FOREWORD

WE BELIEVE that the appearance of a new journal covering the area of plant biochemistry will be generally welcomed by those whose work lies in this field; for, although it is generally agreed that the basic metabolic processes of plants, animals and micro-organisms are similar, we feel that their essential differences must not be lost sight of. Besides the obvious example of photosynthesis, plants show other important dissimilarities, notably their ability to synthesize a whole host of secondary metabolic substances whose function, where known, is unique. Furthermore there are a number of important features of the physiology of higher plants such as the transport of metabolites, the dormancy of seeds, and the response to environment which diverge markedly from those of other life forms. Finally it should not be forgotten that the biochemistry of animals and micro-organisms has been geared to medicine and has thereby reached a more advanced state than that of plants, and consequently every encouragement must be given to enable plant biochemistry to develop as rapidly as possible. We believe that this journal will help towards this end, albeit in a small way, by unifying the science which it sets out to serve, and by giving its protagonists a sense of purpose and pride in their craft. If we succeed in these tasks we shall be well satisfied.

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FORMATION OF *m*- AND *p*-COUMARIC ACIDS BY ENZYMATIC DEAMINATION OF THE CORRESPONDING ISOMERS OF TYROSINE*

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Abstract—An enzyme was found which catalyzes the deamination of L-tyrosine, giving equimolar amounts of *trans-p*-coumaric acid and ammonia as the products. This enzyme (tyrase) was readily detected in sorghum, barley, rice, wheat, oat, corn and sugar cane plants; but not in pea, lupine, alfalfa or white sweet clover plants, or in yeast. Tyrase was concentrated in the stems of barley rather than the leaves, and reached its maximum concentration at about the time the heads were emerging. The crude, soluble protein extracted from an acetone powder of barley stems was purified about forty-fold with respect to tyrase. Tyrase preparations from this source were also found to convert DL-*m*-tyrosine to *m*-coumaric acid and ammonia, and have been shown by Koukol and Conn[1,2] to contain an enzyme (phenylalanase) which can catalyze the conversion of L-phenylalanine to cinnamic acid and ammonia. The data suggest that tyrase is distinct from the enzymes (or enzyme) catalyzing the deaminations of phenylalanine and *m*-tyrosine.

INTRODUCTION

IN RECENT years there has been considerable interest in the formation of secondary growth substances in plants, especially substances with an aromatic structure such as flavanoids, coumarins, lignin and related compounds. Tracer studies on the biosynthesis of these compounds have been reviewed.[3] They are probably derived from phenylalanine and, in some species, from tyrosine as well. Cinnamic acid, *p*-coumaric acid and other phenolic cinnamic acids are likely intermediates. In order to account for the formation of

$$R{-}CH{=}CH{-}COOH \text{ from } R{-}CH_2{-}CH(NH_2){-}COOH$$

the following sequence has been suggested:[3-5]

$$R{-}CH_2{-}CH(NH_2){-}COOH \longrightarrow R{-}CH_2{-}CO{-}COOH \longrightarrow R{-}CH_2{-}CH(OH){-}COOH \longrightarrow R{-}CH{=}CH{-}COOH$$

This postulates formation of the ethylenic bond by dehydration, but the tracer work can be explained just as well by postulating a direct deamination of the amino acid side chain as in the aspartase,[6,7] β-methyl aspartase[8] and histidase[9,10] reactions, i.e.

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$$R{-}CH_2{-}\underset{\displaystyle +NH_3}{\overset{\displaystyle |}{CH}}{-}COO^- \longrightarrow R{-}CH{=}CH{-}COO^- + NH^+$$

Koukol and Conn[1,2] found an enzyme in sweet clover that catalyzes the formation of cinnamic acid from phenylalanine. This enzyme (phenylalanine deaminase) was also found in other legumes and in members of the *Gramineae*. The reaction has been established as a direct deamination of the type shown above (R = phenyl).

The present paper describes two other deamination reactions of the same general type: (1) the conversion of L-tyrosine to *p*-coumaric acid (R = *p*-hydroxyphenyl) and (2) the conversion of DL-*m*-tyrosine to *m*-coumaric acid (R = *m*-hydroxyphenyl). The enzyme catalyzing deamination of L-tyrosine has been named tyrase. It was found in substantial amounts in grasses, but not in any of the legumes examined.

EXPERIMENTAL AND RESULTS

Detection of Tyrase in Plants

The first direct evidence for the existence of tyrase was obtained as follows: A sample of acetone powder (0·2 g) prepared from 6-day-old sorghum seedlings was incubated at room temperature (about 22°) with 3 ml of 0·05 M tris(hydroxymethyl) aminomethane-hydrochloric acid buffer (pH 8·8) containing 0·1 per cent of L-tyrosine. After 3 hr the mixture was diluted by 7 ml of water and filtered in a Buchner funnel. The filtrate was acidified with 0·2 ml of 5 N hydrochloric acid, extracted with 10 ml of ether, the ether evaporated and the residue redissolved in 0·2 ml of 95 per cent ethanol. About one-third of this solution was chromatographed on paper using solvent B (Table 1) as the irrigant.

TABLE 1. COMPARISON OF *cis-* AND *trans-* ISOMERS OF *p*-COUMARIC ACID

	Isomer of *p*-coumaric acid*	
	cis-	*trans-*
Corrected m.p., °C	131	219–220
Absorption peak of salt (mμ)†	290	333
Rf in solvent A‡	0·66	0·35
Rf in solvent B‡	0·64	0·64
Rf in solvent C‡	0·40	0·34
Rf in solvent D‡	0·36	0·29

* Both isomers give the same colored spot with diazotized *p*-nitroaniline, i.e. orange, changing to blue when sprayed with alkali.[22,23]

† Measured in 0·05 N sodium hydroxide. The fluorescence of the *cis-* isomer in this solution is less than one-tenth that of the *trans-* isomer.

‡ Solvent A—2 per cent acetic acid in water.
Solvent B—Upper phase of benzene–acetic acid–water (6 : 7 : 3) (Ibrahim and Towers[23]).
Solvent C—*n*-Propanol–conc. NH_4OH (7 : 3).
Solvent D—Isopropanol–conc. NH_4OH–water (8 : 1 : 1).

A component with the properties of *p*-coumaric acid was observed; it had the correct mobility and gave the same color reactions with diazotized *p*-nitroaniline (i.e. orange changing to blue in alkali). This component was not detected in parallel experiments where

tyrosine was omitted, even if *p*-hydroxyphenyl pyruvic acid or *p*-hydroxyphenyl lactic acid was added.

Acetone powders from other sources were tested in the same way (Table 2). Evidence for an enzyme converting tyrosine to *p*-coumaric acid was obtained for sorghum, wheat, rice, barley, oats, corn and sugar cane. In some of these tests *p*-coumaric acid was detected when tyrosine was omitted, but the parallel experiment with tyrosine always gave a more intense spot. Sorghum seeds did not contain a detectable amount of tyrase. Negative tests were also obtained with acetone powders of yeast and three species of legumes. No *p*-coumaric acid was observed in the negative tests, whether tyrosine was added or not.

TABLE 2. DETECTION OF TYRASE IN ACETONE-DRIED PLANT MATERIALS

Source of plant material	Presence of tyrase
Sorghum vulgare Pers. (sorghum) var. Honey Drip.	
seeds soaked overnight	−
4-day old, etiolated seedlings	+
6–12-day old, non-etiolated seedlings	+
Triticum vulgare Vill. (wheat) var. Ramona.	
4-day old, etiolated seedlings	+
4-day old, non-etiolated seedlings	+
Zea mays L. (corn) var. Minnesota B164.	
6-day old, non-etiolated seedlings	+
Hordeum vulgare L. (barley) var. Mariout.	
4-day old, etiolated seedlings	+
4-day old, non-etiolated seedlings	+
Avena sativa L. (oats) var. Kanota.	
6-day old, non-etiolated seedlings	+
Oryza sativa L. (rice) var. Caloro.	
10-day old, non-etiolated seedlings	+
**Saccharum officinarum* L. (sugar cane).	
stalk from plant about 150 cm tall	+
†*Pisum sativum* L. (peas) var. Alaska.	
6-day old, etiolated seedlings	−
†*Lupinus albus* L. (white lupin).	
6-day old, etiolated seedlings	−
†*Melilotus alba* Desr. (white sweet clover).	
Shoots, 30–40 days old	−
Fleischman's dried bakers' yeast	−

* Obtained from the Department of Agronomy, University of California, at Davis by courtesy of Dr. D. S. Mikkelsen.
† These acetone powders were prepared by Dr. J. Koukol.

A positive test for tyrase was also obtained with soluble protein fractions from sorghum or barley seedlings. These fractions were extracted by 0·05 M tris(hydroxymethyl)-aminomethane-hydrochloric acid (pH 8·8), precipitated by ammonium sulfate at 70 per cent saturation, then dissolved and dialyzed overnight at 0°, using buffer of the same concentration. They lost the tyrase activity on heating at 100° for 3 min. It was concluded that

certain plants, especially members of the *Gramineae*, contain an enzyme that can catalyze the conversion of L-tyrosine to *p*-coumaric acid.

Quantitative Assays for Tyrase

Tyrase was estimated by measuring the rate of formation of *p*-coumaric acid in the presence of excess L-tyrosine. The *p*-coumaric acid was isolated from the reaction mixture by extraction with ether and assayed either spectrophotometrically or fluorometrically in sodium hydroxide solution (Figs. 1 and 2). In most experiments a temperature of 40° was used (see Fig. 3) and the reaction mixture was buffered at pH 8·8 (Fig. 4) by 0·05–0·10 M sodium borate containing 0·1 per cent of L-tyrosine. One unit of tyrase is defined as the amount of enzyme which catalyzes formation of 0·1 μmole of *p*-coumaric acid per hour,

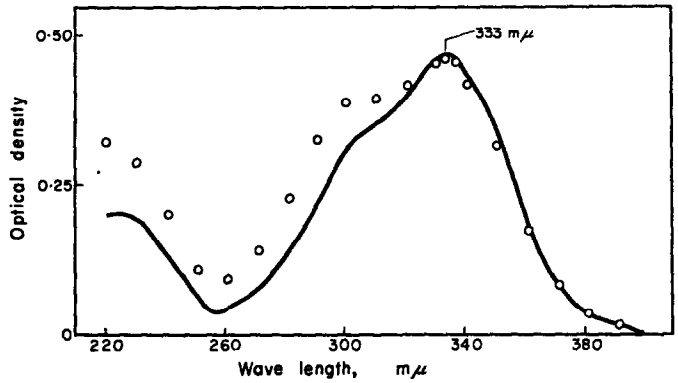

FIG. 1. ULTRAVIOLET ABSORPTION SPECTRUM OF *p*-COUMARIC ACID.
The solid line is for purified *trans-p*-coumaric acid (3·33 μg per ml of 0·05 N sodium hydroxide); the circles are for an ether-extractable acid fraction obtained following treatment of L-tyrosine with barley-stem tyrase (preparation C, Table 6).

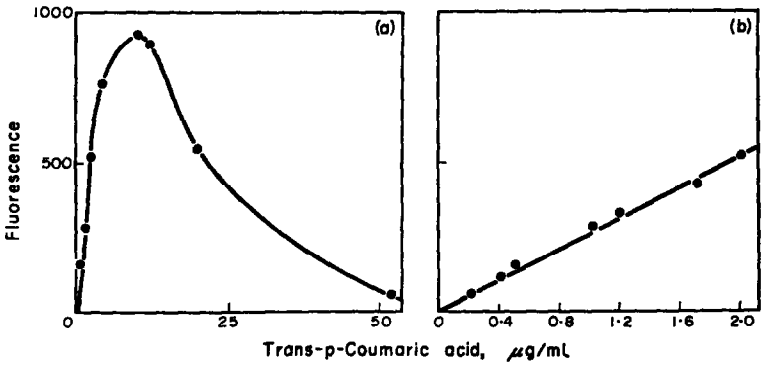

FIG. 2. RELATION BETWEEN CONCENTRATION AND FLUORESCENCE FOR *trans-p*-COUMARIC ACID IN ALKALI.
The acid was dissolved in 0·05 N sodium hydroxide, the solution put in a round quartz cuvette and fluorescence measured with an Aminco–Bowman spectrophotofluorometer using the 350 mμ setting for activation and measuring the output at 440 mμ. Fluorescence is reported in arbitrary units; a reading of unity on the per cent transmission scale at the 0·01 sensitivity setting was defined as one unit of fluorescence. (a)—Curve when concentration was varied over a wide range. (b)—Linear relation obtained for dilute solutions.

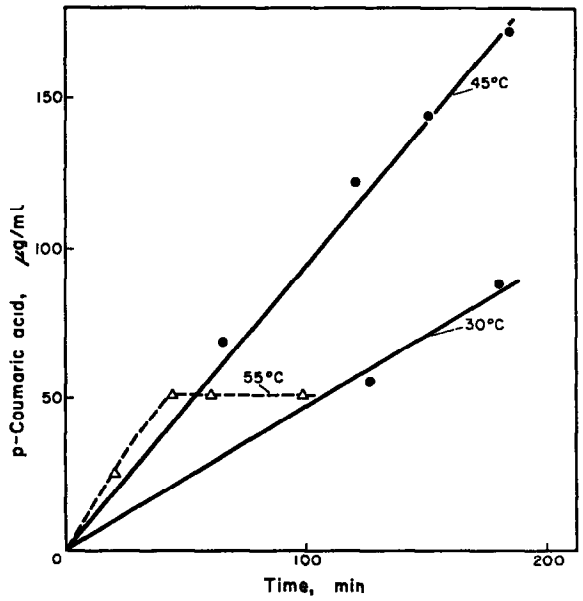

FIG. 3. RATE OF FORMATION OF *p*-COUMARIC ACID BY TYRASE AS A FUNCTION OF TEMPERATURE. The tyrase preparation was a rice seedling protein obtained between 35–70 per cent saturation on the first ammonium sulfate fractionation. Each ml of reaction mixture contained 4·3 mg of protein, 3·1 units of tyrase, 1·0 mg of L-tyrosine and 100 μmoles of sodium borate at pH 8·9.

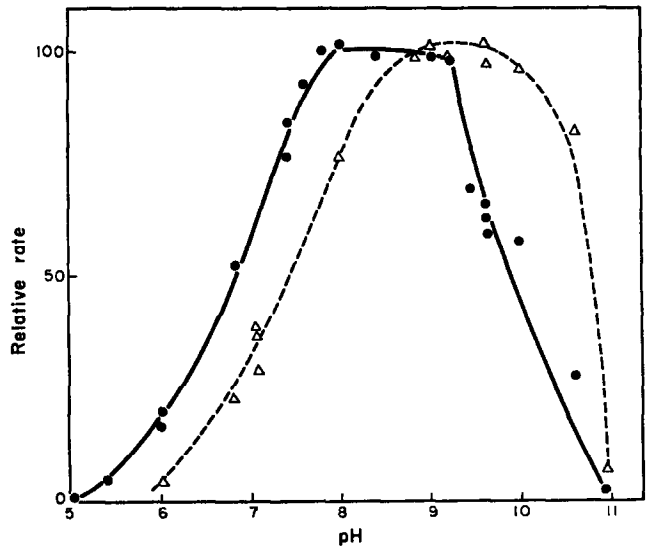

FIG. 4. EFFECT OF pH ON THE ENZYMATIC FORMATION OF *m*- AND *p*-COUMARIC ACIDS FROM THE CORRESPONDING ISOMERS OF TYROSINE.

Barley-stem protein purified through step 4 (Table 5) was used as the enzyme. The solid line (filled circles) is for formation of *p*-coumaric acid from L-tyrosine; the broken line (triangles) is for formation of *m*-coumaric acid from DL-*m*-tyrosine. The rate at any given pH is expressed as the percentage of the rate at pH 8·8 (0·1 M sodium borate) as determined in a parallel experiment. Sodium acetate buffer (0·1 M sodium borate) as determined in a parallel experiment. Sodium acetate buffer (0·1 M) was used for pH 5·0 and 5·4; potassium phosphate buffer (0·1 M) was used for pH 6·0–8·0; sodium borate buffer (0·1 M) was used for pH 8·4–9·6; ethanolamine hydrochloride buffer (0·2 M) was used for pH 10·0 and 10·6 and, methylamine hydrochloride (0·2 M) for pH 10·9.

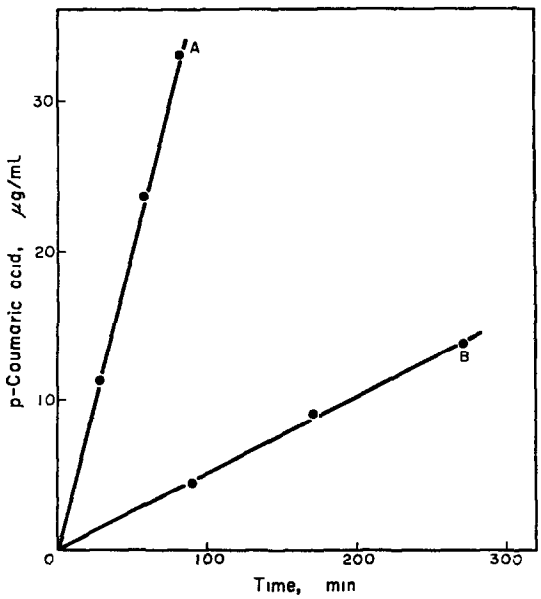

FIG. 5. TIME COURSE OF THE ENZYMATIC FORMATION OF *p*-COUMARIC ACID FROM EXCESS L-TYROSINE. Line A is for preparation C (Table 6) under the usual conditions of assay (i.e. pH 8·8, 40°), using 0·08 mg of protein per ml. Line B is for a crude protein from etiolated barley seedlings obtained between 40–60 per cent saturation on the first ammonium sulfate fractionation. This experiment was run at pH 8·8 and 31° using 2·8 mg of protein per ml.

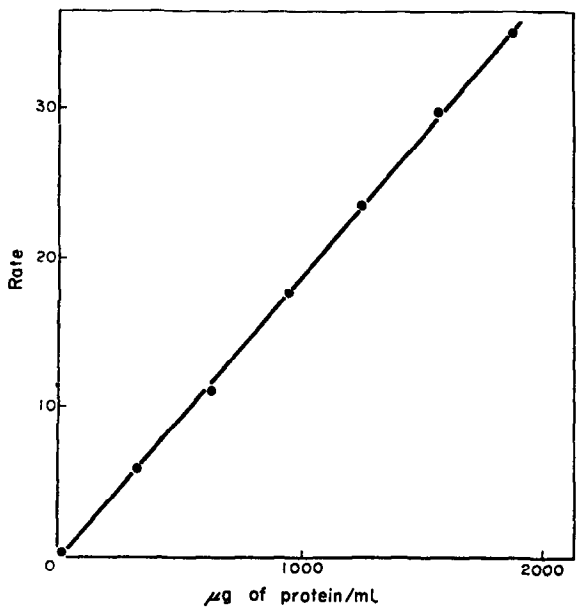

FIG. 6. RATE OF FORMATION OF *p*-COUMARIC ACID AS A FUNCTION OF ENZYME CONCENTRATION. The rate is expressed as μg of *p*-coumaric acid formed in 45 min. Each ml of reaction mixture contained 1·0 mg of L-tyrosine, 100 μmoles of sodium borate (pH 8·8) and enzyme as indicated. The reaction was run at 40°. The enzyme used was a barley-stem protein fraction precipitated at 40–60 per cent saturation on the second ammonium sulfate fractionation.

under these conditions. The rate of formation of *p*-coumaric acid was constant (Fig. 5) and directly proportional to the enzyme concentration (Fig. 6).

Comparison of Sources of Tyrase

Table 3 lists the tyrase contents of acetone powders prepared from the seedlings of several species belonging to the *Gramineae*. Rice seedlings were a good source, particularly the roots. The relatively low tyrase content of acetone-dried whole seedlings is due to dilution by starch and other inert substances in the seed residues. Wheat germ acetone powder was practically free from tyrase. It seems likely that there is little, if any, tyrase in seeds (compare with sorghum seeds, Table 2), but the enzyme is readily formed on germination.

TABLE 3. TYRASE CONTENT OF VARIOUS ACETONE POWDERS

Source*	Age (days)	Yield of acetone powder (% of fresh wt.)	Units of tyrase per g of acetone powder
Rice			
Whole seedlings	12	24	27
" "	11	28	21
Shoots of seedlings	11	12·6	52
Roots of seedlings	11	5·0	95
" " "	11	5·0	138
" " "	12	6·9	104
Barley			
Etiolated whole seedlings	4	11·0	13
Etiolated seedling shoots	5	5·6	36
Non-etiolated seedling shoots	7	6·5	25
" " " roots	7	3·0	31
Wheat			
Shoots of seedlings	7	10·4	43
Sperry wheat germ	—	86	nil

* Seedlings were grown in the laboratory from Caloro rice, Mariout barley and Ramona wheat.

The tyrase content of barley seedlings grown in a field was relatively low, but it increased as the plants developed and reached a peak at about the time the heads were emerging (Table 4). The tyrase content of barley stems was considerably higher than that of the leaves.

Extraction and Partial Purification of Tyrase

(a) *General.* Tyrase was extracted and concentrated from several sources. Acetone powders of wheat seedling shoots, barley seedlings, rice seedlings and roots of rice seedlings were tried, but the best results were obtained with acetone powders from barley stems. Table 5 summarizes the results obtained with barley stems. Each step in the fractionation is described below, with observations on results obtained with other sources. Table 6 describes preparations that were used in a study of the properties of tyrase.

Although rice root acetone powders were rich in tyrase (Table 3), attempts to concentrate the enzyme from this source met with little success because of the instability of the extracts. Sometimes more than half the activity was lost during dialysis overnight at 0·5°. A sample purified through step 3 (Table 5), lyophilized, and stored 83 days at −20°, lost about half of its activity. Tyrase prepared from barley stems was much more stable; a

TABLE 4. CHANGES IN TYRASE CONTENT DURING DEVELOPMENT OF BARLEY*

Date	Stage of development	Yield of acetone powder (% of fresh wt.)	Units of tyrase per g of acetone powder from				Units of tyrase found	
			Whole shoot	Leaves	Stems	Heads	per 100 g† of fresh wt.	per mg of extracted protein‡
Jan. 30	Plants up to 12 cm tall; 2–3 leaves	11·6	5·1	—	—	—	59	0·02
Feb. 26	Plants up to 30 cm tall; 3–4 leaves	10·6	6·0	—	—	—	63	0·04
March 11	Plants up to 40 cm tall; 4 leaves	9·0	17·0	—	—	—	150	0·06
March 18	Plants up to 45 cm tall	9·0	22·0	11	39	—	200	0·46
March 26	Plants up to 65 cm tall	11·8	37·0	9·6	72	—	430	0·67
April 3	Plants up to 80 cm tall; heads ready to emerge	14·7	23·0	7·7	35	—	340	0·47
April 16	Plants up to 90 cm tall; heads just emerged	25·4	32·0	2·8	44	47	810	0·44
April 30	Heads in milk stage	32·1	12·0	4·2	15	12	380	—

* *Hordeum vulgare* L. (var. Aravat) growing in field, University of California at Davis, 1960.
† Calculated for the whole shoot by multiplying the units of tyrase per g of acetone powder by the percentage yield of acetone powder.
‡ The first three figures apply to protein extracted from the whole shoot acetone powder, the others to protein from stem acetone powders. The protein was extracted by 0·10 M sodium borate (at pH 8·8), precipitated by ammonium sulfate (0–70 per cent saturation) and dialyzed overnight against 0·05 M sodium borate (pH 8·8).

TABLE 5. PURIFICATION OF TYRASE FROM AN EXTRACT OF BARLEY-STEM ACETONE POWDER*

Step No.	Description of step	Volume (ml)	Total† protein (mg)	Tyrase (units)	Specific activity (units/mg of protein)	% Recovery‡
1	First ammonium sulfate precipitate, 0–70 per cent	195	3100	1340	0·43	—
2	Supernatant after titrating to pH 6	190	1690	1330	0·80	99
3	Fraction precipitated between 50–60 per cent saturation with ammonium sulfate	21·2	218	725	3·3	54§
4	Chromatographed on DEAE-cellulose	99	33	376	11·4	28
5	Fraction from step 3 purified by calcium phosphate gel	33	49	388	7·9	38
6	Fraction from step 5 chromatographed on DEAE-cellulose	30	14	258	18·4	19

* Barley stem acetone powder (171 g, 5400 units of tyrase) from April 16 (Table 4) was extracted by 2400 ml of 0·10 M sodium borate at pH 8·8. Half of this was purified by steps 1, 2, 3 and 4 and the other half by steps 1, 2, 3, 5 and 6. The results are calculated back to the total.
† The ratio of optical density at 280 mμ to that at 260 mμ was 0·9 after the first ammonium sulfate precipitation, 1·4 after the second ammonium precipitation and 1·6 for the most highly purified sample of tyrase.
‡ Recoveries are based on the tyrase found in the first ammonium sulfate precipitation. This was only 25 per cent of the total tyrase in the acetone powder.
§ The fraction precipitated between 40–50 per cent saturation with ammonium sulfate contained an additional 377 units of tyrase (28 per cent) with a specific activity of 1·14.

sample purified through step 3 and stored 70 days at −20° lost only about 10 per cent of its activity, and when it was lyophilized there was no detectable loss of activity during 70 days. The more dilute solutions obtained from DEAE-cellulose (diethylaminoethyl cellulose) columns (Table 6) lost about half of their activity in the 2 weeks, but during this period they were thawed 8–10 times for sampling. Loss of activity of tyrase preparations may be due to proteolysis, since free amino-acids have been detected, after storage, in preparations purified through step 3.

TABLE 6. DESCRIPTION OF SOME TYRASE PREPARATIONS

Preparation	Source	Steps used in purification*	Composition of solution	Specific activity
A	Rice seedlings, 12 days old, acetone-dried	35–70 fraction of step 1, purified by steps 2, 5 and 6	0·35 mg of protein per ml in 0·025 M potassium phosphate at pH 6·8 and 0·2 M potassium chloride	4·0
B	Barley stems, pre-head stage, acetone-dried	1, 2, 3 and 4	0·31 mg of protein per ml in 0·035 M potassium phosphate at pH 6·8 and 0·2 M potassium chloride	8·0
C	Barley stems just after emergence of heads, acetone-dried	1, 2, 3 and 4	0·34 mg of protein per ml, otherwise as B	11·4
D	Same as C	1, 2, 3, 5 and 6	0·40 mg of protein per ml, otherwise as B	18·4

* The steps are numbered as in Table 5; see Table 5 and text for description.

(b) *Preparation and extraction of acetone powders.* A Waring blendor was used. Fresh plant material was cut into convenient lengths (about 1 in.) by scissors, barely covered with acetone at −20°, blended for 1–1·5 min at full speed, filtered by suction and washed three times with acetone at −20°. After drying for about 5 min on the Buchner funnel, the powder was spread on paper, air-dried in a fume hood for about 15 min and finally dried *in vacuo* at room temperature for at least 1 hr. The powders were stored at 4–6° in containers with tight caps. Although referred to as "powders", some of the acetone-dried materials were rather fibrous, particularly those made from stems of cereals.

The acetone powder was mixed with about 15 times its weight of 0·10 M sodium borate, pH 8·8, at room temperature. The mixture was cooled in an ice-bath and stirred manually from time to time during 30 min, then filtered through a double layer of cheese cloth. The filtrate was centrifuged at 8000 × *g* and 0° for 15 min. The supernatant fluid contained much of the tyrase; the sediment was discarded. Usually only 20–40 per cent of the tyrase present in a sample of acetone powder was obtained in solution. All subsequent steps were carried out at 0–4°.

(c) *First ammonium sulfate precipitation (step 1, Table 5).* The supernatant from the preceding step was cooled in ice, and solid ammonium sulfate, to give 70 per cent saturation, was added gradually, with stirring, during 5–10 min, then the precipitate was collected by centrifugation at 8000 × *g* for 15 min. The precipitate was taken up in 0·05 M sodium borate at pH 8·8, using a volume about one-twentieth the original extract. This was dialyzed overnight against 50 volumes of the same buffer. The precipitate nearly all dissolved giving a slightly turbid solution.

In early experiments the fractions precipitating between 0–35 and 35–70 per cent

saturation were collected separately; tyrase was found chiefly in the 35–70 fraction. Extracts from rice seedlings showed unique behavior; the 35–70 fraction rose to the surface. After allowing about 10 min for separation most of the clear solution was siphoned out from under the surface layer of insoluble material. Centrifugation of this insoluble fraction gave a pellicle containing the enzyme; the clear solution was poured out and the pellicle taken up in 0·05 M sodium borate (pH 8·8) and dialyzed as above.

(d) *Precipitation of inactive protein at pH 6 (step 2, Table 5).* The slightly turbid solution from the preceding step was titrated to pH 5·9–6·1 (glass electrode) using M acetic acid at 0°. A heavy precipitate formed; it was removed by centrifugation at $11{,}000 \times g$. The supernatant fluid contained practically all of the tyrase; it was titrated to pH 8·6 by N sodium hydroxide. This clear solution was used at once for the next step.

(e) *Second ammonium sulfate precipitation (step 3, Table 5).* Solid ammonium sulfate was added carefully to the tyrase solution at 0°; fractions precipitated at 0–40, 40–50, 50–60 and 60–75 per cent saturation were collected separately by centrifugation at $8000 \times g$. Tyrase was always concentrated in the fractions at 40–60 per cent saturation, sometimes chiefly in the 40–50 fraction and other times in the 50–60 cut. The precipitates were dissolved in 0·02 M potassium phosphate at pH 6·8, to give a protein concentration between 1 and 2 per cent. They were then dialyzed overnight against 0–100 volumes of the same buffer at 0°. The dialyzed solution was ready for chromatography on DEAE-cellulose.

(f) *Chromatography of tyrase on DEAE-cellulose (steps 4 and 6, Table 5).* The DEAE-cellulose was a commercial preparation; i.e. Biorad Cellex-D. It was washed with 0·1 N sodium hydroxide, suspended in deionized water, titrated to pH 6·8 by M phosphoric acid, filtered on a Buchner funnel and washed with 0·02 M potassium phosphate at pH 6·8. Only this purified material was used for chromatography.

Two sizes of columns were employed. The small size ($1{\cdot}1 \times 10$ cm) was loaded with about 20 mg of protein; the large size ($2{\cdot}2 \times 17$ cm) was loaded with about 100 mg. The small column was operated at 0°, in an ice bucket, using a flow rate of about 30 ml per hr; the fractions (3 ml) were collected manually. The large column was operated at 4°, in a cold room, using a flow rate of about 90 ml/hr; the fractions (4 ml) were collected automatically. Columns were packed in Pyrex tubes with retaining plugs of fine glass wool. After washing the packing thoroughly with 0·02 M potassium phosphate (pH 6·8) the enzyme solution (see above) was put on and washed in. The column was developed using a linear gradient[11] between equal volumes of 0·02 M potassium phosphate at pH 6·8 and 0·05 M potassium phosphate at the same pH but containing 0·4 M potassium chloride. The total volume in the gradient elution apparatus was 80 ml for the small column and 400 ml for the large one.

Tyrase was detected in the eluate by the direct fluorometric method described in the next paragraph. It emerged from the column after about one-half the contents of the elution apparatus had been used. The enzyme was thus obtained in about 0·035 M potassium phosphate at pH 6·8, 0·2 M with respect to potassium chloride. The protein concentration was usually 0·3–0·4 mg/ml. The active fractions were stored at −20° (Table 6).

The tyrase content of fractions eluted from DEAE-cellulose columns was determined by a rapid fluorometric procedure which omitted ether extraction and zero-time controls. An aliquot (0·1–0·2 ml) of the eluate was mixed with 0·5 ml of 0·1 per cent tyrosine in 0·15 M sodium borate (pH 9·0). This mixture was incubated in a 13×100 mm culture

[11] R. M. BOCK and N. LING, *Anal. Chem.* **26**, 1543 (1954).

tube for 30 min at 40°, then 1·0 ml of 0·13 N sodium hydroxide was added and the mixture poured into a round quartz cuvette for measurement of its fluorescence. All fractions had a fluorescence, but this was augmented 3–5 times in tubes containing appreciable amounts of tyrase. This simple, rapid method showed which fractions could be recombined. The composite fraction was then analyzed for tyrase by one of the more accurate methods outlined above.

(g) *Purification by calcium phosphate gel (step 5, Table 5).* Fractionation by positive adsorption on a calcium phosphate gel was used in some instances. This fractionation was applied to fractions after removal of inert protein at pH 6 or after the second ammonium sulfate precipitation. The calcium phosphate gel[12] contained 37·8 mg of solids per ml. The enzyme solution was dialyzed for at least 4 hr against 100 volumes of 0·01 M potassium phosphate at pH 6·0. The volume was then adjusted to give a protein concentration of 1 per cent; 0·1 volume of the gel was added, the mixture stirred for about 5 min then centrifuged at $6000 \times g$ for 10 min. The supernatant solution was treated with 0·3 volumes of gel, stirred 10 min and centrifuged as before. Most of the tyrase was absorbed on the second portion of gel; this precipitate was washed with 0·01 M potassium phosphate at pH 6·0, and the enzyme eluted by mixing for 10 min with 0·02 M potassium phosphate at pH 7·4, then with 0·05 M potassium phosphate at pH 8·0. These eluates were combined. The enzyme was recovered in a volume about 1·5 times as large as the original volume (Table 5).

(h) *Other fractionation procedures.* A number of other standard fractionation methods were tried in addition to those described above. These procedures were applied to a rice seedling protein fraction obtained between 35–70 per cent saturation with ammonium sulfate (first precipitation). Fractional precipitation by acetone resulted in considerable loss of tyrase with no increase in specific activity. Protamine sulfate (0·01 M potassium phosphate, pH 6·0) was found to remove tyrase from solution; no attempt was made to recover the enzyme from the precipitate. A 2·6 fold purification of tyrase was obtained using alumina C_γ[12] for positive adsorption in the same manner described above for calcium phosphate. Most of the rice seedling proteins, including tyrase, passed through a carboxymethyl-cellulose column (Biorad Cellex-CM) at pH 6·0 (0·02 M potassium phosphate).

Specificity of a Tyrase Preparation

Chromatography on DEAE-cellulose removed practically all the free ammonia from tyrase preparations. It was found that preparations purified in this way formed ammonia and *p*-coumaric acid in equimolar amounts when incubated with tyrosine (see section on stoichiometry below). Other amino-acids were substituted for tyrosine, and ammonia formation was measured, in order to obtain information on the specificity of tyrase (Table 7). Two amino-acids, L-phenylalanine and DL-*m*-tyrosine (i.e. *m*-hydroxy-phenylalanine), induced a more rapid formation of ammonia than did L-tyrosine. The formation of ammonia from L-phenylalanine was expected since Koukol and Conn[1,2] had already demonstrated that barley and other plants contain an enzyme (phenylalanine deaminase) which forms cinnamic acid and ammonia from L-phenylalanine. The deamination of *m*-tyrosine was unexpected; it was investigated further and *m*-coumaric acid identified as the product (see below). There was little, if any, deamination of the other amino-acids. Histidase[9,10] and aspartase[6,7] were either absent or present in very low concentrations.

[12] S. P. COLOWICK, *Methods in Enzymology*, Vol. I, pp. 97, 98. Academic Press, New York (1955).

TABLE 7. DEAMINATION OF VARIOUS AMINO-ACIDS BY A TYRASE PREPARATION*

Amino-acid added	μg of NH_3 found		μmoles of NH_3 formed
	at start	after 3 hr	
L-Tyrosine	4·4	16·6	0·79
DL-*o*-Tyrosine	5·3	6·1	0·05
DL-*m*-Tyrosine	3·0	24·6	1·40
DL-DOPA†	4·7	7·2	0·16
L-Phenylalanine	8·2	72	4·00
L-Aspartic acid	4·1	6·2	0·13
L-Alanine	5·2	6·0	0·05
L-Histidine	4·2	6·2	0·13
L-Tryptophan	4·5	6·5	0·13

* Preparation B (Table 6). The reactions were run in air-filled sealed Thunberg tubes at 40° for 3 hr. Each tube contained 0·61 mg of protein, 150 μmoles of sodium borate (pH 9·0), and 10 μmoles of L-amino acid (or 20 μmole of DL-form) in a total volume of 3·0 ml. The cap of the tube contained 0·3 ml of N sulfuric acid. At the end of the reaction the tubes were cooled in ice, the acid mixed in and a 2 ml aliquot of the mixture removed for estimation of ammonia.

† 3,4-Dihydroxyphenylalanine.

Identification of p-*Coumaric Acid as the Product of the Enzymatic Deamination of Tyrosine*

The product of the tyrase reaction had the same R_f as *trans-p*-coumaric acid in the solvents listed at the foot of Table 1. Tests with the diazotized *p*-nitroaniline spray reagent followed by alkali gave the same colors (orange changing to blue) as were exhibited by an authentic sample. The fluorescence was also the same for both samples; a blue fluorescence was noted on the paper chromatograms when viewed under ultraviolet light (366 mμ) only after the sheet had been sprayed with alkali. The maximum settings of the spectrophotofluorometer were the same, both for activation (350 mμ) and emission (440 mμ), as for *trans-p*-coumaric acid. It is unlikely that *cis-p*-coumaric acid was formed, even transitorally, since it was not observed on paper chromatograms irrigated with solvent A (Table 1) and since *cis-p*-coumarate was not converted to the *trans-* isomer by an active tyrase preparation.

The product of the tyrase reaction was rigorously indentified as *trans-p*-coumaric acid by isolating enough crystalline material for a mixed melting point determination. This was done both with rice and barley preparations. Ten ml of barley stem tyrase purified through step 3 (Table 5), containing 178 units of enzyme and 150 mg of protein, was mixed with 20 ml of 0·15 M sodium borate (pH 9·0) containing 30 mg of L-tyrosine. This mixture was incubated at 40° for 4½ hr, cooled in ice, acidified with 2 ml of 5 N hydrochloric acid, then shaken with 80 ml of ether. The ether phase was separated and back-extracted with 12 ml of 0·1 M sodium bicarbonate. The bicarbonate extract was acidified with 1 ml of 5 N hydrochloric acid, shaken with 15 ml of ether and the ether separated and allowed to evaporate at room temperature. The crystalline residue (9 mg) was recrystallized from water (using charcoal) and the colorless product collected on a glass filter, washed with ice–water and air dried. This gave 7 mg of a crystalline solid, m.p. 219–220°. The melting point was not depressed when the sample was mixed with authentic *trans-p*-coumaric acid, m.p. 219–220°. Both samples evolved gas on melting.

Another crystalline sample was obtained using a rice seedling protein fraction purified only through steps 1 and 2 (Table 5). This tyrase solution (91 ml) contained 100 units of enzyme and 700 mg of protein. The reaction was run essentially as described above. The

crude *p*-coumaric acid, obtained by evaporation of the ether extract, was digested with hot toluene (2 ml), then cooled and 2 ml of 30–60° petroleum ether was added. The solid was filtered out, dried at room temperature, then recrystallized from water as described above. This gave about 4 mg of a colorless crystalline solid m.p. 218–219°. The m.p. was not depressed by admixture with authentic *trans*-*p*-coumaric acid.

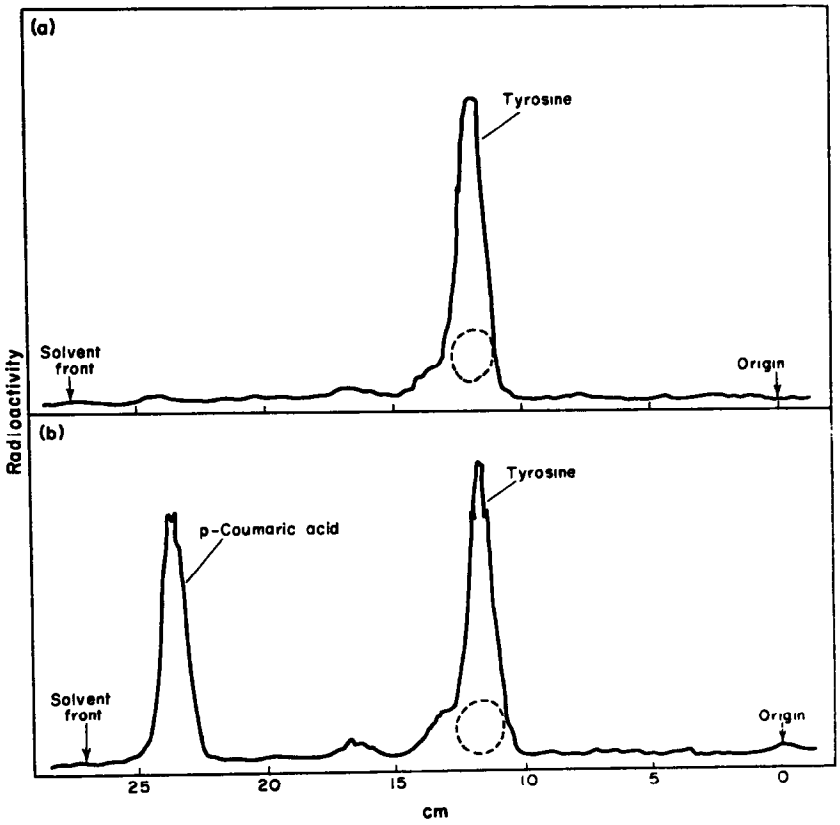

FIG. 7. ENZYMATIC SYNTHESIS OF *p*-COUMARIC ACID-C^{14} FROM L-TYROSINE-C^{14}.

The enzyme was a barley-stem protein fraction precipitated at 40–60 per cent saturation on the second ammonium sulfate fractionation. Each ml of reaction mixture contained 320 μg (1·6 μc) of L-tyrosine-C^{14} (uniformly labelled), 70 μmoles of sodium borate (pH 8·8), 4·4 mg of protein and 7·9 units of tyrase. Samples were chromatographed on Whatman No. 1 paper using *n*-butanol-acetic acid-water (4 : 1 : 1·8) as the irrigant. The chromatograms were scanned by a Nuclear-Chicago Actigraph II. (a)—Tracing of scanner record of 8 μl of reaction mixture, sampled immediately after mixing. (b)—Tracing of scanner record of 15 μl of reaction mixture, sampled after 3 hr incubation at 40°. The broken circles show position of tyrosine as revealed by ninhydrin.

Experiments with L-tyrosine-C^{14} (uniformly labelled) showed that C^{14}-labelled *p*-coumaric acid was formed as the major product and with practically the same specific activity as the tyrosine. The first experiment (Fig. 7) showed one major product with the mobility of *p*-coumaric acid in the irrigant employed. A possible minor component may be an impurity in the tyrosine. The bulk of the reaction mixture was acidified with hydrochloric acid, extracted by ether, and aliquots of the extract were plated and counted under a Nuclear-Chicago gas-flow counter fitted with a micromil window. This showed 42 per cent of the

C^{14} had been converted to an ether-extractable form (i.e. *p*-coumaric acid). Another aliquot of the ether extract was analyzed for *p*-coumaric acid by ultraviolet absorptiometry The specific activity was calculated to be 92 per cent of that expected assuming all the C^{14} to be in *p*-coumaric acid. A similar experiment was run using enzyme preparation *D* (Table 6). The conversion of substrate was 23 per cent and the specific activity of the *p*-coumaric acid formed was 96 per cent of the expected value. Crude preparations probably contain or generate some endogenous tyrosine.

Identification of m-*Coumaric Acid as the Product of the Enzymatic Deamination of* m-*Tyrosine*

The ether-soluble acid formed by incubating tyrase preparations with DL-*m*-tyrosine had the same mobility in solvent D (Table 1) as authentic *m*-coumaric acid, and it gave the same color changes when the paper was sprayed with diazotized *p*-nitroaniline followed by alkali (yellow changing to red). The ultraviolet absorption spectrum in alkali coincided with that of authentic *trans-m*-coumaric acid (Fig. 8). The identity was firmly established by isolation of enough crystalline product for a mixed melting point test. Five ml of barley stem tyrase purified through step 3 (Table 5), containing 87 units of tyrase and 78 mg of protein, was mixed with 10 ml of 0·15 M sodium borate (pH 9·0) containing 100 mg of DL-*m*-tyrosine. This mixture was incubated at 40° for 4½ hr then worked up as described above for isolation of *p*-coumaric acid. The crystalline residue (10 mg) from evaporation of the ether extract was recrystallized from water. This gave 6 mg of colorless crystals m.p. 194–196°; the m.p. was not changed by mixing with authentic *m*-coumaric acid (m.p. 194–196°).

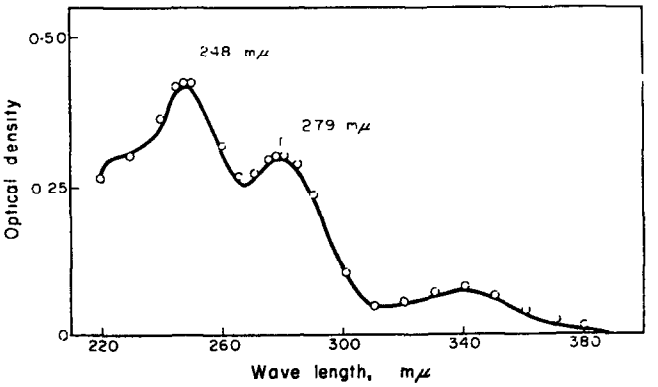

FIG. 8. ULTRAVIOLET ABSORPTION SPECTRUM OF *m*-COUMARIC ACID.
The solid line is for the synthetic material (3·3 μg per ml of 0·05 N sodium hydroxide); the circles are for an ether-extractable acid fraction formed by the action of a barley-stem tyrase on DL-*m*-TYROSINE.

Stoichiometry of the Enzymatic Deamination of Tyrosine and m-*Tyrosine*

Both reactions were found to form one mole each of ammonia and the corresponding coumaric acid for each mole of the amino acid used. The data obtained for the deamination of L-tyrosine by three different enzyme preparations are collected in Table 8. The disappearance of tyrosine was measured only when the amount present initially was low enough to permit utilization of a reasonably large fraction. Enzymes from rice or barley formed *p*-coumaric acid and ammonia in equimolar amounts ±10 per cent. This was also true for the formation of *m*-coumaric acid and ammonia from DL-*m*-tyrosine by a barley-stem preparation (Table 9).

TABLE 8. STOICHIOMETRY OF TYROSINE DEAMINATION

Enzyme*	Protein added (mg)	Incubation time (hr)	μmoles of L-tyrosine		μmoles change on incubation		
			at start	at finish	tyrosine	*p*-coumaric acid	NH_3
A	0·72	5	16·5	—	—	+0·79	−0·77
A	0·72	5	nil	—	—	nil	+0·01
B	0·60	3	1·66	1·01	−0·65	+0·61	+0·54
B	0·60	3	1·53	—	—	+0·97	+0·84
C	0·51	3	1·65	1·05	−0·60	+0·78	+0·87
C	0·51	3	3·31	2·39	−0·92	+1·04	−1·18
C	0·51	3	13·2	—	—	+1·41	−1·50

* The enzyme preparations are described in Table 4. The reactions were run in Thunberg tubes as described in footnote to Table 7. After cooling and acidification one aliquot was analysed for ammonia and another fractionated by ether extraction; *p*-coumaric acid was measured in the ether extract and L-tyrosine in the aqueous residue (see text for further details).

Effect of Inhibitors and Activators on Tyrase

Table 10 summarizes the results of a series of experiments where sulfhydryl compounds or sulfhydryl reagents were added to tyrase reactions. The reactions were run about 1 hr at 40° then the amount of *p*-coumaric acid formed was measured. The relative rate is the amount of *p*-coumaric acid formed, expressed as the percent of the amount formed in a control test where no sulfhydryl compound or inhibitor was added. The sulfhydryl compounds caused a marked decrease in formation of *p*-coumaric acid by crude enzyme preparations, but as the enzyme was purified this effect disappeared. Tyrase was strongly inhibited by *p*-chloromercuribenzoate, but not by iodoacetamide.

Various salts were tested at 0·05 and 1·1 mM concentration, especially those of divalent metals, such as Ca, Mg, Mn, Cu, Co and Fe. None of these gave a marked stimulation; cupric sulfate was rather toxic. Quite large concentrations of ammonium sulfate (0·11 M) had no effect. No evidence could be obtained for participation of a divalent cation in the tyrase reaction. It was not inhibited by EDTA (ethylenediamine tetra-acetate). There is a possibility that a firmly bound cation is involved. The role of monovalent ions was not investigated. The reaction mixtures always contained fairly high concentrations of sodium and potassium ions.

TABLE 9. STOICHIOMETRY OF *m*-TYROSINE DEAMINATION

μmoles of DL-*m*-tyrosine		μmoles change on incubation		
at start	after incubation	*m*-tyrosine	*m*-coumaric acid	NH_3
2·76	2·08	−0·68	+0·72	+0·76
5·52	4·45	−1·07	+0·98	+0·96
22·1	—	—	+1·97	+1·90

Enzyme preparation C (Table 6) was used. The reaction mixture (2 ml) contained 0·34 mg of protein and 150 μmoles of sodium borate (pH 9·0). The reactions were run for 3 hr at 40° in sealed Thunberg tubes with 0·2 ml of N sulfuric acid in the cap. The reaction was terminated by cooling the tubes in ice and mixing in the acid. One aliquot was analysed for ammonia and another was fractionated by ether; *m*-coumaric acid was measured in the ether extract and *m*-tyrosine in the aqueous residue (see text for further details).

TABLE 10. EFFECT OF SULFHYDRYL COMPOUNDS AND INHIBITORS ON BARLEY TYRASE PREPARATIONS AT DIFFERENT STAGES OF PURIFICATION*

Specific activity of enzyme preparation	Compound added	μmoles added per ml	Relative rate (% of control)
0·13	Reduced glutathione	10	20
	$CH_2SH.CH_2OH$	10	41
	$CH_2SH.COONa$	10	18
	Iodoacetamide	7	101
	p-Cl-Hg-Benzoate	0·1	57
	" "	0·01	91
1·8	Reduced glutathione	10	49
	" "	1	94
	Cysteine	10	58
	"	1	82
	"	0·1	91
10·7	Reduced glutathione	10	120
	" "	1	98
	Cysteine	10	104
	"	1	116
	Iodoacetamide	70	89
	"	7	99
18·4	Cysteine	10	90
	"	1	121
	p-Cl-Hg Benzoate	0·1	11
	" "	0·001	93

* The preparation with the lowest specific activity is from etiolated barley seedlings purified through steps 1 and 2, the other preparations are from stems of field grown barley purified through steps 3, 4 and 6 respectively (Table 5).

Tyrase was not inhibited by fluoride, but it was inhibited fairly readily by cyanide (Table 11). Pyridoxal phosphate had no effect on the tyrase reaction whether α-keto acids were added or not. Other cofactors that were without effect include folic acid, adenosine triphosphate and coenzyme A.

Attempts to Reverse the Tyrase Reaction

Two attempts to demonstrate reversal of the tyrase reaction gave negative results. In these experiments *p*-coumarate was incubated with a large excess of ammonium sulfate in the presence of tyrase. In the first experiment the *p*-coumarate concentration was measured;

TABLE 11. INHIBITION OF TYRASE BY CYANIDE*

Enzyme preparation	Specific activity	μmoles of KCN/ml	Relative rate (% of control)
Rice seedling protein purified by steps 1 and 2.	0·58	30	27
		2	91
Preparation D, Table 6.	18·2	10	18
		1	47
		0·1	74

* Reactions run one hour at 40° in 0·07 M sodium borate at pH 8·8.

there was no change even after incubation with 8000 times the theoretical amount of ammonia for one hour either at pH 7·4 or at pH 9·0. In the second experiment *p*-coumaric acid-α-C^{14} was used and measurements were made of the C^{14} fixed in a form not readily extracted into ether. This gave a fairly sensitive method of detecting tyrosine formation. The molar ratio of ammonia to *p*-coumarate was 38 : 1 and the reaction was run at pH 8·8 with a highly purified sample of barley stem tyrase (preparation D, Table 6). No formation of tyrosine could be detected although the counting method was sensitive enough to measure conversion of 0·0005 per cent of the *p*-coumarate.

Comparative Studies on Formation of p-*Coumaric Acid,* m-*Coumaric Acid and Cinnamic Acid by Tyrase Preparations*

Partially purified preparations of tyrase from barley stems have been shown to catalyze the deamination of three aromatic amino-acids, i.e. L-tyrosine, DL-*m*-tyrosine and L-phenylalanine (see above; Koukol and Conn[1,2]). These three activities were found at all stages of purification tested so far. However, the studies reported in this section suggest that tyrase is distinct from the enzymes (or enzyme) catalyzing the deamination of *m*-tyrosine or phenylalanine, although this has not been established beyond doubt.

TABLE 12. VARIABILITY IN RELATIVE RATES OF FORMATION OF *m*- AND *p*-COUMARIC ACIDS

Enzyme preparation*	Buffer	pH	μmoles/mg protein/hr		*meta para* ratio†
			p-coumaric acid	*m*-coumaric acid	
B	Potassium phosphate	7·4	0·27	0·52	1·9
B	Sodium borate	8·8	0·43	1·35	3·1
B	„ „	9·6	0·22	1·30	5·9
C	Potassium phosphate	7·4	0·64	1·73	2·7
C	Sodium borate	8·8	0·84	6·00	7·1
C	„ „	9·6	0·54	4·75	8·8

* Enzyme preparations described in Table 6. Each 1·4 ml of reaction mixture contained 100 μmoles of buffer, 12 μ moles of sodium hydroxide, 1·0 mg of L-tyrosine (or 2·0 mg of DL-*m*-tyrosine) and 65 μg of protein.

† In another experiment barley stem protein fractions, purified through step 3, from plants harvested on March 19, April 4 and April 16 (see Table 4), were tested at pH 8·8 and 40° with 0·1 per cent L-tyrosine or 2·0 per cent DL-*m*-tyrosine. The *meta*/*para* ratios found were 3·4, 4·2 and 6·0 respectively.

The pH curve for the formation of *p*-coumaric acid from tyrosine is displaced about one pH unit towards the acid side relative to the curve for formation of *m*-coumaric acid from *m*-tyrosine (Fig. 4). Because of their rather broad peaks these curves overlap and the optima coincide at pH 8·8–9·0. However, the ratio of these reactions varies with the pH. The data in Table 12 show the variation for two different enzyme preparations. It is evident that the ratio of the two activities varies with the pH and also with the enzyme preparation. It seems likely two enzymes are involved and their relative concentration changes as the plant matures (see footnote to Table 12).

The Michaelis–Menten constants (Fig. 9) show that the affinity of tyrase for tyrosine is about 25 times as great as the affinity of the other enzyme for *m*-tyrosine. Koukol and Conn[1,2] have obtained a Km (Michaelis–Menten constant) for phenylalanine deaminase approximately twice that reported here for tyrase. They have observed a strong inhibition

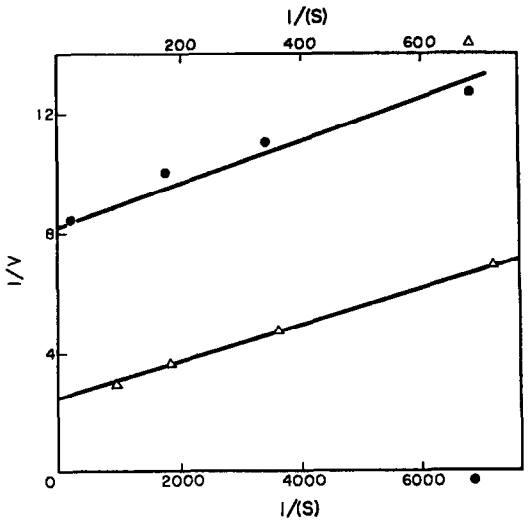

FIG. 9. EFFECT OF SUBSTRATE CONCENTRATION ON THE RATE OF FORMATION OF *m*- AND *p*-COUMARIC ACIDS FROM THE CORRESPONDING ISOMERS OF TYROSINE.

Preparation C (Table 6) was incubated with the substrates 40° and pH 9·0. V = relative rate. (S) = substrate concentration in mole/l. The upper line (filled circles) is for formation of *p*-coumaric acid from L-tyrosine, the lower line (triangles) for formation of *m*-coumaric acid from DL-*m*-tyrosine. Concentrations for *m*-tyrosine were calculated on the assumption that only one enantiomer was active. The Michaelis–Menten constant (Km) for L-tyrosine was found to be $0{\cdot}84\times10^{-4}$ from this data; other determinations gave values of $0{\cdot}74\times10^{-4}$ and $1{\cdot}0\times10^{-4}$. *Km* for *m*-tyrosine was calculated to be $2{\cdot}3\times10^{-3}$; another determination gave a value of $1{\cdot}3\times10^{-3}$.

of phenylalanine deaminase by L-tyrosine. Tyrosine also inhibits the formation of *m*-coumaric acid from *m*-tyrosine (Table 13). On the other hand, the formation of *p*-coumaric acid from L-tyrosine was not inhibited by either L-phenylalanine or DL-*m*-tyrosine (Table 14) even when these substrates were present in a concentration high enough to give an appreciable inhibition, assuming they were competing with tyrosine for the same active center. This supports the view that tyrase is distinct from the enzymes (or enzyme) acting on phenylalanine or *m*-tyrosine.

TABLE 13. EFFECT OF L-TYROSINE ON THE FORMATION OF *m*-COUMARIC ACID FROM DL-*m*-TYROSINE*

Enzyme	μmoles/ml		Relative rate†
	DL-*m*-tyrosine	L-tyrosine	
C (Table 6)	28	5·5	10
D (Table 6)	46	4·6	32

* Run at 40° and pH 8·8 on 0·07 M sodium borate.

† Rate of formation of *m*-coumaric acid as percentage of the control containing no L-tyrosine. The *m*-coumaric acid was extracted by ether and measured at 248 mμ and a correction applied for the absorption by *p*-coumaric acid. This correction was determined in a parallel experiment in which L-tyrosine (but not *m*-tyrosine) was added. This is possible since *m*-tyrosine does not inhibit formation of *p*-coumaric acid (see Table 14).

TABLE 14. EFFECT OF OTHER AROMATIC AMINO-ACIDS ON FORMATION OF *p*-COUMARIC ACID FROM TYROSINE*

Enzyme preparation	Aromatic amino-acid added	μmoles/ml		Relative rate†
		L-tyrosine	Other aromatic amino-acid	
Barley stem protein purified through step 3 Specific activity = 1·8	L-Phenylalanine	3·2	7·2	98
	,,	3·2	1·4	100
Preparation C, Table 6 Specific activity = 11·4	L-Phenylalanine	5·5	11·4	103
	,,	5·5	5·7	102
	DL-*m*-Tyrosine	5·5	28	102
Preparation D, Table 6 Specific activity = 18·4	L-Phenylalanine	4·6	10·1	100
	DL-*m*-Tyrosine	4·6	46	95
	DL-DOPA	4·6	8·1	106

* Run at 40° and pH 8·8 on 0·07 M sodium borate.
† Rate as percentage of the control tube containing L-tyrosine as the only aromatic amino-acid.

Table 15 shows the results of an experiment done in collaboration with Dr. Jane Koukol. Acetone powders from several species were assayed at the same time and under the same conditions for both phenylalanine deaminase and tyrase. The acetone powders from legumes have quite a low tyrase content compared to phenylalanine deaminase; this ratio was not nearly so wide for grasses. It is not proved that legumes contain any tyrase. The figures in Table 15 are maximum values since the ether-soluble C^{14}-labelled acid obtained, in the tyrase assay for acetone powders of legumes (see footnote to Table 15), has not been rigorously identified as *p*-coumaric acid. The ratio of phenylalanine diaminase to tyrase may be even wider than that reported for the legumes.

TABLE 15. COMPARISON OF THE PHENYLALANINE DEAMINASE AND TYRASE ACTIVITIES OF ACETONE POWDERS FROM SEVERAL SPECIES

Source of acetone powder*	μmoles/hr/g†		cinnamic/*p*-coumaric ratio
	cinnamic acid	*p*-coumaric acid	
Barley seedlings, shoots	8·0	2·3	3·5
,, ,, roots	12·2	3·4	3·6
Wheat seedlings, shoots	9·0	2·5	3·6
Rice seedlings, whole	6·5	1·9	3·4
,, ,, roots	31·4	10·4	3·0
White sweet clover, tops	4·5	0·14	32·0
Alfalfa stems	11·0	0·23	48·0

* The alfalfa powder was prepared from *Medicago sativa* L. var. Caliverde, using defoliated stems of shoots about 36 cm long. The other powders were made from the the varieties listed in Table 2.
† Cinnamic acid formation was measured by Dr. J. Koukol using a radiotracer assay method.[1,2] *p*-Coumaric acid formation was measured fluorometrically for barley, rice and wheat, and by a radiotracer method for sweet clover and alfalfa. The tracer assay was based on measurement of the total C^{14}-labelled, ether-extractable acids formed on incubation of L-tyrosine-C^{14} with the acetone powder. All reactions were run at pH 8·8 and 40°.

DISCUSSION

The results of this investigation support the view that lignin and related phenylpropanoid compounds are formed from the aromatic amino acids via the phenolic cinnamic acids,[3] especially when the findings of Koukol and Conn[1,2] are also considered. In view of these new results the scheme suggested previously[3,5,13] for formation of lignin and related compounds from shikimic acid has been revised (Fig. 10). The acids with the

$$-CH=CH-COOH$$

side chain are now shown as originating by direct deamination of the corresponding amino-acid rather than by dehydration of the corresponding α-hydroxy acid. The conversion of the α-hydroxy acids to lignin and related compounds by living plants can be explained by their ready conversion to the amino-acids.[14] There is no longer any reason to postulate these hydroxy acids as having physiological significance in lignification, although it is still possible. All tracer experiments in this field, known to the author, can be explained by Fig. 10, where the α-hydroxy acids are involved in side reactions, if at all.

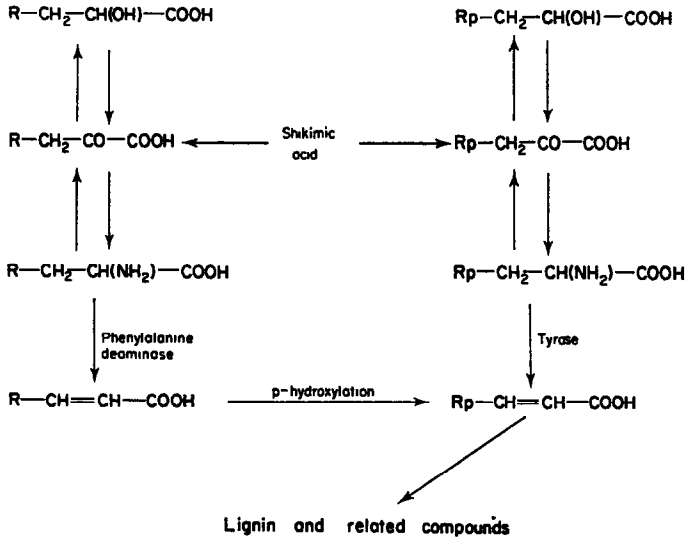

FIG. 10. REVISED SCHEME FOR FORMATION OF LIGNIN AND RELATED COMPOUNDS FROM SHIKIMIC ACID.
R = phenyl; Rp = *p*-hydroxyphenyl.

Conversion of tyrosine-C^{14} to lignin occurs readily only in certain species.[3,15,16] For example, grasses can easily convert tyrosine to lignin while legumes cannot. It is possible that tyrase is necessary for conversion of tyrosine to lignin, since it is found in substantial amounts in grasses with much less, if any, in legumes. This fact agrees with the findings of tracer experiments on living plants, but a more thorough study should be made of the correlation between the ability of a given plant to convert tyrosine to lignin and its tyrase

[13] D. R. MCCALLA and A. C. NEISH, *Can. J. Biochem. and Physiol.* 37, 537 (1959).
[14] O. L. GAMBORG and A. C. NEISH, *Can. J. Biochem. and Physiol.* 37, 1277 (1959).
[15] S. A. BROWN and A. C. NEISH, *Can. J. Biochem. and Physiol.* 34, 769 (1956).
[16] S. A. BROWN, *Can. J. Botany* (in press).

content. At present the correlation is good enough to suggest that *p*-coumaric acid is an obligate intermediate between tyrosine and lignin.

Acerbo *et al.*[17] found that C^{14}-labelled *p*-hydroxyphenyl pyruvic acid was converted to lignin by sugar cane. Its effectiveness was not compared with that of tyrosine or any other potential lignin precursor. However, since sugar cane contains tyrase (Table 2) this result might be explained by the following sequence of reactions:

p-hydroxyphenyl pyruvic acid ⟶ L-tyrosine ⟶ *trans-p*-coumaric acid ⟶ lignin.

The keto-acid fed would be expected to form C^{14}-labelled L-tyrosine.[14] This is presumably accomplished by transamination reactions such as are of general occurrence in all organisms, not merely in lignifying tissues. There is no reason to select *p*-hydroxyphenyl pyruvic acid as an especially important lignin precursor, as has been done by Nord and Schubert,[18] since it is ineffective in many plants.[3,5,19] *p*-Hydroxyphenyl pyruvic acid is probably just an intermediate in tyrosine synthesis and thus convertible to lignin only by plants which can convert tyrosine to lignin. If one is to select an important lignin precursor, *trans-p*-coumaric acid is a much better choice since it is a central intermediate in the formation of lignin and related compounds[3] but is not involved in protein synthesis.[14]

It is difficult to assign a definite physiological role to the deamination of *m*-tyrosine. Neither *m*-tyrosine nor *m*-coumaric acid are of general occurrence in plants. However, it has been recently shown by Winstead and Suhadolnik[20] that *m*-tyrosine may be formed from phenylalanine as a step in the biosynthesis of gliotoxin by *Trichoderma viride*. If this meta-hydroxylation occurred in higher plants, *m*-coumaric acid might be formed and rapidly metabolized to other products. For example, further hydroxylation might give caffeic acid and thus, eventually, lignin.

MATERIALS AND METHODS

Chemicals

L-Tyrosine was obtained from Nutritional Biochemicals Corp. DL-*o*-Tyrosine and DL-*m*-tyrosine were purchased from the H. M. Chemical Co. An additional sample of DL-*m*-tyrosine, synthesized by the procedure of Sealock *et al.*[21] was purified by three recrystallizations from dilute acetic acid–ethanol. These tyrosines all gave a single spot after paper chromatography with *n*-butanol-acetic acid-water (4 : 1 : 1·8) as the irrigant. The sheets were sprayed with ninhydrin and with diazotized *p*-nitroaniline.[22,23] The commercial sample of *m*-tyrosine had an ultraviolet absorption spectrum which agreed closely with that of the synthetic sample.

Some *trans-m*-coumaric acid was prepared from *m*-hydroxybenzaldehyde[24] and purified by two recrystallizations (charcoal) from water; this gave a colorless crystalline solid, m.p. 194–196°. *trans-p*-Coumaric acid (Aldrich Chemical Co.) was purified by recrystallization from water; the final m.p. was 219–220°. A sample of *cis-p*-coumaric acid was prepared by ultraviolet irradiation of a solution of the sodium salt of *trans-p*-coumaric

[17] S. N. ACERBO, W. J. SCHUBERT and F. F. NORD, *J. Am. Chem. Soc.* **80**, 1990 (1958).
[18] F. F. NORD and W. J. SCHUBERT, *Proc. Intern. Congr. Biochem., 4th Congr., Vienna*, **2**, 189 (1958).
[19] K. KRATZL and G. BILLEK, *Monatsh. Chem.* **90**, 536 (1959).
[20] J. A. WINSTEAD and R. J. SUHADOLNIK, *J. Am. Chem. Soc.* **82**, 1644 (1960).
[21] R. R. SEALOCK, M. E. SPEETER and R. S. SCHWEET, *J. Am. Chem. Soc.* **73**, 5386 (1951).
[22] T. SWAIN, *Biochem. J.* **53**, 200 (1953).
[23] R. K. IBRAHIM and G. H. N. TOWERS, *Arch. Biochem. Biophys.* **87**, 125 (1960).
[24] R. ADAMS and T. E. BOCKSTAHLER, *J. Am. Chem. Soc.* **74**, 5346 (1952).

acid[25] as follows. A sample (1·87 mM) of *p*-coumaric acid was dissolved in a slight excess of 0·5 N sodium hydroxide and the volume adjusted to about 10 ml. This solution (pH 9) was put in a 50 ml beaker and irradiated for 18 hr by a 100 W, 366 mμ Hg source shining directly on the surface of the solution, from a distance of about 6 in. When the irradiation was finished an excess of hydrochloric acid was added, the product extracted by shaking with ether, the extract evaporated and the residue recrystallized from toluene. This gave 110 mg (36 per cent) of *cis-p*-coumaric acid. It was a colorless solid, m.p. 128–129°. A portion (42 mg) of this was purified further by chromatography on a 2·2 × 29 cm column of Whatman cellulose powder using 2 per cent acetic acid (Solvent A, Table 1) for elution. *p*-Coumaric acid was detected in the eluate by spotting it on paper and spraying with diazotized *p*-nitroaniline[22,23]. The fractions containing the fast moving band were combined and the ether-soluble acids recovered by extraction, and recrystallized from toluene as described above; 20 mg of purified *cis-p*-coumaric acid were obtained, m.p. 131°. This sample was found to be practically free of the *trans-* isomer by chromatographing with solvent A (Table 1).

L-Tyrosine-C^{14} (uniformly labelled) was purchased from the Volk Radiochemical Co.; *p*-coumaric acid-α-C^{14} was prepared by the malonic acid condensation; *p*-hydroxyphenyl pyruvic acid was prepared by the procedure of Billek and Hermann[26] and reduced to *p*-hydroxyphenyl lactic acid by sodium amalgam.[5] The DL-*p*-hydroxyphenyl lactic acid (m.p. 144–145°) was isolated by ether extraction and recrystallized, first from water, then from toluene–acetic acid.

Other chemicals were of reagent grade, or the best grade commercially available. Deionized distilled water was employed for preparation of solutions used in enzyme investigations.

Culture of Plants

Seeds were soaked overnight in four volumes of tap water enriched by addition of calcium nitrate, magnesium sulfate and potassium dihydrogen phosphate—each at 0·2 mM concentration. Air was bubbled through the mixture. The soaked seeds were washed with tap water by decantation, then drained and spread on cheese cloth for germination. The cheese cloth was laid on a perforated nichrome support inside a plastic dishpan. Tap water, enriched as above, was added until the level was just up to the seeds. The pan was covered with a sheet of glass; this was removed after 2–4 days when germination was general. Etiolated seedlings were obtained in a dark cabinet, non-etiolated seedlings by placing the pans underneath a bank of four 40 W "cool-white" fluorescent light tubes. The lights were about 17 in. from the seeds and 12 hr of illumination were given daily.

In addition to seedlings, plants collected from outdoors were used. A field of barley near the laboratory was particularly useful as a source of tyrase.

Analytical Methods

Ultraviolet absorption measurements were made with a Beckman Model DU spectrophotometer. Fluorescence was measured with an Aminco–Bowman spectrophotofluorometer adapted to use round silica cuvettes (Aloe Scientific Co.). These relatively cheap round cuvettes were as good as the square cuvettes for the fluorometric determination of

[25] W. A. ROTH and R. STOERMER, *Chem. Ber.* **46**, 260 (1913).
[26] G. BILLEK and E. F. HERRMANN, *Monatsh. Chem.* **90**, 89 (1959).

p-coumaric acid. Protein was measured, by absorption at 280 mμ,[27] on extracts that had been purified by dialysis. Ammonia was determined colorimetrically after steam distillation from sodium tetraborate; the ammonia was caught in dilute boric acid (0·1 m moles), the distillate diluted to 9 ml and 1·0 ml of Nessler's reagent[28] added. The optical density at (450 mμ) was then determined using a Coleman junior spectrophotometer.

In studies on stoichiometry (Tables 8 and 9), tyrosine and coumaric acid were separated from each other by ether extraction, then measured by ultraviolet absorption, as follows. The acidified reaction mixture was extracted with three successive half-volume portions of ether, the combined ether extracts were evaporated to dryness by an air stream at room temperature, the residue was dissolved in 0·05 N sodium hydroxide and the optical density determined at 248 mμ, for *m*-coumaric acid, or at 333 mμ, for *p*-coumaric acid (Figs. 1 and 8). The aqueous residue from the ether extraction was neutralized, partially evaporated by an air stream to remove dissolved ether, then adjusted to a known volume with enough sodium hydroxide to give a final concentration of 0·05 N and the optical density determined at 240 mμ for tyrosine or at 238 mμ for *m*-tyrosine. The molar extinction coefficients in 0·05 N sodium hydroxide at these peaks were 22,500 for *p*-coumaric acid, 20,300 for *m*-coumaric acid, 9900 for tyrosine and 6850 for *m*-tyrosine.

Quantitative Assay for Tyrase

When acetone powders were assayed, *p*-coumaric acid was measured fluorometrically. A sample (0·05–0·20 g) of the acetone powder was suspended in 3 ml of a 0·1 per cent solution of L-tyrosine in 0·10 M sodium borate (pH 8·8–9·0) in an 18 × 150 mm culture tube and incubated for 30 to 60 min at 40°. Water (7 ml) was added and the debris removed by filtration with suction through a 7 cm disc of Whatman No. 1 paper. The residue was washed with 2–3 ml of water, the filtrate acidified by 0·2 ml of 5 N hydrochloric acid, then shaken with diethyl ether (10 ml) in a glass stoppered tube with a conical bottom. The aqueous phase was removed with a pipette and discarded. The ether extract was poured into a 20 ml beaker, evaporated to dryness by an air stream at room temperature, the residue dissolved in a suitable volume (5–50 ml) of 0·05 N sodium hydroxide and the *p*-coumarate in this solution estimated fluorometrically as described in the next paragraph. A "zero-time" control was run in parallel; this was exactly as described except the incubation period was omitted. The difference between the fluorescent readings of this control and the sample was proportional to the *p*-coumarate formed during the incubation, and thus to the tyrase present.

p-Coumaric acid is known to fluoresce in dilute aqueous alkali; the maximum instrument setting for activation is 350 mμ and the maximum emission is at 440 mμ.[29] A linear relation between fluorescence and *p*-coumarate concentration was found up to about 2 μg/ml but at higher concentrations there was strong quenching (see Fig. 2). For most fluorescence readings two concentrations are possible. It was necessary to dilute the solutions until they contained less than 2 μg of *p*-coumaric acid per ml. If there was any doubt, readings were made at several concentrations to be sure this condition was met. As the instrument was rather unstable, frequent readings of a standard were made during a set of determinations. A stock solution of *p*-coumaric acid (100 μg/ml) in dilute sodium bicarbonate (1 mM) was stored in a refrigerator. Aliquots of this were diluted 100-fold with 0·05 N

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[28] J. C. BOCK and S. R. BENEDICT, *J. Biol. Chem.* **20**, 47 (1915).
[29] D. E. DUGGAN, R. L. BOWMAN, B. B. BRODIE and S. UDENFRIEND, *Arch. Biochem. Biophys*, **68**, 1 (1957).

sodium hydroxide to give the working standard; this was freshly prepared for each set of analyses.

The *p*-coumaric acid formed by solutions of crude or partially purified tyrase was measured either fluorometrically or by absorption at 333 mμ (Fig. 1). Usually 1·4 ml of reaction mixture contained 1·4 mg of L-tyrosine, 100 μmoles of sodium borate at pH 8·8, and the enzyme being tested. This mixture was incubated in a glass stoppered tube at 40° for 30–60 min, then acidified with 2·3 ml of 0·15 M hydrochloric acid, cooled in an ice bath and shaken manually with 2·0 ml of ice-cold ether for 1 min. The phases were allowed to separate at 0°, 1·0 ml of the ether layer pipetted into an 18 × 150 mm culture tube and the ether evaporated by an air stream at room temperature. The dry residue was dissolved in a suitable volume of 0·05 N sodium hydroxide. *p*-Coumarate was measured in this solution fluorometrically as described in the preceding paragraph or, by its absorption at 333 mμ. A solution of 1·00 μg of *p*-coumaric acid per ml, in 0·05 N sodium hydroxide had an optical density of 0·137 at 333 mμ. A linear relation between optical density and concentration was found up to the highest optical density measured (i.e. 1·50).

The recovery of *p*-coumaric acid in the 1·0 ml of ether layer taken for analysis was 70 ± 3 per cent. The fraction recovered was independent of the concentration of *p*-coumaric acid but depended on the relative volumes of ether and water. An appreciable fraction of the ether dissolves in the aqueous phase, so the ether layer had a volume of only about 1·4 ml. In tyrase assays a correction was applied for the *p*-coumaric acid not recovered by the extraction; zero-time controls were also run.

The fluorometric and absorptiometric methods gave concordant results with the partially purified enzyme preparations listed in Table 6. The fluorometric method was used in early work with crude preparations and found to agree ±10 per cent with a colorimetric method based on diazotized *p*-nitroaniline.[30] The more precise absorptiometric method was used with preparations purified through the second ammonium sulfate precipitation (Table 6).

An assay based on measurement of the rate of ammonia formation could be used with tyrase solutions purified by chromatography on DEAE-cellulose columns although it failed with crude preparations because of the high blanks. The assays based on measurement of *p*-coumaric acid were about 20 times as sensitive.

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THE CAROTENOIDS OF *RHIZOPHLYCTIS ROSEA*

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Abstract—The carotenoids of *Rhizophlyctis rosea* (de Bary and Woronin) Fisher [= *Karlingia rosea* (de Bary and Woronin) Johanson] have been examined, and it has been shown that γ-carotene and lycopene are present in approximately equal quantities. Together they constitute 90 per cent of the total carotenoids. Traces of other carotenoid hydrocarbons are present; it is probable that these are isomers of γ-carotene and lycopene. A xanthophyll is also present in trace amounts; it is possible that this is neurosporoxanthin.

AMONG the Phycomycetes, the organisms whose carotenoids have been studied most extensively are *Mucor hiemalis*[1] and *Phycomyces blakesleeanus*.[2-7] Emerson and Fox have examined the carotenoids of certain species of the aquatic phycomycete, *Allomyces*,[8] and those of *Blastocladiella sp.* have been studied.[9] The pigments of the chytridiaceous *Rhizophlyctis rosea*, the subject of the present study, have only previously been examined in a preliminary manner.[9,10] Haskins and Weston[10] reported the presence of carotenoids without commenting on their nature, and Cantino and Hyatt,[9] state without providing rigorous proof, that the only carotenoid present is γ-carotene.

IDENTIFICATION OF PIGMENTS

Partition of the unsaponifiable lipid of *R. rosea* between 40–60° petroleum ether and 90 per cent aqueous methanol leaves all the pigment in the epiphase. It seems likely, therefore, that if there is any xanthophyll present at all in *R. rosea*, it is present in only trace amounts. The absorption spectrum of the unsaponifiable fraction in light petroleum is very similar to that described by Cantino and Hyatt (see Table 2). When chromatographed on a zinc carbonate–celite column, using increasing concentrations of diethyl ether in petroleum ether (b.p. 40–60°) as the developing solvent, the unsaponifiable residue is resolved into eight distinct coloured zones (Table 1). Spectroscopic examination of the first petroleum ether washings of the column failed to reveal the presence of any of the colourless polyenes, phytoene and phytofluene. The first two fractions were rechromatographed on the stronger adsorbent, alumina, prior to spectrophotometric analysis.

Fraction 1. This pink-yellow zone can be eluted from the zinc carbonate column with petroleum ether. In this solvent it has an absorption spectrum which, with its chromatographic behaviour, suggests that it is γ-carotene (see Table 2). The "Fraction 1" carotenoid

[1] R. CHODAT and W. H. SCHOPFER, *Compt. Rend. Soc. Phys. et Hist. Nat. Genève* **44**, 176 (1927).
[2] W. H. SCHOPFER and A. JUNG, *Compt. Rend. Soc. Biol. Paris* **120**, 1093 (1935).
[3] K. BERNHARD and H. ALBRECHT, *Helv. Chim. Acta.* **31**, 2214 (1948).
[4] G. A. GARTON, T. W. GOODWIN and W. LIJINSKY, *Biochem. J.* **46**, 35 (1950).
[5] G. A. GARTON, T. W. GOODWIN and W. LIJINSKY, *Biochem. J.* **48**, 154 (1951).
[6] T. W. GOODWIN, *Biochem. J.* **49**, 23 (1951).
[7] T. W. GOODWIN, *Biochem. J.* **50**, 550 (1952).
[8] R. EMERSON and D. L. FOX, *Proc. Roy. Soc.*, B **128**, 275 (1940).
[9] E. C. CANTINO and M. T. HYATT, *Am. J. Botany* **40**, 688 (1953).
[10] R. H. HASKINS and W. H. WESTON, *Am. J. Botany* **38**, 739 (1950).

TABLE 1. CHROMATOGRAPHY OF *Rhizophlyctis rosea* UNSAPONIFIABLE RESIDUE ON A ZINC CARBONATE–CELITE, 3 : 1 (W/W) COLUMN

Fraction	Appearance	Eluting solvent (ether in light petroleum)	Probable nature
1	Pink-yellow	100% petroleum ether	γ-carotene
2	Pink	1% ether	Lycopene
3	Pink	2% ether	Probably poly*cis* isomers of lycopene and γ-carotene
4	Orange-yellow	5% ether	
5	Pink	5% ether	
6	Yellow	10% ether	
7	Orange	15% ether	
8	Orange-red	Alkaline CH_3OH	? Neurosporoxanthin

and authentic γ-carotene cannot be separated on a calcium–hydroxide chromatoplate or on kieselguhr-impregnated paper. It can be concluded that the first major chromatographic fraction is γ-carotene.

Fraction 2. This pink zone is more strongly adsorbed on the column than is γ-carotene; it is eluted with 1 per cent ether. It has an absorption spectrum in petroleum ether which suggests that it is lycopene (see Table 2). When this carotenoid is rechromatographed with authentic lycopene on a column, plate or paper, no separation occurs. The second major carotenoid can thus be identified as lycopene.

TABLE 2. ABSORPTION SPECTRA OF CAROTENOID FRACTIONS (IN 40–60° PETROLEUM ETHER)

Carotenoid fraction	Wavelengths of maximal absorption mμ			
Total unsaponifiable fraction	(440·0)	465·0	493·0	mμ
"Fraction 1" carotenoid	437·0	461·5	491·5	mμ
Authentic γ-carotene	437·0	462·0	491·5	mμ
"Fraction 2" carotenoid	443·0	469·5	500·0	mμ
Authentic lycopene	443·5	469·5	501·5	mμ

Fractions 3–7. These five minor zones are eluted from the column with concentrations of ether between 2 and 15 per cent. They were not available in sufficient quantity to allow spectroscopic examination. They are probably isomers of lycopene and γ-carotene, but they could be artifacts.

Fraction 8. This orange-red zone is the most strongly adsorbed zone, and cannot be removed from the basic column with any organic solvent. It can be eluted, however, with alkaline methanol. This suggests that "Fraction 8" is a xanthophyll possibly related to neurosporoxanthin, which has already been identified in certain fungi.[11,12] This pigment was not present in sufficient quantity to allow spectroscopic confirmation of its identity.

QUANTITATIVE DISTRIBUTION

The lipid fraction of *R. rosea* constitutes 19 per cent of the dry weight of cellobiose-grown cultures. This value is in good agreement with those obtained for *Phycomyces blakesleeanus* by other workers.[5] It has been assumed that cellulose-grown cultures have

[11] M. ZALOKAR, *Arch. Biochem. Biophys.* **70**, 568 (1957).
[12] W. RAU and C. ZEHENDER, *Arch. Mikrobiol.* **32**, 423 (1959).

the same lipid content, and calculations of the dry weight of these cultures are based on this value. Of the dry weight of the cultures, 5·4 per cent is unsaponifiable and $1{\cdot}5\times10^{-2}$ per cent is carotenoid. The distribution of the individual carotenoids is shown in Table 3.

TABLE 3. DISTRIBUTION OF CAROTENOIDS IN *Rhizophlyctis rosea*

Carotenoid	Percentage of total carotenoid
γ-Carotene	46
Lycopene	42
Five unidentified isomers	1·5 (each)
? Neurosporoxanthin	1·5

Cantino and Hyatt[9] have reported that the pigment of *R. rosea* is probably almost exclusively γ-carotene, while the above results indicate that lycopene and γ-carotene occur in approximately equal proportions (see Table 3). It is possible that a strain difference could account for this discrepancy, but it is more likely that the use of chromatographic analysis by earlier workers would have revealed the presence of lycopene and the minor carotenoids in their extracts from *R. rosea.*

Lycopene, γ-carotene and neurosporoxanthin have already been shown to be the major carotenoids in *Fusarium aquaeductuum*, and the minor carotenoids in this organism are rhodoxanthin, poly-*cis* lycopene II and other isomers of lycopene.[12] Certain other fungi have lycopene as their major carotenoid. These include *Cantharellus lutescens* and *C. infudibiliformis*[13] and *Anthurus aseroiformis*[14]. Lycopene has recently been shown to be the major pigment in *Cladochytrium replicatum*[15]. In *Phycomyces blakesleeanus* β-carotene is the major polyene, constituting 84 per cent of the total pigment. Lycopene and γ-carotene are present, but they each constitute only 0·6 per cent of the total carotenoids[7]. γ-Carotene, however, is present in high concentrations in Allomyces species,[8] while β-carotene and lycopene in trace amounts are the only other carotenoids present[16]. The present report is thus the first describing the co-existence of lycopene and γ-carotene as major carotenoids in a phycomycete; the acidic carotenoid of *R. rosea* (which may be neurosporoxanthin) also represents the first xanthophyll to be found in a phycomycete.

EXPERIMENTAL

R. rosea was isolated from the soil of the experimental garden of the Department of Agricultural Botany, U.C.W., Aberystwyth. It was cultured either on a liquid medium containing, per litre, cellobiose, 1·0 g; $MgSO_4.7H_2O$, 0·65 g; KH_2PO_4, 1·9 g; $FeSO_4.7H_2O$, 5·0 mg; $ZnSO_4$ 0·5 mg, or on cellulose lens tissue (Green's No. 105) plus a liquid medium containing the same concentrations of inorganic constituents. The cultures were incubated at 25° in the dark for about three weeks, after which time they had become an orange to salmon-pink colour.

The dry weight of the cultures grown on cellulose lens tissue could not be determined by direct weighing, so the value was always calculated from the weight of total lipid. The percentage of lipid in the organism was found by analysis of cellobiose-grown cultures.

[12] H. WILLSTAEDT, *Svensk Kem. Tidskr.* **49**, 318 (1937).
[13] G. TURIAN, *Compt. Rend. Acad. Sci. Paris* **241**, 764 (1955).
[14] M. S. FULLER and J. E. TAVARES, *Biochim. et Biophys. Acta* **44**, 589 (1960).
[15] G. TURIAN and F. T. HAXO, *Botan. Gaz.* **115**, 169 (1954).

The cultures were filtered (if grown on cellulose) or centrifuged (if grown on cellobiose), and the cells were ground up with washed silver sand and acetone. The lipids were transferred from the acetone extract to ether which was dried with anhydrous sodium sulphate and evaporated in a stream of nitrogen to yield the lipid fraction, a soft, brown residue. This was weighed and dissolved in 10 ml ethanol and saponified by boiling with 1 ml 60 per cent aqueous KOH for 5 min. The unsaponifiable residue was extracted with ether, washed free of alkali, dried and isolated as an orange-brown oil.

Column chromatography was carried out on a mixture of zinc carbonate (B.D.H.) and celite (Light's Hyflo Super Cel) 3 : 1 (w/w). The carotenoid mixture (i.e. the unsaponifiable residue) was added to the column in petroleum ether and development of the column was by washing with increasing concentrations of ether. The bands were either eluted or cut out of the column.

Qualitative chromatographic separations were carried out either on chromatoplates or paper. Chromatoplates for general separations were made of silicic acid (Mallinckrodt) and rice starch (B.D.H.) using the method of Demole.[17] The chromatograms were developed with 10 or 20 per cent ethyl acetate in petroleum ether. The less polar carotenes were separated more efficiently by Stahl's method,[18,19] using calcium hydroxide—Kieselgel G (Merck, Darmstadt) chromatoplates, the developing solvent being 2 per cent benzene in 80–100° petroleum ether (O. Isler and A. Winterstein, personal communication to T. W. Goodwin).

Filter paper impregnated with kieselguhr to the extent of 20 per cent (Schleicher and Schüll No. 287) was also used for pigment separations, using the method of Jensen and Jensen.[20] The less strongly adsorbed carotenes were separated with 80–100° petroleum ether, and the more polar pigments with 10 per cent acetone in 80–100° petroleum ether as the developing solvent.

The absorption spectra of the carotenoids in light petroleum were measured with a Unicam S.P. 600 photoelectric spectrophotometer. The $E^{1\%}_{1\,cm}$ of lycopene at 470 mμ was taken as 3100 and that of γ-carotene at 461·5 mμ as 2700.[21] As these two pigments were present in approximately equal quantities, the extinction coefficient of the total carotenoid extract was assumed to be 2900.

Lycopene was isolated from the skin and outer flesh of commercial tomatoes (*Lycopersicon esculentum*). The natural material was extracted with ethanol and saponified. The unsaponifiable fraction was isolated and chromatographed on alumina (Light's Grade H) using increasing concentrations of ether in petroleum ether to develop the chromatogram. Chromatographically pure lycopene was eluted from the column with 30 per cent ether, and was stored in solution in light petroleum under nitrogen at −10°.

The petals of the dark orange variety of the marigold (*Calendula officinalis*) were chosen as the source of authentic γ-carotene, since the carotene fraction of these petals contains 14 per cent γ-carotene.[22] The petals were ground with anhydrous sodium sulphate and ether, and the lipid was transferred from the ether extract to petroleum ether. Saponification prior to chromatography was not necessary.[22] The carotenoids were separated on an

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[18] E. STAHL, *Chemiker Ztg.* **82**, 323 (1958).
[19] E. STAHL, G. SCHRÖTER, G. KRAFT and R. RENZ, *Pharmazie* **11**, 633 (1956).
[20] A. JENSEN and S. L. JENSEN, *Acta. Chem. Scand.* **13**, 1863 (1959).
[21] T. W. GOODWIN, *In Modern Methods of Plant Analysis*. Edited by K. PAECH and M. V. TRACEY. Vol. III, p. 372. Springer, Heidelberg (1955).
[22] T. W. GOODWIN, *Biochem. J.* **58**, 90 (1954).

alumina column using increasing concentrations of ether in petroleum ether as the developing solvent. The γ-carotene was eluted with 15 per cent ether, and was stored in solution in petroleum ether under nitrogen at $-10°$.

Acknowledgements—My thanks are due to Carl Schleicher and Schüll (Einbeck, Germany) for a gift of No. 287 filter paper, to Dr. E. Griffiths and Mr. D. Jones (Department of Agricultural Botany, U.C.W.) for supplying cultures of *R. rosea*, to the Agricultural Research Council for a Research Studentship, and to Professor T. W. Goodwin for his interest and advice during this investigation.

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BIOSYNTHESIS OF TRYPTOPHAN AND GRAMINE IN YOUNG BARLEY SHOOTS

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Abstract—Shikimic acid-G-C^{14} (i.e. generally labelled), anthranilic acid-G-C^{14}, indole-2-C^{14}, serine-3-C^{14} and indolyllactic acid-β-C^{14} were found to be good precursors of bound tryptophan in young barley shoots. It is likely that tryptophan is formed in barley by the same route established for *Escherichia coli*.[1-5] Tryptophan was readily converted to gramine in agreement with the prior observations of Marion *et al.*[10-13] and compounds converted readily to tryptophan were fairly good precursors of gramine. Serine-3-C^{14} gave gramine with about twice as much C^{14} in the methyl groups as in the rest of the molecule, whereas gramine formed from indole-2-C^{14} or anthranilic acid-G-C^{14} was not labelled in the methyl groups. Indolylacetic acid, indolylacetonitrile, indolylacetaldehyde, indolylacrylic acid and tryptamine were not readily converted to either tryptophan or gramine. Gramine was not converted back to tryptophan. None of the compounds tested appeared to be intermediates in the formation of gramine from tryptophan.

