14 to maple shoots and claimed to have found label in hypoxanthine, xanthine, uric acid, allantoin and urea as expected if the pathway shown in figure 2 is involved. However, since Barnes' sole evidence as to the identity of these compounds appears to be their behavior in a poorly described two dimensional paper chromatographic system, his conclusions must be regarded as tentative.

Uric acid has been reported to occur in substantial amounts in many plants (Fosse et al., 1933; Michlin and Ivanow, 1936). Uric acid occurs in a large number of different species scattered throughout the plant kingdom (Tracey, Summary, 1955). Allantoin was first found in Platanus orientalis where it accounts for 1% of the dry weight of sprouting shoots (Schulze and Barbieri, 1881). Since that time allantoin has been reported to be widely distributed in plants as is the enzyme allantoinase which converts it to allantoic acid (Tracey, 1955). Allantoic acid was first found in nature by Fosse (1928) and, while its distribution in plants has not been

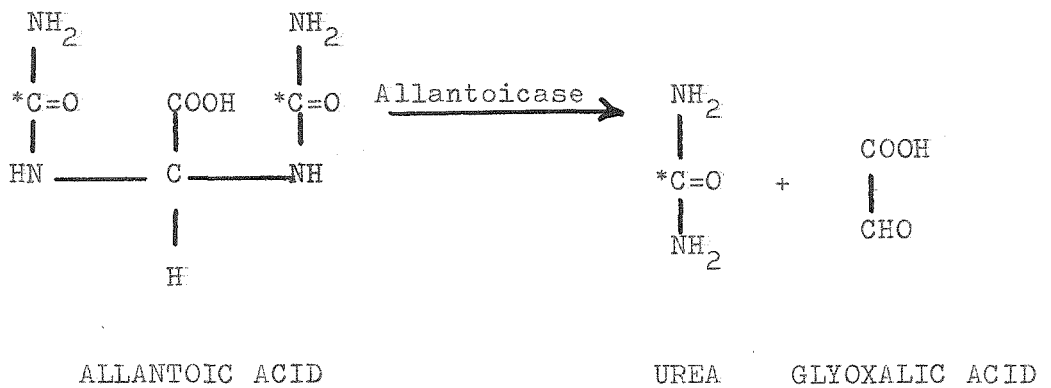
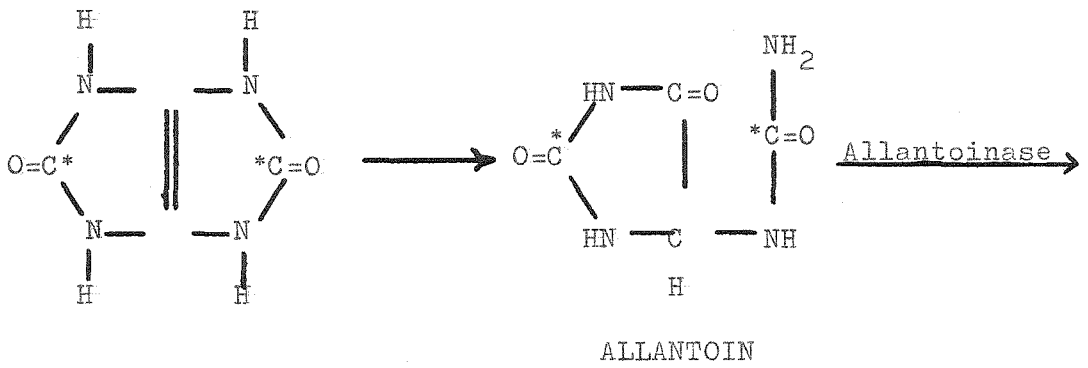
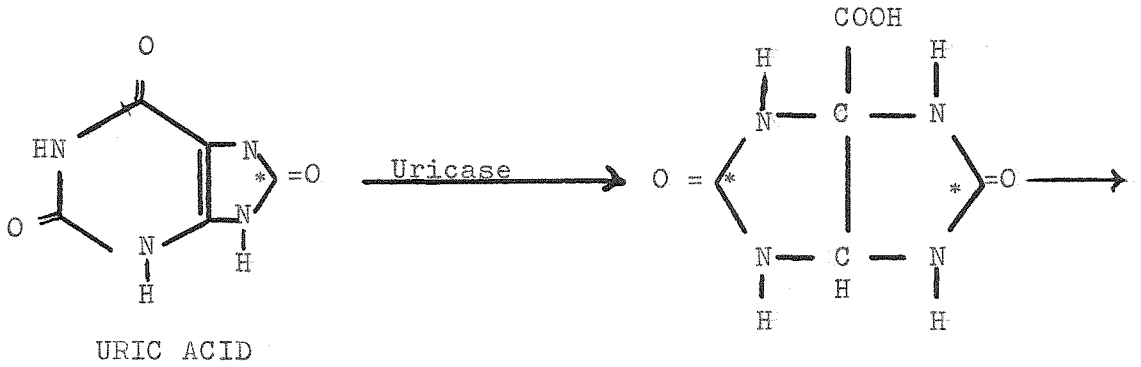


Fig. 3. Degradation of Uric Acid. The fate of purine carbon number 8 is indicated by the asterisk.

so extensively studied as that of allantoin, it is none the less a common plant constituent. The enzyme allantoicase which hydrolyses allantoic acid to urea and glyoxylic acid has been found in Glycine (Soja) hispida seedlings (Brunel and Echevin, 1938) but not in any other higher plant. However, its extreme lability may account for its apparent absence in other plants.

Mothes and Englebrecht (1954) have reported the presence of large amounts of allantoin and allantoic acid in various plants. These substances seem to play a role in upward nitrogen transport in Symphytum officinale. Their work has led to the suggestion that such large amounts of allantoin and allantoic acid cannot arise entirely from purine catabolism. Krupka and Towers (1958) have shown that the carbon-14 of labelled glycine and glyoxylic acid is incorporated into allantoin and allantoic acid in maple cuttings. Unfortunately their data do not exclude the possibility that the compounds fed were first used in the synthesis of purines, which were then degraded to allantoin and allantoic acid. Recently Reinbothe (1961) has published a detailed paper on the biosynthesis of allantoin in some plants of the family Boraginaceae and in the genus Platanus (which contain large amounts of allantoin). He reports that the carbon of glycine is rapidly incorporated into all the carbon atoms of allantoin, whereas an insignificant amount of allantoin was formed from uric acid during the same period. However, when purine antagonists were fed together with labelled glycine, the specific activity of the allantoin isolated was slightly higher than that recovered when glycine-C-14 alone was fed. This observation was interpreted as indicating that purine breakdown does contribute to the allantoin pool. It seems clear from this work that some plants at least form

large amounts of allantoin from simple precursors. Data on the biosynthesis of ureides in a wider variety of plants would be highly desirable.

The one enzyme required for the pathway shown in figure 2 which has never been reported in any higher plant is xanthine oxidase. This enzyme does, however occur in some fungi (Taha et al., 1955). Many of the animal xanthine oxidases which have been reported are exceedingly labile enzymes (De Renzo, 1956) and if the plant enzyme is similarly labile it may have easily been overlooked.

Purpose of the Present Work

The present study was undertaken first, to determine whether kinetin is converted to other soluble compounds in vivo and, if so, to identify such metabolites, and second to determine whether benzyladenine becomes incorporated into the RNA of the leaf.

MATERIALS AND METHODS

Benzyladenine-8-carbon-14

Carbon-14 labelled benzyladenine was a gift from Drs. Loffler and van Overbeek of the Shell Development Corp., Modesto, California. This material was examined in a number of paper chromatographic solvents and found to be pure.

Plant Material

Cocklebur plants (*Xanthium pensylvanicum* Wall.) of the inbred strain maintained at the California Institute of Technology were grown from seed in a greenhouse (maximum temperature 30°C, minimum 20°C; air desmogged with charcoal filters). Normal daylight was supplemented with incandescent light to give a 20 hour photoperiod. Mature leaves were harvested and stored with their petioles in

water, under low light intensity (50 foot candles) at 20°C in Plexi-glass boxes for three days.

Feeding of Benzyladenine

Sixty five discs (diameter 12 mm) were cut from the aged leaves and surface sterilized in 10% "Chlorox" for five minutes. They were then transferred to two petri dishes each containing a thin layer of benzyladenine solution (50 mg/l) to which 100 ppm. streptomycin was added. The dishes were then covered and placed in darkness at 20°C for 22 hours after which the discs were removed, rinsed quickly in distilled water and extracted with 80% ethanol until the residue was colorless.

The combined extracts were evaporated to dryness, filter aid added, followed by sufficient hot water to form a thick paste. The mixture was stirred thoroughly, then more hot water added and the mixture filtered. The filtrate was reduced to a small volume in a rotary evaporator and applied to 3 MM filter paper, which was then chromatographed in the n-butanol-acetic acid-water solvent (BAW).

Periodate Oxidation.

The material to be oxidized was dissolved in about 1.0 ml of water and the pH of the solution adjusted to 4.5 with acetic acid or sodium acetate as required. Sodium metaperiodate (0.3 ml, 0.1 N) was then added. After 30 minutes at room temperature, a further 0.3 ml portion of 0.1 N periodate solution was added and a period of 30 minutes allowed for completion of the reaction. The pH of the solution was adjusted to 12 or 13 by addition of 0.3 N potassium hydroxide. After allowing 40 to 50 minutes for hydrolysis of the dialdehyde, the solution was neutralized with acetic acid and

concentrated. Paper chromatographic techniques were then used to determine the composition of the concentrate. Adenosine treated according to the above procedure was almost quantitatively converted to adenine.

Phosphomonoesterase Treatment

Human prostatic phosphomonoesterase (London, 1955) was obtained through the courtesy of Dr. R. L. Sinsheimer. The enzyme (about 1.5 mg) was dissolved in 2 ml 0.1 M acetate buffer, pH 5.0 and 0.4 ml added to a solution (0.1 to 0.2 ml) of the material to be hydrolyzed. The solution was then incubated at 37°C for one hour after which four volumes of ethanol were added, the precipitate removed by centrifugation and the supernatant evaporated to a small volume for chromatography.

Enzymatic Conversion of Urea-C-14 to C¹⁴O₂

Material tentatively identified as urea was dissolved 1 ml of 0.2 M, pH 7.0 phosphate buffer in the main compartment of a Warburg vessel with two side arms. Urease (Nutritional Biochemical Co.) solution (1 ml phosphate buffer containing 2 mg enzyme) was placed in one of the side arms and 0.5 ml 25% trichloroacetic acid (TCA) in the other. Potassium hydroxide solution (0.2 ml) and a filter paper wick were placed in the center well. The flask was then tightly stoppered and enzyme solution tipped into the main compartment. After two hours incubation at 25°C the reaction was stopped and the CO₂ released from solution by addition of acid. A subsequent 18 hours at room temperature were allowed for diffusion of CO₂ to the center well. The filter paper wick was then removed and carefully washed with distilled water. The center well was

also thoroughly washed. The combined washings were made up to volume and an aliquot analyzed for carbon-14. The amount of radioactivity remaining in solution in the main compartment of the vessel was also determined.

Alkaline Hydrolysis of Residue

The residue, after exhaustive extraction with 80% ethanol, was ground in a mortar with 5% trichloroacetic acid. The ground material was transferred to a 12 ml conical centrifuge tube, washed twice with 5% TCA and then three times with hot 95% ethanol. The tube was then placed in a vacuum desiccator over calcium chloride for several hours after which 2 ml of 0.3 N potassium hydroxide were added and the mixture incubated for about 16 hours at 37°C with shaking (T'so and Sato, 1959). The tube was centrifuged and the pellet discarded. The supernatant was neutralized (pH 5-7) with perchloric acid and allowed to stand in a refrigerator for several hours, after which the precipitated potassium chloride was removed by centrifugation, the supernatant concentrated, applied to strips of paper and electrophoresed at pH 3.7. The presence of salts prevented complete separation of nucleotides so all material moving toward the positive pole was eluted and re-electrophoresed or chromatographed.

Enzymatic Synthesis of C¹⁴-labelled Benzyladenosine

Littlefield and Dunn (1958) and Smith and Dunn (1959) have used E. coli nucleoside phosphorylase described by Paegge and Schlenk (1952) to prepare the β -D-ribofuranosides of a number of purine and pyrimidine bases. This method has now been found suitable for the preparation of the riboside of benzyladenine.

E. coli were grown on 2 l of peptonized milk in a 4 l jar

at 37°C for 20 hours with aeration. The cells were collected by centrifugation and washed twice with distilled water. They were then ground in an "Omnimix" for 20 minutes at 0°C with an equal weight of fine glass beads, and enough 0.05 M phosphate buffer to make a slurry. The debris, unbroken cells and beads, were next removed by centrifugation and the supernatant made up to 55% saturation with saturated ammonium sulfate solution. The mixture was centrifuged and the ammonium sulfate concentration of the supernatant raised to 75% of saturation. After centrifugation, the supernatant was discarded and the pellet redissolved in a few ml. of phosphate buffer. The 55-75% ammonium sulfate fractionation was repeated. The pellet from the final 75% ammonium sulfate cut was dissolved in 3 ml. of phosphate buffer and used as the enzyme solution. The enzymatic activity of the preparation was checked by measurement of its ability to catalyse the formation of adenosine and hypoxanthine from inosine and adenine.

Benzyladenine (0.1 mg, 0.5 μ C of carbon-14) and 1 mg inosine in about 1 ml of water were incubated with 0.5 ml of the enzyme preparation for two hours at 37°C. The solution was boiled, centrifuged to remove protein and concentrated. The concentrated reaction mixture was then chromatographed in BAW, and the rapidly moving zones (Rf 0.7 and faster) were re-chromatographed in water to separate the riboside from unchanged benzyladenine. Paper chromatography in a number of solvents and paper electrophoresis indicated that the benzyladenosine prepared in this way was pure.

RESULTS

A. Soluble Compounds

Chromatography of crude ethanolic leaf extracts clearly

indicated that Xanthium leaves convert benzyladenine-8-C-14 to several labelled compounds. Paper chromatographic examination of the benzyladenine solution in which the discs had been incubated showed that all the label in the solution was still present as unchanged benzyladenine.

The following soluble compounds have been identified in the extract:

Unchanged Benzyladenine--After 22 hours incubation approximately one half of the carbon-14 of the soluble fraction is still present as benzyladenine as identified by chromatography in BAW and in water and by paper electrophoresis.

Benzyladenine Riboside (Benzyladenosine)--It was considered probable that benzyladenine would be converted into its riboside. Benzyladenosine was, therefore, synthesized using E. coli nucleoside phosphorylase to transfer ribose from inosine to benzyladenine. Benzyladenosine, prepared in this way, was found to be incompletely separated from benzyladenine in all organic solvents tested but well separated when the chromatogram was developed in water.

When the Rf 0.70 and faster moving material eluted from BAW chromatograms of the ethanolic extract of benzyladenine-8-C-14-fed Xanthium leaf discs was chromatographed in water, two labelled zones were revealed (Rf 0.33 and 0.57, corresponding to benzyladenine and benzyladenosine respectively). The paper electrophoretic mobility of the Rf 0.57 material at pH 1.9 was found to be identical to that of benzyladenosine. Furthermore, addition of borate to an n-butanol-ammonia-water solvent retarded the movement of these two materials to the same extent (Rf without borate, 0.84; Rf with borate, 0.41). This behavior is due to borate complex formation which is

characteristic of cis glycol containing compounds. The Rf 0.57 material was stable to alkali but was converted to benzyladenine by acid hydrolysis. It was also readily degraded to benzyladenine by treatment with sodium periodate at pH 5.0 followed by hydrolysis at pH 12. Synthetic benzyladenosine reacted to these treatments in exactly the same way. Thus, there is little doubt that the crude ethanolic extract contains benzyladenosine. This compound, in fact, is the major soluble metabolite of benzyladenine.

Benzyladenine Ribotide (Benzyladenylic Acid)

A further compound from the ethanolic leaf extract (Rf in BAW, 0.25), treated with phosphomonoesterase was converted to a compound possessing an Rf in BAW identical to that of benzyladenosine. This material also moved with the same Rf as benzyladenosine in butanol-ammonia-water with added borate; and co-chromatographed with benzyladenosine in water. Therefore, the original Rf 0.25 material is tentatively identified as benzyladenylic acid.

Benzyl-less Purine Derivatives

Electrophoresis of material from the ethanolic extract (Rf in BAW, 0.10 to 0.19) on paper at pH 3.7, indicated the presence of labelled adenylic, guanylic and inosinic acids. Phosphomonoesterase treatment of these compounds gave rise to materials which behave as adenosine, guanosine and inosine respectively on paper chromatography. It is perhaps worth noting that guanylic acid and inosinic acid each contained more carbon-14 than adenylic acid.

Other compounds containing small amounts of label have tentatively been identified as adenine, adenosine and hypoxanthine by chromatography and electrophoresis. Further work is necessary to confirm the identity of these substances.

Ureides

An unidentified compound present in the leaf disc extract (in the zone from Rf 0.15-0.25 in BAW) was readily converted to urea by a variety of treatments including heating and 2N HCl at room temperature. Identification of the product as urea is based on its paper chromatographic properties in BAW, isopropanol-HCl, and 88% phenol as well as its paper electrophoretic behavior. Confirmatory evidence was obtained by urease treatment. As shown in table I, 84% of the carbon-14 of the compound tentatively identified as urea was converted to carbon dioxide by this treatment.

Table I. Decomposition of Putative Urea by Urease

	CPM	%
Original Material	695	100
Carbon Dioxide	580	84
Left in Reaction Mix	100	14
Recovery	680	98

It seems probable that the 14% remaining in the reaction vessel may be the result of incomplete urease action. The ureide originally present in the extract has not been identified. However, it may well be allantoic acid since the latter is a relatively unstable compound and has been reported to have an Rf of 0.15 in a BAW solvent of slightly different composition from the one employed here (Bollard, 1957).

Two other labelled compounds have been tentatively identified as allantoin and free urea by their paper chromatographic behavior. These compounds contain less carbon-14 than the ureide described above, and indeed the free urea found may be an artifact

of allantoinic acid decomposition during extraction.

B. Compounds Derived from Residue

It was of interest to try to determine if benzyladenylic acid is incorporated into the RNA of the leaf. Therefore, the residue left after 80% ethanol extraction of the leaf discs was treated with alkali to hydrolyze the RNA to a mixture of 2'- and 3'-nucleotides. Substantial amounts of adenylic acid and of guanylic acid were liberated by this treatment. (These compounds were identified as previously described.) Also present in the hydrolysate was a very small amount of a labelled compound which chromatographed as benzyladenylic acid in BAW. The amount present (less than 1/10 as much carbon-14 as adenylic acid) was too small for confirmatory tests.

Alkaline hydrolysis also solubilized neutral material which contained as much carbon-14 as did the nucleotides. After acid hydrolysis of the neutral material, adenine, guanine, benzyladenine and an unknown compound with an Rf of 0.80 in isopropanol:HCl were present.

It seems probable that the adenylic and guanylic acids found after hydrolysis were derived from RNA. This, however, cannot have been the origin of the neutral compounds. Possibly this material represents soluble compounds which became occluded in protein precipitated when the discs were extracted with ethanol and then liberated when the protein was partially solubilized by treatment with alkali. In view of the presence of labelled purine-containing material which was not derived from RNA, the small amount of material tentatively identified as benzyladenylic acid cannot be considered to have originated from RNA. In order to obtain a definitive

answer to this question it will be necessary to isolate the RNA from the leaf before hydrolysis and to identify benzyladenylic acid in the hydrolysate with greater certainty than was possible in these experiments.

DISCUSSION

From the results presented in the preceding section it is clear that benzyladenine is converted to its riboside and ribotide by Xanthium leaf tissue. It is of interest in this connection that both the riboside and ribotide of kinetin are as active as kinetin itself in promoting callus growth. The basis of their activity could, of course, simply be their rapid conversion to the free base (kinetin). On the other hand, it may be that the ribotide or riboside, or derivatives thereof, may actually be the physiologically active material and that kinetin is active because it readily forms ribose containing compounds in vivo. The information presently available does not permit a choice between these two alternatives.

It appears to be equally clear that labelled carbon from benzyladenine-8-C-14 also appears in purine compounds which do not contain a benzyl group. Conceivably this conversion could be the result of enzymatic removal of the benzyl group from the intact purine nucleus. On the other hand, the presence of carbon-14 in adenine, guanine, hypoxanthine and their derivatives could also be the result of the removal of the carbon 8 of benzyladenine, its equilibration with the "one carbon" pool of the cells and subsequent incorporation into newly synthesized purines.

While the data obtained are admittedly equivocal, it appears unlikely that benzyladenylic acid becomes incorporated into RNA,

since a rough calculation indicates that, under the conditions of these experiments, the upper limit for benzyladenylic acid incorporation into RNA is of the order of one molecule for every ten molecules of RNA, of molecular weight two million.

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PART III. THE CHEMICAL NATURE OF AN INSECT GALL GROWTH FACTOR

INTRODUCTION

Although insect induced galls have been described for many years, little progress has been made toward understanding their physiology. There are several reports in the literature of extracts from insects or insect organs which stimulate gall formation but the chemical nature of the active material has not been extensively investigated and in no case has a reliable bioassay for the active material been published. Injection of extracts into normal plant tissue is not suitable as a routine method because only a small percentage of such injections lead to gall formation (see literature survey). There are, of course, two aspects of gall development, initiation and growth. Both these processes require stimulation from the insect but nothing is known as to whether the substances responsible for the two processes are identical or not.

The present work is an attempt to define more clearly the chemical nature of the gall-growth promoting material in the accessory glands of Pontania pacifica (Hymenoptera).

LITERATURE SURVEY

Plant galls have been a source of interest since very early times. In ancient Greece they were an item of commerce, used in the manufacture of ink and for medicinal purposes. The scientific literature on galls is vast, widely scattered and predominantly descriptive. Emphasis in this survey will be on work concerning gall initiation and development and even then coverage cannot be comprehensive. Early work is carefully discussed in the book by Küster (1911), while Plumb has covered the literature up to 1953.

Formation of galls on higher plants is known to be induced by various species of bacteria, fungi, Rotifera, Copepoda, Acarina, and by members of almost every order of insects (Wells, 1921). Thus, in the United States alone there are over 2000 gall inducing insects, about 1500 of which are gall wasps and gall midges. The oaks are extremely susceptible and some 600 different gall formers parasitize the leaves, stems, roots or acorns of various oak species (Felt, 1940). According to Wells (1921), gall making forms have evolved independently in many groups of insects. Gall formation is of obvious benefit to the animal species involved as it provides food and shelter for the developing larva but must be considered detrimental to the host plant. Went (1940), in comparing plant parasites to their analogues in human society has said:

I propose to nominate gall parasites as gold-diggers. For they are able to induce the host to excess far beyond anything they provide their own offspring. . . . And as in human society certain plants, such as oaks, are notorious as suckers.

The galls produced can be divided into two main classes: kataplastic galls, in which masses of undifferentiated callus tissue are formed, and prosoplastic galls, in which a definite internal structure is laid down (Küster, 1911). It seems probable that within any given group of insects the ability to produce kataplastic galls evolved first and later prosoplastic forming types developed (Wells, 1921).

Knowledge of the basic biology of galls developed slowly. Hippocrates (406 to 377 B.C.) was aware that "animals" were contained in one kind of gall. Pliny the Elder (23-79 A.D.), while aware that "flies" emerge from galls, did not connect gall growth with the presence of the insects. Martin Lister (1630-1712) is credited with

the discovery that one insect species is always associated with each particular kind of gall while Malpighi (1620-1694) appears to have been the first to suggest that gall formation was induced by substances of animal origin. Reaumur (1683-1759), on the other hand, suggested that mechanical damage to the tissues of the plant by the ovipositor of the insect was responsible for gall formation. The latter hypothesis can now be ruled invalid. Several workers have pointed out that there are many species of insects which damage plant tissue while laying eggs or feeding but which do not cause tumor formation, while other species causing similar damage are able to cause tumor development (Smith, 1926). Rosen (1916) found that insertion of fine glass capillary tubes into young grape leaves did not cause proliferation of plant tissue. Others have since repeated this experiment on other plants with similar results (Smith, 1920; Plumb, 1953).

On a priori grounds alone, the suggestion that the gall forming stimulus is derived from chemicals produced by the insect is much more attractive. For one thing, the occurrence of different types of galls (caused by different insects) on leaves of the same species of plant can be explained by the ability of different compounds, or mixtures of compounds, to make the tissues of the host plants respond in different ways. Some of the experimental evidence which supports this theory will now be considered.

Many workers have attempted to induce the development of galls with extracts of whole insects or insect organs. The results of such work are variable and often negative. Two factors may account for the difficulties. First, the extractive techniques employed have been very unsophisticated; in general no attempt has

been made to remove possible toxic material. Second, the techniques by which extracts have been introduced into the leaves have of necessity been crude. It is impossible to inject material into a leaf with a glass or metal needle, however fine, and not cause a great deal more damage than is caused by the ovipositor of an insect. Also, it is very difficult to know just where within the leaf the tip of a glass needle is located, while insects are able to locate specific tissues very precisely (Zimmermann, 1960).

The literature contains the following reports of successful or partially successful attempts to induce gall growth:

Laboulbene (1892) (quoted by Küster, 1911) claimed partial success from injections of aqueous extracts of Cecidomys larva and from implantation of larval integument into leaf or bud tissue.

Triggerson (1914) extracted dried Malpighian tubules from Dryophanta erinacei with physiological saline solution and injected the filtered extract into the midrib of white oak leaves. While this treatment did not produce galls, the tissue had an appearance similar to that found when gall development starts but ceases at an early stage due to death of the larva.

Martin (1942) induced stem galls of sugar cane by injections of extracts of adult leafhoppers (Draculocephala mallipes) and mealybugs (Trionymus sacchaxi), and showed further that the gall inducing factor was heat stable.

Parr (1939) was able to induce gall growth on pitch pine and chestnut oak with extracts of the salivary glands of Matsucoccus gallicolus and Astirolecanium variosolum respectively. He reports that no growth was produced by extracts treated at 60°C.

Leatherdale (1955) injected filtrate of Dasyneura urticae macerates into immature leaves of Urtica dioica with an ultrafine hypodermic needle. Such treatment produced growth in 12 out of 150 injections of whole larva macerates and in 9 out of 50 head-only macerates. No growth occurred in 50 water injected controls. The growth produced did not resemble normal galls.

Anders (1958) found that the secretions of the larva of Phylloxera contained high concentrations of amino acids, particularly lysine, histidine, tryptophan, glutamic acid and valine. Solutions of pure amino acids induced gall formation on developing grape roots.

Lewis and Walton (1947, 1958) have reported that another aphid, Hormaphis hamamelidis injects virus-like particles into Hamamelidis while feeding and concluded that this agent is responsible for gall initiation and growth.

Hovanitz (unpublished) has shown that saline extracts of the accessory glands of female Pontania pacifica, which contain material which initiates gall formation, also promote the growth of developing galls from which the larvae have been removed. He has further used such growth promotion as the basis of a bioassay for such substances. (For details, see methods section.)

Other workers have drawn attention to the growth promoting properties of larval feces. Küster (1911), working with Pontania proxima galls on willow, observed that where gall tissue was in contact with pellets of excrement, there was greater proliferation of the tissue. La Rue (1937) noted that outgrowths of plant cells occur only around each pellet of feces deposited by leaf miners in their burrows. He demonstrated further that this effect could be

duplicated by application of droplets of indole-3-acetic acid (which is known to be excreted by higher animals) to the inside of the burrow and suggested that auxin was the active component in the feces.

Boysen-Jensen (1948) carried out an extensive study on the initiation and growth of Mikiola fagi galls on beech. He was able to show that mechanical damage alone could not cause gall formation, but that if larvae were placed on lanolin paste on a leaf some proliferation of tissue occurred. In more refined experiments, larvae were placed on lanolin for an unspecified period and then removed. The lanolin paste alone would then induce callus growth on beech leaves and cause curvature of Avena coleoptiles. He concluded that there is no special gall forming substance.

Boysen-Jensen was fully aware of the difficulties besetting those who would carry out experimental work on gall growth and it seems appropriate to quote the following passage:

It is on the whole very hard to reproduce these experiments because their results are strongly influenced by a series of conditions, for instance the rate of development of the buds, the atmospheric humidity, the temperature, and so on, which are difficult to regulate. . . . However imperfect these experiments are, they nevertheless prove that it is possible to regulate the growth of the callus by local influences of substances. There is nothing to prevent the assumption that the natural galls are a sort of a callus produced by the gall larva by means of secreted substances and that the shape of the galls is caused by the gall larva secreting these substances in definite places and in definite concentrations.