INTRODUCTION

THE intermediate steps in the biosynthesis of tryptophan in *Escherichia coli* are known in considerable detail due to studies with biochemical mutants by Davis *et al.*[1,2] and, more recently, by Yanofsky and co-workers.[3-5] A simplified scheme for tryptophan synthesis by *E. coli* is:

$$\text{Carbohydrate} \longrightarrow \text{shikimic acid} \longrightarrow \text{anthranilic acid} \longrightarrow$$
$$\text{indolylglycerol phosphate} \xrightarrow{\text{L-serine}} \text{L-tryptophan} + \text{triose phosphate.}$$

Indole can be substituted for indolylglycerol phosphate in this sequence,[2,4] and indeed this substitution is necessary for *in vivo* experiments since indolylglycerol phosphate does not readily penetrate living cells.[3]

There is some evidence that the enzyme catalyzing the last step, trypophan synthetase, is present in plants. Thus, Kretovich and Polyanovskii[6,7] have shown that unlabelled compounds such as indole, anthranilic acid, serine and indolylpyruvic acid will cause an increase in the level of free tryptophan when they are administered to pea or wheat seedlings. Greenberg and Galston[8] have presented evidence that pea seeding extracts contain trypto-

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[1] B. D. DAVIS, *Advanc. Enzymol.* **16**, 247 (1955).

[2] B. D. DAVIS, *Arch. Biochem. Biophys.* **78**, 497 (1958).

[3] C. YANOFSKY, *Biochem. Biophys. et Acta* **20**, 438 (1956).

[4] C. YANOFSKY and M. RACHMELER, *Biochem. et Biophys. Acta* **28**, 640 (1958).

[5] O. H. SMITH and C. YANOFSKY, *J. Biol. Chem.* **235**, 2051 (1960).

[6] O. L. POLYANOVSKII and V. L. KRETOVICH, *Doklady. Akad. Nauk S.S.S.R.* **12**, 1086 (1957); *Chem. Abstr.* **51**, 12239 (1958).

[7] V. L. KRETOVICH and O. L. POLYANOVSKII, *Biokhimya* **24**, 917 (1959) (in English translation by Consultants Bureau Enterprises, Inc.).

[8] J. R. GREENBERG and A. W. GALSTON, *Plant Physiol.* **34**, 489 (1959).

phan synthetase, and similar results have been obtained by Holmsen and Teas with maize[9]. These observations support the view that tryptophan is formed in plants as shown above, but to obtain more reliable evidence, it is desirable to use C^{14}-labelled precursors, since a net synthesis in such a complex system might be due to an indirect effect. The results of feeding labelled compounds to barley shoots are reported in the present paper.

Barley shoots were chosen as the experimental material because they also form gramine, a simple indole alkaloid that can be readily isolated. Marion *et al.*[10-13] have shown that barley seedlings will convert tryptophan to gramine without rearrangement of the indole ring or loss of the ring-attached carbon (i.e. the β-carbon of tryptophan). Thus any good precursor of tryptophan should also be a fairly good precursor of gramine. In the present investigation this was tested by isolating both gramine and bound tryptophan from each sample of barley shoots after feeding a labelled precursor. This procedure gives a check on the conversion of various labelled compounds to tryptophan.

RESULTS AND DISCUSSION

The results shown in Table 1 support the view that tryptophan synthesis in barley follows the same pathway as in bacteria. This can be seen from the relatively low dilutions of C^{14} during incorporation of shikimic acid, anthranilic acid, indole or serine into tryptophan. The facile conversion of 3-indolyllactic acid to tryptophan is analogous to the conversions of phenyllactic acid to phenylalanine and of *p*-hydroxyphenyllactic acid to tyrosine previously observed in wheat shoots.[14] It seems likely that higher plants can readily oxidize a number of α-hydroxy acids to the corresponding α-keto acids which, in turn, are converted to the α-amino acids by transamination, and in this connection Kretovich *et al.*[7,15] have recently obtained evidence for the formation of aromatic amino acids from corresponding α-keto acids by transamination reactions in plants.

Compounds which were good precursors of tryptophan in the present investigation were also good precursors of gramine, but dilution values for gramine formation were larger, as is evident from Table 1. This suggests that the labelled compounds employed were converted *via* tryptophan. Tryptophan has been shown to be a good precursor of gramine by Marion *et al.*[10-12] and although the intermediate steps between tryptophan and gramine are not yet known, Breccia and Marion[13] have reported that 3-indolylacrylic acid is readily converted to gramine in barley seedlings. However, in the present series of experiments this compound was found to be the poorest gramine precursor of all the compounds tested (Table 1). This discrepancy might be due to the different methods used for administration of the labelled compounds. Breccia and Marion fed the precursor through the roots of whole seedlings over long periods of time, while in the present investigation the precursor was fed directly, and comparatively quickly, into the shoots.

The results in Table 1 do not indicate any of the labelled compounds to be possible intermediates between tryptophan and gramine. Any such intermediate would be expected to form gramine with less dilution of C^{14} than shown for conversion of tryptophan to

[9] T. W. Holmsen and M. J. Teas, *Plant Physiol.* **34**, vi (1959).
[10] K. Bowden and L. Marion, *Can. J. Chem.* **29**, 1037 (1951).
[11] K. Bowden and L. Marion, *Can. J. Chem.* **29**, 1043 (1951).
[12] E. Leete and L. Marion, *Can. J. Chem.* **31**, 1195 (1953).
[13] A. Breccia and L. Marion, *Can. J. Chem.* **37**, 1066 (1959).
[14] O. L. Gamborg and A. C. Neish, *Can. J. Biochem. and Physiol.* **37**, 1277 (1959).
[15] V. L. Kretovich and Z. V. Uspenskaia, *Biokhimya* **23**, 232 (1958) (in English translation by Consultants Bureau, Inc.).

gramine. The only compound to do this was serine-3-C^{14}, in one experiment. The relatively high efficiency of this precursor is due to its ability to contribute C^{14} to all three extranuclear carbons of gramine. It is well known that carbon-3 of serine can enter the "one-carbon" metabolic pool and serve as a source of methyl groups.[16] It also forms the β-carbon atom of tryptophan,[1,2] which is the carbon of gramine that is attached to the indole nucleus.[10,12] Radioactive gramine samples were converted to 3-methoxymethylindole and tetramethylammonium iodide by treatment with methyl iodide in methanolic potassium hydroxide at room temperature.[17] The distribution of C^{14} in gramine formed from serine-3-C^{14} and from

TABLE 1. EFFICIENCY OF C^{14}-LABELLED COMPOUNDS AS PRECURSORS OF TRYPTOPHAN AND GRAMINE

Plant group*	Compound fed	Amount fed†		% of C^{14} found in ‡		Dilution of C^{14} in §	
		μmoles	μc	Tryptophan	Gramine	Tryptophan	Gramine
A	Shikimic acid-G-C^{14}	97	8·87	0·67	0·29	71	440
A	Anthranilic acid-G-C^{14}	99	4·96	1·76	1·70	33	96
A	Indole-2-C^{14}	130	11·1	2·78	0·67	15	169
A	L-Serine-3-C^{14}	123	15·5	0·71	2·81	61	37
B	,, ,,	103	12·7	0·41	0·16	116	490
A	DL-Tryptophan-β-C^{14}	97	9·2	2·89	2·67	18	53
B	,, ,,	98	14·7	8·00	0·52	8·3	192
C	,, ,,	94	16·7	4·80	0·74	10	270
A	Gramine	98	24·9	0·01	76·2	6580	2·8
A	Tryptamine	96	5·91	0·05	0·50	1140	310
A	3-Indolylacetonitrile	78	11·5	0·05	0·43	1540	400
A	DL-3-Indolyllactic acid	92	10·9	4·58	1·21	12	135
B	,, ,,	56	6·64	9·70	0·44	12	410
A	3-Indolylacrylic acid	84	10·5	0·06	0·02	1040	8230
B	,, ,,	90	11·2	0·05	0·01	1540	9800
A	3-Indolylactetaldehyde	85	10·8	0·02	0·05	4300	3900
A	3-Indolylacetic acid	94	10·3	0·04	0·48	1180	345
B	L-Phenylalanine-β-C^{14}	106	6·52	0·02	nil	2020	—
C	,, ,,	100	6·78	0·02	nil	2000	—
C	DL-Phenylalanine-β-C^{14}	102	7·00	0·04	nil	1160	—

* Plants of group A were grown in December, group B in April and group C in July.
† Per 50 g fresh wt. Tryptophan recovered was 40–65 μmoles; gramine recovered was 80–105 μmoles for group B plants and 130–160 μmoles for groups A and C.
‡ % of C^{14} fed found in tryptophan or gramine.
§ Specific activity of compound fed (μc per μmole) divided by specific activity of compound isolated.

other precursors is shown in Table 2. Serine was the only precursor that gave appreciable labelling of the N-methyl groups of gramine. The labelling pattern of the other gramine samples is as expected if they originated via tryptophan. The overall recovery of C^{14} in the serine experiment (Table 2) was about 20 per cent low. This is probably due to poor recovery of C^{14} in the tetramethyl ammonium iodide, since the method gave good recoveries with gramine samples labelled only in the carbon adjacent to the ring (i.e. in the methoxymethylindole).

In conclusion it may be said that aromatic amino acids in plants probably originate by the shikimic acid pathway, as in bacteria.[1,2] The evidence rests mainly on tracer investigations. Previous investigations in this laboratory have shown that it is likely that phenylalanine and tyrosine are formed by the shikimic acid pathway in plants.[18] The present

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results show this is probably true also for tryptophan. However, a considerable amount of work with isolated enzyme systems is now required before more definite conclusions can be drawn.

TABLE 2. DISTRIBUTION OF C^{14} IN GRAMINE ISOLATED FROM BARLEY FED WITH VARIOUS PRECURSORS

Labelled precursor	% of gramine-C^{14} found in 3-methoxymethyl indole	% of gramine-C^{14} found in tetramethylammonium iodide
Anthranilic acid-G-C^{14}	96	—
Indole-2-C^{14}	95	—
L-Serine-3-C^{14}	28	49
DL-Tryptophan-β-C^{14}	101	0·3
Gramine-C^{14}	95	—
3-Indolylacetic acid	95	—
DL-3-Indolyllactic acid	103	—

EXPERIMENTAL

Preparation of C^{14}-labelled Compounds

Sodium formate-C^{14}, formaldehyde-C^{14}, L-serine-3-C^{14}, DL-tryptophan-β-C^{14} and generally labelled samples of L-phenylalanine and toluene were purchased from Atomic Energy of Canada Ltd. Shikimic acid (generally labelled),[19] DL-phenylalanine-β-C^{14} and L-phenylalanine-β-C^{14} were prepared as described previously.[20]

Indole-2-C^{14} was synthesized from sodium formate-C^{14} as described by Leete and Marion.[12] Gramine, labelled in the carbon attached to the ring, was synthesized from formaldehyde-C^{14} by the procedure of Kühn and Stein.[21] Portions of this gramine were used for the preparation of DL-3-indolyllactic acid by the procedure of Gortatowski and Armstrong,[22] for the preparation of 3-indolealdehyde by Thesing's procedure,[23] and for the preparation of 3-indolylacetonitrile by the procedure of Thesing and Schulde.[24] The indolylacetonitrile was used for preparation of tryptamine by lithium aluminum hydride reduction[25] and for preparation of 3-indolylacetic acid by alkaline hydrolysis followed by acid hydrolysis.[26] Hypochlorite oxidation of DL-tryptophan-β-C^{14} gave labelled 3-indolylacetaldehyde.[27] These procedures all give the 3-indolyl derivatives labelled in the carbon attached to the ring. The procedures were scaled down to 0·5 to 1·0 m mole. The yields were approximately as reported for the larger scale described in the literature. The purity was tested by chromatography on paper with an appropriate solvent. Radioautographs of these chromatograms showed one radioactive spot with the correct R_f in each case.

A sample of 3-indolylacrylic acid was prepared from 3-indolealdehyde (100 mg) by the procedure of Bauguess and Berg.[28] The crude acid (96 mg, m.p. 172–175°) was purified by

[19] D. R. MCCALLA and A. C. NEISH, *Can. J. Biochem. and Physiol.* **37**, 531 (1959).
[20] D. WRIGHT, S. A. BROWN and A. C. NEISH, *Can. J. Biochem. and Physiol.* **36**, 1037 (1958).
[21] H. KÜHN and O. STEIN, *Chem. Ber.* **70**, 567 (1937).
[22] M. J. GORTATOWSKI and M. D. ARMSTRONG, *J. Org. Chem.* **22**, 1217 (1957).
[23] J. THESING, *Chem. Ber.* **87**, 507 (1954).
[24] J. THESING and F. SCHULDE, *Chem. Ber.* **85**, 325 (1952).
[25] S. UDENFRIEND, C. R. CREVELING, H. POSNER, B. G. REDFIELD, J. DALY and B. WITKOP, *Arch. Biochem. Biophys.* **83**, 501 (1959).
[26] H. R. SNYDER and F. J. PILGRIM, *J. Am. Chem. Soc.* **70**, 3770 (1948).
[27] R. A. GRAY, *Arch. Biochem. Biophys.* **81**, 480 (1959).
[28] L. C. BAUGUESS and C. P. BERG, *J. Biol. Chem.* **104**, 675 (1934).

chromatography on a 2·5×21 cm column of cellulose powder using isopropanol: conc. ammonium hydroxide: water (20 : 1 : 3) as the eluting agent. Fractions containing the indolylacrylic acid were pooled, evaporated under reduced pressure, the solid residue dissolved in a small volume of 0·2 N sodium hydroxide and the purified product precipitated by hydrochloric acid. This gave 71 mg (55 per cent); m.p. 175–178°. (Found: C,70·47; H,4·89. $C_{11}H_9NO_2$ requires: C,70·58; H,4·85 per cent). The melting point is about 20° lower than reported previously[11,28] but it agrees with that of a sample of 3-indolylacrylic acid purchased from the Sigma Chemical Company. This melting point could not be raised by recrystallization. A sample (m.p. 175–178°) was reduced in ethanol by hydrogen in a simple glass apparatus[29] using palladium (5 per cent)-charcoal as catalyst. The hydrogen uptake was 97 per cent of theory and 3-indolylpropionic acid, m.p. 133–134° was isolated in good yield. This agrees with the m.p. of 3-indolylpropionic acid obtained by Bauguess and Berg.[28] Paper chromatography of the C^{14}-labelled indolylacrylic acid, followed by radioautography, showed only one radioactive spot with the same mobility as unlabelled indolylacrylic acid.

Anthranilic acid-G-C^{14} was made from toluene-G-C^{14} by a procedure involving mononitration of the toluene,[30] oxidation of the mixed *ortho* and *para* nitrotoluenes to the corresponding benzoic acids,[31] reduction of the mixed nitrobenzoic acids to the aminobenzoic acids and chromatographic separation of the aminobenzoic acids. The following procedure gave the best yields of several modifications tried.

Generally labelled toluene (167 mg, 300 μc) in a 1·8×11 cm glass-stoppered tube, was treated with a well-cooled mixture of sulphuric acid (259 mg) and nitric acid (s.g. 1·42, 173 mg). The tube was stoppered and the reaction mixture stirred with a Teflon-covered magnetic stirrer bar, for 18 hr at room temperature. The reaction mixture was diluted with 20 ml of water and extracted with 3×20 ml portions of ethyl ether. The combined ether extract was washed with 25 ml of 0·1 M sodium carbonate, then with water, dried over sodium sulfate, filtered and the ether evaporated, to yield a mixture of *ortho* and *para* nitrotoluene (224 mg). To this mixture in a 50 ml round-bottomed flask, fitted with a reflux condenser, was added 1·6 ml of 5 N sodium hydroxide, 20 ml of water and 210 mg of potassium permanganate. The mixture was refluxed until the pink color disappeared (20 min); three further additions of potassium permanganate (210 mg) were made. After the last addition of potassium permanganate, the color remained after 30 min of refluxing so the oxidation was considered to be finished. The reflux condenser was changed to distillation and 10 ml of distillate, containing the unoxidized nitrotoluene, was collected. The hot residue in the round-bottom flask was filtered and the cake was washed with 2×15 ml portions of boiling water. The filtrate was cooled, acidified with concentrated hydrochloric acid and extracted with 3×30 ml portions of ethyl ether. The combined ether extract was washed with water, dried over sodium sulfate, filtered and the ether evaporated. The remaining residue (*ortho* and *para* nitrobenzoic acids) weighed 160 mg and had a m.p. 132–155°.

The mixture of *ortho* and *para* nitrobenzoic acids was dissolved in 25 ml of ethanol and placed in a low pressure hydrogenation apparatus,[29] 15 mg of Adams platinum oxide catalyst was added, and the mixture was hydrogenated at 15 lb pressure. The reaction stopped in 6 min. The catalyst was removed by filtration and the filtrate evaporated to dryness. The resulting residue, a mixture of *p*-aminobenzoic acid and *o*-aminobenzoic

[29] A. C. NEISH, *Can. J. Biochem. and Physiol.* **37**, 1431 (1959).
[30] W. J. HICKINBOTTOM, *Reactions of Organic Compounds*, p. 37. Longmans Green, London (1936).
[31] L. BIGELOW, *J. Am. Chem. Soc.* **41**, 1599 (1919).

acid (anthranilic acid), weighed 129 mg. The aminobenzoic acid isomers were separated on 2·5×25 cm cellulose column by elution with *n*-butanol saturated with 1·5 N ammonium hydroxide. The anthranilic acid came off the column first and was widely separated from the *p*-aminobenzoic acid. The fractions containing the anthranilic acid were combined, the solvent was removed by evaporation, and the residue sublimed at 95° and 2 mm pressure. The yield of anthranilic acid was 51 mg (20 per cent) m.p. 142–143°. The *p*-aminobenzoic acid was also recovered and crystallized from water. Paper chromatography showed the anthranilic acid to be pure.

Cultivation of Plants

Young plants of barley (*Hordeum vulgare*, L. var. Atlas 1-35-3) were grown in a greenhouse with controlled temperature. The subirrigation gravel culture method was used with a modified Hoagland solution.[32] Additional illumination was supplied by an overhead bank of "cool white" fluorescent tubes to give a daylength of 18 hr and a minimum light intensity of 7000 lux. The temperature was 18–20° during the day and 14–16° at night. The plants were used when 10 days old.

Administration of C^{14}-labelled Compounds

The labelled compound was dissolved in about 20–30 ml of water, the pH adjusted to 6·5–7·0 and the solution divided equally between two 250 ml beakers. The shoots of the seedlings were separated from the roots by cutting under water with a razor blade. These shoots (50 g fresh weight) were distributed equally between the two beakers with the cut ends submerged in the solution of labelled compound. The beakers were put in a growth chamber illuminated by light (12,000 lux) from "cool white" fluorescent tubes. The solutions were nearly all absorbed in 6 hr. Distilled water (10 ml) was then added to each beaker and after it was nearly all absorbed another 10 ml was added. When this "washing-in" procedure was completed an excess of distilled water was added and the shoots allowed to metabolize. The total time allowed, including that for absorption, was 72 hr. During this period a daylength of 18 hr was used and the temperature kept at 23° in the day and 18° at night.

Extraction of Plant Material and Recovery of Gramine

At the end of the metabolic period (72 hr) the plant material (50 g) was extracted with cold methanol (600 ml) in a Waring blender. The homogenate was allowed to stand 24 hr at −5° and was then filtered on a Buchner funnel. The fibrous residue was washed with cold methanol, followed by absolute ethanol, air-dried and saved for recovery of bound tryptophan.

The filtrate was evaporated at 40° and 15 mm pressure, to a volume of about 200 ml, extracted three times with 200 ml portions of petroleum ether (30–60°) and the petrol extracts discarded. The remaining aqueous solution was evaporated to dryness under an air stream at room temperature. The dry residue thus obtained was taken up in 40 ml of N sulphuric acid and extracted four times with 60 ml portions of diethyl ether and the ether extracts discarded. The remaining aqueous solution was cooled in ice and 10 ml of 10 M sodium hydroxide was added. The resulting alkaline mixture was re-extracted

[32] L. JACOBSON, *Plant Physiol.* **26**, 411 (1951).

with four 100 ml portions of diethyl ether. These ether extracts contained the gramine. They were pooled, washed with water, dried over anhydrous sodium sulphate, filtered and the ether evaporated. The residue was rinsed into a sublimation apparatus and the gramine sublimed at 90° and 1·0 mm pressure. The product had a m.p. 122–128°. After recrystallization from benzene the m.p. was 133° and was not depressed when the compound was mixed with an authentic sample of gramine.

Hydrolysis of Protein and Recovery of Tryptophan

The fibrous residue from the above extraction procedure (about 2 g) was mixed with 5 N sodium hydroxide (30 ml) in a 25×100 mm test tube. The tube was sealed and the contents digested for 24 hr at 100°. After cooling to room temperature the tube was opened and the contents filtered through Analytical Celite filter aid, the cake being washed four times with 30 ml of cold water. The filtrate was evaporated under an air stream to about 30 ml. The pH was adjusted to 6·0 with 10 N sulphuric acid. On addition of 50 ml of ethanol a precipitate formed which was removed by filtration after standing 2 hr at room temperature. The filter cake was washed with 80 per cent ethanol, then with absolute ethanol and discarded.

The filtrate was evaporated to dryness, taken up in 20 ml of water and passed through a 1·2×16 cm column of Amberlite 1R-120-H ion exchange resin. The column was washed with water and the washings discarded. The amino acids were eluted from the column with 100 ml of 1·5 M ammonium hydroxide and the eluate evaporated to dryness. The residue was taken up in 0·5 N acetic acid (3 ml) and passed through a 2×60 cm column of Dowex-1-acetate using 0·5 N acetic acid as the eluting agent.[33] The tryptophan fractions were combined and checked for purity by paper chromatography with two solvents: *n*-butanol : acetic acid : water (4 : 1 : 1·8) and isopropanol: conc. ammonium hydroxide: water (20 : 1 : 3). Only one ninhydrin-positive spot could be detected, and it had the same mobility as tryptophan. The tryptophan was measured quantitatively by the method of Moore and Stein.[34] The remainder of the sample (10 mg) was evaporated to dryness and dissolved in 2 ml of hot water, half the water was evaporated under an air stream at room temperature and 0·2 ml of ethanol was added. After standing 18–20 hr at 3° the crystals were filtered off, washed with 10 per cent ethanol, with absolute ethanol and finally with ether, and then dried to constant weight (6 mg) in a vacuum desiccator. Three parts of carrier (DL-tryptophan) was added and the whole dissolved in hot water, evaporated by an air stream at room temperature and dried to constant weight in a desiccator.

Degradation of Gramine

Radioactive gramine samples were converted to 3-methoxymethylindole and tetramethylammonium iodide by treatment with methyl iodide in methanolic potassium hydroxide at room temperature.[17] Both products were recovered and analysed for C^{14}. This procedure was tested on gramine synthesized as described above. The methoxymethylindole contained 99·5 per cent of the C^{14}, whereas the tetramethylammonium iodide was inactive. This procedure seems to be reliable for measuring the C^{14} in the indole ring and the attached carbon, but it was not tested on synthetic gramine labelled in the N-methyl groups, since such a sample was not available.

[33] C. W. H. HIRS, S. MOORE and W. H. STEIN, *J. Am. Chem. Soc.* **76**, 6063 (1954).
[34] S. MOORE and W. H. STEIN, *J. Biol. Chem.* **211**, 907 (1954).

Measurement of C^{14}

The samples were converted to carbon dioxide by combustion with the Van Slyke reagent[35] and the activity of the resulting carbon dioxide was measured by a dynamic condenser electrometer (a Nuclear-Chicago Dynacon).

Acknowledgement—The authors are grateful to Mr. J. Dyck for numerous C^{14} analyses.

[35] D. D. VAN SLYKE, J. FOLCH and J. PLAZIN, *J. Biol. Chem.* **136**, 509 (1940).

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THE ALKALOIDS OF HEMLOCK (*CONIUM MACULATUM* L.).—II

EVIDENCE FOR A RAPID TURNOVER OF THE MAJOR ALKALOIDS

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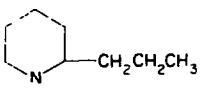
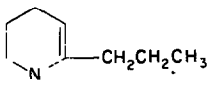
Abstract—Previous work, based on analyses of samples of developing fruits collected at weekly intervals, has been confirmed. Samples were also collected at 4-hourly and 2-hourly intervals and analyses showed that remarkably rapid changes in the alkaloidal picture took place at short intervals during 24 hr. Furthermore, as the coniine (I) content increased, the γ-coniceine (II) content decreased and vice versa. This interrelationship was particularly marked during the critical stages of pericarp development, but was not obvious during the development of the vegetative parts, where γ-coniceine and conhydrine (III) were the only known alkaloids detected. Since coniine and γ-coniceine differ only by two hydrogen atoms, it is suggested that these two alkaloids are involved in oxidation–reduction processes in the developing pericarp. The occurrence of the minor alkaloids conhydrine and N-methyl coniine (IV) and the presence of some unknown alkaloids are also reported and commented on.

INTRODUCTION

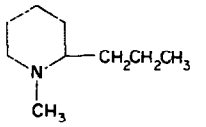
IN A previous communication[1] it was shown that the total alkaloidal content and the



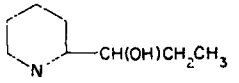
I



II



IV



III

proportions of individual alkaloids in hemlock fruit varied with the development of the fruit and with the season. It was suggested that γ-coniceine (II) was the precursor of the saturated alkaloids, such as coniine (I), and that this change, which appeared to be reversible, is associated with active growth. These conclusions were based mainly on the examination of samples collected at weekly intervals; we have repeated the work over a further two seasons and have also examined samples collected at daily, 4-hourly and 2-hourly intervals. Much of the earlier work has been confirmed but interesting new facts, based on the samples taken at short intervals, have come to light. Sampling and analytical methods are referred to at the end of this paper.

[1] J. W. FAIRBAIRN and S. B. CHALLEN, *Biochem. J.*, 72, 556 (1959).

RESULTS

Weekly samples. Samples were collected at noon on the same day each week and Fig. 1 shows the analytical results for the two major alkaloids in 1958 (a wet season) and 1959 (a dry season). In both seasons a peak of γ-coniceine content preceded a peak of coniine content, and during the first few weeks the increase in coniine content kept pace with the increase in dry weight. The year 1959 was particularly dry; the fruits matured prematurely and dehisced at week 5, in contrast to the three previous seasons when they remained on the plants until week 8.

Daily samples. Samples were collected at noon on each of the 14 days between week 2 and week 4 in 1958. Comparative paper chromatograms of the samples showed no marked changes in the alkaloidal picture, but rather a gradual increase consistent with that shown by the weekly samples in Fig. 1. Quantitative analyses of these samples were not therefore carried out at this stage.

Four-hourly samples. In 1958 samples were collected at 4-hourly intervals between 8 a.m. and 8 p.m., a few days after the week 3 samples had been collected. Chromatograms,

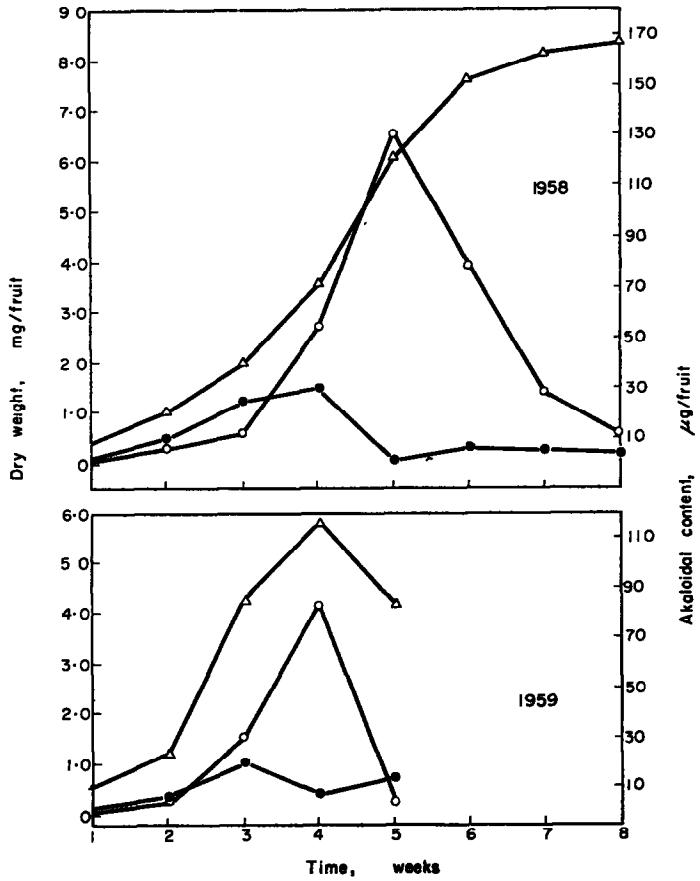


FIG. 1. ALKALOIDAL CONTENT AND DRY WEIGHT OF WEEKLY SAMPLES IN 1958 AND 1959.

Dry weight —Δ—Δ—
Coniine —O—O—
γ-Coniceine —●—●—

prepared from equal numbers of fruits, indicated that considerable variations in the alkaloidal content occurred at short intervals. Consequently 4-hourly samples were collected over a period of 24 hr a few days after the week 4 samples had been collected and a few days after the week 5 samples had been collected. Comparative paper chromatograms (an example of which is given in Fig. 4) again indicated marked changes in the amounts of alkaloid in each sample. Spectrophotometric analyses of all the samples were therefore carried out and the results are shown in Table 1. It will be noted that the amounts of coniine (I) and γ-coniceine (II) vary considerably during the day and that increase in one corresponds to decrease in the other.

TABLE 1. ANALYSES OF FOUR-HOURLY SAMPLES TAKEN DURING A PERIOD OF 24 HR IN WEEK 4 AND IN WEEK 5, 1958

Time	Week 4 (μg/fruit)		Week 5 (μg/fruit)	
	Coniine	γ-Coniceine	Coniine	γ-Coniceine
4 a.m.	90	1	226	0
8 a.m.	2	6	130	2
12, midday	4	15	174	9
4 p.m.	120	1	8	21
8 p.m.	38	10	200	0
12, midnight	132	0	213	0

Two-hourly samples. In 1959 2-hourly samples over a period of 24 hr were collected on the same day as the week 3 and week 4 samples were collected and the results of spectrophotometric analyses are shown graphically in Figs. 2 and 3. In these figures the variations

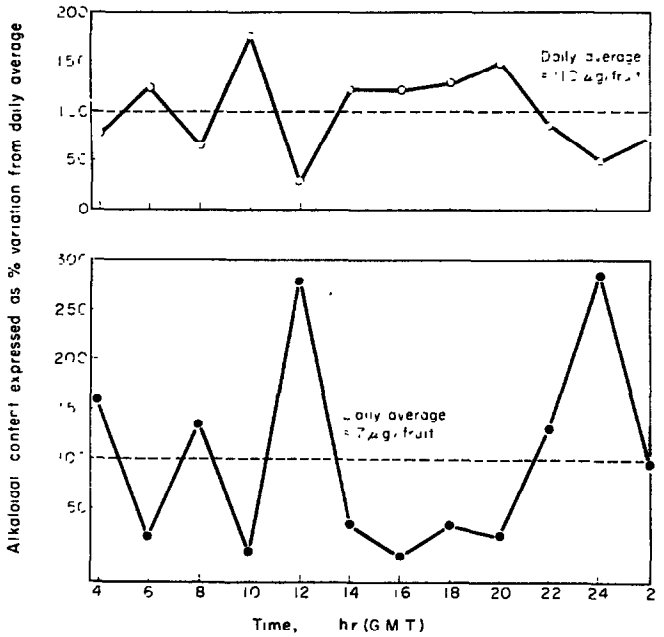


FIG. 2. ALKALOIDAL CHANGES DURING 24 HR; WEEK 3, 1959.
Coniine —O—O—. Average content for 24 hr period = 110 μg/fruit.
γ-Coniceine —●—●—. Average content for 24 hr period = 7 μg/fruit.

in the two major alkaloids are emphasized by representing the variations as percentages of the average content for that particular day, based on analyses of all the samples. Placing both curves on the same diagram makes it clear how they are interrelated, although the average absolute amount of coniine present was generally about 10–20 times that of γ-coniceine.* Four-hourly and 2-hourly samples over a period of 24 hr were also attempted

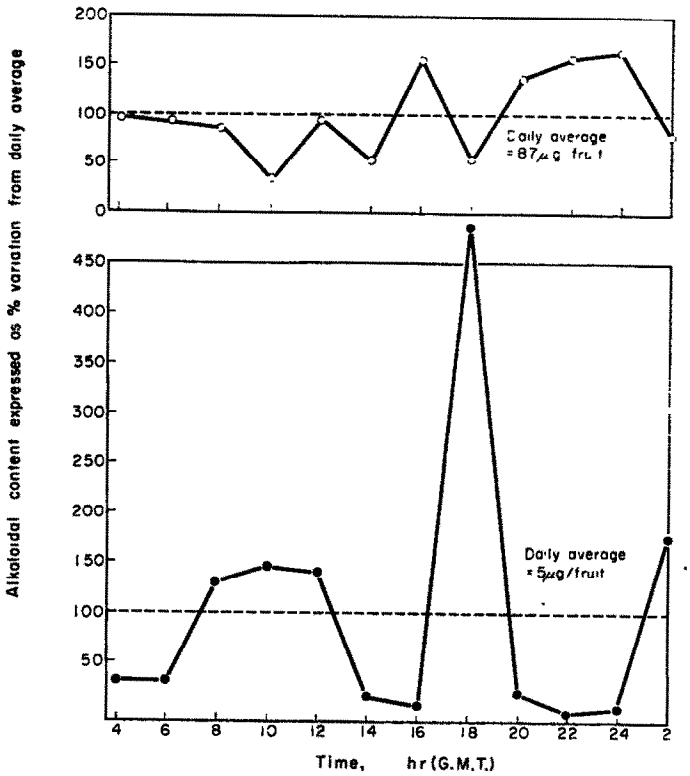


FIG. 3. ALKALOIDAL CHANGES DURING 24 HR IN WEEK 4, 1959.
Coniine —O—O—. Average content for 24 hr period = 87 μg/fruit.
γ-Coniceine —●—●—. Average content for 24 hr period = 5 μg/fruit.

in weeks 1, 2 and 5. In week 1, (flowering stage), the amounts of alkaloid present were too small for quantitative analyses; inspection of chromatograms however showed that γ-coniceine was the predominant alkaloid and indicated close interrelationship between the changes in γ-coniceine and coniine contents. In week 2, more alkaloids were present and quantitative examination was possible for three-quarters of the samples; the results once more indicated close interrelationship between the changes in γ-coniceine and coniine content; the average amount of coniine (23 μg/fruit) however exceeded the average amount of γ-coniceine (4·5 μg/fruit). In neither weeks were the changes so marked as in weeks 3 and 4. In week 5 many of the fruits had dehisced, but seven 2-hourly samples (from

* The values for alkaloidal content given in Fig. 1 are based on midday samples only and do not necessarily correspond to those based on daily averages given in Figs. 2 and 3. Results so far, however, confirm that the trend shown in Fig. 1, and in previous years, would also be shown if figures based on daily averages were used, except that in 1959 maximum coniine content occurs in week 3. This is not surprising in view of the short time required for fruit development in 1959.

8 a.m.–8 p.m.) were collected. The results of quantitative analyses are shown in Table 2 and indicate that little change took place in the alkaloidal picture except for the sample collected at midday.

TABLE 2. ANALYSES OF TWO-HOURLY SAMPLES TAKEN DURING A PERIOD OF 14 HR IN WEEK 5, 1959

Time	Coniine (μg/fruit)	γ-Coniceine (μg/fruit)
8 a.m.	52	traces
10 a.m.	42	traces
12 midday	0	14
2 p.m.	43	traces
4 p.m.	55	0
6 p.m.	60	0
8 p.m.	49	0

Alkaloids other than Coniine and γ-Coniceine

Fig. 4 represents comparative chromatograms prepared from the 2-hourly samples collected in week 3, 1959. This chromatogram shows the same changes presented graphically in Fig. 2. Additional information on some of the minor alkaloids, however, is also given, and has been confirmed by examining similar chromatograms prepared from samples taken on other occasions. When γ-coniceine content is at a maximum small quantities of conhydrine (III) occur; when coniine is at a maximum conhydrine is absent. N-methyl coniine (IV) does occur occasionally in this "Chelsea variety" of hemlock, but only in small quantities and mainly when coniine is at a maximum.

Unknown Alkaloids

An alkaloid of high R_f value (0·73) occurred in four of the samples shown in Fig. 4, but it was not identified nor was it discovered in any other samples examined. In contrast, an alkaloidal spot of low R_f value (0·08) occurred in all samples of fruit, leaf and root examined. Preliminary examination showed that the spot contained several amino-acids and two alkaloid-like substances. These last two gave a red colour with Dragendorff's reagent, a brown colour with iodine in light petroleum, but no red colour with alkaline sodium nitroprusside (distinction from piperideines). They differed from most alkaloids in that they cannot be extracted from alkaline solution with chloroform, and from the normal hemlock alkaloids in that they are sparingly volatile in steam.

Four-hourly Samples of Seedlings

Young seedlings were grown in the open in 1959 and two weeks after the first foliage leaves opened, when they were about 7 cm high, samples of five seedlings were collected at four-hourly intervals during a period of 24 hr, washed rapidly and extracts prepared as for the fruits. Paper chromatographic examination of the extracts showed the presence of γ-coniceine and traces of conhydrine in all samples, but there was no indication of any change in the quantities. Coniine and N-methyl coniine were not detected. Similar experiments were performed on samples from the same crop of seedlings a week later, when they were about 12 cm high. Separate analyses were made on the roots and the aerial

parts. No alkaloids were found in the roots; in the aerial parts γ-coniceine alone was detected and the quantities did not appear to vary markedly during the 24-hr period. As already stated the compound of low R_f value always occurred in all parts of the plant.

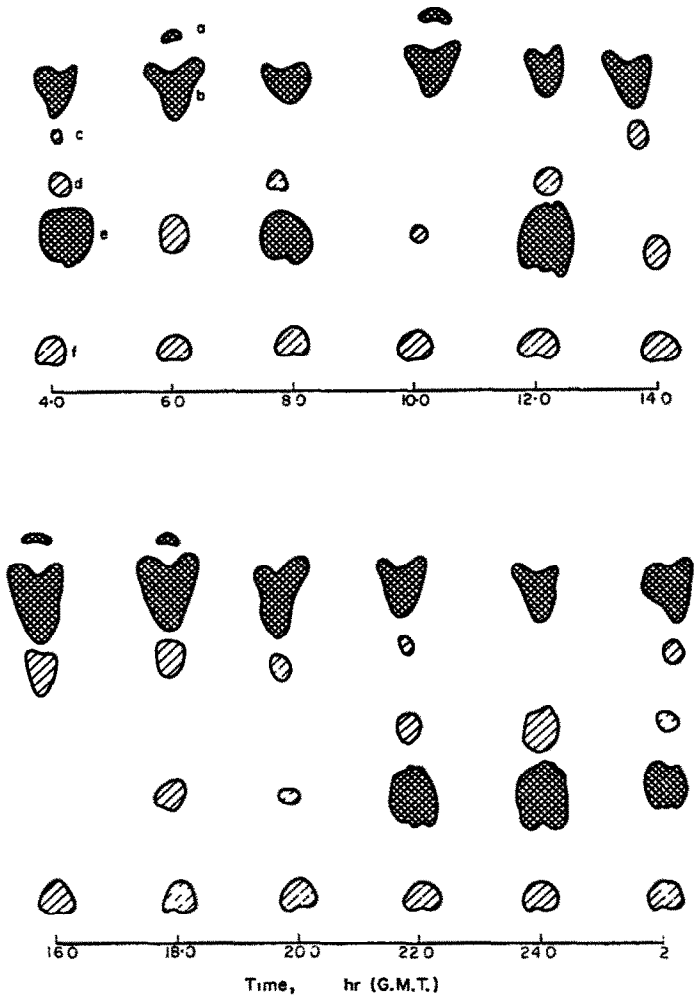


FIG. 4. PAPER CHROMATOGRAMS OF 2-HOURLY SAMPLES EACH REPRESENTING THE SAME NUMBER OF FRUITS; WEEK 3, 1959.

(*a*) Unknown alkaloid of R_f = 0·73. (*b*) Coniine. (*c*) N-methyl coniine. (*d*) Conhydrine. (*e*) γ-Coniceine. (*f*) Unknown alkaloid R_f = 0·08. Depth of shading and area of the spots roughly proportional to amount of alkaloid present.

Second Year Plants

A well-developed plant was removed in March 1958 before signs of spring growth were showing; analysis showed that γ-coniceine alone occurred, both in the aerial parts and in the roots. A similar experiment was carried out in June 1958 when growth was vigorous; γ-coniceine was the only alkaloid detectable in the aerial parts, but no alkaloids were detected in the roots, apart from the low R_f value substance.

DISCUSSION

The results based on weekly and daily samples collected during 1958 and 1959 have confirmed some of the conclusions reached by earlier workers.[1,2] Thus, γ-coniceine is the predominant alkaloid in the vegetative parts and in the very early stages of development from flower to fruit. The overall change from γ-coniceine to coniine is associated with rapid development of the fruits. At the later stages of fruit maturation the coniine content falls and there is a slight increase in the γ-coniceine content, indicating a possible re-conversion of coniine into γ-coniceine (see Fig. 1).

The results of all the work on hemlock alkaloids up to this point would be consistent with a slow accumulation of alkaloids as minor end products of metabolism together with gradual changes in their composition. Our work on 4-hourly and 2-hourly samples has however shown that remarkably rapid changes in the composition and amounts of alkaloid occurred over short periods of time. As far as we know such dynamic changes in the alkaloidal picture have not been demonstrated so clearly before. James[3,4] points out that only in certain seedlings and senescent leaves is there evidence of the return of alkaloidal materials to metabolic circulation and the quantities involved are always extremely small. In a discussion on the physiology of alkaloids, Mothes[5] states: "If, therefore, the alkaloid content, during the course of a year, fluctuates with the changing growth pattern, we may raise the question whether this phenomenon obtains during the course of a day." He then refers to the difficulties involved in answering this question. Hemberg and Flück[6] determined the total alkaloids in samples of *Datura* leaf and root collected at 4-hourly intervals during 24 hr. There was a gradual increase in the amount of total alkaloids during the day and a fall at night, but no information is given on the relative proportions of individual alkaloids. Somewhat similar results were obtained with *Datura* and *Atropa* by Kozlova[7] although the quantities of alkaloids were determined by histochemical means. Miram and Pfeifer[8] determined individual alkaloids in poppy plants at 6-hr intervals, but since the results were expressed as mg per cent dry weight it is not clear whether the absolute amounts of alkaloid present varied. Our results indicate that the change from γ-coniceine to coniine is geared to some active metabolic process; and since only the addition of two hydrogen atoms to γ-coniceine is involved it is possible that the latter acts as a hydrogen acceptor in some oxidation–reduction process. These changes however appear to be restricted to the developing fruit only and are most marked during the weeks when pericarp development is at a maximum.

Quantitative Aspects

Apart from the rapid changes in the two major alkaloids, the following related facts should be noted.

Careful examination of the results given in Figs. 2 and 3 shows that the interchange between γ-coniceine and coniine is not a simple reversible change *quantitatively*. For example, from Fig. 2 it will be seen that between 10 a.m. and 12 noon the coniine content

[1] B. T. CROMWELL, *Biochem. J.*, **64**, 259 (1956).
[2] W. O. JAMES, *The Alkaloids*. Edited by R. H. F. MANSKE and H. L. HOLMES. Vol. I, p. 82. Academic Press, New York (1950).
[3] W. O. JAMES, *Endeavour*, **12**, 76 (1953).
[4] K. MOTHES, *Ann. Rev. Plant. Physiol.*, **6**, 412 (1955).
[5] T. HEMBERG and H. FLÜCK, *Pharm. Acta Helv.* **28**, 74 (1953).
[6] N. A. KOZLOVA, *Izvest. Akad. Nauk. Latv. SSR.*, **6**, (70), 65 (1953).
[7] R. MIRAM and S. PFEIFER, *Sci. Pharm.* **27**, 34 (1959).

fell from 175 per cent to 28 per cent of the daily average (110 μg/fruit); this represents a loss of 162 μg/fruit. During the same period the γ-coniceine content increased from almost zero to 280 per cent of the daily average (7 μg/fruit); this represents an increase of only 19 μg/fruit. This means that during a period of 2 hr 143 μg of coniine have disappeared and have not been accounted for by the formation of γ-coniceine or any other alkaloid. This loss corresponds to about 21 mg/umbel. As the samples were collected on a dry day this relatively large loss cannot be accounted for by rain washing. Neither is it likely to be due entirely to volatilization as there must have been at least 1000 active umbels in the row of plants being visited every 2 hr and such a large loss by volatilization (over 20 g alkaloid) would produce a very marked odour. No very strong odour was noticed on any occasion. Furthermore, it is well known that much of the coniine is stored in the inner layers of the pericarp, in the endocarp or "coniine layer". The question as to what happens to the excess coniine naturally arises; the use of isotopically labelled alkaloids will obviously greatly assist in solving this problem.

Relation to the Development of the Pericarp

During the first few weeks of fruit development there is a rapid increase in the average alkaloidal content. During this period the pericarp, to which the alkaloids are restricted, develops rapidly and marked anatomical changes take place.[1] The increasing "demands" of the developing pericarp for alkaloids may therefore be closely related to the increasing number of living cells. This would be consistent with the view already expressed, that the alkaloids play a significant part in the intracellular metabolism of the pericarp.

Source of the Alkaloids

The fruit does not develop in isolation so that at least three possible sources for its alkaloids exist. (*a*) γ-Coniceine is the precursor of all the alkaloids and enters the fruit by the vascular bundles and schizogenous ducts as suggested previously.[1] If this is so the incoming γ-coniceine must be rapidly converted to coniine, since only small quantities of γ-coniceine occur; at times this conversion is so rapid that the γ-coniceine content falls to zero. This continuous supply of γ-coniceine must result in a considerable daily production of coniine which, in turn, is converted into a non-alkaloidal substance. (*b*) Coniine is formed from incoming γ-coniceine and at certain times combines reversibly with another molecule to form a non-alkaloidal substance; at later stages coniine is released from this loose compound. A small proportion of the coniine may also be re-converted to γ-coniceine. In these circumstances the daily production of coniine may not be large, but would show a gradual increase with increase in size of the pericarp. (*c*) The alkaloids are mainly synthesized in the fruit itself and the incoming γ-coniceine may play only a minor part in the total coniine production.

Minor Alkaloids

The appearance and disappearance of the minor alkaloid conhydrine runs parallel with the increase and decrease of γ-coniceine. This is consistent with the fact that both alkaloids are oxidation products of coniine and, conversely, could be formed into coniine by reduction. On the other hand, the occasional appearance of N-methylconiine and the unknown alkaloid of $R_f = 0{\cdot}73$ is associated with a maximum of coniine and it is not so clear how N-methylation would be connected with a reduction process.

The universal occurrence of the compounds of low R_f value (0·08) and some of their properties might at first sight indicate that they are the precursors of the normal alkaloids, but we found no evidence of the marked changes in quantity shown by the main alkaloids. Cromwell[2] does not mention this compound, but some of his chromatograms show a very small spot of R_f value 0·1. As he used steam distillates for his assay work it is unlikely that much of this compound would be present, as we found it was only sparingly volatile in steam.

EXPERIMENTAL

The "Chelsea variety" of *Conium maculatum* described by Fairbairn and Challen[1] was used throughout. The plants were grown at Myddelton House, Enfield, Middlesex, and the same sampling techniques were used as described previously,[1] week 1 collection being made when the flowers were fully expanded. In view of the large number of samples required, only four compound umbels were collected at a time. These yielded about 250 fruits for chemical analyses and about 250 fruits for dry weight determination. The chromatographic and analytical techniques of these authors were also used except for the following modifications.

Paper chromatograms. An improved method of determining the quantities of alkaloids based on measuring the areas of spots on paper chromatograms was devised.[9] Whatman No. 20 paper, which had been washed with N HCl in a chromatographic tank (descending technique) for 18 hr, followed by a similar washing with distilled water, was used. The solvent system was *tert*-pentanol/*tert*-butanol/N HCl (9 : 3 : 2), development time 15 hr at 20° (ascending technique). After drying, the alkaloidal spots were revealed by spraying with bismuth iodide reagent.[10]

Spectrophotometric methods. The yellow colour referred to previously,[1] which was produced by the interaction of bromethymol blue and the conium alkaloids, was measured in 1 cm cuvettes in a Hilger Uvispek spectrophotometer. The peak of the absorption curve was 402 μ; log ε_{402} for coniine was 4·313 and for γ-coniceine, 4·327. For convenience the alkaloidal contents are expressed as hydrochlorides throughout.

Acknowledgements—The work described in this paper forms part of a thesis presented by one of us, P. N. Suwal, for the award of a Ph.D. degree of the University of London. Financial assistance to P.N.S. from the Colombo Plan Fellowship Scheme is gratefully acknowledged. We would like to thank Mr. J. F. Rogers, Head Gardener at Myddelton House, for his valuable assistance in the cultivation of the hemlock plants.

[9] J. W. FAIRBAIRN and P. N. SUWAL, *Pharm. Acta Helv.* **34**, 561 (1959).
[10] R. MUNIER and M. MACHEBOEUF, *Bull. Soc. Chim. Biol. Paris* **33**, 846 (1951).

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3′-NUCLEOTIDASE ACTIVITY OF RYE GRASS SEEDS

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Abstract—There is no significant change in 3′-nucleotidase activity in rye grass seeds (*Lolium multiflorum*) germinated in the dark over a period of 6 days. In developing seeds, on the other hand, the activity per seed increases during maturation and then falls off as the seed ripens. The significance of these changes is briefly discussed.

INTRODUCTION

AN ENZYME which specifically dephosphorylates 3′-nucleotides, for example adenosine 3′-phosphate, guanylic acid, cytidylic acid, uridylic acid and Coenzyme A, has been purified from germinating barley (*Hordeum vulgare*) and rye grass (*Lolium multiflorum*)[1]. It is present in the seeds of many other cereals but rye grass is undoubtedly the best source. The specificity of this enzyme and its optimum pH (7·4) clearly distinguish it from other non-specific acid phosphatases which have been obtained from seeds.[2,3] It was considered that this enzyme might be a good subject for investigation as part of a general research programme planned to provide information on the biochemistry of seed germination, and that rye grass would be the best source of the enzyme. In a somewhat similar investigation Young and Varner[4] reported on an enzyme in pea cotyledons which specifically dephosphorylates adenosine triphosphate and adenosine diphosphate. They noted an increase in phosphatase activity during germination and concluded that the increased activity represented a net synthesis of enzyme protein.

RESULTS

Enzyme Activity during Seed Germination

The first series of experiments was designed to follow the enzyme activity during the germination of rye grass (*L. multifolium*, v. *Westernwoldicum*). Experiments were carried out on seeds germinated in the dark for a short period (0–24 hr) and for a longer period 0–6 days at room temperature. A typical series of results recorded in Fig. 1 shows that the specific activity of the crude extracts does not alter appreciably during 6 days germination. This behaviour is in marked contrast to the ATP/ADP-specific phosphatase from pea cotyledons, which increased many fold during germination.[4] In one experiment, enzyme activity was followed over a germination period of 16 days, but again no consistent increase in activity was observed during growth. It might be argued that the total amount of protein in the active extract of a given number of seeds would increase during germination and that by measuring the enzyme activity in relation to the protein content of the fraction, any increase in the amount of enzyme would be masked by the simultaneous synthesis of inert

[1] L. SHUSTER and N. O. KAPLAN, *J. Biol. Chem.* **201**, 535 (1953).
[2] B. K. JOYCE and S. GRISOLIA, *J. Biol. Chem.* **235**, 2278 (1960).
[3] M. Z. NEWMARK and B. S. WENGER, *Arch. Biochem. Biophys.* **89**, 110 (1960).
[4] J. L. YOUNG and J. E. VARNER, *Arch. Biochem. Biophys.* **84**, 71 (1959).

portein. However, if the protein levels in the extracts are plotted against germination time, no obvious changes are observed (Fig. 1). Therefore, as expected, the enzyme activity per seed also shows no significant change during germination.

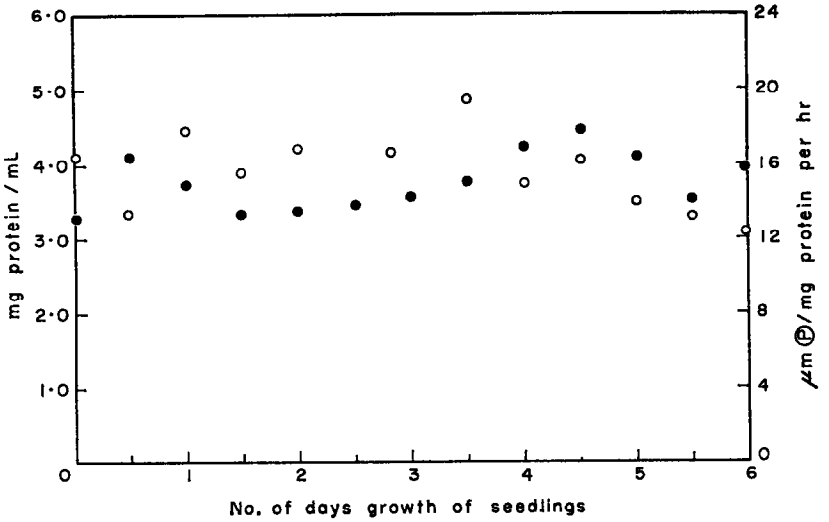

FIG. 1.

—O—O— Specific activity of 3′-nucleotidase in extracts of germinating *L. multifolium* seeds.

●—●—● Protein content of extracts of germinating *L. multifolium* seeds.

[In both curves each point represents the mean of four determinations.]

Enzyme Activity during Seed Development

Seeds were collected from ripening heads and separated into a number of groups according to their degree of maturity. A description of these groups is given in Table 1. The 3′-nucleotidase activity in extracts of seeds at the different stages is given in Fig. 2.

TABLE 1. A DESCRIPTION OF THE STAGES OF DEVELOPMENT AT WHICH SEEDS OF *L. multifolium* WERE EXAMINED, TOGETHER WITH THE PERCENTAGE OF DRY WEIGHT IN THE SEEDS

Stage	Description	% Dry weight
A	Seeds not exceeding 2 mm	39
B	Green seeds, 3–5 mm	43
C	Seeds turning yellow or red	52
D	Red seeds, not fully hardened	56
E	Hard ripe seeds	92
F	Ripe seeds stored 19 weeks	93

It will be seen that during the early stages of maturation the specific activity drops sharply and then recovers slightly; this recovery is then followed by a slow fall as the seeds ripen completely. The activity per seed shows a sharp increase in activity to stages C and D; this is followed by an equally sharp decline. The pattern is similar to that reported for protease activity in germinating wheat.[5] It would appear from the fact that the specific activity of

[5] A. BACH, A. OPARIN and R. WAHNER, *Biochem. Z.* **180**, 363 (1927).

leaves (we find a value of *ca.* 12 for seedling leaves and Shuster and Kaplan report a value of 26) is less than that of immature seeds (*ca.* 60), that in the very early stages of seed formation there is either a stimulated synthesis of this enzyme in the developing seed or a preferential translocation and accumulation in the ovaries. The initial sharp drop in specific activity (Fig. 2) in the transition to stage B, where the amount of enzyme per seed

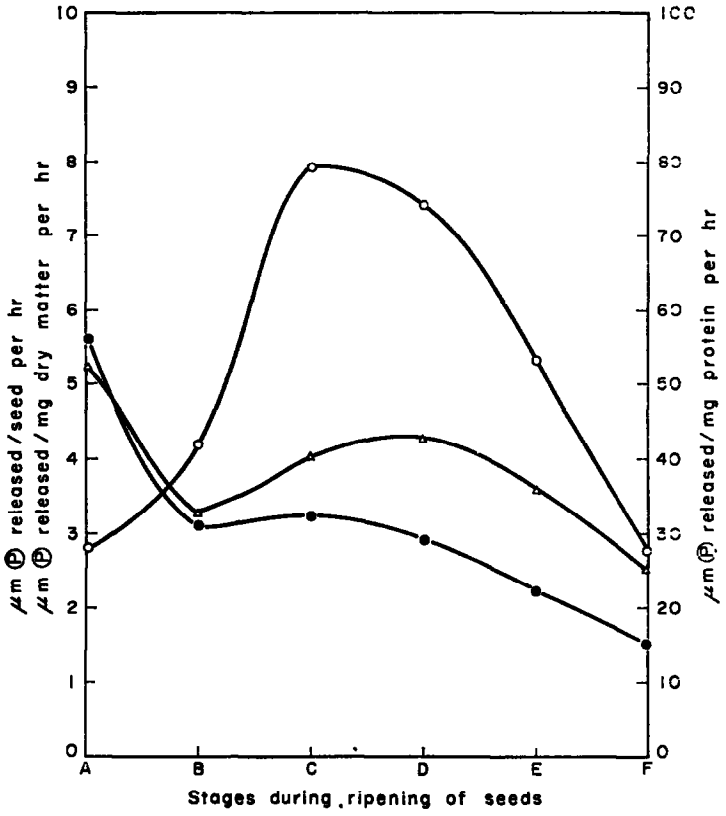

FIG. 2.

—Δ—Δ— Specific activity of 3′-nucleotidase in extracts of developing seeds of *L. multifolium.*

—O—O—O Changes in 3′-nucleotidase activity per seed in developing seeds of *L. multifolium.*

—●—●—●— The 3′-nucleotidase activity of developing *L. multifolium* seeds expressed per unit dry matter.

[The stages of development are described in Table 1: each point represents the mean of two experiments—four determinations in each experiment.]

has increased, shows that other proteins are being synthesized faster than the enzyme itself. Conversely the increase in specific activity observed during the transition to stage C and D, where the amount of enzyme per seed vastly increased, shows that the net synthesis of the enzyme is slightly greater than that of other proteins. The synthesis of all proteins presumably falls off as the maturing seed speeds up its accumulation of starch. The gradual decrease in activity after stage D, whether measured in specific activity or activity per seed, is very typical of changes in other enzyme activities during the later stages of seed matura-

tion. There is no experimentally upheld explanation for this, but it may be due to a gradual denaturation of proteins as the seeds become more dehydrated.

When the graph is considered in which activity per unit dry weight is plotted against degree of maturation (Fig. 2), then it is seen, not unexpectedly, that the general shape of the curve is very similar to the specific activity. The failure ever to observe a rise in activity during the stages C–D is, as already suggested, probably due to the rapid accumulation of starch during this period.

Enzyme Activity of Bracts

It was found that the bracts associated with the developing seeds also contained considerable enzyme activity. For example a sample of bracts from seeds of class D had a total activity of some 40 per cent of that of the corresponding seeds.

Enzyme Activity of Seedling Leaves

Seedling leaves produced by germinating seeds on filter paper in Petri dishes in the light for 14 days had a specific activity of 11·5–12, which is considerably lower than that of the seeds from which they were germinated. This confirms the observations of Shuster and Kaplan[1] and indicates, not unexpectedly, that the gush of protein synthesis association with the onset of photosynthesis is concerned more specifically with other enzymic and structural requirements of the plant.

COMMENT

The precise function, if any, of the specific 3′-nucleotidase in cereal seeds is not clear. This investigation does indicate, however, that it appears not to be concerned in germination, in the sense that germination is not accompanied by any increase in enzymic activity. On the other hand, as indicated earlier, the enzyme appears to be specifically accumulated or synthesized in the developing embryos, so there is an *a priori* case for considering that it is concerned more intimately with seed development than with seed germination.

EXPERIMENTAL

Materials. The seeds of *L. multiflorum* v. *Westernwoldicum* were used throughout. Ripe seeds were germinated on filter paper in the dark at room temperature (approx 15°) for the appropriate time. When seeds were germinated for long periods they were first sterilized by soaking for 1–4 hr in distilled water and then for 10 min in a solution (0·01 per cent) of mercuric chloride containing a few drops of Teepol. After rinsing in distilled water the seeds were allowed to germinate in the usual way. Seeds at various stages of development were obtained from plants grown in boxes in a heated greenhouse.

Preparation of enzyme extract. The method employed was that of Shuster and Kaplan[1] slightly modified. Ripe seeds (0·5 g) were crushed with a pestle and mortar, and transferred to 5 ml 0·1 M Tris buffer pH 7·5, and disintegrated in a M.S.E. Homogenizer fitted with a 10 ml "Vortex" beaker. The extract was then centrifuged at 0–4°C at 8000 $\times g$ for 15 min in a Christy centrifuge. If the assay could not be carried out immediately the supernatant was stored at −20° until it could be used. Immediately prior to assay the enzyme extract was diluted 1 : 3 with 0·1 M Tris buffer pH 7·5.

Phosphatase assay[6]. The diluted enzyme extract (0·05 ml) was added to the substrate (0·45 ml of 2·0 mM adenosine-3′-phosphate in 0·1 M Tris buffer, pH 7·5) at 37° and the

[6] E. J. KING, *Biochem. J.* **26**, 292 (1932).

mixture was incubated for 30 min. The reaction was stopped by adding 2 ml of 10 per cent trichloroacetic acid and then 0·35 ml 60 per cent perchloric acid, 0·35 ml 5 per cent ammonium molybdate and 0·25 ml aminonaphtholsulphonic acid reagent (0·2 g, 1,2,4-aminonaphtholsulphonic acid, 12 g sodium metabisulphite, 2·4 g sodium sulphite in 100 ml H_2O) were added and the volume made up to 5 ml with distilled water. The blue colour was allowed to develop for 10 min and its intensity was read at 750 mμ in a 1 cm cell with a Unicam SP600 photoelectric spectrophotometer. The amount of inorganic orthophosphate present was calculated from a standard curve.

Protein determinations. The protein concentrations in the enzyme extracts were measured following the method of Lowry *et al.*[7]

Specific activity. The specific activity of the preparations was expressed as μmoles of inorganic orthophosphate liberated per hr per mg protein under the conditions just described. This is also the unit used by Shuster and Kaplan.[1]

[7] O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).

Phytochemistry, 1961, Vol 1, pp. 52 to 57. Pergamon Press Ltd. Printed in England

BIOSYNTHESIS OF L-RHAMNOSE FROM D-GLUCOSE IN BUCKWHEAT

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Abstract—D-Glucose-1-C^{14}, -2-C^{14}, and -6-C^{14} were fed to cuttings of buckwheat (*Fagopyrum tataricum* Gaertn.) and 24–48 hr later the rutin was isolated and hydrolysed. This gave D-glucose, L-rhamnose and quercetin all with the same specific activity and suggests that all components of rutin came from the same carbohydrate metabolic pool. Both rhamnose and glucose were degraded by methods which allowed estimation of C^{14} in each of the six carbon atoms. The rhamnose was apparently derived from glucose without rearrangement of the carbon skeleton. L-Rhamnose-1-C^{14} and L-fucose-1-C^{14}, fed to buckwheat, were not incorporated into rutin.

INTRODUCTION

EIGHT methyl pentoses (6-deoxyaldohexoses) are known to occur in plants.[1] These are found in glycosides or polysaccharides rather than as free monosaccharides. The most common ones are L-rhamnose (6-deoxy-L-mannose) and L-fucose (6-deoxy-L-galactose). The other 6-deoxyhexoses are rare and found in the cardiac glycosides.

Three of the 6-deoxyhexoses, namely D-quinovose and D- and L-fucose, can be formally derived from the natural aldohexoses, D-glucose and D- and L-galactose respectively, by loss of the hydroxyl on carbon 6. The parent sugars of the other five 6-deoxyhexoses are not known to occur naturally. Thus on structural grounds it would seem unlikely that the biosynthetic route to these 6-deoxysugars is by formation of the appropriate hexose followed by reduction on carbon 6. We are unaware of any studies on the biosynthesis of rhamnose in higher plants.[2] The present paper reports the biosynthesis of rhamnose found in rutin (quercetin-3-β-rhamnoglucoside) of buckwheat. An improved method of degrading rhamnose gives the C^{14} content of each carbon. Apparently rhamnose is formed from glucose in buckwheat without rearrangement of the carbon chain.

RESULTS

The results of feeding various labelled sugars to buckwheat cuttings (stem plus leaves) on the incorporation of C^{14} into rutin and its hydrolysis products, quercetin, rhamnose and glucose, are shown in Table 1. All the products were isolated and purified chromatographically. The plants used in each experiment were from the same batch. Large plants grown outdoors were used for the first experiment so large amounts of glucose and rhamnose (from rutin) could be obtained for degradation. The amount of labelled glucose fed was roughly equal to the natural pool in the plant.

Previous experience[3] has shown that the per cent incorporation of glucose-C^{14} into rutin

[1] H. BAUMANN and W. PIGMAN, *The Carbohydrates, Chemistry, Biochemistry, Physiology.* (Edited by W. PIGMAN), p. 552. Academic Press, New York (1957).

[2] M. GIBBS, *Ann. Rev. Plant. Physiol.* (Edited by L. MACHLIS), Vol. X, p. 355, Annual Reviews Inc., California (1959).

[3] J. E. WATKIN and A. C. NEISH, *Can. J. Biochem. and Physiol.* (in press).

is roughly equivalent to the percentage of rutin in the plant. This was found in experiment 2. The amount incorporated in experiment 1 was only about one quarter that anticipated, probably because the plants were more mature and the percentage of rutin was beginning to fall.[4] The specific activities of all rutin components (quercetin, glucose and rhamnose) were about the same no matter which labelled glucose was fed (experiment 1).

Free rhamnose was not appreciably incorporated into rutin by buckwheat (experiments 2 and 3). The small amount of C^{14} incorporated from rhamnose in experiment 2 was

TABLE 1. RELATIVE ACTIVITIES OF RUTIN COMPONENTS FROM BUCKWHEAT PLANTS FED C^{14}-LABELLED GLUCOSE, RHAMNOSE OR FUCOSE

	Expt. 1			Expt. 2			Expt. 3	
Compound Fed*	G	G	G	G	Fu	Rh	G	Rh
Position of C^{14}	1	2	6	1	1	1	1	1
Metabolic period (hr)	48	48	48	24	24	24	24	24
Wt. of compound fed (mg)	50	50	100	7·6	3·5	3·8	4·5	6·3
Dry weight of plant† (g)	18·1	21·3	19·1	1·68	2·09	2·36	2·7	4·16
Dose‡ (μmole/g)	15	13	29	25	10	9	9	8
Total C^{14} fed (μc)	51·4	50	37	12·1	9·0	6·8	7·2	11·3
Rutin isolated (mg)	390	468	427	67	75	85	93	102
Specific activity of quercetin (μc/mole carbon)	14·4	9·7	7·5	150	0·3	1·5	12·0	0·68
Specific activity of glucose (μc/mole carbon)	17·2	10·2	8·0	150§	0·1	5·4	35·9	1·9
Specific activity of rhamnose (μc/mole carbon)	13·9	10·8	7·7	150§	0·4	3·3	34·6	1·8
Rutin as % dry weight plant (%)	2·2	2·2	2·2	4·0	3·6	3·6	3·6	2·5
% of C^{14} fed recovered in rutin	0·50	0·41	0·39	3·4	0·01	0·15	1·3	0·05

* G = D-Glucose, Rh = L-Rhamnose, Fu = L-Fucose.
† Calculated from the dry weight of the alcohol extracted marc, the percent solubles being determined on a control plant.
‡ Amount of compound fed expressed in μmoles/g of dry weight of plant.
§ Samples lost, and specific activity assumed to be that of the quercetin on the evidence of experiment 1 and specific activity of the isolated rutin.

only 0·05 per cent of that fed (2 μg) and this might be due to labelling of the general carbohydrate pool by metabolic products of rhamnose, as the glucose and quercetin were labelled to roughly the same extent. The free sugars of a buckwheat plant were isolated and partitioned on a cellulose column but no free rhamnose was found. It is unlikely that free rhamnose occurs naturally in buckwheat.

The isolated sugars were degraded; glucose by fermentation[5,6] and further degradation of the products,[7] rhamnose by an adaptation of the procedure of Boothroyd *et al.*[8] outlined in Fig. 1. The labelling patterns of glucose and rhamnose from experiment 1 are shown in Table 2. The percentages are based on the sum of the activities of the 6 carbons. This total varied, depending on the degradation, from 84 to 98 per cent of the activity of the original sample as measured by combustion of an aliquot before degradation. Generally speaking the rhamnose has the same labelling pattern as the glucose from the same sample, in spite of the fact that appreciable redistribution of the label has occurred in glucose.

[4] J. F. COUCH, J. NAGHSKI and C. F. KREWSON, *Science*, **103**, 197 (1946).
[5] J. HURWITZ, *Biochim. et Biophys. Acta*, **28**, 599 (1958).
[6] H. A. ALTERMATT, A. C. BLACKWOOD and A. C. NEISH, *Can. J. Biochem. and Physiol.* **33**, 622 (1955).
[7] A. C. NEISH and A. C. BLACKWOOD, *Can. J. Biochem. and Physiol.* **33**, 323 (1955).
[8] B. BOOTHROYD, S. A. BROWN, J. A. THORN and A. C. NEISH, *Can. J. Biochem. and Physiol.* **33**, 62 (1955).

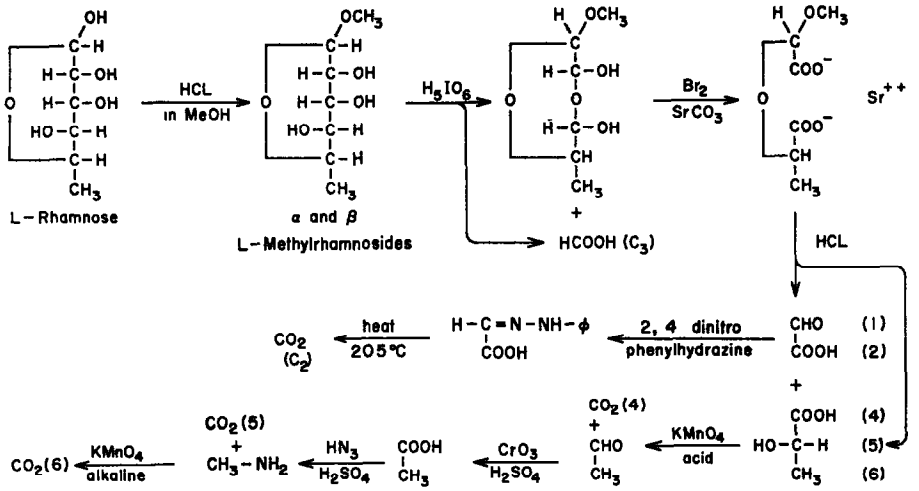

FIG. 1.

DISCUSSION

The main findings of this work are (*a*) all three components of rutin have the same specific activity, (*b*) free rhamnose is not incorporated into rutin although the rhamnose in rutin is formed readily from glucose and (*c*) no appreciable redistribution of the labelling pattern occurs during conversion of glucose to rhamnose.

There have been seven other studies on the conversion of C^{14} labelled glucose to deoxyhexoses in intact organisms—six studies used micro-organisms[9-14] and one the milk of lactating women.[15] In five of these studies bound glucose was isolated as well as deoxyhexose. In each case the labelling pattern of the glucose fed had been redistributed to some extent in the bound glucose but the label in the deoxyhexose in all cases paralleled the bound glucose. An extreme example of redistribution of C^{14} is in the work of Taylor

TABLE 2. % DISTRIBUTION OF C^{14} IN EACH CARBON OF GLUCOSE AND RHAMNOSE OBTAINED FROM RUTIN BY HYDROLYSIS AFTER FEEDING LABELLED GLUCOSE

Fed	G-1-C^{14}		G-2-C^{14}		G-6-C^{14}	
Isolated	G	Rh	G	Rh	G	Rh
C1	70·4	72·8	0·6	11·6	17·0	27·1
C2	2·3	4·5	82·5	64·9	3·1	4·0
C3	4·4	4·5	8·7	7·5	2·2	3·4
C4	3·9	4·1	5·2	6·6	3·0	4·3
C5	1·4	1·3	1·4	7·2	15·6	1·9
C6	17·6	12 8	1·6	2·2	59·1	59·4

[9] G HALSER and M. L. KARNOVSKY, *J. Biol. Chem.* **233**, 287 (1958).
[10] H TAYLOR and E. JUNI, *Bacteriol. Proc.* (*Soc. Am. Bacteriologists*) p. 34, 112 (1959).
[11] W. H. SOUTHARD, J. A. HAYASHI and S. S. BARKULIS, *J. Bact.* **78**, 79 (1959).
[12] E. C. HEATH and S. ROSEMAN, *J. Biol. Chem.* **230**, 511 (1958).
[13] J. F. WILKINSON, *Nature, Lond.* **180**, 995 (1957).
[14] S. SEGAL and Y. J. TOPPER, *Biochim. et Biophys. Acta*, **25**, 419 (1957).
[15] S. SEGAL and Y. J. TOPPER, *Biochim. et Biophys. Acta*, **42**, 147 (1960).

and Juni[10] with an aerobic soil bacterium. When glucose-1-C^{14} or glucose-2-C^{14} were the substrates both glucose and rhamnose in the capsular polysaccharide were unlabelled. However when glucose-6-C^{14} was the substrate both glucose and rhamnose were labelled equally in positions one and six. Another example of the labelling pattern in the rhamnose varying greatly from that of the glucose administered was found in *Pseudomonas aeruginosa*. Hauser and Karnovsky[9] added glucose-6-C^{14} to the medium and isolated rhamnose, labelled in positions one and six, from a rhamnolipid found in the medium. As there was no glucose in the rhamnolipid, bound glucose could not be isolated to check the labelling pattern. Using this same organism Kornfeld and Glaser[16] have recently obtained a cell-free enzyme system that will convert thymidine diphosphate-D-glucose to thymidine diphosphate-L-rhamnose. A similar enzyme system that changes guanosine diphosphate-D-mannose to guanosine diphosphate-L-fucose has been isolated from *Aerobacter aerogenes* by Ginsberg.[17]

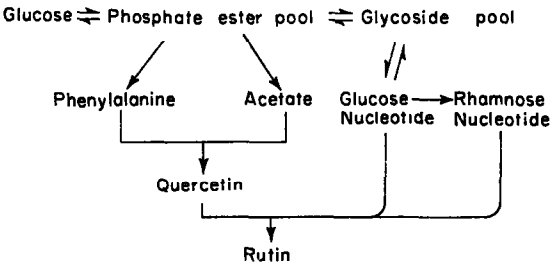

FIG. 2.

In our view our investigation and those mentioned above show a common pattern for deoxyhexose biosynthesis. The first stage is the formation of a hexose nucleotide. During the formation of the nucleotide from glucose extensive redistribution of the C^{14} labelling pattern may occur in the phosphate ester pool depending on the peculiar carbohydrate metabolism of the organism. Buckwheat and some microorganisms[13,14] show a C6-C1 interchange of C^{14} consistent with the EMP type of metabolism. Taylor and Juni's bacterium[10] has a modified Entner–Doudoroff type of metabolism whereby the glucose carbon chain is first split and then the hexose molecule is rebuilt from carbons 4, 5 and 6 before a hexose nucleotide can be formed. In all organisms once the hexose nucleotide is formed it is converted directly without rearrangement of the carbon chain to a deoxyhexose nucleotide. These nucleotides are then used for polysaccharide or glycoside synthesis.

Application of the above facts and ideas leads to a scheme for the biosynthesis of rutin in buckwheat outlined in Fig. 2.

When glucose enters the plant it probably enters the phosphate ester pool.[18,19] This pool contains intermediates of glycolysis and of the pentose phosphate pathway. From this pool may be drawn erythrose-4-phosphate and phosphoenol pyruvate required for synthesis of phenylalanine[20] as well as pyruvate which is used as a source of acetate units. Quercetin is readily formed from phenylalanine and acetate.[21]

[16] S. KORNFELD and L. GLASER, *Biochim. et Biophys. Acta*, **42**, 548 (1960).
[17] V. GINSBURG, *J. Biol. Chem.* **235**, 2196 (1960).
[18] A. C. NEISH, *Proc. Intern. congr. Biochem. 4th Congr.* 1958. Vol. II, p. 82. Pergamon Press, London.
[19] W. Z. HAZZID, E. W. PUTMAN and V. GINSBURG, *Biochim. et Biophys. Acta*, **20**, 17 (1956).
[20] B. D. DAVIS, *Arch. Biochem. Biophys.* **78**, 497 (1958).
[21] J. E. WATKIN, E. W. UNDERHILL and A. C. NEISH, *Can. J. Biochem. and Physiol.* **35**, 229 (1957).

The glycoside pool is postulated to contain glucose-1-phosphate and other glycosidic compounds such as uridine diphosphate glucose. Sugars can be interconverted as nucleotides e.g. uridine diphosphate glucose $\rightleftarrows$ uridine diphosphate galactose, and a rhamnose nucleotide is probably formed here. Nucleotides act as donors for transglycosylation such as occurs in the synthesis of sucrose, oligosaccharides, glucuronides etc. Rutin could be formed by transglycosylation of quercetin from glucose and rhamnose nucleotides. Details of these reactions have not been investigated in rutin formation and it is not known whether a disaccharide is formed before reaction with the flavonol or whether monosaccharides are put on one at a time.

If indeed rhamnose is metabolized in plants as a nucleotide, the absence of a reversible enzyme which would make or break the nucleotide from the free sugar would explain both the absence of free rhamnose and its failure to be used for rutin synthesis. An analogous situation was found in wheat where D-xylose was not converted to xylan except after prior conversion to D-glucose[22] whereas both D-glucose and L-arabinose could give rise to an xylan precursor in the glucoside pool.

The observation that all components of rutin have the same specific activity is now understandable since they all come from the same intermediates in the phosphate ester pool.

Direct formation of L-rhamnose from D-glucose requires three epimerizations and a reduction of $-CH_2OH$ at carbon 6 to $-CH_3$. This reduction has been unknown until now, in the biochemistry of hexoses but an analogy in the triose sugars would be the formation of pyruvate from 2-phosphoglyceric acid. In the hexose series the intermediate would be a 5–6 hexoseen but as the order of the epimerization and dehydration is unknown it is not possible to suggest which hexoseen. However, stereospecific reduction of the hexoseen in the stable pyranose form (carbon 1 being linked to the nucleotide) would enable the formation of the 6 methyl group and the epimerization on carbon 5 to form the L-sugar to take place simultaneously. The epimerization of carbons 3 and 4 may take other enzymes and 3- and 4-epimerases are known, such as galactowaldenase, and L-ribulose-5 phosphate 3-epimerase which occurs in *Aerobacter aerogenes*.[23]

EXPERIMENTAL

General

Measurements of $C^{14}O_2$ were carried out in a gas phase proportional counter using the technique of Buchanan and Nakao.[24] The radioactive sugars were purchased from Atomic Energy of Canada Ltd. Unless stated otherwise, all solutions were evaporated by a stream of dry filtered air at room temperature. This was usually done in a tared beaker.

Administration of Labelled Sugars

Buckwheat (*Fagopyrum tataricum* Gaertn. V. CD 4251) was grown outdoors for the first experiment (Table 1) and in a growth chamber for the second and third experiments. The administration of labelled compounds by absorption of a solution through the cut end of the stem has been described.[25] The cuttings were kept in an incubator at 22°C with 400 ft c. (4000 lx) of fluorescent light for the time indicated in Table 1.

[22] H. A. ALTERMATT and A. C. NEISH, *Can. J. Biochem. and Physiol.* **34**, 405 (1956).
[23] R. L. ANDERSON and W. A. WOOD, *Biochim. et Biophys. Acta*, **42**, 374 (1960).
[24] D. L. BUCHANAN and A. NAKAO, *J. Am. Chem. Soc.* **74**, 2389 (1952).
[25] E. W. UNDERHILL, J. E. WATKIN and A. C. NEISH, *Can. J. Biochem. and Physiol.* **35**, 219 (1957).

Isolation of Products

When the metabolic period was over, rutin was isolated,[26] and hydrolysed by refluxing in 3 per cent sulfuric acid for one hour. The hydrolysate was cooled, filtered to remove quercetin, and the filtrate clarified by passage through a small column (2·5×4 cm) of a mixture of equal volumes of Darco-G-60 and Celite 535. The sulfuric acid was neutralized by barium carbonate, the insolubles filtered, and the solution evaporated to a hard syrup.

This syrup contained both rhamnose and glucose. These sugars were separated by chromatography on a cellulose column (2·6×20 cm). *n*-Butanol one-quarter saturated with water eluted the rhamnose first. The glucose was then eluted by *n*-butanol saturated with water. The sugars were detected by spotting on paper and spraying with *p*-anisidine. The appropriate fractions were pooled, evaporated, taken up in water, extracted twice by ether and evaporated again. The ether-soluble impurities were discarded.

Measurement of C^{14}—distribution in Glucose and Rhamnose

The samples of glucose and rhamnose obtained above were degraded on a one-mmole scale. This required addition of 2–3 parts of carrier. Glucose was degraded by fermentation with resting cells of *Leuconostoc mesenteroides*[5,6] and the products (carbon dioxide, ethanol, lactic acid) isolated and degraded as described by Neish and Blackwood.[7]

Rhamnose was degraded by an adaptation of the procedure of Boothroyd *et al.*[8] for the chemical degradation of glucose. The modified procedure (Fig. 1) incorporates several short cuts; i.e. the α- and β-methyl glycosides were not separated prior to periodic acid oxidation and no attempt was made to isolate a crystalline strontium salt after oxidation. The solution obtained after bromine oxidation was aerated to remove bromine, acidified to pH 1 by hydrochloric acid, concentrated to 2 ml, and then more hydrochloric acid added to make the solution 2N. Hydrolysis and isolation of glyoxylic acid as the 2,4-dinitrophenylhydrazone was carried out as before.[8] The filtrate from the hydrazone was passed through a small column of carbon (Darco-G-60), mixed with Celite 535, then concentrated to 30 ml and the lactic acid isolated by continuous extraction with ether for 3 hr. The ether was evaporated in the presence of a few ml of water and the lactic acid titrated to the phenol red end point with 0·05 N sodium hydroxide. The lactic acid was then purified on a silicic acid column[27] and degraded by oxidation to carbon dioxide and acetic acid as described previously.[7] The acetic acid was degraded further by the Schmidt reaction as described by Phares[28]. Thus the concentrations of C^{14} in carbons 2 to 6 are determined separately whereas the C^{14} of carbon 1 is determined by difference.

Acknowledgements—The authors are indebted to Mr. J. Dyck for radioactivity measurements and to Mr. G. H. Krenz for skilful technical assistance.

[26] J. E. Watkin, *Chem. and Ind. (Rev.)* 378 (1960).
[27] A. C. Neish, *Can. J. Botany* **31**, 265 (1953).
[28] E. F. Phares, *Arch. Biochem. Biophys.* **33**, 173 (1951).

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KINETIN-INDUCED DIRECTED TRANSPORT OF SUBSTANCES IN EXCISED LEAVES IN THE DARK

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Abstract—Kinetin-induced accumulation of amino acids in leaf tissue[1,2] can take place in the dark. Leaves, however, kept in the dark for a long period, or starved, or the white parts of variegated leaves often do not show this effect. It is presumed therefore that ATP is also necessary for the process.

IF KINETIN (6-aminofurfurylpurine) is sprayed on a limited area of an excised mature leaf, numerous substances in the untreated parts will migrate to this "kinetin-locus". This phenomenon can be impressively demonstrated with radioactively labelled amino acids which are applied through the petiole or to the surface of the leaf.[1-3]

This directed migration is caused by an accumulation of substances in the kinetin treated tissue. This accumulation corresponds, at least in its last phase, to an active transport and consequently is an energy-requiring process. Kinetin can also inhibit the outward migration of substances,[3,4] and the treated area, therefore, also represents a locus of predominant synthesis. Accumulation, however, is not the consequence of synthesis, but mass-synthesis of protein, for example, is the consequence of an accumulation of amino acids. This has been proved by the finding that those amino acids which cannot be incorporated into protein, such as α-aminoisobutyric acid, are subjected to the kinetin-directed transport and accumulation.[5]

Kinetin-treated tissue behaves physiologically like young tissue: it accumulates certain substances and arrests others.[3,6] Thus kinetin has been found to be a model-substance of the regulative principle which is of decisive importance for the distribution and exchange of substances within the plant. This finding gives us the possibility to induce and influence certain regulations ourselves.[3] Unpublished experiments (with K. Conrad) have shown that indoleacetic acid is also subjected to this directed transport under the influence of kinetin. The frequently observed polarity of substance transport thus can be augmented, eliminated, or reversed by kinetin.

Our previously published experiments were carried out almost exclusively with green leaves in a weak light. Leaves that have been kept in the dark for a considerable time, or are chlorophyll-deficient show the above mentioned effects either only indistinctly or not at all. This may be due to various causes. One might consider that accumulation preferentially takes place in tissue containing functioning chloroplasts exposed to light. But it may also be possible that a good source of ATP is necessary for the energy-requiring processes of accumulation. Such a source might be represented by photophosphorylation. The

[1] K. MOTHES and L. ENGELBRECHT, *Monatsber. Dtsch. Akad. Wiss.* **1**, 367 (1959).
[2] K. MOTHES, L. ENGELBRECHT and O. KULAJEWA, *Flora, Jena* **147**, 445 (1959).
[3] K. MOTHES, *Naturwissenschaften* **47**, 337 (1960).
[4] L. ENGELBRECHT and K. MOTHES, *Plant Cell Physiol.* (1961).
[5] K. MOTHES, L. ENGELBRECHT and H. R. SCHUTTE, *Physiol. Plant.* **14**, 72 (1961).

following experiments demonstrate that directed transport and accumulation are also possible in leaves kept in the dark.

Excised leaves of *Nicotiana rustica* were first treated with kinetin, and four days later with glycine-1-^{14}C as shown in Fig. 1. After uptake of the amino acid was complete,

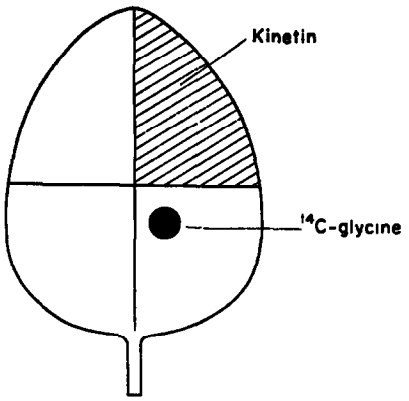


FIG. 1. SCHEME SHOWING THE LOCAL APPLICATION OF KINETIN AND GLYCINE—(1-^{14}C) TO ISOLATED LEAVES OF NICOTIANA RUSTICA.

half the leaves were kept in the dark and the other half exposed to continuous light, and sampled after varying time periods (Table 1). Radioautographs were taken of one leaf from each group (Fig. 2), and samples of kinetin-treated area from corresponding leaves taken for analysis. The samples were extracted with 80 per cent ethanol, and the extracts examined for radioactivity (Table 3) and chromatographed (Fig. 3 and 4). The insoluble residue was hydrolysed and the activity of the hydrolysate determined.

TABLE 1. DISTRIBUTION OF RADIOACTIVITY AT THE KINETIN-LOCUS AFTER APPLICATION OF GLYCINE-(1^{14}-C) TO ANOTHER SITE

Experiment No.	Condition	Duration (hr)	Counts/min			Ratio sol. fract.' insol. fract.
			Sol. fract.	Insol. fract.	Total	
1 (*a*)	light	8½	1443	459	1902	3·2
(*b*)	dark		1426	437	1863	3·3
2 (*a*)	light	23	5824	2050	7874	2·9
(*b*)	dark		3068	784	3852	3·9
3 (*a*)	light	55	2523	1714	4237	1·5
(*b*)	dark		2273	816	3088	2·8

RESULTS

Before discussing the results in detail some observations must be made on the metabolic fate of glycine in leaves. Various publications from our laboratory have demonstrated the rapid transformation of glycine in plants.[6-8] This process gives rise to CO_2 and an active

[6] L. ENGELBRECHT, *Flora, Jena* **150**, 73 (1961).
[7] H. REINBOTHE, *Flora, Jena* **150**, 128 (1961).
[8] H. REINBOTHE and K. MOTHES, *Tetrahedron Letters* No. 25, 32 (1960).

1-C-fragment which by means of folic acid can be used directly, or via methionine or betaine, for methylation and elongation of carbon chains. For example this is the way that the second molecule of glycine is used for the synthesis of serine.

$$\underset{\text{glycine}}{CH_2NH_2COOH} \longrightarrow (1\text{-}C) + CO_2$$

$$(1\text{-}C) + CH_2NH_2COOH \longrightarrow \underset{\text{serine}}{CH_2OHCHNH_2COOH}$$

Thus we find large amounts of serine in plant tissues treated with glycine and since glycine can also readily be formed from serine, we find glycine if the tissue is treated with serine. The CO_2 split off from glycine can be used for carboxylation, and photosynthetic assimilation. and thus rapidly appears in certain organic acids and carbohydrates. It can also be eliminated in the normal manner.

Radioautographs of whole leaves (Fig. 2) show that both in the light and in the dark there is a predominant translocation of radioactivity from the site of application to the kinetin-locus. The activity also spreads through the veins to a certain degree. In the leaves exposed to the light considerable radioactivity also appears in the intervenal regions of the left blade. This latter effect seems to be the consequence of CO_2-assimilation by photosynthesis, since the radioactive CO_2 can readily reach the whole leaf.

The distribution of radioactivity in the soluble and insoluble fractions of the kinetin-treated tissues is shown in Table 1. These results confirm the radioautographs. Activity very rapidly migrates into the kinetin-tissue. After $8\frac{1}{2}$ hr there is not much difference in total radioactivity between the leaf exposed to the light and the one in the dark. Also the ratio of the distribution of activity in the soluble, and hydrolysed insoluble fractions is almost the same in the two leaves. In both cases there is much more activity in the soluble fraction than in the insoluble.

After 23 hr the migration of activity into the kinetin tissue has much increased. The leaf exposed to the light now has a much higher activity due to an increase of both soluble and insoluble radioactive compounds, the latter increasing more rapidly. In this leaf the ratio of radioactivity of the soluble to insoluble fraction has decreased (from 3·2 to 2·9), indicating that incorporation of soluble substances into insoluble ones, has increased. In the leaf kept in the dark, on the other hand, the quotient has increased (from 3·3 to 3·9); in this case therefore the inflow of soluble substances exceeds their further assimilation.

After 55 hr the total activity has decreased. Presumably most activity is lost by disappearance of CO_2, even in the light where the CO_2 can easily be reincorporated.

The radioautographs (Fig. 4) of the paper chromatograms (Fig. 3) of the soluble fraction show that in the darkened leaf (1b, 2b, 3b) most of the radioactivity is fixed as glycine and serine. There is, however, always a little activity in monosaccharides and in sucrose; obviously some of the free radioactive CO_2 is transformed by dark assimilation to carbohydrates. Because of the high specific activity of the two amino acids glycine and serine (spot 6, Fig. 4) it is probable that the activity in the dark leaf migrates primarily in the form in which it was applied, that is as glycine and its rapidly appearing metabolic product serine.