Amongst hymenopterous insects, with the exception of the genus Pontania of the Tenthredinidae (saw flies), the gall forming stimulus appears to be derived from the larval form. Thus, there is commonly no proliferation of plant tissue until after eclosion. The larva of some species even crawl about on the leaf before settling down in a new spot where gall formation then takes place

(e.g. Cecidomyia poae on Poa nemoralis). On the other hand, Beijerinck (quoted by K^uster, 1911) found that small galls developed at sites where Pontania females had injected a small amount of fluid into the leaf but had not deposited an egg or where the egg had been killed with a hot needle. For continued growth and development, however, even the Pontania galls require further stimulation by larval materials.

MATERIAL AND METHODS

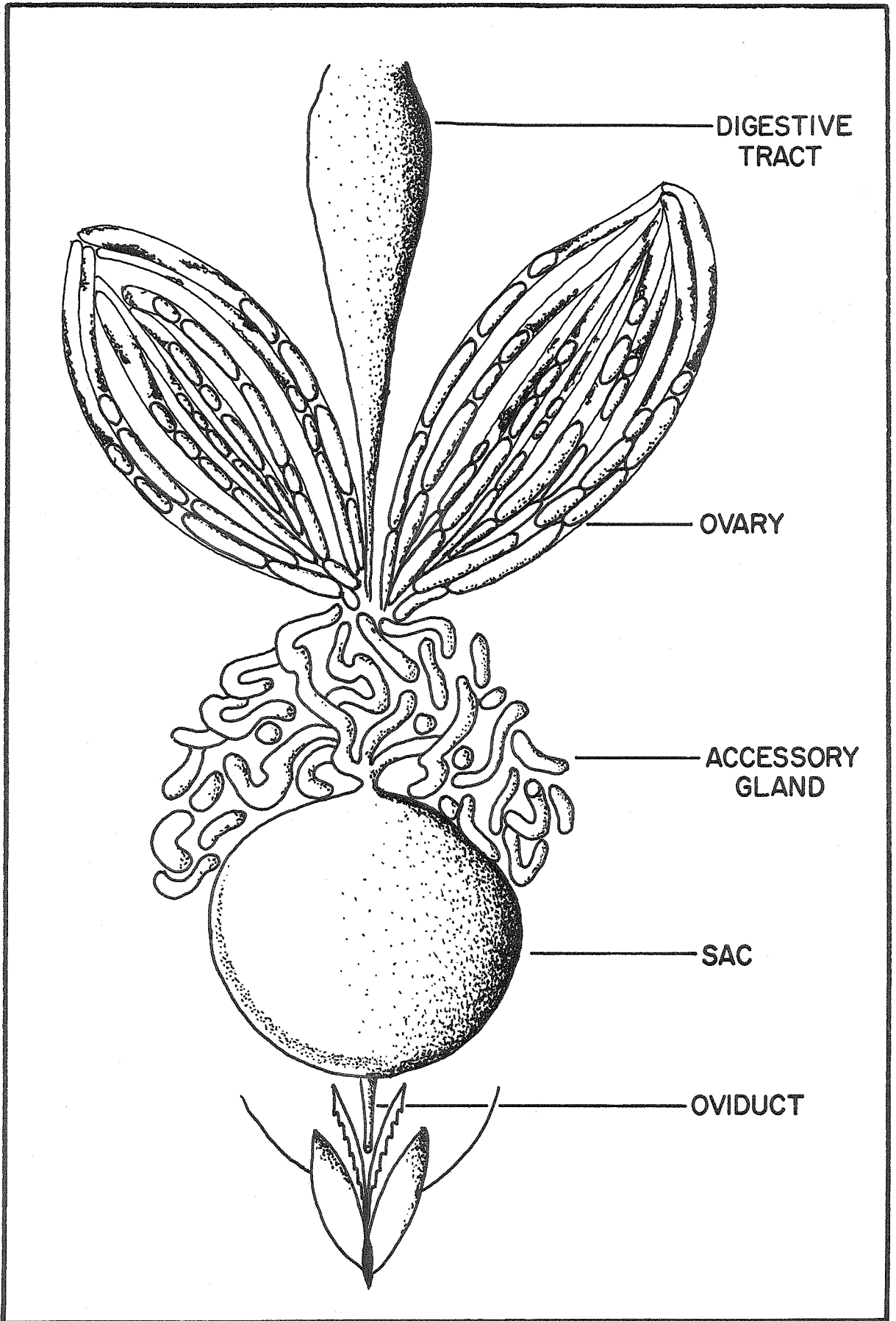
Plants and Insects

Willow leaves (Salix alba) bearing developing Pontania pacifica galls were collected locally and placed in covered plastic boxes containing several layers of paper towel. These boxes were stored at 7°C in the dark for several months. During this time the larva complete their development, and leave the galls to pupate among the leaves and paper at the bottom of the box. The adults begin to emerge after about four to six months of pupation. Since the life span of adult Pontania is very short, daily collections of insects were made. The males were discarded and the females either dissected or frozen.

Preparation of Material

The accessory glands and accompanying sacs (figure 1) were carefully excised from female wasps under a binocular microscope and immediately frozen. They were stored at -20°C until used.

In the initial stages of this work various extractive media were used. These included dilute sodium chloride solution, distilled water, aqueous phenol and 80% ethanol. Physiologically active material could be recovered using each of these techniques.



H. GENTHE

Fig. 1. Accessory gland and reproductive organs of Pontania.

The following method was used in subsequent experiments because of its simplicity.

A small amount of 80% ethanol was added to vials containing 50 or 100 frozen glands. The glands were then macerated in the ethanol, ground in a small, all-glass homogenizer and the grindate centrifuged at 2500 rpm in a clinical centrifuge. The supernatant was decanted and the residue washed with 80% ethanol and again centrifuged. The combined supernatants were evaporated.

In experiments in which whole extract was used, a small volume of water was added to the dried residue, the mixture thoroughly stirred, centrifuged and the pellet discarded.

In some experiments paper chromatography was used to fractionate the material. The dried residue was taken up in water and applied as a narrow band to washed Whatman 3MM filter paper (a band five inches long gave good results with an extract of 400 glands). The initial separation was carried out in butanol-acetic acid-water (BAW) solvent.

Bioassay of Gall Growth Promoters

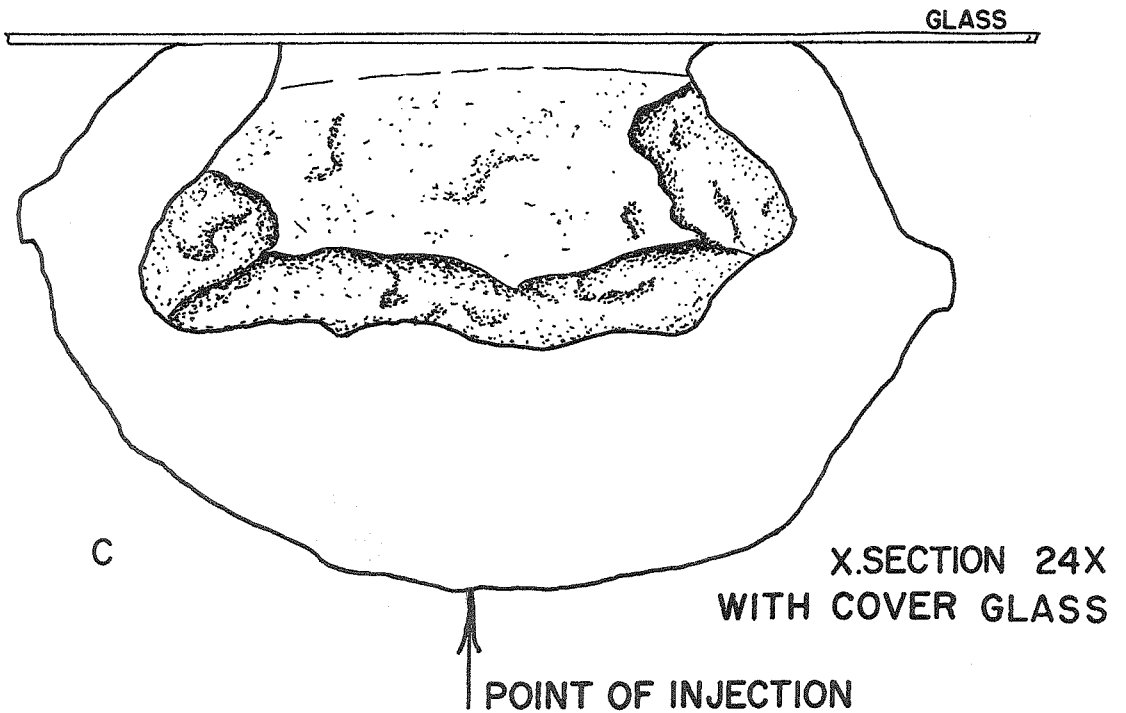
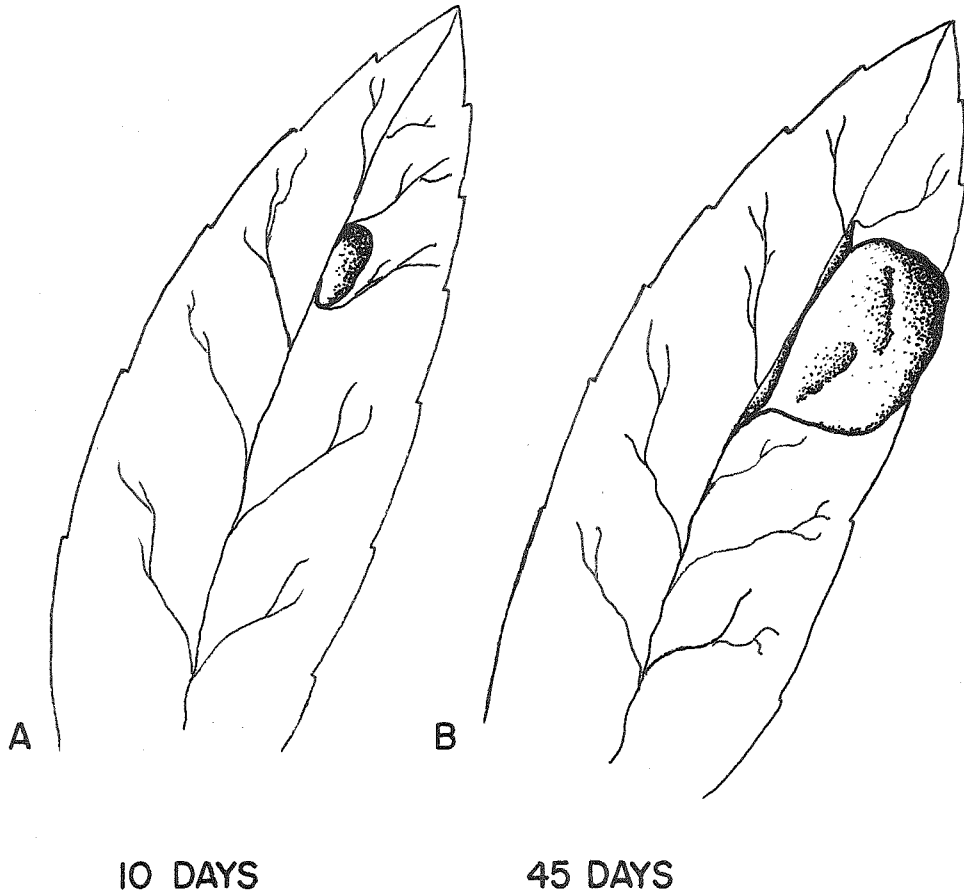
The bioassay devised by Hovanitz (unpublished) was used. Cuttings of the willow trees (Salix alba) from which the insects had been collected were rooted and planted in a quartz-vermiculite mixture in plastic containers. They were watered daily with nutrient solution. Temperature was controlled at 13°C night and 20°C day. The plants were exposed to natural daylight supplemented with artificial light for a total of 16 hours photoperiod. When the cuttings were well established, they were transferred to an artificial light room (light intensity about 500 foot candles, photoperiod 16 hours, temperature 23°C) where they remained until completion of the

bioassay.

The plant was covered with a wire mesh cage into which a newly emerged female Pontania was introduced and allowed to remain for several hours. After a few days small galls could be found on some of the young leaves. After these had developed for 10-12 days (counting from the time of egg deposition) most of the galls were opened and the larva removed from each. The openings of these galls were carefully covered with small pieces of cover glass held in place with casein glue, as shown in figure 2c. The side of the gall opposite the cover glass was pierced with a fine glass needle and a solution of the material to be tested was injected into the hollow center of the gall (about 2 μ l per injection). Injections were repeated every two or three days, usually until a total of seven injections had been made. At the same time, the length, width and height of each gall was measured with a pair of fine calipers which were read to 0.1 mm. The three values thus obtained were multiplied together to give a volume parameter. Since the galls were not all of the same size at the start of the experiment the "volume" was converted to "relative volume" (i.e. volume of gall \div volume at first injection) for comparative purposes.

Galls injected with double glass-distilled water served as controls while other galls from which the larva had not been removed were used as an indication of maximum possible growth under the conditions of a particular experiment.

Experience indicated that best results were obtained when only one gall per leaf was used. When more than one gall occurred on the same leaf all but one was left uncovered after removal of the larva.



H.GENTHE

Fig. 2. a. Young gall. b. Full sized gall. c. Cross section of gall for use in the bioassay.

While no special precautions were taken to prevent microbial contamination (indeed, sterile conditions would be exceedingly difficult to achieve) growth of such organisms was only observed when plants with very high water content were used. In the few cases where mold growth was observed, the experiment was discarded.

RESULTS

Experience with the Bioassay

It must be emphasized at the outset that the bioassay is far from standardized. Different amounts of growth are obtained in successive experiments with the same material in galls on plants originating from cuttings taken from the same tree. Therefore, the results of different experiments can be compared from a qualitative point of view only.

Cuttings from some trees (especially those with soft tissue and high water content) are unsuitable for assay purposes since substantial growth occurred in most distilled water controls. Even when the most suitable plants were used, occasional distilled water controls grew far more than expected. For this reason, in later experiments several distilled water treatments were included.

Preliminary Experiments on the Nature of the Growth Promoting Factor

Figure 3 shows the growth promoting activity of a 0.9% sodium chloride extract of glands. The first experiments were directed towards determining whether the active material in this extract is a small or macromolecule. Glands were macerated in 0.9% sodium chloride solution and the mixture clarified by centrifugation. The extract was then dialyzed against salt solution of the same strength for 20 hours. As shown in figure 4 the dialyzable

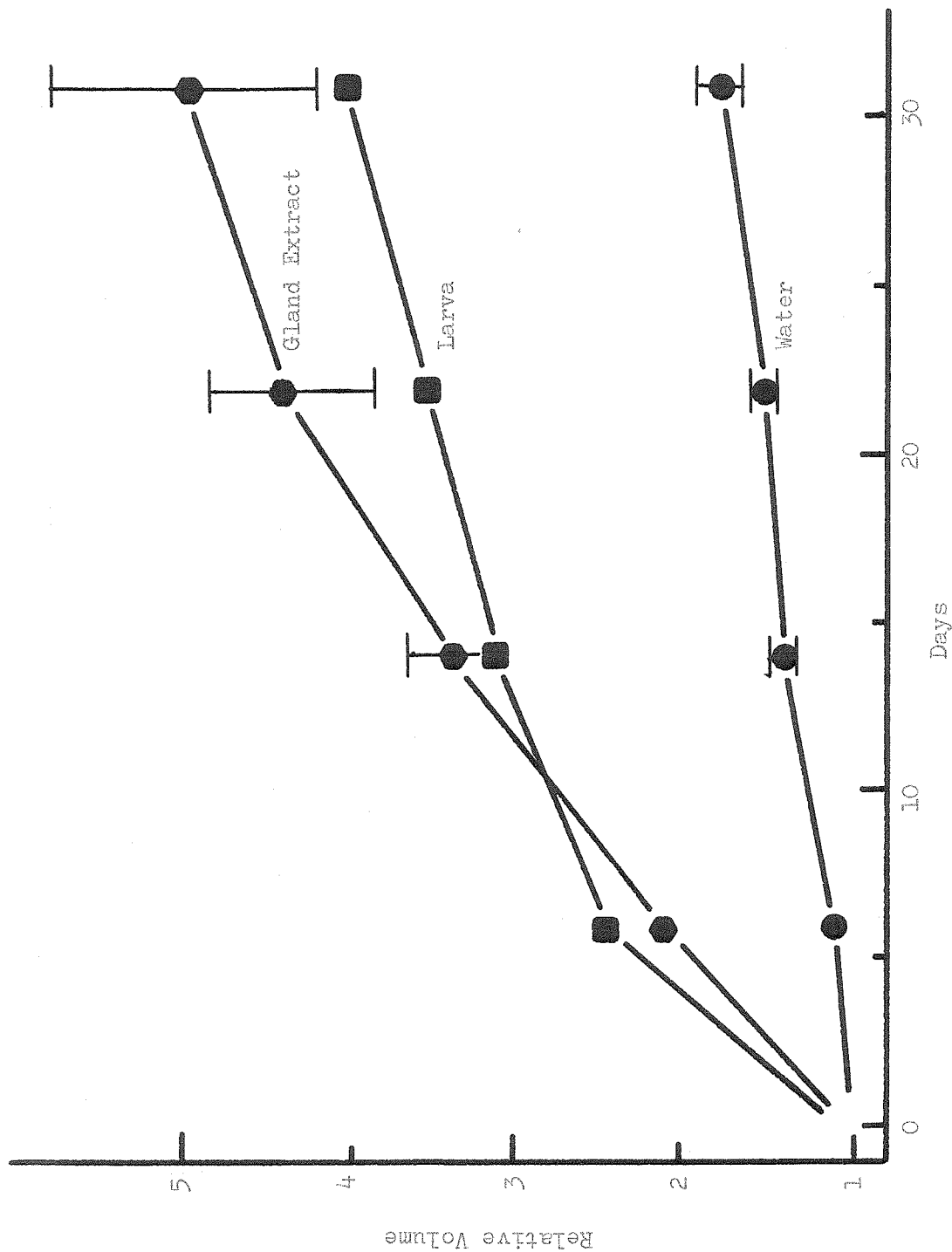


Fig. 3. Stimulation of gall growth by gland extract.

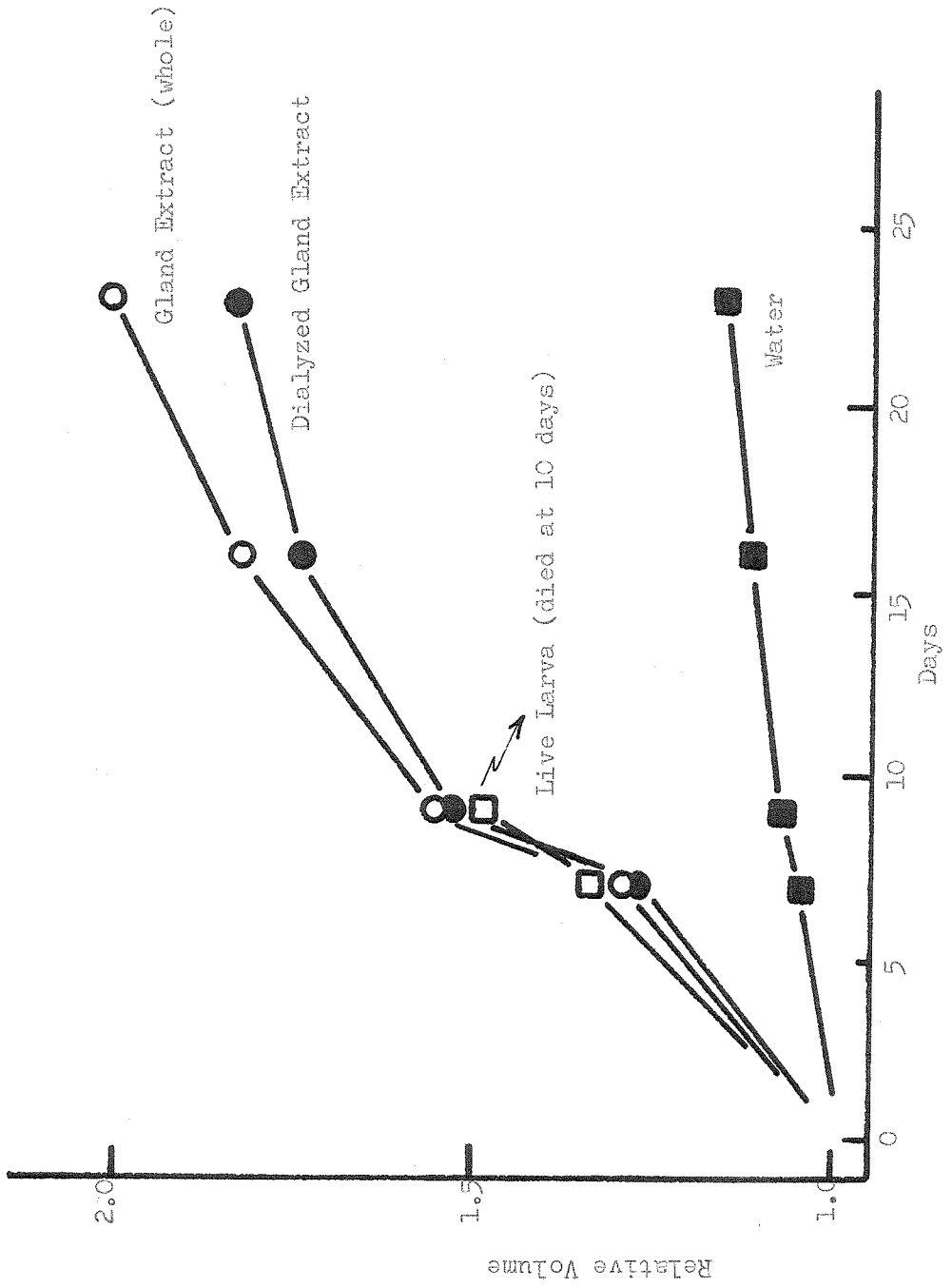


Fig. 4. Gall growth stimulation by dialyzable gland extract.

extract possessed considerable activity although some activity remained in the nondialyzable fraction. In a second experiment the protein of a similar extract was removed by shaking the saline extract with 95% phenol. The phenol was then removed from the aqueous layer with diethylether. Material prepared in this way contained dialyzable growth promoting material. From these results it appears that low molecular weight substances capable of promoting gall growth are present in the extracts.

Figure 5 shows that increasing concentrations of the dialyzable portion of the gland extract cause greater and greater gall growth up to the highest concentrations tested. Concentration is expressed in glands per injection, that is, the number of glands used to obtain the dialyzed material injected in a single injection (2 μ l).

The dialyzable fraction of the extract was subjected to a number of treatments and then assayed, with the following results: The physiologically active material was found to be stable to heating at 100°C for 15 minutes in solution, stable to pH 13 and pH 1 for 15 minutes at room temperature, and to be adsorbed on charcoal from aqueous solutions. Attempts to purify the active factor by selective elution from charcoal failed, although some active material was eluted with 20% ethanol, more with 80% ethanol and still more with 5% aqueous phenol. Similarly, attempts to purify the active factor by means of ion exchange resins were unsuccessful. Active material was found in the acid, basic and neutral eluates from the resin.

Chromatographic Analysis of the Gland Extract

Chromatography, employing the butanol-acetic acid-water

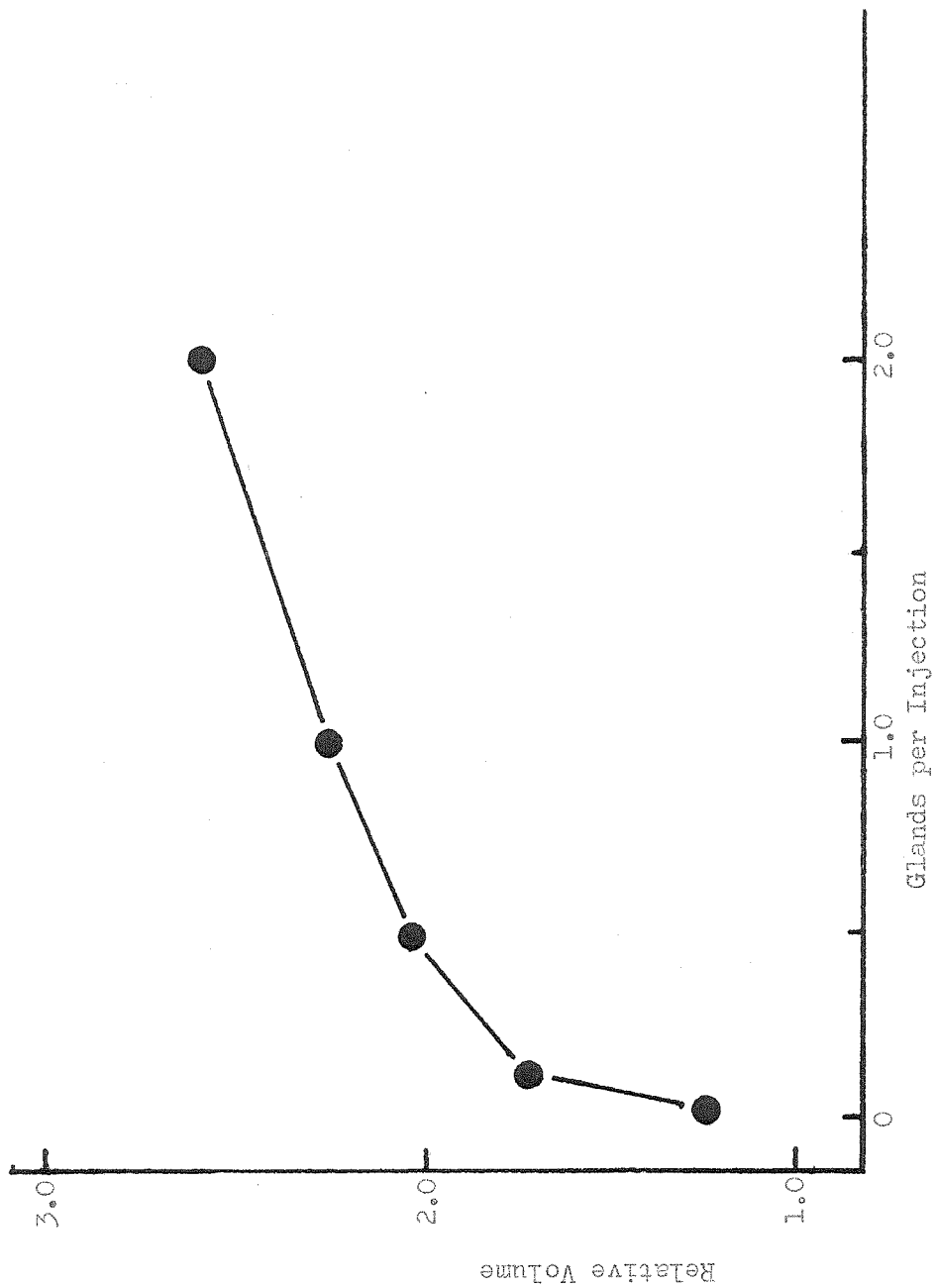


Fig. 5. Dose response curve for the stimulation of gall growth by dialyzable gland extract.

(BAW) solvent, showed the presence of a number of compounds in the extract (figure 6). These include several compounds which absorb ultraviolet light, two phosphate containing compounds, of which the faster moving is inorganic phosphate, one main and several minor ninhydrin positive compounds (amino acids or peptides), and several compounds which gave a faint yellow color with p-dimethylamino-benzaldehyde (ureides or indole-containing compounds). Carbohydrates and phenols are either absent or present in such small quantities that they cannot be detected.

Successive 2 cm zones of a BAW chromatogram were eluted and the eluates assayed for growth promoting activity. Considerable activity was found in several zones from the low Rf regions of the chromatogram, suggesting that the growth promoting material is not a single compound.

A detailed study of the chemical natures and growth promoting activities of the main UV absorbing compounds was begun. Figure 7 shows an isopropanol-HCl chromatogram of the successive UV absorbing zones from the BAW chromatograms. Each of the zones was found to consist of one major compound plus traces of other material. The five major UV absorbing compounds will now be considered individually.