The radioautographs of the leaves exposed to light (1a, 2a, 3a of Fig. 4) show remarkably less activity in the glycine–serine spot, but very high activity in the products of decarboxylation and CO_2 assimilation, sucrose, glucose and fructose, as well as in several amino acids.

The remarkable difference between leaves in the light and leaves in the dark might indicate that metabolism of the glycine and serine is increased by light. But this problem needs further investigation. It is possible, for example, that in the light glycine is partly

transformed at the site of application and that the transformation products, such as carbohydrates, migrate to the kinetin-locus.

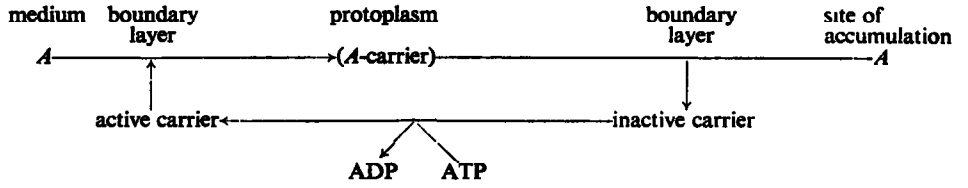
The chemical nature of the insoluble compounds formed from glycine will be reported in another publication (Parthier and Wollgiehn).

DISCUSSION

It has been shown that protein-forming amino acids can be accumulated by kinetin-treated tissue in the dark. This might be of great importance for a closer understanding of the way in which concentrations increase in those organs deficient in chloroplasts, or which are growing in the dark: e.g. the phloem, storage organs, fructified ovula, root tips and in young organs etc.

It is not clear, whether the great difference in the total activity of the kinetin tissue between leaves in the light and in the dark (Table 1, experiments 2a and 2b) is due to an extraordinarily high loss of activity in the form of CO_2 in the darkened leaf, whereas in the light the bulk of this CO_2 can immediately be reassimilated. It is, however, more probable that kinetin-induced directed transport is greatly promoted by light. In leaves which have starved for a longer time than those used in these experiments the directed transport to the kinetin-locus takes place either poorly or not at all. Apparently there is a factor, other than kinetin, necessary to make migration possible. We are inclined to suggest that ATP is this factor.

Since kinetin has a promoting effect on the accumulation of substances with very different chemical constitutions, it seems unlikely to function as a carrier itself; it may, however, contribute to the formation of or regeneration of a natural carrier system. Without having concrete proof as yet in favour of any definite concept, most authors[9,10] incline to a hypothesis which may be expressed in the following scheme:



At the boundary layer an amino acid *A* is transformed to a compound "*A*-carrier" with the help of a specific substance which has been activated in some way by ATP. This "*A*-carrier" dissociates at the boundary of the accumulating system (vacuole membrane, mitochondrial membrane, or plastid membrane) releasing the amino acid and regenerating, not the carrier substance itself, but an energy-poor form of it which is therefore inactive. This form is transformed back to the active carrier by the participation of ATP. The ADP resulting must be regenerated by respiration or photophosphorylation to form ATP. In chlorophyll-deficient or starving leaves it is presumed that the ATP regenerating system is not sufficiently active to carry out the cycle.

Besides the unspecific factors, ATP and kinetin, accumulation would also need specific ones, the carriers. Experiments with animal cells, however, make it improbable that every substance has a specific carrier; rather there exists something like enzyme-group-specificity.[9,10] Perhaps the peculiar differences of translocation direction described by Nelson

[9] E. HEINZ, *J. Biol. Chem.* **211**, 781 (1954).
[10] E. HEINZ and P. M. WALSH, *J. Biol. Chem.* **233**, 1488 (1958).

and Garham[11] are due to such group-specific carriers. We also are inclined to interpret the accelerated efflux of protein degradation products in the presence of asparagine as determined by the more rapid yellowing of the leaves[12] with a blocking of group-specific carriers which are thus rendered incapable of binding other amino acids that are required for protein synthesis.

EXPERIMENTAL

Plants of *Nicotiana rustica* were cultivated in the greenhouse in daylight supplemented with fluorescent light. At the start of the experiment (4 March) the second full-grown leaf (counted from the tip) was excised from a number of plants. The leaves were kept in the laboratory in a humid chamber with moderate diffuse daylight (2 m from a south window) and their right upper quarter was sprayed twice with kinetin (30 mg/l.) (Fig. 1). Four days later, when the untreated parts of the leaves were already distinctly lighter than the kinetin area, 7 μl. of a 0·01 M glycine-1-^{14}C solution (0·7 μC) were applied to a small area about 1 cm below the kinetin-treated quarter, avoiding the larger veins. Since weak light increases the rate of uptake, all the leaves were exposed to a moderate light during this period. After the complete uptake of the solution (1 to 2 hr) half of the leaves were put into total darkness, and the other half into continuous light of fluorescent lamps of about 1000 lx. The leaves were sampled after 8½ hr (1a and b), 23 hr (2a and b) and 55 hr (3a and b). One leaf of each group was oven-dried and radioautographed (Fig. 2), and 450 mg of fresh material from the kinetin-treated area of another leaf was extracted with 80 per cent ethanol. The residue was hydrolysed with 6 N HCl for 14 hr at 100°. The total hydrolysis mixture was evaporated to dryness on a steam bath, and made up to 1 ml with H_2O. The radioactivity of both the soluble fraction and of the hydrolysed ethanol insoluble fraction was determined under comparable conditions with a Geiger–Müller tube in an infinitely thin layer (Table 1). 50 μl. of each of the fractions were chromatographed two dimensionally on Schleicher and Schüll paper 2043b. The chromatograms of the soluble fractions are shown in Fig. 3. The same chromatograms were radioautographed with Diavidox X-ray-film (Fig. 4).

[11] C. D. NELSON and P. R. GARHAM, *Can. J. Bot.* **37**, 431 (1959).
[12] G. MICHAEL, *Z. Bot.* **29**, 385 (1935).

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ON GUANIDINE METABOLISM IN *GALEGA OFFICINALIS*

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Abstract—Galegine has been shown to be synthesized in the seedlings (with or without roots), leaves, flowers, and fruits of *Galega officinalis*. Much of the galegine which accumulates in the seeds is apparently synthesized in the fruit pod and there is no storage of galegine in the roots. Several enzymes of the ornithine-cycle were found to be present in extracts of the seedlings. It was found that arginine can transfer its amidine group to a precursor of galegine by a transamidination reaction.

IN *Galega officinalis*, a leguminous plant, the main soluble nitrogen compound is galegine[1-3], 3-methyl-but-2-enyl-guanidine-(1) (Fig. 1). However, we have also found small amounts of arginine and other galegine-like compounds, one of which has recently been identified as 4-hydroxy-3-methyl-but-2-enyl-guanidine-(1)[4]. We have elaborated new methods to separate such compounds by both ion exchange and paper chromatography, and for their quantitative determination.

We have found that galegine increases during the rapid growth phase of the plant and also during the development of flowers and fruits, and that there is no storage of galegine in the roots during the resting period during the winter. However, since many basic nitrogen compounds are known to be synthesized preferentially in the roots[5], it was of interest to examine where galegine biosynthesis took place. *Galega* seedlings were therefore exposed to air containing $C^{14}O_2$, the roots having been cut off from the one half of the seedlings immediately before the beginning of the experiment. After a few hours galegine was found to be labelled in both sets of plants (Table 1).

TABLE 1. PER CENT ACTIVITY IN THE BASIC COMPOUNDS OF *Galega* SEEDLINGS SEPARATED BY PAPER CHROMATOGRAPHY OF THE BASIC FRACTION OBTAINED BY IONOPHORETIC SEPARATION

Fraction	Activity expressed as a % of that in the basic fraction	
	Complete seedlings	Without roots
1. Arginine	21·6	11·1
2. Hydroxygalegine	5·6	2·8
3. Alkaloid	15·4	2·3
4. Galegine	55·9	80·8
5. Rest	1·5	3·0

As can be seen the galegine obtained from seedlings without roots has more radioactivity than that of intact seedlings, and therefore the main site of galegine synthesis is in the shoots and not in the roots.

The galegine content of the plant increases especially during the ripening of seeds and we may suppose that the seeds are the place of intensive synthesis. This was confirmed by exposing attached fruits to $C^{14}O_2$. Some of the fruits were divided into seed, pod and stalk

[1] G. TANRET, *Compt. rend.* **158**, 1182 (1914).
[2] G. BARGER, F. D. WHITE, *Biochem. J.* **17**, 827 (1923).
[3] E. SPATH, S. PROKOPP, *Ber. deut. chem. Ges.* **57**, 474 (1924).
[4] K. PUFAHL, K. SCHREIBER, *Experientia* **17**, 302 (1961).
[5] K. MOTHES, *J. Pharm. and Pharmcol.* **11**, 193 (1959); and *Symp. Soc. exp. Biol.* **13**, 258 (1959).

before exposure, the others being separated after the treatment and immediately before extraction (Table 2).

TABLE 2. ACTIVITY IN VARIOUS FRACTIONS OF THE FRUITS OF *Galega officinalis* AFTER $C^{14}O_2$-ASSIMILATION

Fraction	Seed	Pod	Stalk
a. Fruits divided before exposure:		COUNTS/MIN 10^{-3}	
1	125	1167	929
2	81	246	60
3	6·6	19·1	2
4	12	4	0·07
Total	224	1436	991
Hydrol.	160	1020	990
b. Whole fruits:			
Fraction			
1	384	466	514
2	119	66	75
3	4·2	2·7	1·4
4	4·4	0·7	0·05
Total	511	535	590
Hydrol.	130	440	450

The plant extracts were separated by ion exchange chromatography (Dowex 50). Fraction 1 = neutral and acid compounds (carbohydrates, fatty acids, etc.); fraction 2 = neutral and acid amino acids; fraction 3 = basic amino acids (mainly arginine); fraction 4 = galegine (and galegine-like compounds). Hydrol. = residues of the samples extracted with ethanol (80% vol./vol.) hydrolysed with 6 N HCl (cf. fractions 1–4)

In the fruits divided before the treatment the activity extracted from the seeds was relatively low, and that of the pod high. But there are no differences in activity of the various parts of the fruits divided after the treatment, presumably because the transport of the assimilates from stalks and pods into the seeds takes place readily.

Most of the galegine appears in the seeds, only a third or less being present built up in the pods. Nevertheless in ripe fruits where the galegine content of the pods is extremely low, a proportion of the galegine accumulated in the ripe seeds will have originated from the pods. There is only little biosynthesis of galegine in the stalks. Arginine appears to be synthesized preferably in the pods, and transported into the seeds, although there is no accumulation of free arginine. The arginine content is only increased when the seeds germinate.

It was found that the synthesis of guanidine compounds can also take place in the dark, albeit in a much reduced manner (Table 3). In all experiments the arginine- and galegine-fractions showed similar behaviour; (arginine being present at a lower concentration has a higher specific activity than galegine).

To get a better understanding of the mode of guanidine biosynthesis we studied the participation of some enzyme systems. We presumed that arginine synthesis in *Galega* would be catalysed by similar enzymes, to those which catalyse urea synthesis via the ornithine-cycle[6,7]. In the supernatant of centrifuged extracts of *Galega* seedlings we

[6] K. KLECZKOWSKI, J. REIFER, *Bull. acad. polon. sci.*, Classe II, 7, 53 (1959).
[7] G. REUTER, *Nature* **190**, 447 (1961).

have found orninthine-carbamyl transferase, which catalyses the transfer of the carbamyl residue from carbamylphosphate to ornithine, yielding citrulline. The "splitting enzyme" which catalyses the reversible formation of argininosuccinate from arginine and fumaric acid has also been found in extracts of *Galega* seedlings. The presence of arginase in such extracts is somewhat uncertain, but we have found high urease activity. None of these

TABLE 3. SPECIFIC ACTIVITY OF ARGININE AND GALEGINE EXTRACTED FROM SEEDLINGS OF *Galega officinalis* EXPOSED TO $^{14}CO_2$ IN LIGHT AND DARK

	Counts/min/mmol × 10^{-6}	
	Light	Dark*
Arginine	67·5	5·2
Galegine	13·4	0·2

* Plants put into the dark 2 hr before exposure to $C^{14}O_2$.

enzymes appeared to be involved in galegine metabolism. Experiments were therefore carried out to determine the relation between arginine and galegine using DL-arginine. HCl-[amidine-^{14}C]. When this was fed to *Galega* seedlings most of it was metabolized within the first hours. Table 4 shows the results of one of these feeding experiments.

TABLE 4. THE METABOLISM OF ARGININE-[AMIDINE-C^{14}] IN *Galega* SEEDLINGS

Fraction*	Counts/min × 10^{-4}	% of total activity
1	2·6	1·2
2	5·1	2·3
3	202·8	92·2
4	9·7	4·3
Total	220·25	

* Fractions 1–4 as in Table 2.

Application of ^{14}C labelled arginine to inflorescences and leaves gave similar results. Since galegine (fraction 4) has more radioactivity than the carbohydrates, fatty acids (fraction 1), and neutral amino acids (fraction 2) it may be supposed that some of the arginine must have transferred its amidine group to a precursor of galegine in a direct manner. It is possible that a transamidination reaction occurs between arginine and isopentenylamine Fig. 1) and further work is now in progress to elucidate this.

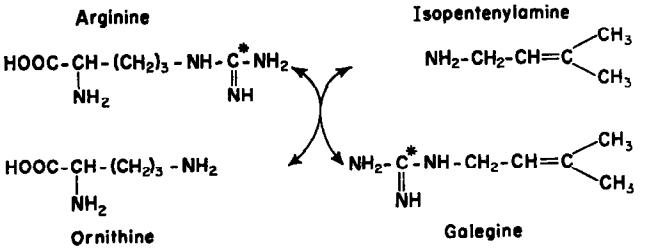

FIG. 1. TRANSAMIDINATION REACTION

Acknowledgement—I wish to express my sincere gratitude to Prof. Dr. K. Mothes for his interest during the course of this investigation and to Dr. Schütte and Mr. Hindorf in our laboratory for the synthesis of DL-arginine-[amidine-C^{14}].

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THE EFFECT OF RED AND FAR-RED LIGHT ON CAROTENOID SYNTHESIS BY ETIOLATED MAIZE SEEDLINGS

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(*Received* 18 *December* 1961)

Abstract—Maize (*Zea mays* var. South African Horse Tooth) seedlings were germinated in darkness for 4–7 days; they were then exposed either to red light (10 min), far-red light (10 min), or to red light (10 min) followed by far-red light (10 min), and returned to darkness for a further period of either 24 or 48 hr. Red light stimulated carotenoid synthesis and growth during this period; far-red light had little effect on either; whilst illumination with far-red light following red light nullified the effect of the latter. The stimulation is most marked in young seedlings. It is concluded that the effects observed are mediated by a phytochrome system, but that the stimulation of carotenoid synthesis is not a primary site of action of the phytochrome system.

HENDRICKS and his colleagues (see e.g. Hendricks[1]) have examined in detail the photic control of flowering, etiolation, germination of light-sensitive seeds, induction of anthocyanin formation and many other growth responses. They all appear to be mediated by phytochrome, which is a soluble chromoprotein. A characteristic of all phytochrome-sensitive reactions is that illumination with a small dose of red light (660 mμ) stimulates the response, whilst illumination with far-red light (730 mμ) reverses the effect. Withrow *et al.*[2] and Virgin[3,4] have observed a phytochrome-controlled effect on chlorophyll synthesis. When normal etiolated seedlings are brought into the light there is a lag period of some hours before chlorophyll synthesis begins; however, if the seedlings are exposed briefly to red light 4 to 6 hr prior to illumination, then the lag period is abolished. Mitrakos[5] and Price and Klein[6] have confirmed these observations and demonstrated that far-red light counteracts this effect.

We decided to see whether carotenoid synthesis was similarly affected, and during this investigation found that carotenoid synthesis in the dark was stimulated by a short exposure to red light.

RESULTS

A. Reproducibility of Results

A number of experiments were carried out to determine the variability in different samples of ten seedlings grown in the same box. The results of one experiment with 4-day seedlings and of another with 5-day seedlings are given in Table 1. These results indicate the extent of scatter usually encountered and indicate how clear-cut are the results presented.

[1] S. B. HENDRICKS, *Comparative Biochemistry of Photoreactive Systems.* (Ed. by M. B. ALLEN), p. 303 Academic Press, New York (1956).
[2] R. B. WITHROW, J. B. WOLFF and L. PRICE, *Plant Physiol.* **31**, (Suppl.) (1956).
[3] H. VIRGIN, *Physiol. Plant.* **10**, 445 (1957).
[4] H. VIRGIN, *Physiol. Plant.* **11**, 347 (1958).
[5] K. MITRAKOS, *Physiol. Plant.* **14**, 497 (1961).
[6] W. H. KLEIN and L. PRICE, *Plant. Physiol.* **36**, 733 (1961).

TABLE 1. VARIATION IN CAROTENE AND DRY WEIGHT LEVELS IN BATCHES OF TEN SEEDLINGS GROWN UNDER IDENTICAL CONDITIONS

Batch No.	4-day seedlings				5-day seedlings			
	Fresh wt. (mg)	Dry wt. (mg)	Carotenoids Total (μg)	Carotenoids Concn. (μg/mg dry wt.)	Fresh wt. (mg)	Dry wt. (μg)	Carotenoids Total (μg)	Carotenoids Concn. (μg/mg dry wt.)
1	170	30	8·0	0·27	290	44	20·8	0·47
2	175	29	11·2	0·39	300	45	20·8	0·46
3	150	28	9·2	0·33	290	43	20·2	0·47
4	200	37	9·8	0·27	370	55	24·0	0·44
Mean	175	31·5	9·55	0·315	312	47	21·4	0·46

B. Effect of Red Light

Seedlings were germinated in the dark for between 4 and 7 days, exposed to red light for 10 min and returned to darkness. They were then examined, along with appropriate controls, 24 and 48 hr later. The results of a series of typical experiments are recorded in Table 2. The main conclusions which can be drawn from these observations are (*a*) red light stimulates both growth (dry wt.) and carotenoid synthesis in young (4–5 day) seedlings: (*b*) the effect is much less marked in older (6–7 day) seedlings; (*c*) that within 24 hr

TABLE 2. THE EFFECT OF RED LIGHT ON CAROTENOID CONTENT OF LEAVES OF MAIZE COLEOPTILES

Seedlings germinated in darkness for time indicated, then exposed to red light for 10 min and returned to darkness for times indicated; batches of 10 seedlings examined in each determination

	Dry wt. (mg/leaf)	Carotenoids Total (μg/leaf)	Carotenoids Concn. (μg/mg dry wt.)	% Increase in Carotenoids Amount	% Increase in Carotenoids Concn.
4-Day seedlings					
Time (hr) in darkness after red light					
0	2·6	0·9	0·35		
24 Red light	6·1	2·6	0·43	53	40
Control	5·6	1·7	0·30		
48 Red light	8·7	5·4	0·62	39	18
Control	7·4	3·9	0·53		
5-Day seedlings					
0	6·5	2·9	0·45		
24 Red light	13·0	7·7	0·59	88	20
Control	8·3	4·1	0·49		
48 Red light	18·3	11·2	0·61	38	9
Control	14·5	8·1	0·56		
6-Day seedlings					
0	10·5	5·0	0·48		
24 Red light	21·9	8·5	0·39	29	2
Control	17·3	6·6	0·38		
48 Red light	30·2	10·3	0·34	−5	−3
Control	30·8	10·9	0·35		
7-Day seedlings					
0	10·2	5·7	0·56		
24 Red light	30·6	13·6	0·44	15	−4
Control	25·4	11·7	0·46		

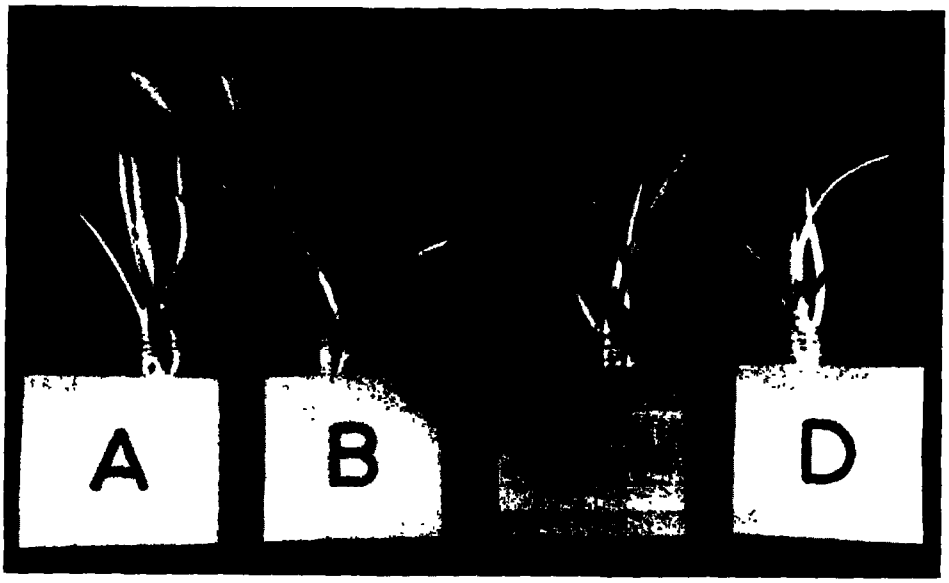

FIG. 1. EFFECT OF RED LIGHT ON GROWTH OF ETIOLATED MAIZE SEEDLINGS.

A. 5-day seedlings grown entirely in the dark.
B. 5-day seedlings grown in the dark except for 10 min exposure to red light after 4 days.
C. 5-day seedlings grown in the dark except for 10 min exposure to red light followed by 10 min exposure to far-red light after 4 days.
D. 5-day seedlings grown in the dark except for 10 min exposure to far-red light after 4 days.

[*facing page 68*]

of red light treatment of young seedlings carotenoid synthesis is stimulated to a greater extent than growth (concentration increases of 40 per cent and 20 per cent in 4- and 5-day seedlings, respectively); (*d*) within 48 hr of red light treatment growth stimulation has caught up with carotenoid stimulation. The reality of the growth-stimulating effect of red light can be seen in Fig. 1.

C. Effect of Far-Red and Red Light

As indicated in the introduction, a characteristic of reactions mediated by phytochrome is that the effect of red light illumination can be nullified by a subsequent exposure to far-red light. The results of a typical experiment involving 4-day seedlings are given in Fig. 2. From this figure it can be seen (*a*) that far-red light has no effect on growth (dry wt.) or

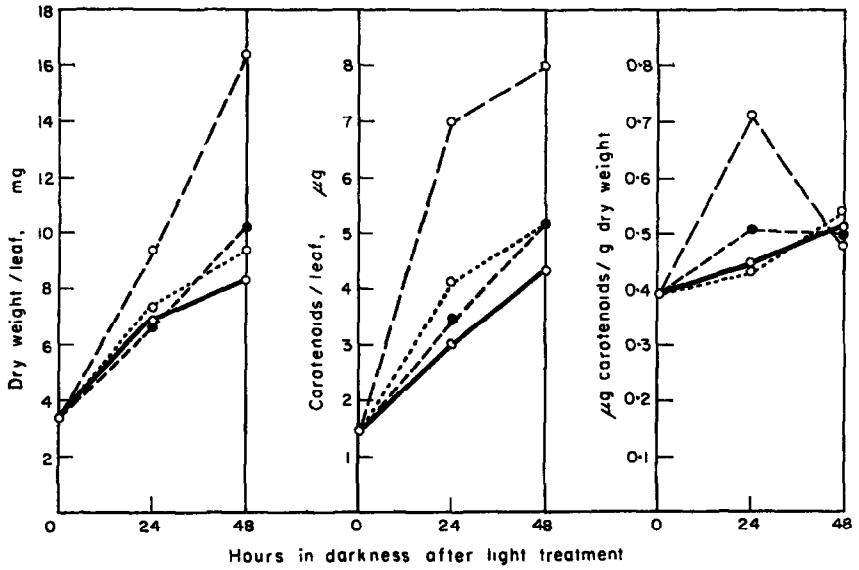

FIG. 2. THE EFFECT OF RED AND FAR-RED LIGHT ON GROWTH AND CAROTENOID PRODUCTION BY 4-DAY ETIOLATED MAIZE SEEDLINGS.

O———O Control—no red or far-red light.
O — — — O Seedlings exposed to red light for 10 min and returned to darkness for times indicated.
● — — — ● Seedlings exposed to far-red light for 10 min and returned to darkness for times indicated.
O ------- O Seedlings exposed to red light for 10 min and then to far-red light for 10 min before being returned to darkness for time indicated.

carotenoid synthesis, and (*b*) that exposure to far-red light following red light illumination nullifies the effect of the red light. It will also be noted that the red light effect observed in these experiments confirms the results already given in Table 2 and Fig. 1.

D. The Nature of the Carotenoids

It has already been shown that the carotenoid pigments present in maize seedlings are almost entirely composed of xanthophylls,[7] with little if any β-carotene present. The absorption spectrum of the total pigments extracted from seedlings which had received

[7] T. W. GOODWIN, *Biochem. J.* **70**, 612 (1958).

red light treatment is essentially the same as that for seedlings kept entirely in the dark (Fig. 3). The two extracts were separated into their components by thin layer chromatography using Kieselgel G/rice starch as adsorbent and hexane/ethyl acetate (4 : 1) as solvent. The chromatograms showed that the extracts were qualitatively identical, with xanthophylls as the major components.

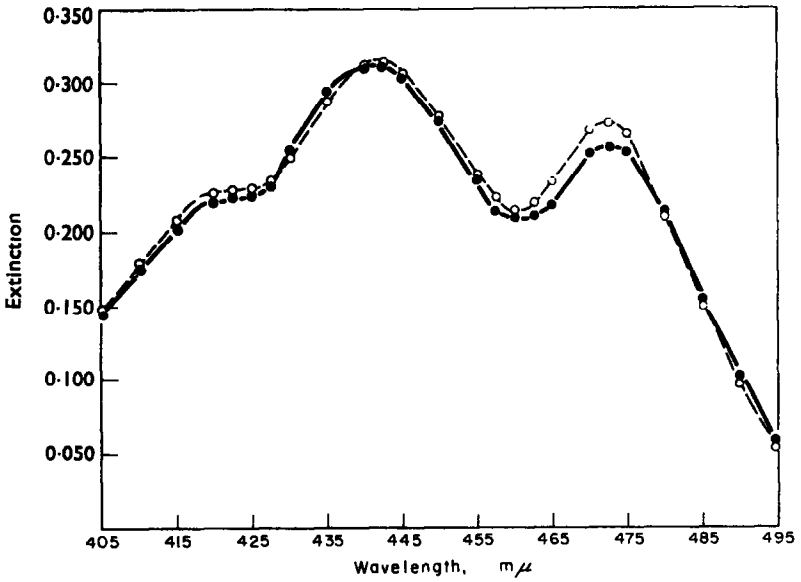

FIG. 3. THE ABSORPTION SPECTRA OF THE CAROTENOID EXTRACTS.

●——● 5-day seedlings grown entirely in the dark.

O— — —O 5-day seedlings grown entirely in the dark except for 10 min exposure to red light after 4 days.

(The extinctions of the peaks at 443 mμ have been made to coincide for easy comparison.)

DISCUSSION

These results clearly indicate that a brief exposure to red light stimulates carotenoid synthesis in etiolated maize seedlings, and the reversal of this effect by far-red light (Fig. 2) shows that it is a phytochrome-mediated reaction. However, growth is also stimulated by red light and although with young (4–5 day) seedlings there is a preferential stimulation of carotenoid synthesis for 24 hr after illumination, the effect is not maintained. It must therefore be concluded that the effect on carotenoids is not a primary locus of action of the red-far-red phenomenon.

It is interesting that in one experiment in a report concerned with phototropism Asomaning and Galston[8] recently recorded that a 10–20 min exposure to red light of etiolated oat seedlings increased the amount of carotenoids present per coleoptile; however, the concentration of pigment on a fresh weight was reduced.

When this manuscript was completed a report by Mego and Jagendorf[9] reached us in which the authors quote, in passing, from their own unpublished work that in *Phaseolus vulgaris* L. var. Black Valentine carotenoids also increase in the leaf during expansion in

[8] E. J. A. ASOMANING and A. W. GALSTON, *Plant Physiol.* **36**, 453 (1961).
[9] J. I MEGO and A. T. JAGENDORF, *Biochim. et Biophys. Acta*, **53**, 237 (1961).

the dark. This observation agrees qualitatively with ours, but quantitatively it appears that the effect in beans is much greater. Mego and Jagendorf quote a six-fold increase in amount per leaf; we have never encountered more than a doubling in amount. Mego and Jagendorf have also clearly demonstrated that plastids of etiolated Black Valentine bean leaves can be induced to grow in the dark by a brief treatment with red (or white) light and that this can be counteracted by subsequent irradiation with far-red light. The growth of plastids is accompanied by a synthesis of protein and lipid; the plastid nitrogen reaches the levels observed in mature chloroplasts, but the final lipid levels are only about two-thirds of those in the mature chloroplast. Although a stimulated synthesis of chlorophyll was observed, this only represented the conversion of the protochlorophyll already present, and it was not reversed by far-red irradiation. The synthesis of carotenoids which we have observed is, similarly, comparatively small and the levels attained are very much less than those in green tissues.[10]

Mego and Jagendorf[9] show by electron microscopy that the stimulation of plastid growth by red light does not involve formation of grana; presumably the massive synthesis of chloroplast pigments, both chlorophylls and carotenoids, occurs along with the formation of lamellar systems and grana.

It is clear from Fig. 3 that the spectrum of the carotenoids which are synthesized in response to red light is the same as that of carotenoids formed in seedlings kept in complete darkness. Chromatography showed that the two extracts are qualitatively the same and consist mainly of xanthophylls. They are not the typical mixture of β-carotene and xanthophylls in the ratio of about 1 : 4 which is found in chloroplasts.

EXPERIMENTAL

Materials

Maize seedlings (South African Horse Tooth) were germinated in Vermiculite (vermiculite : water, 3 : 1 vol./vol.) in darkness in a warm room (25°). When required, they were withdrawn in complete darkness, illuminated for 10 min, with a source of red light; this was followed in some experiments by exposure to far-red light for 10 min. The seedlings were then returned to the growth chamber. Control samples were taken for analysis at this point and further samples were collected after appropriate time intervals. Each sample consisted of ten seedlings, in order to reduce as far as possible errors due to individual variations between seedlings.

The coleoptiles plus sheaths were cut off at the first internode and separated. The coleoptiles were weighed and taken for analysis. The excisions were carried out in the dark, and the weighings in dim green light.

Carotenoid Determinations

The leaves were cut into small pieces in a mortar containing a small quantity of acid-washed silver sand, and extracted by grinding with acetone.[7,10] The acetone extracts were transferred to ethyl ether by adding an equal volume of ether and then water dropwise until two layers formed. The ethereal solution was made up to a known volume and E (442 mμ) measured in 1 cm cells in a photoelectric spectrophotometer (Unicam SP 500). The total carotenoids, which are mainly xanthophylls,[8] were calculated on the assumption that $E^{1\%}_{1\,cm}$ was 2500; that is the same as that of β-carotene.[10] For separation by thin

[10] T. W. GOODWIN, *Handbook of Plant Analysis* (Ed. by K. PAECH and M. V. TRACEY), 3, Springer, Heidelberg (1954).

layer chromatography, the ethereal extract was spotted on to plates holding Kieselgel G/rice starch (20 : 1) as adsorbent. The chromatogram was developed with a 1 : 4 (vol./vol.) mixture of ethyl acetate and hexane.

Dry Weight Determinations

Dry weights were obtained by drying to constant weight at 100°.

Filters

The sources of illumination were (*a*) a red fluorescent tube (Ekco, double life red, 80 W) with no transmission in the far-red, used in conjunction with a red perspex filter as a source of red light, and (*b*) a 75 W tungsten filament lamp used in conjunction with an Ilford Far-red Filter No. 207 as a source of far-red light. The transmission curves of the two filters are recorded in Fig. 4. The seedlings were placed 20 cm away from the red light source and 8–10 cm away from the far-red source.

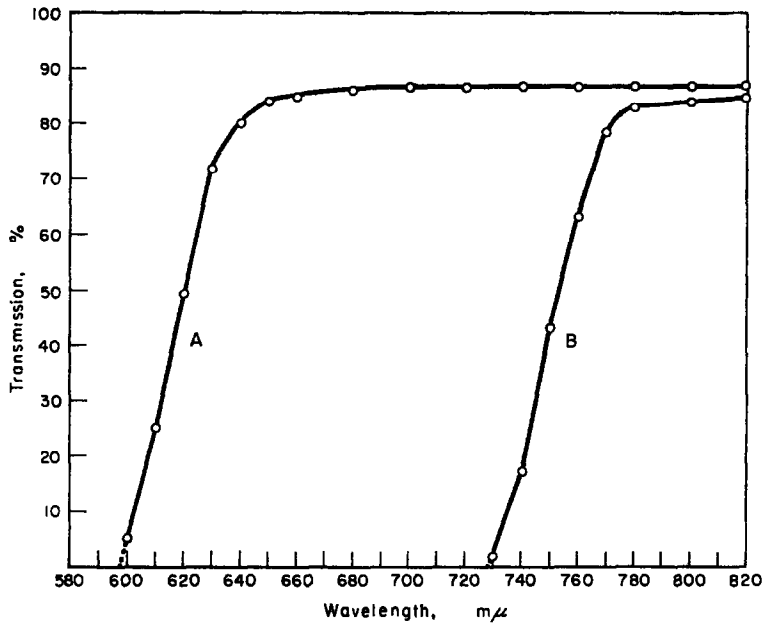

FIG. 4. THE TRANSMISSION CURVES OF THE FILTERS USED.

A. Red filter (red perspex).

B. Far-red filter (Ilford No. 207).

Acknowledgements—We are grateful to Professor P. F. Wareing (Department of Botany, U.C.W., Aberystwyth) for the loan of the Ekco red fluorescent tube, and R. W. Gunston (Seeds) Ltd. for a generous gift of maize seeds.

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PLANT POLYPHENOLS. VII. THE HYDROXYCINNAMOYL ESTERS OF *POLYGALA SENEGA* ROOT*

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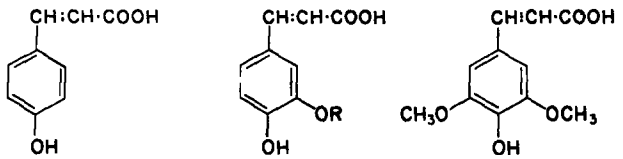
Abstract—At least five hydroxycinnamoyl esters are present in the root of *Polygala senega* (snake root). Structural studies are incomplete, but these esters have been shown to be derivatives of either *p*-coumaric, *p*-methoxycinnamic, ferulic, sinapic, or 3,4,5-trimethoxycinnamic acid. In these esters one or two types of hydroxycinnamoyl residue are present in association with several D-glucose units. The occurrence in plants of a new derivative of 3,4,5-trimethoxycinnamic acid is noteworthy.

INTRODUCTION

RECENTLY an increasing interest has been developing in the derivatives of various cinnamic acids which occur naturally. This is in part due to their wide occurrence in plants,[1] the diversity of structure among the natural products with which they are associated,[2,3] and the recognition that the cinnamic acids are of considerable importance as biosynthetic intermediates in the formation of many types of natural product.[4]



I II III

The cinnamic acids which occur most widely are *p*-coumaric acid (I), ferulic acid (II, R = CH_3), caffeic acid (II, R = H), and sinapic acid (III)' The existence of *O*-glycosides derived from these acids is known,[2,3] but their occurrence in an ester form is much more frequent[3] and includes acyl derivatives of certain flavonoid glycosides, *p*-coumaroyl-quinic acid, and the caffeoyl-quinic acids such as chlorogenic acid.[5] However, it is only recently that simple mono-esters, derived from these four cinnamic acids and either D-glucose, rutinose, or gentiobiose, have been recognized as natural products.[3,6-9] We now wish to describe a preliminary investigation of a group of structurally related esters isolated from the root of *Polygala senega*. Although these substances could not be isolated in a pure state

* Part VI, J. B. HARBORNE, *Biochem. J.*, in press.

[1] E. C. BATE-SMITH, *Chem. and Ind.* (*London*), 1457 (1954); *Sci. Proc. Roy. Dublin Soc.*, **27**, 165 (1956).
[2] W. KARRER, *Konstitution und Vorkommen der Organischen Pflanzenstoffe*, pp. 379–384, Birkhäuser Verlag (1958).
[3] J. B. HARBORNE and J. J. CORNER, *Biochem. J.*, **81**, 242 (1961).
[4] H. GRISEBACH and W. D. OLLIS, *Experientia*, **17**, 4 (1961).
A. C. NEISH, *Ann. Rev. Plant Physiol.*, **11**, 55 (1960).
[5] C. LENTNER and F. E. DEATHERAGE, *Chem. and Ind.* (*London*), 1331 (1958).
[6] J. J. CORNER and J. B. HARBORNE, *Chem. and Ind.* (*London*), 76 (1960).
[7] J. B. HARBORNE and J. J. CORNER, *Arch. Biochem. Biophys.*, **42**, 192 (1961).
[8] J. B. HARBORNE and J. J. CORNER, *Biochem. J.*, **76**, 53P (1960).
[9] J. BIRKHOFER, C. KAISER, W. NOUVERTNÉ and U. THOMAS, *Z. Naturforsch.*, **16b**, 249 (1961).

in sufficient quantity to permit their complete structural elucidation, it has been shown that they are derived from either *p*-coumaric, ferulic, 3,4,5-trimethoxycinnamic, or sinapic acid and D-glucose and that they are not simple mono-esters. They apparently have a structure in which pairs of hydroxycinnamoyl residues are associated with glucose units in molecules of undetermined size.

The study of the esters of sinapic acid in *Polygala senega* was made because although it was known that sinapic acid occurred quite widely in plants,[1] the form in which it was present had not been established except for the choline esters sinapin[10] and sinalbin.[11] The earlier chemical investigations[12] of this plant had been restricted to its constituent triterpenoid sapogenins and it is of interest that the root is still used as a stimulant expectorant in the treatment of chronic bronchitis.

RESULTS

Four compounds, *A*1, *A*2, *A*3, and *E*1, were isolated in a chromatographically homogeneous form by chromatographic fractionation of the methanol extract of *Polygala senega* root. Their chromatographic behaviour, ultraviolet spectra, and colour reactions (see Table 1) indicated that *A*1, *A*2, *A*3, and *E*1 were hydroxycinnamoyl esters. Mild alkaline hydrolysis (see Table 2) of *A*1 gave sinapic and 3,4,5-trimethoxycinnamic acids, *A*2 gave sinapic acid, *A*3 gave ferulic acid, and *E*1 gave ferulic and *p*-coumaric acids. *A*1 and *A*2 also yielded D-glucose on mild alkaline hydrolysis, but the acylated groups in *A*3 and *E*1 were not identified though chromatographic comparison showed that they were not D-glucose or quinic acid. The search for the latter compound was suggested by a possibility that *A*3 and *E*1 had structures analogous to *p*-coumaroyl-quinic or the chlorogenic acids, but as *A*3 and *E*1 were isolated only in minute amounts, their further structural examination was not possible.

TABLE 1. R_f VALUES AND U.V. SPECTRA OF CINNAMIC ACID ESTERS

Ester	R_f values in [a]			Colours [b]		Spectral maxima (mμ) in	
	BAW	BN	BEW	A	B	95% EtOH	95% EtOH (3ml) + 2N NaOH (0·1 ml)
*A*1	0·56	0·24	0·62	db	gr	234, 318	257, 305, 395
*A*2	0·45	0·12	0·50	mb	gr	242, 332	259, —, 392
*A*3	0·34	0·04	0·35	p	gr	233, 327	255, 305, 387
*E*1	0·55	0·21	0·35	mb	gr	235, 326	262, —, 380
*X*1	0·51	0·35	0·14	p	p	230, 307	230, 305
1-(3,4,5-Trimethoxycinnamoyl)-glucose[c]	0·62	0·45	0·83	p	p	230, 307	230, 307
*X*2	0·38	0·03	0·04	mb	gr	245, 334	260, 395
1-Sinapoylglucose[c]	0·50	0·15	0·23	mb	gr	238, 330	255, 397
Sinapin	0·59	0·09	—	mb	gr	245, 270, 330	230, 265, 305, 390

[a] See Experimental for composition of solvents.
[b] Key: A, in u.v. light, B, in u.v. light with NH_3 vapour; p = purple; gr = green; db = dark blue; mb = medium blue.
[c] Data taken from Harborne and Corner.[3]

[10] J. GADAMER, *Arch. Pharm.*, **92**, 235 (1897); *Ber.*, **30**, 2328 (1897).
[11] M. G. ETTLINGER and A. J. LUNDEEN, *J. Am. Chem. Soc.*, **78**, 4173 (1956).
[12] E. WEDEKIND and R. KRECKE, *Ber.*, **57B**, 1118 (1924).
W. A. JACOBS and O. ISLER, *J. Biol. Chem.*, **119**, 155 (1937).
M. SHAMMA and L. P. REIFF, *Chem. and Ind. (London)*, 1272 (1960).

TABLE 2. ALKALINE AND ESTERASE HYDROLYSIS PRODUCTS OF CINNAMIC ACID ESTERS

Ester	Hydrolysis products and molar ratios
*A*1	Sinapic acid (1·0), 3,4,5-trimethoxycinnamic acid (0·97), glucose (0·17).
*A*2	Sinapic acid (2·0), glucose (3·45). (Average of five separate determinations.)
*A*3	Ferulic acid (+), glucose (absent). (Qualitative experiment.)
*E*1	Ferulic acid (+), *p*-coumaric acid (+), glucose (absent) (Qualitative experiment).
*X*1	3,4,5-Trimethoxycinnamic acid (1·0), glucose (0·93).
*X*2	Sinapic acid (1·0), glucose (1·30).

A more detailed study of *A*1 and *A*2 has been made. *A*1 had an ultraviolet spectral maximum (λ_{max} = 318 mμ) almost exactly intermediate between that of methyl sinapate (λ_{max} = 327 mμ) and methyl 3,4,5-trimethoxycinnamate (λ_{max} = 307 mμ). Mild alkaline hydrolysis of *A*1 yielded approximately equivalent amounts of sinapic acid and 3,4,5-trimethoxycinnamic acid and a relatively smaller amount of glucose. Apparently D-glucose was the only carbohydrate present in the alkaline hydrolysis product from *A*1 and no di- or tri-saccharides were detectable. With a trace of alkali in 95% ethanol solution, *A*1 showed a large bathochromic shift indicating that the hydroxyl groups of the sinapoyl residues were free and were not involved in ester or glycosidic bond formation. Enzymatic hydrolysis of *A*1 with an esterase preparation yielded an intermediate ester *X*1 (see Table 1) which was identified as a monoester derived from one 3,4,5-trimethoxy-cinnamic acid unit and a D-glucose unit. The substance *X*1 was different from the known[8] 1-(3,4,5-trimethoxy-cinnamoyl)-D-glucose and was not hydrolysed by β-glucosidase. Thus *A*1 contains a D-glucose residue linked *via* ester bonds to several sinapoyl and 3,4,5-trimethoxycinnamoyl residues which are probably present in equal numbers.

*A*2 differs from *A*1 in that mild alkaline hydrolysis yielded only sinapic acid and a relatively higher proportion of glucose. Enzymatic hydrolysis of *A*2 similarly gave a monoester, *X*2, containing one sinapoyl and one D-glucosyl residue; *X*2 was different from the known 1-sinapoyl-D-glucose.[3] *A*2 is a complex ester derived from glucose and sinapic acid.

Thus there are at least four esters present which are derived from sinapic, 3,4,5-trimethoxy-cinnamic, ferulic, and *p*-coumaric acids and by the direct hydrolysis of the extract of *Polygala senega* it was also possible to isolate *p*-methoxycinnamic acid. It is probable that this acid is also present as an ester, though this was not established.

DISCUSSION

The number of different cinnamic acids present as esters in *Polygala senega* root is unusually large, and although experimental difficulties have prevented the elucidation of the structure of these esters, it is clear that *A*1 and *A*2 are polycinnamoyl derivatives of D-glucose, containing sinapoyl and 3,4,5-trimethoxycinnamoyl residues. In this respect they resemble some of the hydrolysable tannins[13] which are similarly derived from gallic acid and D-glucose.

The esters from *Polygala senega* differ from the other carbohydrate derivatives of hydroxycinnamic acid which occur naturally in two respects. The latter compounds have

[13] R. D. HAWORTH, *Proc. Chem. Soc.*, 401 (1961).

so far only been recognised as mono-esters in which the hydroxycinnamoyl group is located glycosidically, that is in position 1, on either the D-glucose, rutinose, or gentiobiose residue.[3]

The structures of the five cinnamic acids present shows that there is a biosynthetic predisposition towards phenolic *O*-methylation in *Polygala senega*. In this connection the isolation of *p*-methoxycinnamic and 3,4,5-trimethoxycinnamic acid is of interest, particularly as caffeic acid, which is of common occurrence in other plants, was not detected. The isolation of 3,4,5-trimethoxycinnamic acid is quite unusual: it has only been detected previously in two natural products as an esterifying group in the *Rauwolfia* alkaloids, rescinnamine[14] and rescidine.[15]

EXPERIMENTAL

Chemicals and Materials

Sinapin was isolated from the seeds of *Sinapis alba*,[10] and 1-sinapoyl-D-glucose from the leaves of *Brassica oleracea* var. rubra and the flowers of *Matthiola incana*.[3] 1-(3,4,5-Trimethoxycinnamoyl)-D-glucose was prepared by feeding 3,4,5-trimethoxycinnamic acid to the leaves of *Solanum chacoense*.[3]

A sample of anthocyanase, prepared from a strain of *Aspergillus niger*, was supplied by Rohm and Haas Company, Philadelphia, U.S.A. The *Polygala senega* root was obtained from Brome and Schimmer Limited, London.

Paper Chromatography and Spectroscopy

Whatman No. 1 paper was used and the R_f values in Table 1 were determined using the following solvent systems: BAW (*n*-butanol, acetic acid, water—4 : 1 : 5, top layer), BEW (*n*-butanol, ethanol, water—4 : 1 : 2·2), BN (*n*-butanol, 2N ammonium hydroxide—1 : 1, top layer). In measuring the ultraviolet spectra of substances eluted from paper chromatograms, allowance was made for impurities eluted from the paper by a blank correction using the eluates from similar paper. Chromatograms were examined in ultraviolet light in the presence and absence of ammonia (see Table 1).

Extraction of Polygala senega *Root*

Powdered root (11·8 kg) was continuously extracted with hot ether to remove fats and then with hot methanol. The methanol extract was concentrated, cooled to 0°, ethanol added, and the precipitated crude triterpenoid glycoside collected. The filtrate was concentrated and after keeping at 0° crystalline sucrose separated. This was removed and evaporated giving a syrupy residue to which saturated aqueous lead acetate solution was added. The precipitated lead salt was collected (giving filtrate *A*, see below), washed with water and with ethanol, suspended in 60 per cent aqueous ethanol, and then treated with hydrogen sulphide. The precipitated lead sulphide was removed and the filtrate was lyophilized giving a residue (fraction *A*) (165 g).

Saturated basic led acetate solution was added to filtrate *A*, giving a precipitate which when treated as above with hydrogen sulphide yielded a residue (fraction *E*).

*Isolation of the Esters A*1, *A*2, *A*3 *and E*1

Fraction *A* was dissolved in 70 per cent aqueous ethanol and applied as streak to Whatman No. 3 (chromatography grade) paper. Elution with BAW gave three main bands

[14] M. W. KLOHS, M. D. DRAPER and F. KELLER, *J. Am. Chem. Soc.*, **76**, 2843 (1954).
[15] A. POPELAK, E. HAACK, G. LETTENBAUER and H. SPRINGLER, *Naturwissenschaften*, **48**, 74 (1961).

(R_f 0·66, 0·53, and 0·44) which were fluorescent in ultraviolet light. These bands were cut out and separately eluted with 70 per cent aqueous ethanol. Further chromatography of these three fractions using water, BN, and then BEW gave the fractions, *A*1, *A*2, and *A*3, which were homogeneous by chromatographic examination.

Fraction *E* was similarly separated using BEW as the eluting solvent and this gave two main bands (R_f 0·38 and 0·26). The slower-moving material was not investigated as it was strongly absorbed on the paper, but further paper chromatography of the band (R_f 0·38) gave *E*1 which was homogeneous by chromatographic examination.

Hydrolysis of Hydroxycinnamoyl Esters

During hydrolyses using the following methods, paper chromatographic examination of trace samples of the reaction mixtures indicated the extent to which starting materials had been hydrolysed. The following products of these hydrolyses were identified using the methods described below.

(i) *Alkaline hydrolysis.* Two drops of 2N aqueous sodium hydroxide were added to a concentrated aqueous solution of the ester (~ 1 mg) and the solution was kept at 15° for 3 hr in a nitrogen atmosphere. After neutralization with the ion exchange resin Amberlite IR-120(H), the solution was examined[3] both qualitatively and quantitatively (see Table 2) for hydroxycinnamic acids, sugars, and other possible products.

(ii) *Esterase hydrolysis.* The esterase solution (Rohm and Haas anthocyanase at a concentration of 100 mg/ml) was added to a solution of the ester in a phosphate–citric acid buffer (pH 3·9) and the mixture kept at 37° for 24 hr.

(iii) *Attempted hydrolysis with β-glucosidase.* β-Glucosidase (1 mg in 0·1 ml water) was added to a solution of the ester in sodium acetate buffer (pH 5) and the mixture was kept at 37°. Samples of the reaction mixture and a control solution, free of enzyme, were taken at 30 min intervals during 5 hr and chromatographed on Whatman No. 1 paper using BAW and water as solvents.

Examination of Hydrolysis Products

The various hydroxycinnamic acids (see Table 2) were identified[16] by ultraviolet, spectroscopic, and paper chromatographic comparison with authentic samples. 3,4,5-Trimethoxycinnamic acid isolated from an alkaline hydrolysis of a sample of fraction *A* yielded colourless crystals, m.p. 125–126°, which were identified by mixed m.p., infra-red spectral comparison (Nujol mull), and analysis (Found: C, 60·7; H, 5·9; OMe, 38·2. Calc. for $C_{12}H_{14}O_5$: C, 60·5; H, 5·9; OMe, 39·1%).

Glucose was identified and estimated by published methods[3] and the possible presence of other sugars, quinic acid, and other organic acids was investigated using appropriate sprays.[3]

The Formation of the Esters X1 and X2

Esterase solution (5 ml, Rohm and Haas anthocyanase, concentration 100 mg/ml) was added to a solution of fraction *A* (1 g) in phosphate–citric acid buffer (10 ml, pH 3·9) and the mixture kept at 37° for 6 hr. Chromatography (BAW solvent, No. 3 paper) gave two bands (R_f 0·51 and 0·38) which by further paper chromatographic purification yielded the mono-esters *X*1 and *X*2.

Similar hydrolyses of *A*1 gave *X*1 only, and *A*2 gave *X*2 only.

[16] T. A. GEISSMAN and J. B. HARBORNE, *Arch. Biochem. Biophys.*, **55**, 447 (1955).

Phytochemistry, 1962, Vol. 1, pp. 79 to 85. Pergamon Press Ltd. Printed in England

ETUDE DES DIOLS DES CIRES DE POMME ET DE CARNAUBA

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Résumé—Les diols des deux cires, obtenus par chromatographie sur alumine de l'insaponifiable, à 50°C, sont transformés en diacétates de diols par acétylation pyridinée. Ces diacétates sont alors étudiés par chromatographie en phase gazeuse, dans le système hélium–silicone rubber à 300 et 350°. Tous les diols pairs de C_{20}àC_{32} sont ainsi mis en évidence dans la cire de Carnauba; la cire de pomme contient tous les diols pairs de C_{20}àC_{28} L'existence dans les cires, de diols à nombre impair d'atomes de carbone, est discutée.

Abstract—The diols of Carnauba and apple wax, obtained by chromatography on alumina of the unsaponifiable portion of the wax at 50°C, were acetylated and the diol diacetates studied by gas-chromatography, using a helium–silicone rubber system, at 300 and 350°. All even diols from C_{20}–C_{32} were shown to be present in Carnauba wax; apple wax was found to contain all even diols, from C_{20}–C_{28}. The existence of odd carbon-number diols in waxes is discussed.

LES cires végétales sont essentiellement composées de monoesters (cérides) d'acides gras et d'alcools aliphatiques à longue chaîne; les paraffines de haut poids moléculaire forment une catégorie de constituants supplémentaires particulièrement importante pour les cires végétales des pays tempérés (cire de pomme par exemple), tandis que ce sont au contraire les chaînes moléculaires oxygénées (alcools, diols, hydroxyacides, estolides . . .) qui forment en grande partie les cires d'origine tropicale comme la cire de Carnauba.

Dans un article précédent[1], nous avons décrit une microméthode permettant l'étude des acides, alcools et paraffines des cires végétales. Les produits fortement oxygénés (diols et hydroxyacides) avaient alors été laissés de côté. Nous présentons ci-dessous une technique permettant d'étendre la méthode d'analyse proposée aux dialcools aliphatiques (*n*-*α*-*ω*-diols) entrant dans la composition des cires. Ces composés de formule générale $HOH_2C—(CH_2)_n—CH_2OH$ ont été fort peu étudiés jusqu'à présent. La présence de cette catégorie de corps dans les cires a été signalée pour la première fois par Sturcke[2] en 1884, qui isola un diol de la cire de Carnauba. Il lui attribua la formule $HOH_2C—(CH_2)_{23}—CH_2OH$ et put obtenir le diacide correspondant $HOOC—(CH_2)_{23}—COOH$, qu'il appela acide carnaubandioïque. En 1951, Murray et Schoenfeld montrèrent que la cire de Carnauba renferme 3 pour cent de diols et ils réussirent à en isoler trois par distillation fractionnée des dérivés diacétylés: ils identifièrent ainsi les diols en C_{22}, C_{24} et C_{26}.[3]

En 1952, Musgrave, Stark et Spring[4] signalent l'existence de deux diols (le *n*-octadécane-1,18 diol et le *n*-hexacosane 1,26 diol) dans l'insaponifiable d'un extrait de genêt d'Espagne (*Spartium junceum L*). La même année Murray et Schoenfeld signalent que la cire de laine (Woolwax) contient une fraction de diols (de C_{16}–C_{24}) représentant (1,5 pour cent du poids total[5]. Ces diols sont d'ailleurs différents des diols précédemment découverts car il s'agit de 1-2-alkanediol ($CH_3—(CH_2)_n—CHOH—CH_2OH$) comme Horn et Hougen[6]

[1] P. MAZLIAK, *J. Botan. Appl. et agri tropicale.* (1961), **8**, 180–190 (1961).
[2] H. STURCKE, *Ann. Chem. Liebigs*, **223**, 283–314 (1884).
[3] K. E. MURRAY et R. SCHOENFELD, *J. Am. Oil Chemists' Soc.*, **28**, 461–466 (1951).
[4] O. C. MUSGRAVE, J. STARK et F. S. SPRING, *J. Chem. Soc.*, **4**, 4393–4397 (1952).
[5] K. E. MURRAY et R. SCHOENFELD, *J. Am. Oil Chemists' Soc.*, **29**, 416–420 (1952).
[6] D. H. S. HORN et F. W. HOUGEN, *Chem. & Ind.* 670 (1951).

l'avaient déjà signalé en 1951. En 1955 enfin, Murray et Schoenfeld complètent leur étude des diols de la cire de Carnauba en isolant de cette cire un diol supplémentaire en C_{28}.[7] Les auteurs signalent en outre que 39 pour cent des diols n'ont pu être distillés et ont par conséquent plus de 28 atomes de carbone dans la molécule.

Nous avons étudié les diols de la cire de Carnauba par chromatographie en phase gazeuse et nous avons ainsi retrouvé les diols déjà isolés précédemment, plus quelques autres nouveaux. Nous avons également mis en évidence des diols dans la cire de pomme où ces corps n'avaient jamais été trouvés auparavant.

Les diols des deux cires ont été obtenus par chromatographie sur alumine, à 50°C, de la fraction insaponifiable. La transformation préalable des diols en dérivés diacétylés est nécessaire pour l'analyse par chromatographie en phase gazeuse.

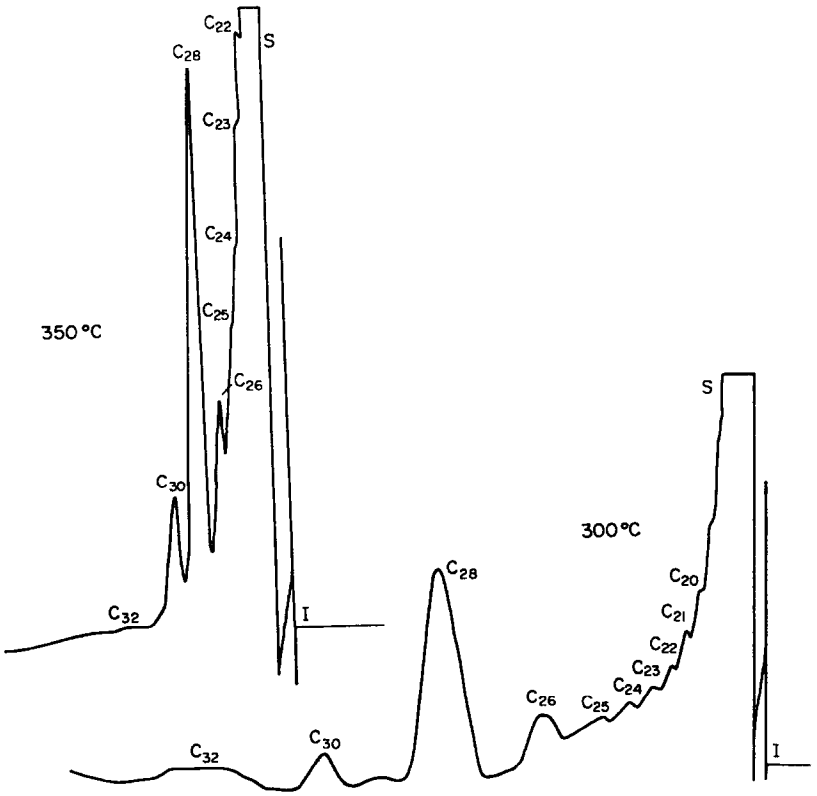

FIG. 1. DEUX CHROMATOGRAMMES DES *n*-α-ω-DIOLS DE LA CIRE DE CARNAUBA, OBTENUS À 300° ET 350°.
(*I*: injection, *S*: solvant.)

Les Diols de la Cire de Carnauba

La Fig. 1 représente deux chromatogrammes en phase gazeuse de diols acétylés de la cire de Carnauba, obtenus à 300° et 350°C. Quatre pics principaux peuvent être observés sur ces chromatogrammes. Les volumes de rétention (Tableau 1) de ces corps les font correspondre aux diols suivants: 1,32-dotriacontanediol, 1,30-triacontanediol, 1,28-octa-

[7] K. E. MURRAY et R. SCHOENFELD, *Australian J. Chem.*, 8, 432–436 (1955).

cosanediol, 1,26-hexacosanediol. Six pics supplémentaires apparaissent sur les chromatogrammes; ces corps nouveaux, en beaucoup plus faible quantité que les quatre diols précédents, ont même volume de rétention que le 1,20-eicosanediol, le 1,21-heneicosanediol, le 1,22-docosanediol, le 1,23-tricosanediol, le 1,24-tetracosanediol et le 1,25-pentacosanediol. L'existence, dans la cire de Carnauba, des diols pairs en C_{20}, C_{22} et C_{24} est très probable, à côté des diols principaux en C_{26}, C_{28}, C_{30} et C_{32}. Par contre la présence, dans cette cire de diols à nombre impair d'atomes de carbone en C_{21}, C_{23} et C_{25} peut être acceptée avec réserve, puisqu'il semble bien que les diols des cires soient généralement pairs. Les pics

TABLEAU 1 *α-ω*-DIOLS DE LA CIRE DE POMME ET DE LA CIRE DE CARNAUBA

Nom systématique	Log V_R du témoin	*Cire de Carnauba* log des V_R mesurés sur le chromatogramme	*Cire de Pomme* log des V_R mesurés sur le chromatogramme
1,20-eicosanediol	—0,17	—0,15	—0,17
1,21-heneicosanediol	—0,10	—0,08	—0,10
1,22-docosanediol	0	0	0
1,23-tricosanediol	0,08	0,08	—
1,24-tetracosanediol	0,15	0,16	0,15
1,25-pentacosanediol	0,25	0,24	—
1,26-hexacosanediol	0,32	0,35	0,29
1,28-octacosanediol	0,50	0,51	0,48
1,30-triacontanediol	0,68	0,67	—
1,32-dotriacontanediol	0,83	0,80	—

Température 300°; phase stationnaire; silicone rubber (20%) sur chromosorb *W*: longueur de la colonne: 2 m: gaz vecteur; hélium. Débit 7 l/hr (surpression à l'entrée de la colonne: 1160 g/cm²—Pression à la sortie: pression atmosphérique).

représentant ces "diols impairs" pourraient aussi correspondre à des impuretés passant en chromatographie sur colonne d'alumine en même temps que la fraction "diols" (il pourrait s'agir, par exemple d'alcools à très longue chaîne ou de diols isomères).

D'un autre côté, il faut bien dire que la loi de Chibnall et Piper[8] fixant un nombre pair ou impair d'atomes de carbone pour chaque catégorie de constituants des cires, n'a jamais été étendue aux diols dont on ignore totalement le métabolisme intermédiaire. En outre, les travaux les plus récents sur la composition chimique des cires (Mazliak[1,9], Waldron *et al.*[10]), ne confirment pas entièrement la validité de la loi de Chibnall et Piper. Ainsi ont été trouvés, comme constituants mineurs des cires, des paraffines paires et des alcools impairs, alors que Chibnall et Piper assignaient, en 1934, à ces deux catégories de constituants, un nombre respectivement impair et pair d'atomes de carbone. Il n'est donc pas totalement impossible que des diols renfermant un nombre impair d'atomes de carbone dans leur molécule soient présents dans la cire de Carnauba, à côté des diols pairs (de C_{20}àC_{32}) beaucoup plus abondants.

Les Diols de la Cire de Pomme

La Fig. 2 représente un chromatogramme en phase gazeuse des diacétates de diols de la cire de pomme. Les volumes de rétention des 6 pics observés (Tableau 1) permettent d'identifier les diols suivants comme constituants de cette cire: 1,20-eicosanediol, 1,21-heneicosanediol, 1,22-docosanediol, 1,24-tetracosanediol, 1,26-hexacosanediol et 1,28-

[8] A. C. CHIBNALL et J. H. PIPER, *Biochem. J.*, **28**, 2189–2208 (1934).
[9] P. MAZLIAK, *Compt. rend.* **252**, 1507–1509 (1961).
[10] J. D. WALDRON, D. S. GONWERS, A. C. CHIBNALL et S. H. PIPER, *Biochem. J.*, **78**, 435–442 (1961).

octacosanediol. La présence de ces corps n'avaient jamais été signalée jusqu'à présent dans la cire de pommes. L'existence du diol impair 1,21-heneicosanediol appelle les mêmes remarques que pour les diols impairs de la cire de Carnauba.

On peut remarquer que la chaîne moléculaire des diols entrant dans la composition de la cire de pomme, d'origine tempérée, est plus courte que celle des diols de la cire de Carnauba, d'origine tropicale. Cette remarque est valable pour toutes les autres catégories de constituants lorsqu'on compare ces deux cires.[1]

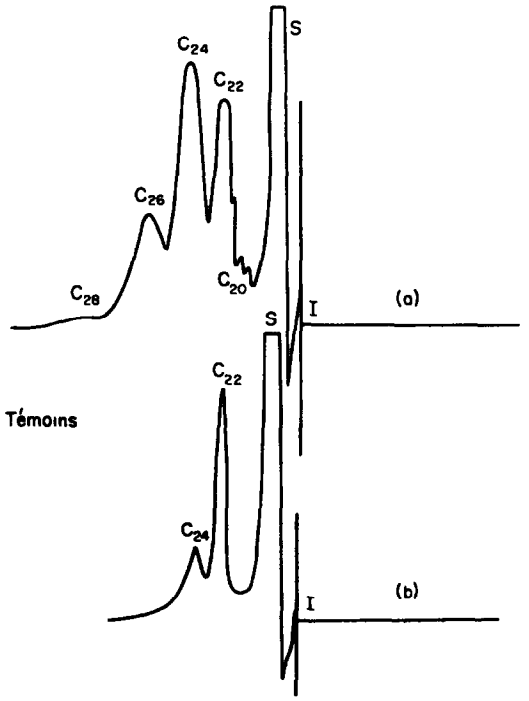

FIG. 2.
(a) CHROMATOGRAMME DES DIOLS DE LA CIRE DE POMME.
(b) CHROMATOGRAMME DU MÉLANGE TÉMOIN 1,22-DOCOSANEDIOL ET 1,24-TETRACOSANEDIOL, OBTENU DANS LES MÊMES CONDITIONS.
(*I*: injection, *S*: solvant.)

CONCLUSION

La chromatographie en phase gazeuse représente certainement une technique de choix pour l'analyse des cires végétales, même si l'on désire étudier des constituants oxygénés lourds, aussi peu volatils que les diols. Cette nouvelle catégorie de constituants des cires peut très bien avoir une répartition très générale puisqu'on trouve des diols dans deux cires aussi différentes que la cire de pomme et la cire de Carnauba. Tout récemment d'ailleurs, Downing *et al.*[11] ont aussi étudié une fraction "diols" dans la cire d'abeille, également par chromatographie en phase gazeuse, mais ces auteurs ont employé un procédé différent: les dialcools étaient transformés en hydrocarbures avant d'être chromatographiés.

[11] D. T. DOWNING, Z. H. KRANZ, K. E. MURRAY et A. H. REDCLIFFE, *Australian J. Chem.*, **14**, 253–263 (1961).

PARTIE EXPERIMENTALE

Obtention des Diols

L'insaponifiable des cires est chromatographié sur une colonne d'alumine, comme nous l'avons décrit précédemment[1]. Deux élutions successives à la température ordinaire (15°) permettent de recueillir: (1) les paraffines en employant l'éther de pétrole seul comme éluant et (2) les *alcools* en employant le mélange éther de pétrole–méthanol (dans les proportions 100 : 3 en volumes). Les diols sont élués de la colonne par le mélange benzène–éthanol (dans les proportions 20 : 1 en volumes) à la température de 50°. Les conditions expérimentales employées pour l'élution des diols sont les mêmes que celles employées précédemment par Murray et Schoenfeld.

Pour réaliser la chromatographie à 50°, nous avons fait construire le dispositif expérimental de la manière suivante. La colonne à chromatographie est entourée d'un manchon d'eau contenu dans une gaîne de verre concentrique à la colonne. L'eau est portée à 50° à l'aide d'un cordon électrique chauffant enroulé en hélice autour de la gaîne de verre et la

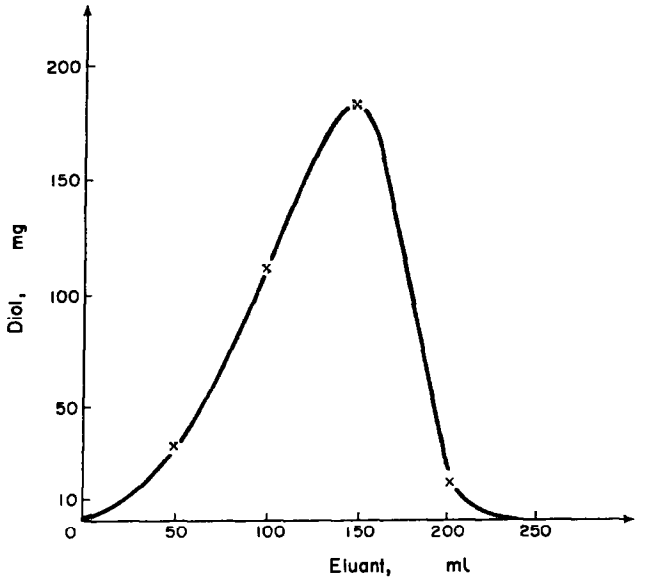

FIG. 3. COURBE D'ÉLUTION DES DIOLS DE LA CIRE DE POMME STAYMAN WINESAP CHROMATOGRAPHIÉS SUR ALUMINE À 50°.

température est maintenue constante (à $\pm$ 0,5°) à l'aide d'un thermostat, compris dans le circuit électrique et dont la sonde plonge dans le manchon d'eau. Le liquide sortant de la colonne à chromatographie est reccueilli par fractions de 50 ml. Le solvant de chaque fraction est ensuite évaporé et les diols présents dans la fraction considérée sont pesés, ce qui permet de tracer approximativement la courbe d'élution des diols hors de la colonne d'alumine. La Fig. 3 représente la courbe d'élution des diols d'un lot de cire de pommes (variété Stayman Winesap).

Preparation des Diols pour la Chromatographie en Phase Gazeuse

Le point d'ébullition des diols purs est beaucoup trop élevé pour que l'on puisse en réaliser directement des chromatographies, même à très haute température. Paquot,

Lefort et Pourchez[12] ont réussi pour les alcools gras, à surmonter la même difficulté en transformant d'abord ces corps en dérivés acétylés. Leur méthode s'applique également aux diols[13] et nous l'avons employée pour tranformer les diols des cires en diacétates de diols $CH_3COO—CH_2—(CH_2)_n—CH_2—OOCCH_3$.

Cette acétylation se fait en présence de pyridine, de la manière suivante. A un poids connu de diol, on ajoute deux fois la quantité théorique d'anhydride acétique nécessaire, soigneusement distillé, et cinq fois le volume correspondant de pyridine déshydratée sur baryte et distillée. Après deux heures de chauffage à 100°, le mélange est refroidi, mis en solution dans l'éther sulfurique et lavé successivement dans une ampoule à décantation par de l'eau distillée, de l'acide chlorhydrique (5N), une solution de carbonate de sodium à 5% et enfin de l'eau distillée. La solution éthérée est ensuite séchée sur du sulfate de sodium anhydre, puis le solvant est évaporé. Le résidu de diacétate de diol est repris par le

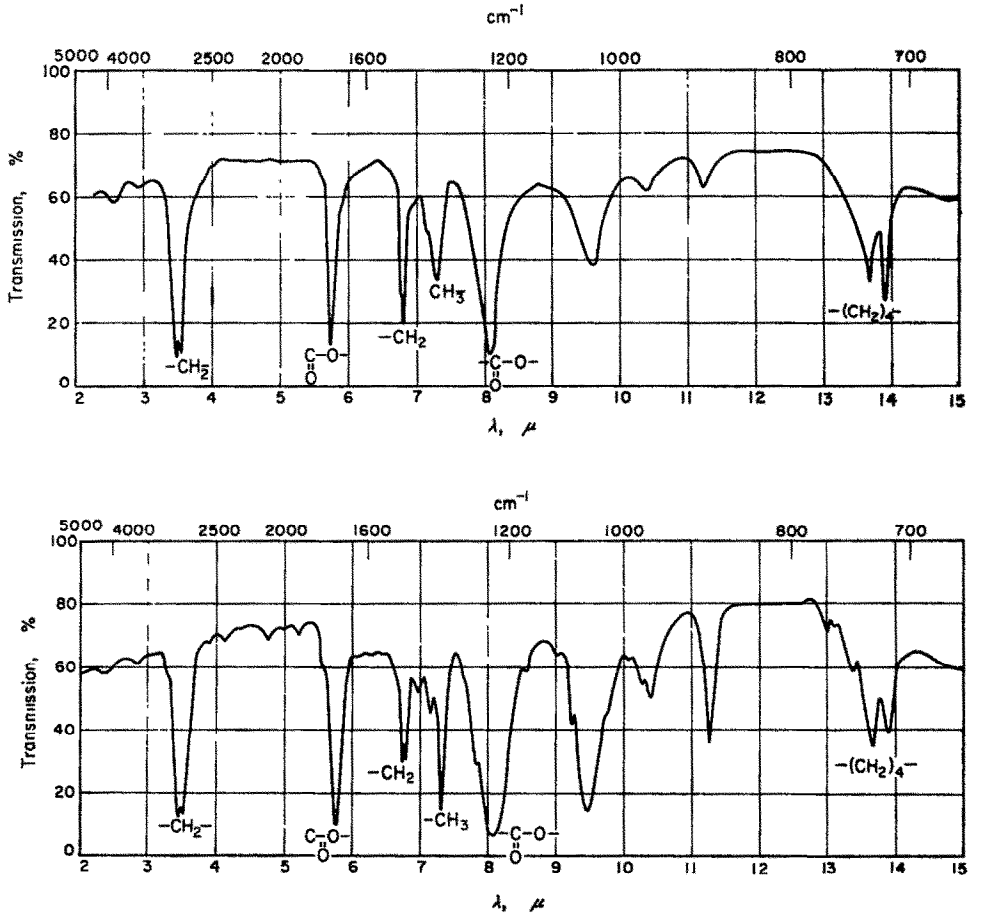

FIG. 4. SPECTRES INFRAROUGES DES DIACÉTATES DE DIOLS.
EN HAUT: spectre des diacétates de diols de la cire de pomme Stayman Winesap.
EN BAS: spectre de mélange témoin des diacétates des deux diols synthétiques: docosane-diol et tetracosane-diol.

[12] C. PAQUOT, D. LEFORT et A. POURCHEZ, *Rev. franç. Corps gras*, 7, 1–5 (1960).
[13] D. LEFORT, C. PAQUOT et A. POURCHEZ, *Oléagineux*, 16, 253–259 (1960).

minimum de benzène; cette solution peut être utilisée directement pour l'analyse chromatographique.

La Fig. 4 présente le spectre infrarouge[14] des diacétates des diols de la cire de pomme. Nous avons figuré au-dessous de ce spectre, le spectre infrarouge d'un mélange témoin des diacétates de docosane-diol et de tetracosane-diol[15]. On constate sur cette figure que les deux spectres sont tout à fait semblables, ce qui confirme bien l'identité de la fraction éluée par le mélange benzène–éthanol.

Technique Chromatographique

L'analyse chromatographique a été effectuée sur un appareil Jobin et Yvon, muni d'une colonne séparatrice, en acier inoxydable, de 2m de long et 4 mm de diamètre intérieur. La phase stationnaire utilisée était la graisse à vide silicone rubber imprégnant des grains de Chromosorb W (nous avons employé 20 g de phase stationnaire pour 100 g de support). La température du four était maintenue à 300°. Le gaz vecteur était l'hélium circulant à travers la colonne avec un débit de 7 l. par heure; ce débit était obtenu grâce à une surpression de 1160 g par cm^2 à l'entreé de la colonne, la sortie du gaz se faisant à la pression atmosphérique. La détection des corps purs à la sortie de la colonne était réalisée par un catharomètre à filaments de tungstène, relié à un dispositif d'enregistrement électronique.

Les corps séparés par chromatographie ont été identifiés en comparant leur volume de rétention respectif aux volumes de rétention de corps purs témoins, chromatographiés dans les mêmes conditions. Nous possédions, comme témoins, un mélange de deux diacétates de diols, en C_{22} et C_{24}, obligemment fournis par M. Lefort, du Laboratoire de Lipochimie du C.N.R.S. Les volumes de rétention des autres diols témoins ont été calculés en appliquant la formule de James et Martin[16] (valable pour une série de corps homologues): $\log V_R = k.\ n.$ (V_R étant le volume de rétention d'un diol, n, le nombre d'atomes de carbone contenus dans la molécule de ce corps, et k, une constante de proportionnalité.)

[14] Les spectres infrarouges ont été obtenus par Mme Cazes au Laboratoire de Chimie Macromoléculaire du C.N.R.S.

[15] Le mélange de ces deux diols synthétiques nous a été obligeamment fourni par Mr Lefort, du Laboratoire de Lipochimie du C.N.R.S.

[16] A. T. James et A. J. P. Martin, *Biochem. J.*, **50**, 679–690 (1952).

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N-ACETYLDIAMINOBUTTERSÄURE, EINE NEUE AMINOSÄURE AUS DEM LATEX VON *EUPHORBIA PULCHERRIMA* WILLD EX KLOTZSCH

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(*Eingegangen* 1 *Dezember* 1961)

Zusammenfassung—Latex verschiedener Arten der Gattung Euphorbia ist durch das Vorkommen seltener und zum Teil noch unbekannter Aminosäuren in ungewöhnlich hoher Konzentration charakterisiert. Aus dem Latex von Euphorbia pulcherrima isolierten wir eine neue Aminosäure und identifizierten sie als N-Acetyldiaminobuttersaure.

Abstract—Latex from different species of the genus *Euphorbia* is characterized by the presence of interesting known and as yet unknown amino acids in unusually high concentration. From the latex of *Euphorbia pulcherrima* a new amino acid has been isolated and identified as N-acetyldiaminobutyric acid.

Wie wir kürzlich schon berichteten[1,2], enthält die lösliche Stickstofffraktion des Latex verschiedener Arten der Gattung *Euphorbia* seltene und auch noch unbekannte Aminosäuren in auffällig hoher Konzentration.

Aus dem Latex von *Euphorbia pulcherrima* isolierten wir die Hauptaminosäure (Abb. 1) und charakterisierten sie als Monoacetyldiaminobuttersäure. Die Aminosäure ist offenbar auf den Latex beschränkt und in den Parenchymen von Blatt, Stengel und Wurzel höchstens in Spuren nachweisbar. Sie ist in Varietäten mit roten, weissen und rosa Hochblättern dieser Art während der ganzen Vegetationsperiode vorhanden.

Die Verbindung wurde mit 80%igem Äthanol aus dem Latex extrahiert, störende Begleitsubstanzen an einer Dowex-50-×4-Säule (H^+-Form) entfernt, und an einer Zellulosesäule mit Phenol–Wasser (4 : 1), anschliessend an einer gleichgrossen Zellulosesäule mit Propanol–Wasser (3 : 1) abgetrennt. Die Substanz kristallisierte beim Einengen der entsprechenden Propanol-Fraktionen im Vakuum in Nadeln aus. Aus 200 ml Milchsaft wurden 82 mg Aminosäure isoliert; sie wurde 2 mal aus heissem Propanol–Wasser (3 : 1) umkristallisiert. Der Schmelzpunkt liegt bei 208–211°C (Heiztisch "Boëtius"). Die Substanz schmilzt unter allmählicher Zersetzung.

In papierelektrophoretischen Untersuchungen reagiert die Aminosäure neutral; sie hat in eindimensionaler aufsteigender Papierchromatographie in Propanol–Wasser (3 : 1), Phenol–Wasser (4 : 1), Pyridin–Isoamylalkohol–Wasser (1 : 1 : 2), Methyläthylketon–Pyridin–Wasser (5 : 1 : 1), Methanol–Ammoniak–Wasser (9 : 0·2 : 0·8), *n*-Butanol– Ameisensäure–Wasser (7·5 : 1·5 : 1·0) und *n*-Butanol–Eisessig–Wasser (4 : 1 : 7) die gleichen R_f-Werte wie δ-Acetylornithin. Das Infrarotspektrum der isolierten Substanz beweist ihre Verwandtschaft mit δ-Acetylornithin, ohne mit ihm identisch zu sein.

Nach Hydrolyse in 6 N HCl entsteht eineel ektrophoretisch stark basisch reagierende Aminosäure; diese wurde in absteigender Papierchromatographie mit dem Lösungsmittel Phenol–Citronensäure–Phosphatpuffer pH 4 und papierelektrophoretisch bei pH 10

[1] I. Liss, *Naturwissenschaften* **48**, 304 (1961).
[2] I. Liss, *Flora, Jena.* **151**, 351 (1961).

(0·05 M Na_2CO_3) eindeutig von den basischen Aminosäuren Diaminopropionsäure, Ornithin, Arginin, Lysin und Histidin abgetrennt und als α, γ-Diaminobuttersäure charakterisiert. Die DNP-Verbindung des Hydrolyseproduktes verhält sich wie DNP-Diaminobuttersäure. Mit Ninhydrin gibt die hydrolysierte Verbindung eine braunviolette Färbung, wie sie ebenfalls für Diaminobuttersäure charakteristisch ist.

Acetat wurde nach Hydrolyse der Verbindung in wasserfreiem HCl–Methanol als Methylacetat überdestilliert und quantitativ als Hydroxam–Eisen–Komplex kolorimetrisch bestimmt[3]. In 8·75 μmol (1·40 mg) Substanz wurden 8·57 μmol (0·51 mg) Essigsäure nachgewiesen, was einer Ausbeute von 98 Prozent entspricht, wenn man eine Monoacetyl-Verbindung zugrunde legt.

Die Analyse C, 44·98; H, 7·62; N, 17·12 Prozent entspricht der Formel $C_6H_{12}O_3N_2$ und der theoretischen Zusammensetzung C, 44·99; H, 7·55; N, 17·49 Prozent von Monoacetyldiaminobuttersäure. Ob Acetat in α-oder γ-Stellung substituiert ist, kann erst durch die Synthese entschieden werden.

Diaminobuttersäure ist ausser in Mikroorganismen in Tieren und Pflanzen erst jeweils einmal nachgewiesen worden[4,5]. Die Frage, ob die acetylierte Aminosäure in *Euphorbia pulcherrima* eine Entgiftungsform der stark basischen Diaminobuttersäure darstellt und die Anreicherung der Verbindung im Latex als eine Exkretion aufzufassen ist, bedarf weiterer Untersuchungen.

Beziehungen, wie sie zwischen Glutaminsäure, δ-Acetylornithin und Prolin nachgewiesen wurden, dürften auch zwischen Asparaginsäure, Acetyldiaminobuttersäure und der von Fowden[6] in vielen Liliaceen nachgewiesenen Azetidin-2-carbonsäure bestehen. Azetidin-2-Carbonsäure konnte mit den von uns angewandten papierchromatographischen Methoden im Latex von *Euphorbia pulcherrima* nicht aufgefunden werden.

Anerkennung—Herrn Prof. Dr. K. Mothes danke ich für die stete Förderung dieser Untersuchungen herzlich.

[3] J. Ludowieg and A. Dorfman, *Biochim. Biophys. Acta* **38**, 212 (1960).
[4] L. Fowden and M. Bryant, *Biochem. J.* **70**, 626 (1958).
[5] P. Ackermann and H. G. Menssen, *Z. physiol. Chem. Hoppe-Seyler's* **318**, 212 (1960).
[6] L. Fowden, *Biochem. J.* **64**, 323 (1956).

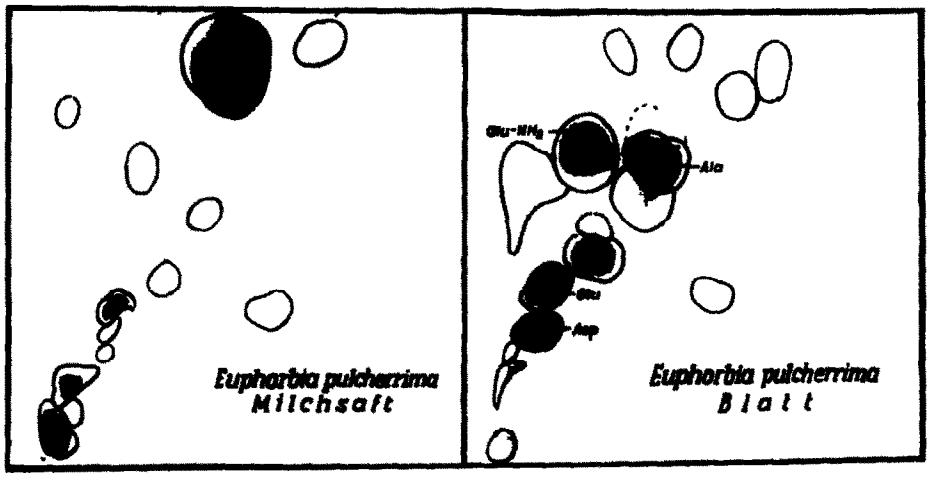

ABB. 1. PAPIERCHROMATOGRAMME DER ALKOHOLLÖSLICHEN AMINOSÄUREN. Die aufgetragenen Extraktmengen von Milchsaft und Blatt entsprechen dem gleichen Frischgewicht. Als Lösungsmittel diente in der 1 Richtung (von links nach rechts) Propanol–Wasser (3 : 1) in der zweiten, Phenol–Wasser (4 : 1). Die Chromatogramme wurden mit Ninhydrin entwickelt. Die Hauptaminosaure de Milchsaft-Chromatogramms ist α-γ-Diaminobuttersäure.

[*facing page 88*]

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HYDROCARBON CONSTITUENTS OF THE WAX COATINGS OF PLANT LEAVES: A TAXONOMIC SURVEY

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Abstract—A gas-chromatographic study has been made of the hydrocarbon content of the leaf waxes of the sub-family Sempervivoideae (Crassulaceae), through the study of a compact grouping of closely related genera endemic to the Canary Islands. Within the limits of the investigation, it has been shown that a single species possesses a fairly constant hydrocarbon distribution pattern, thereby justifying the use of this criterion for taxonomic purposes. The variations from one species to another reveal a rough parallelism with the botanical classification of the sections within a genus. *Iso*alkanes are sometimes present to the extent of more than 50 per cent of the total alkane content.

INTRODUCTION

THE occurrence of a waxy coating on plant leaves is an almost universal phenomenon, but the detailed chemical investigation of the constituents of this protective covering has attracted less attention than might be expected. This is due in large part to the formidable problems of separation involved in the study of such a complex mixture of closely related long-chain aliphatic components. The most outstanding contribution has been that due to Chibnall and his collaborators[1] in the 1930's using the classical method of fractional crystallisation, and employing as purity criteria precise melting points coupled with X-ray determination of crystal long-spacings. Their careful work demonstrated that many previously-isolated, "pure" components were in fact mixtures.[1] The main constituents of leaf waxes as determined from this and later work[2-9] are shown in Table 1. This later work, employing the combined techniques of gas-liquid chromatography and mass spectrometry, has complicated the original picture derived from the earlier work. To take one example, the idea of the exclusive occurrence of odd carbon number *n*-alkanes has had to be modified in view of the detection of even-numbered *n*-alkanes[6,9] and of branched-chain alkanes.[4,9] It is certain that more widespread application of the above techniques will result in considerable future modification of the information contained in Table 1.

The use of chemical constituents of plants as an aid to their classification is now a familiar concept,[10,11] the outstanding example being the extensive work of the Erdtman[12]

[1] A. C. CHIBNALL, S. H. PIPER, A. POLLARD, E. F. WILLIAMS and P. N. SAHAI, *Biochem. J.*, **28**, 2189 (1934); A. C. CHIBNALL and S. H. PIPER, *Biochem. J.*, **28**, 2209 (1934).

[2] J. T. MARTIN and R. F. BATT, *Ann. Appl. Biol.*, **46**, 375 (1958); D. V. RICHMOND and J. T. MARTIN, *Ann. Appl. Biol.*, **47**, 583 (1959); J. T. MARTIN, *J. Sci. Food Agri.*, **11**, 635 (1960).

[3] B. WIEDENHOF, *J. Am. Oil Chemists' Soc.*, **36**, 297 (1959).

[4] W. CARRUTHERS and R. A. W. JOHNSTONE, *Nature* **155**, 500 (1945).

[5] Z. H. KRANZ, J. A. LAMBERTON, K. E. MURRAY and A. H. REDCLIFFE, *Australian J. Chem*, **13**, 498 (1960); *ibid*, **14**, 264 (1961).

[6] G. G. WANLESS, W. H. KING and J. J. RITTER, *Biochem. J.*, **59**, 684 (1955).

[7] K. S. MARKLEY, C. E. SANDO and S. B. HENDRICKS, *J. Biol. Chem.*, **132**, 641 (1938).

[8] A. H. WARTH, *Chemistry and Technology of Waxes* (2nd Ed.), Reinhold, New York (1956).

[9] J. D. WALDRON, D. S. GOWERS, A. C. CHIBNALL and S. H. PIPER, *Biochem. J.*, 78, 435 (1961).

[10] E. SHAW and R. D. GIBBS, *Nature* **190**, 463 (1961).

[11] E. LODGE, *Sci. Progr. Twent. Cent.*, **49**, 497 (1961) (and references cited therein).

[12] H. ERDTMAN, W. E. HARVEY and J. G. TOPLISS, *Acta Chem Scand*, **10**, 1381 (1956); H. ERDTMAN, *Perspectives in Organic Chemistry*, p. 473, Interscience New York (1956).

TABLE 1. MAJOR CONSTITUENTS OF LEAF WAXES[1,9]

Type	Range	Frequency
Alkanes	Normal: odd C_{21}–C_{37}	Common (especially C_{29} and C_{31})
	Normal: even C_{20}–C_{34}	Common minor constituents
	Branched: C_{27}–C_{33}	Infrequent
Alcohols (usually as esters)	Primary: even C_{22}–C_{32}	Common
	Primary: odd C_{25}–C_{31}	Infrequent
	Secondary: odd C_{21}–C_{31}	Common
	Diols and ketols	Rare
	Terpene alcohols	Infrequent
Aldehydes (as polymers)	Normal: C_{24}–C_{34}	Rare
Ketones	Di-*n*-alkyl ketones	Rare
Acids (usually as esters)	Normal. even C_{14}–C_{34}	Common
	Normal: odd C_{15}–C_{33}	?
	Ketoacids	Rare
	Dibasic acids	Rare
Esters	Between *n*-acids and primary and secondary alcohols	Common
	Estolides of hydroxyacids	Infrequent?

school on conifers. The employment[13] of leaf waxes as an advantageous taxonomic criterion is suggested by the following considerations:

(a) The universality of the occurrence of these waxy coatings; (b) The already observed species variation in the wax composition; (c) The reported[1] lack of seasonal variation in the wax composition. This may be due to the fact that the wax is extracellular and is almost certainly an end-product insulated from the regular essential metabolic functions of the plant; (d) The simplicity of sampling; (e) The present-day availability of the precise and rapid micro-analytical tools of gas-liquid chromatography, mass spectrometry and infrared spectrometry.

This taxonomic possibility seemed worthy of further investigation in spite of forebodings that the differences between the waxes might not be sufficiently discriminating, too "conservative" as Erdtman has put it.[12] To simplify the approach it seemed reasonable to concentrate on one class of wax constituent, i.e. the hydrocarbon content, which in most waxes accounts for a significant portion of the total wax. Such a hydrocarbon mixture is very amenable to exact and rapid analysis by gas-liquid chromatography[4,5,14-16] and its

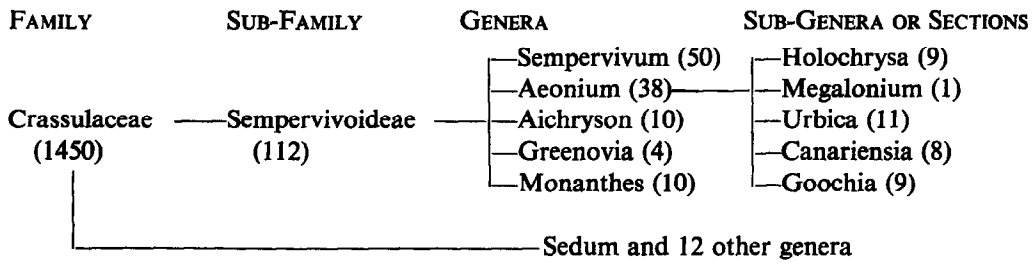

FIG. 1. THE CRASSULACEAE FAMILY SHOWING THE SUB-DIVISION[17] OF THE SUB-FAMILY SEMPERVIVOIDEAE; NUMBER OF SPECIES IN PARENTHESES.