Compound A

Rf in BAW = 0.10, in isopropanol = 0.06, in butanol-ammonia = 0.00. Upon hydrolysis of compound A in 6N HCl for one hour at 105°C, adenine is liberated. The absorption spectrum of A is shown in figure 1b in Appendix II, p.102. When this compound is electrophoresed at pH 4.7 it moves toward the negative pole considerably more rapidly than does adenine or adenosine. Tests for

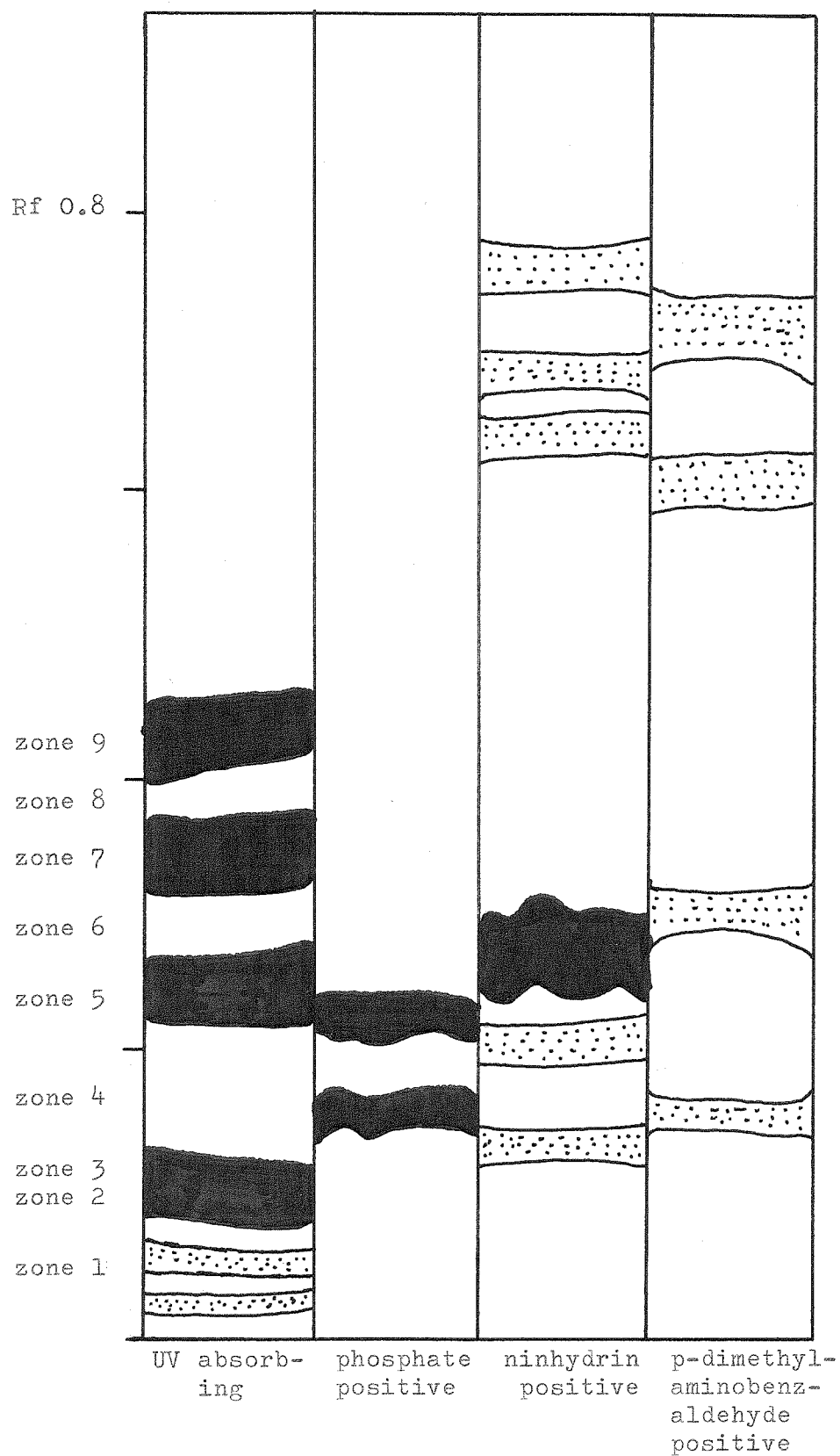


Fig. 6. BAW chromatogram of extract from Pontania glands.

Rf 0.90 →

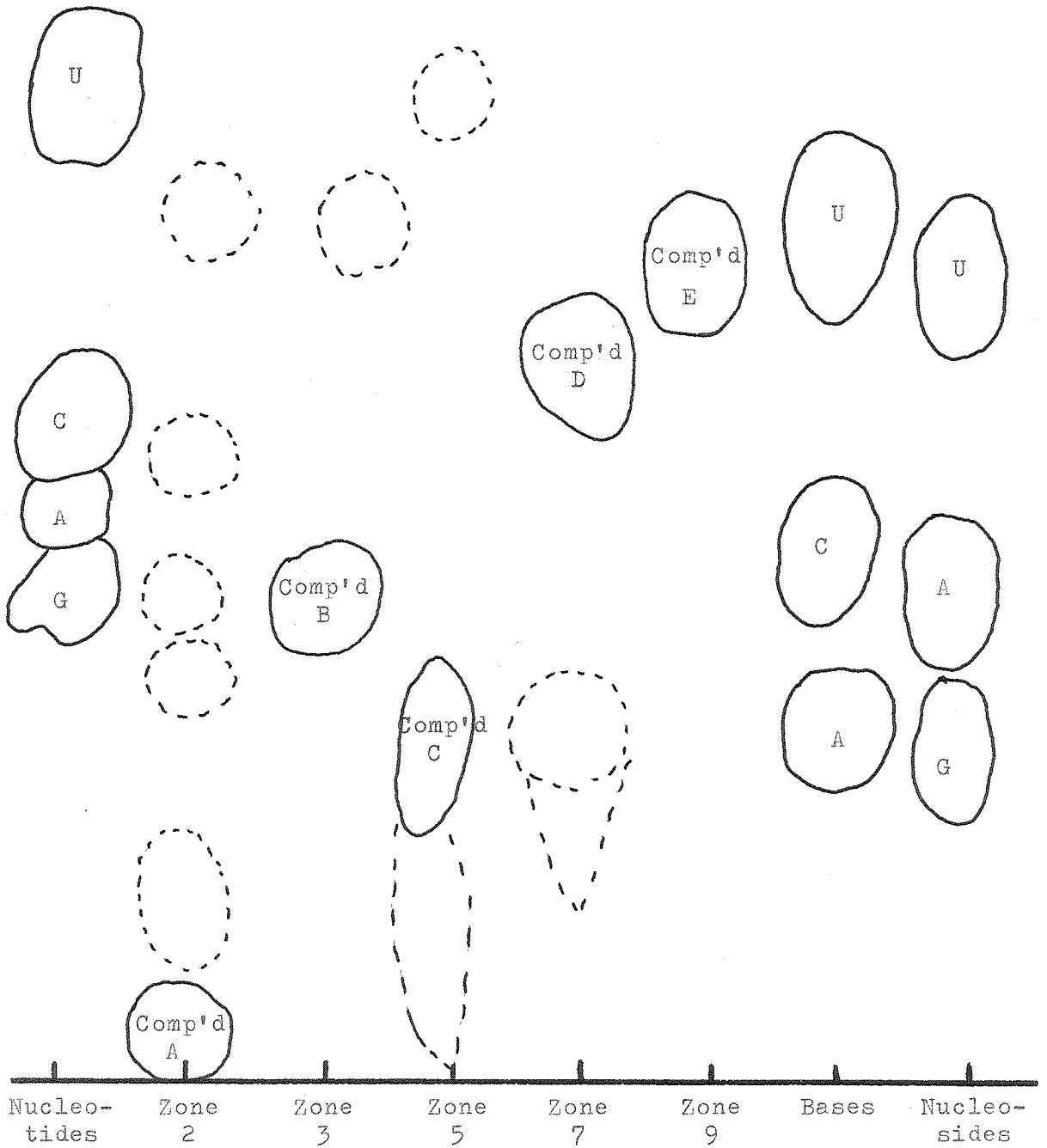


Fig. 7. UV absorbing material in zones from a BAW chromatogram of Pontania extract chromatographed in isopropanol, HCl.

phosphate and for the ability to oxidize periodate were negative so that A cannot be a nucleoside or nucleotide. Its chromatographic properties are such as to exclude the possibility that it is deoxyadenosine.

Figure 8 shows the gall-growth promoting activity of a mixture of compounds A and B. Each of these compounds has since been found to have activity when tested alone.

Compound B

Rf in BAW = 0.15, in isopropanol-HCl = 0.34. The spectrum of compound B is similar to that of adenosine (figure 1c in Appendix II, p.102). However, the chromatographic properties of this compound do not match those of adenosine or deoxyadenosine nor does it oxidize periodate. Compound B does not contain phosphorous. As mentioned previously this compound has gall-growth stimulating activity.

Compound C

This compound has been identified as uric acid on the basis of its chromatographic properties and UV spectrum (figure 3c in Appendix II, p.104). As shown in figure 9, compound C possesses gall-growth activity. Similarly, pure uric acid stimulates the growth of larva-free galls.

Compound D

Rf in BAW = 0.33, in isopropanol-HCl = 0.50. Figure 2a in Appendix II, p.103, shows the spectrum of this compound. Treatment with 6N HCl for 2½ hours at 105°C converts compound D into two other compounds. One of these (Rf in isopropanol-HCl = 0.48) shows blue fluorescence under UV light. The other (Rf 0.70) contains UV absorbing material. The spectrum of these two compounds is shown in

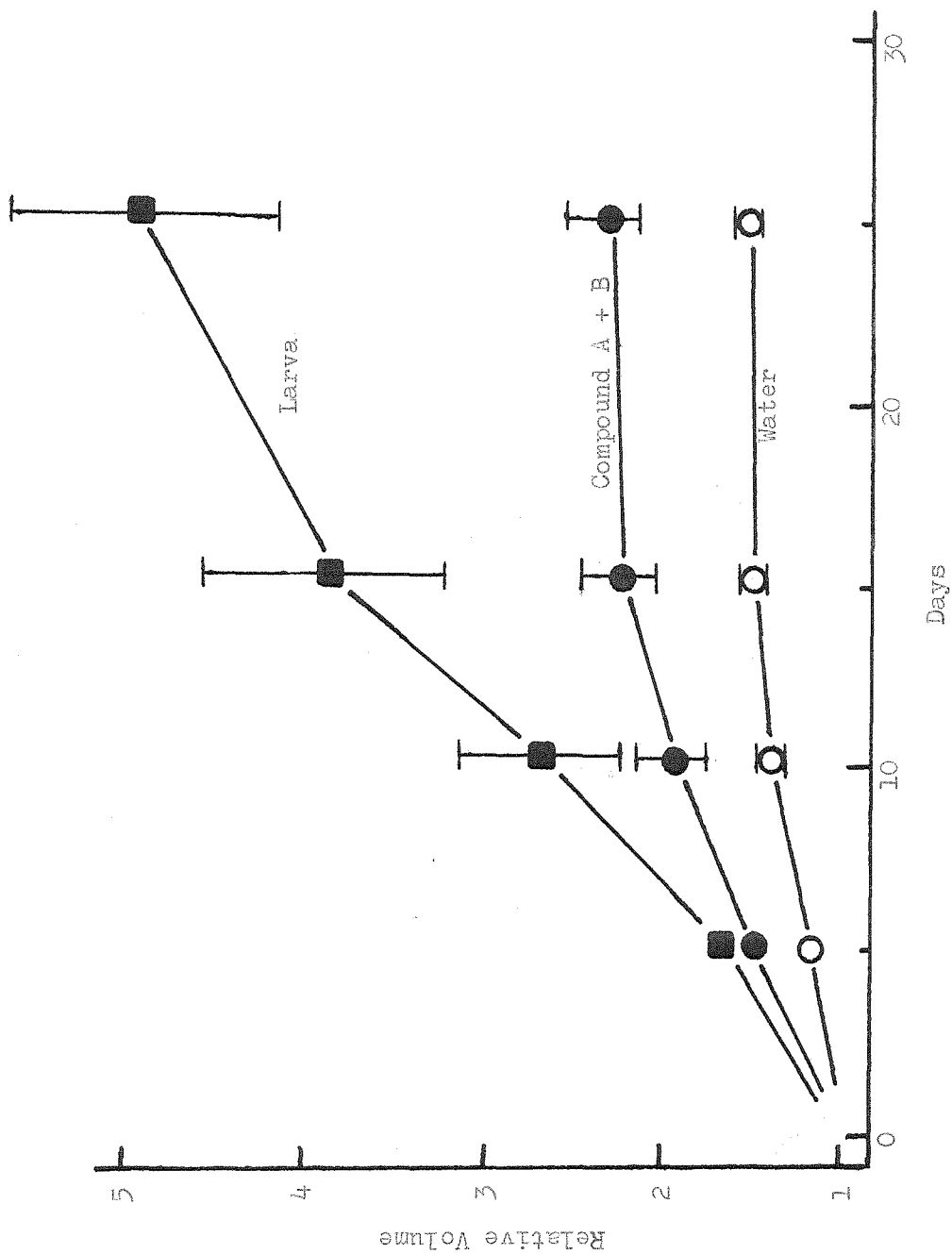


Fig. 8. Stimulation of gall growth by a mixture of compounds A and B.

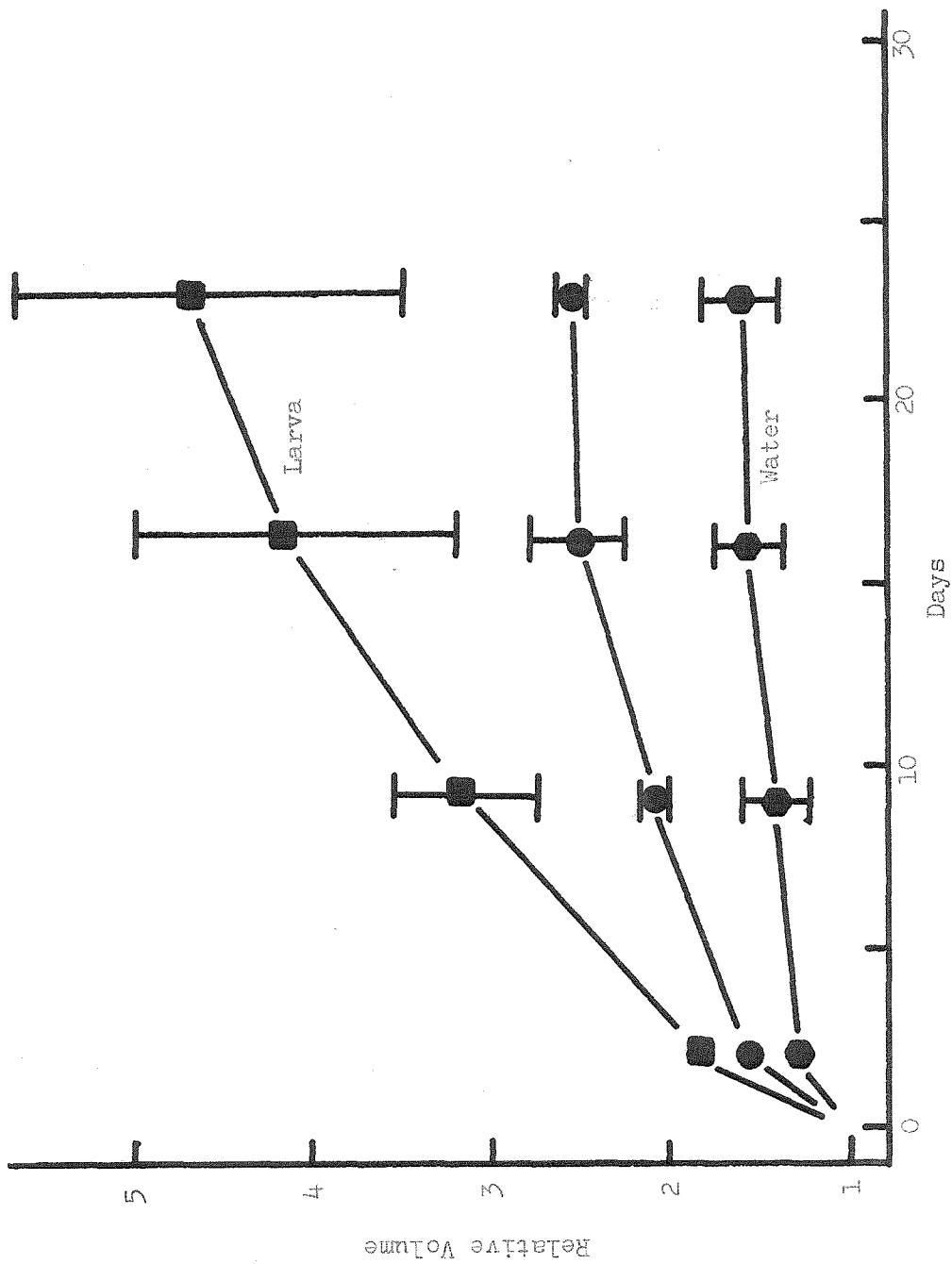


Fig. 9. Stimulation of gall growth by Compound C.

figures 2b and 2c in Appendix II, p. 103. Compound D has insignificant gall-growth promoting activity.

Compound E

The chromatographic properties of compound E are identical to those of uridine as is its UV spectrum both in acidic and basic solutions (figure 3d in Appendix II, p. 104). Further, treatment with periodate (cf. part III) followed by alkali converted E to a compound with the chromatographic properties of uracil. Uridine has some growth promoting activity but is less active than compounds A, B, or C.

Trace UV Absorbing Compounds

Both adenosine-5'-phosphate and adenosine-5'-triphosphate appear to be present in the low Rf material of the BAW chromatogram but in amounts too small to work with. As shown in figure 10 there are several minor UV absorbing compounds in the extract. Judging from their movement in isopropanol-HCl, some of these may be identical with known purines, pyrimidines or their derivatives.

Tests on Known Material

A number of purines and their derivatives have been tested for their ability to promote gall growth. The first of these, kinetin, shows considerable activity when about 0.1 μ g is applied per injection (figure 10). Subsequent tests, however, indicate that kinetin is no more effective than adenine or adenosine applied in the same amount. Other purine-containing compounds possessing activity include guanosine and uric acid. Some pyrimidines and their derivatives (uracil, uridine, and cytosine have slight growth promoting activity as does indole-3-acetic acid.

It should also be noted that no single compound consistently

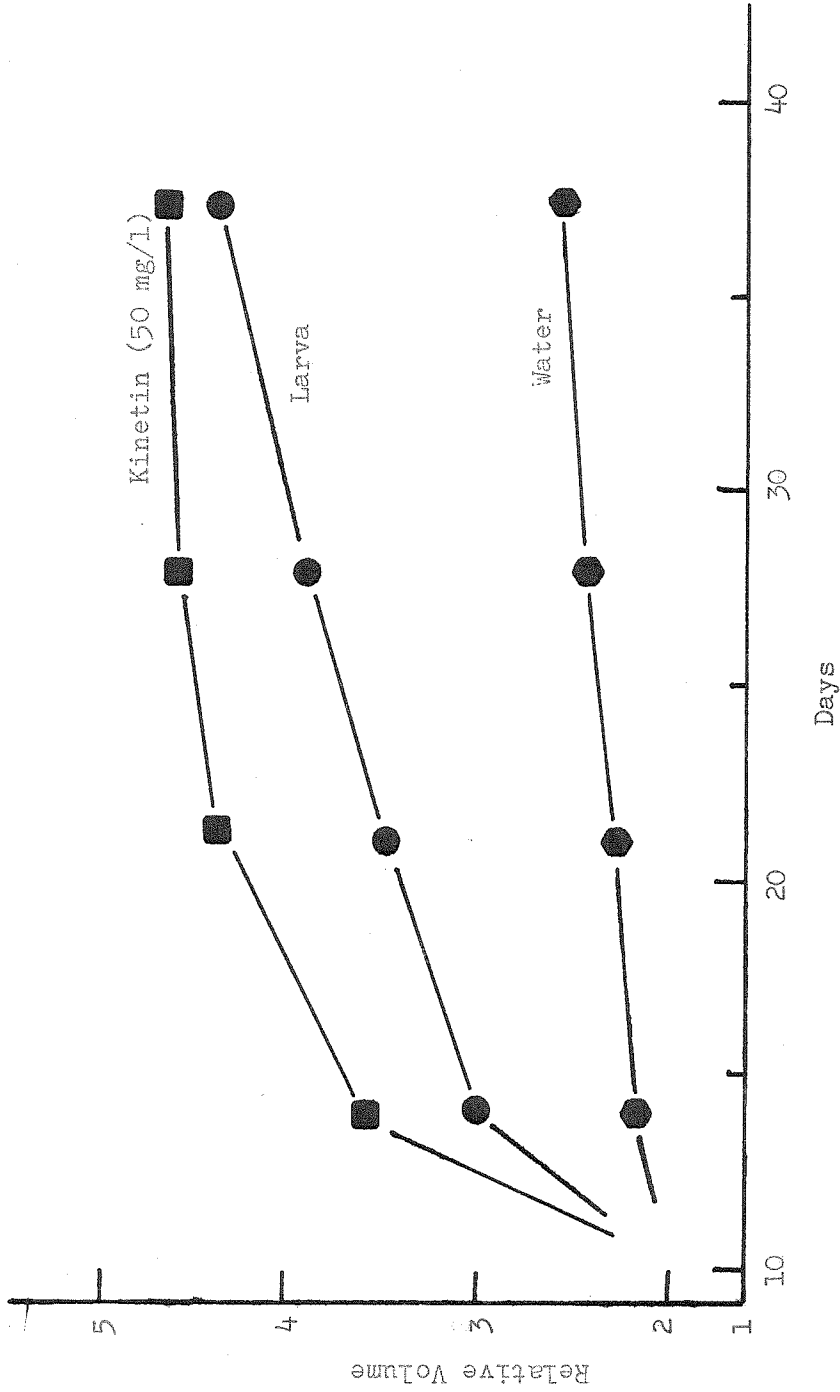


Fig. 10. Stimulation of gall growth by kinetin.

produced as great a response as the mixture of materials in the glandular fluid. Rapid and sustained growth was, however, obtained in one experiment in which a mixture containing adenine (50 mg/l), kinetin (10 mg/l) and IAA (10 mg/l) was applied.

Test for Kinetin in the Extract

The Xanthium leaf assay for kinetin (Osborne and McCalla, 1961, Appendix III) was applied to chromatographed Pontania gland extract. While some areas of the chromatogram gave slight preservation of chlorophyll, higher concentrations of material did not increase the response. Such behavior is characteristic of a number of purines and other common metabolites. Even if the response elicited by the gland extract in this assay were entirely due to kinin, the maximum amount present could not exceed the equivalent of 4×10^{-3} μg per gland. Pure kinetin was effective in promoting gall growth of a concentration of 0.1 μg per injection but almost no growth was obtained when only 0.01 μg was supplied. It is, therefore, apparent that the maximum amount of kinin present in the glandular fluid is too small to be of importance in gall growth.

Extract of Pontania Larva

Young Pontania larva were removed from developing galls and extracted with 80% ethanol as described above. The extract showed considerable growth promoting activity and yielded several UV absorbing zones when chromatographed in BAW. These zones were not further examined.

DISCUSSION

Low molecular weight compounds present in the glandular fluid of Pontania are clearly effective in promoting gall growth.

Chromatographic examination of the fluid indicates that five ultra-violet absorbing compounds are prominent constituents of the fluid. Two of these compounds, uridine and uric acid, have been satisfactorily identified while two others appear to be adenine derivatives. Large scale chemical work on these partially characterized compounds is prohibited by the labor involved in obtaining the glands. The quantity of each of these UV absorbing materials present in a single gland is of the order of 0.5 to 1.5 μg (determined spectrophotometrically) and is sufficient to produce one full sized larva-less gall. A comparable amount of pure kinetin or adenine has roughly equivalent growth promoting activity. Under natural conditions each female Pontania initiates many small galls which develop to a diameter of about 1 to 2 mm with no further stimulus.

From the present study as well as from much earlier work it is apparent that the growth of insect galls continues only so long as stimulus is supplied either from the larva or from experimentally applied material. Pelet et al. (1960) have shown that both auxin and kinetin are required for growth of insect gall tissues in vitro and that addition of casein hydrolysate and adenine further stimulates growth. This is in contrast to bacterial crown galls in which the plant cells become "transformed" so that they are nutritionally independent.

Work in several laboratories has provided some insight into the problem of the control of differentiation in plants. Skoog and Tsui (1948) found that the ratio of adenine to auxin determines whether roots or buds are formed on tobacco pith callus cultures cultivated in vitro, adenine promoting bud formation, auxin root formation. Further work by Skoog and his collaborators (Skoog and

Miller, 1957) has shown that the auxin-kinetin ratio is also important in bud and root development. Wickson and Thimann have shown that the ratio of kinetin to auxin determines whether the development of lateral buds on stem sections cultured in vitro proceeds or remains inhibited. In this system also, kinetin promotes bud growth while auxin tends to inhibit this growth. There is also good evidence for the interaction of gibberellins with kinetin and with auxin (Phinney and West, 1960).

Results such as these have led Skoog to reject the idea of specific organ forming chemicals in favor of the concept of control of organ formation by the relative amounts of various growth substances present in various tissues.

Gall formation must be regarded as a result of the release of plant cells from these normal developmental controls. This is most clearly illustrated by simple kataplastic galls which are merely undifferentiated callus-like tissue covered with epidermis. In the more complex prosoplastic galls, growth substances derived from the insects must also impose new controls which result in the appearance of characteristic tissue patterns within the gall. It is important to note that no new cell types appear in insect galls (Went, 1940).

It seems reasonable to assume that the growth substances supplied by the insects interact with the endogenous growth regulators of the plant. In this connection it is worth noting that while the Pontania glandular fluid does not appear to contain material with kinin activity, these insects deposit their eggs adjacent to the mid-rib of the leaf, a known source of kinin.

It is apparent from the literature on gall formation that

different species of insects use different compounds to stimulate gall growth on plants. Normal growth can undoubtedly be disturbed by a variety of chemicals and it is not surprising that in the course of evolution various species have developed different techniques by which they mold plant tissue to their own uses.

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APPENDIX I. PAPER CHROMATOGRAPHIC TECHNIQUES

Paper

Whatman no. 1 and 3 MM papers were used. For most experiments the paper was washed in either 0.5% acetic acid, 0.2% versene, or ethylacetate, acetic acid, water (10:5:2;v/v) followed by distilled water before use. The ethylacetate, acetic acid, water mixture was very effective and removed much more organic material than did either of the other two washing techniques.

Apparatus

Ascending chromatograms were run in pyrex glass jars (dia. 5½ inches, height 18 inches). The papers were either hung on glass from the lid of the jar or held into cylinders by means of polyethylene clips.

Descending chromatograms were run either in a 9 inch diameter glass jar fitted with glass troughs or in a "Chromatocab."

Except where noted below, chromatograms were run without prior equilibration with the vapor phase of the developing solvent.

Elution of Material from Chromatograms

A square or rectangular area including the spot to be eluted was cut from the chromatogram and folded down the center. The folded piece of paper was then placed upright in a petri dish containing a small amount of the eluting solvent (usually water) which ascended the paper by capillary action. When the eluting solvent reached the top, the sheet of paper was rolled in a sheet of heavy duty aluminum foil (considerably larger than the paper) so that the top edge of the paper was coincident with one edge of the foil. The roll was suspended (paper down) in a 15 ml conical centrifuge tube by folding the other end of the roll over the lip of the tube. The

tube was then placed in a clinical centrifuge and spun at about 1000 rpm. This process was repeated a second and occasionally a third time. In this way a nearly quantitative recovery of material from the paper was achieved using minimal amounts of solvent.

Determination of Carbon-14 on Chromatograms

The location of radioactive compounds on chromatograms was determined by means of a Nuclear Chicago "Actigraph I" fitted with a "Micromil" window. This instrument records counts per minute vs. position on the chromatogram. The relative amount of radioactivity in various compounds on the chromatogram was determined from the area under the peaks.

Solvents (arranged alphabetically by major organic component):

1. n-Butanol-acetic acid-water (4:1:1.8;v/v) (Neish, 1959) (BAW). In the proportions given above it is a single phase mixture, not quite saturated with water, so that temperature changes are not troublesome. Ascending and descending techniques gave similar results.

2. n-Butanol-acetic acid-water (74:19:50;v/v) (Schwink, 1960). This solvent was used to separate phosphate containing compounds. To get good separations the descending technique was used and the solvent allowed to run off the paper for a total development time of 40 hours.

3. n-Butanol-conc ammonium hydroxide-4% aqueous boric acid (96:1:13;v/v). In this solvent the movement of compounds which contain cis glycol groups is greatly retarded over that in a similar borate-free solvent: n-butanol-conc ammonium hydroxide-water (96:1:13;v/v).

4. n-Butanol-formic acid-water (1:1:1;v/v) (Romberger,

1960). This solvent gave good separations of carboxylic acids and was used for this purpose only. Best results were obtained when the solvent was made up 24 hours prior to use and when the chromatogram was equilibrated overnight with the aqueous phase and subsequently developed with the organic phase. Only the descending technique gave satisfactory separations.

5. t-Butanol-picric acid-water (80:2:20;v/w/v) (Loughman and Martin, 1957). Used for nucleotides and sugar phosphates in some experiments. Abandoned because picric acid interfered with spray reagents.