[13] S. J. PURDY and E. V. TRUTER, *Nature* **190**, 554 (1961).
[14] G. BAKER, J. H. PEPPER, L. H. JOHNSON and E. HASTINGS, *J. Insect Physiol.*, **5**, 47 (1960); D. T. DOWNING, Z. H. KRANZ, J. A. LAMBERTON, K. E. MURRAY and A. H. REDCLIFFE, *Australian J. Chem*, **14**, 253 (1961).
[15] E. J. LEVY, R. R. DOYLE, R. A. BROWN and F. W. MELPOLDER, *Analyt. Chem.*, **33**, 698 (1961).
[16] R. ROWAN, *Analyt. Chem.*, **33**, 658 (1961); B. T. WHITHAM, *Nature* **182**, 391 (1958).
[17] K. LEMS, *Ecology*, **41**, 1 (1960).

very complexity serves as a positive advantage in providing a taxonomic "fingerprint".

In order to test the validity of these ideas a study of certain plants of the Canary Islands has been undertaken. This choice was made because of the availability of a compact grouping of closely related genera of the sub-family Sempervivoideae (Crassulaceae; Fig. 1), which had already been extensively studied botanically. They are believed to be descended from a common ancestor which initially colonized the islands and to have been developed in isolation from the mainland. Because of the wide climatic variation on these islands of diverse terrain, the variety of forms presents an excellent example of "adaptive radiation".[17] The members of this family are all xeromorphic and generally possess quite substantial waxy coatings. Several botanists, including Pitard and Proust,[18] Burchard[19] and particularly

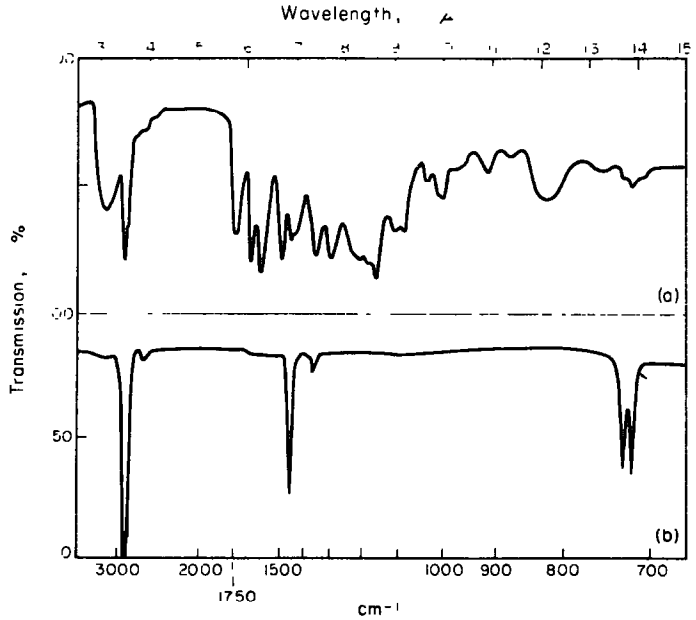

FIG. 2. THE INFRARED SPECTRUM (SOLID FILM) OF (a) A LEAF WAX AS EXTRACTED FROM THE PLANT (*Aeonium manriqueorum*, TABLE 2; 1) AND OF (b) THE HYDROCARBON FRACTION DERIVED FROM THIS WAX.

Praeger[20] have discussed the classification of these plants and Lems[17] devotes a recent lengthy paper to the evolutionary aspects of the group in which he states his belief that the *Aeoniums* present "a situation comparable in many ways to the finches of the Galapagos". His study concerns the plant forms of the Sempervivoideae and his conclusion is that "this group is composed of species of many forms, from shrub to biennial and annual herb; it is possible to derive all of them from a shrubby ancestor". The results of our own study are considered in relation to this approach.

RESULTS

The plants studied are listed in Table 2. They include examples from all the constituent genera of the Sempervivoidae (Fig. 1), with the exception of *Sempervivum*. For reasons discussed later seven species of the genus *Euphorbia* (Euphorbiaceae, 4,000 species) and two

[18] J. PITARD and J. PROUST, *Les Iles Canaries*, Klincksieck, Paris (1908).
[19] O. BURCHARD, *Bibliotheca Botanica*, Schweitzbart, Stuttgart (1929).
[20] R. L. PRAEGER, *An Account of the Sempervivum Group*, Roy. Horticult. Soc., London (1932).

TABLE 2.

SPECIES	No.*	Leaf Wax			Distribution in mole percentage of the hydrocarbons in the hydrocarbon fraction of the leaf wax ‖																			
					C_{25}		C_{26}		C_{27}		C_{28}		C_{29}		C_{30}		C_{31}		C_{32}		C_{33}		C_{34}		C_{35}	
		Wet (wt. %)†	Dry (wt. %)‡	H/C (%)§	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*
AEONIUM																										
Section Holochrysa																										
A. manriqueorum	1	0·95	5 5	5		•		•		1	•	•	2	2	1	1	10	21	1	4	15	38		1	1	2
A. holochrysum	2	0·15	1·5	65		•		•		•		•	1	1	•	•	4	18	1	4	12	48	•	2	6	3
A. rubrolineatum	3	0·15	1	60				•		•		•		•		•	2	17	•	3	14	54		2	3	4
Section Urbica																										
A. percarneum	4	0·2		35		•		•	•	•		•		•		•	1	13		2	1	74		2		6
A. percarneum	5	0·3	4	15		•		•	•	•		•		•		•		9		3	4	79		2		4
A. percarneum	6	0·1	1	55		•		•	•	•		•		•		•	1	8		2	4	80		1		4
A. haworthii	7	0·1	1	20		•		•		•		1				•	•	21		3	3	68		•		2
A. decorum	8	0·1		15		•		•		•		1					1	25		5	5	59	1	1		2
A. castello-paive	9	0·1		60		•		•		•		1				•	1	30		3	1	59		1		5
A. urbicum	10	0·25	1·5	25		•		•		•		1				•	1	20		3	3	68		2		3
A. urbicum	11	0·1		55		•		•		•		1				1	1	21		4	2	63		•	1	6
A. urbicum	12	0·1		60		•		•		•		•				•	•	15		2	4	69		3		6
A. urbicum	13	0·2		50		•		•		•		•				•	•	16		3	6	70		1		3
A. urbicum	14	0·2		35		•		•	•	1		•		1		•	1	22		2	1	68		1		2
A. urbicum	15	0·1	1	65		•		•	•	•		•		•		•	1	15		4	4	72		1		2
A. urbicum	16	0·1		55						•		•		•			•	12		3	1	69		2	•	13
A. urbicum	17	0·05				•		•		1		•	•	1		•	•	12		3	2	74		1	•	5
A. urbicum	18	0·1		25		•		•		•		•	•	1		•	1	21		3	1	64		2		5
Section Megalonium																										
A. nobile	19	0·2	2·5							•		•		1		•	•	12	•	3	1	76		2		5
Section Canariensia																										
A. cuneatum	20	0 1	0·5	35		•		•		•	•	•		1		•	•	27		3	2	63		•		4
A. cuneatum	21	0·05	0 5	35		•		•		1	•	•	•	1		•	•	29		2	2	60		•		4
A. canariense	22	0·05	0·5	55		•		•		•	•	•	•	1	•	•	9	21	1	4	23	36		•	1	2

A. canariense	23					1		•	•	2		•	1	3			11	10	1	1	39	25			•	4
A. palmense	24	0·25	2·5	30		•		•	•	2		•	1	3	•	•	9	14	1	3	26	35	•	1	2	2
A. subplanum	25	0·05	1	45		1		•		1		•	•	1		•	3	36		3	4	45		1	1	3
A. virgineum	26	0·05	0·5	40		1		1		2	2	2	1	1	•	•	7	14	1	2	23	33			3	4
A. tabulaeforme	27	0·05		45						•		•		2		1		79		2		17				
Section Goochia																										
A. goochiae	28	0·7	9	10		1		•		3			8	7		2	16	14	3	2	17	21			3	4
A. viscatum	29	1·1		5		2		•	2	8			16	12			13	18			12	16			•	•
A. lindleyi	30	1·2		1		•		•		1		•	3	8	•	•	11	22	1	2	18	27			2	2
A. lindleyi	31			5	•	2		•	•	2	•	•	12	9	1	1	16	17	1	1	15	19				2
A. lindleyi	32				•	2		•		2		1	11	8	1	1	16	17	1	2	17	19				•
A. saundersii-Bolle	33			15		•		•	•	1	•	1	13	5	1	1	20	16	1	3	20	16	•	•	2	1
A. saundersii-Bolle	34					•		•	•	1	•	1	13	5	1	1	22	16	1	2	20	14		•	2	1
A. sedifolium	35	0·9	12	5		1				1			1	2		•	10	17	1	3	25	34			2	2
A. smithii	36	0·1	1	20						•			1	1	•	•	30	17	1	3	22	19	•	•	3	1
A. cruentum	37	0·4	4·5	25				•		•		•		3		1	2	53		3	4	29				3
A. spathulatum	38	3·8	7·5	2				•		•		•		2		1	7	56		4	5	24				
A. spathulatum	39					•	•	•	•	•		•	•	2		•	15	54		2	8	18				
A. spathulatum	40			3		•		•		•		•	•	3		1	15	59		2	5	14				
A. spathulatum	41					•		•		•			•	3		1	13	51	•	2	7	21				
A. caespitosum	42	0·25		25									1	1			4	13	1	3	16	57		1	1	2
A. caespitosum	43	0·25	3	20						•		•		•			3	13		2	16	58		•	2	3
Hydrocarbon mixture	44					1				1			1	2			10	13	1	2	35	35				
Repeat run	45					1				2			1	2			10	12	1	2	35	34				
Repeat run	46					•				2			1	3			11	12	1	3	34	33				
AICHRYSON																										
Ai. dichotomum	47	0·2	2	55		•		•		•		•	1	1		1	2	12	4	2	4	62	2	1	•	6
Ai. punctatum	48	0·05	0·6	35										1		•	1	17		4	3	62		1	3	6
Ai. unidentified	49	0·05		35						1			2	2		4	7	13	7	2	14	41		2	2	3
GREENOVIA																										
G. aurea	50	0·1						•		•				•			•	9		1	1	80		1		7
G. aurea	51	0·05	1	35		•		•		•				•		•	•	8		1	1	76		2		12
G. diplocycla	52	0·05	1	45		•		•	•	•		•		•			•	4		1	2	78		2		9
G. unidentified	53	0·05	1	20					•	1		•	2	2		•	2	8		1	3	72		1		7
G. unidentified	54	0·3		15		•		•		•				•				4		1	3	75		1	2	14
G. unidentified	55		1	50														7		3	2	77		1		10
G. unidentified	56	0·05	1	20		•		•	•	1		•		1			•	5		1	1	82		1	1	8
G. unidentified	57	0·05		30		•		•		•		•		•			•	5		2	2	81		2		8

TABLE 2 *continued.*

SPECIES	No.*	Leaf Wax			Distribution in mole percentage of the hydrocarbons in the hydrocarbon fraction of the leaf wax ‖																			
		Wet (wt.%)†	Dry (wt.%)‡	H/C (%)§	C_{25}		C_{26}		C_{27}		C_{28}		C_{29}		C_{30}		C_{31}		C_{32}		C_{33}		C_{34}		C_{35}	
					iso	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*
MONANTHES																										
M. amydros	58			45						1		•	•	1		3		52		2	1	43				
M. laxiflora	59	3·1		65			•			•		•		•		•		2		1		94		1		1
M. polyphylla	60	0·4	4	30		•		•		•		•	•	•				12		2	2	84		•		1
M. muralis	61			15						•				•				9		1		87		1		2
M. muralis	62			25		•		•		1		•	•	1				15		2	1	77				4
M. anagensis	63	2·0	12	35						•				•				4		1		89		1		3
M. brauchycaula	64	0·1		70		•		•	•	•			•	1			•	10		1	•	84		1		2
M. pallens	65	0·25		25		•		1		1			1	1			1	13		2	1	80		1		3
SEDUM																										
S. anglicum	66									1				8		1		80		1		9				
DRACAENA																										
D. draco	67			1		4		5		12		7		22		6		31		1		4				
LOLIUM																										
L. multiflora	68			10		4		1		7		1		42		1		40				3				
EUPHORBIA																										
E. peplus	69	0·2	1	3		3		2	1	9	•	1	1	17		1		45		1		18				
E. balsimifera	70	1·2		5		20		1		51		1		24												
E. atropurpurea	71	0·4		10		1	•	•		5		•	•	13	1	1	•	70		1		8				
E. regis-jubae	72	0·2	1	2		1		•		3		•		11		1		67		1		15				
E. aphylla	73	0·8		2		14		2		15		3		23		3		33		•		2				
E. bourgaeanea	74					•		•		17		1		25		1		56								

* The species were collected at the following stations: (i) on the island of *Tenerife* at Buenavista, 7, 17, 18, 59, 65 and 74; La Laguna, 10, 11, 12, 13 and 67; Las Mercedes, 14 and 15; the Forestry Gardens, La Laguna, 20 and 21; San Juan de la Rambla, 27; Santa Ursula, 16; Bajamar, 30, 31, 32; Canadas, 36; Aguamansa, 38, 39, 40, 41; Punta de Hidalgo, 48; La Esperanza, 50 and 51; Playa de Martianez, 60 and 64; Punta de Anaga, 63; Taco, 72; and at undetermined locations, 2, 22, 23, 47, 69, 70 and 71: (ii) on the island of *Gran Canaria* at Tafira, 1; Santa Brigida, 5 and 6; Firgas, 26; Lagunetes, 4, 42 and 43; San Mateo, 54 and 55; and at an undetermined location, 49: (iii) on the island of *Gomera* at Chejelipe, 8, 33, 34, 57 and 58; Agulo, 53; Tunel, 56; and at undetermined locations, 3, 9, 25, 29, 52 and 73: (iv) on the island of *La Palma* at Tazacorte, 35; Mazo, 37 and 62; and at undetermined locations, 19, 24 and 28: (v) on the island of *Hierro* at Sabinosa, 61: and (vi) at *Portencross, Scotland*, 66 and 68.

The species were collected during the following months: September 1960, 1, 2, 3, 4, 5, 9, 10, 11, 12, 13, 20, 22, 25, 26, 27, 30, 42, 47, 48, 49, 54, 55, 70 and 71; October, 38, 52, 53, 56, 57 and 63; November, 6, 14, 15 and 43; December, 7, 8, 17, 18, 19, 24, 28, 29, 35, 37, 59, 65, 74; January 1961, 21, 36, 60, 62, 64, 69 and 73; February, 16, 50, 51 and 72; March, 39, 40 and 41; April, 23, 31, 32, 33, 34, 58, 61 and 67; May, 66 and 68.

Specific points concerning the origins of the wax extracts are as follows:

Species numbers: 10–13 are repeat extractions of fresh leaves from the same batch of individuals of that species; 15, immature leaves; 16 and 55, dead leaves in a naturally desiccated condition; 17 and 18 differ in that they were collected at different altitudes in the same district; 32 and 34, hydrocarbon fractions isolated solely by column chromatography; 39, the plant was in flower; 41 is the hydrocarbon fraction from sp. 40 after treatment with concentrated H_2SO_4 for 3 hrs at 120°C; 44–46 are replicate analyses of the same hydrocarbon mixture; 51, young individuals; 53 and 54, possible *G. diplocycla* and *G. aurea*, respectively but identification awaits appearance of flowers; 69 and 73, stalk wax in addition to leaf wax—all other species numbers were derived from leaf surface waxes only.

† *Wet wt.* (%) = wt. of wax extract/wt. of undried leaves. Values are approximated to nearest 0·05 per cent.

‡ *Dry wt.* (%) = wt. of wax extract/wt. of leaves after air drying to constant weight. Values are approximated to nearest 0·5 per cent. Data are incomplete due to difficulties in drying many species.

§ *H/C* (%) = wt. hydrocarbon fraction/wt. of wax extract. Values greater than 5 per cent are approximated to nearest 5 per cent.

‖ *The content* of an individual hydrocarbon is expressed as a mole percentage of the total hydrocarbon content from C_{25}–C_{35} inclusive. The mole percentage is taken as being equivalent to the area percentage, i.e. $A_n / \Sigma_{n=25}^{n=35} A_n$, where A_n is the area of the peak corresponding to the hydrocarbon C_nH_{2n+2} in the gas-liquid chromatograms (cf. Fig. 3). The values are approximated to the nearest 1 per cent and peaks of relative area <1 per cent are indicated by • . The species 67–74 were measured over the range C_{23}–C_{35} as there is an appreciable content of these hydrocarbons C_{21} and C_{24} thus: 67 has *n*-C_{23} 1 per cent, *n*-C_{24} 2 per cent; 68 has traces of *n*-C_{23} and C_{n-24}; 70 has *n*-C_{23} 2 per cent, *n*-C_{24} 1 per cent; 73 has *n*-C_{24} 5 per cent.

monocotyledons: *Dracaena draco* (Liliaceae, 3,700 species) and *Lolium multiflora* (Gramineae, 4,500 species) were included.

The waxes were extracted[2] from both surfaces of the leaf samples by dipping the leaves either singly or in small bunches in three successive portions of cold chloroform. This treatment was found sufficient to remove all the surface wax without removing cytoplasmic constituents. The i.r. spectrum of the residue remaining after removal of the solvent was then recorded (e.g. Fig. 2a).

The hydrocarbon fraction was isolated in 95 per cent yield from the waxes by saponification followed by extraction with light petroleum and chromatography on basic alumina. (In two cases, Table 2, nos. 32, 34, the saponification step was omitted with equally good results.) The i.r. spectrum of the isolated fraction (e.g. Fig. 2b) showed no contaminants of different functional type, and repetition of the alkali treatment caused no change in the proportions of hydrocarbon present. Replicate extractions of leaves from the same batch gave hydrocarbon concentrates of almost identical composition (Table 2, Nos. 10–13).

The hydrocarbon fraction was analysed by gas chromatography (e.g. Fig. 3) and the peaks of the *n*-alkanes assigned both by co-chromatography with known compounds and from the relationship of the retention times (Fig. 4). The remaining peaks have all been assigned to isoalkanes (2-methylalkanes)[15,21] on the grounds of the relationship of the retention times (Fig. 4) and the enhancement of the appropriate peak by addition of a genuine sample of 2-methyldotriacontane.[22] Although the presence of other types of saturated hydrocarbon cannot be rigorously excluded on the basis of the above techniques, it is certain that the components of the hydrocarbon mixtures belong overwhelmingly to the *n*- and *iso*-alkane series in the range C_{22} to C_{35} inclusive.

The quantitative results are given in Table 2; they were obtained by measurement of peak areas and are expressed as a percentage of the sum of the peak areas of all compounds lying between C_{25} and C_{35}. The results were found to be reproducible to $\pm$ 5 per cent by replicate analyses (Table 2 Nos. 44–46); and evaluation of synthetic mixtures showed that the areas were proportional to molar concentrations for each type of hydrocarbon.

DISCUSSION

Some of the main findings of this study on the Sempervivoideae may be summarised as follows:

(a) Alkanes of carbon number less than C_{25} and more than C_{35} are not present to any detectable extent;
(b) The content of odd carbon-number alkanes is greater than that of even carbon number alkanes by a factor of more than ten. The distribution ratio of the odd and even series shows no parallelism;
(c) Some species contain quite high proportions of *iso*alkanes which occasionally even outweigh the *n*-alkane content. When present, an *iso*alkane constituent invariably accompanies its straight chain isomer, and for the major odd carbon number constituents (e.g. C_{31} and C_{33}) of any given leaf wax there are indications of a parallelism in the *iso*- to normal-hydrocarbon ratio.

This demonstration of the occurrence of branched chain structures makes it clear that this phenomenon should no longer be regarded as a biological "freak"[23] although the only

[21] D. T. DOWNING, Z. H. KRANZ and K. E. MURRAY, *Australian J. Chem.*, **13**, 80 (1960).
[22] Kindly provided by Shell Ltd., Thornton Research Station, Cheshire.
[23] D. E. GREEN, *Sci. Am.*, **202**, 46 (1960).

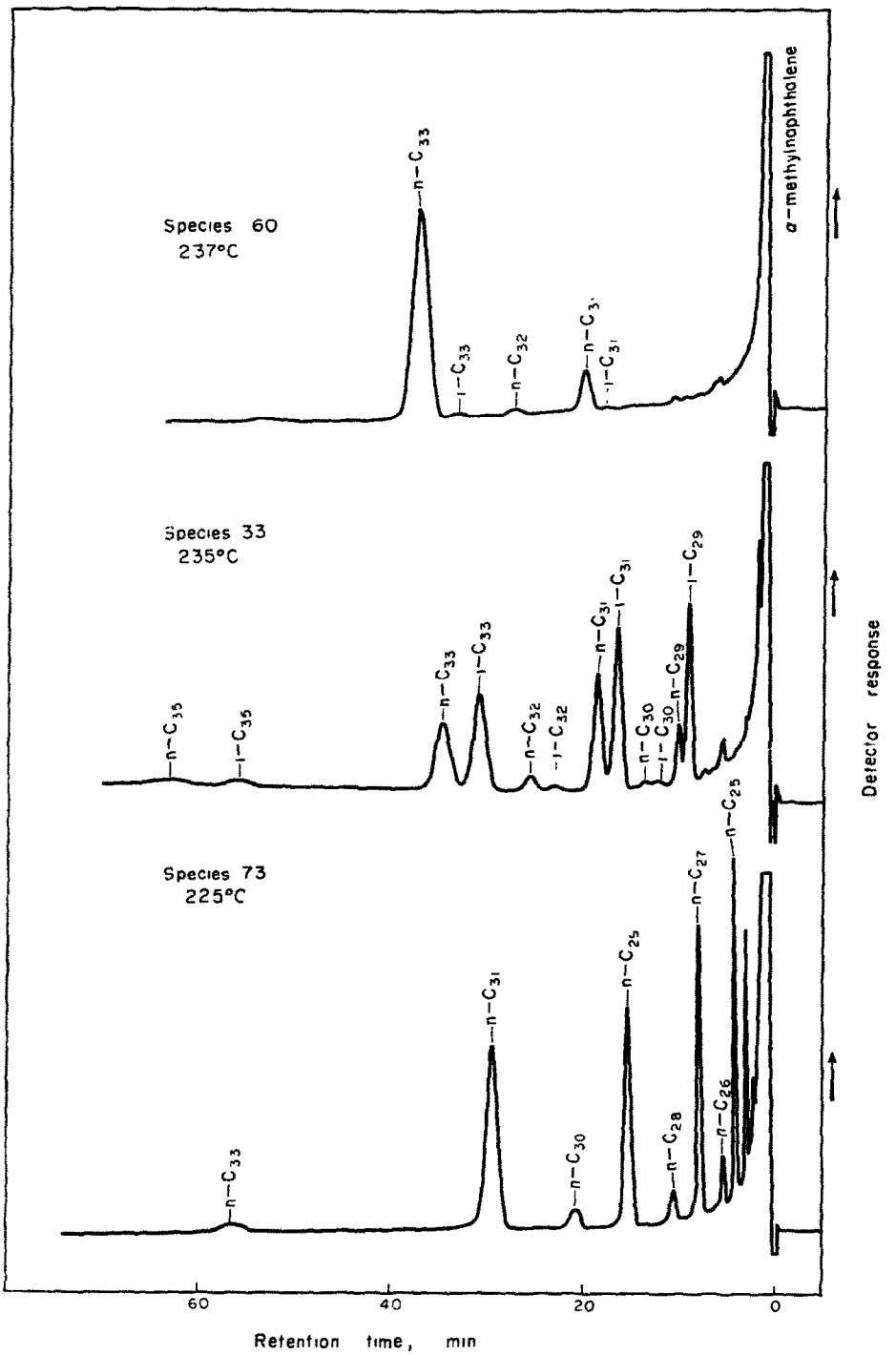

FIG. 3. GAS-LIQUID CHROMATOGRAMS OF HYDROCARBON FRACTIONS DERIVED FROM THE SURFACE WAXES OF THE LEAVES OF *Monanthes polyphylla* (Table 2; 60), AND *Aeonium saundersii-Bolle* (33) AND OF THE LEAVES AND STEMS OF *Euphorbia aphylla* (73). Load, 0·1 μl of ~5% solution of the hydrocarbon fraction in α-methylnaphthalene; column, 0·5% Apiezon 'L' on Celite, 80–100 mesh; gas flow, 45 ml of argon per min; detector voltage 1750 V, attenuation × 10.

other reported occurrence of branched chain hydrocarbons is that found for tobacco leaf wax.[4]

The principal requirement for a taxonomic criterion is that it be species specific. The present results indicate that the hydrocarbon composition of the leaf wax is not appreciably dependent on the position of the leaves with respect to the meristem (Table 2; 14 and 15) the age (20 and 21, 50 and 51) or the station (4 and 5, 13 and 17, 61 and 62) of the individual plant, provided that the species is the same. This was found to hold in spite of the differences in, for example, leaf size (e.g. 30 and 31, 38 and 40, 42 and 43) shown by those species which exhibit great seasonal variation in growth. Thus, within the limitations of this investigation

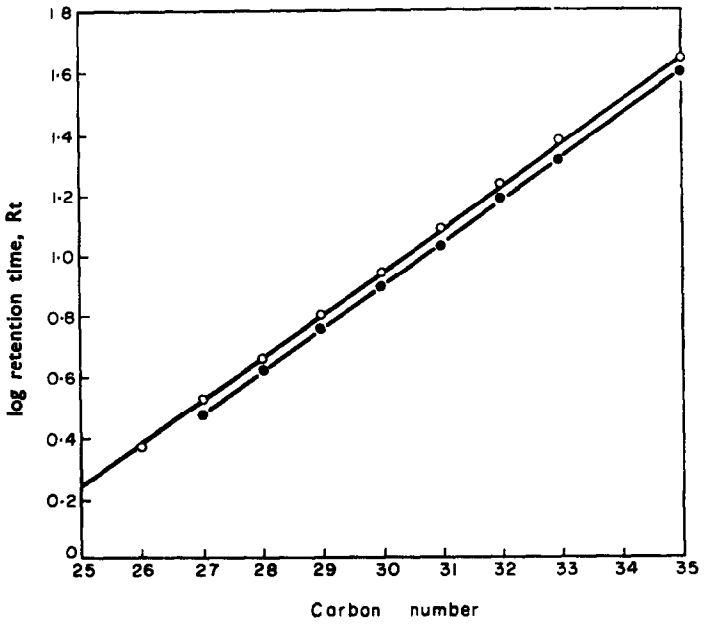

FIG. 4. PLOT OF THE LOG OF THE RETENTION TIME AGAINST CARBON NUMBER FOR THE PEAKS ASSIGNED TO THE *n*- AND *iso*-ALKANE SERIES. Data from the gas-liquid chromatogram (Fig. 3) of the hydrocarbon fraction of the leaf wax of *Aeonium saundersii-Bolle* (Table 2; 33). O = *n*-alkanes, ● = *iso*-alkanes.

and, in the absence of sufficient data for a thorough statistical treatment, it seems reasonable to conclude that the hydrocarbon pattern of the leaf wax of a species is a property characteristic of that species. Such fluctuations as there are (for example, with the *A. urbicum* examples 10–18) may be due to the occurrence of genetic and/or environmental factors, and do not invalidate the general conclusions.

In Fig. 5 the hydrocarbon constituents of the leaf waxes studied are shown in histogram form, a single diagram for each authenticated species. The botanical classification is delineated, and within this the species are arranged such that, in general, the branched-chain isomer content increases from left to right and from top to bottom; the proportion of C_{31} to C_{33} seems to increase in a similar progression.

The most extensive survey was centred on the genus Aeonium (Fig. 1) with the prime concern of unearthing any relationship between the hydrocarbon patterns and the botanical classification. Within the section *Holochrysa* the three species examined show closely

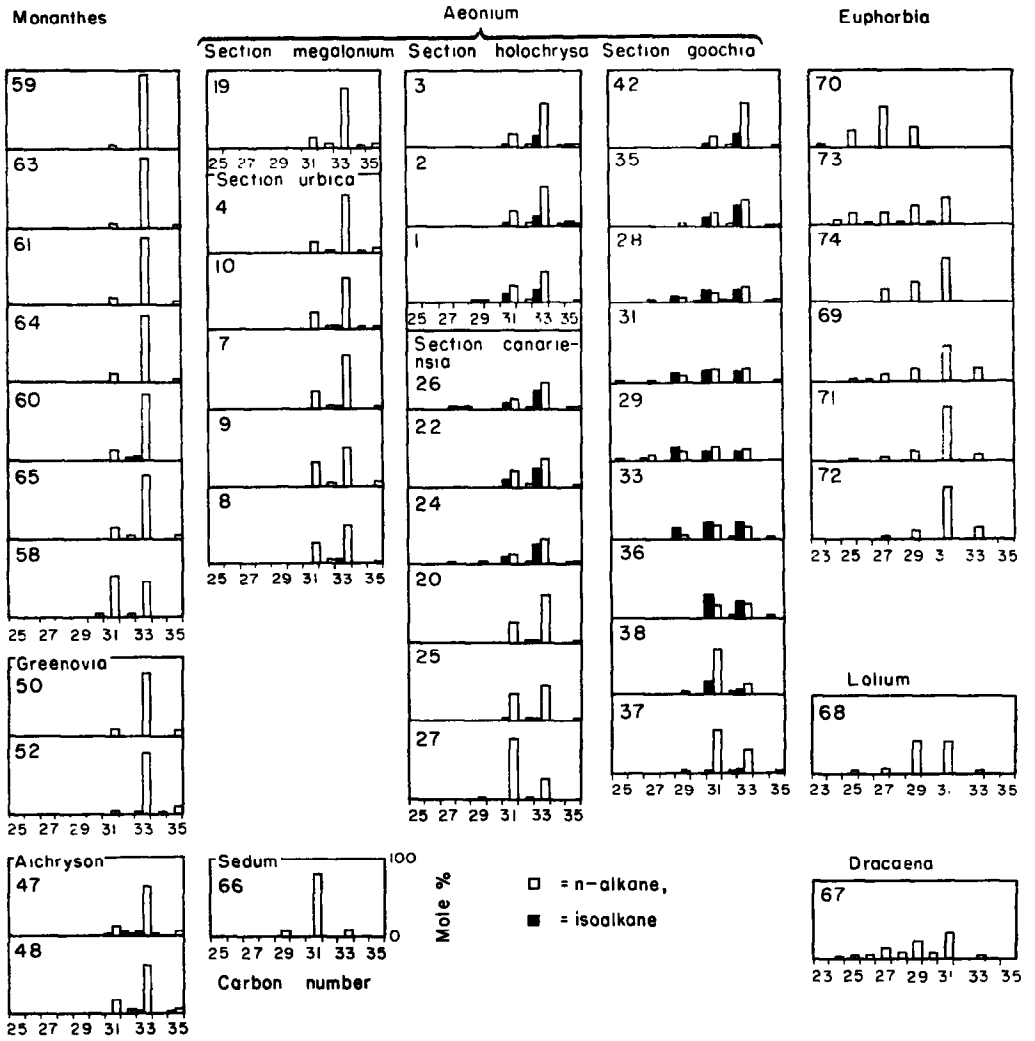

FIG. 5. DISTRIBUTION, IN MOLE PERCENTAGE (TABLE 2) OF *n*- AND *iso*-ALKANES C_{23}–C_{35} IN THE HYDROCARBON FRACTION OF THE SURFACE WAXES FROM THE LEAVES OF INDIVIDUAL SPECIES OF THE GENERA *Monanthes, Greenovia, Aichryson, Aeonium, Sedum, Euphorbia, Lolium* and *Dracaena*. (In two species, *Euphorbia aphylla* and *E. peplus*, the wax originates from stalk and leaves.) Alkanes present as less than 2 mole per cent have been omitted.

similar hydrocarbon patterns and the same is true for the different hydrocarbon pattern obtained for the species in the section *Urbica*. The botanical sub-division of *Urbica* proposed by Lems[17] is not paralleled by these results. Six of the species (35, 28, 31, 29, 33, 36) of the section *Goochia* give mutually similar hydrocarbon patterns but the remaining three seem out of step in this respect, *A. caespitosum* (42), *A. spathulatum* (38) and *A. cruentum* (37). The species of the section *Canariensia* fall into two groupings. The first three members (26, 22 and 24) show a close hydrocarbon pattern relationship allied to *Holochrysa* while the patterns of the next two (20 and 25) are much more akin to those

of the section *Urbica*; one member of the *Canariensia*, the distinctive plate-like *A. tabulaeforme* (27), is quite anomalous in the reversal of its C_{31} to C_{33} *n*-alkane ratio.

In the less extensively examined genera *Greenovia*, *Aichryson* and *Monanthes* the hydrocarbon patterns are internally consistent with the exception of one of the last, *M. amydros* (58). Lems has suggested that the genus *Greenovia* is evolutionarily related to the *Canariensia* section of the genus *Aeonium* but the hydrocarbon patterns of the two are quite different and on this latter basis one might rather propose a relationship between the genera *Greenovia* and *Monanthes* and the sections *Urbica* and *Megalonium* of the genus *Aeonium*. On the other hand, the branched hydrocarbon content of the *Aichryson* species examined does give some support to Lems' contention that this genus is related to the *Goochia* section of the genus *Aeonium*.

Although such comparisons of hydrocarbon patterns may serve to establish relationships, it would seem that the differences between related genera may be insufficiently discriminating to act as "fingerprints", e.g. in the cases of the genera *Monanthes* and *Greenovia* studied in this work.

To show the results obtained in a completely different plant family a few readily available species of the genus *Euphorbia* have been included. This genus (750 species) is not closely knit botanically and the observed hydrocarbon distributions are of at least two types. In comparison with the Sempervivoideae, where the C_{31} and C_{33} hydrocarbon content generally approaches about 90 per cent of the whole, the *Euphorbiae* examined are much more variable in this respect and include a higher proportion of C_{27} and C_{29} hydrocarbons.

The monocotyledons *Lolium multiflora* (Italian rye grass) and *Dracaena draco* (Canary dragon tree) possess hydrocarbon patterns strikingly different from those of the Sempervivoideae representatives. From this and other work[4,5,9,24] it is thus far apparent that the major leaf-wax hydrocarbon constituents are C_{27}, C_{29}, C_{31}, C_{33} and C_{35}.

CONCLUSIONS

Even the present limited investigation on a restricted area has shown at least some glimmerings of a taxonomic relevance in the leaf-wax hydrocarbon pattern. The power of gas chromatography for work of this kind is rapidly increasing with development[25] of temperature-programmed, high-efficiency, high-speed columns and of automatic tabulation of quantities and retention times of the separated constituents. Such refined techniques should render surveys of the type reported here readily feasible on a very large scale, not only for the hydrocarbons, but for the oxygenated constituents.[25,26] Such investigations would not only be of taxonomic interest but could be used in the study of the possible relationships existing between plant waxes and such matters as plant metabolism,[1] water balance,[27] plant diseases,[2,28] weather damage,[2,27,28] atmospheric pollution damage[29], efficacy of crop-spraying,[2,30] the carcinogenetic effect of tobacco smoke,[4] and the origin of petroleum.[31] Other waxy coatings such as those possessed by micro-organisms,[32] insects[14] and animals[21] should certainly be amenable to similar study.

[24] P. MAZLIAK and COMBES, *Compt. Rend.*, **251**, 2393 (1960).
[25] W. J. A. VANDENHEUVEL and E. C. HORNING, *J. Org. Chem.*, **26**, 634 (1961).
[26] E. J. HEDGELEY and W. G. OVEREND, *Chem & Ind.*, 378 (1960).
[27] D. M. HALL and R. L. JONES, *Nature* **191**, 95 (1961); A. C. CHIBNALL, *J. Biol. Chem.*, **55**, 333 (1923).
[28] B. E. JUNIPER, Doctoral Thesis, Botany School, Oxford (1959).
[29] F. W. WENT, Proc. 1st national air pollution symp., California, 148 (1949).
[30] G. E. BLACKMAN, *Nature* **155**, 500 (1945).
[31] R. ROBINSON, *Catalyst*, **6**, 41 (1961).
[32] J. PUDLES and E. LEDERER, *Bull. Soc. Chim. biol.*, **36**, 759 (1954).

EXPERIMENTAL

Collection and Identification of Plant Material

The plants listed in Table 2, with the exception of the *Sedum* and the grass, were collected during the period September 1960 to April 1961 in the Western group of the Canary Islands—Tenerife, Gran Canaria, Gomera, La Palma and Hierro. A few species were collected from several stations and at different seasons. All were identified by Dr. S. Sventenius of the Jardin del Aclimataçion at Orotava, Tenerife. In order to prevent contamination of the surface waxes, contact between the leaves of different species was carefully avoided and stations were chosen which were not adjacent to road traffic or subject to urban atmospheric pollution.

Extraction of Surface Waxes[2]

In all cases, except certain *Euphorbia* species, only leaf waxes were extracted. Although the quantity and appearance of the leaf wax is known[2,28] to differ on the abaxial and adaxial surfaces, the present work concerns the total wax from both surfaces. The leaves were generally examined as soon as possible after collection of the entire plant which, in most cases, survived even prolonged storage.

A representative sample (100 g) of leaves (picked from several individual plants of the particular species, collected at the same station) was extracted by dipping small bunches or individual leaves for 30 sec in each of three successive small volumes (50 ml, 25 ml and 25 ml) of chloroform. In some cases complete buds, unopened or partially opened, of small leaves were extracted. The combined extract was filtered to remove suspended matter and the filtrate and washings evaporated to dryness under reduced pressure. The residue was weighed and its infrared spectrum recorded as a solid film (Perkin–Elmer Model 13 or 137; cf. Fig. 2a).

Tests with further volumes of chloroform and also with light petroleum demonstrated that this rapid and simple procedure adequately dissolved the surface wax without removing any of the cytoplasmic constituents. After this treatment the fleshy-leaved species frequently exuded in a dramatic manner considerable quantities of water.[27]

Isolation of the Hydrocarbon Content

The wax (50–250 mg) was heated for 2 hr with methanolic potassium hydroxide (1 g in 30 ml); the ethanol was removed under reduced pressure and the residue extracted consecutively with three 120 ml portions of 60–80° light petroleum. After concentration to small bulk, the petroleum extract was passed down a column of basic alumina (10 cm by 1 cm, Brockmann, Grade II) followed by elution with light petroleum. The solvent was removed from the first 50 ml of eluate under reduced pressure; the residue was weighed and its infrared spectrum recorded (solid film). The residue represented about 95 per cent of the total hydrocarbon content of the wax and contained no contaminants of different functional type (Fig. 2b). Repetition of the alkali treatment on this product caused no change in the hydrocarbon proportions. An alternative simpler procedure in which the hydrolysis step was omitted, was employed in two cases (Table 2; 32 and 34). The residue from the chloroform extract was chromatographed directly[3,33] as above. The first 50 ml of the eluate furnished a hydrocarbon fraction identical in composition with the product obtained by the above alkali treatment.

[33] L. SAVIDAN, Doctoral Thesis, L'Universite de Paris (1956).

Gas-Liquid Chromatography of the Hydrocarbon Extracts

A Pye "Argon Gas Chromatograph" (^{90}Sr detector) was used with a 120×0·5 cm column of Celite (80–100 mesh)[34] coated with 0·5 per cent of Apiezon L grease deposited from 60–80° light petroleum. Generally, the hydrocarbon mixture (~ 2 mg) in warm α-methylnaphthalene (~ 50 mg) was applied to the heated column (~ 230°) in 0·1 μl loads. Several columns were used as prolonged operation at this temperature resulted in denudation of stationary phase. Typical chromatograms are illustrated in Fig. 3. The peaks were assigned on the basis of addition of genuine *n*-alkanes which resulted in intensification of the appropriate peaks. Further, a linear relationship was obtained between the log of the retention time (R_t) and the assigned *n*-alkane carbon number (Fig. 4). Nearly all the remaining peaks showed a parallel straight line relationship (Fig. 4) and were consequently attributed to *iso*alkanes (2-methylalkanes).[15,21] Confirmation of this latter conclusion was derived from the enhancement of the appropriate peak by addition of a genuine sample[22] of 2-methyldotriacontane and from the infrared characteristics of the hydrocarbon mixture itself. The absence of unsaturated hydrocarbons and oxygen-containing components was further checked in one case (41) by the virtual constancy of the gas chromatogram pattern even after several hours' treatment of the hydrocarbon mixture (40) with hot concentrated sulphuric acid.[1]

The content of individual hydrocarbons in the mixtures is expressed in Table 2 as an area percentage derived from the area of the relevant peak and the total area of all the peaks between C_{25} and C_{35} inclusive. Alkanes below and above these limits have been ignored as control experiments with weighed mixtures show them to be present in only trace amounts. Areas were determined by planimeter (six determinations) for chromatograms with all peaks on scale and with the major constituents registering almost full scale. Accuracy was checked by repeat analyses (e.g. 44–46) and the percentages in Table 2 seem to be reproducible to ± 5 per cent, a variation due to detector-amplifier and planimetering errors. While not negligible this deviation is definitely too small to invalidate the general conclusions. Evaluation of synthetic mixtures of *n*- and *iso*alkanes confirmed the detector response to be proportional to the molar concentration and was virtually the same for the two types of hydrocarbon.

"Column efficiencies"[35] were of the order of 1500–2500 theoretical plates for the hydrocarbons in the C_{30} region (retention times ca 35 min). In Fig. 3, the "peak resolutions"[35] were, for example, 1·3 for the *n*- and *iso*-C_{33}-alkane peaks and 2·9 for the *n*-alkane C_{31} and C_{32} peaks.

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[34] J. and J's, Ewell, Surrey.
[35] R. P. W. SCOTT, *Gas Chromatography*, p. 429, Butterworths, London (1960).

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ÜBER γ-METHYLENGLUTAMIN UND γ-METHYLENGLUTAMINSÄURE IN KEIMLINGEN VON *AMORPHA FRUTICOSA* L.

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Zusammenfassung—γ-Methylenglutaminsäure und ihr Amid, das γ-Methylenglutamin, wurden in Keimlingen verschiedener *Amorpha*-Arten nachgewiesen und als γ-Methylenglutaminsäure aus Keimlingen von *Amorpha fruticosa* isoliert. *Amorpha* ist damit nach *Arachis* die zweite γ-Methylenglutamin führende Leguminose. Die Übereinstimmungen im Stickstoffmetabolismus der beiden Pflanzen werden im Zusammenhang mit der taxonomischen Stellung von *Arachis* und *Amorpha* diskutiert.

Abstract—γ-Methyleneglutamic acid and its amide, γ-methyleneglutamine, have been shown to be present in seedlings of several *Amorpha* species, and have been isolated as γ-methyleneglutamic acid from seedlings of *Amorpha fruticosa*. Thus *Amorpha*, like *Arachis*, is another γ-methyleneglutamine containing leguminous genus. The similarities in the nitrogen metabolism of the two genera are discussed in connection with their taxonomical position.

γ-METHYLENGLUTAMIN und γ-Methylenglutaminsäure wurden bisher nur in wenigen Pflanzen beobachtet, nähmlich in der Erdnuss,[1] in Tulpen[2] und in einigen weiteren Liliaceen[3] sowie im Hopfen.[4] Done und Fowden[1] haben im Zusammenhang mit ihren Untersuchungen an *Arachis* einige weitere Leguminosen ohne Erfolg getestet. Wenn von den Untersuchungen von Hyde[5] und von Engelbrecht[6] abgesehen wird, die nur Spuren von γ-Methylenglutamin in Erbsen bzw. Bohnen auf papierchromatographischem Wege gefunden haben, so ist *Arachis* bisher die einzige, wirklich nennenswerte Mengen von γ-Methylenglutamin führende Leguminose.

In den reifenden und ausgereiften Samen von *Arachis* sind γ-Methylenglutaminsäure und ihr Amid nicht nachweisbar. Erst während der Keimung der Samen erscheinen beide Verbindungen. Es wird vermutet, dass sie massgeblich an den dabei stattfindenden Stickstoffverlagerungen beteiligt sind.[7] Die hohe Konzentration des γ-Methylenglutamins im Blutungssaft junger Keimlinge spricht ebenfalls für diese Annahme. Alle anderen Aminosäuren treten in wesentlich geringerer Konzentration auf. Im Gegensatz zu diesem Befund werden bei normaler Extraktion des Gewebes mit 70% Äthanol eine Anzahl weiterer Aminosäuren in ähnlich hoher Konzentration wie γ-Methylenglutamin erhalten. Der Blutungssaft junger Erdnusspflanzen ist also ein besonders günstiges Ausgangsmaterial für die Darstellung des γ-Methylenglutamins.

Bei Untersuchungen über die während der Keimung der Samen von *Amorpha fruticosa* und einiger weiterer *Amorpha*-Arten ablaufenden Veränderungen der Stickstoff-Fraktionen haben wir ganz ähnliche Verhältnisse beobachten können. Die Samen dieser Leguminosen

[1] J. DONE und L. FOWDEN, *Biochem. J.* **51**, 451 (1952).
[2] R. M. ZACHARIUS, J. K. POLLARD und F. C. STEWARD, *J. Am. Chem. Soc.* **76**, 1961 (1954).
[3] L. FOWDEN und F. C. STEWARD, *Ann. Botany (London)* **21**, 53 (1957).
[4] G. HARRIS und R. A. TATCHELL, *J. Inst. Brewing* **59**, 371 (1953).
[5] T. G. HYDE, *Biochem. J.* **55**, 21 (1953).
[6] L. ENGLEBRECHT, *Flora, Jena* **142**, 25 (1954).
[7] L. FOWDEN, *Ann. Botany (London)* **18**, 417 (1954).

besitzen weder γ-Methylenglutamin noch γ-Methylenglutaminsäure. Erst im Zustand der Keimung gelingt es u.a. zwei Verbindungen nachzuweisen, von denen wir auf Grund ihres Verhaltens bei der papierchromatographischen Untersuchung annahmen, dass es sich um γ-Methylenglutamin und um γ-Methylenglutaminsäure handelt. Denn mit *Arachis* durchgeführte vergleichende Untersuchungen zeigten, dass sowohl hinsichtlich der Konzentrationsverhältnisse als auch in der Reaktionsweise (gelbbraune Färbung mit Ninhydrin, R_f-Werte in verschiedenen Lösungsmitteln, Verhalten gegenüber Hydrolyse mit N HCl) zwischen den in *Arachis* enthaltenen γ-Methylen-Aminosäuren und den in *Amorpha* beobachteten Verbindungen kein Unterschied besteht. Durch die Isolation der γ-Methylenglutaminsäure konnte schliesslich der endgültige Beweis für diese Vermutung erbracht werden.

Obwohl auch im Blutungssaft von *Amorpha*-Keimlingen das γ-Methylenglutamin die Hauptaminosäure bildet, war es auf Grund der geringen Grösse dieser Keimlinge nicht möglich, eine für die Darstellung ausreichende Menge an Blutungssaft zu gewinnen. Wir beschränkten uns deshalb auf die Isolation der γ-Methylenglutaminsäure. Dazu wurden die Keimlinge in einem Mixer zerkleinert und mit 70% Äthanol extrahiert. Der so gewonnene Rohextrakt wurde nach der Methode von Plaisted[8] an einer Dowex-50-Säule gereinigt und die in ihm enthaltenen Amide—es handelt sich hauptsächlich um γ-Methylenglutamin und um Asparagin—durch Hydrolyse mit N HCl in die entsprechenden Säuren überführt. Mit Hilfe einer Dowex-1-Säule wurden die im Hydrolysat enthaltenen Dicarbonsäuren von den neutralen und basischen Aminosäuren abgetrennt. Die weitere Fraktionierung der Dicarbonsäuren erfolgte durch Chromatographie mit einem Phenol–Wasser–Gemisch an einer Cellulosepulver-Säule. Die so gewonnene γ-Methylenglutaminsäure wurde mit Aktivkohle entfärbt und nach mehrmaligem Umkristallisieren aus heissem Wasser in Form feiner Kristallnadeln erhalten. Die Verbindung erwies sich als papierchromatographisch rein und zeigte in bezug auf den Schmelzpunkt (195–198°) eine gute Übereinstimmung mit dem in der Literatur angegebenen Verhalten.[1]

Zur weiteren Charakterisierung der Verbindung wurden 5 ml einer gesättigten Lösung von γ-Methylenglutaminsäure tropfenweise mit einer 1% $KMnO_4$-Lösung versetzt. Es erfolgte eine Entfärbung der Lösung als Hinweis auf die Anwesenheit einer Doppelbindung. Durch weitere Permanganatzugabe wurde eine bleibende Rotfärbung erreicht. In dem Reaktionsgemisch konnte nach Filtration und Entsalzung Asparaginsäure neben einer geringen Menge der Ausgangssubstanz nachgewiesen werden. Weitere 5 ml der gesättigten γ-Methylenglutaminsäurelösung wurden mit Zink und Salzsäure reduziert. Die nach einer entsprechenden Reinigung durchgeführte papierchromatographische Untersuchung des Reaktionsgemisches führte zum Nachweis einer Aminosäure, die im Gegensatz zu der mit Ninhydrin eine gelbbraune Färbung ergebenden γ-Methylenglutaminsäure mit demselben Reagens eine rotviolette Färbung zeigte, und die im R_f-Wert mit γ-Methylglutaminsäure übereinstimmte.

Diese bisherigen Ergebnisse lassen erkennen, dass in *Amorpha* die isolierte γ-Methylenglutaminsäure—hauptsächlich in Form ihres Amides—offensichtlich die gleiche Rolle spielt wie in *Arachis*. Es ergibt sich somit die Tatsache, dass zwei Gattungen der Papilionaceen, die in dem System von Engler und Prantl an verschiedenen Stellen stehen, grosse Ähnlichkeit im Stickstoff-Stoffwechsel aufweisen. Die Leguminosen sind nicht allein durch hohen N-Gehalt, sondern auch durch das Vorkommen aussergewöhnlicher, nicht proteinogener Aminosäuren in reichem Masse ausgezeichnet. Die Erforschung dieser

[8] P. H. PLAISTED, *Contrib. Boyce Thompson Inst.* **19**, 231 (1958).

interessanten Substanzen steht sicherlich noch in den Anfängen (z.B. Canavanin:[9-13]). Es handelt sich meist um stoffwechselaktive Verbindungen, die als solche stetig vor sich gehenden Aufbau—und Abbauprozessen unterliegen, und die Speicher—oder Transportformen des Stickstoffs darstellen mögen. Es scheint bei der Lückenhaftigkeit unserer phytochemischen Kenntnisse vorläufig noch sehr gewagt, taxonomische Schlüsse aus diesem Vorkommen zu ziehen.

[9] E. A. BELL, *Biochem. J.* **70**, 617 (1958).
[10] E. A. BELL, *Biochem. J.* **75**, 618 (1960).
[11] B. A. BIRDSONG, R. A. ALSTON und B. L. TURNER, *Can. J. Botany* **38**, 499 (1960).
[12] B. TSCHIERSCH, *Flora, Jena* **147**, 405 (1959).
[13] B. TSCHIERSCH, *Flora, Jena* **150**, 87 (1961).

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TRANSITIONAL STATES OF RIBONUCLEIC ACID AND MORPHOGENESIS IN SYNCHRONOUS SINGLE GENERATIONS OF *BLASTOCLADIELLA EMERSONII*

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Abstract—The biochemistry of morphogenesis in the water fungus, *Blastocladiella emersonii*, was studied in cultures in which from ten million to four hundred million plants were grown synchronously for a single generation. Thus, chemical changes per cell were established at different stages in ontogeny. The point of no return in morphological differentiation was associated with a biochemical differentiation of the internal pool of the cell's ribonucleic acid. The results are discussed in terms of previous studies on the induced synthesis of enzymes presumed to play a roll in morphogenesis in *Blastocladiella*.

THE morphological point of no return in the bicarbonate-dependent differentiation of a resistant sporangium (R.S.) of the water fungus, *Blastocladiella emersonii*, is associated with (a), cessation of deoxyribonucleic acid (DNA) synthesis, and (b), a marked change in the nature of ribonucleic acid (RNA). In particular, a NaCl-soluble RNA ($RNA_{NaCl\text{-}sol}$) fraction, which contains a small amount of cytidylic acid relative to adenylic acid, begins to decrease; simultaneously, a NaCl-insoluble, KOH-soluble (RNA_{insol}) fraction, which sediments at $15{,}000 \times g$ and contains all four nucleotides in almost identical proportions, begins to increase sharply. When the transformation is completed, and net synthesis of RNA has ceased, the $RNA_{NaCl\text{-}sol}$ which remains no longer possesses the composition of the original $RNA_{NaCl\text{-}sol}$; instead, all four nucleotides occur in equimolar amounts. The data suggest that the $RNA_{NaCl\text{-}sol}$, synthesized during exponential growth, contains the "soluble" and microsomal components; that the latter, at the morphological point of no return, undergoes degradation (turnover) with consequent resynthesis of RNA_{insol}; and that the RNA_{insol} is similar to the remaining "soluble" fraction of $RNA_{NaCl\text{-}sol}$ in nucleotide composition, but different from it in size and/or physical state. When the exogenous inducer of morphogenesis, bicarbonate, is removed before the point of no return (at which time, synthesis of certain enzyme systems such as α-ketoglutarate dehydrogenase, isocitric dehydrogenase, and morphogenesis itself, are still plastic and reversible), the cell's RNA_{insol}, which is in the process of increasing, decreases sharply. After the point of no return (when it is no longer possible to reverse synthesis or degradation of these enzyme activities, nor morphogenesis, by removal of bicarbonate), the cell's RNA_{insol} remains similarly unaffected by removal of bicarbonate. At maturity, the 2-celled R.S. plant can be made to germinate, whereby the protoplast is cleaved into some 6500 uninucleate spores which start the next generation. This does not involve "growth", in the usual sense (i.e. increase in mass), but it is associated with a rate of RNA synthesis almost equal to that associated with exponential growth. Conversion of $RNA_{NaCl\text{-}sol}$ to RNA_{insol} may provide a focal point for some of the manifold reactions associated with the biochemical and morphological point of no return in the differentiation of a resistant sporangium in *Blastocladiella emersonii*.

METHODS

Ordinary Colorless (O.C.) Plants

O.C. plants,[1] grown in liquid medium PYG (Difco Laboratories, Detroit, Mich.) at 25° and harvested between 50 and 90 per cent of their generation time, were pooled and homogenized 5 min at 0–2° in a Waring Blender (75 g wet wt./250 ml H_2O). Homogenates were mixed with pre-chilled trichloroacetic acid (TCA; final concn. 10%), incubated 30 min at 0–2° with intermittent agitation, and centrifuged (5 min, 500×*g*). The supernatant was frozen for subsequent analyses. The ppt. was re-extracted 4 times, 15 min each, with 5 vol. 10% TCA, refluxed[2] 20 min with ethanol–ethyl ether (3 : 1), 2 hr with chloroform–methanol (1 : 1), washed with ether, and dried *in vacuo* at 2°.

Following Volkin and Cohn[3] with slight modification (see also Davidson and Smellie[4]), the dried ppt. was washed with 10% NaCl (1·0 g/30 ml 10% NaCl containing 0·002% $NaHCO_3$; pH 7·5). After centrifugation (nucleic acid content of supernatant, negative), the ppt. was resuspended in 4 vol. NaCl and extracted with agitation for one hr at 100°. The extract was collected by centrifugation and the ppt. re-extracted twice. Supernatants were combined, and the sodium nucleate therein was precipitated with 3 vol. ethanol (8 hr, 2°), washed with ethanol, and dried *in vacuo* at 2°.

The nucleotide content of the sodium nucleate was established by incubation with 0·3 N KOH (18 hr, 37°). DNA was generally removed by addition of an equal vol. of ethanol at 2°, either before or after treatment with Dowex-50 (see below). Following Cohn,[5] but modifying column sizes to suit the problem (generally, 15 cm×0·8 cm), Dowex-1 (×8, 100–200 mesh, Cl′ form) was washed successively with H_2O, H_2O-acetone (1 : 1), acetone, petroleum ether, acetone, acetone-H_2O, and H_2O, charged with 1N HCl, and washed exhaustively with H_2O. The KOH hydrolysate was neutralized with well-washed Dowex-50 (×8, 100–200 mesh, H form) to pH 7·8 (thus removing K) and passed through the Dowex-1 column (with prior removal of DNA by alcohol precipitation if not done previously). Nucleotide fractions were eluted according to Cohn,[5] using 0·002 N HCl for the mixed cytidylic acids (CMP) and the 2′ and 3′ adenylic acids (AMP), followed by 0·003 N HCl for uridylic acids (UMP), and 0·005 N HCl for the 2′ and 3′ guanylic acids (GMP); the elution pattern was followed by measuring optical density (O.D.) in ten ml fractions at 260mμ. After GMP, no other u.v.-absorbing components were detected with additional elution (several l., up to 0·2 N HCl). Nucleotide fractions were neutralized with NH_4OH, re-concentrated on small columns (1–2 cm×0·5 cm) of Dowex-1 (Cl′ form), and re-eluted with a small vol. of 0·1 N HCl. Completeness of recovery was established spectrophotometrically.

All nucleotides were identified and determined quantitatively by several methods. The extinction coefficients used were taken from Cohn.[5] Complete spectra (220–300 mμ), 250/260, 280/260, and 290/260 mμ ratios, phosphorus/nucleotide molar ratios, and (for purine nucleotides) pentose/nucleotide molar ratios were obtained. Pentose was determined with Norit-decolorized, recrystallized (benzene, petroleum ether) orcinol.[6] Nucleotide R_{uracil} (movement relative to uracil) values, and the absence of contaminating materials, were established by chromatography in isopropanol-12 N HCl-H_2O (65 : 17 : 18)[7] and *iso-*

[1] E. C. CANTINO and M. T. HYATT, *Leeuwenhoek Ned. Tijdschr.* **19**, 25 (1953).
[2] G. SCHMIDT, *Meth. Enzymol.* **3**, 671 (1957).
[3] E. VOLKIN and W. E. COHN, *Meth. Biochem. Anal.* **1**, 287 (1954).
[4] J. N. DAVIDSON and R. N. SMELLIE, *Biochem. J.* **52**, 594 (1952).
[5] W. E. COHN, *Meth. Enzymol.* **3**, 724 (1957).
[6] W. C. SCHNEIDER, *Meth. Enzymol.* **3**, 680 (1957).
[7] A. BENDICH, *Meth. Enzymol.* **3**, 715 (1957).

propanol-15 N NH_4OH-H_2O (70 : 7 : 30; chamber vol. for one dimens., 40 l.) in one and two dimensions. Also, all four nucleotides (as mixed 2′ and 3′ isomers) were isolated from Dowex-1 eluates as Ba salts (neutralization to pH 8·2, addition of ten fold molar excess of Ba acetate and 3 vol. ethanol, incubation at 2°, 24 hr), washed with 50% ethanol, and their spectra and chromatographic properties established.[8]

Resistant Sporangial (R.S.) Plants

Synchronized, single-generation cultures of R.S. plants were grown at 24° and harvested as previously described.[9,10,11] Per-plant (i.e. per-cell; see Discussion) calculations were made as before.[9] The organism was dried *in vacuo* at 2°, ground to a fine powder, and frozen. Dried powders were homogenized at ca. 0° in a Servall Omnimixer with glass beads as abrasive[9] (575 mg/15 ml H_2O and 15 g beads, 10–20 min; time required for complete fragmentation of R.S. depends upon stage of development and was established microscopically). Homogenates were freed of glass beads, mixed with pre-chilled TCA (final concn., 10%), and incubated several hr at 2°. After centrifugation (5 min, 500 × *g*). TCA-supernatants and TCA-insoluble residues were treated as follows:

(a) *TCA-soluble fraction.* This fraction was analyzed for total nitrogen (N) by wet combustion[12] (H_2SO_4 and H_2O_2, followed by Nesslerization) to further ensure that complete breakage of all sporangia had occurred; i.e. total, soluble, non-protein N was compared with that previously established[9] for R.S. plants at different stages of development. Analyses for orthophosphate (P_i) and total P on extracts, nucleotide fractions, etc. were adapted from conventional methods (the Fiske-Subbarow procedure and wet combustion analyses) as outlined by Umbreit *et al.*[12] After removing TCA by ether extraction (see below), the pool of free, basic purines (adjusted to pH 1 with HCl) was isolated on Dowex-50 (H form),[13] eluted with 10% NH_4OH, concentrated *in vacuo* (flash-evaporator), chromatographed 2-dimensionally on Whatman No. 1 paper with ethanol-acetic acid-H_2O (81 : 5 : 14)[13] and butanol-H_2O (86 : 14),[13] eluted with 0·1 N HCl, and quantitized spectrophotometrically, using appropriate extinction coefficients,[14] authentic samples as standards, and the usual paper blanks. For isolation of the pool of free nucleotides, the TCA-soluble fraction was extracted 4 times with an equal vol. of ethyl ether, neutralized to pH 8 with NH_4OH, diluted to a suitable ionic strength,[5] and run through a Dowex-1 column as used for O.C. plants. A preliminary fractionation was made with 0·003 N HCl, 0·03 N HCl, and 1·0 N HCl such that CMP and AMP were collected in the 0·003 N eluate, and GMP and UMP in the 0·03 N HCl. These fractions were re-concentrated and re-eluted twice from smaller columns, using dilute HCl of various strengths. Thus, other u.v. absorbing materials, amino acids, etc. particularly abundant in young (i.e. 32 hr) R.S. plants, were effectively eliminated as shown by paper chromatography with *iso*propanol-HCl and *iso*propanol-NH_4OH systems. The purity and identity of these TCA-sol. nucleotides were established by the methods already described for O.C. plants (i.e. relative R_f values in several solvents, complete spectra, O.D. ratios, nucleotide/P ratios, and when applicable, nucleotide/pentose ratios). Soluble, undegraded RNA was not detected in TCA extracts.

[8] A. R. Todd, *Meth. Enzymol.* **3**, 811 (1957).
[9] J. S. Lovett and E. C. Cantino, *Am. J. Bot.* **47**, 550 (1960).
[10] J. S. Lovett and E. C. Cantino, *J. Gen. Microbiol.* **24**, 87 (1961).
[11] E. C. Cantino and J. S. Lovett, *Physiol. Plantarum.* **13**, 450 (1960).
[12] W. W. Umbreit, R. H. Burris, and J. F. Stauffer, *Manometric Techniques*, (3rd Ed.) Burgess Publ. Co. Minneapolis, Minn. (1957).
[13] F. Bergmann and S. Dikstein, *Meth. Biochem. Anal.* **6**, 79 (1958).
[14] Properties of the Nucleic acid derivatives; *Calif. Corp. Biochem. Res.*, Los Angeles, Calif. (1955).

(b) *TCA-insoluble residue.* This was re-extracted twice with 10% TCA, refluxed with ethanol-ethyl ether and chloroform-methanol, washed with ether, and dried as for O.C. plants. Similarly, the NaCl extraction for nucleic acids, removal of DNA, alkaline hydrolysis of RNA, and Dowex-50 treatments were like those used for O.C. plants, as were the major fractionations of RNA nucleotides on columns of Dowex-1. In addition, somewhat smaller columns were frequently used to expedite fractionations when it was not essential to separate the 2′ and 3′ isomers.

The RNA_{insol} in the residue from NaCl extractions was estimated as follows: residual salt was washed out with 50% ethanol, and the residue was incubated (18 hr, 37°) with 0·3 N KOH. The KOH hydrolysate was treated with ethanol (final concn. 50%) to remove certain non-RNA materials, and analyzed for nucleotides by chromatography on Dowex-1 (see above) and pentose analysis.[6,15] The molar purine/pyrimidine ratio in RNA_{insol} is almost exactly one; thus, the ribose value represents one-half the total RNA-nucleotide level.

Finally, a very small residue of NaCl-insoluble, KOH-insoluble RNA was extracted by digestion with 0·6 M perchloric acid (20 min, 70°). Additional extraction with KOH and perchloric acid did not yield nucleic acids or nucleotides.

The thrice TCA extracted and defatted TCA-insoluble residue was digested with 5% TCA (30 min, 90–95°), and the DNA estimated with the *p*-nitrophenylhydrazine method.[15] Both 45 sec and 1 min intervals between addition of alkali and O.D. measurements at 560 mμ were used; the rapidity of the O.D. change made critical timing essential. Highly-polymerized DNA (Na salt; 12·7% N, 7·9% P; *Calif. Corp. Biochem. Res.*) and chromatographically-pure deoxyadenosine were used as standards.

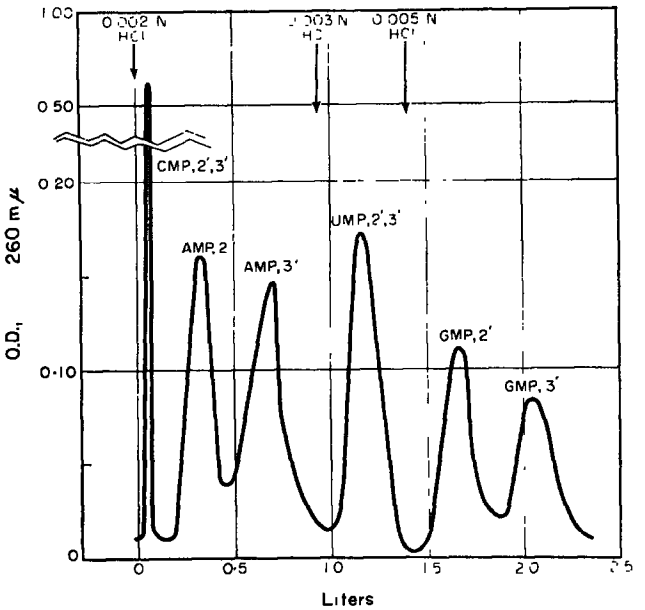

FIG. 1. REPRESENTATIVE RESULTS FROM THE COLUMN FRACTIONATION OF A KOH HYDROLYSATE OF $RNA_{NaCl\text{-}sol}$

$RNA_{NaCl\text{-}sol}$ derived from dry, TCA-extracted and defatted O.C. plants (orig. wt., ca. 1 g) of *B. emersonii*, using Cohn's[5] procedure on Dowex-1-×8, 100–200 mesh.

[15] J. M. WEBB and H. B. LEVY, *Meth. Biochem. Anal.* 6, 1 (1958).

RESULTS

O.C. Plants

Representative results from the Dowex-1 fractionation of $RNA_{NaCl\text{-}sol}$ nucleotides from O.C. plants are shown in Fig. 1 to illustrate the applicability of Cohn's method[5] for *Blastocladiella* and, thus, its probable utility for investigations of water fungi. No other u.v.-absorbing ingredients were recovered after the last nucleotide (3′ GMP) had been collected. The molar ratios of the nucleotides in this RNA fraction from O.C. plants reveal (Table 1) that pyrimidines constitute the lesser fraction (43·8 per cent) of the RNA (with CMP the minor component), and purines, the greater one (56·2 per cent).

TABLE 1. COMPOSITION OF RNA FRACTIONS FROM *B. emersonii* AT SEVERAL DIFFERENT STAGES OF DEVELOPMENT (see Fig. 2 for additional data)

	CMP	AMP	UMP	GMP
RNA fraction and source	Moles per 100 Moles			
$RNA_{NaCl\text{-}sol}$				
O.C. plants	20·5	28·1	23·3	28·1
	(ratio: 1·00 : 1·37 : 1·14 : 1·37)			
R.S. plants (av. for 32 and 36 hr; see Fig. 2)	21·4	27·4	23·1	28·2
	(ratio: 1·00 : 1·28 : 1·08 : 1·31)			
$RNA_{NaCl\text{-}sol}$				
R.S. plants (av. for 60 and 84 hr; see Fig. 2)	25·3	25·2	23·0	26·7
	(ratio: 1·00 : 1·00 : 0·91 : 1·05)			
RNA_{msol}				
R.S. plants, 60 hr	24·7	24·8	25·0	25·5
R.S. plants, 84 hr	24·8	24·8	24·9	25·5
Av.	24·8	24·8	25·0	25·5
	(ratio: 1·00 : 1·00 : 1·01 : 1·03)			

TABLE 2. RANGE OF VARIATION IN CHARACTERISTICS OF THE RNA NUCLEOTIDES (MIXED 2′ AND 3′ ISOMERS) ISOLATED BY COLUMN CHROMATOGRAPHY (SEE METHODS) FROM R.S. PLANTS OF *B. emersonii* AT DIFFERENT STAGES (FROM 38% OF GENERATION TIME TO MATURITY) IN DEVELOPMENT

	CMP	AMP	UMP	GMP
Absorption data at pH 1:				
O.D., 250/260 mμ	0·46 ± 0·02	0·85 ± 0·01	0·77 ± 0·02	0·99 ± 0·03
O.D., 280/260 mμ	1·91 ± 0·04	0·24 ± 0·02	0·33 ± 0·02	0·68 ± 0·01
O.D., max (mμ)	278–279	257–258	260–261	256
O.D., min (mμ)	240–241	ca. 230	ca. 230	ca. 228
P and N analyses:				
μMole nucleotide/μMole P	1·0 : 0·9–1·0	1·0 : 1·0–1·1	1·0 : 0·9–1·0	1·0 : 1·0
μMole nucleotide/μM pentose	—	1·0 : 0·95–1·0	—	1·0 : 0·95–1·0
Chromatography:				
R_{uracil} in *iso*propanol-HCl-H_2O (65 : 17 : 18)*	0·88	0·73	1·17	0·65

* Whatman No. 1, descending, ca. 25°; migration path, 50 cm. All R_{uracil} values ca. ±0·04. No detectable contaminants with u.v., ninhydrin, *p*-anisidine-HCl.

R.S. Plants

The narrow range of variation in absorption characteristics for all the re-concentrated nucleotide fractions isolated from R.S. plants at different stages of differentiation (Table 2), as well as other parameters, show that the nucleotides were isolated in reasonably clean form.

The composition of the $RNA_{NaCl\text{-}sol}$ fraction undergoes a marked change during morphogenesis (Fig. 2). In the $RNA_{NaCl\text{-}sol}$ derived from a young (32 hr) R.S. plant, nucleotide ratios are essentially the same as those found in the $RNA_{NaCl\text{-}sol}$ from O.C. plants (Table 1); indeed, such an R.S. plant will switch developmental pathways and grow into an O.C. plant if the exogenous bicarbonate is removed.[9,10,16] However, at the point of no return in R.S. differentiation (ca. 36 hr at 24°; 46 per cent of the generation time), the nucleotide ratios in the RNA begin to change; the proportion of CMP rises sharply, that of AMP dips almost equally fast, the GMP displays a less pronounced, gradual decrease, and the UMP undergoes no change at all (Fig. 2). Thus, the composition of $RNA_{NaCl\text{-}sol}$ changes drastically during morphogenesis, the final product containing more nearly equimolar quantities of all four nucleotides (Table 1). In view of a recent report,[17] it should be added that no 5′ nucleotides were detected in these RNA hydrolysates.

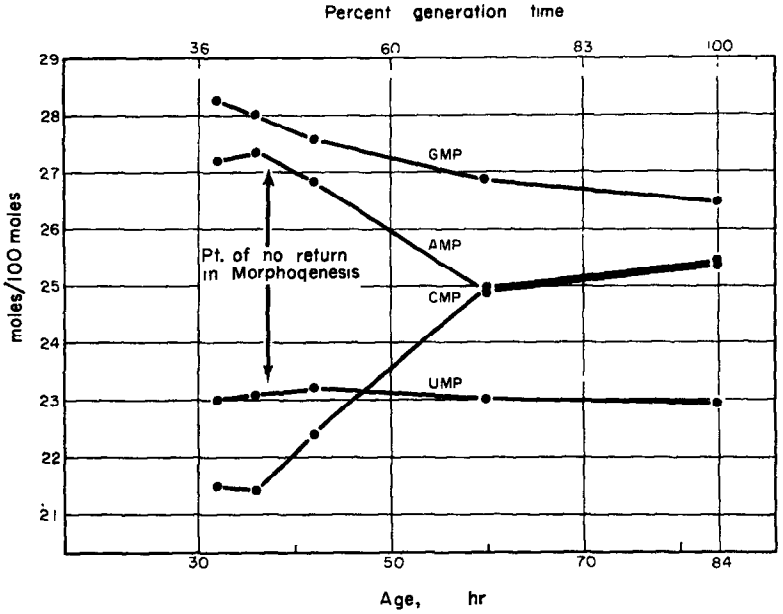

FIG. 2. THE MOLAR COMPOSITION OF $RNA_{NaCl\text{-}sol}$
Isolated at different stages during differentiation of an R.S. plant of *B. emersonii*.

During development (Fig. 3), the total $RNA_{NaCl\text{-}sol}$ per cell rises just as the organism itself increases exponentially in dry wt., vol., dia., etc.[9,10,11,16] up to approximately the morphological point of no return; at this time, the $RNA_{NaCl\text{-}sol}$ begins to decrease again to a level which approximates that in a young, 32 hr cell. Separate analyses on 15,000 × g and 20,000 × g supernatants derived from aqueous homogenates of 32 hr and 36 hr plants revealed that, at these early stages in ontogeny, over 95 per cent of the $RNA_{NaCl\text{-}sol}$ is recovered in the supernatant; thus, prior to the point of no return, this $RNA_{NaCl\text{-}sol}$ (which

[16] E. C. CANTINO, *Sympos. Soc. Gen. Microbiol.* **11**, 243 (1961).
[17] F. J. FINAMORE and E. VOLKIN, *J. Biol. Chem.* **236**, 443 (1961).

probably consists of microsomal and "soluble"[18] RNA) represents almost all the RNA in a young R.S. plant.

Before the maximum quantity of $RNA_{NaCl\text{-}sol}$ per cell has been formed, a NaCl-insoluble but KOH-soluble (i.e. by hydrolysis to nucleotides) fraction, RNA_{insol}, is detectable in very small amounts. But, when the point of no return is reached, and the $RNA_{NaCl\text{-}sol}$ begins its

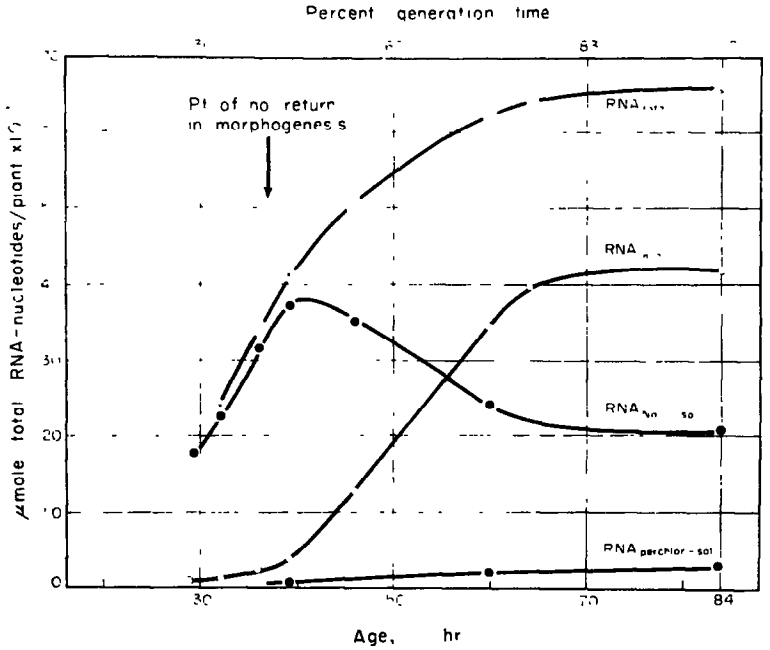

FIG. 3. THE PATTERN FOR NET SYNTHESIS OF DIFFERENT RNA'S IN AN R.S. PLANT DURING DIFFERENTIATION.

Values for RNA are expressed as total μMoles of all nucleotides derived from the RNA by KOH hydrolysis.

TABLE 3. THE EFFECT OF REMOVING AND NOT REMOVING THE BICARBONATE* FROM THE ENVIRONMENT OF A YOUNG R.S. PLANT (29 HR; 35% OF ITS GENERATION TIME) UPON THE RNA/CELL

	A 29 hr plant, growing on medium PYG containing bicarbonate[10]	B 29 hr plant, as in (A), transferred to H_2O for 3½ hr	C 29 hr plant, as in (A), grown for an additional 3½ hr
RNA_{insol} plant, μMoles	$1{\cdot}20 \times 10^{-6}$	$0{\cdot}64 \times 10^{-6}$	$1{\cdot}80 \times 10^{-6}$
Molar ratios of nucleotides in this RNA	(1·00 CMP	1·00 AMP : 1·00 UMP	1·03 GMP)
Net change	—	47% loss	50% increase
$RNA_{NaCl\text{-}sol}$ plant, μMoles†	$18{\cdot}0 \times 10^{-6}$	$15{\cdot}2\text{–}17{\cdot}9 \times 10^{-6}$	$24{\cdot}8 \times 10^{-6}$
Molar ratios of nucleotides in this RNA	(1·00 CMP	1·33 AMP : 1·08 UMP	1·27 GMP)
Net change	—	0–15% loss	38% increase

* Actually, in these experiments, all the ingredients of the medium, along with the bicarbonate, were removed. However, a young R.S. plant this far along in ontogeny will revert to an O.C. plant whether or not the other ingredients of the medium are replaced after the bicarbonate is removed.

† I.e. total μMoles of all nucleotides derived from the RNA by KOH hydrolysis.

[18] G. N. COHEN and F. GROS, *Ann. Rev. Biochem.* **29**, 525 (1960).

downward trend, the RNA_{insol} displays a striking rise in total quantity per cell (Fig. 3). In this kind of RNA, all four nucleotides are present in very nearly equimolar quantity (Tables 1, 3). A very small amount of perchloric acid-extractable but otherwise insoluble RNA also begins to appear (Fig. 3). Separate analyses on 15,000 × g supernatants derived from aqueous homogenates of plants of various ages beyond the morphological point of no return reveal that the supernatants now contain only an amount of RNA equivalent to the $RNA_{NaCl\text{-}sol}$ extractable at these various ages. Thus, the RNA_{insol} appears to consist of, or to be attached to, aggregates of greater than the usual, microsomal dimensions.

The total RNA/cell reaches its peak at ca. 75 per cent of the generation time of the R.S. plant, and then it levels off and remains constant throughout the remaining 25 per cent. Similarly, from 75 per cent of the generation time on, the RNA remains essentially uniform in composition; i.e. both the insoluble and soluble forms of RNA possess the four nucleotides in equal and almost-equal quantities, respectively (Table 1).

During the period immediately preceding and following the initiation of the RNA transformations described above, the free TCA-soluble, nucleotide pool/cell also undergoes rapid changes (Fig. 4). The CMP/cell, in particular, undergoes a sharp, 5-fold rise at the time the $RNA_{NaCl\text{-}sol}$/cell begins to decrease and the RNA_{insol}, with its relatively higher CMP content, begins to increase. The free AMP (and UMP; see legend, Fig. 4) pool/cell changes relatively little, and the GMP is altered even less. The behavior, at this time, of the free purines/cell is shown in Fig. 5. It is noteworthy that the corresponding nucleotide di- and triphosphates were not detected when attempts were made to find them, using appropriately different eluting systems.[5] Thus, while adenosine triphosphate, guanosine

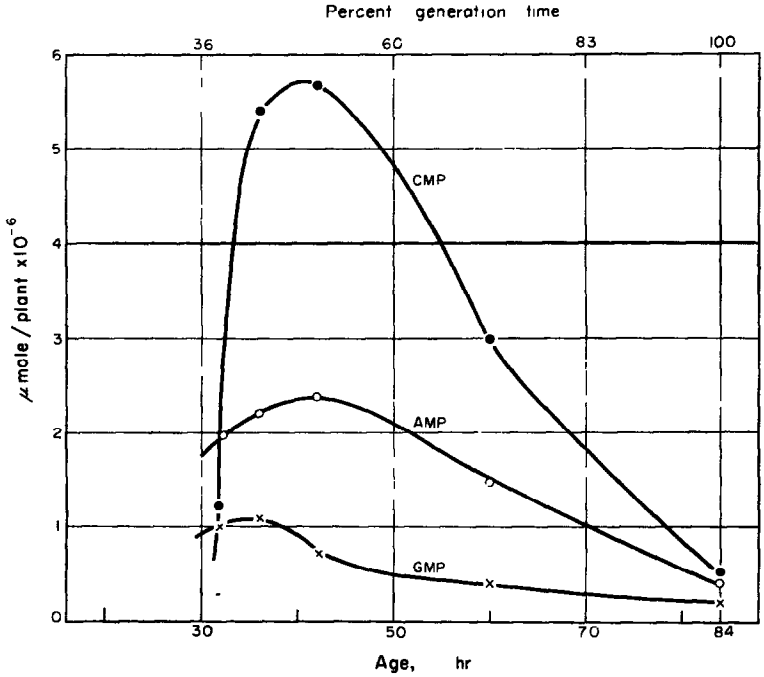

FIG. 4. THE POOL OF FREE NUCLEOTIDES/R.S. PLANT DURING DIFFERENTIATION.
The UMP values (not plotted) could not be established accurately because of the presence of an interfering material which could not be completely removed from TCA extracts. In general, however, the amount of UMP appeared to be roughly similar to that of the AMP.

triphosphate, etc. may be presumed to occur in *Blastocladiella*, they must be present at much lower levels than those reported here (i.e. 0·2–5·7 × 10^{-6} μMoles/plant) for mononucleotides.

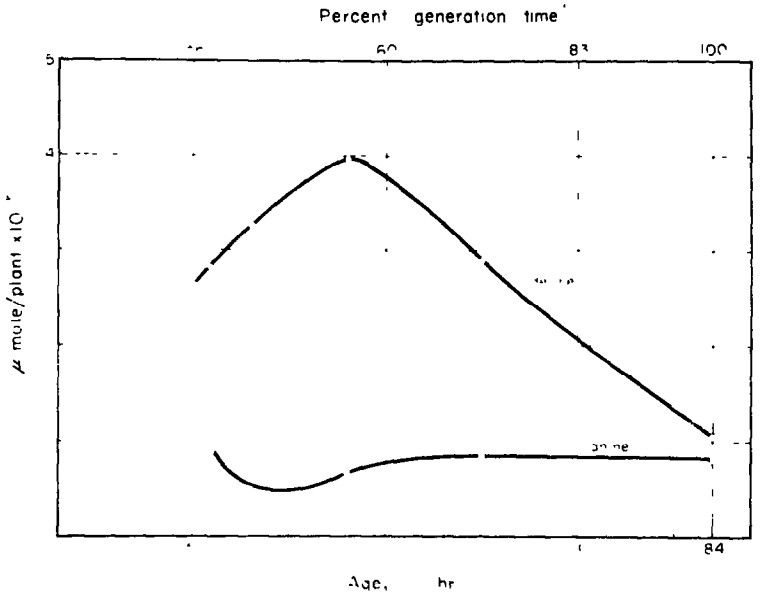

FIG. 5. THE POOL OF FREE ADENINE AND GUANINE, R.S. PLANT DURING DIFFERENTIATION.

Finally, estimates of the DNA/cell show that it levels off (Fig. 6) just beyond the point of no return in morphogenesis—very nearly at the same time that the $RNA_{NaCl\text{-}sol}$ (but not the RNA_{insol} nor total RNA) reaches its peak and then begins to decrease again. The final ratio of DNA/total nucleic acid (μMole values for total RNA nucleotides, Fig 3, converted to μg RNA) is ca. 7·5 per cent. Since, at maturity, the whole protoplast of an R.S. plant is converted to spores (if induced to do so) for the next generation, this value corresponds nicely with the percentage DNA/total nucleic acid (5·3 per cent–7·4 per cent) previously reported[19] for spores of *B. emersonii*.

Reversal of RNA Transformations by Removal of Bicarbonate

Using procedures described previously[10], young (29 hr) R.S. plants which had not yet reached the point of no return in morphogenesis were induced to embark upon the path leading to O.C. plants by removing exogenous bicarbonate (i.e. by washing the plants and then placing them in H_2O). The data (Table 3) show that: (a), before the point of no return, when the cell's low level of RNA_{insol} is just beginning to rise, reversal of the morphogenetic pathway by removal of bicarbonate is associated with a sudden loss of about half of the RNA_{insol} fraction, while without reversal, it continued to rise in normal fashion. On the other hand, the $RNA_{NaCl\text{-}sol}$, which is in the process of rising much more rapidly, drops slightly or simply levels off. However, when similar experiments are performed after the point of no return has passed (i.e. when removal of bicarbonate no longer causes the reversal of R.S. differentiation), the RNA pattern continues to change (as in Fig. 2, 3) whether or not bicarbonate is removed. These changes in the quantity of the RNA/cell before, but not after, the point of no return correspond to similar changes in the activity of certain enzymes believed to be directly involved in differentiation in *B. emersonii*.[10,16]

[19] G. TURIAN and E. C. CANTINO, *J. Gen. Microbiol.* **21**, 721 (1959).

DISCUSSION

At maturity, the water mold *B. emersonii* consists of two cells. But, by the time the young, single-celled, multinucleate thallus has begun to lay down a cross wall, thus subdividing itself into two units, its protoplasmic contents migrate in apparently unidirectional fashion into the apical region of the plant; that is clearly seen in photomicrographs of synchronized cultures at different stages of development.[9] Thus, when the two-celled stage is reached, the terminal, spherical cell contains essentially all of the protoplasm, while the sub-terminal, root-like, rhizoidal cell appears to contain none of it. In effect, then, the term "per plant" can be considered to mean "per cell"; an appreciation of this fact will facilitate interpretation of the results discussed below.

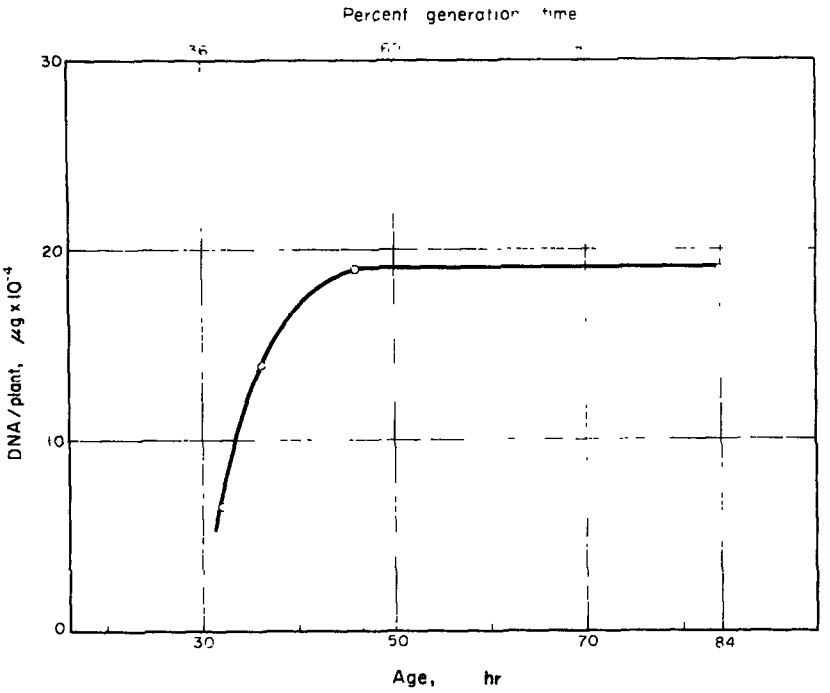

FIG. 6. THE DNA/R.S. PLANT DURING DIFFERENTIATION.

The two, major[16] morphogenetic pathways which may be taken by a spore of *B. emersonii* (Fig. 7) can be manipulated experimentally; synchronous, single generations of the organism can be induced to develop along either path, using populations of 10 million to 500 million or more cells per growth chamber. Furthermore, cultures started from motile spores are automatically synchronized without special treatment. That is, a spore inoculum consists of millions of swimming, uninucleate cells, all of which are derived from an immediately preceding, essentially simultaneous division of thousands of multinucleate protoplasts. Thus, with careful time scheduling, *Blastocladiella* produces very large, newly-formed cell populations of the sort that can be obtained from bacterial cultures only with appropriate selection procedures.[20] Synchronous growth does not depend upon an environmental change (heat shocks, etc.), and there is no question of creating experimental artifacts of the sort discussed by Williamson and Scopes.[21] Furthermore, because synchronous cultures

[20] F. E. ABBO and A. B. PARDEE, *Biochem. Biophys. Acta*, **39**, 478 (1960).
[21] D. H. WILLIAMSON and A. W. SCOPES, *Sympos. Soc. Gen. Microbiol.* **11**, 217 (1961).

involve only one generation, no complications are introduced by death and lysis (as in multiple-generation bacterial cultures[22]) resulting in proteolysis.

In the presence of added bicarbonate, the fungus spore develops into a thick-walled, brown, pitted R.S. plant; in the absence of bicarbonate, a distinctly different, thin-walled, colorless, papillate plant is formed (Fig. 7). Many differences in metabolic pools, storage products, enzyme activities, etc. associated with these two, different, morphological forms have been described.[16,23,24,25] In particular, recent studies[10,16] re-emphasized the belief that

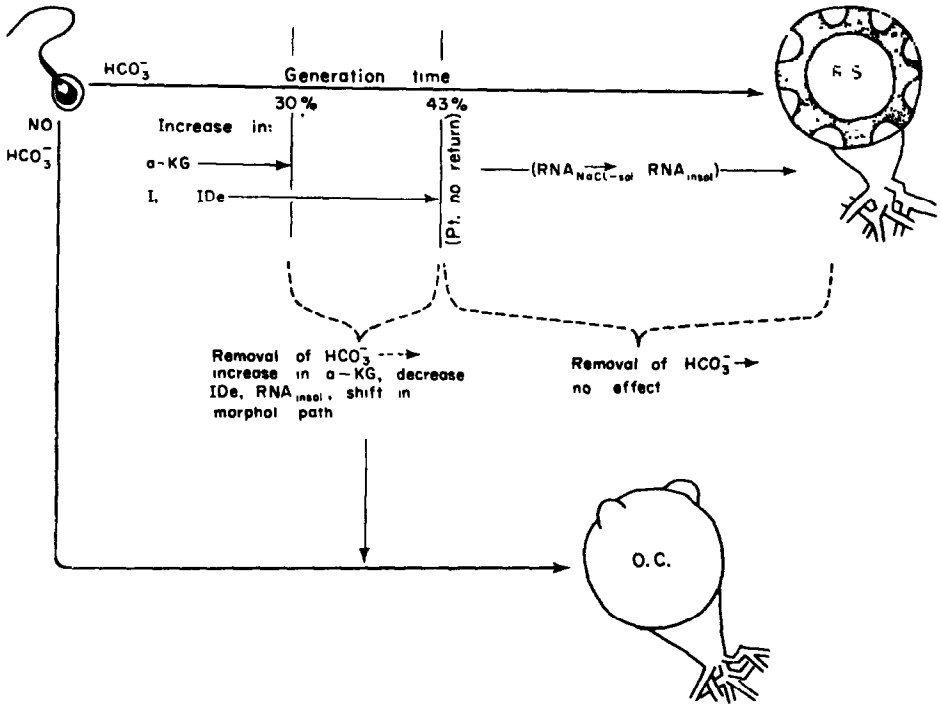

FIG. 7. SCHEMATIC PICTURE OF CERTAIN PERTINENT ASPECTS OF THE BICARBONATE-INDUCED DIFFERENTIATION OF R.S. PLANTS.