6. Diethylether-acetic acid-water (13:3:1;v/v) (Dennison and Phares, 1952). This solvent gave good separation of carboxylic acids when used in the cold room. The solvent is so volatile that it is useless at room temperature.

7. Diisobutyl ketone-acetic acid-water (40:25:5;v/v) (Marienette et al., 1957). This solvent was used for the separation of lipids on silicic acid impregnated paper.

8. Ethanol-conc ammonium hydroxide-water (80:5:15;v/v) (Osteux and Laturaze, 1954). This solvent was used to check the identity of carboxylic acids.

9. Ethylacetate-acetic acid-water (10:5:2;v/v) (Romberger, 1960). This solvent gave good separation of sugars when the chromatogram was developed in the descending direction after equilibration for a few hours. It also proved to be an extremely effective solvent for washing filter paper.

10. isoPropanol-conc HCl-water (170:41:39;v/v) (Wyatt, 1951). This was an extremely useful solvent for separating purines and pyrimidines and their derivatives.

11. Isopropyl alcohol-pyridine-acetic acid-water (8:8:1:4; v/v) (Gordon et al., 1956). This solvent was used as a further check on the identification of the sugars.

12. Propanol-30% acetic acid (3:1;v/v) and propanol-1% ammonium hydroxide (3:1;v/v). These solvents were used to develop two dimensional chromatograms of amino acids.

13. 88% Aqueous phenol (0.1% KCN added, ammonia atmosphere). This solvent was used to check the identity of purines and urea. However, material streaked rather badly so it was not extensively used.

Electrophoretic Separations

All electrophoretic separations were carried out in a Spinco-Durum-type apparatus at room temperature. The following electrolytes were used:

1. Pyridine-acetic acid-water (5:0.2:90;v/v) pH 6.4 (modified from Ingram, 1958). This buffer gave excellent separation of aspartic acid, glutamic acid, histidine, lysine and asparagine from neutral amino acids and from each other.

2. 0.05 M pH 3.7 acetate buffer (Werkheister and Winzeler, 1953). Gave good separation of nucleotides.

3. 3% Formic acid (pH 1.8). Gave good separation of purines and purine ribosides.

4. 0.1 M Formic acid (pH 2.3). Gave separation of some phosphate containing compounds.

Dip and Spray Reagents

1. p-Anisidine spray for sugars (Hough et al., 1950). p-Anisidine (2.32 gm) is dissolved in 3.8 ml 5 N HCl and the solution made up to 100 ml with n-butanol. The chromatogram is allowed

to dry at room temperature after spraying and then heated at 90-100° for a few minutes. The color produced by various compounds is diagnostic:

brown	aldohexose
yellow	ketohexose
green	methylpentose
cherry red	uronic acid
bright red	aldopentose
yellow	ketopentose
brown	

2. Molybdate dip for phosphates (Burrows et al., 1952).

Ammonium molybdate (1 gm) is dissolved in 8 ml of water and 3 ml of hydrochloric acid. Three ml of 70% perchloric acid is then added and the solution made up to 100 ml with reagent grade acetone. Chromatograms are dipped in this solution and allowed to dry at room temperature. Inorganic phosphate spots give a light yellow color at this stage. The chromatogram is then exposed to light from a germicidal UV lamp (2537 Å max emission). With this treatment all phosphate containing materials give blue spots.

3. Ferricyanide spray for phenols (Barton et al., 1952).

Soln. A: 1% ferric chloride in water. Soln. B: 1% potassium ferricyanide in water. Solutions A and B are mixed just before use. After the chromatogram has been sprayed it is washed in dilute acid and then in distilled water in order that the background remains white. This reagent gives a very sensitive test for phenols which appear as blue spots.

4. Diazotized p-nitroaniline (Bray et al., 1950).

Soln. A: 0.5% p-nitroaniline in 2 N HCl.

Soln. B: 5% sodium nitrite solution.

Soln. C: 20% sodium acetate solution.

Mix 5 ml A, 0.5 ml B and 15 ml C in that order. The colors produced are characteristic for various types of phenols.

5. Ninhydrin dip for amino acids and peptides. Ninhydrin (0.5 gm) was dissolved in 100 ml of reagent grade acetone. Chromatograms were dipped in this solution, and kept for several hours at room temperature in subdued light. Ninhydrin positive spots were marked and the paper heated at 70°C for a few minutes in order to insure complete reaction.

6. Bromphenol blue reagent for acids (Palmer, 1955). Bromphenol blue (0.2 gm) was dissolved in 500 ml of 95% ethanol and sodium hydroxide added until the solution was a deep red purple.

7. Periodate-KI spray for periodate oxidizing compounds (Metzenberg and Mitchell, 1954). The chromatogram was first sprayed with a 0.01M solution of potassium metaperiodate allowed to dry for about ten minutes at room temperature and then sprayed with a 35% saturated sodium tetraborate solution containing 0.8% potassium iodide, 0.9% boric acid and 3% soluble starch. Periodate-oxidizable compounds showed up as white spots on a blue background.

Table I. Rfs and Mobility of Amino Acids

Compound	BAW	Rf x 100 in:		Mobility (Cm. Migrated pH 4.7; 400 v; 140 min.)
		Propanol Acetic Acid	Propanol Ammonia	
Alanine	36	32	34	-0.9
Arginine	27	19	19	-6.0
Aspartic Acid	27	17	10
Asparagine	20
Cysteine	5	12
Glutamic Acid	33	25	14	+4.9
Glutamine	26
Glycine	29	18	23
Histidine	22	14	24
Leucine	73	60	64	-0.8
Lysine	20	14	14	-6.8
Methionine	62	20	50
Phenylalanine	68	52	58
Proline	43	35	58
Serine	26	18	23
Threonine	30	26	31
Tryptophan	63	38	44
Tyrosine	52	36	39
Valine	59	46	50

Table II. Rf and Mobility of Purines, Pyrimidines and Related Compounds

Compound	BAW	100 x Rf in:			100 x M _{adenine} at pH 1.9	100 x M _{uridylic} at pH 3.7
		88% Phenol	Iso- propanol HCl	Water		
Adenine	54	81	29	35	100
Adenosine	41	27	50	61
Deoxyadenosine	65	30
Adenylic Acid	40	40
Guanine	18
Guanosine	25	27	32
Guanylic Acid	34	80
Cytosine	34	50
Cytidylic Acid	45
Uracil	48	70
Uridine	38	58
Uridylic Acid	71	100
Hypoxanthine	40	82	22	60	05
Inosine	26	22	73	05
Xanthine	30	52	19
Xanthosine	22	21
Uric Acid	20	22	17
Benzyladenine	93	90	33	65
Benzyladenosine	83	57	43
Allantoin	26	36	45	05
Urea	57	65	72	05

Table III. Rf of Phosphate Containing Compounds

Compound	100 x Rf in Picric Acid t-Butanol Water	100 x $\frac{Rf}{Rf'PO_4}$	100 x M_{PO_4}	
		Butanol Acetic Acid Water	pH 2.3	pH 4.7
Adenosine triphosphate	03	16	68	73
Adenosine monophosphate	23	46	50
Glucose-1-Phosphate	34	62	67
Glucose-6-Phosphate	61	67
Fructose-6-Phosphate	49	68	70
Fructose-1,6-diphosphate	27	91	88
3-Phosphoglyceric Acid	122	126
Orthophosphate	65	100	100	100

Table IV. Rf of Sugars

	Rf x 100 BAW	M sucrose	Rf x 100	Rf
		Ethanol Acetic Acid	Pyridine Isopropanol	Ethanol Acetic Acid
Glucose	22	158	52	12
Fructose	33	190
Ribose	38	63	23
Arabinose	31	54	15
Xylose	34	59	18
Sucrose	13	100	48	05
Raffinose	09	35	01
Cellobiose			43	03
Melibiose			35	02
Galacturonic			20	07
Glucuronic			23	08

Table V. Rf of Acids x 100

	Butanol	Diethylether	BAW
	Formic Acid	Acetic Acid	
Tartaric	13	07
Citric	20	24
Malic	32	35
Glycolic	47	44
Malonic	58	62
Succinic	68	71
Fumaric	90	92
Caffeic Acid	76	79

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APPENDIX II. ULTRAVIOLET ABSORPTION SPECTRA OF COMPOUNDS

ISOLATED FROM PONTANIA ACCESSORY GLANDS

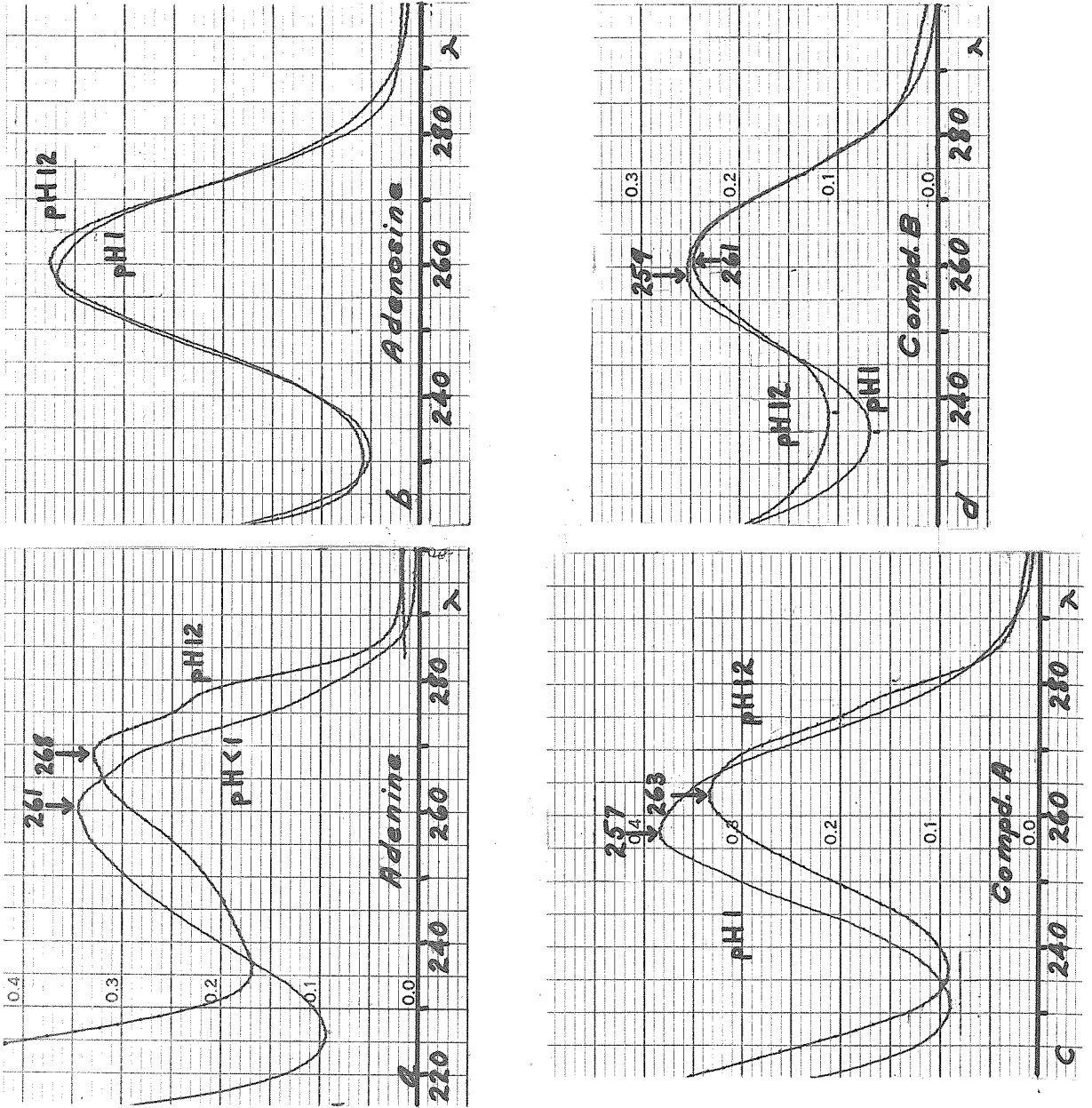


Fig. 1. UV absorption spectra of: a) adenine; b) adenosine; c) and d) compounds A and B isolated from Pontania accessory glands.