Legend: I., IDe, and α-KG = total isocitritase, isocitric dehydrogenase, and α-ketoglutarate dehydrogenase activities per plant.

at least four critical enzymes—α-ketoglutarate dehydrogenase system, isocitric dehydrogenase, isocitritase, and glycine-alanine transaminase—are involved in the bicarbonate trigger mechanism which leads to differentiation of an R.S. plant (Fig. 7). In brief, they seem to operate as follows:[16]

Isocitric dehydrogenase mediates fixation of the added bicarbonate via reductive carboxylation of ketoglutarate to isocitrate; the latter is removed by cleavage to succinate and glyoxylate via isocitritase; and the glyoxylate is metabolized by transamination via glycine-alanine transaminase. Exponential synthesis of isocitritase and isocitric dehydrogenase occurs during exponential growth of the R.S. plant. Simultaneously, accumulation

[22] J. MANDELSTAM, *Bact. Rev.* **24**, 289 (1960).
[23] E. C. CANTINO, *Handb. Pflphysiol.* **15**, 213 (1961).
[24] E. C. CANTINO and A. GOLDSTEIN, *Arch. Mikrobiol.* **39**, 43 (1961).
[25] H. D. MCCURDY and E. C. CANTINO, *Plant Physiol.* **35**, 463 (1960).

of the ketoglutarate dehydrogenase system (which, if present, would compete for ketoglutarate) is inhibited and brought to a halt at ca. 30 per cent of the generation time But, by removing the morphogenetic inducer, bicarbonate, before it is too late (Fig. 7), the total isocitric dehydrogenase activity/plant (which is in the process of increasing) is quickly reduced, the total ketoglutarate dehydrogenase activity/plant (which has levelled off) increases sharply, and the morphogenetic pathway (which is leading to an R.S. plant) simultaneously changes to that which leads to an O.C. plant. However, beyond a critical point (under our conditions 43–44 per cent of the generation time), the total activities/plant of α-ketoglutarate dehydrogenase and isocitric dehydrogenase are no longer reversible by removing bicarbonate; similarly, the differentiation process leading to an R.S. plant loses its apparent plasticity and can no longer be altered. These relationships between morphological and biochemical differentiation, discussed in greater detail elsewhere,[16] are summarized here because it was in an attempt to study these phenomena further that the experiments reported here were done.

With comparative biochemistry the guide post, it followed that the synthesis and apparent plasticity of these enzymes before the point of no return, and the lack of plasticity afterwards, would be under at least partial control of "soluble" and/or microsomal RNA.[18,25,26,27,28] Experiments then showed that the RNA in *Blastocladiella* undergoes major alteration at the point of no return in differentiation, and that the very early stages in such transformations remain plastic and reversible as do both the morphological and biochemical differentiations discussed above.

The Different "Kinds" of RNA in Blastocladiella

The total RNA cannot be extracted completely by any one procedure. On the basis of NaCl solubility, two main classes of RNA occur, and the quantity of each depends upon the stage in development of the cell. The RNA_{insol}/RNA_{total}, for example, increases from ca. 5 per cent in a young cell to ca. 65 per cent in a mature R.S. Parenthetically, these results re-emphasize the need, (a) for establishing the applicability of extraction procedures previously untried with the organism in question (e.g. as pointed out by Smillie and Krotkov[30] for *Euglena* and *Chlorella*); and (b) for specifying chemical composition in terms of the stage in development of an organism or population (see Herbert[31]).

Before the morphological point of no return, almost all of the RNA/cell is $RNA_{NaCl\text{-}sol}$, and its nucleotide composition is like that of the $RNA_{NaCl\text{-}sol}$ extractable from an O.C. plant. This observation lends support to the conclusion[9,16] that, whatever the changes induced by bicarbonate early in R.S. ontogeny, they are not irreversible changes—and that, early in development, a young R.S. plant is, in fact, potentially either an O.C. plant or an R.S. plant. Synthesis of $RNA_{NaCl\text{-}sol}$/cell is exponential,[32] and coincides with exponential growth[9] of the R.S. cell. On these bases, and judging also from the constancy of the base ratios in the $RNA_{NaCl\text{-}sol}$ up to the point of no return, and the low, free-nucleotide pool/cell, this RNA appears to be "stable" (as it is, for example, during exponential growth of *E. coli*[22]). But at the point of no return, only 65 per cent of the RNA/cell has been formed,

[26] G. D. NOVELLI, *Ann. Rev. Microbiol.* **14**, 65 (1960).
[27] P. BERG and E. J. OFENGAND, *Proc. Nat. Acad. Sci., Wash.* **44**, 173 (1958).
[28] H. K. SCHACHMAN, A. B. PARDEE, and R. Y. STANIER, *Arch. Biochem.* **28**, 245 (1952).
[29] K. MCQUILLEN, R. B. ROBERTS, and R. J. BRITTEN, *Proc. Nat. Acad. Sci. Wash.* **45**, 1437 (1959).
[30] R. M. SMILLIE and G. KROTKOV, *Can. J. Bot.* **38**, 31 (1960).
[31] D. HERBERT, *Sympos. Soc. Gen. Microbiol.* **11**, 391 (1961).
[32] E. C. CANTINO unpubl. data.

although almost 90 per cent of the DNA/cell and 100 per cent of the soluble protein/cell[9] has been synthesized. Then, there occurs an explosive rise in the RNA_{insol}/cell. This kind of RNA, with its different nucleotide composition, is synthesized after the point of no return and constitutes the bulk of the RNA in a mature R.S. plant. Thus, the beginning of morphological differentiation is associated with the beginning of RNA differentiation.

The picture is not unlike that reported for bacteria where, for example,[33] net synthesis of nucleic acid stops when sporogenesis begins although turnover of nucleic acid continues. It differs, however, in that both turnover and net synthesis of RNA/cell occur in *Blastocladiella* after the point of no return—at which time "growth" ceases and the irreversible trend toward R.S. formation commences.

Speculation on the RNA Transformation and the Nature of the RNA_{insol}

(1) *The RNA transformation.* In the young (less than 36 hr) R.S. cell, over 95 per cent of the total RNA is $RNA_{NaCl\text{-}sol}$, and essentially all of this is found in $15{,}000 \times g$ supernatants from aqueous homogenates. Thus, although we have not separated the "soluble" and microsomal[18,26] fractions by high-speed centrifugation, nor finger-printed possible differences in minor components such as pseudouridine,[34] it seems a reasonable certainty that at these early stages in ontogeny (when active protein synthesis is occurring[9]), the $RNA_{NaCl\text{-}sol}$ contains the cell's "soluble" and microsomal RNA.

How, then, should the decrease in $RNA_{NaCl\text{-}sol}$/cell after the point of no return be interpreted? Two critical events which occur at this time bear directly upon the case:

> (a) when the $RNA_{NaCl\text{-}sol}$/cell reaches its maximum and then begins to decrease, net synthesis of soluble protein/cell ceases, and the RNA_{insol}/cell begins to increase rapidly;
>
> (b) simultaneously, the nucleotide composition of $RNA_{NaCl\text{-}sol}$ begins to change, the proportion of CMP increasing and that of AMP decreasing.

Our interpretation is this (see Figs. 2–4): at the point of no return, the microsomal component (17×10^{-6} μMoles/plant*) of the $RNA_{NaCl\text{-}sol}$ (38×10^{-6} μMoles/plant) undergoes decomposition into short-lived building blocks (CMP, AMP, etc. in soluble pool); the undegraded $RNA_{NaCl\text{-}sol}$ which remains (21×10^{-6} μMoles/plant) is "soluble" RNA. In mature R.S., the latter amounts to 31 per cent of the total RNA, a value not greatly out of line with that (10–20 per cent) for *E. coli*.[35] Simultaneously, the short-lived nucleotides are re-utilized for synthesis of a new RNA (38×10^{-6} μMoles/plant)—the RNA_{insol}—with a nucleotide composition like that of the residual, undegraded "soluble" RNA, but in a state no longer extractable with NaCl. The interpretation is strengthened by stoichiometric data on P turnover. When the transformation of $RNA_{NaCl\text{-}sol}$ occurs, the free TCA-sol. P_i/plant drops precipitously.[16] During these RNA interconversions (Table 4), the sum of (a), the P_i plant which disappears but is not converted to TCA-sol. organic-P, and (b), the free nucleotide-P/plant which disappears, is almost equivalent to the total RNA-P synthesized. Also, the interpretation is consistent with observations (Berg, personal communication in

* Expressed as total μMoles of nucleotides derived from the RNA (Fig. 3).

[33] I. E. YOUNG and P. C. FITZ-JAMES, *J. Biophys. Biochem. Cytol.* **6**, 467, and 483 (1959).
[34] D. B. DUNN, *Biochem. et Biophys. Acta.* **34**, 286 (1959).
[35] A. TISSIERES, J. D. WATSON, D. SCHLESINGER, and B. R. HOLLINGSWORTH, *J. Mol. Biol.* **1**, 221 (1959).

TABLE 4. CHANGES IN THE P POOL/PLANT BETWEEN 46% OF THE GENERATION TIME (WHEN THE $RNA_{NaCl\text{-}sol}$ AND RNA_{Insol} BEGIN THEIR RAPID DISAPPEARANCE AND APPEARANCE, RESPEC.) AND 75% OF THE GENERATION TIME (WHEN THE FULL COMPLEMENT OF THE RNA_{Insol} AND TOTAL RNA HAVE FORMED, AND THE $RNA_{NaCl\text{-}sol}$ HAS BEEN REDUCED TO ITS FINAL LEVEL/PLANT)

Component	μMoles · 10^{-6}/plant at:			
	46% of generation time	75% of generation time	Net change	
			Gain	Loss
(1) P_i	58	12		−46
(2) TCA-sol. organic-P	5	40	+35	
(3) P_i used but not accountable as TCA-sol. organic P; (1)–(2), above	—	—	—	−11
(4) Total RNA (as μMoles RNA-nucleotides)	42	63	−21	
(5) Total μMoles P used in synthesis of total RNA; from (4), above			+21	
(6) Free pool of CMP	5·7	2·4		−3·3
" " AMP	2·4	1·3		−1·1
" " GMP	1·0	0·4		−0·6
" " UMP	*ca.* 2·3	*ca.* 1·3		*ca.* −1·0
(7) Total μMoles nucleotide-P disappearing from free pool in cell; from (6), above	—	—	—	−6·0

Final balance:		
Total RNA-P synthesized per plant		21
Total P_i (not appearing as sol. org. P) and free nucleotide P used; (3) plus (7), above		17

Cohen and Gros;[18] Cox and Littauer,[36]) that "soluble" RNA (*E. coli*) contains a higher cytosine- and a lower adenine-mole fraction than does "intact" or high mol. wt. RNA.* The apparent lability of the low CMP component in $RNA_{NaCl\text{-}sol}$, and the *in vivo* stability of both high CMP RNA in mature R.S. cells is in keeping with suggestions[36] that RNA stability may depend upon the relative abundance of cytosine-guanine pairs. Finally, initiation of $RNA_{NaCl\text{-}sol}$ degradation by R.S. plants which have ceased to increase in size[9] and to synthesize soluble protein[9] is similar to the RNA degradation mediated by stationary bacterial and yeast populations (reviewed in Mandelstam[22]).

(2) *The nature of RNA_{Insol}.* RNA fractions with different base ratios have been reported for algae,[37] rat liver,[38] thymus tissue,[39] etc. Ribonucleoprotein particles with different sedimentation characteristics occur in bacteria, the relative proportions depending upon the stage in development (i.e. resting vs. growing), and environmental conditions.[40] In *E. coli*, in particular, small-sized microsomes are quickly converted to larger ones when growth stops.[35]

* In the high mol. wt. RNA of Littauer and Eisenberg (in Cox and Littauer, above), the molar cytosine/guanine ratio is 76 per cent, and in the "intact" RNA of Berg (personal communication in Cohen and Gros[18]), it is *ca.* 72 per cent; in our $RNA_{NaCl\text{-}sol}$ from young plants, which is presumed to be mainly microsomal RNA, it is also 76 per cent. In the low mol. wt. RNA of Spahr (pers. com. in Cox and Littauer, above), the cytosine/guanine ratio is 90 per cent, and in that of Berg (see above), it is *ca.* 95 per cent; in our $RNA_{NaCl\text{-}sol}$ from mature R.S. plants (which is assumed to be a "soluble" kind of RNA), and our RNA_{Insol} from mature R.S. plants (which is assumed to be an aggregate of "soluble" RNA), the ratios are 95 per cent and 97 per cent respectively.

[36] R. A. Cox and U. Z. LITTAUER, *J. Mol. Biol.* **2**, 166 (1960).
[37] T. IWAMURA and J. MYERS, *Arch. Biochem. Biophys.* **84**, 267 (1959).
[38] T. IWAMURA, *Biochim. et Biophys. Acta.* **42**, 161 (1960).
[39] G. DELAMIRANDE, C. ALLARD, and A. CANTERO, *J. Biol. Chem.* **214**, 519 (1955).
[40] B. J. MCCARTHY, *Biochim. et Biophys. Acta.* **39**, 563 (1960).

In *Blastocladiella*, perhaps the solubility of RNA in, or its displacement from protein binding by 10% NaCl decreases as the molecular weight increases (as it may be, for example, in rat liver RNA;[41] see also Markham[42]). Thus, the RNA_{insol} which accumulates during morphological differentiation may be a substance similar to "soluble" RNA in overall composition (i.e. nucleotide ratios) but with greater molecular dimensions. However, this cannot result simply from aggregation of pre-existing $RNA_{NaCl\text{-}sol}$ into larger units, because the decrease in $RNA_{NaCl\text{-}sol}$ involves loss of a component with non-uniform nucleotide ratios, while the RNA_{insol} which builds up simultaneously possesses equimolar nucleotide ratios. Therefore, the transformation must involve turnover (see previous section)—degradation of the low CMP component of the $RNA_{NaCl\text{-}sol}$ to smaller units, and their re-utilization for synthesis of the RNA_{insol}. (It would be interesting to know if the particularly large, transient build up of CMP is related to a requirement[43] for synthesis of a cytosine–cytosine-adenine terminus).

Our interpretation—and it must be recognized as speculation—is that RNA_{insol} is laid down during differentiation in the form of protein-bound organelles, which are finally converted to (and appear, microscopically, as) the well-known, Blastocladeaceous nuclear caps in the spores (produced when the mature R.S. protoplast is cleaved into several thousand motile swarmers). The reasoning is as follows:

(a) The large nuclear cap in *B. emersonii*, which overlies the spore nucleus but is not present during exponential growth, is a ribonucleoprotein organelle.[16,44] In the close relative, *Allomyces*, the nuclear cap is thought to be derived from a cytoplasmic structure (the "chromosphere") which contains ca. 12 per cent RNA and 60 per cent protein.[45]

(b) In *B. emersonii*, the RNA_{insol}, $RNA_{NaCl\text{-}sol}$, and total RNA/cell is constant during the last stages of R.S. maturation (ca. 75 per cent–100 per cent of the generation time). Thus, nucleoprotein required for the nuclear cap is produced before 75 per cent of the generation time.

(c) It is, in fact, during the immediately preceding interval (ca. 45 per cent–75 per cent of the generation time, when the RNA transformations in *B. emersonii* occur) that an insoluble, non-chitin N fraction (N_{insol}) is produced;[9] N_{insol}, like RNA_{insol}, is sedimented at 15,000 × g. The RNA_{insol}-N amounts to ca. 9 per cent[46] of the N_{insol}, and this is of the same order of magnitude as the RNA-N/total N (= ca. 15 per cent) for chromospheres of *Allomyces*.[45]

(d) Presumably, *B. emersonii* would require a source of energy and additional pentose (beyond that provided by turnover of $RNA_{NaCl\text{-}sol}$) for all this activity. Glucose-6-phosphate dehydrogenase is the only enzyme whose specific activity (and total activity/plant) is known to rise during maturation of an R.S. plant.[9] Simultaneously, a large glycogen-like polysaccharide pool[24] is used up. Thus, *Blastocladiella* possesses both the energy reservoir and the potential source of the pentose moiety.

[41] R. LIPSHITZ and E. CHARGAFF, *Biochim. et Biophys. Acta.* **42**, 544 (1960).
[42] R. MARKHAM, *Mod. Meth. Plant Anal.* **4**, 246 (1955).
[43] E. S. CANELLAKIS, *Ann. N.Y. Acad. Sci.* **81**, 675 (1959).
[44] E. C. CANTINO and G. TURIAN, *Ann. Rev. Microbiol.* **13**, 97 (1959).
[45] E. S. ROREM and L. MACHLIS, *J. Biophys. Biochem. Cytol.* **3**, 879 (1957).
[46] (a) N_{insol}/mg. mature R.S. plant: 32 μg. (b) RNA_{insol} N mg. mature R.S. plant (basis: RNA = 16% N): 2·8 μg. (c) RNA_{insol}-N/N_{insol}: ca. 9 per cent.

RNA Transformations and the Start of the New Generation

During R.S. germination, a surprising amount of synthetic activity occurs. For example, when the protoplast of an R.S. cell is cleaved into motile spores, the total RNA increases about 7·4 fold.[47] The N_{insol} present in the protoplast before spore formation[9] is almost equal to the new RNA-N which finally appears in the spores (i.e. the N_{insol} fraction apparently provides the N for synthesis of ca. $25{\cdot}0 \times 10^{-6}$ μg RNA/spore unit). Thus, the

TABLE 5. CHANGES WHICH OCCUR DURING GERMINATION OF AN R.S. PLANT; I.E. CLEAVAGE OF THE PROTOPLAST INTO "SPORE UNITS"* AND LIBERATION OF MOTILE SPORES

	R.S. plant (6480 spore units of protoplasm)	Spores (6480 spores)[24]	Increase
Total RNA/spore unit (μg)[47]	$3{\cdot}5 \times 10^{-6}$†	$26{\cdot}0 \times 10^{-6}$	7·4 fold
Total SPN/spore unit (μg)[48]§	$2{\cdot}26 \times 10^{-6}$	$5{\cdot}42 \times 10^{-6}$	2·4 fold
Oxygen consumption/spore unit per hr (μl)‡	$0{\cdot}063 \times 10^{-6}$	$11{\cdot}7 \times 10^{-6}$	186 fold

Increase in endogenous lactic acid[11] is of the same order of magnitude as the oxygen consumption; increase in specific activity of enzymes such as glucosamine synthetase,[9] cytochrome oxidase,[10] and the α-ketoglutarate dehydrogenase system[10] varies from 5 to 30 fold or more.

* A spore unit is defined as the amount of protoplasm which, after cleavage of the R.S. protoplast into spores, becomes the protoplast of the spore itself.

† Since a mature R.S. plant contains RNA with equimolar amounts of nucleotides, conversion of μMoles of RNA[47] was based on an av. nucleotide mol. wt. of 339.

‡ Derived from raw data.[9,11,16,25]

§ SPN = sol. protein N.

7·4-fold increase in RNA appears to result from utilization of the insoluble protein (see previous section) presumably associated with the RNA_{insol} aggregates in the cell. Nucleic acid, however, is not the only component to increase. Soluble protein-N (SPN) also rises—albeit only 2·4 fold—during germination.[48] Since other processes also increase, some of great magnitude (Table 5), perhaps the increase in nucleic acid is not too surprising. But, it *is* surprising that the rate of synthesis of RNA/cell during germination—a process not commonly thought of as a "growth" phenomenon—is almost on a par with the maximum rate of RNA synthesis during exponential growth of an R.S. plant (Table 6). It is quite possible that the intimate relation between the RNA-rich nuclear cap and the nucleus immediately underlying it[44,49,50]—a situation which exists only at this stage in the life history—represents the device, *par excellence*, for ensuring immediate and efficient transfer of material and information for the successful initiation of the next generation of Blastocladiellas.

[47] (a) Wt. of spore[9]: $1{\cdot}13 \times 10^{-4}$ μg. (b) Av. RNA in spores[19]: 230 μg/mg. (c) RNA/spore ($a \times b/10^3$): 26×10^{-6} μg. (d) Total RNA/R.S. plant: $2{\cdot}24 \times 10^{-3}$ μg. (e) Av. number of spores/R.S. plant: 6480. (f) RNA/"spore unit" in R.S. plant (d/e): $3{\cdot}5 \times 10^{-6}$ μg. (g) Increase in total RNA/spore unit during germination (c/f): 7·4 fold.

[48] (a) SPN in R.S.[9]: 18 μg/mg. (b) Wt. of mature R.S. plant: 0·8137 μg. (c) SPN/R.S. ($a \times b/10^3$): $14{\cdot}7 \times 10^{-3}$ μg. (d) Spore units/R.S.: 6480. (e) SPN/spore unit in R.S. (c/d): $2{\cdot}26 \times 10^{-6}$ μg. (f) SPN in spores[9]: 48 μg/mg. (g) Wt. of spore[9]: $1{\cdot}13 \times 10^{-4}$ μg. (h) SPN/spore ($f \times g/10^3$): $5{\cdot}42 \times 10^{-6}$ μg. (i) Increase in total SPN/spore unit during germination (h/e): 2·4 fold.

[49] G. TURIAN and E. KELLENBERGER, *Exp. Cell Res.* 1, 417 (1956).

[50] B. BLONDEL and G. TURIAN, *J. Biophys. Biochem. Cytol.* 7, 127 (1960).

TABLE 6. COMPARATIVE RATES OF SYNTHESIS OF TOTAL RNA BY *BLASTOCLADIELLA*

	During:	
	Exponential growth*	Germination†
(a) RNA synthesized/plant (μg/hr)	$2.0 \cdot 10^{-3}$	$24.6 \cdot 10^{-3}$
(b) Size/plant (μ^3)	$7.0 \cdot 10^{4}$‡	115.0×10^{4}§
(c) RNA synthesized/μ^3 of protoplasm (μg/hr; a/b, above)	$2.8 \cdot 10^{-8}$	2.2×10^{-8}

* During 12–24 hr period, 24°.[32]
† Based on 5 hr av. germination time for mature R.S.
‡ Size at 18 hr; i.e. av. size during exponential growth from *ca.* 12–24 hr.
§ Size of plant less thick R.S. wall; i.e. size of protoplast.

Reversibility of RNA Transformations and Morphological Differentiation

Since RNA is involved in protein synthesis in other organisms,[18,26] it probably plays a role in synthesis of enzyme-protein in *Blastocladiella*. If enzymes[10,16] involved in R.S. differentiation undergo shifts in activity during morphogenesis, the RNA which controls these shifts should exhibit some change in quantity, quality, or activity. In *Blastocladiella*, such changes are demonstrable; it is at the point of no return in morphogenesis, when the total α-ketoglutarate dehydrogenase activity/cell levels off and the total isocitric dehydrogenase activity·cell is rising rapidly, that the apparent transformation of $RNA_{NaCl\text{-}sol}$ to RNA_{insol} accelerates greatly. But if, in fact, RNA is related in cause and effect fashion to the synthesis, turnover, or activity of these enzymes—as a repressor substance might be, for example[51]—is should be possible to show that alteration of an existing RNA pattern is reflected in a corresponding change in the enzyme activity pattern. This, in part, has been demonstrated for *Blastocladiella*. Before the point of no return, reversal of the morphogenetic path (by removal of bicarbonate) brings about an increase[10] in total ketoglutarate dehydrogenase activity/cell, a decrease in total isocitric dehydrogenase activity/cell, and simultaneously, a decrease in the RNA_{insol} pool/plant. Thus, RNA turns over during synthesis and degradation of inducible[10] enzymes in a "resting" cell of *Blastocladiella*; perhaps, this is not unlike RNA turnover during enzyme induction in resting cells of yeast.[52] In the absence of morphological reversal (i.e. bicarbonate not removed), the pool of RNA_{insol}/cell continues to rise. We suggest, therefore, that (a) formation of RNA_{insol}/cell controls or influences, either in a negative or positive fashion, the synthesis and/or activity of the isocitric dehydrogenase and α-ketoglutarate dehydrogenase systems which are believed[10,16] to be directly involved in the control of morphogenesis; and (b), once the point is reached where the accumulation of RNA_{insol} can no longer undergo turnover and be re-utilized, the rise and fall of enzyme systems under its control becomes similarly irreversible, and R.S. morphogenesis must continue to completion.

The Relation Between DNA and RNA

In other biological entities, RNA is thought to be formed in the nucleus, after which it moves out into the cytoplasm.[53] There is also good reason to believe[54] that not all RNA

[51] A. B. PARDEE and L. S. PRESTIDGE, *Biochim. et Biophys. Acta.* **36**, 545 (1959).
[52] H. CHANTRENNE, *Nature, Lond.* **177**, 579 (1956).
[53] J. H. TAYLOR, *Amer. Scient.* **48**, 365 (1960.)
[54] D. B. STRAUS and E. GOLDWASSER, *J. Biol. Chem.* **236**, 849 (1961), and refs. therein.

need be synthesized in the nucleus, and that it need not be directly dependent upon synthesis of new DNA.[55,56] In *Blastocladiella*, some 36 per cent of the total RNA/cell is manufactured after net DNA (and soluble protein) synthesis has stopped. Of this RNA, some 70 per cent appears to be derived from building blocks resulting from turnover of pre-existing RNA, and 30 per cent is formed *de novo*. Thus, the message responsible for the suddenly accelerated synthesis of the RNA_{insol} and the message responsible for initiation of the apparently selective degradation of the NaCl-soluble, low CMP type of RNA seems to originate at the time that net DNA synthesis ceases. We have not yet embarked upon an analysis of the DNA in *Blastocladiella* on the scale used for RNA. It may be, however, that the DNA is not "uniform" in composition and/or structure (as in some other systems[41]), and that at the point of no return in morphogenesis, when net DNA synthesis ceases, it too—like RNA—undergoes differential changes (but not a change in total quantity) which are reflected in the kind of RNA transformations which occur during differentiation. Alternatively, some of the RNA itself may be carrying this information.

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[55] T. OKAZAKI and R. OKAZAKI, *Biochim. et Biophys. Acta.* **29**, 211 (1958).
[56] S. S. COHEN and H. D. BARNER, *Proc. Nat. Acad. Sci. Wash.* **40**, 885 (1954).

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ISOLATION AND CHARACTERIZATION OF A COMPLEX POLYSACCHARIDE FROM AGED BURLEY TOBACCO

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Abstract—A soluble polysaccharide, characterized as the calcium–magnesium salt of a complex acid composed of glucuronic acid, galactose, arabinose and rhamnose, was isolated from the nondialysable fraction of an aqueous extract of aged Burley tobacco. The amount so isolated corresponded to 0·9 per cent of dry leaf weight. The qualitative composition of the free acid is similar to that of arabic acid. The polysaccharide contained a minor protein component of which hydroxyproline was a constituent amino acid. Characterization of the polysaccharide was made by paper chromatography of the products of hydrolysis, by quantitative analysis of the constituent carbohydrates, and by ash analysis.

INTRODUCTION

MOST investigations on tobacco carbohydrates, excluding simple sugars, have been concerned with water-insoluble polysaccharides of the structural type—e.g., celluloses, hemicelluloses, and pectic substances—and with oligosaccharides.[1,2] As a consequence there is a paucity of information about the soluble polysaccharides, including gums.

During an investigation of the soluble browning reaction pigments of aged Burley tobacco a polysaccharide was detected in the nondialysable fraction.[3] The compound was subsequently characterized as the calcium–magnesium salt of a complex polysaccharide acid qualitatively similar in composition to arabic acid. There is no previous known report describing the occurrence of this polysaccharide in tobacco. The compound is of interest and importance from the standpoint of current knowledge of tobacco composition.

This paper presents qualitative and quantitative data on the composition of the soluble nondialysable polysaccharide, and describes procedures for its isolation and characterization.

RESULTS AND DISCUSSION

Carbohydrates

Hydrolysis of the polysaccharide with 0·1 N hydrochloric acid and subsequent separation by two-dimensional paper chromatography of the carbohydrate products of hydrolysis resulted in the qualitative identification of galactose, arabinose, rhamnose and glucuronolactone. Under the conditions of acid hydrolysis glucuronolactone is produced from glucuronic acid by lactonization. The carbohydrate composition of the polysaccharide is thus qualitatively similar to that of gum arabic. The constituent carbohydrates of the polysaccharide cochromatographed with authentic compounds, individually and when mixed with acid hydrolysates of gum arabic, in all solvent systems (Table 1).

Protein Component

Hydrolysis of the polysaccharide with 6 N hydrochloric acid yielded a mixture of eleven amino acids: lysine, aspartic acid, glutamic acid, hydroxyproline, serine, glycine, threonine,

[1] W. G. FRANKENBURG, *Advances in Enzymology* (Ed. by F. F. NORD), Vol. 6, pp. 309–387. Interscience, New York (1946).
[2] R. A. W. JOHNSTONE and J. R. PLIMMER, *Chem. Rev.* **59**, 885 (1959).
[3] H. E. WRIGHT, Jr., W. W. BURTON and R. C. BERRY, Jr., *Arch. Biochem. Biophys.* **86**, 94 (1960).

alanine, valine, leucine and isoleucine. The amino acids cochromatographed with authentic compounds in all solvent systems (Table 2). Tryptophan was not found after hydrolysis either with acid or with 14% barium hydroxide solution. Alanine appeared to be the predominant amino acid. No ninhydrin-positive spots were obtained when the polysaccharide was chromatographed prior to hydrolysis.

TABLE 1. *Rf* VALUES OF CONSTITUENT CARBOHYDRATES OF THE TOBACCO POLYSACCHARIDE

Compound	Solvent*				
	A1	A2	B1	B2	C1
Galactose	0·22	0·40	0·54	0·59	0·40
Arabinose	0·31	0·48	0·64	0·62	0·47
Rhamnose	0·46	0·60	0·79	0·69	0·61
Glucuronolactone†	0·42	0·56	0·77	0·72	0·65

* See Experimental section.
† Derived from glucuronic acid.

The identification of bound hydroxyproline in the protein moiety of the polysaccharide is interesting in view of the infrequent occurrence of this amino acid in plant proteins. Although there is no known previous report describing its occurrence in Burley tobacco, it has been found in Maryland tobacco, U.S. type 32.[4] Burley and Maryland tobaccos are both air-cured types; hydroxyproline has not been reported so far in tobaccos cured by the application of artificial heat. Steward and Pollard[5] have reported the occurrence of bound hydroxyproline in tumours which grow on *Nicotiana* hybrids by virtue of their genetic constitution.

TABLE 2. *Rf* VALUES OF AMINO ACIDS FROM PROTEINACEOUS MOIETY OF THE TOBACCO POLYSACCHARIDE

Compound	Solvent*			
	D1	D2	E1	F1
Lysine	0·14	0·08	0·12	0·17
Aspartic acid	0·27	0·02	0·25	0·29
Glutamic acid	0·34	0·02	0·32	0·33
Hydroxyproline	0·30	0·07	0·29	0·31
Serine	0·25	0·13	0·24	0·26
Glycine	0·26	0·08	0·25	0·29
Threonine	0·32	0·37	0·32	0·30
Alanine	0·38	0·15	0·39	0·34
Valine	0·55	0·29	0·53	0·48
Leucine	0·70	0·44	0·78	0·61
Isoleucine	0·67	0·41	0·77	0·58

* Solvent: see Experimental section.

The Polysaccharide

Qualitative tests for sulphur and phosphorus, made after fusion of the polysaccharide with sodium, indicated trace amounts of these elements. Nitrogen content was determined by Kjeldahl, from which value the protein content was calculated by the factor N × 6·25.

[4] G. GIOVANNOZZI-SERMANNI, *Il Tabacco* **59**, 335 (1955); **60**, 400 (1956).
[5] F. C. STEWARD and J. K. POLLARD, *Nature* **182**, 828 (1958).

Quantitative analyses for calcium and magnesium were determined on the polysaccharide ash. Qualitative tests on the ash for potassium, iron and copper were negative. The equivalent weight, from which the glucuronic acid content was calculated, was determined on the free acid precipitated by acetone from an acidified aqueous solution of the polysaccharide. The quantitative determinations of galactose, arabinose and rhamnose were also carried out on the free acid. Quantitative data on the composition of the polysaccharide are presented in Table 3.

TABLE 3. COMPOSITION OF THE TOBACCO POLYSACCHARIDE

Elemental analysis of polysaccharide	%	Carbohydrate analysis of polysaccharide free acid	
Carbon	39·26	Equivalent weight	924
Hydrogen	6·37	Glucuronic acid	21·0%
Nitrogen	1·22	Galactose	37·2%
Protein	7·63	Arabinose	25·7%
Calcium	2·98	Rhamnose	15·6%
Magnesium	0·43		

In accordance with the evidence it was concluded that the polysaccharide, a pale buff amorphous powder freely soluble in water, was the calcium–magnesium salt of a complex acid composed of glucuronic acid, galactose, arabinose and rhamnose. On the basis that the equivalent weight of the free acid represents the molecular weight of the repeating unit, the molecular ratio of the constituent carbohydrates was: glucuronic acid, 1 mole; galactose, 2 moles; arabinose, 2 moles; rhamnose, 1 mole. The free acid comprised 89 per cent of the polysaccharide, which also contained a minor proteinaceous moiety, the mode of linkage of which is unknown. Chemical and physical properties of the polysaccharide suggest it may be a gum, and natural polysaccharides of this class are known to be unhomogeneous and usually contain proteinaceous matter.[6]

The procedure by which the polysaccharide was isolated from the nondialysable fraction of the aged Burley tobacco precluded an accurate measurement of its content. However, based on the calcium content of this fraction, the amount of the polysaccharide in the tobacco was estimated to be 0·9 per cent of dry leaf weight. Experimental evidence indicated that the polysaccharide was the only calcium-containing component of the fraction. The polysaccharide and the two browning reaction pigments previously reported[3] comprised the total soluble nondialysable fraction of the aged Burley tobacco.

EXPERIMENTAL

Materials

The tobacco (*Nicotiana tabacum*) used in this investigation was the lamina portion of the leaf of field-grown Burley, U.S. type 31, 1953 crop, aged. In preparation for extraction, the tobacco, 7 per cent moisture, was ground to a particle size of 1 mm. All reagents used were of the highest purity obtainable.

Extraction and Isolation of the Polysaccharide

Isolation of the total nondialysable fraction from the Burley tobacco, in which fraction the polysaccharide was found, has been described in detail.[3] Briefly, the tobacco, pre-

[6] F. SMITH and R. MONTGOMERY, *The Chemistry of Plant Gums and Mucilages*, p. 494, Reinhold, New York (1959).

extracted successively with pentane, ether, chloroform, acetone and ethanol, was next extracted with deionized water. The aqueous extract was dialysed against running deionized water for 72 hr. The dialysis residue was concentrated, and 9 vols. of acetone were added, to precipitate the total nondialysable fraction. A 400 mg sample of the dried precipitate was dissolved in 80 ml of deionized water, 40 ml of ethanol were added, and the solution was centrifuged. The supernatant solution was decolorized with activated carbon (Norit A), filtered through a pad of diatomaceous earth (Celite Analytical Filter-Aid), and the filtrate concentrated *in vacuo* to ca. 40 ml and 9 vols. of acetone added, thereby precipitating the polysaccharide. After refrigeration overnight the precipitate was centrifuged, washed with acetone, and dried *in vacuo* at room temperature.

Hydrolysis of the Polysaccharide

For qualitative carbohydrate chromatography a 40 mg sample of the polysaccharide in 25 ml of 0·1 N hydrochloric acid was refluxed for 1 hr. The hydrolysate was evaporated to dryness on the steam bath. Excess hydrochloric acid was removed by adding a small quantity of water to the residue and evaporating it to dryness three successive times. The residue was finally taken up with 1 ml of absolute ethanol, allowed to stand 1 hr at room temperature, and centrifuged. The final volume of supernatant solution was adjusted to 1 ml. Carbohydrate components of the hydrolysate were separated by two-dimensional paper chromatography. Authentic gum arabic was hydrolysed in a similar manner for use in comparative chromatography.

Chromatographic Procedure. Whatman No. 1 chromatography paper was used in all chromatographic separations. Chromatograms were run at room temperature for 18–20 hr in glass tanks 12×12×24 in. by the ascending technique. For two-dimensional chromatography the sheets were rolled into cylinders and fastened with glass hooks. The tentative identification of each compound was substantiated by cochromatographing the sample with an authentic specimen of the compound. The solvent systems, all single phase, were prepared on a vol./vol. basis. A1. *first direction*: *n*-butanol–acetic acid–water (12 : 3 : 5); 2. *second direction*: *iso*propanol–*n*-butanol–water (7: 1: 2). B1.: *iso*propanol–water (4 : 1); 2.: ethyl acetate–pyridine–water (12 : 5 : 4). C1.: *n*-butanol–pyridine–water (2 : 2 : 1); 2.: *iso*propanol–*n*-butanol–water (7 : 1 : 2). Carbohydrates were detected on the chromatograms with *p*-anisidine phosphate spray.[7]

Quantitative Analysis

The quantitative determinations of galactose, arabinose and rhamnose were performed on the polysaccharide free acid. A 100 mg sample of the polysaccharide was dissolved in 15 ml of deionized water and filtered through a pad of diatomaceous earth (Celite Analytical Filter-Aid). The pH of the filtrate was adjusted to 1 with 3 N hydrochloric acid and, after standing 15 min, the polysaccharide free acid was precipitated by addition of 9 vols. of acetone. Following refrigeration for ca. 3 hr, the precipitate was centrifuged, washed with acetone until the supernatant solution was free of hydrochloric acid, and dried *in vacuo* at room temperature. A suitable sample of the free acid was hydrolysed by refluxing in 0·5 N hydrochloric acid for 2 hr. The carbohydrate components of the hydrolysate were separated by one-dimensional paper chromatography in *n*-butanol–pyridine–water (2 : 2 : 1), sprayed

[7] R. J. BLOCK, E. L. DURRUM and G. ZWEIG, *Paper Chromatography and Paper Electrophoresis*, 2nd ed., p. 182. Academic Press, New York (1958).

with *p*-anisidine hydrochloride, eluted, and their concentrations determined spectrophotometrically in accordance with the procedure of Pridham.[8]

For amino acid chromatography, a 25 mg sample of the polysaccharide in 25 ml of 6 N hydrochloric acid was refluxed for 20 hr. The hydrolysate was evaporated to dryness on the steam bath, and the residue was taken up in 1 ml of 0·1 N hydrochloric acid and centrifuged. The final volume of supernatant solution was adjusted to 1 ml. Amino acids in the hydrolysate were separated by two-dimensional paper chromatography using the following solvent systems.

D1. *first direction*: *n*-butanol–acetic acid–water (12 : 3 : 5); 2. *second direction*: *n*-butanol–methyl ethyl ketone–ammonium hydroxide–water (5 : 3 : 1 : 1) run twice. E1.: *sec*-butanol–formic acid–water (15 : 3 : 2); 2.: *n*-butanol–methyl ethyl ketone–ammonium hydroxide–water (5 : 3 : 1 : 1) run twice. F1.: methyl ethyl ketone–propionic acid–water (15 : 5 : 6); 2.: *n*-butanol–methyl ethyl ketone–ammonium hydroxide–water (5 : 3 : 1 : 1) run twice.

Amino acids were detected by a ninhydrin spray,[9] prepared by dissolving 0·2 g of ninhydrin in 90 ml of acetone and adding 10 ml of glacial acetic acid.

Acknowledgements—The authors are grateful to Mrs. Joyce B. Booth for the elemental analyses, and to Mrs. Louise H. Bowman for technical assistance.

[8] J. B. PRIDHAM, *Anal. Chem.* **28**, 1967 (1956).
[9] J. B. JEPSON and B. J. STEVENS, *Nature* **172**, 772 (1953).

Phytochemistry, 1962, Vol. 1, pp. 131 to 136. Pergamon Press Ltd. Printed in England

PROPERTIES OF A PYRIDINE NUCLEOTIDE-MENADIONE OXIDOREDUCTASE FROM MUNG BEAN SEEDLINGS

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Abstract—A soluble NAD(P)H_2 : 2-methyl-1,4-naphthoquinone oxidoreductase (menadione reductase) has been partially purified from etiolated mung bean (*Phaseolus aureus*) seedlings. The enzyme is equally active with $NADH_2$ and $NADPH_2$, and the K_m for $NADH_2$ is $2{\cdot}14\times10^{-4}$ M. It shows diaphorase activity and can be assayed conveniently with ferricyanide ($K_m = 2{\cdot}27\times10^{-4}$ M). A number of 1,4-naphthoquinone derivatives were tested as electron acceptors, and of these menadione was the most effective; only the related amino-naphthol, Vitamin K_5, was more active. The enzyme also reduces 1,4-benzoquinone, but not Vitamin K_1 or ubiquinone UQ_{10}. The menadione reductase is completely inhibited by 10^{-4} M dicumarol, but it is not affected by 10^{-4} M DNP.

INTRODUCTION

RECENT work on both animals and plants has focused attention on the possible roles of the Vitamin K- and ubiquinone-type quinones in electron transport.[1] A number of different enzymatic systems which catalyse the reduction of quinones by reduced pyridine nucleotides have been isolated. These vary in their electron acceptor specificities, and some of the enzymes have even been described as diaphorases. Wosilait and Nason[2] first obtained an enzyme from pea seeds that reduces benzoquinone. They then found that pyridine nucleotide-2-methyl-1,4-naphthoquinone oxidoreductase (menadione reductase) activity is widely distributed in higher plants.[3] This activity has since been measured using soluble extracts from potato tubers,[4] wheat germ,[5] and tobacco roots,[6] and the enzyme has been partially purified from the last source. The present paper describes some properties of a soluble mung bean enzyme which catalyses the reduction of a variety of quinones and is particularly active with menadione (Vitamin K_3).

EXPERIMENTAL AND RESULTS

Enzyme Purification

Fifty lb of 3- to 5-day-old etiolated mung bean (*Phaseolus aureus* Roxb.) seedlings were macerated in 1½ lb batches with water in a Waring blender for 15 sec. The homogenate was squeezed through two layers of cheesecloth to give the crude extract (approximately 18 l.), which was adjusted to pH 5 with 1 N acetic acid and allowed to stand overnight at 5°. The pH 5 supernatant fraction (pH 5-super) was siphoned off from the precipitated material, adjusted to pH 7 with NH_4OH, and enough ammonium sulphate (AS) added to it to make a 60 per cent saturated solution. After standing overnight, the sediment was collected by filtration on a Buchner funnel, dissolved in 150 ml of 0·1 M phosphate buffer, pH 7·5, and dialysed against distilled water overnight at 5°. The dialysed solution (AS 0–60 per cent

[1] G. E. W. WOLSTENHOLME and C. M. O'CONNOR, *Quinones in Electron Transport*, Little, Brown, Boston (1960).
[2] W. D. WOSILAIT and A. NASON, *J. Biol. Chem.* **206**, 255 (1954).
[3] W. D. WOSILAIT and A. NASON, *J. Biol. Chem.* **208**, 785 (1954).
[4] D. P. HACKETT, *Plant Physiol.* **33**, 8 (1958).
[5] H. H. CLUM and A. NASON, *Plant Physiol.* **33**, 354 (1958).
[6] E. C. SISLER and H. J. EVANS, *Tobacco Sci.* **2**, 132 (1958).

sat.) was refractionated with ammonium sulphate and the fraction precipitating between 45 and 55 per cent saturation at pH 7 was collected. This was dissolved in buffer, dialysed against distilled water overnight at 5°, and centrifuged to remove any insoluble residue. To the dialysed solution (AS 45–55 per cent sat.), 30 per cent by volume of cold (−30°) acetone was added with constant stirring at 0°. Ten minutes after the addition of acetone, the precipitate was collected by centrifugation and discarded. The acetone concentration of the supernatant fraction was increased to 60 per cent by adding more cold acetone; the resultant precipitate was then collected by centrifugation and dissolved in 5 ml of 0·1 M phosphate buffer, pH 7·5 (acetone 30–60 per cent). This was used as the final preparation. For the routine enzyme assay ferricyanide was used as the electron acceptor.

TABLE 1. SUMMARY OF ENZYME PURIFICATION*

Fraction	Specific activity (units/mg protein) Electron donor		
	(A) $NADH_2$	(B) $NADPH_2$	B/A
pH 5 supernatant	70	35	0·50
AS 0–60% sat.	254	308	1·21
AS 45–55% sat.	254	254	1·00
acetone 30–60%	545	636	1·17

* See text for details of assay. One unit of activity defined as a change in extinction of 0·01 in a 1 cm cell at 340 mμ measured during the first 20 sec.

Preliminary studies showed that the major fraction of the activity was precipitated when the pH 5 supernatant was 60 per cent saturated with ammonium sulphate; none was precipitated by 40 per cent saturation. With acetone, all of the activity was precipitated when the concentration (by volume) was between 30 and 60 per cent. The results of the fractionation procedure described above are given in Table 1: the enzyme was purified either 8-fold or 18-fold, depending on whether $NADH_2$ or $NADPH_2$ was the electron donor. Only 1·4 per cent of the total activity in the pH 5 supernatant was recovered in the final fraction.

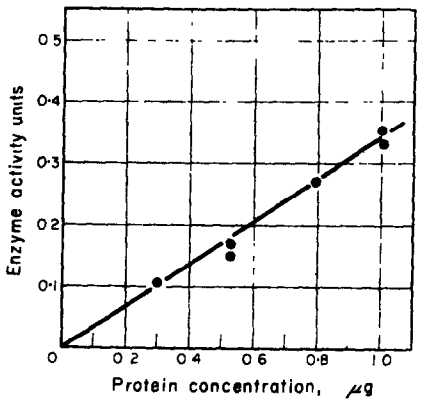

FIG. 1. EFFECT OF ENZYME CONCENTRATION ON RATE OF $NADH_2$ OXIDATION.
One unit of enzyme activity defined as a change in extinction of 0·01 in a 1 cm cell at 340 mμ during the initial 20 sec. Assay mixture as described in text.

Assay Conditions

For the standard assay, the reaction mixture contained 1·0 ml of 0·2 mM $NADH_2$ or $NADPH_2$ dissolved in 0·12 M phosphate buffer (pH 7·5), 0·01 ml of enzyme solution, and 0·01 ml of 0·01 M potassium ferricyanide or some other acceptor. The rate of decrease in extinction at 340 mμ was followed at room temperature and was corrected for any non-enzymatic oxidation of the coenzyme by the acceptor. The rate of the reaction was directly proportional to the enzyme concentration (Fig. 1); the pH optimum is 7·8 (Fig. 2); and the Michaelis constant (K_m) for ferricyanide in this reaction is $2{\cdot}27\times10^{-4}$ M (Fig. 3).

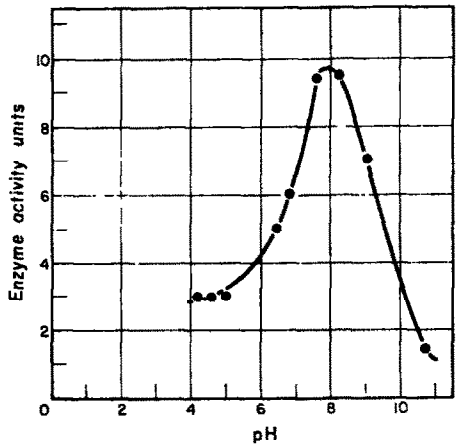

FIG. 2. EFFECT OF pH ON ENZYME ACTIVITY.
A series of 0·12 M Tris(hydroxymethyl)aminomethane buffers used and activity expressed as indicated in Fig. 1.

Electron Donors

As shown in Table 1, the purified enzyme is almost equally active, on a protein basis, with $NADH_2$ and $NADPH_2$. This is characteristic of a number of menadione reductases and diaphorases. The dependence of the reaction rate on the concentration of $NADH_2$ was determined and the K_m for this donor was calculated to be $2{\cdot}14\times10^{-4}$ M (Fig. 4). This

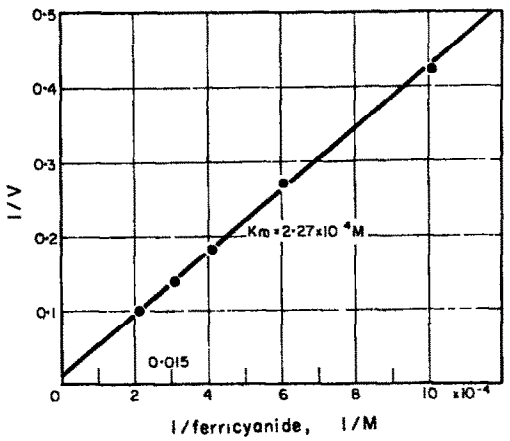

FIG. 3. DETERMINATION OF ENZYME AFFINITY FOR THE ELECTRON ACCEPTOR (FERRICYANIDE).

value is close to the comparable Michaelis constants for pea seed quinone reductase[2] and tobacco root diaphorase.[6]

Electron Acceptors

The abilities of a number of compounds to act as electron acceptors for the oxidation of $NADH_2$ by this enzyme are compared in Table 2. Both unsubstituted benzoquinone and α-naphthoquinone are active, whereas anthroquinone is not. The effect of substitution in the 2- and 3-positions of naphthoquinone was examined. Menadione, the 2-methyl derivative, is particularly active, and it was assigned an arbitrary value of 100. Activity is completely abolished by substitution in the 2-position with the electron-repelling methylamino

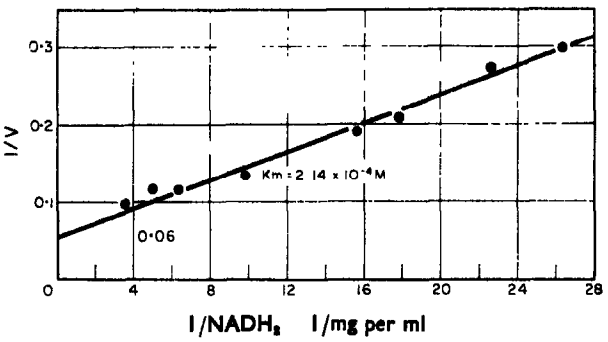

FIG. 4. DETERMINATION OF ENZYME AFFINITY FOR THE ELECTRON DONOR ($NADH_2$).

or hydroxy groups. Of the several 2,3-disubstituted 1,4-naphthoquinones, only the dichloro-compound showed significant activity. This indicates that the 3-position does not necessarily have to be free in order for the quinone to react. There was no reaction with phthiocol (2-hydroxy-3-methyl-1,4-naphthoquinone), a compound which is very effective in catalysing photosynthetic phosphorylation.[7] Vitamin K_5, 4-amino-2-methyl-1-naphthol, was even more active than menadione; K_5 must be very rapidly converted to the quinonimine form. It is of interest that this nitrogen analogue is more effective than the natural vitamins in the promotion of prothrombin formation. Since the naturally-occurring quinones UQ_{10} and Vitamin K_1 are extremely insoluble, special methods must be used to solubilize or emulsify them for use in aqueous systems. Solubilization by Tween 80 (polyoxyethylene sorbitan monoöleate) and BRIJ-35 (polyoxyethylene lauryl alcohol) did not make possible the reduction of these quinones by the mung bean enzyme.

As shown in Table 2, ferricyanide is a considerably less effective acceptor than several of the quinones. At equal concentrations, 2,6-dichloro-phenol-indophenol is even less effective than ferricyanide. The low diaphorase activity of the mung bean enzyme distinguishes it from some other menadione reductases. Molecular oxygen, cytochrome *c*, α-lipoic acid, and acetyl-pyridine NAD did not serve as electron acceptors (Table 2). This indicates that the enzyme shows no oxidase, cytochrome *c* reductase, lipoate dehydrogenase, or transhydrogenase activity.

Inhibitors

One of the properties which has been most useful for the characterization of the diaphorases and quinone reductases is their response to certain inhibitors. 2,4-Dinitrophenol

[7] D. I. ARNON, *Federation Proc.* **20**, 1012 (1961).

TABLE 2. RELATIVE ACTIVITIES OF ELECTRON ACCEPTORS*

Acceptor†	Solvent‡	Relative activity
1,4-benzoquinone	ethanol	41
1,4-naphthoquinone (NQ)	ethanol	70
2-methyl-NQ (K_3)	ethanol	100
2-sulphonyl-NQ	water	38
2-methylamino-NQ	chloroform	0
2-anilino-NQ	chloroform	0
2-hydroxy-3-methyl-NQ	ethanol	0
2,3-dihydroxy-NQ	ethanol	1·4
2-hydroxy-3-methoxy-NQ	chloroform	2·2
2-hydroxy-3-(3′-phenylpropyl)-NQ	ethanol	0
2,3-dichloro-NQ	chloroform	25
anthraquinone	ethanol	0
2-methyl-3-phytyl-NQ (K_1)	BRIJ-35	0
4-amino-2-methyl-1-naphthol (K_5)	ethanol	122
ubiquinone UQ_{10}	BRIJ-35	0
ferricyanide	water	23
cytochrome *c* §	water	0
DL-α-lipoic acid ‖	water	0
3-acetylpyridine-NAD	water	0
oxygen		0

* Standard assay for $NADH_2$ oxidation as described in text. Final concentration of acceptors = 5×10^{-5} M.

† NQ equals 1,4-naphthoquinone.

‡ Solvent in which acceptor was dissolved before addition to reaction mixture. K_1 and UQ_{10} solubilized in BRIJ-35 according to method of Wosilait.[9]

§ Reduction of cytochrome *c* (10^{-5} M) measured at 550 mμ.

‖ Reaction mixture contained 0·01 μmole $NADH_2$ and 3 μmoles lipoic acid in 1 ml pH 6·0 phosphate buffer.

(DNP), at 10^{-4} M, did not inhibit the menadione reductase activity. The response to dicumarol (10^{-5} M) depends on the nature of the electron acceptor: the oxidation of $NADH_2$ was inhibited 60 per cent when menadione was the acceptor, but only 8 per cent with ferricyanide. At 10^{-4} M, dicumarol inhibited the reaction 97 per cent. Several other inhibitors were tested, using ferricyanide as electron acceptor: activity was inhibited 26 per cent by 10^{-4} M PCMBA (parachloromercuribenzoic acid), 26 per cent by 10^{-5} M Atabrine and 15 per cent by 10^{-4} M Amytal.

DISCUSSION

The properties of the mung bean menadione reductase distinguish it from other enzymes isolated from higher plant tissues. The pea seed quinone reductase reduces benzoquinone but not menadione, and it is very sensitive to DNP.[8] The wheat germ diaphorase is active with menadione but not with 1,4-naphthoquinone.[5] Phylloquinone reductase from spinach leaves shows some activity with both Vitamin K_1 and cytochrome *c*, and it is not inhibited by dicumarol.[8] The tobacco root diaphorase is almost equally active with 1,4-benzoquinone, 1,4-naphthoquinone, menadione, and 2,6-dichlorophenol-indophenol as electron acceptors.[6] The pH optima of the tobacco and mung bean enzymes differ by one unit. More definitive comparisons of all these enzymes must await their further purification.

In some, but not all, respects the mung bean enzyme is similar to the mammalian enzymes

[8] G. WOLF, F. KIEFFER and C. MARTIUS, *Federation Proc.* **18**, 354 (1959).

which can act as pyridine nucleotide-menadione oxidoreductases.[9-12] All of them are inhibited by dicumarol, but they differ in their responses to DNP and PCMBA. A comparison of the inhibitor sensitivities and the electron acceptor specificities suggests that the mung bean enzyme most closely resembles the Vitamin K-reductase of Märki and Martius.[10] This enzyme has been extensively purified and shown to be a flavoprotein.

The physiological roles of the various menadione reductases have not been definitely established. It is significant that in both plant and animal homogenates most of the activity is found in the soluble fraction, and it was from a particle-free fraction that the mung bean enzyme was prepared. This intracellular localization suggests that the enzyme is not a part of the normal respiratory chain and that it probably functions in hydrogen transport in the cytoplasm itself. The fact that naturally occurring quinones were not effective with this enzyme might be the result of our inability to duplicate the physiological state in which the electron acceptors may be bound in some intracellular membrane. Several of the mammalian enzymes are active with Vitamin K_1 and/or ubiquinone (UQ_{10}),[9,11,12] but neither of these natural quinones was active with Martius' highly purified enzyme.[10] The natural acceptor for the soluble mung bean enzyme might even be menadione itself, although this quinone has not yet been clearly identified in Nature.

Acknowledgements—The authors are indebted to Drs. M. W. Foote and R. Whatley for samples of some of the naphthoquinones.

[9] W. D. WOSILAIT, *J. Biol. Chem.* **235**, 1196 (1960).
[10] F. MÄRKI and C. MARTIUS, *Biochem. Z.* **333**, 111 (1960).
[11] T. E. CONOVER and L. ERNSTER, *Biochem. Biophys. Res. Com.* **2**, 26 (1960).
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THE ALKANE CONSTITUENTS OF SOME NEW ZEALAND PLANTS AND THEIR POSSIBLE TAXONOMIC IMPLICATIONS*

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Abstract—The compositions of the hydrocarbon fractions of certain New Zealand plants have been determined by gas-liquid chromatography. The results may be of taxonomic value.

INTRODUCTION

A QUANTITATIVE, gas-liquid chromatographic study of the alkane components of the leaf-surface waxes of a number of plant species belonging to the sub-family Sempervivoideae (Crassulaceae) was recently reported.[1] The provisional conclusions reached were firstly that the alkane distribution pattern could be regarded as a species characteristic, despite slight seasonal and regional variation. There are analogies for this conclusion in the fat and essential oil fields, though the taxonomic possibilities have been largely ignored. For example, gas liquid chromatograms of seed oils or, more usually, of the methyl esters of the constituent fatty acids may be used as guides to the origin of particular samples.[2] It is well known that the composition of the essential oil of a given species may vary with season, geographic location and climatic conditions.[3] External protective plant waxes, being "conservative"[4] constituents, might not necessarily be expected to show similar variations.

Secondly, it was decided that the patterns given by closely related species, belonging to certain compact botanical sub-groups, were sometimes alike. This work[1] had its inception in the observation that the alkane content of some New Zealand plants showed marked species variation, and these earlier studies are now reported in full.

RESULTS AND DISCUSSION

The plants studied are listed in Table 1, together with the pertinent experimental data and the alkane distribution patterns represented diagrammatically in Fig. 1A. The distribution patterns in other species (Fig. 1B) have been constructed for comparison from data in the literature, further details being given in Table 2. The examples chosen for the construction of Fig. 1B are restricted to results which have been obtained, either by mass

* Joint contribution from the Department of Chemistry and the Experimental Pharmacology Division, Institute of Physiology.

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[1] G. EGLINTON, A. G. GONZALEZ, R. J. HAMILTON and R. A. RAPHAEL, *Phytochemistry* **1**, 89 (1962); G. EGLINTON, A. G. GONZALEZ, R. J. HAMILTON and R. A. RAPHAEL, *Nature* **193**, 739 (1962).

[2] E. VON RUDLOFF, *Can. J. Chem.* **39**, 1200 (1961).

[3] See for example H. FLÜCK, *J. Pharm. and Pharmacol.* **7**, 361 (1955), **6**, 153 (1954); N. T. MIROV, *J. Am. Pharm. Assoc., Sci. Ed.* **47**, 410 (1958); Symposium in *Pharm. Weekblad* **92**, 762 (1957).

[4] H. ERDTMAN, *Perspectives in Organic Chemistry* (Ed. by R. ROBINSON), p. 473, Interscience (1956).

TABLE 1. DISTRIBUTION IN MOLE PERCENTAGE OF THE ALKANES*

No	Plant†	Family	Portions extracted	Total petrol extractives‡	Total alkane fraction§	C_{23}		C_{24}		C_{25}		C_{26}		C_{27}		C_{28}		C_{29}		C_{30}		C_{31}		C_{32}		C_{33}		C_{34}		C_{35}	
						iso	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*	*iso*	*n*
1	*Hebe odora* (Hook f.) Ckn.	Scrophulariaceae	Stems and leaves	3·9	4		+		1		2		2		10		3		52		2		25		2		3				
2	*Hebe parviflora* (Vahl.) var. *arborea* (Buchan.) L. B. Moore	,,	,,	2·5	10						1		+	+	4		1	+	17		2	+	65		3		10				
3	*Hebe diosmifolia* (A. Cunn.) Ckn. and Allan	,,	,,	3·2	10										1		2	+	15		6	+	40		3	+	18		+		16
4	*Hebe stricta* (Benth.) L. B. Moore	,,	,,	1·7	4·5						+		1		2		2	+	10		4	+	24		1	1	37		1	1	16
5	*Gaultheria subcorymbosa* Col.	Ericaceae	,,	2·0	13 5						4		3		8		1		16		3		56		4		7				
6	*Gaultheria antipoda* Forst. f.	,,	,,	1·6	11						1		1		8		3		26		3		52		2		7				
7	*Phormium tenax* J. R. and G. Forst. var. S.S.	Liliaceae	Rhizomes	1·0	5·5				2	+	9	+	4	2	16	+	5	3	47	+	2	3	8		2						
8	*Phormium tenax* J. R. and G. Forst. var. Ngaro	,,	,,	1·1	1					+	15	+	4	2	20	+	3	4	40	+	3	2	5								
9	*Cordyline australis* (Forst. f.) Hook f.	,,	,,	1·3	3·5						21		2	1	42		3	1	15		3	1	10								
10	*Pimelea prostrata* (J R. and G. Forst.) Willd.	Thymelaeaceae	Stems and leaves	1 25	8						3		2		13		3	+	65		2	+	13								
11	*Acaena anserinifolia* (J. R. and G. Forst.) Druce.	Rosaceae	,,	2 0	4						+		1		4		2	1	11	1	2	1	59		2	+	19				
12	*Arundo conspicua* Forst. f‖	Cortaderia	Leaves	2·5	4		+		+		4		2		9		5		60		5		12		2		3				

* The content of an individual alkane is expressed as a mole percentage of the total hydrocarbon content from C_{23}–C_{35} inclusive. The mole percentage is taken as being equivalent to the area percentage i.e. $100\,A_n/\sum_{n=23}^{35} A_n$, where A_n is the area of the peak corresponding to the hydrocarbon $C_nH_{(2n+2)}$, as measured by planimeter. The values are approximated to the nearest 1 per cent and peaks of relative area less than 1 per cent are indicated by +. The branched chain alkanes are designated *iso*-, but see text.

† The species were collected at the following locations. Porters Pass, Canterbury, 1; Rimutaka Saddle, Wellington, 2, 5; Ngaiotonga, North Auckland, 3; Blockhouse Bay, Auckland, 4; Akatarawa Valley, Wellington, 6, 11; Moutoa Development Area of the Department of Agriculture, Wellington, 7, 9; Foxton Plantation of N.Z. Woolpack and Textiles Ltd., 8; Houghton Bay, Wellington, 10; Plimmerton, Wellington, 12.

Collection was made during the following months: July 7, 8, 9, 10, 11, 12; August 2, 3, 4, 5; September 1, 6.

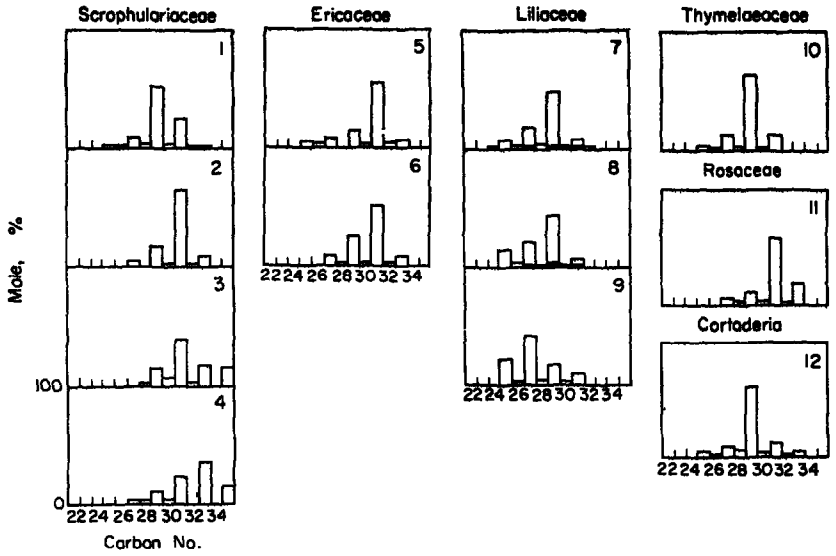
Names used are, for dicotyledons as in Allan H. H. *Flora of New Zealand*, Vol. 1, 1961, Wellington, Government Printer; for monocotyledons as in Cheeseman, T. F., *Manual of the New Zealand Flora*, 2nd edition, Wellington, Government Printer.

Grateful acknowledgement is made to the following for aid in the collection of certain species: The director and staff, Botany Division, D.S.I.R., Christchurch, 1; Dr. R. C. Cooper, Auckland Museum, 4; Mrs. W. Reynolds, 3; Messrs. G. B. Miller, W. R. Boyce and W. E. Hale, 7, 8, 9; Miss Peggy Martin-Smith, 12.

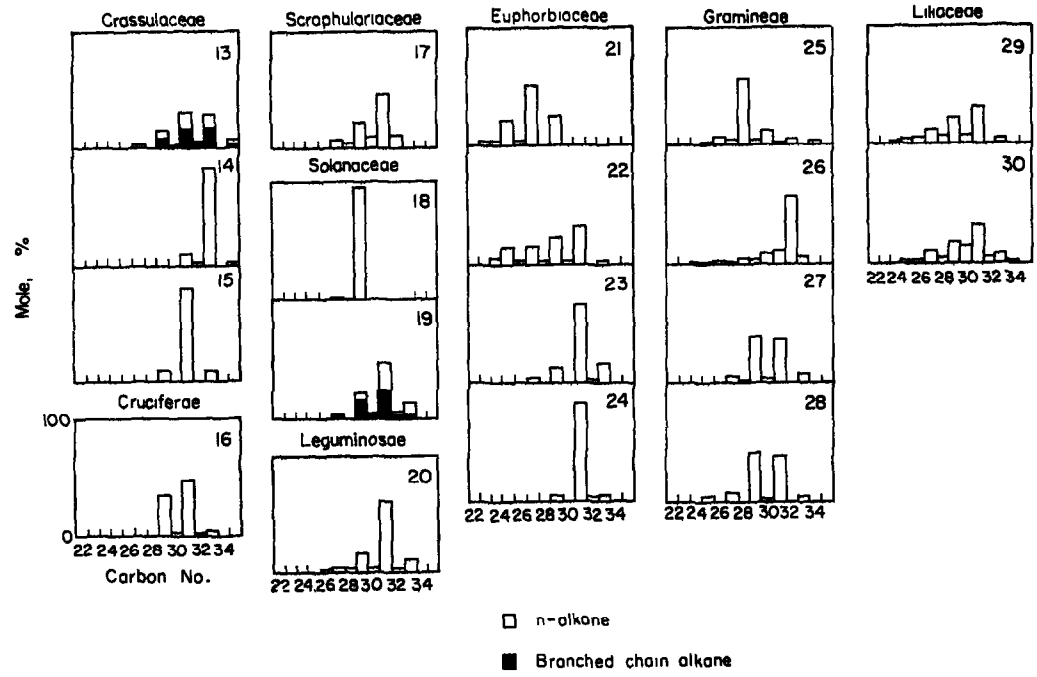
‡ As per cent dry weight-values to the nearest 0·05 per cent.

§ Per cent total alkane hydrocarbons calculated on total weight of petro lextractives to the nearest 0·5 per cent.

‖ A separate sample of Species 12 has been examined: The leaves were collected from the same station in late December 1961 and the surface wax removed by brief immersion of the fresh leaves in chloroform. The alkane fraction was examined with the following results: C_{23}, +; C_{24}, +; C_{25}, 7%; C_{26}, 6%; C_{27}, 14%; C_{28}, 9%; C_{29}, 50%; C_{30}, 4%; C_{31}, 10%. This correlates quite well with the previous result



A. THE PRESENT WORK—NEW ZEALAND PLANTS (Table 1)



B. SOME LITERATURE DATA (Table 2)

FIG. 1. DISTRIBUTION IN MOLE PERCENTAGE OF *n*- AND BRANCHED CHAIN ALKANES C_{22-35} IN THE HYDROCARBON FRACTION OF THE WAXES FROM INDIVIDUAL PLANT SPECIES.

Alkane percentages less than 2 mole per cent have been omitted.

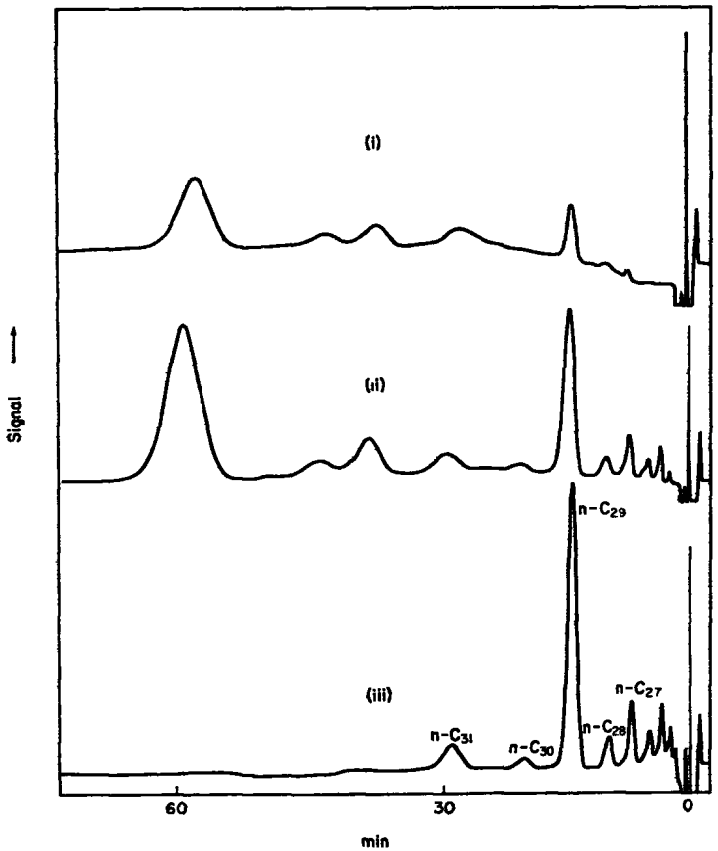
spectrographic[5-7] or gas–liquid chromatographic analysis[7-10] of unfractionated plant alkanes. Little reliance can be placed upon claims for the separation and identification of plant alkanes by other methods, as shown by the outstanding collaborative work of Chibnall and Piper,[11,12] and the later mass spectrographic analysis of some of their original samples.[5]

The New Zealand plants were collected primarily on account of their pharmacological interest, but at the suggestion of Miss Lucy B. Moore, Botany Division, D.S.I.R. (New Zealand), four *Hebe* spp. were included in the collection in order to ascertain whether it would be possible to secure a taxonomical differentiation on the basis of their chemical constituents. The genus *Hebe* is well known for the ease with which hybridization occurs,[13] making botanical classification extremely difficult, and the species chosen had been carefully selected by Miss Moore as representative of definite groups in her re-classification of the genus.[14] The versatility of gas–liquid chromatographic techniques[15] suggested their application to the problem, as it was felt that analysis of a wide range of components might afford more clear-cut distinctions than observations as to the presence or absence of single components. Originally, investigations were performed on the total light-petroleum extractives, but as interpretation of the gas–liquid chromatograms was exceedingly difficult, the study was restricted to the alkane fraction, since this could be readily separated from the accompanying acids, alcohols, carbonyl compounds and esters. The results indicated the potentialities of the method as a means of "fingerprinting" individual species and this aspect was subsequently examined in more detail in the study of the Sempervivoideae already reported.[1]

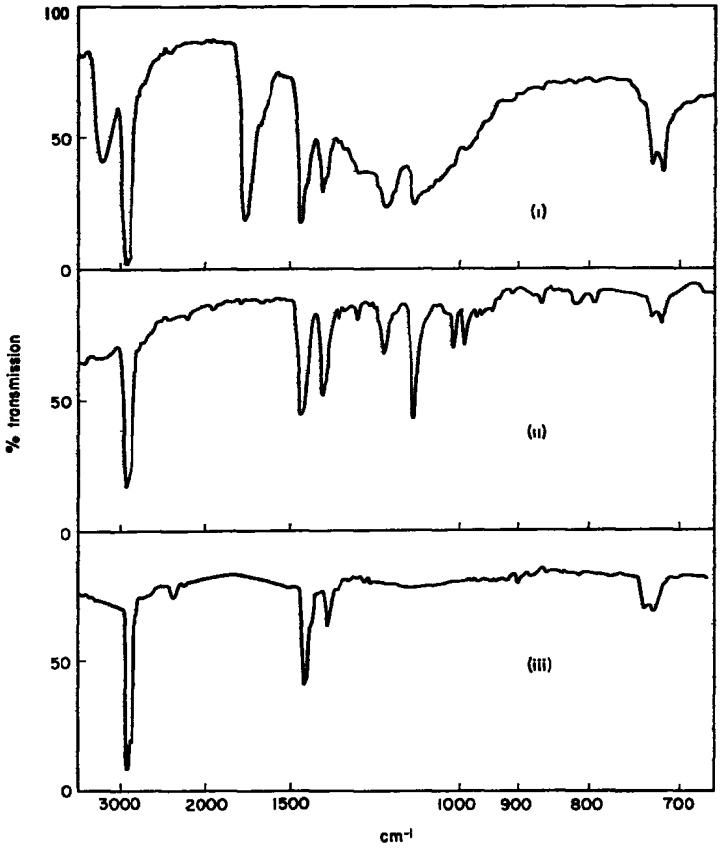
The results of the present studies may not be strictly comparable with those obtained with the Sempervivoideae, since the plant material was pre-dried to facilitate transportation and the portions of plants employed were not confined to the leaves. Although the alkane components are probably restricted to the surface coatings as evidenced by the fact that the alkane patterns of the leaf-surface wax and of the total alkane extractives of the leaves of *Arundo conspicua* were virtually identical (see footnote Table 1), there is no reason to suppose that the alkane distribution patterns are the same for different anatomical portions of the plant. In fact the marked difference between the alkane distribution in the rhizomes of *Cordyline australis* (9, Fig. 1A) and that in the leaves of *Dracaena draco* (29, Fig. 1B), plants which are considered to be closely related botanically, shows the need for studies on the relative alkane distribution within the same plant. In the present work comparison of the alkane distribution patterns of the different species within the same genus would appear to be valid *inter se*, as in all cases the comparisons are made using corresponding portions of the plants. In the case of the *Gaultheria* and *Hebe* spp. twigs and leaves were employed, and in the case of the two varieties of *Phormium tenax*, rhizomes were used.

It was found that the alkane analysis could be adequately performed on 40 g of dried plant. A crude fraction containing the total alkanes was obtained from the finely-ground

[5] J. D. WALDRON, D. S. GOWERS, A. C. CHIBNALL and S. H. PIPER, *Biochem. J.* **78**, 435 (1961).
[6] G. G. WANLESS, W. H. KING and J. J. RITTER, *Biochem. J.* **59**, 684 (1955).
[7] W. CARRUTHERS and R. A. W. JOHNSTONE, *Nature* **184**, 1131 (1959).
[8] Z. H. KRANZ, J. A. LAMBERTON, K. E. MURRAY and A. H. REDCLIFFE, *Australian J. Chem.* **14**, 264 (1961).
[9] Z. H. KRANZ, J. A. LAMBERTON, K. E. MURRAY and A. H. REDCLIFFE, *Australian J. Chem.* **13**, 498 (1960).
[10] P. MAZLIAK and R. COMBES, *Compt. rend.* **251**, 2393 (1960).
[11] S. H. PIPER, A. C. CHIBNALL, S. J. HOPKINS, A. POLLARD, J. A. B. SMITH and E. F. WILLIAMS, *Biochem. J.* **25**, 2072 (1931).
[12] A. C. CHIBNALL, S. H. PIPER, A. POLLARD, E. F. WILLIAMS and P. N. SAHAI, *Biochem. J.* **28**, 2189 (1934).
[13] L. COCKAYNE and H. H. ALLAN, *Ann. Bot.* **48**, 39 (1934).
[14] L. B. MOORE, *Flora of New Zealand* (Ed. by H. H. Allan), Vol. 1, Government Printer, Wellington (1961).
[15] G. EGLINTON, R. J. HAMILTON, R. HODGES and R. A. RAPHAEL, *Chem. & Ind. (London)* 955 (1959).



A. GAS–LIQUID CHROMATOGRAMS. Load ca. 5 μg of solid wax; Column 130 cm × 0·4 cm, 0·5% Apiezon "L" on Embacel, 80–100 mesh at 225°; gas flow, 45 ml of argon per minute; detector voltage 1750V, attenuation X10.



B. INFRA-RED SPECTRA. Solid films.

FIG. 2. ISOLATION OF THE HYDROCARBON FRACTIONS FROM THE PLANT WAX OF ARUNDO CONSPICUA.

(i) The crude plant wax as extracted from the dried plant. (ii) The wax remaining after successive treatments with alcoholic sodium hydroxide and 2,4-dinitrophenylhydrazine reagent, followed by chromatography over alumina. (iii) The wax remaining after treatment of fraction (ii) with hot concentrated sulphuric acid.

dried plant material by continuous extraction with boiling light petroleum (b.p. 40°–60°) for 24 hr. Infra-red analysis of the crude extract, which varied considerably in quantity (see Table 1), indicated the presence of compounds containing hydroxyl, carbonyl and carboxyl functions. As chromatography on alumina did not completely remove these contaminants, the esters were saponified and the carbonyl compounds were converted to their dinitrophenylhydrazone derivatives. After this pretreatment, alumina chromatography afforded the pure alkane fraction (as evidenced by infra-red analysis) in all cases except that of *Arundo conspicua* which showed a prominent band at 1120 cm^{-1} in the infra-red. This peak can be ascribed to the presence of ethers, one of which has since been isolated in pure form and will form the subject of a separate communication. The ethers were readily removed from the alkanes by treatment of the mixture with concentrated sulphuric acid. The infra-red spectra and gas–liquid chromatograms of the light petroleum extractives of *Arundo conspicua* at the various stages of purification are shown in Fig. 2. A separate experiment established that the ethers were present in the true leaf-surface wax.

Complete analysis of alkanes possessing between 23 and 35 carbon atoms was achieved, those of higher and lower chain length which may have been present in small amounts being ignored. Eight of the plants studied (Nos. 2–4 and 7–11 in Table 1) appeared to contain branched alkanes as well as *n*-alkanes, although the former were present in very small amounts, usually less than 4 per cent of the total, except in the cases of Nos. 8 and 9. Direct identification of the branched chain alkanes was not possible on the scale employed in the present work, but further experiments are in progress. By analogy with the previously reported work[1] and other studies, the odd-numbered, branched hydrocarbons are assumed to be isoalkanes (2-methyl-*n*-alkanes). However, isomeric iso- and anteiso- (3-methyl-*n*-alkanes[8]) alkanes are reported to have almost identical retention times on Apiezon columns[8,16] and it is possible that the even-numbered branched alkanes belong in fact to the anteiso- series. Should this be so, the situation would be the inverse of that reported for the branched hydrocarbons of wool wax.[17] A high content of branched alkanes is a relatively rare occurrence in leaf waxes. Very few of the examples in Fig. 1B show the presence of branched alkanes, although trace quantities may have been overlooked by the original workers. Careful investigation of spp. 21–23 and species 29 in Fig. 1B, however, failed to show the presence of any isoalkanes.[1] It is therefore all the more interesting that *Nicotiana tabacum* (19, Fig. 1B) has a high iso-alkane content[7] and that certain *Aeonium* spp., e.g. 13, Fig. 1B, show appreciable quantities of iso-alkanes.[1]

In the gas–liquid chromatograms of several of the plants (viz. those of Nos. 1, 3, 6–10 in Table 1) small peaks were present which could not be assigned to either *n*-alkanes or iso-alkanes. It is possible that these peaks are due to the presence of cycloalkanes or of compounds possessing double bonds or other functional groups which are present in such small amounts as to be not detectable in the infra-red spectra. The intensity of these peaks is, however, so low that they in no way interfere with the interpretation of the major peaks.

In all cases *n*-alkanes with an odd number of carbon atoms form the major components of the paraffin fraction, which is in agreement with the results obtained by other workers.[5,12] Of the mixtures given in Figs. 1A and 1B the major constituent is either the C_{27}, the C_{29}, the C_{31} or the C_{33} *n*-alkane, but the C_{35} *n*-alkane is the major constituent of cactus leaf wax.[5] It is of considerable interest that within the genus *Hebe* the major constituent is C_{29} in *H. odora*, C_{31} in *H. parviflora* and *H. diosmifolia*, and C_{33} in *H. stricta*, thus giving an

[16] R. ROWAN, *Anal. Chem.* **33**, 658 (1961).
[17] D. T. DOWNING, Z. H. KRANZ and K. E. MURRAY, *Australian J. Chem.* **13**, 80, (1960).

TABLE 2. LITERATURE SOURCES OF THE DATA DEPICTED IN FIG. 1B

No.	Plant species	Family	Common Name	Portion extracted	Method of determination*	Reference
13	*Aeonium goochia* W.B.	Crassulaceae	—	Leaf surface	g.l.c.	1
14	*Monanthes brauchycaula* Lowe	„	—	„ „	g.l.c.	1
15	*Sedum anglicum* Huds.	„	—	„ „	g.l.c.	1
16	*Brassica nigra* Koch.	Cruciferae	Black mustard	Aerial parts	m.s.	5
17	*Bacopa monnieri* (L) Pennell.	Scrophulariaceae	—	„ „	g.l.c.	18
18	*Solanum tuberosum* L.	Solanaceae	Potato	Tuber cuticle	g.l.c.	10
19	*Nicotiana tabacum* L.	„	Tobacco	Whole leaf	g.l.c. and m.s.	7
20	*Phaseolus aureus* Roxb.	Leguminosae	String bean	Aerial parts	m.s.	6
21	*Euphorbia balsimifera* Ait.	Euphorbiaceae	—	Leaf surface	g.l.c.	1
22	*Euphorbia aphylla* Brouss.	„	—	Aerial parts	g.l.c.	1
23	*Euphorbia regis-jubae* W.B.	„	—	„ „	g.l.c.	1
24	*Euphorbia cerifera* Alcocer.	„	Candelilla	„ „	g.l.c.	19
25	*Saccharum officinarum* L.	Gramineae	Sugar cane	Stem surface	g.l.c.	9
26	*Leptochloa digitata* Domin.	„	—	„ „	g.l.c.	8
27	*Lolium perenne* Aitch.	„	Perennial rye grass	Aerial parts	m.s.	6
28	*Lolium multiflora* Lam.	„	Italian rye grass	Leaf surface	g.l.c.	1
29	*Dracaena draco* L.	Liliaceae	Dragon tree	„ „	g.l.c.	1
30	*Copernicia cerifera* Mart.	„	Carnauba palm	„ „	g.l.c.	20

* g.l.c. = gas-liquid chromatography.
m.s. = mass spectrometry.

[18] I. BROWN and M. MARTIN-SMITH, *J. Chem. Soc.* 2783 (1960).
[19] R. A. ROBERTSON and S. ROBERTSON, personal communication.
[20] J. S. ROBERTS and A. A. ROBERTSON, personal communication.

immediate chemotaxonomic distinction. In the case of the two *Gaultheria* spp. the differences in the alkane distribution patterns are not nearly so clear cut but they are still easily distinguishable. The fact that the two varieties of *Phormium tenax* give virtually superimposable patterns may strengthen the utility of plant hydrocarbon analysis rather than weaken it, as distinction between species without differentiation between varieties would be useful in taxonomy.

In most cases the shortest and longest *n*-alkane chains differ by about ten carbon atoms with varying proportions of each homologue between these limits. In certain waxes such as that of *Solanum tuberosum* (18, Fig. 1B) and *Pyrus communis*,[5] however, there appear to be only one or two individual alkanes present in appreciable quantities. An intriguing general point which emerges is that when the odd and even members are considered separately, in both cases the plot of percentage of constituent present against the number of carbon atoms is a simple distribution curve with a single maximum. This generalization also seems to apply with the odd and even branched alkanes, though less data are available. Since the alkanes can be assumed to be end products of plant metabolism this may be of significance in terms of the specificity of the enzyme systems which are involved in the elaboration of the alkanes from acetate units.[9]

The variety of the hydrocarbon patterns is striking when Fig. 1, in which 12 families and 21 genera are represented, is considered as a whole and augurs well for the future taxonomic application of plant hydrocarbon analysis. As yet no general patterns are apparent which might permit:—

(*a*) monocotyledons (e.g. Fig. 1, Gramineae spp. 12, 25–28, and Liliaceae spp. 7–9, 29 and 30) to be distinguished from dicotyledons (the remaining families illustrated in Figs. 1A and 1B),

(*b*) assignment of a species to a given family, as there is marked variation in the hydrocarbon patterns from one constituent genus to another (e.g. within the Crassulaceae spp. 13–15, and the Gramineae and Liliaceae given in Fig. 1, although, incidentally, there is a marked resemblance between the two species *Dracaena draco* and *Copernicia cerifera*),

(*c*) assignments of species to a particular genus, since again there is little constancy in the patterns (e.g. the diversity within the genus *Hebe* spp. 1–4, the genus *Aeonium*[1] and the genus *Euphorbia* spp. 21–24, but there is a similarity between the *Lolium* species, *L. perenne* and *L. multiflora*, spp. 27 and 28 respectively).

EXPERIMENTAL

Materials

The plant material was collected from July to September 1959 by one of us (M.M-S.) with the aid of generous financial assistance from the Royal Society and the Pharmaceutical Society of Great Britain. All the plants were identified by botanists whose names appear in the acknowledgements, and specimens have been lodged with the Auckland Museum and the Pharmaceutical Society of Great Britain.

Extraction and Purification of the Alkane Fraction

The plants were dried at 80° in a forced draught oven for 24 hr. The finely ground plant material (40 g) was extracted in a Soxhlet apparatus with light petroleum (b.p. 40°–60°) for 24 hr. After removal of the solvent under reduced pressure the extractives (usually of the

order of 0·4–1·0 g, see Table 1) were refluxed with 2,4-dinitrophenylhydrazine (1·0 g) and conc. HCl (0·5 ml) in EtOH (20 ml) for 2 hr to convert carbonyl compounds into 2,4-dinitrophenylhydrazones. The solvent was then removed under reduced pressure and the residue exhaustively extracted with light petroleum. After removal of the solvent the petrol-soluble material was refluxed for 2 hours in aqueous EtOH (1 : 2, 20 ml) containing NaOH (1·0 g). The solution was taken to dryness under reduced pressure and the residue thoroughly extracted with light petroleum. The petrol extract was filtered through alumina (Brockmann grade I) and the hydrocarbon fraction completely eluted with further light petroleum and freed from solvent.

Infra-red spectra of the hydrocarbon fractions were recorded in order to establish the absence of compounds other than alkanes (Fig. 2). In the case of *Arundo conspicua* (Table 1 and Figs. 1 and 2, sp. 12) the hydrocarbon fraction showed strong infra-red absorption at 1120 cm^{-1}, indicative of an ether grouping. The compounds responsible for this absorption were removed by treatment with hot conc. H_2SO_4 (140°, 4hr). The light petroleum extract of the reaction mixture furnished the pure alkane fraction after alumina chromatography.

Gas Chromatography

Gas–liquid chromatography of the alkane fractions was generally accomplished by chromatographing 0·1 μl of a solution of the fraction (2 mg in 40 mg of α-methylnaphthalene) on a column of Embacel (80–100 mesh) coated with Apiezon L (0·5%), as previously described.[1]

Acknowledgements—In addition to acknowledging the generous aid towards the collection of the plant material by those mentioned in the footnote to Table 1, the authors wish to express their indebtedness to Professor R. A. Raphael for his interest and to Miss L. B. Moore and Mr. A. P. Druce of Botany Division, D.S.I.R. (New Zealand), and to Dr. R. Dawson, Victoria University of Wellington, for confirming the identity of the plants studied. We thank Dr. A. C. Chibnall, F.R.S., for kindly providing reference specimens of pure alkanes. It is a pleasure to acknowledge the technical assistance of Miss Doreen Barclay, Miss June Galbraith, Miss Pamela Pellitt and Miss Irene Wilson, and to thank Dr. A. T. Johns, Plant Chemistry Division, Grasslands Research, D.S.I.R., Palmerston North, for the use of the drying ovens. One of us (R.J.H.) is indebted to the Department of Scientific and Industrial Research for a maintenance grant, and another (M.M-S.) gratefully acknowledges generous financial support from the Royal Society and the Pharmaceutical Society of Great Britain and thanks the University of Glasgow for leave of absence.

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STUDIES ON PLASTOQUINONE—1. DETERMINATION OF THE CONCENTRATION AND OXIDATION–REDUCTION STATE OF PLASTOQUINONE IN ISOLATED CHLOROPLASTS

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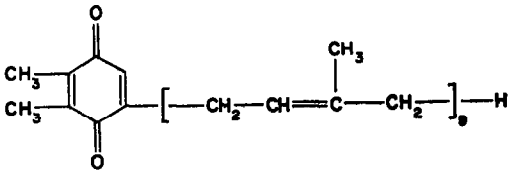
Abstract—In order to study the possible function of plastoquinone in the photosynthetic electron transport system, a method for extracting the endogenous quinone from isolated chloroplasts and measuring its concentration and oxidation–reduction state has been devised. Using this method it has been shown that the plastoquinone in freshly isolated chloroplasts is predominantly in the oxidized form. The concentration of plastoquinone was 0·1 mole per mole chlorophyll (mean value). Comparison of this concentration with that of the published concentration of cytochrome *f* indicated that plastoquinone was present in a considerable excess on a molar basis.

INTRODUCTION

PLASTOQUINONE (koflerquinone) is a lipid-soluble trisubstituted benzoquinone, 2,3-dimethyl-5-solanesyl-1,4-benzoquinone, (I).[1,2] It possesses a characteristic absorption spectrum in the oxidized form with peaks at 255 and 262 mμ, while on reduction a single absorption of reduced intensity at 290 mμ is obtained.



I

It was shown by Crane[3] that plastoquinone is localized in the chloroplasts of higher plants, and there is now a good deal of evidence which suggests that it functions as an oxidation–reduction carrier in the photosynthetic electron transport system. Bishop[4] showed that when lyophilized chloroplasts were extracted with organic solvents they lost their Hill-reaction activity, but that this could be restored by adding plastoquinone. Furthermore, Crane *et al.*[5] have shown that the endogenous plastoquinone is reduced to the quinol when isolated chloroplasts are illuminated. It was of interest therefore to devise a method for the determination of the concentration and oxidation–reduction state of plasto-

[1] N. R. TRENNER, B. H. ARISON, R. E. ERICKSON, C. H. SHUNK, D. E. WOLF and K. FOLKERS, *J. Am. Chem. Soc.* **81**, 2026 (1959).
[2] M. KOFLER, A. LANGEMANN, R. RÜEGG, U. GLOOR, U. SCHWIETER, J. WÜRSCH, O. WISS and O. ISLER, *Helv. Chim. Acta* **42**, 2252 (1959).
[3] F. L. CRANE, *Plant Physiol.* **34**, 128 (1959).
[4] N. I. BISHOP, *Proc. Natl. Acad. Sci., U.S.* **45**, 1696 (1959).
[5] F. L. CRANE, B. EHRLICH and L. P. KEGEL, *Biochem. Biophys. Research Communs.* **3**, 37 (1960).

quinone in isolated chloroplasts. A preliminary report of part of this work has already appeared.[6]

EXTRACTION AND PURIFICATION OF PLASTOQUINONE

Using a chloroplast preparation from sugar beet prepared in a similar way to that described previously,[7] it was found that plastoquinone could be extracted and its oxidation-reduction state determined spectrophotometrically by a method similar to that used for ubiquinone in mitochondrial preparations.[8] The chloroplast preparation (1 ml) was placed in a glass-stoppered test-tube and cold (—20°) methanol (4 ml) containing pyrogallol (1 mg/ml) added rapidly from a hypodermic or pipetting syringe. The purpose of the pyrogallol was to prevent the reoxidation of any quinol present during the isolation procedure. Light petroleum (40°–60°, 4 ml) was then added and the mixture shaken rapidly by hand for 1 min. After separation of the layers by low-speed centrifugation the light petroleum layer was removed and another extraction with light petroleum (3 ml) was made. The light petroleum extracts were combined in a glass-stoppered test-tube or 50 ml separating funnel and aqueous methanol (90 per cent (vol./vol.) 5 ml) added (in later experiments 95 per cent (vol./vol.) methanol (2 ml) was used and found to be satisfactory). The mixture was shaken five times and the layers allowed to separate; the methanol layer was removed and the partitioning process with methanol and water continued until the methanol layer was colourless. This procedure removes all the chlorophyll and most of the hypophasic carotenoids, which would otherwise interfere seriously with the spectrophotometric determination of plastoquinone. The yellow light petroleum layer which contained plastoquinone and epiphasic carotenoids (mainly β-carotene) was evaporated to dryness in a vacuum desiccator and the residue dissolved in spectroscopically pure ethanol (3 ml).