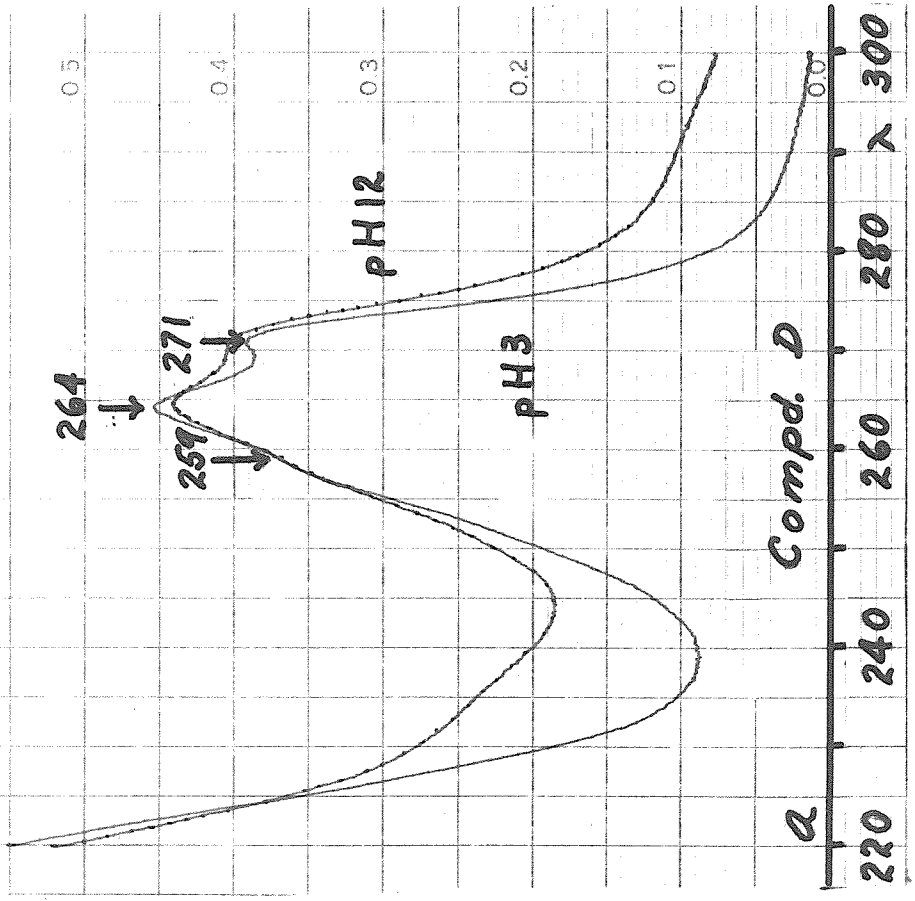
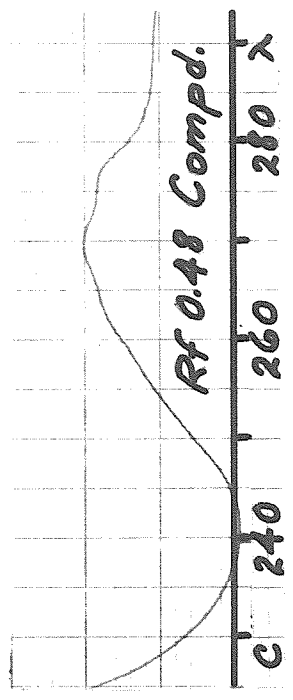
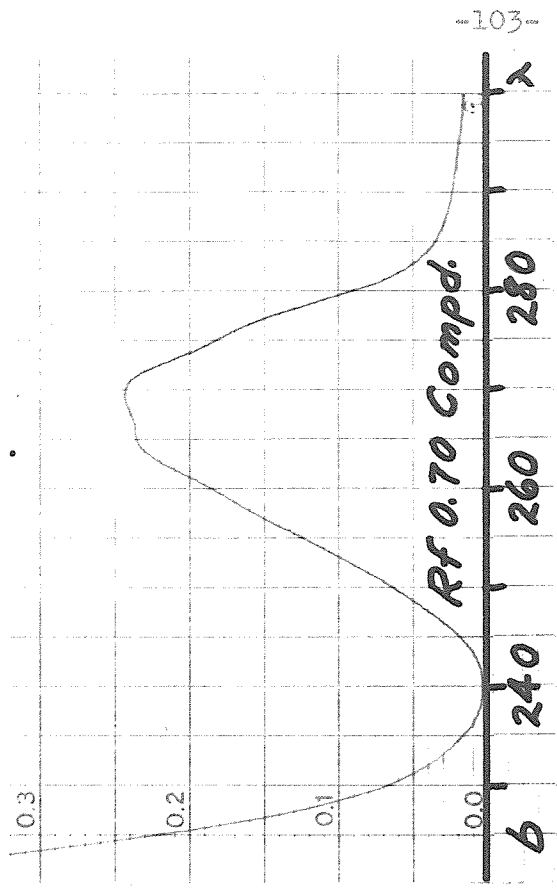


Fig. 2. a. UV absorption spectrum of compound D isolated from Pontania accessory glands. b. and c. Spectra of compounds formed on acid hydrolysis of compound D.

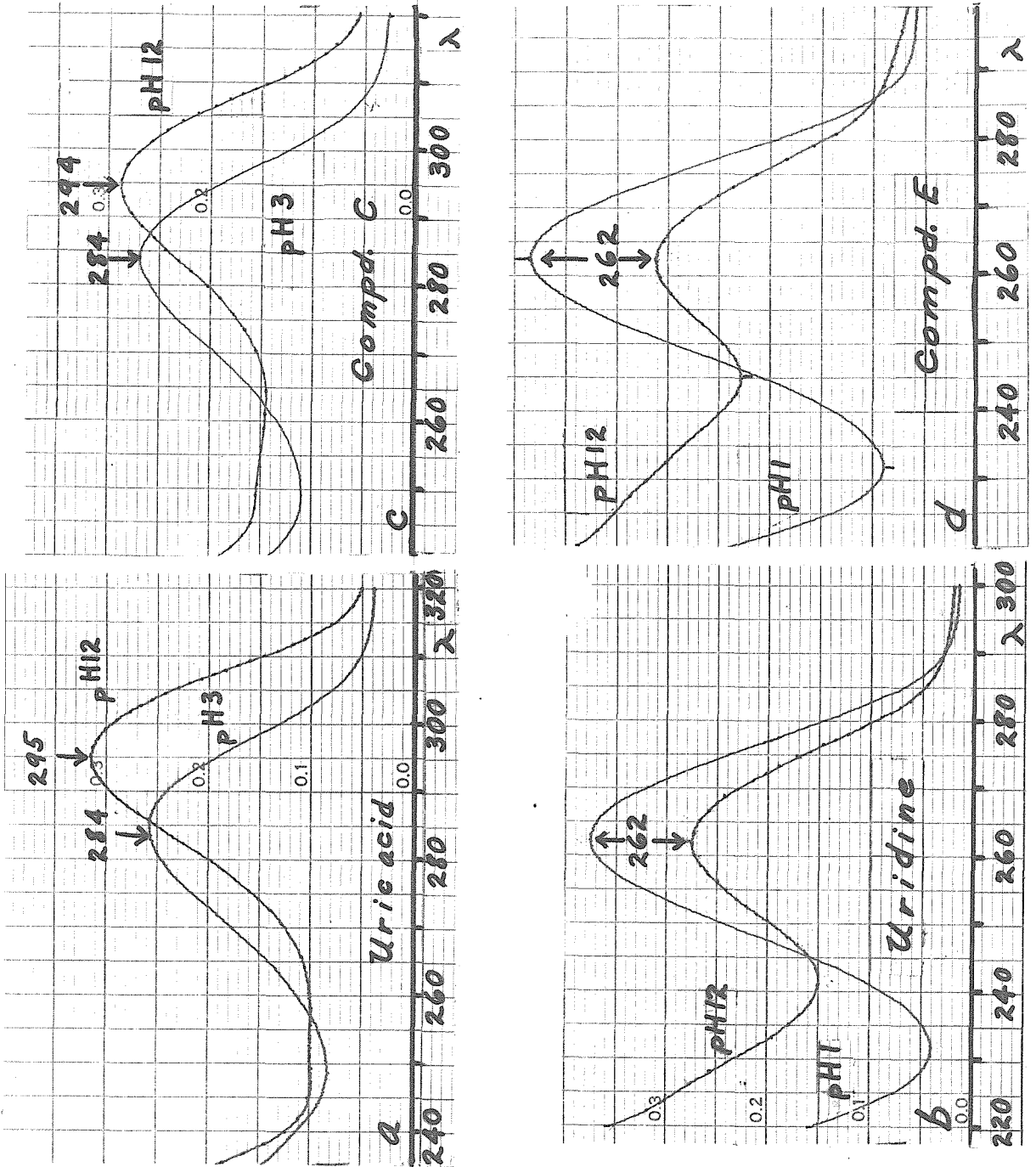


Fig. 3. a) UV absorption spectrum of uric acid; b) spectrum of uridine; c) and d) spectra of compounds C and D isolated from Pontania accessory glands.

APPENDIX III

RAPID BIOASSAY FOR KINETIN & KININS USING SENESCING LEAF TISSUE¹

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The isolation and characterization of kinetin (6-furfurylaminopurine) was first reported in 1955 by Miller, Skoog, von Saltza, and Strong (4). Since then both kinetin and a number of its analogs have been shown to have remarkable effects on plant growth (1, 2, 5, 7). So far, however, no natural kinin has been isolated and identified. The search for natural kinins and their subsequent isolation has been hampered by lack of a fast and simple bioassay. To meet this need we have developed the following quantitative assay which is based upon the ability of kinetin to retard the degradation of chlorophyll in the cells of

senescing leaf tissue (7). It is quantitative over the range from 0.05 to 5.0 μ g of kinetin and may be used for detecting still larger amounts, although it is then no longer quantitative. The assay may be used either to test zones cut directly from paper chromatograms or for material in solution. Unlike tests for kinetin based on growth responses (3), the present method does not require the addition of auxin.

MATERIAL & METHODS

Cocklebur plants (*Xanthium Pensylvanicum* Wall.) of the inbred strain used in the California Institute of Technology are grown from seed in a greenhouse (max temp 30° C, min 20° C; air de-smogged with charcoal filters). Normal daylight is supplemented with incandescent light to give a 20 hour photoperiod. Under these conditions the mature leaves are thin and soft in texture and attain an area

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of about 150 cm². When the plants have produced ten leaves in all, the fifth fully-expanded leaf (counting back from the apex) is suitable for the bioassay. The leaves are harvested and stored with the petioles in water under conditions of low light intensity (50 ft-c) for 3 days in Plexiglas boxes at 20° C. During this period, metabolic processes leading to senescence are initiated and there is a fall in the total chlorophyll content of the leaf. The final selection of suitable leaves is made on the basis of a uniform pale green color of the blade.

Kinetin standards are prepared as follows: 0.5 ml of aqueous solutions of kinetin at concentrations ranging from 16 to 0.063 mg/l are applied uniformly to circles of Whatman no. 1 filter paper, 4.25 cm in diameter placed in the base of a 5 cm petri dish. Control circles receive 0.5 ml of distilled water. Paper for the chromatography of extracts must be thoroughly washed to avoid accumulation of toxic materials just behind the solvent front. Ethyl acetate, acetic acid, water, (10:5:2, V/V) (8) is very effective for this purpose. Uniform discs 12 mm in diameter are cut from the interveinal blade tissue of previously aged and selected cocklebur leaves. Some 60 discs may easily be obtained from a suitable leaf. Four discs are placed on each filter-paper circle or portion of chromatogram so that the abaxial surface of the leaf tissue is in contact with the liquid. (0.5 ml is a suitable volume of water to add to portions of chromatogram strip large enough to accommodate four discs.) Duplicate sets of four untreated discs may be extracted immediately in boiling 80% ethanol and the extract retained for determining the original chlorophyll content of the discs. Each small dish is placed on two layers of damp filter paper inside a larger closed petri dish. The larger dishes are stacked in enamel trays lined with damp filter paper and covered with aluminum foil. They are then maintained in darkness for 48 hours at 24° C. By the end of this period most of the chlorophyll in the controls is degraded and the discs are yellow. Those in the higher kinetin treatments are still green. (If the leaves have been insufficiently aged prior to use the duration of the assay may have to be extended.)

Each group of discs is then dropped into a graduated centrifuge tube containing 5 to 6 ml of hot 80% ethanol, and boiled gently on a water bath until the chlorophyll has been extracted. The tubes are cooled and the volume made up to 10 ml with 80% ethanol. The optical density of each solution was measured against 80% ethanol in a Beckman spectrophotometer, Model B at 665 m μ and 645 m μ , the absorption maxima in the red region for chlorophyll a and chlorophyll b, respectively.

RESULTS

The optical densities of extracts of leaf discs which had been exposed to kinetin standards ranging from 0.031 to 25 μ g are presented in figure 1. It is clear that since the chlorophyll a/chlorophyll b ratio

remains approximately the same in the presence of kinetin the determination of the optical density of the extract at either the chlorophyll a maximum or the chlorophyll b maximum is suitable as a measure of the retention of total chlorophyll in the leaf tissue. However, since it is advantageous to cover a wide range of optical densities, measurement at the wave length of the maximum absorption of the chlorophyll a (665 m μ) is recommended.

Some absorption at both these wave lengths is found even when all the chlorophyll has disappeared. This is due to the presence of yellow pigments which are not degraded as rapidly as chlorophyll.

The retention of chlorophyll bears a linear relation to the logarithm of the kinetin concentration over a range of approximately 0.05 to 5.0 μ g. These values correspond to concentrations of 0.1 to 10 mg/l. The relationship is not linear for quantities of kinetin

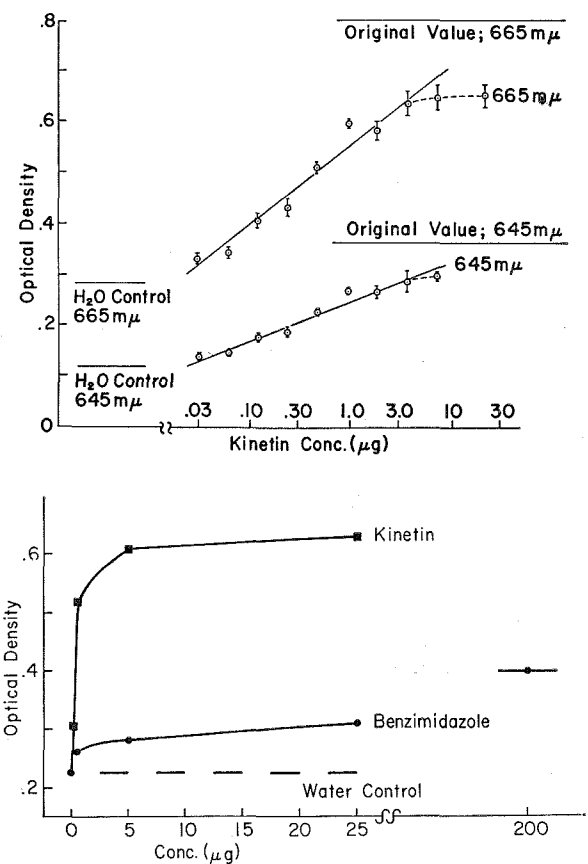


FIG. 1. Retention of chlorophyll by kinetin in discs of senescing Xanthium leaves. Optical densities of chlorophyll extracts in 80% ethanol determined at the chlorophyll a and chlorophyll b maxima 48 hours after treatment.

FIG. 2. Comparison of the effectiveness of benzimidazole and kinetin in preventing loss of chlorophyll in senescing Xanthium leaf discs. Optical density of 80% ethanol extract determined at 665 m μ .

above 5 μg because a maximum value for chlorophyll retention is obtained. Under these conditions the assay still provides a qualitative test for kinetin at least up to the maximum amount tested (25 μg).

6-Benzylaminopurine which is active in promoting cell divisions in tobacco pith cultures (9) is also active in the leaf disc assay, as is its riboside. Person et al (6) have shown that chlorophyll degradation in detached wheat leaves is prevented by benzimidazole as well as by kinetin. Figure 2 shows that with Xanthium leaf discs benzimidazole is not nearly so effective as kinetin.

A number of other compounds have been tested for possible interference in this assay. These include auxins (IAA & 2,4-D) and amino acids which are inactive, purines (adenine, adenosine, & guanosine) and 1,3-dimethylurea which interfere slightly but even at high concentrations give values lower than those obtained with 0.05 μg kinetin. Sugars in large amounts (2 mg) give values approaching those for 0.05 μg kinetin.

DISCUSSION

This bioassay offers a rapid and simple screening test for material with kinetin-like properties. If desired, materials giving positive results in this assay could be tested using the tissue culture techniques. The leaf disc assay has several advantages. It does not require the use of complex media and sterile conditions and it gives quantitative results very rapidly.

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