DETERMINATION OF THE OXIDATION-REDUCTION STATE OF THE ENDOGENOUS PLASTOQUINONE

The spectrum of the ethanol solution was determined from 230 to 310 mμ. The plastoquinone was then reduced by the addition of a small crystal of sodium borohydride (0·1–0·5 mg) followed by rapid stirring, and the spectrum redetermined over the same range. A typical result showing the spectra of the original and the reduced solutions is shown in Fig 1a and the difference spectrum in Fig. 1b. It will be seen that the difference spectrum has peaks at 255 and 262 mμ characteristic of plastoquinone, and a negative peak at 290 mμ of lower intensity characteristic of plastoquinol. Isosbestic points occur at 276 and 308 mμ. These values were found to be identical with those given by solutions of pure plastoquinone and plastoquinol (Fig. 1c and d). The plastoquinone concentration was calculated from the difference in extinction at 255 mμ (ΔE_{255}) using the molecular extinction coefficient for the difference in absorption of the oxidized and reduced[3] forms of plastoquinone, $(\varepsilon_{ox.} - \varepsilon_{red.})_{255} = 14{,}800$.

To determine the total oxidized level of the quinone, 2, 6-dichlorophenolindophenol or potassium ferricyanide (1 μmole) was first added to the chloroplast suspension and the mixture left to stand in the dark for 5 min before extraction and estimation as before.

The validity of the method was tested by taking an ethanolic solution of pure plastoquinone (125 μg in 0·5 ml) through the complete extraction and partitioning procedure. This concentration is of the same order as that of the endogenous plastoquinone in a typical

[6] E. R. REDFEARN and J. FRIEND, *Nature* **191**, 806 (1961).
[7] A. M. MAYER and J. FRIEND, *J. Exp. Bot.* **11**, 141 (1960).
[8] A. M. PUMPHREY and E. R. REDFEARN, *Biochem. J.* **76**, 61 (1960).

determination. After treatment with methanol and extraction with light petroleum, the combined light petroleum extract was partitioned with 95 per cent (vol./vol.) methanol as before.

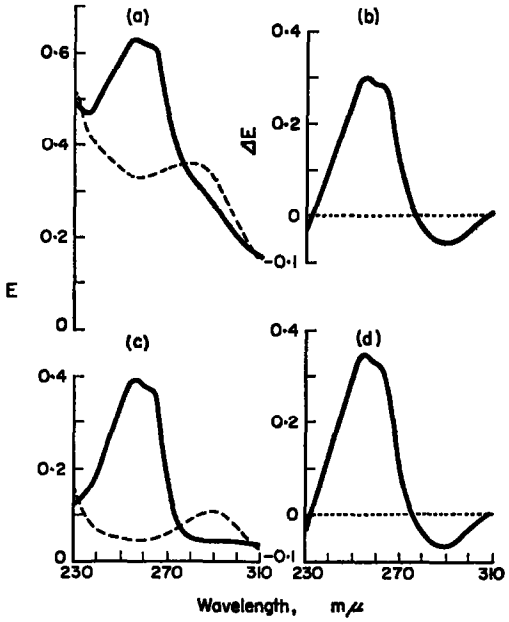


FIG. 1. ABSORPTION SPECTRA OF CHLOROPLAST LIPID EXTRACT AND PURE PLASTOQUINONE.
(*a*) ———spectrum of chloroplast lipid extract; - - - - - - - same after reduction with $NaBH_4$ using ethanol as blank); (*b*) difference spectrum (oxidized *minus* reduced) of chloroplast lipid extract; (*c*) ———plastoquinone, - - - - - - - plastoquinol (using ethanol as blank); (*d*) difference spectrum (plastoquinone *minus* plastoquinol).

After twelve partitions 95 per cent of the plastoquinone was recovered. A similar experiment done with an ethanolic solution of plastoquinol (0·5 ml containing 0·2 mg) showed that the recovery depended upon the number of partitions with 95 per cent methanol, indicating that the quinol was more soluble in the methanol phase than was plastoquinone. The recovery value for 12 extractions (mean of three determinations) was 65 per cent. However, since in the method the total concentration of the quinone is measured as the oxidized form, the recovery of the quinol can be disregarded.

RESULTS AND DISCUSSION

The procedure described is a rapid and convenient method for determining the concentration and oxidation–reduction state of the endogenous plastoquinone of isolated chloroplasts. The difference spectrum of the oxidized and reduced chloroplast extract shows that plastoquinone is the principal chromophoric substance capable of undergoing oxidation–reduction. The isosbestic point for plastoquinone → plastoquinol reduction at 276 mμ[9] is close to the absorption maximum of ubiquinone (275 mμ). Thus the lack of any change in extinction at 276 mμ indicates the absence of ubiquinone in the chloroplast preparations. Although the extracts also contain carotenes which possess absorption bands in the same spectral region as plastoquinone these are not reduced by sodium borohydride and thus do not affect the difference spectrum.

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Extraction of a number of chloroplast preparations immediately after they had been prepared showed that the oxidation-level of the endogenous plastoquinone varied from 45 to 90 per cent (Table 1). The mean value of 0·1 mole of plastoquinone per mole chlorophyll agrees well with Crane's result for spinach chloroplasts.

Quinones have previously been implicated as oxidation–reduction carriers in the photosynthetic electron transport system on the assumption that the chloroplasts of higher plants contained vitamin K_1.[10–12] Recent attempts, however, to isolate vitamin K_1 from chloroplasts have been unsuccessful[13] and in the present work vitamin K_1 could not be detected spectrophotometrically in the chloroplast lipid extracts. Thus it now seems that vitamin K_1 is either completely absent or present in such small quantities so as to make a functional role in electron transport seem very unlikely. Plastoquinone, however, is present in the

TABLE 1. CONCENTRATION AND OXIDATION–REDUCTION STATE OF ENDOGENOUS PLASTOQUINONE IN SUGAR-BEET CHLOROPLAST PREPARATIONS

Date of preparation 1960	Plastoquinone concentration		Percentage in oxidized form
	chlorophyll moles/mole	protein μmoles/g	
11 May	0·072	17·3	59
9 June	0·132	13·5	62
16 June	0·097	—	81
17 Aug.	0·124	15·7	51
19 Aug.	0·075	9·15	46
6 Sep.	0·095	8·87	97

chloroplasts in a relatively high concentration. It is interesting to compare the molar ratio of chlorophyll to plastoquinone (mean value of 10) with that of chlorophyll to cytochrome *f* whose value has been given as 200–400.[14,15] Thus there is 20 to 40-fold excess of plastoquinone compared to cytochrome *f* on a molar basis. It is interesting also to recall that the related mitochondrial quinone, ubiquinone, is present in a considerable excess compared with the individual cytochromes in the mitochondria of non-photosynthetic plant tissues and of animal tissues.

There was considerable variation in the oxidation–reduction level of the quinone in freshly isolated chloroplasts, but the oxidized form usually predominated. The reason for this variation is not clear at the moment but it probably depends upon a number of factors such as the environmental conditions during the growth of the plant and the time of storage of the detached leaves before isolation of the chloroplasts.

It is considered that the method described will be a useful technique for studying the possible function of plastoquinone as an oxidation–reduction carrier in the photosynthetic electron transport system.

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EXPERIMENTAL

Solvents

Light poetroleum, b.p. 40–60°, methanol and ethanol were purified as previously described.[8]

Chloroplasts

These were isolated from the leaves of sugar-beet (var. Sharpe's Klein E) grown in a greenhouse. The method used was that previously described[8] except that the isolation medium was buffered to pH 7·8 with 0·05 M Na_2HPO–KH_2PO_4 buffer instead of tris-HCl. After one washing, the chloroplasts were suspended in a medium consisting of 0·4 M sucrose, 0·01 M KCl and 0·05 M Na_2HPO–KH_2PO_4 buffer, pH 7·0, to give a chlorophyll concentration in the range 0·4–0·8 mg/ml. The chlorophyll and protein concentrations were determined by the methods of Mackinney[16] and Cleland and Slater[17] respectively.

Spectra

These were determined in 1 cm matched quartz cells using a Unicam SP500 spectrophotometer.

Acknowledgements—We are indebted to Mr. J. K. Hulme, Director, Liverpool University Botanic Gardens, and Mr. G. E. Russell, Plant Breeding Institute, Cambridge, for generous supplies of sugar-beet plants.

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MULTIPLE MOLECULAR FORMS OF 4-GLUCOSYL TRANSFERASE (PHOSPHORYLASE) IN *OSCILLATORIA PRINCEPS*

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Abstract—The separation of purified preparations of starch phosphorylase (α-1,4-glucan : D-glucose-4-glucosyl transferase) from *Oscillatoria princeps* was achieved on polyacrylamide gel columns by the new technique of disc electrophoresis. A total of five components, making up a chromatographically homogeneous phosphorylase preparation, was obtained by the method. Of these five proteins, four exhibited varying degrees of polysaccharide-synthesizing activity. Fraction a_1 synthesized a linear polyglucoside from D-glucosyl phosphate; fraction a_2 did so only in the presence of adenosine-5-phosphate; fraction a_3 showed both 4-glucosyl transferase activity and 6-glucosyl transferase action (Q enzyme activity, α-1,4-glucan : 1,4-glucan-6-glucosyl transferase); while fraction a_4 had only Q enzyme activity and was unable to synthesize linear dextrins from D-glucosyl phosphate even when adenosine-5-phosphate was present as cofactor; fraction a_5 was devoid of polysaccharide-synthesis action both as to the formation of linear or branched sugars. These various fractions are discussed with a view to the possibility of the evolution of Q enzyme from an original intermediate type of phosphorylase.

INTRODUCTION

THE SYNTHESIS of polyglucosides in plants is brought about by two enzymes: "phosphorylase" (α-1,4-glucan : D-glucose-4-glucosyl transferase) which links glucose residues in α-1,4-linkages, thus forming linear chains of polyglucoside from glucosyl phosphate, and Q enzyme (or branching enzyme α-1,4-glucan : 1,4-glucan-6-glycosyl transferase) which acts upon the polyglucoside resulting from starch phosphorylase action, forming α-1,6- linkages, thereby giving rise to branching of the linear chain.

In the blue-green alga, *Oscillatoria princeps*, two strains have been reported,[1,2] one of which (*n*-strain) forms a ramified, amylopectin-like component, and the other (*LTV*-strain), which forms a linear, amylose-like glucan.[3] To date, no significant differences have been detected in the physico-chemical[4,5] or biological[6] properties of the starch phosphorylases and Q enzymes of the two strains.

Multiple forms of plant enzymes have been reported, such as the different peroxidases of corn[7] and horse-radish,[8] the fumarases of *Torula* yeast,[9] and the multiple phosphatases and esterases of the green alga, *Acetabularia*.[10] Multiple forms of phosphorylase have been reported in groups other than algae. For example, two forms of the enzyme in mammalian skeletal muscle are well documented.[11-13] Immunological studies have revealed an even greater number of different forms of phosphorylase both inter and intra species in

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animals.[14] In plants, using paper electrophoresis, at least five proteins having phosphorylase activity have been reported in potato extracts.[15]

Therefore, substantial evidence exists for inter-specie differences, intra-specie differences, and even differences within the same tissue of the same organism with regard to phosphorylase.

In *Oscillatoria*, as well as in other plants,[16-18] the type of polysaccharide synthesized ultimately depends upon the relative activities of phosphorylase and Q enzyme in the tissue. Because of the relative ease of removal of the essential manganese bound to the protein constituting this algal enzyme,[19,20] it has been postulated that inactivation of the phosphorylase component of a natural mixture by demetallization with a chelating agent might serve as a mechanism whereby the Q enzyme/phosphorylase ratio were controlled, thereby acting as the determinant of the type of polyglucoside eventually synthesized.[20,21]

Hitherto, all methods used for the detection of possible differences in the enzymes of these two strains of *Oscillatoria* have resulted in no observable deviations. With the advent of column electrophoresis, more sensitive techniques have been made available, particularly with the observation that proteins can be resolved in extremely small quantities if the electrophoresis takes place in a polyacrylamide gel whose pore size is rigidly controlled by the conditions of its formation in a minute glass column. This technique, known as disc electrophoresis,[22,23] because the protein fractions separate as thin wafers in the gel, makes it possible to resolve as little as 0·01 μg of protein from dilute solutions. Therefore, a study of the purified enzymes of this blue-green alga was undertaken using this new technique.

RESULTS

Five bands were observed in the purified enzyme preparations which had been subjected to disc electrophoresis and stained by naphthalene black 12B (Fig. 1). These bands were designated a_1 through a_5 because movement was towards the anode. The band positions for crude extracts and purified preparations from *n* and *LTV* strains are indicated in the shadowgram shown in Fig. 1.

Four of these five bands showed polysaccharide-synthesizing activity, both when electrophoretically eluted from the polyacrylamide gel, and when the column was incubated after immersion in buffered D-glucosyl phosphate or buffered linear dextrins. The glucan formed by fractions a_1 and a_2 (Fig. 1C and D) stained a deep blue with iodine; that formed by a_3 stained violet. Of the three fractions exhibiting polysaccharide synthesis from D-glucosyl phosphate, only fraction a_2 was able to accomplish this without the addition of adenosine-5-phosphate (AMP). Both a_1 and a_3 showed polysaccharide synthesis from the hexose phosphate only in the presence of 0·001 M AMP. It was known that while animal phosphorylase *b* required AMP or ATP as cofactor[24,25] plant phosphorylases did not.[26]

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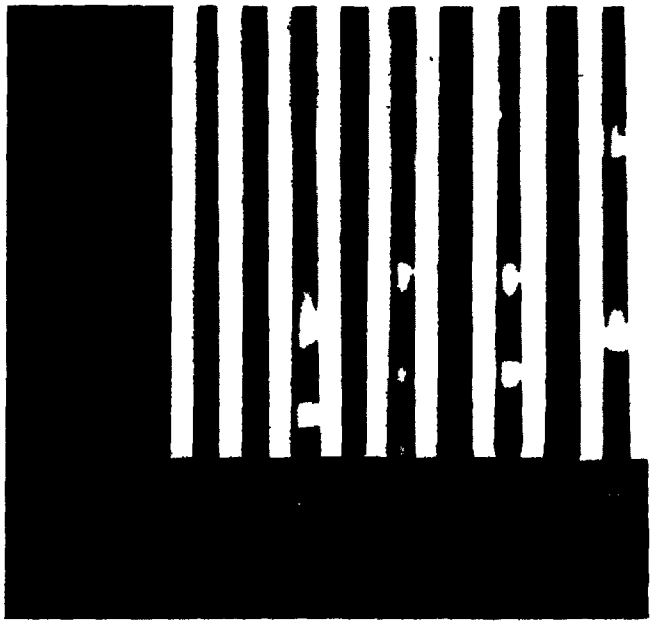

FIG. 1. CONTACT PRINT OF POLYACRYLAMIDE GEL COLUMNS AFTER DISC ELECTROPHORESIS. The fractions are indicated to the left of the photograph. Migration was towards the anode (+). A and B show crude extract of *LTV* and *n* strains; C shows a pure preparation from *LTV* strain; D shows a pure preparation from *n* strain; E shows a purified preparation from the green alga, *Spirogyra*.

[*facing page 154*]

Fraction a_4 (cf. Fig. 1C and D) showed only Q enzyme activity, rapidly branching linear dextrins. But this activity was also present in fraction a_3 of the *LTV* preparation (Fig. 1C), though completely absent from fractions a_1 and a_2 of this preparation. It was not possible, even after the isolation of fraction a_3 of this preparation in a dialysis bag and its re-electrophoresis, to separate the Q enzyme activity from this fraction. This discrete band of protein continued to exhibit both phosphorylase and Q enzyme activities when immersed in each respective substrate. This fraction was absent from *n* strain preparations (Fig. 1D).

The crude extracts from *n* and *LTV* strains of this alga are shown in Fig. 1A and B. Although many diffuse fractions are present in these crude unfractionated extracts, only the same four bands as are present in the purified preparations showed any type of polysaccharide synthesis.

It should be noted that fraction a_4, while present in both purified preparations from *n* and *LTV Oscillatoria*, is much weaker in *LTV* than in *n* strains (cf. Fig. 1C and D). This may be indicative of a difference in absolute concentrations of this branching enzyme in these two strains.

DISCUSSION

While the phosphorylases of *Oscillatoria princeps* had previously showed no marked chromatographic[4,19] or immunological[6] differences, the present study indicates that what was thought to be a homogeneous enzyme preparation contains at least three enzyme proteins (a_1, a_2, a_3) with identical functions. Of these three, one (a_3) had both the ability for the synthesis of linear glucans and branching action. In this light the reported properties of the phosphorylases and related enzymes of this alga must be re-examined. For example, while the pH optima, isoelectric points, electrophoretic rates of migration, etc. (Table 1), show no great differences between the *n* and *LTV* strains of this *Cyanophyceas*, the minute differences present in this data may very well reflect the presence of the a_3 fraction and diminution of the a_4 fraction in *LTV* strains, both of which were only detected by this technique of disc electrophoresis.

TABLE 1. COMPARISON OF PHYSICO-CHEMICAL CONSTANTS OF GLUCOSYL TRANSFERRING ENZYMES OF *Oscillatoria Princeps.**

Property	*n*-strain P	*n*-strain Q	*LTV*-strain P	*LTV*-strain Q
pH Optimum	6·9	7·0	6·9	7·2
pI (isoelect.)	5·9	5·1	6·0	5·1
Mobility†	−3·4	−3·0	−3·6	−3·0

* All data J. FREDRICK, *Physiol. Plant.* **12**, 511 (1959).
† As 10^{-5}cm²/V sec.

Five proteins having polysaccharide-synthesizing action were separated in potato extract by means of paper electrophoresis.[15] The polysaccharides synthesized by two of these proteins showed evidence of being branched polymers rather than the linear polymers associated exclusively with phosphorylase activity. Undoubtedly, the branching was caused by Q enzyme action. It is also worth considering that perhaps proteins similar to fraction a_3 of *LTV* strain *Oscillatoria* are involved.

The situation in so far as polyglucoside synthesis is concerned is complicated by the presence of two enzymes with distinctive actions but with physical, chemical, and possibly biological, similarities (cf. Table 1). Indeed, this intimate association is obvious by the invariable coprecipitation by ammonium sulphate of phosphorylase and Q enzyme, even in relatively "pure" phosphorylase preparations.

Of greater importance is the fact that fraction a_3 of *LTV Oscillatoria* appears to be a single protein having both Q enzyme and phosphorylase activities. All attempts to separate the distinctive activities of this fraction have been unsuccessful. If both activities do reside in the same enzyme molecule, some very interesting points are raised for possible evolutionary paths of associated enzymes comprising so-called enzyme "systems".

Large areas of enzyme molecules can vary to a great extent without resulting in loss of activity,[28,29] but it has been postulated that the "active centre" of enzymes possessed rather rigorous structural limitations.[30,31] Recently, however, this orthodox view has had to be modified; it has been found that even within the "active centre" some variations may be perfectly compatible with identical enzyme function.[32,33] It would seem, therefore, that this large pool of molecularly different proteins with identical catalytic functions may be convenient for the evolution of enzymes normally associated within an enzyme system.

The presence of three types of phosphorylases in *LTV* strains of *Oscillatoria* raises the possibility that in this alga we have evidence for the evolution of associated enzymes (Q enzyme). For example, it is interesting to ponder whether Q enzyme, because of its close association with phosphorylase, could have evolved from an intermediate phosphorylase molecule (possibly like fraction a_3 of *LTV* strains) to serve the particular function of branching a linear glucan in order to conserve storage space in the plant cell.

The dependency of animal phosphorylases on coenzymes (such as AMP), and the expendability of AMP in the case of most plant phosphorylases, may possibly be traced via fractions a_1 and a_2 of these *Oscillatoria* preparations. Whereas a_1 is AMP-dependent, the a_2 fraction is able to function without this coenzyme.

Of interest is the fact that in the green alga, *Spirogyra* (cf. Fig. 1E) only fraction a_3 is present, while a_4 (the Q enzyme fraction) is completely absent. This might very well account for the amylose-like polysaccharides synthesized by this alga as compared with the sugars formed by *Oscillatoria*.

EXPERIMENTAL

Extracts were prepared by maceration of washed strands of *Oscillatoria* in Tris buffer[34] at pH 7·5. The extracts were fractionated with ammonium sulphate and the phosphorylase fractions obtained as described previously.[35] The purified fractions were separated by chromatography on a centrifugally-accelerated paper disk,[19] and refractionated with ammonium sulphate until a chromatographically-homogeneous preparation was obtained. This fraction was dissolved in Tris-hydrochloric acid buffer (0·01 M Tris) at pH 7·6 to give a protein concentration of 1·4 mg per ml.

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A 0·20 ml sample of the preparation was then subjected to disc electrophoresis[22,23] in a freshly prepared polyacrylamide gel column contained in a glass tube (5 mm × 70 mm) on a Canalco Disc Electrophoresis Model P/S 150 unit. The entire apparatus was refrigerated at 5° and the current was kept at 2·8 mA for exactly 2 hr. Bromophenol blue was added to the buffer (Tris, pH 8·2, ionic strength 0·01), and the run was considered complete when the dye had concentrated at the "front" and moved into the lower gel to a depth of 2 cm. Identical columns containing the same concentrations of enzyme proteins were run at the same time under the same conditions.

After removal from the glass tubes, the gels were either fixed and stained with saturated naphthalene black 12 B 200 in methanol and 10 per cent acetic acid for 1 hr, or placed in a buffered substrate containing 0·03 M dipotassium D-glucosyl phosphate, 0·01 M Tris buffer pH 7·1 and 1·0 per cent maltoheptaose (prepared after Whelan[24]), and incubated at 30° for 2 hr. At the end of this time the gels were removed from the substrate, washed in cold water and stained in an aqueous solution of 0·2 per cent iodine in 1 per cent potassium iodide. The gels were then placed into small micro test-tubes for storage.

Some of the gel columns were subjected to electrophoretic elution after the column had been resolved under the conditions of electrophoresis described. The fractions were eluted into small dialysis bags tied to the lower end of the glass column and immersed in the lower buffer reservoir of the apparatus. Under these conditions the fractions were obtained electrophoretically pure in 1/2–2 hr with a current of 2·5 mA.

The eluted fractions were tested for phosphorylase activity using the buffered D-glucosyl phosphate described above. Each fraction was also tested for phosphorylase activity in the presence of 0·001 M adenosine-5-phosphate. Each fraction was also tested for Q enzyme and amylase activity.[17]

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THE OXIDATION OF SOME AROMATIC α-HYDROXY ACIDS BY GLYCOLLATE : O_2 OXIDOREDUCTASE*

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Abstract—Glycollate : O_2 oxidoreductase (glycollate oxidase) present in extracts from salvia, wheat, angelica, rape and buckwheat plants oxidizes phenyllactate and *p*-hydroxyphenyllactate to the corresponding keto acids. A convenient method employing Sephadex and hydroxyapatite was developed for purifying the enzyme from wheat. The purified enzyme had an optimum pH of 7·0–7·8 and required flavin mononucleotide for maximum activity although it could be partially replaced by flavin adenine dinucleotide. The enzyme from wheat was specific for the L-isomer of phenyllactic acid and also oxidized indolelactic acid to indolepyruvic acid.

INTRODUCTION

TRACER experiments with plants have shown that β-phenyllactic acid is readily converted to phenylalanine,[1] phenolic acids,[2] quercetin,[3] and lignin.[4] Similarly, *p*-hydroxyphenyllactate and indolelactate are good precursors of tyrosine and tryptophan, respectively.[1,5] The aromatic α-hydroxy acids are presumably oxidized to the corresponding α-keto acids, but the enzymic basis for this reaction has not been established.

Plants contain an α-hydroxy acid oxidase known as glycollate oxidase.[6] This enzyme has been isolated as a crystalline protein from spinach plants by Frigerio and Harbury.[7] It contains FMN which is required for its activity.[6,7] The enzyme catalyses the oxidation of glycolic acid, L-lactic acid, α-hydroxybutyric acid and glyoxylic acid.[6,8]

The results presented in this report demonstrate that glycollate oxidase also catalyses the oxidation of phenyllactate, *p*-hydroxyphenyllactate and indolelactate to the corresponding α-keto acids.

RESULTS

Identification of Products

The oxidation of phenyllactate and *p*-hydroxyphenyllactate was first observed by using crude plant extracts. The corresponding substituted pyruvic acids and their 2,4-dinitrophenylhydrazones were isolated from the reaction mixtures and identified by paper chromatography, as shown in Table 1, by u.v. absorption[9,10] and by the boron test.[11]

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TABLE 1. THE *Rf* VALUES OF KNOWN α-KETO ACIDS AND THEIR 2,4-DINITROPHENYLHYDRAZONES (2,4-DNPH) COMPARED WITH *Rf* VALUES OF OXIDATION PRODUCTS FROM VARIOUS α-HYDROXY ACIDS OXIDIZED BY GLYCOLLATE OXIDASE

	Rf values					
	Free acids		2,4-DNPH		Spray on free acids	
	BFW[1]	BAW[2]	MBBW[3]	BA[4]	DNA[5]	$FeCl_3$
Authentic keto acids:						
Phenylpyruvic acid	0·86	0·63	0·85	—	brown-yellow	blue
p-Hydroxyphenylpyruvic acid	0·77	0·42	—	0·63	purple-brown	blue
Indolepyruvic acid	—	—	0·84	0·78	—	—
Reaction product from:						
L(−)Phenyllactic acid	0·86	0·63	0·85	—	brown-yellow	blue
DL-*p*-Hydroxyphenyllactic acid	0·77	0·42	—	0·63	purple-brown	blue
DL-Indolelactic acid	—	—	0·84	0·78	—	—

Solvents

[1] Butanol : formic acid : water 50 : 2·5 : 10 (ascending).
[2] Butanol : acetic acid : water 4 : 1 : 1·8 (ascending) on Whatmann No. 1 paper treated with borate.[12]
[3] Methanol : butanol : benzene : water 4 : 2 : 2 : 2 (descending).
[4] Butanol saturated with 3% NH_4OH (descending).

Sprays

[5] Diazotized *p*-nitroaniline (DNA).

The products from indolelactate oxidation were isolated and identified spectrophotometrically as shown in Fig. 1. The products were obtained by incubating 2 μmoles FMN, 145 μmoles DL-indolelactate, 200 μg catalase, 400 μmoles potassium phosphate buffer pH 8·0 and 60 mg enzyme protein from wheat purified on a Sephadex G-25 column, in a total volume of 5·0 ml. Incubation time was 110 min at 26°. The ether extractions were

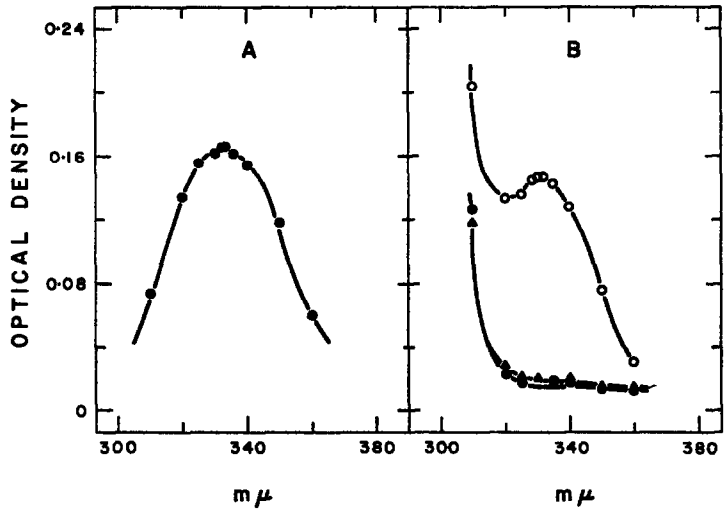


FIG. 1. IDENTIFICATION OF THE PRODUCT OF INDOLELACTATE OXIDATION.

A. Keto-enol difference spectra with and without borate. The reference cuvette contained 0·84 mg ether-extractable material in 0·2 ml ethanol and in 2·3 ml 1 M arsenate; the sample cuvette contained the same amount in 2·3 ml 1 M arsenate+1 M boric acid.

B. Absorption in 1 N NaOH. The cuvettes contained 0·42 mg DL-indolelactate in 0·1 ml ethanol +2·4 ml 1 M NaOH ●-●-●; 0·42 mg ether-extractable material in 0·1 ml ethanol+2·4 ml 1 N NaOH. No enzyme added, ▲-▲-▲; enzyme added, O-O-O. The reference cuvette contained 1 M NaOH.

[12] T. SWAIN, *Biochem. J.* 53, 200 (1953)

carried out immediately at 0–4°, and the ether evaporated *in vacuo*. Residual ether and moisture were removed by drying in a vacuum desiccator. For the spectrophotometric analyses 4·2 mg of indolelactate and acid–ether-extractable material from reaction mixtures with and without enzyme were dissolved in 1 ml ethanol at 0°C. The boron test, Fig. 1A, and the analysis in alkali, Fig. 1B, show an absorption peak at 332 mμ and 330 mμ, respectively. These values are in good agreement with the values reported by Lin *et al.*[11] and by Schwarz[10] for the enol tautomer of indolepyruvic acid.

Sources of Glycollate Oxidase

Glycollate oxidase from wheat, rape, buckwheat and salvia oxidized a number of substrates as shown in Table 2. Wheat and rape appeared to be better sources of the enzyme than salvia; however, the values should not be considered a quantitative comparison because the enzyme is very labile and loss of activity may vary somewhat depending on the plant source. The keto acids also were identified from reaction mixtures containing *p*-hydroxyphenyllactate or phenyllactate and the preparations from rape, salvia and angelica plants.

TABLE 2. OXIDATION OF α-HYDROXY ACIDS BY GLYCOLLATE OXIDASE FROM VARIOUS SOURCES

Source of enzyme	Units/mg protein‡		
	PL	HPL	GA
Wheat var. Selkirk* (7 wks.)	0·46	0·46	3·4
Wheat var. Thatcher* (3 wks.)	0·48	—	3·6
Rape* (Argentine) mature plants	0·45	—	3·5
Buckwheat† (4 wks.)	0·30	—	—
Salvia* var. Globe of Fire (6 wks.)	0·05	—	—

Note: * The preparation was used after gel filtration on Sephadex G-25.

† The fraction was obtained after repeated fractionations with ammonium sulphate (0–40%).

Abbreviations: PL = Phenyllactate; HPL = *p*-Hydroxyphenyllactate; GA = Glycollate.

‡ The activity with GA was determined manometrically in the Warburg apparatus. The vessel contained 0·4 μmoles FMN, 50 μg catalase, 20 μmoles glycollate, enzyme and 280 μmoles K-phosphate buffer pH 8·0 in a total volume of 2·0 ml. The centre well contained 0·1 ml 5 N NaOH. The reaction was carried out at 25°C and in pure O_2. The other substrates were tested spectrophotometrically as outlined under Assays and Analyses.

Identification of Glycollate Oxidase

Table 3 shows the specific activity with three substrates. The data were obtained by assaying various fractions from the hydroxyapatite column. It also gives the ratio of specific activities and the extent of purification. The data indicates a striking uniformity in the ratio of specific activities of two substrates (columns 4 and 5). It suggests that one enzyme catalyses the oxidation of all three substrates.

Properties of Glycollate Oxidase from Wheat

The data presented in Fig. 2 show the formation of phenylpyruvate from phenyllactate as a function of time (A) and of enzyme concentration (B). The effect of pH on the oxidation

TABLE 3. OXIDATION OF GLYCOLLATE (GA), L(−)PHENYLLACTATE (PL), AND DL-*p*-HYDROXYPHENYLLACTATE (HPL) BY GLYCOLLATE OXIDASE BEFORE AND AFTER PURIFICATION WITH HYDROXYAPATITE

Enzyme source	Relative specific activity			Ratio of specific activities		Relative specific activity		
	GA	PL	HPL	GA/PL	PL/HPL	GA	PL	HPL
After gel filtration	3·4	0·56	0·47	6·1	1·19	=1	=1	=1
Eluate from hydroxyapatite column:								
Tube 4		1·6	1·37		1·19		2·8	2·9
5	63·0	10·6	8·4	6·0	1·25	18·5	18·8	17·9
6	10·6	1·9	1·85	5·7	1·03	3·2	3·4	3·9
7		1·12	0·77		1·46		2·0	1·7

Note: For procedure see Table 2 and Assays and Analyses.

of phenyllactate is illustrated in Fig. 3. It appears that the enzyme has an optimum pH between 7·0 and 7·8. This approximates to values obtained in studies with glycollate oxidase from other sources which oxidized lactate and glycollate.[6,13]

The effect of varying the concentration of FMN on the oxidation of phenyllactate is shown in the double reciprocal plot in Fig. 4. A Michaelis constant of $5{\cdot}6\times10^{-5}$ M was calculated from the graph. There was no measurable formation of phenylpyruvate in the absence of FMN. In another experiment it was shown that FAD could replace FMN to the extent of 25 per cent in the oxidation of phenyllactate. Similar observations have been made by Zelitch and Ochoa in a study on the oxidation of glycolic acid.[6]

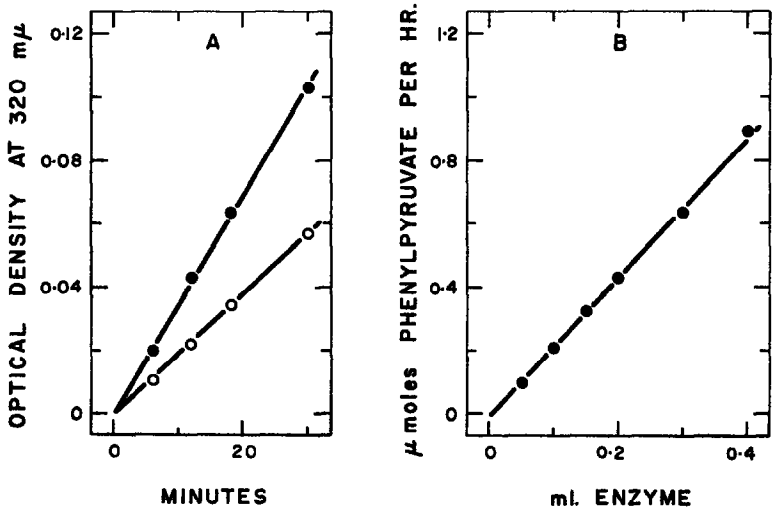


FIG. 2. KINETICS OF THE CONVERSION OF L(−)PHENYLLACTIC ACID TO PHENYLPYRUVIC ACID BY GLYCOLLATE OXIDASE FROM WHEAT.

Conditions were as described under Assays and Analyses.

A. Formation of phenylpyruvate as a function of time. O–O–O 0·1 ml enzyme, ●–●–● 0·2 ml enzyme.

B. Formation of phenylpyruvate as a function of enzyme concentration.

[13] C. O. CLAGETT, N. E. TOLBERT and R. H. BURRIS, *J. Biol. Chem.* **178**, 977 (1949).

Figure 5 shows the effect of varying the concentration of phenyllactate on phenylpyruvate formation. A Michaelis constant of $6{\cdot}7 \times 10^{-3}$ M was calculated from the graph. The values which have been obtained with other substrates are: glycollate $3{\cdot}8 \times 10^{-4}$ M[6], L-lactate 2×10^{-3} M[6] and glyoxylate $5{\cdot}4 \times 10^{-3}$ M.[8] The enzyme is specific for the L-isomer of phenyllactic acid. (+)— Phenyllactic acid was entirely inactive. This was to be expected since D-lactate has been shown to be inactive.[6] Blanchard *et al.*[14] have reported an L-amino

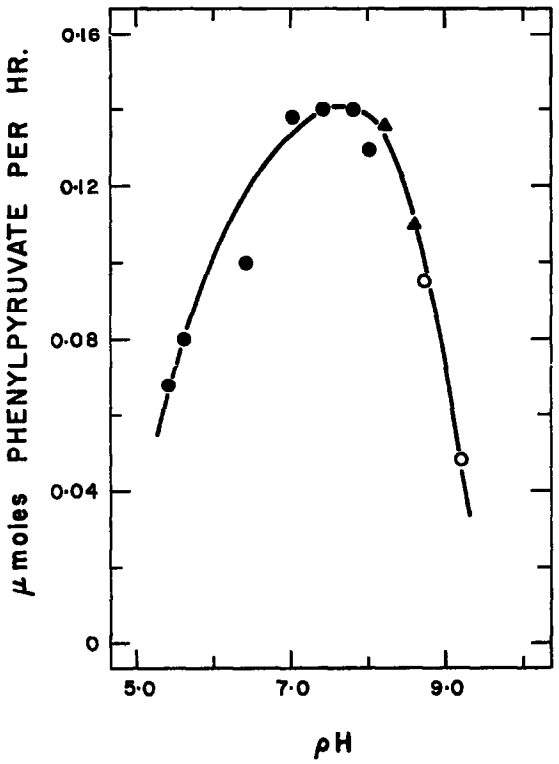


FIG. 3. THE EFFECT OF pH ON THE OXIDATION OF L(−)PHENYLLACTIC ACID BY GLYCOLLATE OXIDASE. The reaction mixture consisted of 0·4 μmoles FMN, 20 μmoles phenyllactate, 0·07 mg protein (Sp. Act. 3·2), and buffer to a volume of 2·5 ml. The buffers were 0·2 M K-phosphate (●–●–●), 0·1 M Tris-HCl (▲–▲–▲) and 0·2 M glycine-NaOH (O–O–O).

acid oxidase from rat kidneys which oxidized a number of α-hydroxy acids including phenyllactic acid. Thus the possibility existed that an L-amino acid oxidase might be involved in these experiments, but the addition of phenylalanine to an active preparation from wheat failed to yield measurable amounts of phenylpyruvic acid. Ochoa and Zelitch[6] have tested other amino acids with negative results.

Kolesnikov *et al.*[15] observed an inhibition of glycollate oxidase by a catechol oxidase (*o*-diphenol : O_2 oxidoreductase) from barley. In the present experiments highly purified catechol oxidase from mushroom* had no inhibitory effect on the oxidation of phenyllactic acid by glycollate oxidase from wheat. However, the enzyme was inhibited 50 per cent in

* Prepared by Mr. M. D. Chisholm of this laboratory and contained 43 units of catecholase and 264 units of tyrosinase per mg protein.

[14] M. BLANCHARD, D. E. GREEN, V. NOCITO-CARROLL and S. RATTNER, *J. Biol. Chem.* **163**, 137 (1946).
[15] P. A. KOLESNIKOV, E. I. PETROCHENKO and S. V. ZORE, *Fiziol. Rastenii, Acad. Nauk U.S.S.R.* **6**, 598 (1959). *Chem. Abst.* **54**, 14375 (1960).

the presence of $2{\cdot}4 \times 10^{-3}$ M benzaldehyde. A similar observation was made by Kun *et al.*[16] with the glycollate oxidase from rat liver.

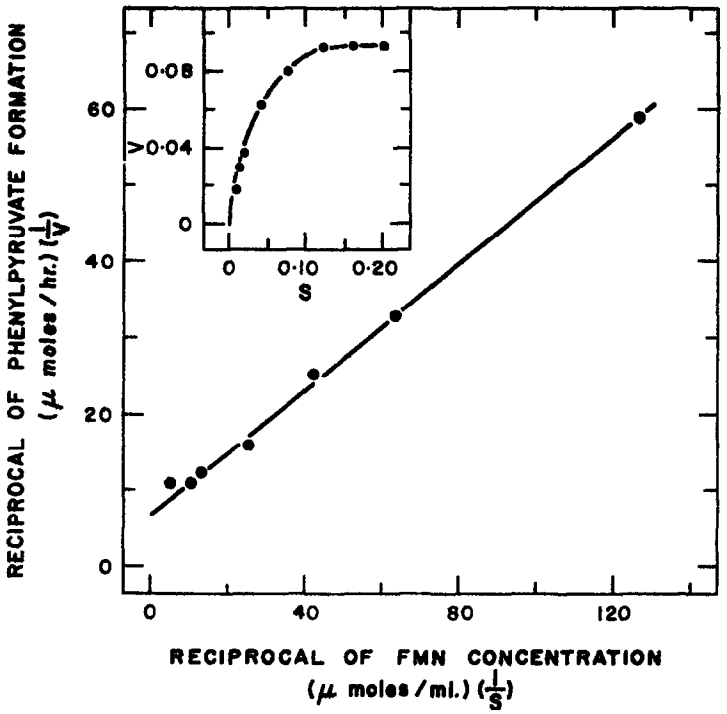
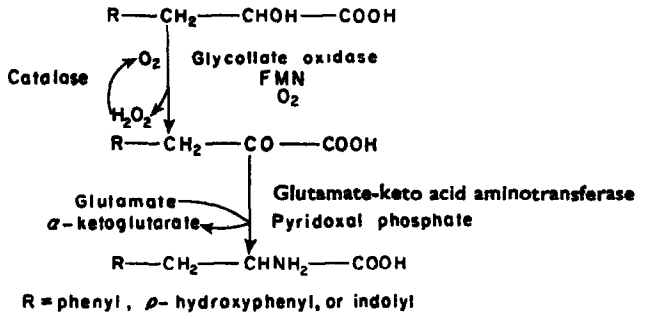


FIG. 4. THE EFFECT OF CHANGES IN THE CONCENTRATION OF FLAVIN MONONUCLEOTIDE ON THE OXIDATION OF L(−)PHENYLLACTATE.

Conditions were as described under Assays and Analyses except 30 μmoles of phenyllactate and 0·1 mg protein (Sp. Act. 1·0) were used.

DISCUSSION

The results obtained in this study demonstrate that glycollate oxidase catalyses the oxidation of phenyllactate, *p*-hydroxyphenyllactate and indolelactate to the corresponding α-keto acids. Thus the conversion of aromatic lactate derivatives to alanine derivatives probably involves the participation of glycollate oxidase followed by a transaminase reaction.[17,18] The interconversion of these compounds is illustrated below:



[16] E. KUN, J. M. DECHARY and H. C. PITOT, *J. Biol. Chem.* **210**, 269 (1954).
[17] V. L. KRETOVICH and Z. V. USPENSKAIA, *Biokhimiya* **23**, 232 (1958). *Eng. trans. Consultants Bureau Inc.* (1958)
[18] V. L. KRETOVICH and O. L. POLYANOVSKII, *Biokhimiya* **24**, 917 (1959). *Eng. trans. Consultants Bureau Inc.* (1959).

This synthesis of α-amino acids from the corresponding α-hydroxy acids aids in the interpretation of results obtained by feeding C^{14}-labelled α-hydroxy acids to plants.[19] There is now no reason to believe that these α-hydroxy acids are natural intermediates in aromatic metabolism. The non-nitrogenous aromatic compounds formed when the hydroxy acids are fed probably arise by known enzyme reactions via the α-keto and α-amino acids.[20,21]

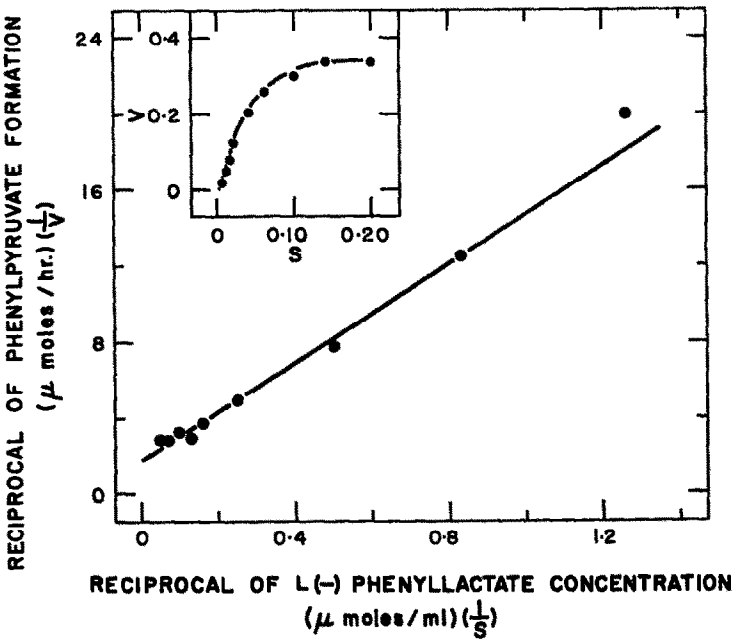


FIG. 5. RECIPROCAL PLOT OF THE RATE OF OXIDATION OF L(−)PHENYLLACTIC ACID (V) AND THE CONCENTRATION OF L(−)PHENYLLACTIC ACID (S).
The reaction mixture contained 0·4 μmole FMN, substrate, enzyme purified on hydroxyapatite, 0·35 mg protein (Sp. Act. 1·0), and 300 μmoles phosphate buffer pH 8·0.

The versatility of glycollate oxidase is somewhat comparable to that of beef heart D-lactate dehydrogenase which converts a great variety of α-keto acids to the α-hydroxy acids.[22] It resembles the L-amino acid : O_2 oxidoreductase from rat kidney in that both enzymes have FMN as prosthetic group and have some substrates in common. However, glycollate oxidase is unable to oxidize malate and glycerate[6,7] and it does not appear to function as an amino acid oxidase.[6]

EXPERIMENTAL

Materials

Glycolic acid, DL-indolelactate, phenylpyruvate and *p*-hydroxyphenylpyruvate were obtained from commercial sources. L(−)Phenyllactate was prepared from L-phenylalanine by treatment with nitrous acid.[4,23] FMN and FAD were obtained from Sigma Chemical

[19] A. C. NEISH, *Ann. Rev. Plant. Physiol.* **11**, 55 (1960).
[20] A. C. NEISH, *Phytochemistry* **1**, 1 (1961).
[21] J. KOUKOL and E. E. CONN, *J. Biol. Chem.* **236**, 2692 (1961).
[22] A. MEISTER, *J. Biol. Chem.* **184**, 117 (1950).
[23] A. C. NEISH, *Can. J. Biochem. and Physiol.* **39**, 1205 (1961).

Company and catalase was purchased from General Biochemicals Incorporated. The 2,4-dinitrophenylhydrazones were prepared by treating the keto compounds with a slight excess of 0·5 per cent 2,4-dinitrophenylhydrazine.[24] Sephadex G-25 (medium) was obtained from Pharmacia, Uppsala, Sweden, and hydroxyapatite prepared according to the method of Anacker and Stoy.[25] Other compounds used in these experiments were prepared as indicated in other reports from this laboratory.[1,4]

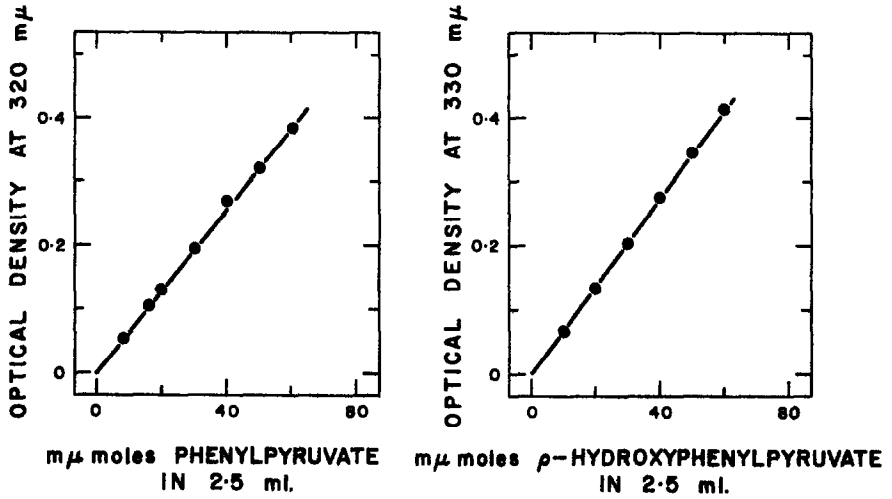


FIG. 6. GRAPHS SHOWING ABSORPTION IN 1 M NaOH WITH INCREASING CONCENTRATIONS OF (*a*) PHENYLPYRUVATE AND (*b*) *p*-HYDROXYPHENYLPYRUVATE.

Assays and Analyses

Oxidation of phenyllactate and *p*-hydroxyphenyllactate was measured spectrophotometrically by following the increase in absorption at 320 mμ and 330 mμ, respectively. The increase in absorption is due to the enol-tautomer of the α-keto acids which are known to exhibit absorption peaks in alkali at these wave lengths.[26,27] The molar extinction coefficient for phenylpyruvate in 1 M NaOH at 320 mμ was 16,000 and the value for *p*-hydroxyphenylpyruvate in 1 M NaOH at 330 mμ was 17,200. Standard curves are shown in Fig. 6.

The enzyme was assayed as follows: a reaction mixture consisting of 0·4 μmole FMN, 20 μmoles L(−)phenyllactate or 40 μmoles DL-*p*-hydroxyphenyllactate, enzyme, and 360 μmoles potassium phosphate buffer at pH 8·0 in a total volume of 2·5 ml was incubated in a 20 ml beaker at 26°C. Samples of 0·2 or 0·5 ml were then withdrawn at 0 time (addition of substrate) and at 5 min intervals, added to a test tube containing 2·3 or 2·0 ml 1 M NaOH and read in a Beckman DU spectrophotometer. Phenylpyruvate appeared to be stable in alkali for about 1 hr. Since *p*-hydroxyphenylpyruvate decomposes in alkali in air,[28] the samples were added to 0·1 ml 5 N acetic acid. The readings were then taken immediately after adding the appropriate volume of 1 M NaOH. Standard curves are shown in Fig. 2. One unit of enzyme is defined as the amount required to catalyse the formation of one μmole

[24] P. K. STUMPF and D. E. GREEN, *J. Biol. Chem.* **153**, 387 (1944).
[25] W. F. ANACKER and V. STOY, *Biochem. Z.* **330**, 144 (1958).
[26] I. SCHWINCK and E. ADAMS, *Biochim. et Biophys. Acta* **36**, 102 (1959).
[27] U. WEISS, C. GILVARG, E. S. MINGIOLI and B. D. DAVIS, *Science* **119**, 774 (1954).
[28] C. H. DOY, *Nature* **186**, 529 (1960).

of α-keto acid per hour. Oxidation of glycollate was measured manometrically by the standard Warburg method.

The free α-keto acids were obtained by ether extraction of the acidified reaction mixtures, and the 2,4-dinitrophenylhydrazones were prepared by adding a slight excess of 2,4-dinitrophenylhydrazine to the acidified reaction mixture followed by isolation of the acid hydrazones as described by Towers and Mortimer.[29]

Protein was measured by the method of Lowry *et al.*[30] or spectrophotometrically according to Warburg and Christian.[31]

Extraction and Purification of Glycollate Oxidase

The enzymes were obtained from plants grown in the greenhouse or in growth rooms in subirrigated gravel culture as previously described.[1,4] The following plants were used as sources of glycollate oxidase: shoots of wheat (*Triticum vulgare* Vill. var. Thatcher and var. Selkirk), shoots of buckwheat (*Fagopyrum tataricum* (L) Gaertn.), young leaves of mature plants of rape (*Brassica napus* L.), shoots of salvia (*Salvia splendens* Sello var. Globe of Fire) and leaves of angelica (*Angelica archangelica* L.).

The fresh plant material was ground in 2–3 volumes of cold water in a mortar or in a Waring blendor. During grinding the pH was adjusted to about 7·0 with 0·2 M K_2HPO_4. After grinding the mixture was filtered through several layers of cheese cloth and centrifuged at 30,000 × *g* for 20 min in a Servall Superspeed Angle Centrifuge. (Centrifugations and other procedures were carried out at 0–4°C.) The clear supernatant was then added to a column of Sephadex G-25. In a typical experiment 75 ml of the supernatant was put on a 2 × 18 cm column previously equilibrated with 0·02 M potassium phosphate buffer pH 8·0. Elution was carried out with the same buffer at a rate of about 0·6 ml per min. The protein solution was then lyophilized and 500 mg of a pale green powder was obtained. The powder contained approximately 70 per cent protein. When kept in a desiccator in the cold, the lyophilized powder was stable for several months.

Further purification was effected on a column of hydroxyapatite. For this process 70 mg of lyophilized powder was dissolved in 4 ml cold water, centrifuged at 30,000 × *g* for 20 min and the supernatant added to the 1 × 10 cm column of hydroxyapatite. The column

TABLE 4. PURIFICATION OF GLYCOLLATE OXIDASE FROM WHEAT ON COLUMNS OF SEPHADEX G-25 AND HYDROXYAPATITE

Fraction	Volume (ml)	Total activity (units)	Protein (mg/ml)	Specific* activity (units/mg)	Recovery	Purification
Crude	75	120	7·4	0·22	100	1·0
Sephadex G-25	80	127	4·3	0·37	106	1·68
Hydroxyapatite:						
Expt. I Initial	4·0	16·9	9·4	0·45	100	1·68
Tube a	2·0	10·2	0·71	7·2	60	26·9
„ b	2·0	4·8	2·4	1·0	28	3·7
Expt. II Initial	4·5	26·9	12·00	0·56	100	1·68
Tube a	2·0	13·1	0·62	10·6	50	31·6
„ b	2·0	4·2	1·12	1·9	16	5·5

Note: For assay procedure refer to Assays and Analyses.
* The substrate was phenyllactate.

[29] G. H. N. TOWERS and D. C. MORTIMER, *Can. J. Biochem. and Physiol.* **34**, 511 (1956).
[30] O. H. LOWRY, N. L. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).
[31] O. WARBURG and W. CHRISTIAN, *Biochem. Z.* **310**, 384 (1941).

was prepared by dispersing 2·5 gm of hydroxyapatite powder in cold 0·02 M potassium phosphate buffer pH 6·5, and pouring the slurry into a glass tube. After washing the column with the same buffer, the protein solution was added. To the column was then added 15 ml of 0·02 M potassium phosphate buffer pH 6·5, followed by 0·08 M potassium phosphate buffer pH 8·0 which eluted the enzyme. Purification up to 18-fold was obtained by this method. The enzyme was used shortly after elution from the column.

The results from two experiments are presented in Table 4. The data show that 60–80 per cent of the total enzyme can be recovered from the hydroxyapatite column. The procedure of gel filtration, instead of dialysis,[32] and adsorption and elution on hydroxyapatite is particularly useful for this enzyme because these steps can be carried out in a relatively short time. This is important because of the instability of the enzyme.[7]

Acknowledgements—The authors are indebted to Mr. J. Kirkpatrick for valuable technical assistance and to Dr. C. S. McArthur, Department of Biochemistry, University of Saskatchewan, for permission to publish these results from a thesis.[33]

[32] P. FLODIN, *J. Chromat.* **5**, 103 (1961).
[33] O. L. GAMBORG, Ph.D. Thesis, Department of Biochemistry, University of Saskatchewan, 1962.

Phytochemistry, 1962, Vol. 1, pp. 169 to 174. Pergamon Press Ltd. Printed in England

HYDROXYCINNAMIC ACIDS AND THEIR GLUCOSE ESTERS IN HYBRIDS OF *LILIUM* SPECIES AND THEIR RELATION TO GERMINATION

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(*Received* 16 *March* 1962)

Abstract—Ferulic, sinapic, and *p*-coumaric acids as well as their glucose esters were isolated from seeds and pericarps of the inter-specific hybrid *Lilium speciosum* Thumb. "Album" by *Lilium auratum* Lindl. The abnormal physiology of these seeds was ascribed to the relatively high concentration of free ferulic acid in these tissues and a possible limiting of the mechanism for detoxification by esterification.

INTRODUCTION

TUMOURS induced on embryos excised from seeds of the inter-specific hybrid *Lilium speciosum* Thumb. "Album" by *Lilium auratum* Lindl. were associated with the exogenous supply of ferulic acid available to these tissues.[1] Since many phenolic compounds are phytotoxic and their toxic effects can be greatly reduced by glycosylation of one or more hydroxyl groups,[2] a study was initiated to isolate and identify the endogenous hydroxycinnamic acids in the seeds and in the pericarps of this interspecific hybrid and to determine whether the hydroxycinnamic acids were present in these tissues as free acids or as sugar derivatives.

RESULTS AND DISCUSSION

Three hydroxycinnamic acids were isolated from seeds and pericarps of the inter-specific hybrid *Lilium speciosum* "Album" by *Lilium auratum*. Their properties are shown in Table 1. Bands 1, 2, and 3 correspond to *p*-coumaric, sinapic, and ferulic acids, respectively.

Also isolated from these tissues were three sugar derivatives of hydroxycinnamic acids. Their properties are shown in Table 2. Bands 1, 2, and 3 correspond to the glucose esters of sinapic, ferulic, and *p*-coumaric acids, respectively. All three sugar derivatives yielded only glucose on acid hydrolysis (Table 3) and the relevant hydroxycinnamic acid (Table 1).

Since tumours were induced on embryos excised from seeds of the inter-specific hybrid *Lilium speciosum* "Album" by *Lilium auratum* with exogenous supplies of ferulic acid, quantitative measurements of this compound were made. The seeds of the inter-specific hybrid contained 11 μg of free ferulic acid/g wet weight. The seeds from *Lilium speciosum* "Album" by *Lilium speciosum* "Rubrum", an intra-specific hybrid whose seeds germinated readily and whose embryos showed no signs of tumour formation, contained only 1 μg free ferulic acid/g wet weight. When the glucose ester of ferulic acid was expressed as the free acid, the inter-specific hybrid seeds contained 8 μg/g wet weight as compared to 18 μg/g wet weight in the intra-specific hybrid seeds. In pericarps of both these hybrids ferulic acid was present primarily as the glucose ester with only a trace of the free acid.

[1] S. L. EMSWELLER, S. ASEN and J. UHRING, *Science* **136** 266 (1962).
[2] J. B. PRIDHAM, *Phenolics in plants in health and disease*, p. 13. Pergamon Press, New York (1960).

TABLE 1. *Rf* VALUES, WAVE LENGTHS OF MAXIMUM ABSORPTION, AND COLOUR OF HYDROXYCINNAMIC ACIDS IN SEEDS AND PERICARPS OF THE INTER-SPECIFIC HYBRID *Lilium speciosum* "ALBUM" BY *Lilium auratum*

Compounds	*Rf* value in			λ max. mμ in		Fluorescence in		Colour with diazotized *p*-nitroaniline	
	*Toluene–acetic acid–water (4 : 1 : 5 by vol., upper phase)	Phenol–water (73 : 27 by wt.)	1-butanol–2*N* ammonia (1 : 1 by vol., upper phase)	95% ethanol	0·002 M sodium ethoxide	Ultraviolet light	Ultraviolet light + NH_3 vapour	+ sodium acetate	+ Na_2CO_3
Authentic hydroxy-cinnamic acids:									
p-Coumaric	0·08	0·70	0·14	311 227	335 310	None	Blue	Brown	Purple
Sinapic	0·24	0·89	0·09	324 238	352 245	Blue	Blue-green	Red-brown	Gray-brown
Ferulic	0·40	0·80	0·13	324 296 233	347 305 240	Blue	Blue	Red-brown	Gray-blue
Unknowns:									
Band 1	0·08	0·69	0·14	311 227	335 310	None	Blue	Brown	Purple
Band 2	0·24	0·88	0·08	324 238	352 245	Blue	Blue-green	Red-brown	Gray-brown
Band 3	0·40	0·80	0·13	324 296 233	347 305 240	Blue	Blue	Red-brown	Gray-blue

* Papers were equilibrated for 24 hr in an atmosphere saturated with the aqueous phase of the solvent.

TABLE 2. *Rf* VALUES, WAVE LENGTHS OF MAXIMUM ABSORPTION, AND COLOUR OF THE SUGAR DERIVATIVES OF HYDROXYCINNAMIC ACIDS IN SEEDS AND PERICARPS OF *Lilium speciosum* "ALBUM" BY *Lilium auratum*

Compounds	*Rf* value in				Fluorescence in		λ max. mμ in	
	1-butanol–acetic acid–water (4 : 1 : 5 by vol., upper phase)	1-butanol–2*N*-ammonia (1 : 1 by vol., upper phase)	1-butanol–ethanol–water (20 : 5 : 11 by vol.)	Water	Ultraviolet light	Ultraviolet light + NH_3 vapour	95% ethanol	0·002 *M* sodium ethoxide
Glucose esters of*								
Sinapic acid	0·47	0·16	0·66	0·47 ; 0·57	Blue	Green	238 330	255 397
Ferulic acid	0·57	0·19	0·68	0·64 ; 0·73	Blue	Green	240 300 330	250 382
p-Coumaric acid	0·66	0·30	0·75	0·73 ; 0·81	None	Blue	235 313	240 365
Unknowns:								
Band 1	0·48	0·15	0·65	0·50 ; 0·58	Blue	Green	238 330	255 397
Band 2	0·58	0·17	0·67	0·66 ; 0·76	Blue	Green	240 300 330	250 382
Band 3	0·65	0·28	0·72	0·71 ; 0·84	None	Blue	235 313	240 365

* *Rf* values, wave lengths of maximum absorption, and colour reported by Harborne and Corner.[5]

TABLE 3. R_g* VALUES AND COLOUR WITH ANILINE HYDROGEN PHTHALATE OF SUGARS FROM ACID HYDROLYSIS OF THE SUGAR DERIVATIVES OF HYDROXYCINNAMIC ACIDS IN SEEDS AND PERICARPS OF *Lilium speciosum* "ALBUM" BY *Lilium auratum*

Compounds	R_g value in			Colour with aniline hydrogen phthalate
	Ethyl-acetate–pyridine–water (8 : 2 : 1 by vol.)	Phenol–water (73 : 27 by wt.)	1-butanol–ethanol–water (40 : 11 : 19 by vol.)	
Authentic sugars:				
Galactose	0·84	1·08	0·92	Brown
Glucose	1·00	1·00	1·00	Brown
Arabinose	1·33	1·39	1·21	Red
Lyxose	1·61	1·33	1·43	Red
Xylose	1·51	1·25	1·31	Red
Rhamnose	2·02	1·63	1·78	Brown
Unknowns:				
Band 1	0·98	1·00	1·01	Brown
Band 2	0·99	1·00	1·00	Brown
Band 3	1·00	1·00	1·00	Brown

* R_g is relative to glucose = 1.

Apparently 60 per cent of the total ferulic acid in seeds of the inter-specific hybrid, but only 6 per cent of that in seeds of the intraspecific hybrid, was present as the free acid. Seeds of the inter-specific hybrid when planted in soil or vermiculite failed to germinate, whereas seeds of the intra-specific hybrid germinated readily and produced normal seedlings. Most of the mature seeds of the inter-specific hybrid, when cultured on nutrient agar, initiated growth. Approximately 8 per cent developed tumours and died. The others developed to various seedling stages but none survived.

Many phenolic compounds are phytotoxic and their effects can be greatly reduced by glycosylation. Pridham and Saltmarsh[3] reported that hydroquinone and arbutin enter the tissues of broad bean seeds. With arbutin germination proceeds normally but with hydroquinone the seeds rapidly blacken and die. Van Sumere[4] showed that ferulic acid-β-glucoside had no effect on the germination of wheat rust uredospores whereas free ferulic acid was strongly inhibitory.

Harborne and Corner[5] reported that the main products of feeding the leaves of a variety of plants species with hydroxycinnamic acids were glucose esters. The esters formed from *p*-coumaric, ferulic, and sinapic acids were identical with those isolated from natural sources. It is conceivable that the formation of glucose esters might also be a function for the detoxification of compounds harmful to plants. Thus the abnormal physiology of the inter-specific hybrid seeds could be attributed to the relatively high concentration of free ferulic acid and the limiting of the mechanism for detoxification by esterification in these tissues.

EXPERIMENTAL

Preparation of tissue extracts. Mature fruits (500 g) were separated into seeds and pericarps and were macerated in a blendor with 95% ethanol. The macerated tissue was extracted by refluxing for 30 min in boiling 95% ethanol. The ethanol extracts were decanted and the residue was re-extracted with an equal volume of 95% ethanol. Ethanol

[3] J. B. PRIDHAM and M. J SALTMARSH, *Biochem. J.* **74**, 42 (1960).
[4] C. F. VAN SUMERE, *Phenolics in plants in health and disease*, p. 25. Pergamon Press. New York (1960).
[5] J. B. HARBORNE and J. J. CORNER, *Biochem. J.* **81**, 242 (1961).

was evaporated from the combined extracts under reduced pressure at 50°. The remaining aqueous solution was extracted in a continuous liquid–liquid extractor, first with petroleum ether and finally with ethyl ether to remove free hydroxycinnamic acids. The ethyl ether extract was taken to dryness under reduced pressure at 50° and the residue was dissolved in a minimum volume of 90% ethanol for chromatography.

After extracting with ethyl ether the volume of the aqueous residue was reduced under reduced pressure at 50° and then dried on powdered polyamide.[6,7] The polyamide containing the aqueous residue was placed on top of a 2×30 cm polyamide column, and the column was thoroughly washed with water. The sugar derivatives of hydroxycinnamic acids were then eluted with 40% ethanol. The eluant was taken to dryness under reduced pressure at 50° and the residue was dissolved in a minimum volume of 70% ethanol for chromatography.

Hydroxycinnamic acids. Sinapic and *p*-coumaric acids were synthesized by standard procedures from malonic acid and the appropriate aldehyde.[8] Ferulic acid was obtained from a commercial source.

Isolation of hydroxycinnamic acids and their sugar derivatives

Hydroxycinnamic acids were resolved by paper chromatography. Chromatograms were prepared by streaking the extracts in a band approximately 1 cm wide across the narrow width of Whatman No. 3 MM filter paper (46×57 cm). The papers were irrigated in a chromatographic cabinet by the descending method with toluene–acetic acid–water (4 : 1 : 5 by vol., upper phase).[9] The resolved bands were located by fluorescence in ultraviolet light and were cut out and eluted with 95% ethanol.

The separated bands were concentrated under reduced pressure at 50° and were further purified by successive paper chromatography with 1-butanol-2*N*-ammonia (1 : 1 by vol., upper phase) and benzene–acetic acid–water (6 : 7 : 3 by volume, upper phase).[10]

Isolation of sugar derivatives of the hydroxycinnamic acids was similar to that described from the free acids except for different developing solvents and eluant.[5] Prepared chromatograms were first irrigated with 1-butanol–acetic acid–water (4 : 1 : 5 by vol., upper phase) and the resolved bands were eluted with 70% ethanol. Solvents for successive paper chromatography were water; 1-butanol-2*N*-ammonia (1 : 1 by vol., upper phase); 1-butanol–ethanol–water (20 : 5 : 11 by vol.); and finally again in 1-butanol–acetic acid–water (4 : 1 : 5 by vol., upper phase).

Identification of hydroxycinnamic acids and their sugar derivatives

Hydroxycinnamic acids were identified by direct comparison with authentic samples for colour reaction,[11] absorption spectra, and R_f values. Bathochromic shifts of the isolated hydroxycinnamic acids and their sugar derivatives were obtained by the addition of sodium ethoxide to a final concentration of 0·002*M*. Allowance for filter paper impurities was made by using the eluants of an appropriate filter paper blank.

The sugar derivatives of the hydroxycinnamic acids were identified by comparing their colour reaction, absorption spectra, and R_f values with values reported by Harborne and

[6] B. Carelli, A. M. Liquori, and A. Mele, *Nature* **176**, 70 (1955).
[7] L. Hoerhammer, H. Wagner and W. Leeb, *Naturwissenschaften* **44**, 513 (1957).
[8] J. R. Johnson, *Organic reactions*. Vol. I, p. 248. Wiley, New York (1942).
[9] E. C. Bate-Smith, *Sci. Proc. Roy. Dublin Soc.* **27**, 165 (1956).
[10] R. H. Ibrahim and G. H. N. Towers, *Arch. Biochem. Biophys.* **87**, 125 (1960).
[11] T. Swain, *Biochem. J.* **53**, 200 (1953).

Corner.[5] Further identification was made by hydrolyzing a 1 ml portion of each concentrated purified extract with 1 ml of 2*N* HCl at 100° under reflux for 1 hr. The aglycones were extracted with ethyl ether and the aqueous fractions were examined for sugars. The aglycones were identified by direct comparison with authentic samples. The aqueous fractions containing the sugars were deacidified with di-*n*-octylmethylamine,[12] concentrated under reduced pressure at 40°, and chromatographed with authentic samples in ethyl acetate–pyridine–water (8 : 2 : 1 by vol.), phenol–water (73 : 27 by wt.), and 1-butanol–ethanol–water (40 : 11 : 19 by vol.). The sugars were located on the air-dried chromatograms by momentarily dipping them in aniline hydrogen phthalate,[13] removing the excess by blotting, and then heating them at 100° for 5 min.

The amount of ferulic acid in the purified extracts was determined with Folin–Dennis reagent by the procedure of Swain and Hillis.[14]

[12] E. L. SMITH and J. E. PAGE, *J. Soc. Chem. Ind.* **67**, 48 (1948).
[13] S. BAAR, *Biochem J.* **58**, 175 (1954).
[14] T. SWAIN and W. E. HILLIS, *J. Sci. Food Agric.* **10**, 63 (1959).

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β-1,3-GLUCAN HYDROLASES FROM THE GRAPE VINE (*VITIS VINIFERA*) AND OTHER PLANTS

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Abstract—The activities of enzymes hydrolysing sodium carboxymethylpachyman, sodium carboxymethylcellulose and salicin have been measured in extracts from a number of higher plants. The properties of the β-1,3-glucan hydrolase system from xylem and phloem of grape vine canes have been investigated and its relationship to the seasonal removal of sieve tube callose explored.

INTRODUCTION

ENZYME systems capable of hydrolysing β-1,3-glucosidic linkages in glucans* have been reported from the digestive tract of invertebrates[1] from fungi[2-6] and from algae.[7,8] Records of the occurrence in higher plants of enzyme systems hydrolysing glucans containing β-1,3- linkages are listed in Table 1.

The early investigations on the enzymic depolymerization of lichenin have been included, although at the time, it was used as an alternative substrate to cellulose. It has since been shown to contain both β-1,4- and β-1,3-glucosidic linkages and could, depending on the linkage sequence, be a substrate for both β-1,3- and β-1,4-glucan hydrolases.[9,31,32]

To avoid possible uncertainties in definition of enzyme specificity, arising from the use of mixed linked substrates, e.g. cereal glucans, yeast glucan and laminarin, it is desirable to use a wholly β-1,3-linked glucan. As no water soluble glucan containing only β-1,3-linkages is readily available, the soluble carboxymethyl derivative of the β-1,3-glucan pachyman has been prepared and introduced as a substrate for assay of β-1,3-glucan hydrolase. It should be noted that the use of this sodium carboxymethylpachyman (SCMP) is subject to the same limitations as sodium carboxymethylcellulose (SCMC).[33-35]

The known distribution of β-1,3-glucan hydrolases in higher plants is extended by the work reported here, in which the β-1,3- and β-1,4-glucan hydrolase levels of extracts from a variety of plant species have been measured.

The physiological significance of the β-1,3-enzymes in invertebrate digestive tract and the extracellular glucan hydrolases of fungi is presumably digestive, while the intracellular

* β-1,3-glucan hydrolase is used to denote the enzyme systems which hydrolyse glucans or oligoglucosides containing β-1,3-glucosidic linkages without defining the point(s) of attack. β-1,4-glucan hydrolase is used in the same way.

[1] B. A. STONE and J. E. MORTON, *Proc. Malacological Soc. Lond.* **33**, 127 (1958).
[2] E. T. REESE and M. MANDELS, *Can. J. Microbiol.* **5**, 173 (1959).
[3] C. R. KRISHNA MURTI and B. A. STONE, *Biochem. J.* **78**, 715 (1961).
[4] M. MANDELS, G. L. MILLER and R. W. SLATER, *Arch. Biochem. Biophys.* **93**, 115 (1961).
[5] Y. SATOMURA, M. ONO and J. FUKUMOTO, *Bull. Agr. Chem. Soc. Japan* **24**, 317 (1960).
[6] R. A. AITKEN, B. P. EDDY, M. INGRAM and C. WEURMAN, *Biochem. J.* **64**, 63 (1956).
[7] W. A. M. DUNCAN, D. J. MANNERS and A. G. ROSS, *Biochem. J.* **63**, 44 (1956).
[8] J. FELLIG, *Science* **131**, 832 (1960).
[9–30] See Table 1.
[31] F. W. PARRISH, A. S. PERLIN and E. T. REESE, *Can. J. Chem.* **38**, 2094 (1960).
[32] W. L. CUNNINGHAM and D. J. MANNERS, *Biochem. J.* **80**, 42P (1961).
[33] M. HOLDEN and M. V. TRACEY, *Biochem. J.* **47**, 407 (1950).
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[35] P. K. DATTA, K. R. HANSON and D. R. WHITAKER, *Biochim. Biophys. Acta* **50**, 113 (1961).

TABLE 1. RECORDED OCCURRENCE IN HIGHER PLANTS OF ENZYMES HYDROLYSING GLUCANS CONTAINING β-1,3-GLUCOSIDIC LINKAGES

Plant	Substrates*	Reference
Soya bean seeds	laminarin	10
Almonds	laminarin	10
Papaya latex	callose	11
Spinach	lichenin	12
Spear grass seeds	lichenin	12
Beans	lichenin	12
Peas	lichenin	12
Hyacinth bulbs	lichenin	12
Hyacinth bulbs	laminarin	13
Potato	laminarin	13
Lucerne seeds	laminarin	14
Mung bean seeds	laminarin	55
Maize embryo and endosperm	lichenin	12
Maize embryo and endosperm	barley glucan	15
Wheat	barley glucan	16
Wheat	laminarin	15
Oats	laminarin	13
Oats	barley glucan	15
Rye	barley glucan	15
Bromus *spp.*	barley glucan	17
Barley	lichenin	12, 18–24
Barley	laminarin	13, 15, 25, 26, 28, 29
Barley	barley gum	27
Barley	barley glucan	15, 30

* For structure of substrates see reference 9.

enzymes of algae and fungi may be concerned with the mobilization of β-1,3-glucan storage polysaccharides, e.g. laminarin, paramylon and sclerotinia glucan[5]. The role of the enzymes in most higher plants is obscure. In germinating cereals they may assist in the dissolution of the endosperm walls by breaking down the wall glucans prior to the removal of the enclosed starch granules.[36] It is also possible that they are involved in the physiological events of phloem reactivation in certain plants.

[9] B. A. STONE, *Nature* **182**, 687 (1958).
[10] S. PEAT, G. J. THOMAS and W. J. WHELAN, *J. Chem. Soc.* 722 (1952).
[11] W. ESCHRICH, *Naturwissenschaften* **46**, 327 (1959).
[12] P. KARRER and M. STAUB, *Helv. Chim. Acta* **7**, 916 (1924).
[13] T. DILLON and P. O'COLLA, *Chem. and Ind.* p. 111 (1951).
[14] H. COLIN and P. RICARD, *Bull. Soc. Chim. Biol.* **12**, 88 (1930).
[15] I. A. PREECE and J. HOGGAN, *J. Inst. Brewing*, **62**, 486 (1956).
[16] T. DILLON and P. O'COLLA, *Nature* **166**, 67 (1950).
[17] A. M. MACLEOD and R. SANDIE, *New Phytologist* **60**, 117 (1961).
[18] H. PRINGSHEIM and K. SEIFERT, *Z. Physiol. Chem. Hoppe-Seyler's*, **128**, 284 (1923).
[19] H. PRINGSHEIM and J. LEIBOWITZ, *Z. Physiol. Chem. Hoppe-Seyler's*, **131**, 262 (1923).
[20] H. PRINGSHEIM and W. KUSENACK, *Z. Physiol. Chem. Hoppe-Seyler's*, **137**, 265 (1924).
[21] H. PRINGSHEIM and A. BEISER, *Biochem. Z.* **172**, 411 (1926).
[22] H. PRINGSHEIM and K. BAUR, *Z. Physiol. Chem. Hoppe-Seyler's* **173**, 188 (1928).
[23] G. OTTO, *Biochem. Z.* **209**, 276 (1929).
[24] B. DRAKE, *Biochem. Z.* **313**, 388 (1942).
[25] D. J. MANNERS, *Biochem. J.* **61**, xiii (1955).
[26] I. C. MACWILLIAM and G. HARRIS, *J. Inst. Brewing* **63**, 29 (1957).
[27] E. J. BASS and W. O. S. MERIDITH, *Proc. Am. Soc. Brewing Chem.* p. 38 (1960).
[28] I. A. PREECE, N. K. GARG and J. HOGGAN, *J. Inst. Brewing* **66**, 331 (1960).
[29] I. A. PREECE, N. K. GARG, *J. Inst. Brewing* **67**, 267 (1961).
[30] W. W. LUCHSINGER, D. G. COCHRANE and E. KNEEN, *Cereal Chem.* **37**, 525 (1960).
[36] A. M. MACLEOD, *Wallerstein Lab. Commun.* **23**, 87 (1960).

Esau[37] has described the deposition of callose pads on the sieve plates in the phloem of the grape vine at the onset of winter dormancy and has observed the partial removal of this callose during natural and artificial reactivation. The recent demonstration[38,39] that sieve tube callose is a β-1,3-glucan leads directly to the suggestion that specific β-1,3-glucan hydrolases may be involved in the removal of the dormancy callose.

Experiments to characterize grape vine β-1,3-glucan hydrolases and to explore their relationship to the removal of dormancy callose are described.

RESULTS AND DISCUSSION

β-Glucan- and β-glucoside hydrolase levels in higher plants

Table 2 shows the results obtained for the levels of activity towards SCMP, SCMC and salicin in plant extracts. In all cases they represent results of single experiments.

The enzyme levels recorded in the table refer only to the extract and should not be equated with the actual levels in the original plant tissues. The amount extracted under the conditions used will depend on such factors as the number of cells broken, the optimum pH and time for extraction, and the effect of other cell components which may cause partial precipitation of proteins or present surfaces for adsorption. With the exception of grape vine canes no attempt was made to define the optimum conditions for extraction of the hydrolases. Furthermore, the physiological state, season of collection, variety, etc., have not been controlled. In certain cases no activity was detected in our extracts. This should not be taken to mean that the plant tissue is deficient in this enzyme. Thus we failed to detect β-D-glucoside-glycohydrolases in potato juice whereas Baruah and Swain[40] have found the enzyme in acetone powders of potato. With these reservations the results suggest that β-1,3-glucan hydrolases are present in all the species tested and provide some indication of the type of system present. Thus the viscometric assay would be sensitive to the presence of β-1,3-glucan 3-glucanohydrolase (endo-enzyme), whilst the reductometric assay would, depending on the influence of carboxymethyl groups,[33] reflect the presence of endo- and exo-(β-1,3-glucan glucohydrolase) enzymes as well as non-specific exo-β-glucan hydrolases. This would also apply to the reductometric assay of β-1,4-glucan hydrolases using SCMC substrate.

Potato, lily spadix, cauliflower, Moreton Bay fig latex and five-day germinated wheat and particularly tobacco leaves had a very high level of extractable β-1,3-enzymes as measured viscometrically, suggesting the presence of endo-type hydrolases. Potato and cauliflower also had a high activity as measured reductometrically and no detectable β-glucohydrolase and are thus probably good sources of both endo- and exo-β-1,3-glucan hydrolases. Lily spadix which is metabolically very active showed high values for all the enzymes measured. The sharp rise in specific activity of the β-D-glucoside glucohydrolase during germination of wheat seed is similar to that shown for germinating barley.[15] Tracey's[41] demonstration of the presence of β-1,4-glucan hydrolase activity in a number of higher plants has been confirmed and extended to a number of other species.

Glycoside and β-Glucan hydrolases from grape vine canes

Extraction of enzymes. Table 3 shows the effect of pH of the extracting buffer on the level of activity towards SCMP in phloem homogenates prepared from dormant canes.

[37] K. ESAU, *Hilgardia* **18**, 217 (1948).
[38] G. KESSLER, *Ber. Schweitz. Bot. Ges.* **68**, 5 (1958).
[39] G. O. ASPINALL and G. KESSLER, *Chem. and Ind.* p. 1296 (1957).
[40] P. BARUAH and T. SWAIN, *Biochem. J.* **66**, 321 (1957).
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TABLE 2. LEVELS OF ACTIVITY OF GLUCOSIDE AND β-GLUCAN HYDROLASES IN PLANT EXTRACTS
Results are expressed in terms of units of activity per g wet tissue and also per mg protein.

Tissue	β-1,3-glucan hydrolase				β-1,4-glucan hydrolase		β-D-glucoside glucohydrolase	
	Units / mg protein / ml	Units / g tissue / ml	Units / mg protein / ml	Units / g tissue / ml	Units / mg protein / ml	Units / g tissue / ml	Units / mg protein / ml	Units / g tissue / ml
	Viscometric		Reductometric					
Potato (*Solanum tuberosum* L.), tubers	105	—	390	—	133	—	0	0
Pineapple (*Ananas comosus* (L) Merr.), ovular tissue and basal parts of bracts	3·4	5·6	205	340	200	330	0	0
Onion (*Allium cepa* L.), bulb scales	4·8	9·1	310	600	150	280	54	104
Carrot (*Daucus carota* L.), root	2·7	5·6	137	290	114	240	0	0
Parsnip (*Pastinaca sativa* L.), root	45	141	350	1080	130	410	0	0
Radish (*Raphanus sativus* L.), root	3·7	9·1	200	500	130	310	0	0
Lily (*Zantedeschia aethiopica* Spreng.), spadix	80	800	99	990	75	750	57	570
Cauliflower (*Brassica oleracea* L.), immature inflorescences	89	800	134	1200	0	0	0	0
White clover (*Trifolium repens* L.), flowers	22	57	120	310	77	200	28	72
Silver beet (*Beta vulgaris* L.), leaves	7·4	225	36	1050	13	390	3·4	104
Tobacco (*Nicotiana glutinosa* L.), leaves	1170	12,000	300	3100	31	320	13	136
Willow (*Salix babylonica* L.), leaves	2·2	32	37	540	37	550	6·5	96
Poplar (*Populus alba* L.), leaves	8·4	134	77	1220	26	420	270	4200
Grape vine (*Vitis vinifera* L.), leaves	36	150	132	550	19	79	420	176
Grape vine (*Vitis vinifera* L.), xylem	—	—	35	1250	24	860	0	0
Grape vine (*Vitis vinifera* L.), phloem	—	—	75	1250	65	1060	0	0
Clover (*Trifolium repens* L.), leaves	—	520	—	1220	—	152	—	900
Soya beans (*Glycine max* Mer.), Hexane extracted meal	0·68	160	11	2660	3·3	770	0·07	18
Almond (*Prunus amygdalus* Batsch), Hexane extracted meal	0·23	56	5·9	1420	3·8	920	14	340
Papaya (*Carica papaya* L.), dried latex	2·7	530	8·6	1700	1·6	310	0	0
Moreton bay fig (*Ficus macrophylla* Roxb.), latex	240	—	420	—	0	—	0·39	—
Wheat (*Triticum vulgare* Vill.), seed	13	270	99	2000	91	1800	43	860
Wheat, 12 hr steeped seed	9·5	160	72	1200	79	1300	39	660
Wheat, 5 day germinated seed	370	6000	2300	29,300	190	3100	94	1520

Over the pH range investigated the total activity (residue plus supernatant) did not vary markedly but maximum extraction of activity into the supernatant was obtained at pH 7·8. Dithionite reduced browning of the extracts but had little effect on the amount of enzyme extracted at each pH. Complete extraction from the residue was obtained by more prolonged homogenization.

TABLE 3. EFFECT OF PH OF THE EXTRACTING BUFFER ON THE LEVEL OF ACTIVITY TOWARDS SCMP IN HOMOGENATES OF PHLOEM OF GRAPE VINE

Homogenates of phloem from dormant canes were prepared using ten homogenization periods each of 10 sec in the buffers indicated.

	Activity in supernatant (units)	Activity in residue (units)	Total activity in supernatant (%)
Phosphate–citrate pH 3·0	25·2	19·0	57
Phosphate–citrate pH 5·0	21·4	15·8	57
Phosphate–citrate pH 5·0, 0·1% dithionite	22·5	12·7	64
Phosphate–citrate pH 7·8	33·0	9·0	78
Phosphate–citrate pH 7·8, 0·1% dithionite	35·7	8·3	81
Boric acid–KCl–NaOH pH 9·2	16·8	20·6	45
Boric acid–KCl–NaOH pH 9·2, 0·1% dithionite	18·5	21·0	47

Table 4 shows the effect of homogenization time on the extraction of enzymes hydrolysing SCMP, SCMC and sucrose from phloem and xylem. By increasing the duration of each period of homogenization from 10 to 15 sec all the SCMP and SCMC hydrolysing activity could be extracted but the invertase (β-fructofuranosidase) activity remained distributed between the supernatant and the residue. Increasing the number of periods of homogenization above ten did not increase the total activity extracted, indicating that this mechanical treatment could not release more hydrolases under the conditions used. Microscopical examination of the phloem and xylem homogenates prepared by ten homogenizations each of 15 sec showed that only fibres and fibre tracheids remained intact.

TABLE 4. EFFECT OF TIME OF HOMOGENIZATION ON EXTRACTION OF HYDROLASES FROM XYLEM AND PHLOEM OF GRAPE VINE

Homogenates of phloem and xylem in phosphate–citrate buffer (pH 7·8) were prepared using the times of homogenization indicated.

Homogenization (No. of 15 sec periods)		*PHLOEM*			*XYLEM*		
		β-1,3-glucan hydrolase (units)	β-1,4-glucan hydrolase (units)	Invertase (units)	β-1,3-glucan hydrolase (units)	β-1,4-glucan hydrolase (units)	Invertase (units)
10	Supernatant	53	47	70	48	45	243
	Residue	0	0	82	0	0	200
20	Supernatant	53	48	77	48	50	240
	Residue	0	0	78	0	0	230
30	Supernatant	52	48	76	48	45	245
	Residue	0	0	82	0	0	224
50	Supernatant	55	48	74	48	44	250
	Residue	0	0	68	0	0	187

In all subsequent experiments phloem and xylem homogenates were prepared in phosphate–citrate buffer, pH 7·8 by ten 15 sec homogenizations.

Properties of phloem and xylem extracts. Dialysis of both the phloem and xylem extracts against frequently changed distilled water at 4° in 10/32 Visking tubing for 5 hr removed all the reducing sugars present without loss of β-1,3- or 1,4-glucan hydrolase activity. Storage of the dialysed or undialysed extracts at 4° resulted in the complete loss of hydrolase activities in 4 days.

pH-activity relationships. Both phloem and xylem extracts showed bell-shaped curves with a broad range of optimum activity in the region pH 5–6.

Action on β-1,3-glucosidic substrates. Both phloem and xylem extracts hydrolysed the β-1,3-linked substrates, laminaribiose, laminarin, SCMP, and pachyman. The products of hydrolysis of laminaribiose and pachyman were investigated by paper chromatography of deionized and concentrated enzyme–substrate mixtures. Laminaribiose was hydrolysed to produce glucose, and pachyman hydrolysates showed a strong spot corresponding to the glucose marker and two slower moving spots with R_g 0·63 and 0·39 and a series of spots of lower R_g. The R_g values of laminaritriose and laminaripentaose are 0·63 and 0·39 respectively in the propan-1-ol/ethyl acetate/H_2O (6 : 1 : 3 v/v) solvent system used. Only a very weak spot corresponding to laminaribiose was detected.

Reduction of viscosity of SCMP. Xylem and phloem extracts showed very low activity in the viscosity reduction test with SCMP as substrate. Phloem extracts were concentrated by precipitating the enzyme with 80% $(NH_4)_2SO_4$, centrifuging at 10,000 *g* for 30 min, dissolving and dialysing against distilled water at 4°. The ammonium sulphate precipitate contained 70 per cent of the total activity. The concentrated phloem extract (3·5 mg protein/ml) reduced the viscosity of SCMP in the standard test by 10 per cent in 20 min. Over the same period the production of reducing sugar was equivalent to 0·5 μg of glucose.

Inhibition of SCMP-hydrolysing activity. The effect of various enzyme inhibitors on the activity of exhaustively dialysed phloem and xylem extracts acting on SCMP is recorded in Table 5.

TABLE 5. EFFECT OF INHIBITORS ON SCMP HYDROLYSING ACTIVITY OF EXTRACTS OF XYLEM AND PHLOEM OF GRAPE VINE

The solution of inhibitor (0·4 ml) in phosphate–citrate buffer at pH 5·0 was incubated with the dialysed enzyme (0·2 ml) for 30 min prior to the addition of substrate SCMP 1·5% (0·4 ml). The activity remaining was determined under the standard conditions.

Inhibitor	Final concentration of inhibitor in incubation mixture (μM)	Inhibition % Phloem	Xylem
Mn^{++}	1	62	64
Hg^{++}	0·1	100	100
Cu^{++}	0·1	6	4
p-Chloromercuribenzoate	0·1	88	90
D-glucono-1→4-lactone	2·5	29	34
Iodoacetate	0·1	24	22

Heat Stability. Table 6 records the activity towards SCMP remaining in phloem and xylem extracts following heating at various temperatures.

Localisation of SCMP-hydrolysing enzymes. Homogenates of both phloem and xylem tissues were made by the standard procedure in a medium consisting of 0·6 *M* mannitol,

TABLE 6. EFFECT OF HEAT ON SCMP HYDROLYSING ACTIVITY OF EXTRACTS OF XYLEM AND PHLOEM OF GRAPE VINE

Samples of dialysed phloem and xylem extracts were held at the temperature shown for 10 min and the β-1,3-glucan hydrolase activity remaining was determined under the standard conditions.

Temperature (°C)	Activity remaining (%) Phloem	Xylem
20	100	100
30	100	100
40	95	94
50	65	62
60	15	24
70	7	12
80	2	3
90	0	0

1·0 m*M* EDTA, 50 m*M* phosphate buffer, pH 7·2 and 1% human serum albumin. The homogenates were fractionated by centrifugation as shown in Fig. 1.

The homogenizing medium was used for each washing and final suspension and all manipulations were performed at 4°. Each fraction was assayed for β-1,3- and β-1,4-glucan hydrolase, glutamate dehydrogenase, malate dehydrogenase and succinate dehydrogenase activities. The results are given in Table 7. In a similar experiment in which the homogenizing medium did not contain serum albumin no fraction showed dehydrogenase activities (cf. Stickland[42]), although the distribution of the glucan hydrolases was comparable to the distribution in the presence of serum albumin. Succinate dehydrogenase activity could

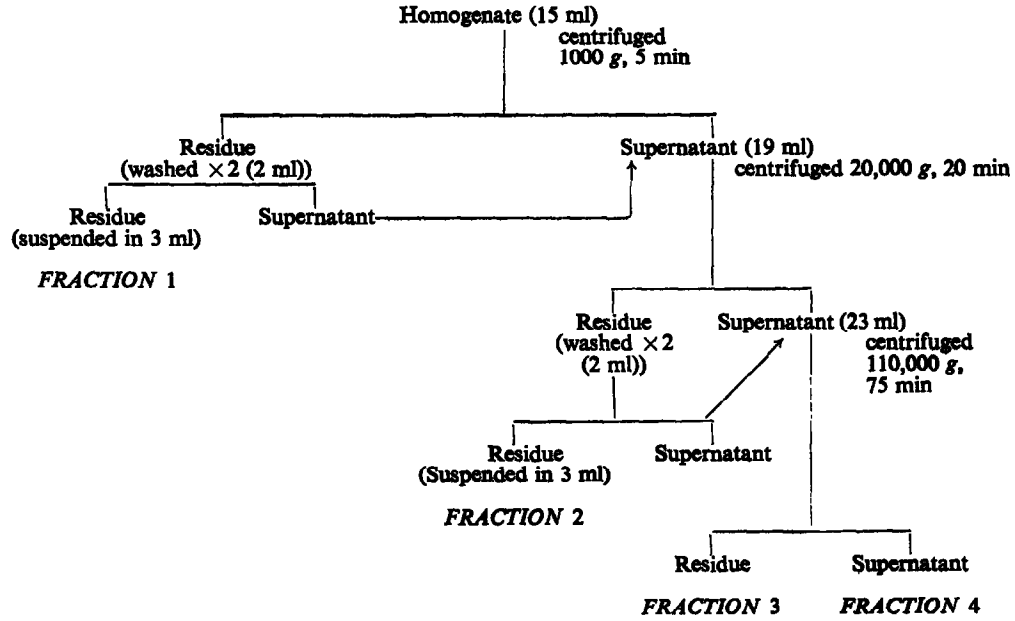

FIG. 1.

[42] R. G. STICKLAND, *Biochem. J.* 77, 636 (1960).

not be detected in these homogenates although it has been demonstrated in grape vine phloem strips.[43]

These experiments show that both the phloem and xylem of the grape vine contain extractable enzyme systems which can hydrolyse a number of 1,3-β-linked glucan substrates. The properties of both phloem and xylem extracts with respect to pH activity, heat stability and inhibitor behaviour are similar to each other and resemble the β-1,3-glucan hydrolases from algae and fungi. The presence in the systems of an enzyme hydrolysing laminaribiose was demonstrated directly and explains the relatively small amount of laminaribiose detected in the enzymic hydrolysates.

TABLE 7. DISTRIBUTION OF ENZYME ACTIVITIES IN FRACTIONATED HOMOGENATES OF GRAPE VINE PHLOEM AND XYLEM

Phloem and xylem samples were homogenized in a medium containing 0·6 M mannitol, 1 mM EDTA, 50 mM phosphate buffer pH 7·2, and 1% human serum albumin. The homogenates were fractionated by centrifugation as described in the text.

	Total activity Recovered (%)							
	Phloem				Xylem			
Fraction No. ——→	1	2	3	4	1	2	3	4
Succinate dehydrogenase	0	0	0	0	0	0	0	0
Glutamate dehydrogenase	6·2	56·3	37·5	0	0	66·6	33·3	0
Malate dehydrogenase	0	62·2	37·8	0		Not assayed		
β-1,3-glucan hydrolase	9·2	5·6	5·2	80·0	13·2	4·6	2·2	80·0
β-1,4-glucan hydrolase	1·2	3·2	1·4	94·2	16·8	1·1	1·1	81·0

The β-1,3-glucan hydrolase, in contrast to invertase, can be completely extracted from the insoluble residue which represents the cell-wall and adhering material sedimenting at low speeds. The fractionation studies under conditions chosen to maintain the structure of the subcellular components confirmed this. The β-glucan hydrolases were almost entirely confined to the soluble fraction obtained after high-speed centrifugation while the dehydrogenases were located in the sub-cellular fractions sedimenting at intermediate speeds. The small amount of glucan hydrolase activity retained in the insoluble fraction can probably be accounted for by incomplete breakage of the cells under the conditions used for the preparation of the homogenates.

Activity of invertase and β-glucan hydrolases in the xylem and phloem of dormant and reactivating canes

Single canes were used to detect changes in enzyme level during reactivation since the time of onset of reactivation varies from cane to cane. Experiments were first carried out to determine the variation in enzyme activity in extracts of the phloem and the xylem taken along the length of both dormant and reactivated canes. The results indicated that there was no significant variation in the invertase and hydrolase activities along the length of the canes. It was assumed that any change in the level of activity induced by reactivation would occur uniformly over the length of the cane, so that the activities in pieces of cane cut successively from one end of a reactivating cane would represent the level of activity of the cane as a whole.

[43] M. D. DULOY and F. V. MERCER, *Aust. J. Biol. Sci.*, **14**, 391 (1960).

Changes in enzyme level in xylem and phloem during reactivation

Three canes were removed from the cold room and after cutting a 4 cm piece from the apical end of each they were reactivated under the conditions described in the Experimental Section. After periods of reactivation indicated in Table 8 samples, two internodes in length, were cut from the apical end and the upper internode discarded. From the remaining internode, sections were cut for microscopical assessment of the stage of reactivation and from a 4 cm piece of the same internode homogenates of the xylem and phloem were prepared. The results are shown in Table 8.

TABLE 8. HISTOLOGICAL AND ENZYMIC CHANGES IN XYLEM AND PHLOEM OF GRAPE VINE, DURING REACTIVATION
Three canes were activated in a warm room at 27°, at the times indicated samples were cut from the apical end, assayed for hydrolase activities, and assessed microscopically for degree of reactivation as described.

Time of activation (days)	Histological assessment	β-1,3-glucan hydrolase sp. act. (units)		β-1,4-glucan hydrolase sp. act. (units)		Invertase Supernatant sp. act. (units)		Invertase Residue sp. act. (units)	
		Phloem	Xylem	Phloem	Xylem	Phloem	Xylem	Phloem	Xylem
Cane A									
0	—	30	77	20	60	124	776	156	534
1	—	35	78	24	65	131	750	142	500
3	—	32	82	26	67	38	800	115	485
7	+	36	78	22	62	38	710	116	372
10	++	37	84	24	61	33	444	130	533
16	++	39	78	19	60	30	500	115	495
23	+++	13	30	10	14	31	445	101	510
Cane B									
0	—	47	60	34	42	133	945	121	410
1	—	50	98	37	47	130	965	117	422
3	—	52	66	38	46	51	563	83	410
7	—	50	67	38	49	35	240	75	352
10	+	54	63	33	49	32	174	71	352
16	++	46	60	36	43	25	252	63	380
23	+++	14	42	7	13	30	260	59	400
Cane C									
0	—	37	85	22	54	191	770	100	362
1	—	43	89	24	55	201	770	111	345
3	—	39	90	26	58	42	530	86	326
7	+	40	89	26	56	23	317	54	240
10	+	43	87	21	58	30	530	98	370
16	++	39	82	20	55	23	375	61	360
23	+++	14	27	5	25	33	310	58	250

— No visible connecting strands and callose maximal thickness.
\+ Connecting strands visible and callose maximal thickness.
++ Thickened connecting strands, callose pads thinner.
+++ Much thickened connecting strands, callose pads much thinner.

No significant increase in the β-glucan hydrolase activities in the xylem or phloem occurred during the period of reactivation. There was, however, a marked decrease in the activities after 23 days. By contrast the soluble invertase level especially in the phloem decreased sharply after the first day in the warm room and reached a fairly constant level prior to the appearance of connecting strands in the sieve plates. No such marked change was noticeable in the level of bound invertase.

There have been suggestions that invertase is bound to the cell walls of pollen tubes

(*Nicotiana alata*),[44] wheat roots,[45] oat coleoptiles[46] and Canna leaves,[47] and sugar beet root.[48] At least part of this enzyme is associated with the cell wall fractions in the homogenates of grape vine phloem and xylem, but whether all types of phloem and xylem cells carry invertase cannot be decided from these experiments. Wanner[49] was unable to demonstrate the enzyme in sieve tube exudates from *Robinia pseudoacacia* but the present demonstration of bound invertase in the phloem indicates the possibility that sieve tubes also have the enzyme in a bound form.

Van Fleet[50] suggested in 1952 that ". . . seasonal changes in the callus of phloem . . . may be related to seasonal enzyme syntheses and hydrolysis of the callose by carbohydrases." If there is a seasonal increase in the level of β-1,3-glucan hydrolase activity either by an enzyme synthesis, by the release of an enzyme from an inactive bound form, or activation of a proenzyme, an increase in the activity in the phloem extracts might be expected.

The results presented here show that although β-1,3-glucan hydrolases are indeed present in the phloem, their level did not detectably change during the removal of dormancy callose. There are a number of possible interpretations of these results.

Although a soluble β-1,3-glucan hydrolase system of low but sufficient activity to remove the dormancy callose can be detected in phloem homogenates, it is not certain whether this is the system which is involved in the *in vivo* process. Both the phloem and xylem tissue used were heterogeneous with respect to cell types so that the β-glucan hydrolase activity cannot with any certainty be localized in a particular cell type. In view of the similarity of the enzyme systems from xylem and phloem it is possible that the enzymes have their origin in cells common to both tissues viz. parenchymatous cells, especially those of the rays. This does not exclude the possibility that other living cells in the phloem, e.g. sieve tubes, may also contain the enzyme. If the enzyme studied is in fact localized in the parenchymatous cells it is unlikely to be concerned with callose removal as relatively few of these cells abut on to the sieve tubes.

As the substrate (callose) is localized on the sieve plates the system operating in its removal may also be found localized there. This localized system may be diluted by cell contents and obscured by any β-1,3-glucan hydrolase originating in other cells, so that any increase would not be detected by the method of assay used. It is also possible that the enzyme is present at all times but that the substrate callose is made available only during reactivation by removal of some physical barrier, e.g. a lipid membrane; this would involve a different enzyme system. There is some evidence from electron microscope studies of several species including *Vitis* that the callose plug is surrounded by a membrane.[51-53]

Alternatively, in the dormant cane, all or part of the β-1,3-glucan hydrolase may be confined in a sub-cellular particle from which they could be released during the reactivation process. If this particle were disrupted during homogenization only total activity would be measured, any change in the relative amounts of free and associated enzyme would not be detected. Although the cell fractionation did not indicate that such a situation exists,

[44] J. TUPY, *Biologia Plantarum* **2**, 169 (1960).
[45] H. BURSTRÖM, *Physiol. Plantarum* **10**, 741 (1957).
[46] H. BURSTRÖM, *Physiol. Plantarum* **11**, 771 (1958).
[47] W. Z. HASSID, *Handbuch der Pflanzen physiologie Bd. VI* Ed. W. RUHLAND Springer-Verlag (1958).
[48] J. S. D. BACON, *Biochem. J.* **79**, 20 (1961).
[49] H. WANNER, *Ber. Schweiz. Bot. Ges.* **63**, 201 (1953).
[50] D. S. VAN FLEET, *Bot. Rev.* **18**, 354 (1952).
[51] M. DULOY, F. V. MERCER and N. RATHGEBER, *Aust. J. Biol. Sci.* **14**, 506 (1961).
[52] N. RATHGEBER, personal communication.
[53] K. ESAU and V. I. CHEADLE, *Proc. Nat. Acad. Sci.* **47**, 1716 (1961).

the possibility is not excluded. Further investigation is necessary before any decision can be made between these possibilities and also before the enzyme system in the heterogeneous phloem and xylem tissues can be definitively localized.

Callose, a potential substrate for β-1,3-glucan hydrolases, is not confined to sieve tubes, but occurs in small amounts in a variety of situations in higher plants, e.g. pollen tubes, root hairs, pollen mother cells, lactifers, cystoliths, etc.[54] and a β-1,3 glucan synthesizing system has been described in a number of higher plants.[55] Whether there is any direct relationship between the β-1,3-hydrolase systems described and these substrates remains to be determined.

EXPERIMENTAL

Substrates

Salicin was purchased from L. Light and Co. Ltd. Laminaribiose was isolated from a laminarin hydrolysate by charcoal column chromatography. Soluble laminarin from *Laminaria digitata* was purchased from the Institute of Seaweed Research, Inveresk, Scotland. Sodium carboxymethylcellulose (Edifas "B") controlled metals content, normal viscosity, 0·45–0·55 degree of substitution was a product of Imperial Chemical Industries Ltd., Stevenston, Ayrshire, Scotland. Sclerotia of *Poria* (*Pachyma*) *cocus* were a gift of Dr. K. Aoshima, Ministry of Agriculture and Forestry, Tokyo, Japan. The inner portions of the sclerotia were ground to pass the 0·5 mm mesh of a Wiley mill and extracted twice with chloroform–ethanol (2 : 1 v/v). The extracted material is referred to as pachyman.[56] Carboxymethylpachyman was prepared by grinding 100 g of pachyman with 240 ml of 10·5 *N* NaOH in the cold to form a doughy paste. A solution of 50 g of monochloroactic acid in 60 ml H_2O was added dropwise with constant stirring. The mixture was heated at 70° for 4 hr, cooled and 500 ml of 2 *N* HCl added with stirring. The viscous solution was dropped portionwise into 2 l. aqueous ethanolic HCl (20 ml conc. HCl in 80 ml ethanol) in a Waring blendor. The product was dialysed against distilled water until chloride free. The acid form of carboxymethylpachyman was purified by shaking for 4 hr with 4 l. acid methanol (100 ml 75% HNO_3 diluted to 1 l. with methanol). The product was centrifuged and washed with 70% MeOH until neutral to methyl red. (The degree of substitution determined by the acid-wash method of Eyler[57] and also by direct estimation of free glucose in an acid hydrolysate after removal of the substituted residues by anion ion-exchange was 0·3. The p*K* determined by electrometric titration was 3·45). Before use the carboxymethylpachyman was converted to its sodium salt as follows: to 20 g of CMP swollen in 200 ml of water for 1 hr was added 50 ml of 5 *N* NaOH and the paste stirred until all the CMP had dissolved. A further 250 ml of 10 *N* NaOH and 1500 ml of water was added and the solution stirred for 1 hr. The pH of the solution was adjusted to 7·0 with H_2SO_4 and dialysed.

Sources of plant material

Unless otherwise stated the materials used were recent purchases from the local market, were greenhouse plants or were collected from trees within the University grounds. The almonds used were from the 1960–61 crop. The soya beans were commercially obtained. The dried papaya latex was obtained from Wheeler and Huisking Ltd., London. Wheat samples, germinated and ungerminated were supplied through the courtesy of Barrett

[54] W. Eschrich, *Protoplasma*, **47**, 487 (1956).
[55] D. S. Feingold, E. F. Neufeld and W. Z. Hassid, *J. Biol. Chem.* **233**, 783 (1958).
[56] S. A. Warsi and W. J. Whelan, *Chem. and Ind.* p. 1573 (1957).
[57] R. W. Eyler, E. D. Klug, F. Diephuis, *Anal. Chem.* **19**, 24 (1947).

Hudson Pty. Ltd., Melbourne. Dormant grape vine canes (Waltham Cross) were cut from vines growing at Swan Hill, Victoria, between June and August, 1961. The canes were transferred as soon as possible after cutting (4–8 hr) into polythene bags and stored at 4° until used.

Preparation of tissues

The various materials were treated as follows prior to the preparation of the enzyme extract. Onions, carrots, parsnips, radishes—were washed and the epidermis removed. Pineapple—the shell was removed and the underlying ovular tissue and basal portion of the bracts used. Cauliflower—the washed immature inflorescences were used. Almonds were expressed in a hydraulic press and the kernels ground to a meal in an edge-runner mill and extracted with hexane at room temperature. Soya beans were similarly treated but without prior expression of the oil. Leaves were washed and the petioles and mid-ribs removed.

Preparation of enzyme extracts

The soft tissues were suspended in 2 vol., and the leaves in 3 vol. of 0·1 *M*—phosphate buffer (pH 7·0) containing 0·004 *M* cysteine and homogenized in a Waring blendor. The homogenate was squeezed through cheese cloth. The juice of the potato was pressed out in a hand-operated press. The soya bean meal, almond meal, wheat and ground dried papaya latex were extracted with 4 vol. of buffer by shaking mechanically for 2 hr. The extract was then squeezed through cheese cloth. All the extracts were dialysed overnight in cellophane tubing against the extracting buffer and centrifuged at 1200 *g* for 15 min. The latex obtained by tapping a Moreton Bay fig tree was diluted six times with buffer, dialysed overnight and centrifuged at 25,000 *g* for 30 min. All manipulations were carried out at 4°.

Preparation of grape vine extracts

Pieces (4 cm long) of grape vine canes were sectioned and dissected as described by Canny and Markus[58] to obtain phloem and xylem tissue (40μ thick). Strips of the separated tissues (about 80 mg) were cut into approximately 25 mm pieces immediately after sectioning and transferred to 3 ml of phosphate-citrate buffer (pH 7·8) containing 0·1% sodium dithionite in a 15 ml centrifuge tube. The tissue was homogenized in the tube using a nylon pestle rotating at 1600 rev/min with cooling in an ice bath between each homogenizing period. The homogenates were centrifuged at 1000 *g* for 15 min. Where used, the residue was washed twice by centrifugation with 3 ml of buffer and resuspended in 3 ml of buffer. The supernatant is referred to as the phloem or xylem extract.

Reactivation of grape vine phloem

The method of artificially reactivating the phloem in grape vines by standing dormant canes in a warm room has been described by Esau.[37] Reactivation under these conditions closely follows the pattern of natural reactivation of the phloem in the spring when the appearance of connecting strands, disappearance of callose and appearance of shoots are used as indices of the activation.

In this study canes were placed in a warm room at 27°. The canes were enclosed in a length of wide bore glass tubing, the basal ends of the canes were dipped into a beaker of tap water through the open end of the glass tubing, the other end of the tubing was stoppered. The water in the beaker was changed daily during the reactivation period.

[58] M. J. CANNY and K. MARKUS, *Aust. J. Biol. Sci.* 13, 292 (1960).

Preparation and staining of grape vine sections

Transverse sections 20μ thick of dormant or reactivating canes were cut on a horizontal microtome, stained with aniline blue and KI-I_2, and mounted in glycerine jelly.

Enzyme assays. β-Glucan and β-glycoside hydrolase activities were measured by determining the reducing sugar liberated under standard conditions by the Nelson[59] colorimetric method with the Somogyi[60] alkaline copper reagent or with laminaribiose as substrate, by the glucose aerodehydrogenase (Sigma) method.[61]

Reactions were stopped by the direct addition of the alkaline copper reagent or with laminaribiose by adding 1 ml of $ZnSO_4$ followed by 1 ml of $Ba(OH)_2$.[62] Stoppered tubes were used in reactions lasting 24 hr and longer and a drop of toluene was added. Blanks without substrate and without enzyme were run. In all cases the pH of the reaction mixture was 5·0 and the incubation temperature 40°. The reaction mixtures incorporating the individual substrates had the following compositions:

(*a*) sucrose: 0·8 ml of 0·1 *M* sucrose in phosphate–citrate buffer, pH 5·0, 0·2 ml enzyme solution; incubation time 5 hr.
(*b*) SCMP: 0·8 ml of 0·75% SCMP in phosphate–citrate buffer, pH 5·0, 0·2 ml enzyme solution; incubation time 24 hr.
(*c*) SCMC: as for SCMP. The SCMC solutions were prepared as previously described.[3]
(*d*) laminaribiose: 0·5% in phosphate–citrate buffer, pH 5·0, 0·2 ml enzyme solution, incubation time 24 hr.

Activity in each case was recorded as μg reducing sugar (as glucose) per ml enzyme-substrate mixture produced under standard conditions described above. Specific activity units were obtained by dividing these values by the protein concentration of the enzyme solution (mg/ml). Protein concentration was determined by the method of Gornall *et al.*[63] Succinate dehydrogenase activity was measured by the colorimetric method of Redfearn and Dixon.[64] Malate and glutamate dehydrogenase activity was measured by rate of reduction of NAD.

Viscometric assay of β-1,3-glucan hydrolase. The reduction in viscosity of a SCMP solution was used as an additional measurement of β-1,3-glucan hydrolase activity. Viscosities of the enzyme–substrate mixtures were measured in a No. 1 B.S.S. Ostwald viscometer which had been modified for use with a total volume of 2 ml by reducing the capacity of the bulb carrying the markings to 0·7 ml and that of the reservoir bulb to 1·5 ml. The flow time for water was 27·3 sec.

The substrate solution contained 0·75% SCMP in phosphate–citrate buffer, pH 5·0; 5 m*M* EDTA (to minimize the effect of any metal ions added on the viscosity of the substrate) and 0·25 *M* NaCl (to reduce the effect of added salt). Preliminary experiments showed that above 0·2 *M* NaCl the specific viscosities of SCMP solutions were unchanged. In the assay procedure 1·6 ml of substrate was equilibrated at 40° and mixed with 0·4 ml of similarly equilibrated enzyme solution, 1·95 ml of the mixed solutions was transferred to the viscometer and the flow time observed at frequent known intervals from the time of mixing during incubation at 40°. To obtain an expression for the activity of the enzyme the results

[59] N. Nelson, *J. Biol. Chem.* **153**, 375 (1944).
[60] M. Somogyi, *J. Biol. Chem.* **195**, 19 (1952).
[61] A. St. G. Huggett and D. A. Nixon, *Lancet* **2**, 368 (1957).
[62] M. Somogyi, *J. Biol. Chem.* **160**, 61 (1945).
[63] A. G. Gornall, C. J. Bardawill and M. M. David, *J. Biol. Chem.* **177**, 751 (1949).
[64] E. R. Redfearn and J. M. Dixon, *Biochem. J.* **81**, 19P (1961).

were treated as described by Tracey.[41] Plots of $\frac{100}{T}$ (where T is the time required to reduce the initial specific viscosity by 10 per cent, 33·3 per cent or 50 per cent) against various concentrations of an *Aspergillus niger* enzyme are shown in Fig. 2. The approximately linear relationship provides a suitable means of measuring enzyme activity. For measure-

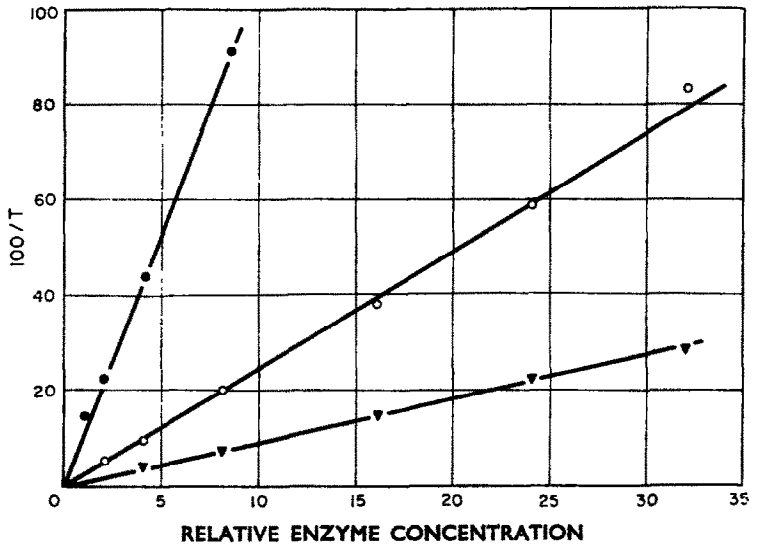

FIG. 2. REDUCTION OF VISCOSITY OF SCMP BY AN *Aspergillus niger* ENZYME.

Plots of $\frac{100}{T}$, where T is the time required to reduce the initial specific viscosity by 10 per cent —●—●—, 33·3 per cent —O—O—, or 50 per cent —▼—▼—, against various enzyme concentrations are shown.

ment of the β-1,3-glucan hydrolase activities of various plant extracts the time required for a 10 per cent reduction of the initial specific viscosity of SCMP was used. Unit activity was taken as the amount of enzyme required to cause a 10 per cent reduction in initial η_{sp} in 100 min.

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EXTRACTION AND IDENTIFICATION OF LIPIDS FROM LOBLOLLY PINE POLLEN

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Abstract—Loblolly pine (*Pinus taeda*) pollen was found to contain about 7·5 to 9 per cent lipids, most of which were triglycerides of oleic, linoleic, and palmitic acids. The triglycerides were not easily extracted from pollen unless the grains were fractured. Lipids, which were easily extracted and were probably part of the outer spore coat, comprised 1·6 per cent of the pollen. These were mainly octaconsanol and hexacosanol with smaller amounts of wax esters and free acids.

INTRODUCTION

In his reviews of pollen chemistry, Lunden[1,2] expressed the hope that the availability of pollen would not be a limiting factor in the advancement of pollen chemistry. The continued scarcity of published research on this subject indicates that chemists are not generally aware that pollen of many species can be obtained easily in pound quantities. Many trees, particularly pines, are prolific producers of pollen. For the work reported here, 20 lb of loblolly pollen were collected in about 6 working days.

Pollen is an interesting biological material to use for chemical studies. Its primary function, the transport of the male gamete, is sustained by a number of special structural features, and one can expect that the chemical constitution of pollen will also be found to support its genetic role. In the case of pines, which are wind pollinated, the outer spore coat, the exine, includes two air sacks outside the central, roughly spherical section. The ground material of the exine, sporopollenin, is a material so resistant to strong alkalies and to concentrated acids that recognizable pollen grains can be isolated from ancient deposits by dissolving other materials away.[3] Sporopollenin's chemical structure is still unknown although it was isolated by Berzelius in 1837.

Under suitable conditions, a pollen grain germinates with the growth of a long pollen tube. The raw materials to support this physiological change are contained within the pollen grain. An understanding of this germination process is important for its likely contributions both to physiology and to the practical problems of storage of viable pollen. These and other studies may be expected to benefit by greater chemical knowledge of pollens.

The emphasis of this report is on the lipids of loblolly pine pollen. Some general remarks on other extractives and on other pine pollens are included. In the literature, the following pollen lipids have been reported: 1-tetracosanol, 1-hexacosanol and 1-octacosanol in *Pinus montana*;[4] pentacosane, heptacosane, and palmitic, stearic, oleic, linoleic, and linolenic acids in *Zea mays*;[4,5] heptacosane and nonacosane in *Alnus glutinosa*;[4] tricosane

* Maintained at Madison, Wis., in co-operation with the University of Wisconsin.

[1] R. Lunden, *Svensk Kem. Tidskr.*, **66**, 201 (1954).
[2] R. Lunden, *Grana Palynologica* (*N.S.*) **1** and **2**, 3 (1956).
[3] A. Kwiatkowski and K. Lubliner-Mianowska, *Acta Soc. Botan. Polon.*, **26**, 501 (1957).
[4] M. Nilsson, R. Ryhage and E. von Sydow, *Acta Chem. Scand.* **11**, 634 (1957).
[5] C. R. Barr, C. D. Ball and H. M. Sell, *J. Am. Oil Chemists Soc.* **36**, 303 (1959).

and palmitic acid in *Corylus avellana*;[6] oleic, linoleic, palmitic, and other lower molecular weight, saturated acids, in ragweed;[7] and pentacosanol and heptacosanol in *Cedrus atlantica* and *Cedrus deodara*.[8] Amounts of lipids found in pollens vary: 1·5–2·6 per cent in corn,[5,9] 9–10 per cent in pine and alder[4,10] and 11–15 per cent in ragweed.[7,11]

RESULTS AND DISCUSSION

Solvent extraction of pine pollens

Because of the uniform sizes of intact pollen grains, solvents flow through pollen beds at relatively rapid rates. Therefore, extraction with refluxing solvents is very conveniently used on intact pine pollen. It was found that benzene rapidly removed soluble materials to the point where successive volumes of solvent eluted less than 1 per cent of the total extract, but that diethyl ether and methyl alcohol, used in succession after benzene, continued to elute material slowly even after large volumes of solvent had been used.

The continued elution of extractives by ether and by methanol was partly due to the slow extraction of triglycerides which separated as oils during the concentration of the ether and alcohol extracts. It was found that the oils could be extracted more easily by lipid solvents if the pollen grains were first fractured by ball-milling for 30 min while they were suspended in a solvent.

It is reasonable to suppose that the material which lipid solvents quickly removed from intact pollen was associated mainly with the exine; extraction of the triglycerides, most likely contained in the protoplast, was probably restricted by one or both of the spore coats. The slow elution of triglycerides may have been augmented by the fact that the pollen grains swelled (without bursting) when benzene was displaced by ether or methanol.

Pollen of several pine species was extracted for comparative purposes. The benzene-extractable material was 1·5–2·3 per cent of the air-dried, intact pollen for the following pine species: loblolly (*Pinus taeda*), slash (*P. eliottii*), longleaf (*P. palustris*), shortleaf (*P. echinata*), jack (*P. banksiana*), red (*P. resinosa*) and ponderosa (*P. ponderosa*). The ether extractives from the benzene extracted pollen were between 0·15 and 0·25 per cent; and the succeeding methanol extracts were all about 25 per cent of the dry pollen.

As mentioned above, the benzene extracts of intact pollens did not contain all of the lipids because of the unextracted triglycerides. In the case of loblolly pollen collected in Georgia in 1958, the triglyceride, obtained as an oil from the ether and methanol extracts, was about 7·5 per cent of the pollen. Loblolly pollen collected in 1960 from the same location was washed three times with petroleum ether (65°–69°) and then was milled and re-extracted with petroleum ether. The first washings removed 1·5 per cent of the pollen. The extraction after milling removed triglyceride oil which was 6·1 per cent of the pollen. Although this was not determined for other pollens, there was sufficient oil to indicate that several pine species had pollens with similar amounts of triglycerides.

Identification of constituents of loblolly pine pollen

The material in the benzene extract (1·6 per cent) was fractionated first by column chromatography on silicic acid. The individual fractions of the chromatograms were then

[6] C. Sosa-Bourdouil and A. Sosa, *Bull. soc. chim. biol.* **36**, 393 (1954).
[7] F. W. Heyl, *J. Am. Pharm. Assoc.* **12**, 669 (1923).
[8] A. Spada, D. Coppini and A. Monzani, *Ann. chim. (Rome)* **48**, 181 (1958).
[9] R. J. Anderson and W. L. Kulp, *J. Biol. Chem.* **50**, 433 (1922).
[10] E. Havivi and J. Leibowitz, *Bull. Research Council Israel* **9A**, 157 (1960).
[11] R. P. Mariella, T. B. Bernstein and A. L. Mosher, *Bull. col. quim. Puerto Rico* **9**, 15 (1952).

examined by infrared spectroscopy to determine what further fractionation was necessary. In this procedure, free acids and alcohols were determined separately from wax esters. The lipids which were identified among the benzene extractives are listed in Table 1. The main criterion for identification of the components in the acid and alcohol fractions was retention time in gas chromatography. In all cases, the melting points of the mixtures before and after chemical conversions, and the neutral equivalents of free and derived acid mixtures substantiated the gas chromatographic analyses. A further check on the identification of the free-alcohol mixtures was made by gas chromatography of the free alcohols before oxidation to the corresponding acids.

TABLE 1. COMPONENTS IN THE BENZENE EXTRACT OF INTACT LOBLOLLY POLLEN EXPRESSED AS APPROXIMATE WEIGHT PERCENTAGES OF THE BENZENE EXTRACT*

Free alcohols		Free acids		Wax esters	
Alcohol	%	Acid	%	Constituents	%
C_{28}	11·4	C_{26}	3·1	C_{24} alcohol	1·2
C_{26}	8·2	C_{28}	1·3	C_{26} ,,	0·7
C_{24}	0·9	C_{16}	0·6	C_{20} ,,	0·3
C_{30}	0·7	C_{18}	0·6	C_{22} ,,	0·3
C_{27}	0·5	C_{24}	0·6	C_{28} ,,	0·3
		C_{28}	0·4		
		C_{22}	0·1	C_{20} acid	1·0
				C_{18} ,,	0·7
				C_{16} ,,	0·4
Totals	21·7		6·7		5·1

* All of these lipids were normal, aliphatic, saturated compounds. This benzene extract was 1·6 per cent of the dried pollen and did not contain the triglycerides. This analysis is only of Georgia-grown loblolly pollen of the 1958 season.

In addition to the large triglyceride fraction, about one third of the compounds present in the total benzene extract of whole pollen was identified. Small amounts of unsaturated acids, acids with lower molecular weights, and acids and alcohols with odd numbers of carbons were also shown to be present by gas chromatography, but are not included in Table 1. The unidentified lipids were mostly in the smaller fractions obtained from the first column chromatogram; the infrared spectra of several of these fractions showed the presence of aromatic compounds. Qualitative tests on the unidentified lipid fractions showed the absence of significant amounts of nitrogen and phosphorus. Only a trace amount of hydrocarbons was found.

Table 2 lists the molar ratios of the fatty acids in the triglyceride fraction. The data, calculated from the gas chromatogram, agreed with the neutral equivalent of the acid mixture from hydrolysis and with the amount of unsaturation determined by hydrogenation of a sample in glacial acetic acid over platinum at room temperature and at atmospheric pressure.

Whatever other properties the triglycerides may give to pollen, they can act as efficient sources of raw material for growth. Also present for growth purposes is a store of carbohydrate. In many cases, enough sucrose was eluted during methanol extractions to form large crystals of sucrose directly in the extract. The sucrose crystals obtained from loblolly pollen collected in 1960 were 10·3 per cent of the whole dried pollen. Sucrose was identified

by paper chromatography before and after hydrolysis. A similar amount of sucrose has been found in *Pinus canariensis*.[9]

A gross analysis of pollen was not attempted but the results obtained on loblolly pine pollen are summarized in the following approximation: sucrose, 10 per cent; triglycerides, 6–7·5 per cent; other lipids, 1·5–2·0 per cent; sporopollenin, about 23 per cent. This value

Table 2. Molar Proportions of Fatty Acids in the Triglycerides*

Linoleic	37
Palmitic	27
Linolenic	25
Oleic	2
Others	9

* The triglycerides constituted 6–7·5 per cent of the total pollen.

for sporopollenin is the amount of residue remaining from the solvent extracted pollen after an acid hydrolysis procedure which completely removes carbohydrates from wood[12] followed by hydrolysis in hot 5 per cent potassium hydroxide.

EXPERIMENTAL

Pollen collection

Male flowers were collected in the green condition at the time when a few flowers were showing signs of opening to shed pollen. The flowers were spread out on screens in a well-ventilated building. Pollen was shaken through the screens on to collection papers as it was shed during the air-drying process and then was stored in a refrigerator. Male flowers can be collected by the bushel easily if one can find a logging operation at the proper time. When collecting such quantities, care must be taken to spread the flowers apart as soon as possible to prevent the generation of excess heat and high moisture due to metabolism.

Preparation and fractionation of the benzene extract of loblolly pollen

Air-dried, intact pollen was extracted in a tubular, continuous-infusion type of extractor with redistilled benzene, followed by diethyl ether, and finally by methanol. The amounts of solvents refluxed through 1·6 kg of pollen were estimated to be 16 l. of benzene, 60 l. of ether and 45 l. of methanol. Except for the previously described slow extraction of triglycerides by ether and by alcohol, most of each extract could have been obtained with one third or less solvent. Each extract was recovered by distillation of most of the solvent at atmospheric pressure and by complete removal at 15 mm pressure and 60°.

The benzene extract was fractionated on two successive columns of reagent-grade silicic acid which was dried overnight at 110°. Samples for chromatography were put into solution and then dried on to three or four times their weight of silicic acid. On the first column of 270 g of silicic acid, a 36 g sample of the benzene extract was eluted with the following series of solvents: 60% petroleum ether; 5, 50, and 90% diethyl ether in petroleum ether; 25 and 50% methanol in diethyl ether; and finally with methanol. About half of the sample was eluted by the 5% diethyl ether and about one fourth was eluted by 50% diethyl ether.

The fraction eluted with 5% Et_2O was recrystallized from hexane at −17° giving an

[12] J. F. Saeman, W. E. Moore, R. L. Mitchell and M. A. Millett, *Tappi* **37**, 336 (1954).

87 per cent yield of insoluble material. A 13 g sample of this precipitate was placed on the second chromatographic column, which had a diameter of 4·5 cm and contained 800 g of silicic acid. This column was eluted with hexane, diethyl ether in increasing concentrations in hexane, ether, 5% methanol in ether, and methanol. The methanol desorbed a fatty alcohol mixture which was then eluted with 5% ether in hexane.

The lipids eluted in the second chromatogram were in four well-separated fractions. The infrared spectra of these showed in order: ester, probable acid, a mixture of acid and alcohol and finally the desorbed alcohol. The acids, as the insoluble sodium soaps, were separated from the hexane solutions of the second and third fractions. The ester fraction was saponified by 0·6% sodium hydroxide in ethanol–benzene (5 : 1) for 2 hr at 90° and the resulting sodium salts were separated from a hexane solution of the alcohols.

Gas chromatographic analyses

Alcohol fractions were oxidized with sodium dichromate in a mixture of glacial acetic acid with sulfuric acid.[13] Because of esterification during this reaction, three successive saponifications and oxidations were necessary to convert over 90 per cent of each alcohol mixture to the corresponding acids. Each free and derived acid fraction was analyzed separately by gas chromatography* after conversion of the acids to methyl esters with diazomethane. The acids and alcohols from the saponified esters were similarly analyzed.

Gas chromatography was done on columns of ethylene glycol–isophthalate on acid- and base-washed diatomaceous earth at 240°. Since the ionization detector response was reported to be approximately linear with weight per cent, the figures in Tables 1 and 2 were calculated from the areas under the curves and from the weights of fractions in the chromatograms off the silicic acid columns.

Triglycerides

The triglyceride oil which separated from the alcohol solvent during concentration of the alcohol extract was divided into nearly equal parts of acetone-soluble triglycerides and acetone-insoluble triglycerides at −17°. These samples were converted to glycerol and a mixture of methyl esters by methanolysis at room temperature in 68 ml of methanol and 0·9 ml of 0·5 *N* KOH. The acetone-insoluble sample, which contained the higher content of saturated acids, dissolved after 3 hr of stirring and then stood overnight. The acetone-soluble sample, however, required two periods of 22 hr of stirring under a nitrogen atmosphere with a change of reagent between periods in order to dissolve all but a small amount of the oil. The esters were removed and analyzed by gas chromatography. The figures in Table 2 represent the sum of the two triglyceride fractions.

The glycerol was quantitatively determined by periodate oxidation[14] and the tribenzoate (m.p. 70·5–71°) was prepared. A sample of the water phase after saponification was paper chromatographed in butanol–pyridine–water (10 : 3 : 3). The developed chromatogram sprayed with ammoniacal silver nitrate had a single spot identical in R value to that of glycerol.

Acknowledgements—The author is indebted to several people in U.S. Forest Service field stations for pollen collections, and particularly to John C. Barber, Southeastern Forest Experiment Station.

* All gas chromatographic analyses were done at Applied Science Laboratories, Inc., 140 North Barnard Street, State College, Pa.

[13] A. H. Blatt, *Organic Syntheses*, Collective Vol. II, p. 139. Wiley, New York (1943).
[14] E. L. Jackson, *Organic Reactions*, Vol. 2, p. 341. Wiley, New York (1944).

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GAS–LIQUID CHROMATOGRAPHY OF TERPENES VI. THE VOLATILE OIL OF *THUJA PLICATA* DONN.*

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Abstract—The neutral, volatile oil of the foliage of western red cedar was analysed by means of gas-liquid chromatography and infrared spectroscopy. The major constituent was found to be *l*-thujone accompanied by nearly 10 per cent *d*-isothujone. The main hydrocarbons were *d*-sabinene and tentatively identified car-4-ene. Smaller amounts of *d*-α-pinene, *d*-limonene, *d*-terpinen-4-ol and an unidentified aromatic ester were also isolated. Trace amounts of α-fenchene, camphene, γ-terpinene, terpinolene, *p*-cymene and 1,8-cineole were identified by retention characteristics only. The presence of thujyl alcohol and its acetate could not be confirmed. The oil from small branchlets from different locations on a young tree was analyzed separately. The qualitative composition was found to be the same, but the younger leaves contained a noticeably smaller amount of hydrocarbons than older ones. The oil may lend itself to a study of the biosynthesis of some of its components and possible pathways are discussed.

IN THE previous paper of this series[1] the analysis of the volatile oils of the leaves of black, white and Colorado spruce (*Picea mariana* (Mill.) BSP., *P. glauca* (Moench) Voss, and *P. pungens* Engelm.) was described. It was shown that for a complete analysis by means of gas–liquid chromatography (GLC) only 0·1-0·2 g of the oil were required and that quantitative comparisons of oils having known chemical composition could be carried out with as little as 5–20 mg. The technique was applied to the study of some seasonal variations in the composition of these spruce oils and it was suggested that variations in the composition of volatile oils from different parts of a single tree or plant could now be studied. This communication describes the analysis of the chemical composition of the leaf oil of western red cedar (*Thuja plicata* Donn.) A comparison was made between the oil of a mature tree grown under natural conditions and that of a young tree grown in a greenhouse, and possible changes in this composition at different locations in a single tree were examined.

The oil of western red cedar leaves was first investigated by Brandel[2] who reported yields of 0·8-1·4 per cent and the presence of thujone, fenchone, and esters of borneol. In 1912, Rose and Livingston[3] confirmed the major constituent to be *l*-thujone (80–85 per cent), and identified *d*-α-pinene (3–5 per cent), *d*-thujyl alcohol (*syn.* tanacetyl alcohol, 1–3 per cent), and thujyl acetate (1–2 per cent), but could not isolate fenchone or bornyl esters. A more recent investigation with more modern analytical techniques does not appear to have been made of this oil. Guenther[4] points out that thujone occurs in nature as a mixture of the stereoisomers *l*-thujone and *d*-isothujone. Shaw,[5] and more recently von Rudloff,[6] have shown the thujone fraction of the leaf oil of eastern white cedar (*Thuja*

* Issued as N.R.C. No. 6920.

[1] E. VON RUDLOFF, *Tappi* **45**, 181 (1962).
[2] I. W. BRANDEL, *Pharm. Rev.* **26**, 248 (1909).
[3] R. E. ROSE and C. LIVINGSTON, *J. Am. Chem. Soc.* **34**, 201 (1912).
[4] E. GUENTHER, *The Essential Oils*, Vol. II, p. 423. Van Nostrand, New York (1952).
[5] A. C. SHAW, *Can. J. Chem.* **31**, 277 (1953).
[6] E. VON RUDLOFF, *Can. J. Chem.* **39**, 1200 (1961).

occidentalis L.) to be such a mixture. Thus, it could be expected that the leaf oil of *Thuja plicata* would also contain isothujone. Also, GLC analysis[6] of the leaf oil of *Thuja occidentalis* showed the presence of at least 32 terpenoid compounds. Thus, it seemed likely that the leaf oil from *Thuja plicata* would have a more complex chemical composition than the data of Rose and Livingston[3] indicates.

The chemical components and the average percentage composition of the oils isolated in the present study from a mature tree from the Prince Rupert district, British Columbia, and the locally grown young tree are shown in Table 1. A typical chromatogram, obtained with a 6 ft polyethylene glycol column (PEG 1540) at 100°, is shown in Fig. 1. The present findings confirm the earlier reports (*loc. cit.*) that *l*-thujone is the major component (about 80 per cent) of the oil, but do show in addition the presence of about 8 per cent of the stereoisomer *d*-isothujone. The major hydrocarbons found were *d*-sabinene, α-pinene and component 4. Only α-pinene had been reported previously. Small amounts of *d*-limonene and tentatively identified camphene, 1,8-cineole, γ-terpinene, terpinolene and *p*-cymene were also detected. Myrcene, car-3-ene, α-phellandrene were not found. Besides the major components, thujone and isothujone, only two oxygenated components were recorded in detectable amounts, and of these terpinen-4-ol (component 13) was positively identified. Thujyl alcohol, thujyl acetate, borneol, bornyl acetate, fenchone and camphor could not be detected. These latter findings differ from those of Brandel[2] and Rose and Livingston.[3] Component 4 differed from myrcene, car-3-ene, and α-phellandrene and may be car-4-ene, but the latter hydrocarbon was not available for direct comparison. Component 12 could not be correlated with a known monoterpene and its infrared spectrum suggests it to be an aromatic acetate.

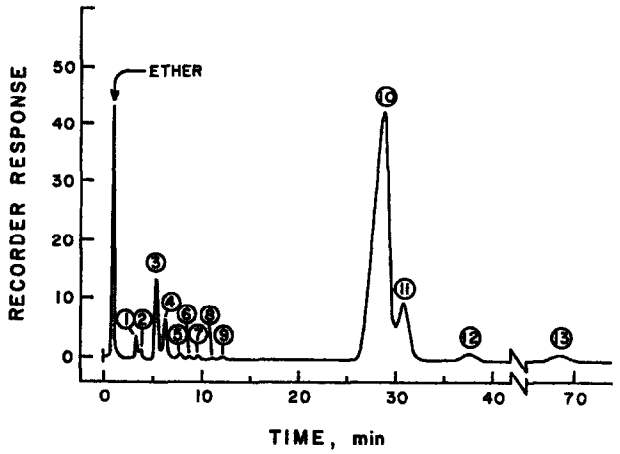


FIG. 1. CHROMATOGRAM OF THE LEAF OIL OF *Thuja plicata* AS OBTAINED ON A 6 FT. × ¼ IN. O.D. POLYETHYLENE GLYCOL (PEG 1540) COLUMN AT 100° AND 60 ML HELIUM PER MIN.

1. α-Pinene; 2. camphene (+α-fenchene); 3. sabinene; 4. (?) car-4-ene; 5. limonene; 6. 1,8-cineole; 7. γ-terpinene; 8. *p*-cymene; 9. terpinolene; 10. thujone; 11. isothujone; 12. (?) aromatic ester; 13. terpinen-4-ol.

The qualitative and quantitative data for the oil from the young tree were practically identical with those obtained for the oil from the mature tree (see Table 1). It was, therefore, concluded that the biosynthesis of the terpenes found in the young tree was similar to that

TABLE 1. AVERAGE COMPOSITION OF THE OIL OF THE LEAVES OF *Thuja plicata*

Compound (Peak No.)*	RRT†	Leaf oil sample‡														
		Prince Rupert	Saska-toon	1	2	3	4	5	6	7	8	9	10	A	B	C
(a) Hydrocarbons																
1. *d*-α-Pinene	0·29	2·0	1·5	0·8	0·6	0·4	0·8	tr.	tr.	tr.	0·3	0·1	tr.	0·3	0·3	0·3
2. (Camphene)	0·41	0·4	0·2	tr.	tr.	tr.	tr.	—	—	—	—	—	—	0·2	0·2	tr.
3. *d*-Sabinene	0·59	6·4	6·3	6·5	8	5·2	3·5	1·3	1	2·2	2·7	2	2·2	1·5	2	1·5
4. (Car-4-ene)	0·78	2·8	2·8	1·5	2	1·9	2·5	0·5	0·3	0·5	1·8	0·6	0·5	0·6	0·8	0·8
5. *d*-Limonene	1·00	0·5	0·3	0·4	0·6	0·1	0·2	tr.	tr.	0·1	tr.	0·1	0·2	tr.	tr.	tr.
6. (1,8-Cineole)	1·08	0·1	tr.	0·8	0·8	0·2	0·5	tr.	tr.	0·3	tr.	0·2	0·2	0·2	tr.	tr.
7. (γ-Terpinene)	1·30	0·1	tr.													
8. (p-Cymene)	1·55	0·2	0·1													
9. (Terpinolene)	1·65	0·4	0·2													
(b) Oxygenated																
10. *l*-Thujone	0·63	76·0	77·5	79	77	82	82	85·5	86	86	84	86	85	86	88	87
11. *d*-Isothujone	0·68	7·5	7·8	8	7	8	7	8·5	9	8	8	7	7	9	7·5	8·5
12. (Arom-ester)	0·82	1·9	1·5	0·5	1	1	2·5	1·5	1	0·8	2·5	1	1	1·3	0·5	0·5
13. *d*-Terpinen-4-ol	1·39	1·7	1·8	2	3	1	1	1·5	1	1·5	0·8	2·4	3	0·5	0·5	0·5
Age of branchlets (years):		mixed	mixed	4–5	3–4	3–4	3	1–2	1	1–2	1	2	1	3–4	2–3	1–2

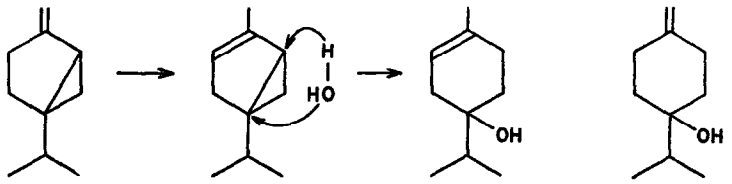
* Names in parentheses refer to compounds identified by retention times only.

† Relative retention times on the adipate polyester column: (a) hydrocarbons at 60° and 150 ml helium per minute.
(b) oxygenated terpenes at 110° and 100 ml helium per minute.

‡ Composition given as % of total in sample.

of the mature tree growing under natural conditions, and that analysis of the oil from individual branchlets would give a fair answer to the question whether or not there exists a considerable variation in the composition of the oil from branchlets at different locations on a tree. The branchlets were picked at sites 1 to 10 during January 1962 and A, B and C on March 12th, as indicated in the schematic Fig. 2, and the analytical results (GLC data only) are shown in Table 1. Samples 1 to 4 were older leaves (approx. 3–5 years old) whereas samples 5 to 10 were younger (approx. 1–2 years). The oils from these leaflets were found to be rather similar in composition, but a distinctly lower amount of hydrocarbons is characteristic of the younger leaves. The recorded values are outside of the experimental error and consistent in this trend. No other characteristic difference could be detected. Samples A, B and C were all three low in hydrocarbon content. This may be due to the return of the growing season before this branch was collected.

The relatively simple composition of the oil from *Thuja plicata* foliage should lend itself to a study of the biosynthesis of the thujones and possibly of sabinene. Earlier attempts to explain the formation of monoterpenes in plants have been reviewed by Haagen-Smit.[7] It is generally thought that a non-cyclic monoterpene, e.g. geraniol or citronellal, cyclizes specifically to the cyclic monoterpenes[7a] found in a particular plant. However, practically no data on this cyclization step in the sequence acetate → mevalonic acid → isopentyl pyrophosphate → geranyl pyrophosphate → monoterpenes[8,9] are available and no pathway for the formation of a cyclopropane ring, as found in the thujones and sabinene, has been put forward as yet. The absence of thujyl alcohol in the leaf oil of *Thuja plicata* suggests that thujone is not derived by oxidation of this alcohol, nor do the two *Thuja* species discussed here appear to have a reductive mechanism of the type ketone → alcohol → acetate as was found to occur by Reitsema[10] in the oil of peppermint. This type of sequence appears to be present in the oils from spruce leaves.[1] However, it has to be demonstrated as yet that thujyl alcohol is not formed during other times of the year. The formation of terpinen-4-ol (III) may be explained by isomerization of sabinene (I) to α-thujene (II) followed by hydrolytic cleavage of the cyclopropane ring. Hydrolytic cleavage of sabinene to 1-isopropyl-4-methylene cyclohexanol (IV) followed by isomerization to terpinen-4-ol



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seems less likely, since α-thujene was identified in the oil from *Tuja occidentalis*, whereas IV was not found in either oil, nor is it known to occur in nature. Experiments are in progress in this laboratory to determine whether radioactive precursors can be converted efficiently to the terpenoid constituents in the oil of *Thuja plicata* and it is hoped to report on the biosynthetic pathways of the thujones and possibly other constituents in the future.

[7] A. J. HAAGEN-SMIT, *Ann. Rev. Pl. Physiol.* **4**, 305 (1953).
[7a] L. RUZICKA, EXPERIENTIA, **9**, 357 (1953).
[8] F. LYNEN and U. HENNING, *Angew. Chem.* **72**, 820 (1960).
[9] A. TODD, *Nature* **187**, 819 (1960).
[10] R. H. REITSEMA, *J. Am. Pharm. Assoc., Sci. Ed.* **47**, 267 (1958).

EXPERIMENTAL

The GLC experiments were carried out with a modified Beckman GC-2 chromatograph and an instrument of similar design built in this laboratory.[11] The columns used for both the analytical runs (0·1–10 μl) and preparative runs (10–100 μl) have been described previously.[1,6] The quantitative composition of the oils was determined by measuring the area under the peaks by means of the triangulation method. Janak[12] has reported the mean error of this method to be 0·9, 3·4, 9·5 and 16·5 per cent respectively for peaks of 301–1200, 101–300, 51–100, and 0–50 mm^2 area. Runs with mixtures of known terpene hydrocarbons, alcohols, and ketones on the columns used in this study showed the errors to be within these limits. However, as noted before,[6] the values for the hydrocarbons tended to be high whereas those for the oxygenated components were on the low side. The best analytical runs were obtained on a 365×0·6 cm (12 ft×¼ in. o.d.) rapeseed oil (RO) column and 183×0·6 cm adipate polyethylene glycol polyester (APEG) and polyethylene glycol 1540 (PEG 1540) columns. A typical run on the RO column with the leaf oil of *Thuja occidentalis* has been shown previously.[6] Figure 1 is a reproduction of the recorder chart obtained with the oil (3 μl.) from *Thuja plicata* on the PEG 1540 column. Relative retention times (RRT) of hydrocarbons were measured with reference to limonene (1·00) and those of oxygenated monoterpenes with reference to camphor (1·00) and the values obtained on the APEG column are shown in Table 1. As was done previously, the retention times were measured from the time of injection to the initial emergence of the peaks.[13] Samples were injected either with Hamilton microsyringes or the Beckman liquid sampler No. 224,000.

Reference compounds were either commercial samples or those obtained previously in the analysis of conifer leaf oils. Thujyl alcohol was prepared from thujone (90% *l*-thujone, 10% *d*-isothujone) by reduction with lithium aluminum hydride in the usual manner. Thujyl acetate was obtained by acetylating thujyl alcohol with acetic anhydride–pyridine mixture at room temperature. α-Phellandrene, myrcene and car-3-ene were gifts. Infrared spectra were recorded with a Perkin–Elmer model 21 double beam spectrophotometer, liquid samples being placed between two sodium chloride plates. Optical rotations of the oils were measured undiluted, whereas those of individual components were determined as 1–10 per cent solutions in chloroform.

Isolation of the oils

The foliage (1500 g) of a mature western red cedar tree, harvested on January 3, 1962, in the Prince Rupert area, B.C., and water (5 l.) were blended in a Waring blender. The mixture was transferred to a 10 l. distillation flask and was steam-distilled for 3 hr. The distillate was saturated with sodium chloride and was extracted with three portions (25 ml) of ether. The ether layer was washed with saturated sodium bicarbonate solution (5 ml) to remove traces of free acids and then with water (5 ml). The ethereal solution was dried over anhydrous sodium sulphate and was then evaporated on a steam bath in a 100 ml flask having a long neck. The residual oil (30·5 g, 2·03 per cent) had n_D^{25} 1·4560, d_4^{25} 0·912, $[\alpha]_D^{25}$ +2·25 and an ester number of 0·6.

The branchlets (290 g) of a 5-year-old tree growing in the greenhouse of the Prairie Regional Laboratory (transferred from a Vancouver nursery in April 1961) were picked at random on December 6, 1961. Steam distillation gave a slightly better yield of oil (6·7g)

[11] B. M. Craig and N. L. Murty, *J. Am. Oil Chem. Soc.* **36**, 549 (1959).
[12] J. Janák, *J. Chromatog.* **3**, 308 (1960).
[13] E. von Rudloff, *Can. J. Chem.* **38**, 631 (1960).

2·31 per cent) on a fresh weight basis, but this was practically the same on a dry weight basis (5·5 per cent). The oil had n_D^{25} 1·4565, d_4^{25} 0·911, $[\alpha]_D^{25}$ +1·85 and ester number 0·7.

In small scale experiments the individual small branchlets (1–2 g) were picked from the young local tree at specific locations 1 to 10 as shown in Fig. 2 during the second and third week of January 1962. They were ground to a fine powder in a mortar with solid carbon dioxide (1–2 g) which was transferred to a Markham steam distillation apparatus.[14] Steam was passed through for 0·5 hr and the neutral volatile oil was recovered as above. After evaporation of most of the ether in the long neck flask the oil was transferred quantitatively to a weighed vial (10 ml) and the residual ether was boiled off on a steam bath. Heating

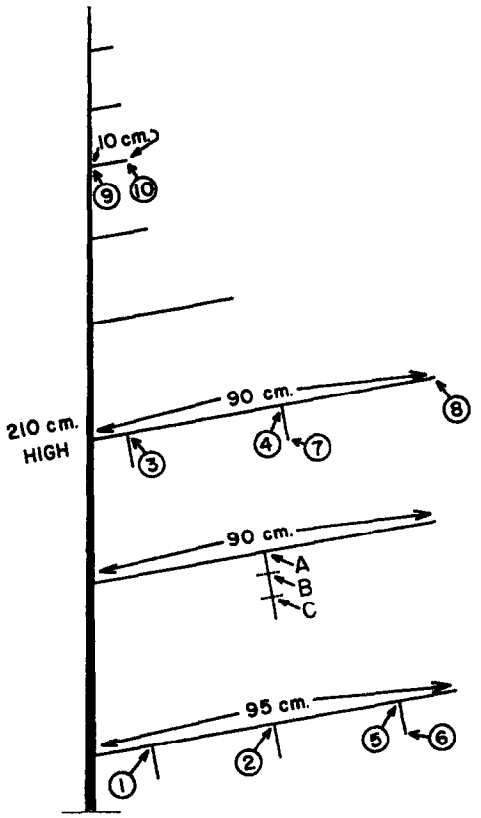

FIG. 2. SCHEMATIC DRAWING OF LOCATION OF BRANCHLETS PICKED FROM 5-YEAR-OLD *Thuja plicata* TREE.

Samples 1 to 10 picked during second and third week of January 1962; samples A to C on March 12, 1962.

was continued 3–5 min after visual examination showed the ether to have been evaporated. Trial experiments with α-pinene, limonene and thujone (10–20 mg) showed that losses due to volatility were less than 5 per cent under these conditions. The amount of residual ether varied from 3–10 per cent. If heating was continued until the residual ether was less than

[14] R. MARKHAM, *Biochem. J.* 36, 790 (1942).

1 per cent, losses of the terpenes rose to over 10 per cent. Because of these difficulties in small-scale experiments it was not possible to determine the yield of oil from the individual branchlets accurately. However, the trial runs did show that if heating was continued for only 3–5 min the small losses of hydrocarbons and oxygenated terpenes were about the same and practically within the error of the method. On the basis of the lower boiling points of terpene hydrocarbons (160–190°) as compared with those of terpene ketones and alcohols (190 to about 230°) one would expect the losses due to volatility to be somewhat larger with the former compounds. However, this appears to be cancelled out by the tendency of the calculated GLC values for the hydrocarbons to be somewhat higher than those of oxygenated terpenes (see above). Thus no correction factor was found to be necessary and aliquots (1–10μl) of the oils from single branchlets were analysed in the same manner as the oils from the larger scale experiments on the APEG, PEG 1540, SE-30 silicone and RO columns.

Samples A, B and C (see Fig. 2) were from a secondary branch, picked on March 12, which was divided into three parts (3–4 g each).

Identification of components

Analysis of the oils on the APEG and SE-30 silicone columns at 160°[1] showed that no sesquiterpene hydrocarbons, alcohols, or ketones were present. The oils were then analysed for their content of monoterpene hydrocarbons at 60 and 80° and that of oxygenated monoterpenes at 80° to 130°. The RRT values were determined by comparison of retention times with those of the reference compounds (limonene, camphor) and these were used for the tentative identification of individual components. The values obtained on the different columns for components 1 to 3, 5 to 11, and 13 corresponded with those of α-pinene, camphene, sabinene, limonene, 1,8-cineole, γ-terpinene, *p*-cymene, terpinolene, thujone, isothujone, and terpinen-4-ol respectively. Individual components were separated on a preparative scale (10–50 mg) and the collected fractions were used for recording the infrared spectra, optical rotation and for reinjection on to analytical columns to confirm RRT values and purity. Because of the large amounts of the thujones present in this oil the RRT values of the components eluted after thujone and isothujone were found to be somewhat high, and the values reported for components 12 and 13 are those obtained after reinjection of isolated fractions. The infrared spectra and rotational measurements confirmed the identity of *d*-α-pinene, *d*-sabinene, *d*-limonene, *l*-thujone, *d*-isothujone and *d*-terpinen-4-ol. Insufficient amounts of the other components were obtained in the pure state and their identity could not be confirmed. Reinjection of a crude fraction of component 2 indicated (by RRT) the presence of α-fenchene besides camphene. Component 4 had RRT values close to those of myrcene on all columns except on the PEG 1540 column. Its infrared spectrum, however, differed from that of myrcene and suggests the presence of a cyclopropane ring (3042 cm^{-1}). Since myrcene and car-4-ene have nearly the same boiling points, component 4 could be the latter compound. Component 12 was also isolated fairly pure. The infrared spectrum had strong bands at 1515 and 1245 cm^{-1}, and bands of medium intensity at 2950 (shoulders at 3060 and 2830), 1725, 1637, 1610, 1150, 1035, 905 and 805 cm^{-1}. This spectrum (as well as the RRT values) could not be correlated with a known terpene, but it suggests the structure of an aromatic acetate. A very weak positive optical rotation was measured.

Comparison of retention data (allowing for possible displacement by the large thujone peaks with components being eluted later than thujone and isothujone) showed that

β-pinene, car-3-ene, α-phellandrene, thujyl alcohol, thujyl acetate, borneol, bornyl acetate, fenchone and camphor were not present in measurable amounts.

Acknowledgements—The gift of terpene samples by Dr. Sukh Dev, National Chemical Laboratories, Poona, India, and Dr. P. J. Porcaro, The Givaudan Corp., Delawana, U.S.A., and the shipment of western red cedar foliage by Messrs. Columbia Cellulose Company Ltd., Prince Rupert, B.C., is gratefully acknowledged. Technical assistance was rendered by Mr. M. Granat and the infrared spectra were recorded by Mr. W. C. Haid.

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PLANT POLYPHENOLS. VIII. CHALCONE AND FLAVONOL GLYCOSIDES OF GORSE FLOWERS*

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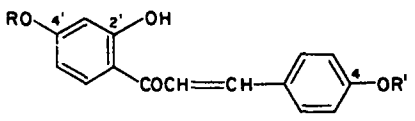
(*Received* 19 *March* 1962)

Abstract—The 4'-glucoside, the 4'-glucosylglucoside, the 4,4'-diglucoside and the 4-glucoside-4'-glucosylglucoside of 4,2',4'-trihydroxychalcone have been found in the flowers of gorse, *Ulex europaeus*. Five flavonol glucosides are also present: the 7-, the 4'-glucoside and the 3,7-diglucoside of quercetin and two glucosides of 3,7,4'-trihydroxyflavone. The biogenetic and taxonomic significance of these findings are discussed.

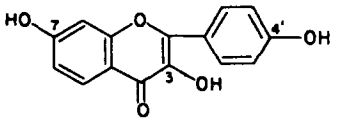
INTRODUCTION

PREVIOUS papers on the glycosidic pattern of plant phenols have dealt mostly with the anthocyanins, flavonol glycosides and cinnamic acids.[1,2,3,4] The opportunity to study the glycosidic pattern of chalcones arose from a chance discovery, made while surveying plants for their cinnamic acid esters,[4] that flowers of gorse, *Ulex europaeus*, contain a mixture of pigments belonging to the chalcone class. In view of the relative rarity of chalcones in nature (*cf.*[5]) and also because of the interest that is being taken in the Leguminosae as material for combined chemical and taxonomic studies,[6,7,8] the gorse pigments



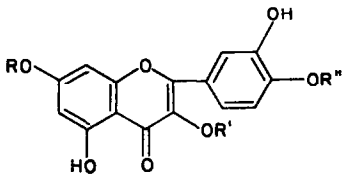
(I)

4,2',4'-trihydroxychalcone (R=R'=H)



(II)

3,7,4'-trihydroxyflavone



(III)

Quercetin (R=R'=R"=H)

* Part 7: J. J. CORNER, J. B. HARBORNE, S. G. HUMPHRIES and W. D. OLLIS, *Phytochemistry*, **1**, 73 (1962).

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TABLE 1. SPECTRAL PROPERTIES OF GORSE PIGMENTS

Pigment	λ max in 95% EtOH (mμ)* Alone	+2NNaOH (2 drops/3 ml)	ΔO.D.alk (as %)	+5% AlCl (2 drops/3 ml)†	Satd with NaOAc‡	Satd. with NaOAc and H_3BO_3†
*B*1.1	240,373	422	+6	378,420	240	—
*B*1.2	242,372	425	−3	378,415	242	—
*B*3.1	237,360	410	−63	378,415	237	—
*B*3.2	237,361	410	−49	377,410	239	—
*B*2.2	254,367	429	—	350,423	269	367
*B*3.11	257,373	decomp.	—	350,425	257	389
*B*4.1	258,363	420	—	370,402	259	385
*B*2.1	261,357	404	—	352,405	264	357
*B*4.2	260,354	—	—	352,395	—	—

* — not measured.
† Long wavelength peak only.
‡ Short wavelength peak only.

TABLE 2. COLOURS AND *Rf* VALUES OF GORSE PIGMENTS

Pigment	Colours in Ultra-violet	Ultra-violet +ammonia	*Rf* values in* BAW	H_2O	PhOH	BEW
*B*1.1	dull dark brown	bright orange	0·61	0·06	0·80	0·64
*B*1.2			0·53	0·24	0·56	0·31
*B*3.1		dull brown	0·34	0·19	0·70	0·30
*B*3·2			0·36	0·80	0·40	0·18
*B*2.2	dull yellow	bright yellow	0·48	0·01	0·35	0·46
*B*3.11	bright yellow		0·34	0·01	0·31	0·29
*B*4.1	dull ochre		0·30	0·33	0·32	0·24
*B*2.1	fluorescent yellow-green	fluorescent yellow-green	0·42	0·04	0·71	0·32
*B*4.2			0·27	0·10	0·58	0·17

* On Whatman No. 1 paper.

TABLE 3. HYDROLYSIS PRODUCTS OF GORSE PIGMENTS

Pigment	Hydrolysis products	Aglycone : sugar ratio	Identified as the	Concentration in fresh flowers†
*B*1.1	2'4'4-trihydroxy-chalcone and glucose	1 : 1·06	4'-glucoside	+++
*B*1.2		*	4'-glucosylglucoside	++
*B*3.1		1 : 1·99	4,4'-diglucoside	+++
*B*3.2		†	4-glucoside, 4'-glucosylglucoside	++
*B*2.2	quercetin and glucose	1 : 0·98	4'-glucoside	+++
*B*3.11		‡	7-glucoside	+
*B*4.1		1 : 2·15	3,7-diglucoside	++
*B*2.1	3,7,4'-trihydroxy-flavone and glucose	‡	4'(?)-glucoside	+
*B*4.2		‡	7(?)-glucoside	+

* Not hydrolysed by β-glucosidase.
† Gave *B*1.2 and glucose (1·16 equivs.) on hydrolysis by β-glucosidase.
‡ Present in concentration too low for this determination.
\+ Abundant (+++), scarce (++), trace (+).

were examined in some detail. In earlier studies[9,10] three pigments (two being called "ulexoside" and "ulexflavone") were isolated from gorse. While it was established that these pigments were flavonoid in nature, no structures were advanced for them.

RESULTS

Four chalcone glucosides and five flavonol glucosides have been isolated and identified. The R_f values and the spectral and other properties of these compounds are listed in Tables 1 and 2. Four of the pigments yield 4,2',4'-trihydroxychalcone (*iso*-liquiritigenin) (I, R = R' = H) and glucose on hydrolysis (Table 3). Two of these, *B*1.1 and *B*1.2, exhibit a characteristic orange fluorescence on paper chromatograms in the presence of ammonia, indicating that the 4-hydroxyl groups are free. Spectral measurements show that in *B*1.1 and *B*1.2 the 2'-hydroxyl group is free (positive $AlCl_3$ shift)[11] and that the 4-hydroxyl is also free (the spectral maxima in alkaline solution is equal to or greater than that measured at neutral pH).[11]

Since *B*1.1 contains one equivalent of glucose, it must be the 4'-glucoside (I, R = Glc, R' = H), a compound that has previously been found in *Dahlia variabilis* petals.[12] Indeed *B*1.1 agrees in all its properties with those described by Nordstrom and Swain[12] for the *Dahlia* pigment. It is clear from R_f values that *B*1.2 contains two equivalents of glucose and it must be accordingly the 4'-glucosylglucoside (I, R = Glc–Glc, R' = H), which has also been isolated previously from *Dahlia* flowers.[12]

The other two chalcone glucosides, *B*3.1 and *B*3.2, are new pigments and are identified as the 4,4'-diglucoside (I, R = R' = Glc) and the 4-glucoside, 4'-glucosylglucoside (I, R = Glc–Glc, R' = Glc) on the basis of the following observations. (1) Neither substance, when placed on filter paper, changes colour (initially dark brown) in the presence of ammonia vapour, evidence that both the 4- and 4'-hydroxyl groups are substituted. This conclusion is confirmed by spectral measurements in alkaline solution (the long wavelength peak is of low intensity, *cf.*[11]). (2) In neutral solution, the spectral maxima of these glucosides exhibit a hypsochromic shift of 10–11 mμ, when compared with the aglycone, 4,2',4'-trihydroxychalcone. A similar shift is shown by 2'-hydroxy, 4'4-dimethoxychalcone.[11] (3) The spectra of *B*3.1 and *B*3.2 display bathochromic shifts in the presence of aluminium ion, so that both substances must have unsubstituted 2'-hydroxyl groups. (4) *B*3.1, which contains two glucose residues, is readily and completely hydrolysed by β-glucosidase, proving that the two sugars are not combined in a disaccharide unit. By contrast, *B*3.2 must contain a disaccharide, since it is not hydrolysed completely by this enzyme; in fact, enzymic hydrolysis of *B*3.2 yields the 4'-glucosylglucoside (*B*1.2) and glucose (1·16 equivalents). (5) The R_f values in several solvent systems are consistent with the proposed structures.

Of the five flavonol glucosides present, two (*B*2.2 and *B*3.11) are known compounds, i.e. the 7- and 4'-monoglucosides of quercetin. Identifications are based on direct comparisons with authentic materials. The 7-glucoside (quercimeritrin) (III, R = Glc, R' = R'' = H) has been isolated from at least six plants (e.g. *Helianthus annuus*[13]) and the 4'-glucoside (spiraeoside) (III, R'' = Glc, R = R' = H) has recently been found in the skin of *Allium cepa*[14] and in the flowers of *Spiraea ulmaria*,[15] *Hamamelis japonica*[16]

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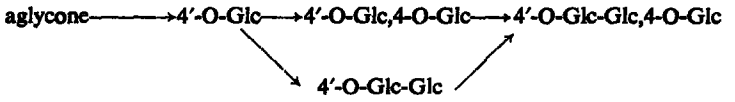
and *Aesculus hippocastanum*.[17] A third quercetin glucoside (*B*4.1) is present in gorse and this has been identified as the previously unknown 3 : 7-diglucoside (III, R = R′ = Glc, R″ = H). Thus, it shows all the characteristic properties of 3 : 7-diglycosides,[3] yields on partial hydrolysis the related 3- and 7-monoglucosides and, on methylation and hydrolysis, is converted to 5,3′,4′-tri-O-methylquercetin. It is of interest that the related 3 : 7-diglucoside of kampferol has recently been found in the petals of *Paeonia albiflora*.[18]

The remaining two flavonol glucosides (*B*2.1 and *B*4.2) are unusual in possessing the characteristic intense yellow green fluorescence on filter paper, found only among the flavonols lacking a 5-hydroxyl substituent or having protected 5-hydroxyl groups. The aglycone formed on hydrolysis of *B*2.1 and *B*4.2 also had a yellow green fluorescence and was readily identified as 3,7,4′-trihydroxyflavone (II). This substance has been isolated only once before; it occurs in non-glycosidic form in Quebracho (*Schinopsis*) wood.[19] Both the glucosides of 3,7,4′-trihydroxyflavone are present in gorse in such small amounts that it has not been possible to prove conclusively the position of their glucose substituents. However, from the R_f values and spectral data, it is likely that *B*2.1 is the 4′-glucoside and *B*4.2 is the 7-glucoside.

DISCUSSION

From the systematic viewpoint, it is of interest that *Ulex europaeus*, a member of the Leguminosae, contains chalcone derivatives. Indeed, chalcones appear to be almost confined in their occurrence to two families, the Compositae and the Leguminosae. Previous records of chalcones in legumes are as follows: 4,2′,4′-trihydroxychalcone (*Glycyrrhiza glabra*, root[20]); butein (*Butea frondosa*, flowers[21]); neoplathymenin (*Plathymenia reticulata*, heartwood[22]); and okanin (*Cylicodiscus gabunensis*, heartwood[23]). Chalcones thus seem to be uncommon in the family and this impression is supported by the failure to find other examples in the course of a (restricted) survey of other members. Examination of petal extracts of plants belonging to other genera, and particularly of other species with yellow flowers (i.e. *Cytisus scoparius*, *C. graminifolia*, *Genista hispanica*, *G. tinctoria*, *Laburnum anagyroides* and *Spartium junceum*) failed to reveal the presence of any chalcone pigments. The chalcones are thus less distinctive of the Leguminosae than are the isoflavones, which are virtually confined to the group and are of rather common occurrence in it.[24]

From the biosynthetic viewpoint, the co-occurrence in gorse of four related chalcone glucosides strongly suggests that the glucosylation of chalcones takes place in the same way as that of anthocyanidins[1] and flavonols,[3] that is by the stepwise transfer of glucose from uridinediphosphateglucose to the appropriate chalcone derivative. It is not possible, at the moment, to say which of the following two alternative routes occur:



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The occurrence of flavonols and chalcones with similar, but not identical, glucosidation patterns suggests that some of the enzymes in gorse are used in the synthesis of both classes of pigment glycoside. Finally, the co-occurrence in gorse of a chalcone (4,2',4'-trihydroxy) (I) and a flavone (3,7,4'-trihydroxy) (II) with the same hydroxylation pattern supports a current theory of flavonoid biosynthesis in which chalcones are regarded as precursors of flavonols and anthocyanidins.[25]

EXPERIMENTAL

Authentic materials

Samples of 3,7,4'-trihydroxyflavone and quercetin 7-glucoside were provided by Dr. T. Swain, Cambridge and by Dr. W. Siegelman, USDA, Beltsville. Quercetin 4'-glucoside was isolated from onions[15] and 3,5,7,3'-tetra-*O*-methyl quercetin was prepared from it by standard procedures.[15]

Paper chromatography

The solvents used, with their abbreviations, are as follows: BAW, butan-1-ol–acetic acid–water (4 : 1 : 5, vol./vol.); BEW, butan-1-ol–ethanol–water (4 : 1 : 2·2, vol./vol.); BW, butan-1-ol–water (1 : 1, vol./vol.); PhOH, water-satd.–phenol; H_2O, water; and 15% HOAc, acetic acid–water (15 : 85, vol./vol.).

Isolation and separation of pigments

Fresh gorse flowers were plunged into boiling 95% ethanol, and the resulting solution was concentrated to a small volume *in vacuo* and this was applied as streaks on to Whatman No. 3 filter paper. Chromatography in BAW gave four main bands: *B*1, R_f 0·71; *B*2, R_f 0·60; *B*3, R_f 0·49; and *B*4, R_f 0·34. *B*1 and *B*3 were yellow in visible light and dull brown in the ultra-violet; *B*2 and *B*4 were bright yellow in ultra-violet light. *B*1 was rechromatographed in BEW and H_2O and finally in BAW, when it separated into *B*1.1, R_f 0·71 and *B*1·2, R_f 0·67. *B*2 separated, when run in BEW, into *B*2.1 (R_f 0·55) and *B* 2.2 (R_f 0·50) the latter being distinguished by its brilliant yellow-green fluorescence. Similarly, *B*3 in BEW separated into *B*3·1 (R_f 0·46) and *B*3.2 (R_f 0·40). *B*3.1 separated in H_2O into pure *B*3.1 (R_f 0·15) and *B*3.11 (R_f 0·03). *B*4 separated in H_2O into *B*4.1 (R_f 0·41) and *B*4.2 (R_f 0·16). All these bands were then purified by repeated chromatography in two or more of the solvent systems listed above.

Identification of pigments

This was carried out using the methods described in previous papers in this series[1-4] (see also[12] and refs. therein). The more important spectral and chromatographic date are collected in Table 1. Although R_f values are only quoted for four solvent systems, chromatography was, in fact, carried out in six different solvents when comparing the pigments and their aglycones with authentic compounds. In view of the ease with which 2'-hydroxy chalcone derivatives cyclise to flavanones in the presence of acid, special care was taken to avoid this complication when dealing with pigments *B*1.1, *B*1.2, *B*3.1 and *B*3.2. For the same reason, enzyme (β-glucosidase) rather than acid hydrolysis was used in the determination of aglycone : sugar ratios. Similarly, methylation and subsequent hydrolysis of the pigments gave complex mixtures of substances (see also: Nordstrom and Swain[12]) in which it was not possible to identify the relevant partially methylated chalcone.

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Acknowledgements—The author is grateful to Dr. T. Swain for a gift of 3,7,4'-trihydroxyflavone and to Dr. W. Siegelman for a gift of quercetin 7-glucoside.

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THE BIOGENESIS OF STACHYDRINE*

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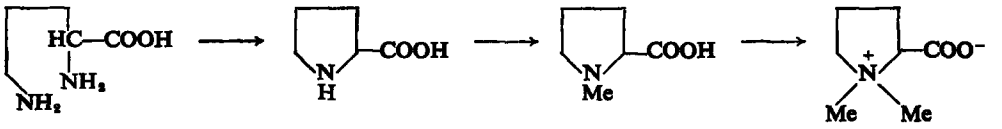
(*Received* 29 *March* 1962)

Abstract—The amino-acids ornithine and proline have been shown to serve as precursors of the alkaloid stachydrine in the mature 5½–6 month old alfalfa plant. Degradation of the alkaloid obtained from experiments with DL-ornithine-2-^{14}C has shown that no randomization occurs and that the label is entirely located on carbon atom 2.

INTRODUCTION

THE biogenesis of the alkaloid stachydrine (IV) in alfalfa (*Medicago sativa* L. Grimm) has been the subject of several communications from this laboratory.[1-5] Experiments have been carried out in order to demonstrate the occurrence of the following biogenetic sequence:



I ornithine — II proline — III hygric acid — IV stachydrine

The results obtained have been summarized and discussed by Robertson and Marion.[4]

It has been shown that in plants aged 2–3 weeks, radioactive stachydrine is produced by feeding Me-^{14}C-methionine[4] or carboxyl labelled hygric acid,[5] but not by feeding radioactive ornithine,[1] proline[4] or glutamic acid.[3]

RESULTS

It has now been demonstrated that in 3-week old alfalfa plants even $^{14}CO_2$ is not utilized in the synthesis of the alkaloid. On the other hand, radioactive stachydrine can be isolated from mature flowering alfalfa plants which have been exposed to an atmosphere of $^{14}CO_2$ for one hour. This supplies further evidence that the young plant does not normally synthesize stachydrine which, however, is produced in the mature plant just before seed formation.

In another qualitative experiment two mature plants were fed via the roots with proline labelled with ^{14}C in the carboxyl group. The alkaloid subsequently isolated from these plants was radioactive. It thus appears that in the mature plant the synthesis of the alkaloid does follow the biogenetic pathway outlined above.

Following these results information has now been obtained concerning the role of

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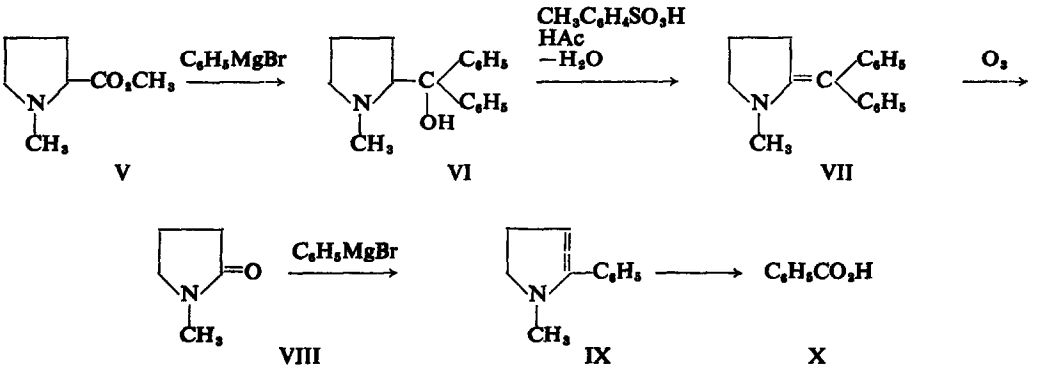
[4] A. V. ROBERTSON and L. MARION, *Can. J. Chem.* **37**, 1197 (1959).
[5] A. V. ROBERTSON and L. MARION, *Can. J. Chem.* **38**, 396 (1960).

ornithine in stachydrine biogenesis in the mature plant from experiments in which firstly ornithine-2-^{14}C and pyridoxine, and secondly ornithine-2-^{14}C alone were fed to 5½–6 month old plants by means of a glass capillary tube plugged into the base of each stem. It was found that feeding of the labelled precursor by direct injection into the plant stem by such means results in much better radioactive yields than when the precursor is added to a hydroponic solution to which the plants have been transferred. In each case the stachydrine isolated from the plants was radioactive. The active stachydrine obtained by feeding ornithine-2-^{14}C was degraded so as to isolate carbon 2 of the stachydrine ring. Within experimental error, all of the incorporated activity was found to be associated with this carbon atom.

The method used to isolate carbon 2 was that described by Leete, Marion and Spenser[1] with the exception that the carbinol (VI) was dehydrated with *p*-toluenesulphonic acid in glacial acetic acid since, in our hands, dehydration under the conditions described led mainly to the formation of the acetate of the carbinol.



DISCUSSION

It may thus be concluded that in the mature plant, ornithine is the precursor of stachydrine, and proline is an intermediate in the biogenetic pathway. It is known that in 3 week old alfalfa plants ornithine is converted into glutamic acid but not into proline, although this conversion can be induced by feeding pyridoxine.[2] The conversion of glutamic acid into proline can also be induced by the same method.[3] Even in the presence of pyridoxine, however, proline does not give rise to stachydrine in the 3 week old plant[4] and the previous conclusion of Wiehler and Marion[3] that this could be induced by folic acid has been shown to be in error.[4]

Since the young plant can synthesize stachydrine from hygric acid[5] the methylation of the latter is possible, but it appears that the 3-week old plant lacks the coenzymes necessary for the steps involved in the reactions: ornithine→hygric acid. By the time the plant reaches the flowering stage, however, all the necessary coenzymes are present and the alkaloid is synthesized from the precursor ornithine.

Proline is present in the 3-week old plant and like stachydrine, which is also present, has likely been translocated from the seed, because it is not then being formed from ornithine[2] or glutamic acid.[3] It is noteworthy that in the mature plant, which actively synthesizes the alkaloid, proline can no longer be detected[6] and hence must be used up immediately it is formed for the synthesis of the alkaloid.

[6] D. J. McCaldin and L. Marion, unpublished results.

EXPERIMENTAL

Incorporation of radioactive carbon dioxide by alfalfa plants

Approximately 200 alfalfa seedlings (22 days old) were enclosed in a polyethylene envelope and treated with $^{14}CO_2$ (approx. activity 660×10^6 d.p.m.) for 1 hr under strong light. They were then removed from the envelope and set aside for 4 hr. The plants were collected, washed with cold water, shaken to remove excess moisture and plunged into a mortar containing liquid nitrogen. When the nitrogen had evaporated, the plant material was ground to a fine powder, and boiled with 70% aqueous ethanol for 30 min. The mixture was filtered, the filtrate concentrated until material began to separate, and extracted with 1·5 vol. of chloroform. The chloroform extract was discarded, and the aqueous ethanolic layer concentrated to less than 1 ml in a stream of air. This residue was separated by two-dimensional paper chromatography using two single phase systems:[7] *n*-butyl acetate–glacial acetic acid–water (3 : 3 : 1) and pyridine–conc. ammonia–isobutylalcohol (4 : 2 : 1). The radioactive substances on the chromatogram were detected photographically by contact with an X-ray plate (Kodak No-screen) for 6 days. The amino-acids were detected by the ninhydrin method, and stachydrine by Dragendorff's reagent.

A similar experiment was conducted on one flowering plant (5½ months old). In this case the chromatographic procedure revealed the presence of interfering radioactive material and a further purification of the ethanolic plant extract was introduced. The ethanolic solution was concentrated to a volume of ca. 5 ml and placed on an acid-washed ion-exchange (Dowex 50) column. The radioactive materials were eluted from the column as shown in Table 1. Fraction 4 was subjected to two-dimensional paper chromatography as described above. A major portion of the radioactivity was contained in a spot which had an R_f

TABLE 1.

Fraction	Eluant	Synthetic mixture containing stachydrine ($^{14}CH_3$)	Sample from plant (5½ months)
1	Water	2100	11,960
2	HCl in ethanol (*N*)	659	5650
3	HCl in water (*N*)	714	18,600
4	HCl in water (5*N*)	52,900	49,430

value similar to that of stachydrine hydrochloride developed on a separate chromatogram. These spots were eluted from the papers and co-chromatographed: the materials proved to be identical.

Feeding experiment with radioactive proline

Two alfalfa plants were grown in soil and when in bloom at the end of 5½ months they were transferred to hydroponic solutions.[8] A solution of proline labelled with ^{14}C in the carboxyl group (5 ml, total activity $2{\cdot}2 \times 10^8$ d.p.m.) was divided into two portions and added to the solutions. The quantity of nutrient was kept to a minimum (150 ml) to ensure efficient uptake of the radioactive proline. After 8 hr, less than 5 per cent of the activity remained in the solution. The plants were then kept overnight with their roots immersed

[7] D. P. BURMA and D. C. MORTIMER, *Arch. Biochem. Biophys.* **62**, 16 (1956).
[8] E. LEETE, *J. Am. Chem. Soc.* **78**, 3520 (1956).

in distilled water and extracted in the usual way. The aerial portions of the plant yielded the most satisfactory chromatograms. The proline spots were found to be inactive whereas the stachydrine spots were radioactive.

Feeding experiment with radioactive ornithine and pyridoxine

Twenty-two alfalfa plants (*Medicago sativa* L. Grimm) were grown in soil in pots to an age of 5½ months. An aqueous solution (0·1 ml, $4\times10^{-4}M$) of pyridoxine was introduced into each plant by means of a bent glass capillary tube plugged into the base of each plant stem. After one day, an aqueous solution of DL-ornithine-2-^{14}C hydrochloride (16·9 mg in 2·2 ml distilled water) was distributed equally among the plants by the same method. The total added activity was $1\cdot2\times10^8$ d.p.m. After 3 days 11 of the plants were harvested and the stachydrine isolated as described by Robertson and Marion[9] (at this plant age stachydrine is the only alkaloid present). This gave 48 mg stachydrine hydrochloride of specific activity 790 d.p.m./mg. This represents a total activity of $3\cdot8\times10^4$ d.p.m., i.e. an incorporation of 0·13 per cent of the administered activity.

The remaining 11 plants were grown for a further 3 days before being extracted, and yielded 53 mg stachydrine hydrochloride of specific activity 1100 d.p.m./mg. The total activity is thus $5\cdot8\times10^4$ d.p.m. or 0·19 per cent of the added activity. The radioactive samples were counted on aluminium planchets with a Radiation Counter Laboratory Nucleometer counting about 45 per cent of the disintegrations.

Feeding experiment with radioactive ornithine

Thirty-two alfalfa plants (*Medicago sativa* L. Grimm) were grown in soil in pots to an age of 6 months. An aqueous solution of DL-ornithine-2-^{14}C hydrochloride (23·1 mg in 3·2 ml distilled water) was administered to the plants via bent glass capillary tubes. The total activity thus introduced was $3\cdot0\times10^8$ d.p.m. After 3 days 16 of the plants were harvested and the alkaloid isolated as before. The stachydrine hydrochloride obtained (40 mg) had specific activity 2820 d.p.m./mg. This corresponds to an incorporation of 0·15 per cent of the added tracer. After a further 3 days the remaining 16 plants yielded 48 mg of stachydrine hydrochloride of specific activity 3120 d.p.m./mg. This is a total activity of $1\cdot5\times10^5$ d.p.m. or 0·2 per cent of the administered activity. These samples were counted on aluminium planchets using a Baird-Atomic proportional flow counter measuring 21 per cent of the disintegrations.

*Degradation to isolate carbon 2 of stachydrine**

The L-stachydrine hydrochloride obtained from the experiment with labelled ornithine was mixed with an inactive synthetic sample prepared by the method of Cornforth and Henry.[10] This mixture was repeatedly recrystallized from ethanol–ethyl acetate until successive samples with identical activity were obtained. The degradation was carried out on 850 mg L-stachydrine hydrochloride of specific activity $2\cdot1\times10^4$ d.p.m./millimole. Conversion to methyl hygrate (V) was carried out in 75 per cent yield by the method of King.[11] The methyl hygrate (510 mg) was allowed to react with phenylmagnesium bromide in dry ether under an atmosphere of nitrogen to yield the carbinol (VI) (575 mg, 62 per cent

* All melting points are uncorrected.

[9] A. V. ROBERTSON and L. MARION, *Can. J. Chem.* 37, 1043 (1959).
[10] J. W. CORNFORTH and A. J. HENRY, *J. Chem. Soc.* 602 (1952).
[11] H. KING, *J. Chem. Soc.* 337 (1941).

yield) as a colourless oil, b.p. 40–43/0·9 mm. The carbinol and *p*-toluenesulphonic acid (600 mg) were heated under reflux for 2 hr in glacial acetic acid (5 ml). After cooling, the solution was made alkaline and extracted with chloroform. Evaporation of the solvent from the dried extracts left a gummy residue from which the ethylene derivative (VII) (390 mg, 71 per cent yield) was distilled as a colourless oil, b.p. 102–104°/1 mm. This was dissolved in dry methylene dichloride (6 ml) and an excess of ozone bubbled through the solution which was cooled to −30°. The solvent was then removed and the ozonide decomposed with dilute sulphuric acid. Extraction with ether afforded *N*-methyl-pyrrolidone-2 (VIII) (94 mg, 60 per cent yield) as a colourless oil, b.p. 82–85°/12 mm. The method of Craig[12] was used to prepare 2-phenyl-*N*-methyl-Δ^2-pyrroline (IX), 99 mg, 71 per cent, b.p. 104–112°/11 mm. This yielded a picrate, m.p. 139–140°, reported m.p. 139°;[11] 141.[1] The pyrroline (IX) (90 mg) was heated under reflux with an excess of potassium permanganate (1·2 g) in water (90 ml). After cooling, ethanol was added to destroy the excess permanganate, the precipitated manganese dioxide was removed by filtration and the acidified filtrate was extracted with ether. Evaporation of the solvent from the dried extracts and sublimation of the residue gave benzoic acid (X) (41 mg, 55 per cent yield), m.p. 121–122°, no depression of melting point on admixture with an authentic specimen. The acid was purified by repeated sublimation at 100–105°/15 mm until samples of constant activity were obtained. The acid had a specific activity of 165 d.p.m./mg or $2{\cdot}01 \times 10^4$ d.p.m./millimole. Thus the activity associated with carbon 2 of stachydrine is 96 per cent of that incorporated.

[12] L. C. Craig, *J. Am. Chem. Soc.* **55**, 295 (1933).

Phytochemistry, 1962, Vol. 1, pp. 215 to 221. Pergamon Press Ltd. Printed in England

THE ISOLATION OF HARMANE AND NORHARMANE FROM TOBACCO AND CIGARETTE SMOKE

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(*Received* 18 *May* 1962)

Abstract—Harmane and norharmane were isolated from cured tobacco and its smoke. This establishes the presence of indole alkaloids in tobacco. Quantitative studies showed that cigarette smoke contained between 15 and 20 μg of combined harmane and norharmane per gram of tobacco smoked. The tobacco itself contained only about 1 per cent of the amount found in smoke. Thus, the pyrolytic formation of the harmane alkaloids was strongly indicated. The addition of radioactive tryptophan to the tobacco resulted in the isolation of radioactive harmane alkaloids in the smoke. Further studies indicated that enough tryptophan was present in the tobacco to account for the quantities of the harmane alkaloids found in the smoke.

INTRODUCTION

DURING a study of the basic fraction of cigarette smoke (Fig. 4) two strongly fluorescent compounds were observed on paper chromatograms. These were subsequently identified as harmane (I) and norharmane (II) by ultraviolet and infrared spectrophotometry. No reference to these compounds was found in recent reviews of tobacco chemistry.[1,2]

The isolation of these alkaloids was achieved by the acidic extraction of smoke, followed by cellulose column chromatography of the free bases and subsequent resolution by paper chromatography. The isolated compounds gave identical ultraviolet, infrared, and fluorescence spectra with those given by authentic harmane and norharmane.

RESULTS AND DISCUSSION

The alkaline fraction of smoke contained considerable blue fluorescent material, most of which remained in the steam pot during steam distillation. Examination of this pot residue in numerous paper chromatographic systems showed that 15% wt./vol. aqueous NaCl gave good separation of the slow moving fluorescent material from the faster running colored pigments and the nicotine alkaloids. The non-steam-volatile basic fraction was therefore separated on a cellulose column using 15% NaCl to elute most of the latter more mobile compounds; the blue fluorescent material was then readily eluted from the column with methanol. A *tert*-amyl alcohol–acetate buffer system resolved the fluorescent fraction into several zones, two of which were intensely blue in fluorescence. The elution and rechromatography of the two blue zones resulted in chromatographically pure alkaloids for analysis.

The R_f values of the zones isolated from smoke were shown to agree with those of the known reference compounds, harmane and norharmane, in several solvent systems as shown in Table 1. The infrared spectra of the compounds isolated from smoke were determined and shown to agree very closely to the spectra of authentic reference alkaloids, harmane and norharmane (Fig. 1). The ultraviolet spectra in both 0·1 *N* HCl and diethyl

[1] R. A. W. JOHNSTONE, and J. R. PLIMMER, *Chem. Rev.* **59**, 885 (1959).
[2] H. R. BENTLEY and E. G. N. BERRY, *The Constituents of Tobacco Smoke:* An Annotated Bibliography. (Research Paper No. 3.) The Tobacco Manufacturers Standing Committee (1959).

ether of the compounds also showed close agreement with the published spectra of the known alkaloids.[3-7]

The fluorescence spectra of the zones isolated from smoke also agreed with the spectra of authentic harmane and norharmane. The fluorescence excitation spectra of the zones isolated from smoke agreed with the ultraviolet absorption spectra in all peak positions.

TABLE 1. PAPER CHROMATOGRAPHIC COMPARISON OF ZONES ISOLATED FROM SMOKE WITH REFERENCE HARMANE AND NORHARMANE

	Rf Values		
Solvent system	I	II	III
Authentic norharmane	0·73	0·10	0·91
First zone from smoke	0·74	0·11	0·91
Authentic harmane	0·60	0·10	0·94
Second zone from smoke	0·60	0·10	0·94

I. *Tert*-amyl alcohol saturated with pH 5·7 acetate buffer. The paper is pretreated with 0·2*M* ammonium tartrate buffer.
II. 15% NaCl water solution
III. *n*-butanol–HCl–water (10 : 2 : 3)

A quantitative method was developed to determine the concentration of the harmane alkaloids in smoke and in tobacco. The results obtained for the amounts of harmane alkaloids in the smoke of several types of cigarettes and in leaf are given in Table 2. Smoke from burley tobacco was shown to contain more of the harmane alkaloids than the smoke from bright tobacco; the alkaloid concentration in smoke from the standard commercial blend closely resembled that of the smoke from the bright tobacco sample. Only traces of the harmane alkaloids were found to be present in the uncured leaf. Their concentration in smoke was approximately one hundred times greater than that found in the leaf, and thus, apparently, they were formed during the burning of the cigarette.

Harmane has been prepared by reacting tryptophan and acetaldehyde under oxidative conditions.[8] Since tryptophan and acetaldehyde are present in the cigarette during burning, it seemed likely that tryptophan could be a precursor of the harmane alkaloids. Radioactive tryptophan was therefore added to tobacco leaf, from which cigarettes were subsequently

TABLE 2. CONCENTRATION OF HARMANE ALKALOIDS IN THE SMOKE AND LEAF OF SEVERAL TYPES OF CIGARETTES

	Concentration (μg/g)	
Samples	Harmane	Norharmane
Bright cigarette smoke	3·6*	12·6*
Burley cigarette smoke	5·8*	14·1*
A standard commercial blend	3·3*	12·3*
Bright leaf	0·02	0·20
Burley leaf	0·02	0·18

* based on wt. of cigarette = 1·0 g

[3] RAYMOND-HAMET, *Compt. Rend.*, 232, 507 (1951).
[4] G. R. CLEO and D. G. I. FELTON, *J. Chem. Soc.*, 1658 (1952).
[5] B. WIKTOP, *J. Am. Chem. Soc.*, 75, 3361 (1953).
[6] J. D. SPENSER, *J. Chem. Soc.*, 3659 (1956).
[7] N. SCHMID, A. EBNOTHER and P. KARRER, *Helv. Chim. Acta.*, 33, 1486 (1950).
[8] R. H. F. MANSKE and H. L. HOLMES, *The Alkaloids*, Vol. II, p. 396, 1st Ed. Academic Press, New York (1952).

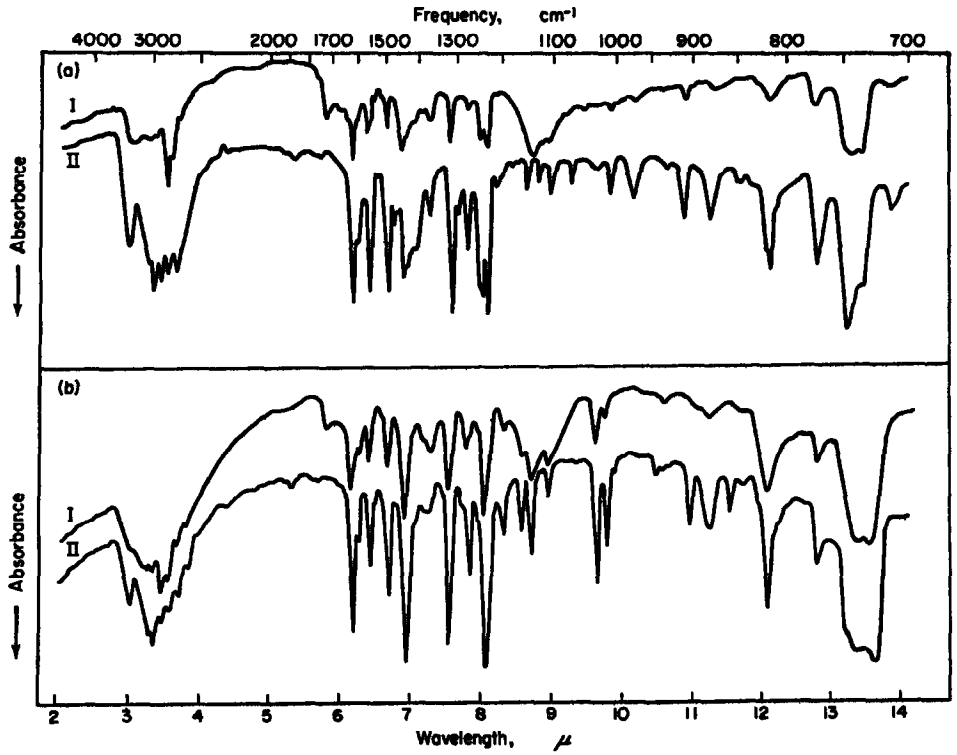


FIG. 1. INFRARED SPECTRA OF REFERENCE ALKALOIDS, HARMANE AND NORHARMANE AND THE COMPOUNDS ISOLATED FROM SMOKE

A–I. Compound isolated from smoke.
A–II. Harmane.
B–I. Compound isolated from smoke.
B–II. Norharmane.

made and smoked. The harmane alkaloids isolated from this smoke were found to be radioactive. By knowing the amount of radioactivity originally added to the leaf, and by measuring the amount present in the harmane alkaloids isolated from the smoke, the percent conversion of tryptophan to harmane alkaloids during burning was calculated. Another study was conducted by adding both untagged and radioactive tryptophan to the tobacco leaf and measuring the total increase of the harmane alkaloids in the smoke. The results of both experiments are shown in Table 3. From these data it is seen that the increase found in the harmane alkaloids in smoke due to the addition of tryptophan to the leaf agrees quite well with the increase calculated from the radiochemical conversion.

Based on the percent conversion of tryptophan to the harmane alkaloids during burning, approximately 3 mg of tryptophan is necessary in the tobacco leaf of one cigarette (i.e. 1·0 g) to form the amount of harmane alkaloids found in the smoke. The concentrations of both free tryptophan and protein-bound tryptophan were determined by a microbiological method,[9] and the results given in Table 4 show that the concentration of tryptophan in the bright leaf agreed well with the predicted amount necessary to produce the harmane alkaloids during burning. The concentration of the harmane alkaloids in burley smoke, on the other hand, was lower than expected from the concentration of the

[9] R. D. GREENE and A. BLACK, *J. Biol. Chem.* **155**, 1 (1944).

tryptophan found in the burley leaf. This difference may be due to different rates of conversion of protein-bound tryptophan, as the two types of tobacco are different in their chemical composition and their burning characteristics.

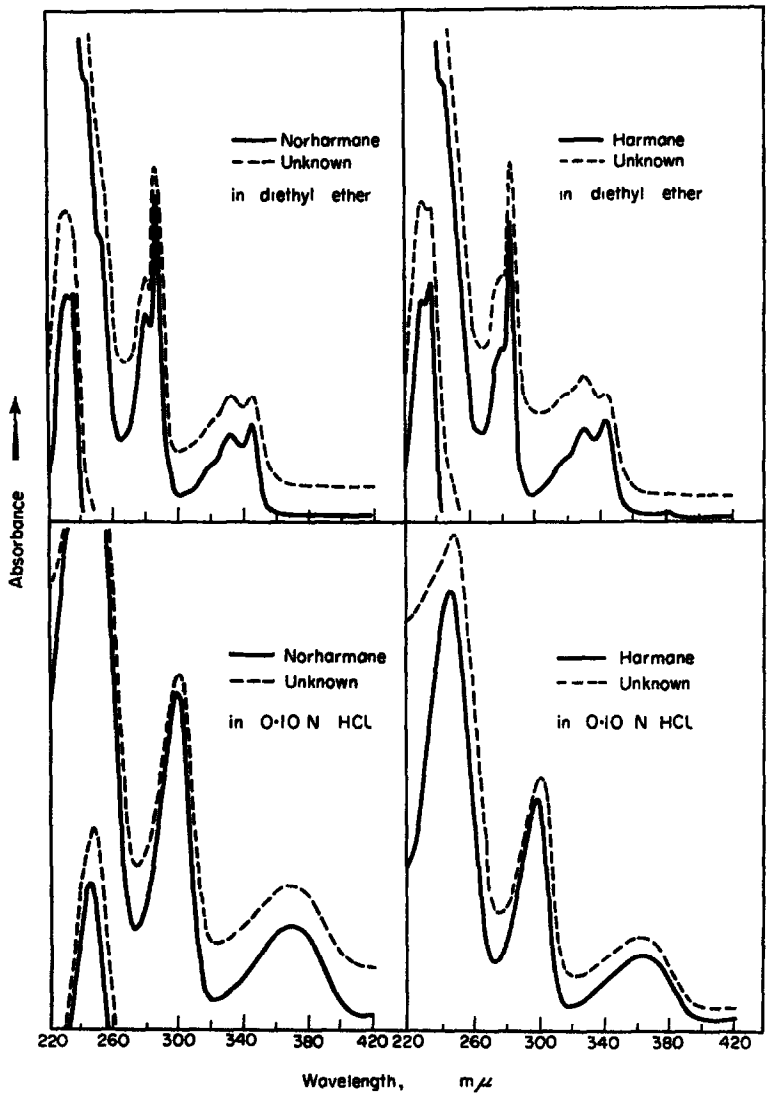


FIG. 2. ULTRAVIOLET SPECTRA OF REFERENCE ALKALOIDS, HARMANE AND NORHARMANE, AND THE COMPOUNDS ISOLATED FROM SMOKE

EXPERIMENTAL

Determination of i.r., u.v., and fluorescence spectra

The infrared analyses of the two isolated compounds were carried out using a Perkin–Elmer Model 221 double-beam spectrophotometer equipped with a sodium chloride prism. The samples were examined as micro potassium bromide pellets, 1½ mm in diameter, with the Perkin–Elmer 6X microsampling unit attached. Large blanks were observed initially,

TABLE 3. THE CONVERSION OF TRYPTOPHAN IN TOBACCO TO HARMANE ALKALOIDS IN SMOKE

	I*
Activity added by way of tryptophan to filler, (mμc/cigt)	169
Activity found in norharmane in the smoke, (mμc/cigt)	0·80
Activity found in harmane in the smoke, (mμc/cigt)	0·26
Percent activity in norharmane	0·47
Percent activity in harmane	0·15

	Concentration found in smoke	
	Harmane	Norharmane
I. Only radioactive tryptophan added to the filler (2 μg/cigt)	4·3 μg/cigt	11·2 μg/cigt
II. Radioactive tryptophan (2μg/cigt)+untagged tryptophan (820 μg/cigt) added to the filler	5·9 μg/cigt	15·7 μg/cigt
Increase	1·6 μg/cigt	4·5 μg/cigt
Predicted increase from the radiochemical studies	1·2 μg/cigt	3·9 μg/cigt

* 2 μg/cigt of radioactive tryptophan and 820 μg/cigt of untagged tryptophan added to the filler.

and it was found important to clean all glassware by the procedure recommended by Perkin–Elmer when using the potassium bromide ultra-micro die.[10] All ether and distilled water were redistilled to eliminate solvent impurities.

The chromatographic paper was also found to contain impurities which were eliminated from the alkaloids by the following procedure: the acid eluant (containing the alkaloids from the chromatographic zones) was extracted with ether. The solution was then made basic and the alkaloids extracted into fresh ether. After drying over anhydrous sodium sulfate, the ether solution was concentrated to 0·5 ml just prior to preparing the potassium bromide micropellet.

The ultraviolet spectra were determined with a Cary Model 14 spectrophotometer. The fluorescence spectra were determined with an Aminco-Keirs spectrophosphorimeter, employing a 1P21 phototube detector. A 1 cm rectangular quartz cell was used to measure the fluorescence. The fluorescence spectra were recorded on an Electro Instruments Model 101 X–Y recorder.

Radioactivity measurements were made in a Liquid Scintillation Counter, model LSC-10B, manufactured by Tracerlab Inc.

TABLE 4. CONCENTRATION OF TRYPTOPHAN IN TOBACCO, AND A COMPARISON OF THE PREDICTED CONCENTRATION OF HARMANE ALKALOIDS IN SMOKE WITH THE CONCENTRATION FOUND

	Bright	Burley
Free tryptophan (mg/g)	0·07	0·45
Protein bound tryptophan (mg/g)	3·0	9·0
Predicted concentration of harmane (μg/cigt)*	4·6	14·2
Predicted concentration of norharmane (μg/cigt)†	14·4	44·4
Concentration of harmane found in smoke (μg/cigt)	3·6	5·8
Concentration of norharmane found in smoke (μg/cigt)	12·6	14·1

* Based on 0·15 per cent conversion of total tryptophan.
† Based on 0·47 per cent conversion of total tryptophan
Weight of cigarette = 1·0 g.

[10] Perkin–Elmer Corporation: Instruction Manual for KBr Ultra-Micro Die. 186–0007.

Chromatography

The chromatographic columns (16×1·5 cm) were packed by adding small amounts of Whatman cellulose powder at a time, and packing tightly with a tamping rod to prevent solvent channelling. The column was eluted with 15% wt./vol. aqueous NaCl, to remove

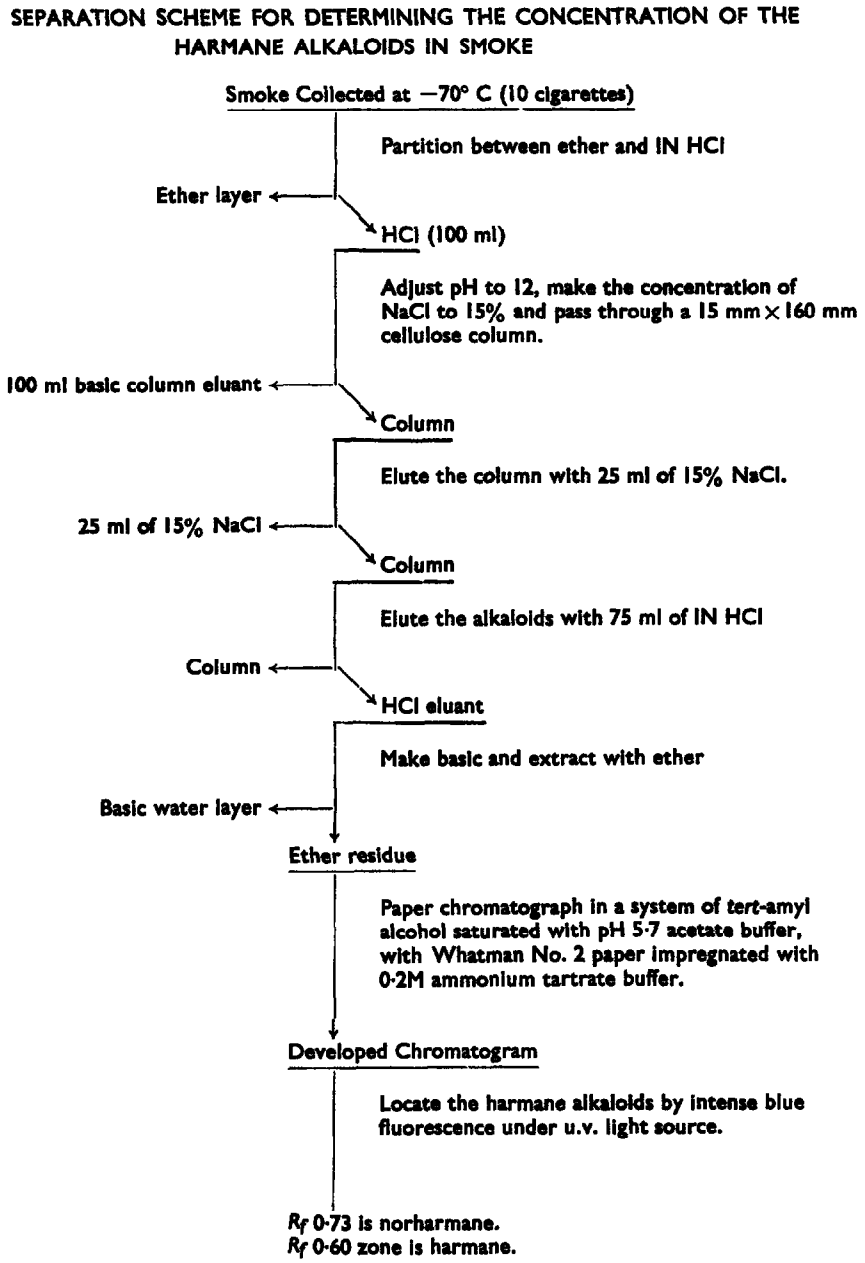
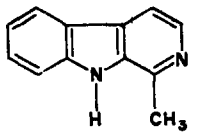


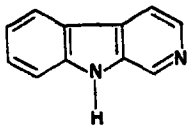
FIG. 3. SEPARATION SCHEME FOR DETERMINING THE CONCENTRATION OF THE HARMANE ALKALOIDS IN SMOKE

nicotine and the colored material present in the smoke, and the harmane alkaloids were displaced with methanol. The isolated harmane and norharmane fraction was resolved in a system consisting of *tert*-amyl alcohol saturated with pH 5·7 sodium acetate buffer



I Harmane

II Norharmane



STRUCTURAL FORMULAS OF HARMANE AND NORHARMANE

(0·2 *M*) on Whatman No. 2 paper pretreated with 0·2 *M* ammonium tartrate and dried before chromatography. The harmane alkaloids were located on the paper chromatogram by u.v. light.

Quantitative determination of the harmane alkaloids

The outline of the quantitative method for the harmane alkaloids in smoke is shown in Fig. 3. After final paper chromatographic development, the alkaloid zones were cut out and eluted with 1·0 *N* HCl. The HCl was extracted with ether to remove impurities, the aqueous phase was made alkaline to pH 12, and the alkaloids extracted quantitatively with one volume of ether. The alkaloids were then re-extracted into 1·0 *N* HCl and made up to a known volume prior to ultraviolet analysis. For harmane the peak at 365 mμ ($E^{1\%}_{1\,cm}$, 248) and for norharmane, the peak at 370 mμ ($E^{1\%}_{1\,cm}$, 237) was measured by using a baseline correction technique drawn from the minimum of each curve to the minimum at 399 mμ or 408 mμ respectively.

Experiments with known quantities of the harmane alkaloids showed that 81 per cent recovery was achieved with the method. This factor was used to find the correct concentration of the harmane alkaloids isolated from smoke.

Acknowledgement—The authors wish to express their thanks to Dr. E. W. Robb for his valuable advice and suggestions, to Mr. Gunars Vilcins and Mrs. Margaret Bill Vilcins for their assistance with the infrared spectra, to Mr. E. P. Crowell for his assistance with the ultraviolet and fluorescence studies and to Mr. G. Segura and Mr. J. E. Hardcastle for aid with the radiochemical study. The authors are grateful to Philip Morris Inc. for permission to publish this work.

Phytochemistry, 1962, Vol. 1, pp. 223 to 224. Pergamon Press Ltd. Printed in England

BOOK REVIEW

Recent Developments in the Chemistry of Natural Phenolic Compounds; Ed. by W. D. OLLIS. Pp. 237. Pergamon Press Ltd., 1961. 70s.

A SERIES of papers read at a meeting arranged by the "Plant Phenolics Group" in 1960 have been collected in this valuable volume. They cover important recent advances in the Chemistry of Natural Products and deal with biogenetic, biosynthetic and synthetic problems as well as the structural elucidation of new plant phenolics.

R. W. Richards has written an excellent review on the role of acetic acid in the biosynthesis of phenolic compounds. The "acetate hypothesis" was proposed by Collie and has frequently been used by Sir Robert Robinson to explain the hydroxylation pattern in several phenolic natural products. It has recently been developed in considerable detail by Birch and now ranks as a useful theory allowing some predictions to be made; these have actually been confirmed in several cases by structural reinvestigations and biochemical studies. W. B. Whalley's thorough discussion of structural relations in the phenol series is very stimulating. These mainly speculative contributions which are coloured by the biogenetic optimism of the organic chemist are followed by H. Griesebach's report on what is known to date about the biosynthesis of flavonoids, particularly the isoflavones. W. D. Ollis and I. O. Sutherland deal with the large number of phenolic compounds containing isoprenoid components. The actual combination of the phenolic and isoprenoid moieties poses an important biochemical problem. C. H. Hassal and A. L. Scott discuss recent work on the *in vitro* synthesis of a series of compounds which, at least formally, appear to result from the oxidative coupling of simple phenols.

The chemistry of tannins has recently undergone rapid development due to improved analytical methods and brief summaries of the work of the Sheffield school are given by R. D. Haworth and E. Haslam. O. Th. Schmidt describes the brilliant work which has been accomplished in Heidelberg on algarobilla tannins. The interesting story of the discovery and structural elucidation of the "biflavonyls" is told by W. Baker and W. D. Ollis who, together with Japanese chemists, have contributed a great deal to this field. The literature on biflavonyls is somewhat inaccessible to western chemists since many papers are in Japanese. This paper introduces a new feature in the book, that of chemotaxonomy. The biflavonyls have mainly been isolated from the leaves of gymnosperms and their occurrence appears to be limited to certain groups. The non-methylated amentoflavone has recently been found in a pteridophyte (*Selaginella*). Strangely enough hinokiflavone has been isolated from an angiosperm (*Casuarina*). The statement that among the angiosperms this genus is specially closely related to the gymnosperms should perhaps be taken with a pinch of salt. A systematic search for biflavonyls in angiosperms is, however, to be recommended.

The curious "carbon-glycosides" found in several plant families are discussed by L. Hörhammer and W. Wagner. They furnish interesting biosynthetic problems.

Dreiding's contribution on "betacyanines" such as the "nitrogenous anthoycanin", from red beet, is very fascinating. The structure of these compounds has long remained obscure, again mainly because of isolation difficulties. However, they have now been revealed as complex indole derivatives. Their occurrence in the vegetable kingdom appears to be restricted to the orders Cactales and Centrospermae (with the notable exception of

Caryophyllinae which contains normal anthocyanins). This is very interesting since botanists (e.g. Schumann, Engler) have long assumed that these orders are phylogenetically related in spite of the fact that they are, systematically, far apart.

W. D. Ollis and I. O. Sutherland finally discuss a series of tetracene quinones of fungal origin in relation to the "acetate theory".

Reviews like the present one, written by experts engaged in active research in their particular fields, are invaluable to their colleagues and highly stimulating for the younger generation of chemists that has just started research or is preparing for scientific careers. Unfortunately, however, in many of the reviews only the very latest papers are referred to and this sometimes obscures the historical perspective. Science is not merely a collection of facts and theories. It is easy to see that phytochemistry leads directly into the domains of biochemistry and biology. The present book is written by chemists most of whom have demonstrated the wide scope of their interests. It will be interesting for future chemists to return to this book and see which of the speculations have survived the biochemical ordeal.

The Plant Phenolics Group is to be congratulated on the success of its efforts.

University of Stockholm,
Sweden.

H. ERDTMAN

PAPERS RECEIVED

J. L. RAMAUT: Contribution à l'étude des produits du métabolisme d'*Aspergillus versicolor* (Vuill.) Tiraboschi. II. Fraction soluble dans l'éther sulfurique. (*Received* 25 *June* 1962.)

S. MARUMO and R. W. CURTIS: Chemical studies on Malformin. Malformin A. (*Received* 7 *May* 1962.)

M. D. WILDING and M. A. STAHMANN: Hydroxylysine from alfalfa roots. (*Received* 26 *May* 1962.)

M. D. WILDING and M. A. STAHMANN: Hydroxyaspartic acid from alfalfa and clover roots. (*Received* 26 *May* 1962.)

R. GMELIN, A. KJAER and P. OLESEN LARSEN: *N*-acetyl-1-djenkolic acid. A novel amino acid isolated from *Acacia farnesiana* Willd. (*Received* 7 *June* 1962.)

A. M. MAYER: Inhibition and substrate specificity of lettuce phenolase. (*Received* 25 *June* 1962.)

C. PEAUD-LENOEL and C. DE GOURNAY-MARGERIE: Some effects of chloramphenicol on isolated wheat roots. (*Received* 24 *June* 1962.)

H. M. HURST, A. BURGES and P. LATTER: Some aspects of the biochemistry of humic acid decomposition by fungi. (*Received* 3 *April* 1962.)

H. ENRES and M. HILAL: Die gerbstoffe der *Acacia arabica*. (*Received* 22 *June* 1962.)

T. SHIROYA: Metabolism of raffinose in cotton seeds. (*Received* 26 *June* 1962.)

H. G. KRISHNAMURTY, V. KRISHNAMOORTHY and T. R. SESHADRI: Preparation of anthocyanins and their glycosides from related flavonoids. (*Received* 9 *July* 1962.)

J. B. HARBORNE: Plant polyphenols. Part 9: The glycosidic pattern of anthocyanin pigment. (*Received* 29 *May* 1962.)

M. A. JOHNSON and D. S. FREAR: L-malate : NADP oxidoreductase (decarboxylating) from germinating flax rust uredospores. (*Received* 9 *July* 1962.)

A. KJAER and H. THOMSON: Isothiocyanate producing glucosides in species of the *Capparidaceae*. (*Received* 18 *July* 1962.)

M. MAZELIS: Demonstration and characterisation of cysteine sulphoxide lyase in the *Cruciferae*. (*Received* 24 *July* 1962.)

L. R. G. VALADON: Carotenoid pigments of *Protomyces inundatus*. (*Received* 16 *July* 1962.)

J. E. WILLIS and H. J. SALLACH: Serine biosynthesis from hydroxypyruvate in plants. (*Received* 28 *July* 1962.)

ERRATUM

Phytochemistry, **Vol. 1**, No. 2, April 1962.

I. Liss: *N*-Acetyldiaminobuttersäure, eine neue Aminosäure aus dem Latex von *Euphorbia Pulcherrima* Willd ex Klotzsh. Caption to Fig. 1. Last word of figure caption, for α-γ-Diaminobuttersäure read Acetyldiaminobuttersäure.

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SOME ASPECTS OF THE BIOCHEMISTRY OF HUMIC ACID DECOMPOSITION BY FUNGI

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(*Received* 3 *April* 1962)

Abstract—Twenty-nine strains of fungi, representing 20 species of basidiomycetes, 3 fungi imperfecti and 1 ascomycete were tested for their ability to decolourize humic acid and to reduce the carboxyl group of *m*-hydroxybenzoic acid. There was an absolute correlation between the ability to decolourize humic acid and to reduce the carboxyl group. It is suggested that decomposition of humic acid may include a reductive step, the reductive power having been produced by aerobic growth on a substrate other than humic acid.

DESPITE the numerous studies on humic acids extracted from soil these substances have so far proved highly resistant to conventional chemical methods of analysis. Savage and Stevenson[1] justly pointed out that whereas information concerning the structure of complex biological materials such as polysaccharides, protein and lignin has been obtained by hydrolytic and oxidative procedures, which convert the material into units amenable to chemical study, no satisfactory procedure has yet been developed for the conversion of humic acid into its constituent components. Such evidence as is available on the chemical nature of humic acid has led most workers to the tacit assumption that it is a hetero-polycondensate of various aromatic nuclei.[2] Low yields of aromatic compounds have been obtained by various degradative techniques; anthraquinone by Kumada *et al.*;[2] syringyl, guaiacyl and *p*-hydroxyphenyl residues by Morrison;[3] veratric and hemipinic acid by Esh and Guhar-Sircar;[4] vanillin, protocatechuic acid and resorcinol by Steelink *et al.*[5] The smallness of the yields, however, makes it difficult to place any confidence in structures based on such evidence. The isolation of organisms which actively decompose humic acid,[6] may provide an alternative line of investigation in which biological degradation may be substituted for chemical degradations. Since the initial report[7] the number of fungi found capable of decomposing humic acid has been considerably increased.

Initial attempts to obtain decomposition of humic acid by cell-free preparations were unsuccessful. A preliminary study was therefore made of the action of the fungi on simple mono-hydroxy benzoic acids. In this study none of the compounds usually found prior to ring-fission of benzoic acid derivatives by micro-organisms was detected. Instead, large amounts of the corresponding primary alcohols were produced. The reduction of ring-conjugated carboxylic acid groups by *Polystictus versicolor* has been previously reported by Shimanzo and Nord[8] and by Farmer, Henderson and Russel.[9] The reduction of a

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[1] S. M. SAVAGE and F. J. STEVENSON, *Proc. Am. Soil Sci. Soc.*, **25**, 35 (1961).
[2] K. KUMADA, A. SUZUKI and A. KAZUYUKI, *Nature*, **191**, 415 (1961).
[3] R. I. MORRISON, *J. Soil Sci.*, **9**, 130 (1958).
[4] G. C. ESH and S. S. GUHA-SIRCAR, *J. Ind. Chem. Soc.*, **17**, 405 (1940).
[5] C. STEELINK, J. W. BERRY, A. HO and H. E. NORDBY, *Sci. Proc. Roy. Dublin Soc. Ser.*, **A.1**, 59 (1960).
[6] P. LATTER and A. BURGES, *7th Int. Cong. Soil Sci., Madison*, **2**, 643 (1961).
[7] A. BURGES and P. LATTER, *Nature*, **186**, 404 (1960).
[8] K. SHIMANZO and F. F. NORD, *Arch. Biochem. Biophys.*, **87**, 140 (1960).
[9] V. C. FARMER, M. E. K. HENDERSON and J. D. RUSSELL, *Biochim. Biophys. Acta*, **35**, 202 (1959).

carboxyl group is a highly endergonic reaction of considerable significance in biological reactions. It is of importance in the synthesis of carbohydrate from pyruvate, in the reduction of β-hydroxy-β-methyl glutaric acid to mevalonic acid in the *iso*pentenyl-pyrophosphate pathway, and in the formation of carbohydrate during photosynthesis. The discovery that the reductive property was shared by other fungi all capable of decomposing humic acid made it seem worth while to investigate these two functions in a number of fungi.

RESULTS

The results of the main tests are given in Table 1. In these tests the humic acid medium contained sodium nitrate as a nitrogen source. With other nitrogen sources mycelial growth was greatly stimulated but varying degrees of inhibition of decolourization were observed. The order of inhibition was approximately ammonium sulphate > tyrosine > asparagine > phenylalanine > glycine > lysine > $NaNO_3$. The reason for this inhibition is not understood.

TABLE 1. ACTION OF VARIOUS FUNGI ON HUMIC ACID AND *m*-HYDROXYBENZOIC ACID

Fungus	Humic acid decolourized	Aromatic carboxyl reduced
Polystictus versicolor (L.) Fr. Strain A*	++	++
Polystictus versicolor (L.) Fr. Strain B†	++	++
Hypholoma fasciculare (Huds. ex Fr.) Kummer Strain A‡	++	+
Hypholoma fasciculare (Huds. ex Fr.) Strain B‡	++	+
Hypholoma fasciculare (Huds. ex Fr.) Strain C§	++	+
Trametes suaveolens (L.) Fr. Strain A‡	++	++
Trametes suaveolens (L.) Fr. Strain B†	—	—
Trametes rubescens (A and S) Fr.†	++	++
Polyporus brumalis (Pers. ex Fr.)‖	+	++
Polyporus arcularius (Batsch ex Fr.)†	+	++
Polyporus resinosus (Schrad. ex Fr.)†	+	++
Polyporus schweinitzii Fr.‖	+	++
Ustulina zonata (Lev.)†	+	++
Nidularia denudata Fr.‡	—	—
Polyporus squamosus (Huds.) Fr.‡	—	—
Polyporus adustus (Wild. ex Fr.)	—	—
Polyporus betulinus (Bull. ex Fr.)‡	—	—
Polyporus dryophilus (Berk.)‖	—	—
Coniophora cerebella (Pers.)†	—	—
Cyathus striatus (Huds.) Pers.‡	—	—
Collybia velutipes (Curt. ex Fr.)‡	—	—
Marasmius graminum (Libert Berk.)**	—	—
Schizophyllum commune (Fr.)‡	—	—
Fomes pinicola (Sw. Cooke)‖	—	—
Poria ferrea (Pers. Bourd. and Getz)‖	—	—
Penicillium spinulosum Thom.¶	—	—
Aspergillus niger van Tieg.¶	—	—
Penicillium janthinellum Biourge¶	—	—
Boletus variegatus Sow. ex Fr.‡	—	—

* Isolated from soil, Hartley Botanical Laboratories, Liverpool.
† Obtained from Forest Products Research Laboratory, Princes Risborough, England.
‡ Isolated from naturally occurring fruit bodies, Hartley Botanical Laboratories, Liverpool.
§ A mutant from Strain B.
‖ Obtained from Dr. M. R. Noble, Canada.
¶ Obtained from Commonwealth Mycological Institute, Kew.
** Obtained from Dr. Lindiberg, Göteburg, Sweden.

Complete decolourization of humic acid is shown as ++, partial decolourization as +. Where — is shown there was no evidence of decolourization even after 6 weeks.

Table 1 also shows whether *m*-hydroxybenzyl alcohol was detected in the cultures. In all cases where reduction occurred, with the exception of *Hypholoma fasciculare*, strong alcohol spots were detected after chromatographic separation of the ether extract and the alcohol was detectable in the growth solutions for at least 2 weeks. *Hypholoma* produced much weaker but still clearly identifiable alcohol spots after 4 days, but later both acid and alcohol disappeared from the growth solution. This organism obviously possesses further systems for the metabolism of the alcohol and this was confirmed by demonstrating that a 0·05 per cent solution of *m*-hydroxybenzyl alcohol was completely metabolized after 8 days contact with a fungal mat. Trials with a wide range of other substrates, including 2,4-dimethoxybenzoic acid, showed that with most substrates tried *Polystictus versicolor* was able to reduce the carboxyl group. Those fungi listed as incapable of reducing the carboxylic group were tested both for the presence of the aldehyde as well as the alcohol, at 4 day intervals over a 5 week culture period.

TABLE 2. ABILITY TO METABOLIZE *m*-HYDROXYBENZOIC ACID AND *m*-HYDROXYBENZYL ALCOHOL BY ORGANISMS APPARENTLY INCAPABLE OF REDUCING AN AROMATIC CARBOXYLIC GROUP

Fungus	Acid	Alcohol
Nidularia denudata	—	—
Polyporus squamosus	—	—
Polyporus adustus	—	—
Polyporus betulinus	++	—
Polyporus dryophilus	—	—
Coniophora cerebella	—	—
Cyathus striatus	—	—
Trametes suaveoleus Strain B	—	—
Collybia velutipes	+	+
Marasmius graminum	+	·
Boletus variegatus	+	+
Schizophyllum commune	—	+
Fomes pinicola	—	—
Poria ferrea	—	+

— no action + metabolized slowly ++ metabolized rapidly

The failure to detect the alcohol in the cultures might perhaps have been due to its rapid metabolism. A number of the non-reducing fungi were therefore tested for their ability to metabolize the acid and the alcohol. The results of these tests are given in Table 2. None of these fungi was able to decolourize 0·1% humic acid agar even after a period of 6 weeks. Only four fungi were able to metabolize the acid. Of these, *Polyporus betulinus* had no effect on the alcohol and therefore it would have been readily detected if the fungus had been able to reduce the acid. *Collybia*, *Marasmius* and *Boletus* which can utilize both the acid and the alcohol may possibly bring about the reduction, and the alcohol may disappear so rapidly as to escape detection. This, however, is considered unlikely.

DISCUSSION

The strong correlation between the ability to decolourize humic acid and to reduce the aromatic carboxyl group is clearly shown in Table 1. The importance of the correlation is strengthened by the results obtained with the two strains of *Trametes suaveolens*.

Field evidence strongly indicates that decomposition of humic acid under natural conditions is associated with good aeration of the soil. Laboratory experiments have also shown

that good decomposition is correlated with good aeration of the cultures. It might be considered therefore that decomposition of humic acid is primarily an oxidative process. The present work gives strong grounds for believing that a reductive step may well be implicated. Such knowledge as we have of the chemical structure of humic acid indicates that carboxylic acid groups form the more important reactive groups. The first step in the decomposition of the humic molecule may be the reduction of the carboxylic group, the reductive capacity having been produced by active aerobic growth on substrates other than humic acid.

METHODS

Decolourization of humic acid

In the early work decomposition of humic acid was measured by adding a known amount of humic acid either to shake cultures in which the fungus grew as pellets or in penicillin flasks under stationary mats of mycelium and then to extract and weigh the residual humic acid on the hyphae and in the culture medium.[6] For routine screening it has been found more convenient to use ordinary $30 \times 1{\cdot}0$ cm growth tubes containing 10 ml of nutrient agar with 0·1% humic acid. The nutrient agar contained KH_2PO_4, 0·4; K_2HPO_4, 0·5; $NaNO_3$, 1·7; glucose, 5·0 g/l. with trace elements added. Where comparative tests were made with different nitrogen sources these were added so as to give a final concentration equivalent to 0·2 M per atom of nitrogen. The humic acid was extracted from the B_1 horizon of a podzol under *Pinus sylvestris* as previously reported.[6]

A clear indication of decolourization of the humic acid was generally obtained two weeks after inoculation. Exposure to light was found to have a slight inhibiting effect on the decolourization process; accordingly all cultures were kept in the dark.

With some fungi declourization of the humic acid was complete, with others only a partial decolourization of the humic acid agar was obtained.

Reduction of m-*hydroxybenzoic acid*

Tests for the reduction of the carboxyl group were made using a modification of the Klujver and van Zijp replacement technique as described by Henderson.[10] 100 ml conical flasks containing 30 ml of 2% (w/v) liquid malt extract were inoculated with the organism being studied and incubated at 25°. When a suitable mat had been formed, the growth solution was poured away and the fungal mat washed with three changes of sterile distilled water. 30 ml of an aqueous 0·1% (w/v) solution of *m*-hydroxybenzoic acid neutralized to pH 6·5 was poured under the mat and the culture again incubated at 25°. The culture media or extracts were examined for phenolic materials by acidifying the media to pH 2·0 with 2 N HCl and extracting with two separate 10 ml portions of diethyl ether. After removal of ether from the combined extracts, the residue was taken up in 1 ml of ethanol and used for chromatography.

Chromatographic separations

Chromatographic separations were carried out either on paper, Whatman No. 4, using *n*-butanol/ammonia (4:1) or preferably on the faster running "Desaga" chromatostrips using Kieselgel G. (Merck, Darmstadt) and chloroform/10% (v/v) acetic acid. R_f values for *m*-hydroxybenzoic acid, aldehyde and alcohol are 0·24, 0·71, 0·80 on paper, and 0·34, 0·55, 0·15 on chromatostrips.

[10] M. E. K. HENDERSON, *J. Gen. Microbiol*, **16**, 686 (1960).

Phenolic compounds were located by spraying with diazotized sulphanilic acid followed by 10% Na_2CO_3. This reagent reveals *m*-hydroxybenzoic acid and alcohol as strong orange spots, and *m*-hydroxybenzaldehyde as a much weaker light brown. The latter compound is more easily detected by its strong green fluorescence in u.v. light after spraying with diazotized sulphanilic acid, or by its reaction with 2,4-dinitrophenylhydrazine.

Culture of fungi

Cultures of all the fungi except the Penicillia and Aspergilli were maintained on 2% malt extract agar. The remainder were grown on Czapek–Dox agar.

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N-ACETYL-L-DJENKOLIC ACID, A NOVEL AMINO ACID ISOLATED FROM *ACACIA FARNESIANA* WILLD.

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(*Received* 7 *June* 1962)

Abstract—In the course of an analysis by paper chromatography of the amino acids of a series of seed samples of species belonging to the family *Mimosaceae*, a sulphur-containing amino acid, different from those previously found in plants, was observed as the major constituent in a few species. The new amino acid was isolated in pure form from seeds of *Acacia farnesiana* Willd. Its elemental composition, and hydrolysis to L-djenkolic acid and acetic acid, indicate that the amino acid is *N*-acetyl-L-djenkolic acid (I). This conclusion was confirmed by synthesis.

INTRODUCTION

OF THE more than sixty non-protein plant amino acids known at present, about a dozen have been encountered in genera belonging to the family *Mimosaceae*.[1]

In the course of analyses by paper chromatography of seed extracts of numerous species of this family a strong ninhydrin-positive spot was occasionally observed due to an amino acid obviously different from those previously encountered in the plant kingdom. Hence, efforts were directed towards its isolation in pure form.

For this purpose seeds of *Acacia farnesiana* Willd., which are rich in the unknown amino acid and readily accessible, were employed. *A. farnesiana* is a shrub or tree widely distributed through tropical and sub-tropical regions of both hemispheres, reputed for its tannin-rich bark and fragrant flowers ("cassie flower oil"). The present communication describes the isolation, structure determination and synthesis of the new amino acid.

RESULTS AND DISCUSSION

When the amino acid fraction of a seed extract of *A. farnesiana* was subjected to two-dimensional paper chromatography, the pattern shown in Fig. 1 was observed. The predominant amino acid gave an orthodox ninhydrin reaction and a positive response to the platinic iodide reagent diagnostic for sulphur-containing amino acids.[2] It was different from djenkolic acid, S-(2-carboxyethyl)-L-cysteine,[3] S-(2-carboxyisopropyl)-L-cysteine[4] and dichrostachic acid[1] (II), the four sulphur amino acids formerly recognized in members of *Mimosaceae*, as well as from other previously known plant amino acids. In addition, the chromatograms revealed the presence of several protein amino acids as well as of other acids formerly encountered in *Mimosaceae* (djenkolic acid,[5] pipecolic acid[6,7] and 4-hydroxy-pipecolic acid[6,7]) as indicated in Fig. 1.

On ion exchange resins the unknown amino acid behaved as an acidic substance, a property subsequently utilized in its isolation. When the total amino acid fraction, isolated

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[1] R. GMELIN, *Z. physiol. Chem., Hoppe-Seyler's*, **327**, 186 (1962).
[2] G. TOENNIES and J. J. KOLB, *Anal. Chem.*, **23**, 823 (1951).
[3] R. GMELIN, G. STRAUSS and G. HASENMAIER, *Z. Naturforsch.*, **13b**, 252 (1958).
[4] R. GMELIN and P. K. HIETALA, *Z. physiol. Chem., Hoppe-Seyler's*, **322**, 278 (1960).
[5] A. G. VAN VEEN and A. J. HYMAN, *Rec. trav. chim.*, **54**, 493 (1935).
[6] A. I. VIRTANEN and S. KARI, *Acta Chem. Scand.*, **9**, 170 (1955).
[7] J. W. CLARK-LEWIS and P. I. MORTIMER, *J. Chem. Soc.*, 189, (1961).

from 250 g of seeds of *A. farnesiana* by means of a cation exchange resin, was applied to a weakly basic resin, the total acidic amino acid fraction could be eluted with acetic acid and further fractionated by gradient elution with acetic acid from a strongly basic resin to give the desired amino acid (6·8 g), virtually uncontaminated with other ninhydrin-reacting components.

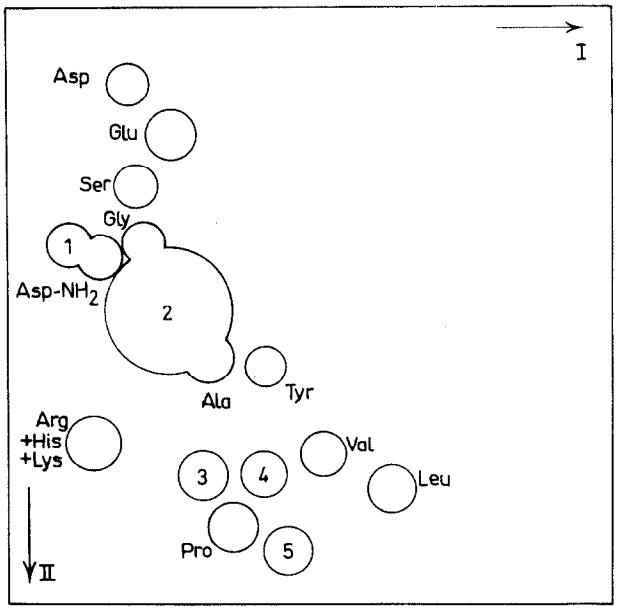


FIG. 1. TWO-DIMENSIONAL PAPER CHROMATOGRAM OF SEED EXTRACT OF *Acacia farnesiana* WILLD. Solvent I: butanol:acetic acid:water (12:3:5) Solvent II: phenol:water:conc. ammonia (120:30:1); Spraying reagent: ninhydrin. (1) Djenkolic acid. (2) *N*-Acetyl-L-djenkolic acid. (3) 4-Hydroxypipecolic acid. (4) γ-Aminobutyric acid. (5) Pipecolic acid.

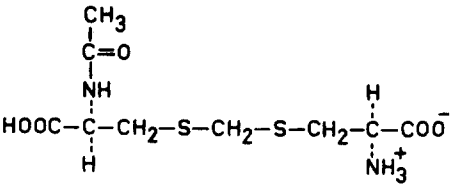
A purified sample possessed the elemental composition $C_9H_{16}N_2O_5S_2$ and was acted on by the C-S-lyase of *Albizzia lophantha* seeds which cleaves L-cysteine derivatives.[8,9] On hydrolytic fission in hot acid, equimolecular quantities of L-djenkolic acid and acetic acid were produced, suggesting that the amino acid was the previously unknown *N-acetyl-L-djenkolic acid* (I). This conclusion was corroborated by comparison with an authentic, synthetic specimen, produced by partial acetylation of L-djenkolic acid with acetic anhydride, albeit in mediocre yield. On critical comparison, the two preparations proved identical with regard to melting point, mixed m.p., optical rotation and infra-red spectra.

N-acetyl-L-djenkolic acid appears to be present also in seed extracts of *Acacia horrida* Willd., *Acacia karroo* Hayne and *Mimosa acanthocarpa* Benth., which all give paper chromatograms very similar to that of *A. farnesiana* (Fig. 1).

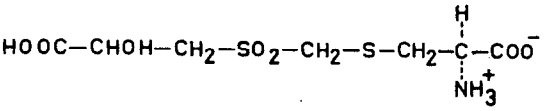
Only a few *N*-acetylated amino acids have previously been found in Nature. *N*-acetylglutamic acid and α-*N*-acetylornithine are accepted intermediates in the conversion of glutamic acid into ornithine in *Escherichia coli*. In higher plants, δ-acetylornithine has been found in *Corydalis ochotensis*[10] and numerous other members of *Fumariaceae*,[11] as well as

[8] R. GMELIN, G. HASENMAIER and G. STRAUSS, *Z. Naturforsch.*, **12b**, 687 (1957).
[9] S. SCHWIMMER and A. KJÆR, *Biochim. Biophys. Acta*, **42**, 316 (1960).
[10] R. H. F. MANSKE, *Can. J. Res.*, **15B**, 84 (1937).
[11] L. FOWDEN, *Nature*, **182**, 406 (1958).

in the fern *Asplenium nidus*,[12] the grass *Brachypodium sylvaticum* and a number of common grasses of the tribe *Festuceae*.[11] Very recently, the occurrence of a not further characterized *N*-acetyl-α,γ-diaminobutyric acid in the latex of *Euphorbia pulcherrima* was reported.[13] The biogenesis of these monoacetyl-diamino acids seems to be unknown. The *in vivo* formation of *N*-acetyl-L-djenkolic acid (I), possibly from L-djenkolic acid, the origin of which is unknown, poses a problem of biochemical interest.



I



II

EXPERIMENTAL

General

Rotations were measured in a 1 dm tube. Infra-red spectra were determined in potassium bromide pellets on a Perkin–Elmer "Infracord" instrument. Melting points were determined in capillary tubes in an Anschütz–Hershberg apparatus equipped with fully immersed thermometers. The standard rate of heating was 2° per min.

L-djenkolic acid was purchased from California Corporation for Biochemical Research, Los Angeles 63, Calif.

Isolation of N-*acetyl*-L-*djenkolic acid from seeds of* Acacia farnesiana *Willd.*

Finely ground seed material (250 g) was refluxed for 30 min with carbon tetrachloride (1 l.). After filtration, the air-dried material was refluxed for 2 hr with methanol–water (1 l., 7:3) and filtered. The extraction was repeated three times and the combined filtrates were evaporated *in vacuo* to a brown syrup (57 g), which was partly dissolved in water (0·5 l.) and filtered. The total amino acid fraction in the filtrate was bound to a strongly acid ion exchange resin (Zeokarb 215, 4×50 cm) in the acid form. After washing with water (1·5 l.), the amino acids were eluted with ammonia (1·5 l., 1 *N*). The eluate was evaporated *in vacuo* to a syrup (13 g), which was dissolved in water (125 ml). The solution was passed through a weakly basic ion exchange resin (Dowex 3, 20–50 mesh, $3{\cdot}5\times64$ cm) in the acetate form. After washing with water (900 ml), the acidic amino acids were eluted with acetic acid (3·6 l., 1 *N*). The acid eluate was concentrated *in vacuo* to a yellow syrup (9 g) which was dissolved in water (75 ml) and applied to a strongly basic ion-exchange resin (Dowex 1×8, 200–400 mesh, 3×60 cm) in the acetate form. The column was then eluted with acetic acid, progressively increasing in concentration from zero to 1 *N* over a

[12] A. I. VIRTANEN and P. LINKO, *Acta Chem. Scand.*, **9**, 531 (1955).
[13] I. LISS, *Phytochemistry*, **1**, 87 (1962).

volume of 4 l. Fractions of 250 drops (*ca.* 20 ml) were collected. The fractions 76–128 contained glutamic acid, fractions 184–197 aspartic acid, and fractions 240–314 the unknown amino acid, virtually free from other ninhydrin reacting substances. The latter fractions were combined and evaporated *in vacuo* to a yellowish semi-solid material (6·8 g). After four recrystallizations from ethanol (96%), a pure specimen of *N*-acetyl-L-djenkolic acid was obtained (Found: C, 36·29; H, 5·60; N, 9·21; S, 21·33. $C_9H_{16}N_2O_5S_2$ requires: C, 36·47; H, 5·44; N, 9·45; S, 21·64%), $[\alpha]_D^{23}$ −22·0° (*c* 1·0, water), m.p. 170° (decomp.).

Hydrolysis of N-*acetyl*-L-*djenkolic acid to acetic acid and* L-*djenkolic acid*

N-acetyl-L-djenkolic acid (300 mg) was refluxed with 1 *N* hydrochloric acid (10 ml) for 4 hr. Water (10 ml) was added and 10 ml of the solution was distilled. After this step had been repeated the combined distillates were adjusted to pH 6 with NaOH and evaporated to dryness. The residue was dissolved in water (1 ml) and added to a solution of S-benzylisothiuronium chloride (200 mg) in water (1 ml) at 90°. On cooling, S-benzylthiuronium acetate (130 mg) separated and was recrystallized from water, m.p. 134–136°, alone or in admixture with an authentic specimen. The infra-red spectrum coincided with that of an authentic specimen.

After distillation, the residual solution was adjusted to pH 6 with NaOH causing L-djenkolic acid (205 mg) to separate, $[\alpha]_D^{25}$ −49·0° (*c* 2·0, 1% HCl), $[\alpha]_D^{25}$ −60·2° (*c* 1·0, 1 *N* HCl). Literature values[14]: $[\alpha]_D^{25}$ −47·5° (*c* 2, 1% HCl), $[\alpha]_D^{21}$ −65·0° (*c* 1, 1 *N* HCl). The infra-red absorption spectrum was identical with that of an authentic specimen of L-djenkolic acid.

Synthesis of N-*acetyl*-L-*djenkolic acid*

To an ice-cooled and stirred solution of L-djenkolic acid (1·27 g) in 2 *N* NaOH (5 ml) and water (2·5 ml), acetic anhydride (600 μl) and 2 *N* NaOH (4.8 ml) were added in small portions in the course of 40 min. After standing for 50 min at room temperature, the solution was cooled and adjusted to pH 6 with HCl. Precipitated L-djenkolic acid (390 mg) was removed by filtration and the filtrate was applied to a strongly acid ion exchange resin (Zeokarb 215, 1×8 cm) in the acid form. After washing with water, amino acids were eluted with ammonia (1 *N*). The eluate was taken to dryness, the residue dissolved in water (5 ml) and applied to a strongly basic ion exchange resin (Dowex 1×8, 200–400 mesh, 1×8 cm) in the acetate form. After washing with water, *N*-acetyl-L-djenkolic acid was eluted with acetic acid (1 *N*). The eluate was again taken to dryness (440 mg), and pure *N*-acetyl-L-djenkolic acid was obtained upon recrystallization from ethanol (96%) (Found: C, 36·28; H, 5·63; N, 9·21; S, 21·37), $[\alpha]_D^{25}$ −22·1° (*c* 1·0, water), m.p. 170° (decomp.), alone or in admixture with the natural product. The infra-red absorption spectrum was identical with that of the natural product.

Paper chromatography. The following R_f-values were determined for the new amino acid by descending chromatography on Whatman paper No. 1 at 25°: butanol:acetic acid:water (12:3:5): 0·26; phenol:water:conc. ammonia (120:30:1 (w/v/v)): 0·51.

Acknowledgement—The authors are indebted to Mr. G. Cornali for the microanalyses. Seed material of *A. farnesiana* was obtained through the good offices of Mr. P. Garnier, Bouaké, Côte d'Ivoire, and Dr. Q. Jones, United States Department of Agriculture, New Crops Research Branch, Beltsville, Md. Support from The Carlsberg Memorial Foundation for Brewer I. C. Jacobsen to one of us (P.O.L.) is gratefully acknowledged. The work is supported by Kai Hansen's Fond.

[14] M. D. ARMSTRONG and V. DU VIGNEAUD, *J. Biol. Chem.*, **168**, 373 (1947).

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INHIBITION AND SUBSTRATE SPECIFICITY OF LETTUCE PHENOLASE

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Abstract—The effect of phenylthiourea and carbon monoxide on a soluble and a mitochondrial phenolase from lettuce is described. Differences in inhibition, depending on the substrates oxidized, were observed and the implications of this, as far as deductions about metabolic pathways are concerned, are discussed. The rate of oxidation of a number of *p*-substituted dihydroxy phenols was determined. It appears that oxidation is by electrophilic attack. The possible reaction mechanism and the probable importance of enol-keto tautomerism of the phenol are discussed.

REACTIONS involving phenolases are increasingly of interest to plant physiologists. Evidence for difference in response of phenolase to inhibitors, when coupled oxidations are compared with direct oxidation reactions, has been found for phenolase prepared from lettuce seeds.[1,2] The inhibition of phenolase in the soluble and mitochondrial fractions, prepared from lettuce seeds, variety Grand Rapids, germinated for 48 hr[1], was studied. The enzymic activity was determined[1,3] in crude extracts, in extracts after dialysis, or in dialyzed freeze dried preparations. The inhibitors used were phenylthiourea or carbon monoxide.

Phenylthiourea inhibited the soluble enzyme in both crude and dialyzed extracts, less than the mitochondrial enzyme (Table 1). The extent of inhibition depended on the substrate oxidised, the oxidation of 4-methylcatechol being less inhibited than that of dopa(DL-3,4-dihydroxyphenylalanine). Using carbon monoxide as the inhibitor, no difference was observed when either 4-methylcatechol or dopa was used as the substrate, the oxidation of both being inhibited 50–60 per cent by a 90% carbon monoxide–10% oxygen mixture. However when the oxidation of *p*-cresol was investigated in the presence of carbon monoxide, some peculiar results were observed. Direct oxidation of *p*-cresol only occurred when the crude extract was used. In this case carbon monoxide initially stimulated the oxidation, but as the reaction proceeded, this stimulation decreased and

TABLE 1. INHIBITION OF ACTIVITY OF PHENOLASE BY PHENYLTHIOUREA
(Results as % inhibition. All substrates 5×10^{-3}M)

	Soluble enzyme					Mitochondrial enzyme	
	Crude			Dialyzed			
Substrate	4-Me. cat.	Dopa	*p*-cresol	4-Me. cat.	Dopa	4-Me. cat.	Dopa
Phenylthiourea concn. M							
5×10^{-4}	60	100	100	60	—	100	100
$2{\cdot}5\times10^{-4}$	40	100	80	50	95	—	—
$1{\cdot}0\times10^{-4}$	0	—	0	—	—	0	85

[1] A. M. MAYER, *Physiol. Plantarum*, **14**, 322 (1961).
[2] L. STAVY and A. M. MAYER, *Bull. Research Council Israel*, **11D**, 31 (1962).
[3] A. M. MAYER and J. FRIEND, *J. Expt. Bot.*, **11**, 141 (1960).

eventually inhibition occurred, (Table 2). When, however, dialyzed extracts were used, in the presence of ascorbic acid, inhibition occurred from the start of the reaction (Table 2). The inhibition only appears to decrease somewhat with time because the substrate was exhausted earlier in the control. The reason for the difference in response between the crude and dialyzed extracts is not entirely clear. In both cases two reactions occur, hydroxylation due to the cuprous form on the enzyme, and dehydrogenation due to the cupric form, which is produced as a result of hydroxylation. The rates of oxidation using the two preparations are however very different, the coupled reaction in the presence of ascorbic acid being about five times as fast as the direct oxidation by the crude enzyme. The use of a gas mixture containing only 10 per cent oxygen seems to be permissible since, for example, the oxidation of 4-methylcatechol by the mitochondrial enzyme only decreased by 30 per cent when decreasing oxygen concentration from 20 per cent to 10 per cent.

TABLE 2. EFFECT OF CARBON MONOXIDE ON THE OXIDATION OF *p*-CRESOL BY THE CRUDE ENZYME AND BY DIALYZED ENZYME

Time from initiation of reaction (min)	Crude enzyme	Time from initiation of reaction (min)	Dialyzed enzyme
18	+30 (stimulation)	3	83
27	+17 (stimulation)	9	76
36	+2 (stimulation)	12	63
46	3	15	47
61	6		
103	37		

Crude enzyme: *p*-cresol concn. 5×10^{-3}M. Dialysed enzyme *p*-cresol $2{\cdot}5\times10^{-3}$M and ascorbic acid $5{\cdot}6\times10^{-3}$M. Gas mixture contains 90% CO and 10% O_2 and the controls 90% N_2 and 10% 0. Results are given as inhibition by CO as % of control.

The difference in response of the enzyme to different inhibitors depending on the substrate, suggested that a study of the substrate specificity of the enzyme might help eludicate some of the reactions involved during oxidation. Various possible substrates of phenolase were tested and the results related to the rate of oxidation of 4-methylcatechol (Table 3). In most cases the rate of reaction was constant for about 12 min, except for catechol where the reaction stopped after 6 min so that the initial rate was probably higher than that actually recorded. When crude extract was used, coupled oxidation of the substrate under examination by the endogenous phenols occurred, so that no very clear cut differences in rates of oxidation were observed. The results shown in Table 3 are of some interest. Previously some natural substrates for phenolases have been investigated qualitatively by Baruah and Swain,[4] but apparently *p*-substituted *ortho*-dihydric phenols have not been previously compared by determinations of the rates of reaction. The rate of oxidation of the substrates can be arranged in the order of the effect of para substitution on the rate of oxidation as follows: Me > H ? > Cl > CH:CHCOOH > CH_2CHNH_2COOH > CNS > COOH > CHO. This series corresponds reasonably well with the values of σ, (the substituent constant), in the Hammet equation,[5] which are in the order CH_3 > H > Cl > CHO > COOH. This indicates the probability that an electrophilic, cationoid type of attack on the dihydric phenol is involved in the oxidation reaction. Dopa and 3,4-dihydroxy-

[4] P. BARUAH and T. SWAIN, *J. Sci. Food Agri.* **2**, 125 (1959).
[5] H. H. JAFFE, *Chem. Rev.*, **53**, 191 (1953).

cinnamic acid probably fall in the sigma series at about the place at which they occur in the oxidation series. Blocking one of the positions adjacent to the two *o*-hydroxyl groups by methyl (3-methylcatechol) or hydroxyl (pyrogallol) groups also reduced the rate of oxidation. Apparently both these positions must be left free for oxidation to occur. A

TABLE 3. RATE OF OXIDATION OF VARIOUS SUBSTRATES BY SOLUBLE PHENOLASE PREPARATIONS FROM DRY LETTUCE SEEDS

Substrate	% Rate of oxidation of 4-methylcatechol		
	Crude enzyme	Dialyzed enzyme	Freeze dried enzyme
4-methylcatechol	100	100	100
4-chlorocatechol	100	89	82
3,4-dihydroxyphenyl-alanine	100	75	90
3,4-dihydroxycinnamic acid	100	87	100
3,4-dihydroxybenzoic acid	59	39	1
Catechol	70	43 (reaction stops after 6 min)	·10 (reaction stops almost immediately)
4-cyanothiocatechol	45	64	55
3,4-dihydroxybenzaldehyde	30	19	18
p-cresol*	18	0	—
p-hydroxycinnamicacid	5	0	—
pyrogallol	—	0	0
3-methylcatechol	47	8	0
3-methylcatechol plus 4-methylcatechol	—	95	55
1-naphthol	0	0	0
2-naphthol	0	0	0
resorcinol	0	0	0
orcinol	0	0	0
2,3-dihydroxynaphthalene	0	0	0

* Oxidized by dialyzed and freeze dried enzyme only in presence of ascorbic acid (results not quoted here).

All substrates at concentration of 5×10^{-3}M. Activity of the enzyme preparations towards 4-methylcatechol were as follows: crude enzyme, 4·1 μl O_2/mg protein/min, dialyzed enzyme 7·6 μl O_2/mg protein/min, and freeze dried enzyme 30·0 μl O_2/mg protein/min.

possible reason for this may be in the ketonisation which all phenols undergo to some extent.[6] Thus it is perhaps not unreasonable to suggest that it is the keto form of the phenol that is attacked during the oxidation of dihydric phenols by phenolase. However, undoubtedly additional factors, such as steric effects, are involved in the enzymic oxidations reactions, which will also affect the rate of reaction.

Acknowledgement—My thanks are due to Professor S. Patai for valuable discussion.

[6] G. W. WHELAND, *Advanced Organic Chemistry* (3rd Ed.), Wiley, New York (1960).

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HYDROXYASPARTIC ACID FROM ALFALFA AND CLOVER ROOTS

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Abstract—Hydroxyaspartic acid has been identified in extracts of the free amino acids from field grown alfalfa and clover root tissue. It was identified by chromatography and co-chromatography on ion exchange columns and the amount of ammonia liberated on periodate oxidation. It occurs in these legume root tissues in amounts ranging from 0·6–2 μmoles/g of dry root tissue. This is much higher than the concentration of some of the more common amino acids.

INTRODUCTION

DURING the past ten years many new amino acids have been isolated from plants by the use of paper or ion exchange chromatography. Consden, *et al.*[1] in 1944 first described the use of paper chromatography in the separation of amino acids. Dent, *et al.*[2] demonstrated the potentialities of paper chromatography to plant biochemistry in a study of the amino acid complex of potato tubers and supplied definite evidence that other unknown ninhydrin positive substances (possibly amino acids) were present in green plants. Wilding, *et al.*[3] have compared paper and ion exchange chromatography in a study of free amino acids in alfalfa and clover roots and have shown the advantages of the latter method.

In 1921 Dakin[4] synthesized the amino acid, hydroxyaspartic acid, and described its chemical and physical properties. Sallach and Peterson[5] observed the formation of hydroxyaspartic acid by transamination between oxaloglycolate, the keto form of dihydroxyfumarate, and glutamic acid. Virtanen in 1957[6] reported hydroxyaspartic acid in extracellular material produced by *Azotobacter* during nitrogen fixation by the organism. It appeared to be combined with other commonly occurring amino acids in *Azotobacter*. Sallach and Kornguth[7] have reported the occurrence of hydroxyaspartic acid in pancreatic digests of casein.

During recent work on the biochemistry of cold hardiness in legumes[3,8] a very well resolved ninhydrin reacting component was observed preceding aspartic acid by approximately 36–37 ml of eluant in an ion exchange chromatogram of the free amino acid of both alfalfa and red clover root extracts.

The recognition of this new component as hydroxyaspartic acid was facilitated by simultaneous collaborative work with Dr. H. J. Sallach and Mrs. M. L. Kornguth, for whom we were analyzing the amino acids in pancreatic digests of casein. A ninhydrin reacting peak which they obtained from this source, the one the authors had observed from legume extracts, and an authentic sample of synthetic hydroxyaspartic acid all occurred at an identical location on the elutograms and gave us strong evidence for believing that the

[1] R. CONSDEN, A. H. GORDON and A. J. P. MARTIN, *Biochem. J.*, **38**, 224 (1944).
[2] C. E. DENT, E. STEPKA and F. C. STEWARD, *Nature*, **160**, 682 (1947).
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[4] H. D. DAKIN, *J. Biol. Chem.*, **48**, 273 (1921).
[5] H. J. SALLACH and T. H. PETERSON, *J. Biol. Chem.*, **223**, 629 (1956).
[6] A. I. VIRTANEN, *Act. Chem. Fennica*, B, **30**, 100 (1957). (Cited from *Biol. Revs.* **33**, 393 (1958)).
[7] H. J. SALLACH and M. L. KORNGUTH, *Biochim. Biophys. Acta.*, **34**, 582 (1959).
[8] M. D. WILDING, M. A. STAHMANN and D. SMITH, *Plant Physiol.*, **35**, 733 (1960).

component from the root tissue was indeed hydroxyaspartic acid. Moore, *et al.*[9] show an unknown compound at the same position in their elutogram of an extract of rat liver. The results presented in this paper provide evidence that hydroxyaspartic acid occurs uncombined in protein free extracts of alfalfa and clover roots.

RESULTS AND DISCUSSION

Figure 1 is typical of the results obtained in the amino acid analysis of the free amino acids in alfalfa root tissue showing the sharp ninhydrin positive peak (labelled OH-Asp)

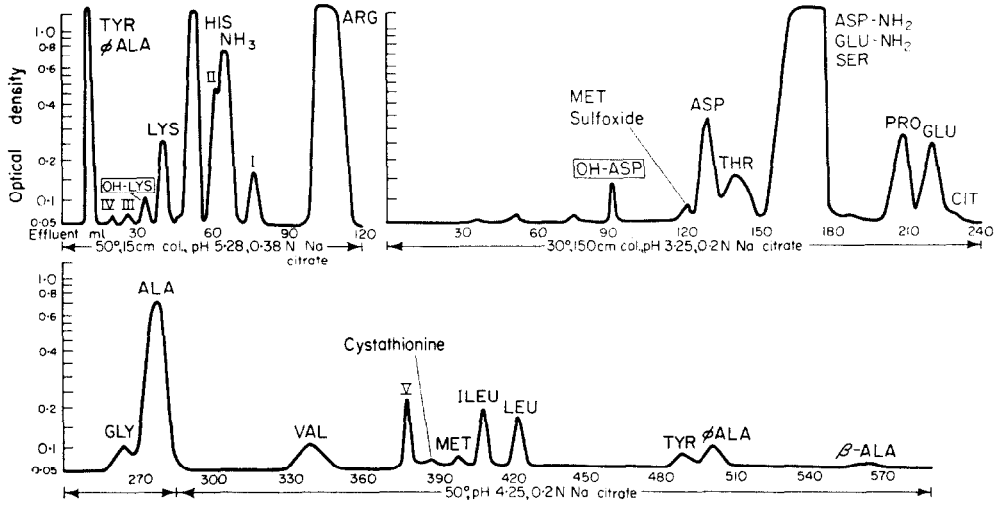

FIG. 1. A TYPICAL ELUTOGRAM OF FREE AMINO ACIDS FROM A VERNAL ALFALFA ROOT EXTRACT. The upper right hand portion shows the position of the hydroxyaspartic acid peak in relation to the other acidic components.

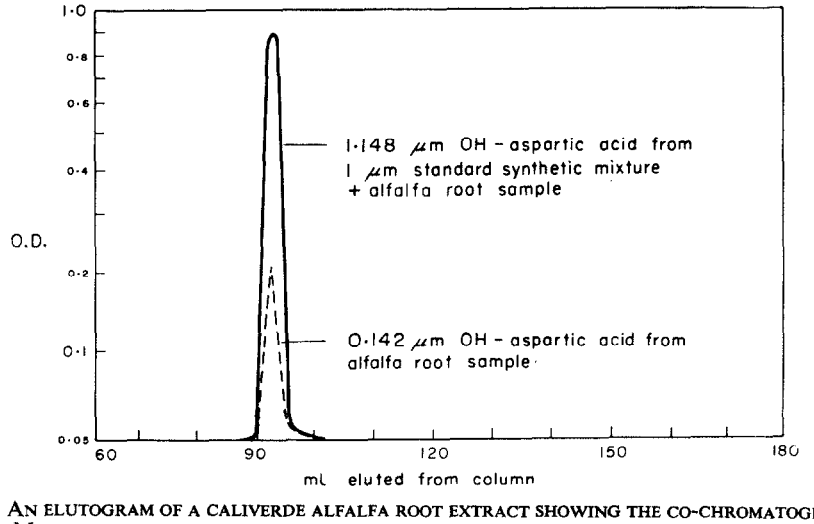

FIG. 2. AN ELUTOGRAM OF A CALIVERDE ALFALFA ROOT EXTRACT SHOWING THE CO-CHROMATOGRAPHY OF 1·0 μM SYNTHETIC HYDROXYASPARTIC ACID WITH AN UNKNOWN NINHYDRIN REACTING PEAK FROM THE ALFALFA EXTRACT. This extract was analyzed on the 150 cm column of the Beckman/Spinco amino acid analyzer.

[9] S. MOORE, D. H. SPACKMAN and W. H. STEIN, *Federation Proc.*, **17**, 1107 (1958).

that came off the column before aspartic acid. Figure 2 shows a portion of the elutogram obtained by co-chromatography of 1 μM of authentic hydroxyaspartic acid and 2 ml of the alfalfa root extract in relation to the position of this peak. From a quantitative determination using the ninhydrin colour value of the authentic sample, we estimated that the alfalfa extract contained 0·142 μmoles of hydroxyaspartic acid. The results obtained on co-chromatography show that the authentic hydroxyaspartic came off the column at exactly the same position as the unknown and that it produced the expected theoretical increase in ninhydrin colour.

Nicolet and Shinn[10] showed that ammonia is quantitatively liberated from molecules having vicinal amino and hydroxyl groups upon oxidation with periodate. The ammonia release following periodate oxidation of an authentic sample of synthetic hydroxyaspartic acid and of an aliquot of the effluent containing the unknown peak thought to be hydroxyaspartic was determined. Based upon an equal ninhydrin colour value for the amino acid in the effluent and for the synthetic hydroxyaspartic acid; the ammonia released by the isolated amino acid was 100·7 per cent of that recovered from the authentic synthetic sample of hydroxyaspartic acid.

The amount of free hydroxyaspartic acid found in alfalfa root tissue varied from 0·58–1·17 μM/g of dry root tissue.[3] There was some slight differences between the two alfalfa varieties. The lowest values were found in those root samples collected in October; both the August and December values were somewhat higher. Clover roots contained a little less; values from 0·42–0·58 μM/g of clover root tissues were obtained with samples collected in November.[8] It is interesting to note that the concentration of free hydroxyaspartic acid in alfalfa or clover roots was often considerably higher than that of the common amino acids, valine, methionine, *iso*leucine, leucine, tyrosine, phenylalanine, or lysine.

Our results show that these alfalfa and clover roots contained free hydroxyaspartic acid. Further work is needed to determine whether this amino acid is formed by the green plant or if it is produced solely by the nitrogen-fixing bacteria that may have been present in the roots of these legumes.

EXPERIMENTAL

Extraction and chromatography of free amino acids

Alfalfa (*Medicago sativa* L., var. Caliverde and Vernal) and Red Clover (*Trifolium pratense* L., var. Wisconsin common) roots were harvested in the fall from field grown plants, washed free of soil and the upper 4 in. frozen immediately in liquid air to prevent enzymatic change. The root tissue was ground while frozen, lyophilized and the free amino acids extracted with acetone–water as previously described.[3,8] Protein in the extracts was removed with picric acid according to the method of Stein and Moore.[11] The free amino acids in the extracts then were separated by ion exchange chromatography according to the method described by Spackman, Stein and Moore.[12] Two ml of each extract, which was equivalent to 120 mg of dry tissue was chromatographed on a Beckman/Spinco amino acid analyzer. The 150 cm column was employed for acidic and neutral amino acids using sodium citrate buffer at pH 3·25 at 30°–50° as the eluting agent. A typical elutogram is shown in Fig. 1.

[10] B. H. Nicolet and L. A. Shinn, *J. Am. Chem. Soc.*, **61**, 1615 (1939).
[11] W. H. Stein and S. Moore, *J. Biol. Chem.*, **211**, 915 (1954).
[12] D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

Other chromatograms were run using 2 ml of an extract from alfalfa to which was added 1 μM of authentic, synthetic hydroxyaspartic acid[4] kindly supplied by Dr. Sallach. The results of the co-chromatography are shown in Fig. 2.

Periodate oxidation

An amount of extract equivalent to 4·8 g of dry root tissue was chromatographed in 4 runs and the effluent containing the ninhydrin positive material thought to be hydroxyaspartic acid was collected and concentrated to 4 ml. Two ml of the concentrated effluent was placed in the outer well of a Conway diffusion dish containing 2 ml of 0·2 M periodate and 0·1 ml of 5 per cent glycine solution to bind the formaldehyde released. The center well contained 2 ml of 2 per cent boric acid solution with indicator. The contents were stirred gently, incubated 3 hr at room temperature and the ammonia released then titrated. Standards containing 1 μM of authentic synthetic hydroxyaspartic acid and controls without periodate were run simultaneously.

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CHEMICAL STUDIES ON MALFORMIN[1]. 1. MALFORMIN A

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(*Received* 7 *May* 1962)

Abstract—The optical configuration and sequence of amino acids of malformin A has been determined. From partial hydrolysates of malformin A, desthiomalformin A, and oxidized malformin A, eleven peptides were isolated. The structure *cyclo*-L-*iso*leucyl-D-cysteinyl-L-valyl-D-cysteinyl-D-leucyl was assigned to malformin A. The complete racemization of D-half-cystine to DL- cysteic acid after performic acid oxidation and acid hydrolysis made it difficult to postulate the presence of an S–S bond in the molecule. The structure surrounding the sulfur was investigated. A new isomer of malformin A was isolated by alumina chromatography.

INTRODUCTION

MALFORMIN, a metabolic product of the fungus *Aspergillus niger*, induces curvatures and grotesque malformations of bean plants[3,4] and severe curvatures of corn roots.[5] In a previous paper[6] it was shown that at least two kinds of malformin are produced by various strains of *A. niger*. Malformin A (referred to earlier as "old" malformin[7]), $C_{23}H_{39}N_5O_5S_2$, m.p. over 300° (decomp.), is produced by *A. niger* strain 56–39 and is a cyclic pentapeptide consisting of one mole of leucine, isoleucine and valine, and two moles of half-cystine. Malformin B (referred to earlier as "new" malformin[7]) is produced by *A. niger* strain 56–30 and has the same physical and biological properties as malformin A. However, malformin B liberates low yields of allo-*iso*leucine (0·22 moles) and *iso*leucine (0·08 moles) after acid hydrolysis. The present paper reports further chemical studies on malformin A with emphasis on the amino acid sequence and the structure surrounding the sulfur.

RESULTS AND DISCUSSION

Optical configuration of amino acid components

The molar ratios of amino acids in malformin A and several of its derivatives are summarized in Table 1. Valine was isolated from acid hydrolysates of malformin A and from the specific rotation was shown to be L-valine. Leucine and *iso*leucine were isolated as a mixture from acid hydrolysates of desthiomalformin A by cellulose column chromatography. The $[\alpha]_D^{25}$ of +9·2° agreed with the calculated value of a mixture of authentic D-leucine and L-*iso*leucine. These results were confirmed by enzymatic methods when the isolated mixture was treated with L-amino acid oxidase.[8]

Malformin A was oxidized with performic acid and completely hydrolyzed. From the hydrolysate cysteic acid (II) was isolated. The $[\alpha]_D^{25}$ was zero, indicating a racemized form. This suggested to us that of the two moles of half-cystine in the molecule one was L- and

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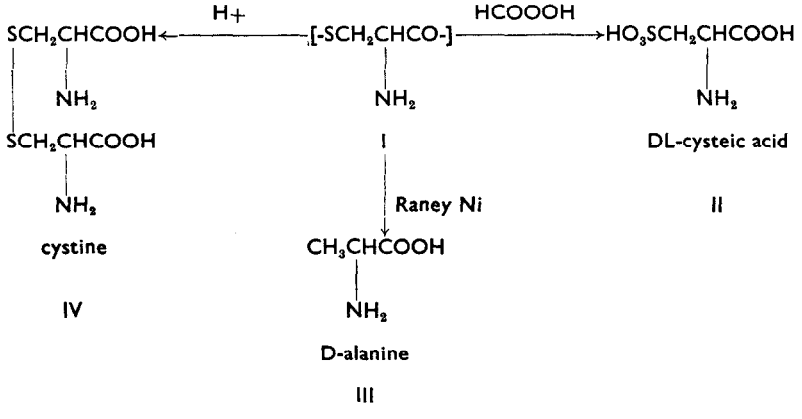
[2] Institute of Physical and Chemical Research, Bunkyo-Ku, Tokyo, Japan.
[3] R. W. CURTIS, *Plant Physiol.*, **33**, 17 (1958).
[4] R. W. CURTIS, *Plant Physiol.*, **36**, 37 (1961).
[5] R. W. CURTIS, *Science*, **128**, 661 (1958).
[6] N. TAKAHASHI and R. W. CURTIS, *Plant Physiol.*, **36**, 30 (1961).
[7] S. MARUMO and R. W. CURTIS, *Plant Physiol.*, **36**, xiii (1961).
[8] E. A. ZELLER, *Advances in Enzymol.*, **8**, 459 (1948).

TABLE 1. MOLAR RATIOS OF AMINO ACIDS IN MALFORMIN A, OXIDIZED MALFORMIN A AND DESTHIOMALFORMIN A

Amino Acid	Molar ratio		
	Mal A[6]	Oxid. Mal A[6]	Desthiomal. A
D-leucine	1·00	1·00	1·00
L-*iso*leucine	0·79	0·76	0·77
L-valine	0·80	0·99	1·13
Half-cystine	1·04	—	—
DL-Cysteic acid	0·08	1·97	—
D-alanine	—	—	1·89

the other D- form. Then DL-cysteic acid would be obtained from oxidized malformin after acid hydrolysis. However, when malformin A was desulfurized by Raney Ni and desthiomalformin was hydrolyzed, D-alanine (III), and not DL-alanine, was obtained. Obtaining DL-cysteic acid from oxidized malformin A and D-alanine from desthiomalformin A is apparently conflicting. It is generally stated that Raney Ni reduction does not induce racemization of cystine and cysteine during desulfurization except in compounds in which the sulfur is attached directly to an asymmetric carbon.[9] We have concluded that both moles of half-cystine in the original molecule are of the D-form and are racemized during performic acid oxidation and acid hydrolysis (see later). Malformin A contains one mole of D-leucine, L-*iso*leucine and L-valine, and two moles of D-half-cystine.



Amino acid sequence of malformin A

To determine the amino acid sequence of the molecule, malformin A, oxidized malformin A and desthiomalformin A were partially hydrolyzed and the N-terminal amino acid of each purified peptide was determined by the DNP-method. Malformin A was difficulty soluble in conc. HCl and 6*N* HCl-CH_3COOH at room temperature, conditions considered favorable for partial hydrolysis of peptidic compounds. Accordingly malformin A was partially hydrolyzed with 2*N* HCl–CH_3COOH at 100° for 2 hr. The partially hydrolyzed peptides were separated by cellulose column chromatography using butanol–acetic acid–water as the solvent system. Each fraction was purified further into single spots by multiple paper chromatography. Oxidized malformin A was partially hydrolyzed

[9] W. A. BONNER, *J. Am. Chem. Soc.*, **74**, 1034 (1952).

in conc. HCl for 22 days at 25°, and each peptide was purified by multiple paper chromatography. Desthiomalformin A was hydrolyzed in the same manner for 18 days.

The data from the partial hydrolysis studies are summarized in Table 2. Five peptides were isolated from malformin A and three each from oxidized and desthiomalformin A. Only one sequence of amino acids is possible which satisfies the occurrence of the peptides. Because of these results and earlier data[6] which indicated that malformin A is a neutral cyclic peptide, we have assigned *cyclo*-L-*iso*leucyl·D-cysteinyl·L-valyl·D-cysteinyl·D-leucyl as the structure of malformin A.

TABLE 2. AMINO ACID SEQUENCE OF MALFORMIN A*

Source	Peptide	Amino acid components	N-terminal amino acids	Amino acid sequence
Malformin A	P_1	Leu, *i*Leu, Val, Cys	Leu	*i*Leu-Cys-Val-Cys Leu
	P_2	Leu, *i*Leu, Cys	Leu	*i*Leu-Cys Leu
	P_3	Leu, Val, Cys	Val	Val-Cys-Leu
	P_4	Val, Cys	Cys	S—S Cys-Val Cys
	P_5	Val, Cys	Val, Cys	S—S Cys Val-Cys
Oxidized Malformin A	P_6	Leu, Val, Cys-SO_3H	Cys-SO_3H	SO_3H SO_3H Cys-Val-Cys-Leu
	P_7	Val, Cys-SO_3H	Cys-SO_3H	SO_3H Cys-Val
	P_8	*i*Leu, Cys-SO_3H	*i*Leu	SO_3H *i*Leu-Cys
Desthio-Malformin A	P_9	Leu, *i*Leu, Val, Ala	Ala	*i*Leu Ala-Val-Ala-Leu
	P_{10}	*i*Leu, Ala	*i*Leu	*i*Leu-Ala
	P_{11}	Val, Ala	Val	Val-Ala
Amino acid sequence of Malformin A				*i*Leu-Cys-Val-Cys-Leu

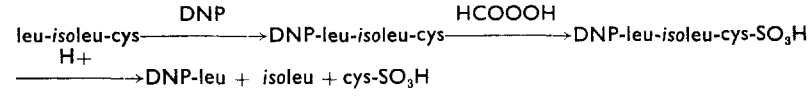
* Cys = both half cystine and cysteine since the structure surrounding the sulfur is not known.

If an S–S bond were present in malformin A, peptides P_2 and P_3 could not be obtained. Peptide P_2 was dinitrophenylated to produce DNP-P_2 which was a single spot on the paper chromatograms. DNP-P_2 remained as a single spot after performic acid oxidation as shown in Scheme 1. Acid hydrolysis of oxidized DNP-P_2 liberated DNP-leucine, *iso*leucine and cysteic acid. If an S–S bond were present two DNP-derivatives should have been produced after oxidation with performic acid as shown in Scheme 2. This was confirmed by determining the amount of half-cystine in the peptide. Less than one mole of cysteic acid was liberated from P_2 after performic acid oxidation and hydrolysis.

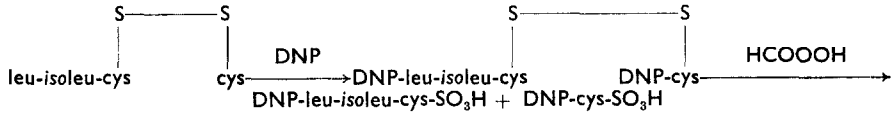
Similar experiments with peptide P_3 also indicated the absence of an S–S bond in malformin A. As with peptide P_2, DNP-P_3 remained as a single compound following performic acid oxidation. On the other hand, peptides P_4 and P_5 were shown to contain

S-S bonds. DNP-P_4 which occurred as a single yellow spot on the paper chromatograms, was split into two yellow compounds after performic acid oxidation. These compounds were DNP-cysteic acid and DNP-β-sulfoalanylvaline (see Scheme 3). However, the occurrence of cystinylvaline and valylcystine does not necessarily indicate the presence of S–S bonds in malformin A, because the formation of S–S bonds from thiol groups occurs readily during acid hydrolysis and isolation procedures.

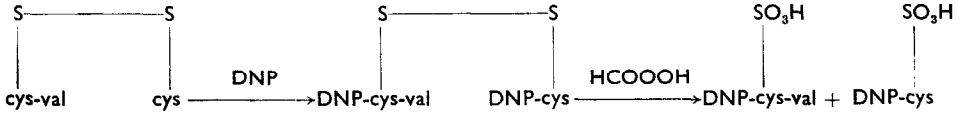
Scheme I



Scheme II



Scheme III



Generally, sulfur-containing peptides which contain two moles of half-cystine which can be reduced to cysteine by zinc in acetic acid or sodium cyanide, are considered to contain an S–S bond. But in the case of malformin A, complete racemization of D-half-cystine into DL-cysteic acid after performic acid oxidation and acid hydrolysis, as well as the lack of S–S bonds indicated by two of the partial hydrolysis products (P_2, P_3), does not allow us to make this conclusion. We have oxidized authentic cystine and cysteine with performic acid and found that they did not racemize. Furthermore, L-cysteine in normal protein retains its configuration during oxidation.[10] We have been unable to find references which describe racemization of optically active cystine in proteins during oxidation. Therefore, we have investigated possibilities other than an S–S bond as well as attempted to show the presence of this bond in malformin A.

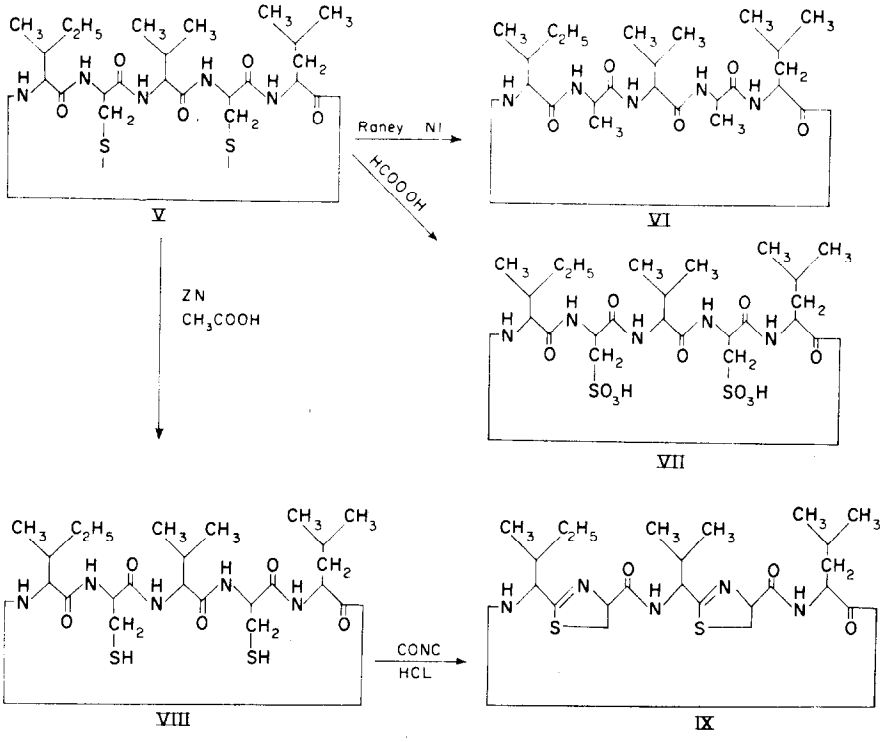
Investigations on the structure surrounding sulfur in malformin A

Nitroprusside,[11] phosphotungstic acid[12], and iodine[13] tests on malformin were negative, indicating the lack of free thiol groups. It was easily reduced to an SH-form by boiling with Zn dust in glacial acetic acid and a positive nitroprusside test was obtained after 5 min. boiling, reaching maximum coloration after 15 min. Malformin A was not reduced with sodium cyanide. These data indicated that the sulfur of malformin A was masked but could be readily converted to cysteine by Zn reduction.

After direct acid hydrolysis of malformin A, 1·04 moles of half-cystine was obtained

[10] L. C. CRAIG, Rockefeller Institute for Medical Research, N.Y., personal communication (1961).
[11] G. TOENNIES and J. J. KOLB, *Anal. Chem.*, **23**, 823 (1951).
[12] F. D. SNELL and C. T. SNELL, *Colorimetric Methods of Analysis*, pp. 176, 237, Van Nostrand, New York (1937).
[13] M. P. SCHUBERT, *J. Biol. Chem.*, **114**, 341 (1936).

from two moles of half-cystine (Table 1). The low yield (approx. 50 per cent) was attributed to the instability of half-cystine in acid solution. When malformin A (V) was boiled with Raney Ni in methylcellosolve, desthiomalformin (VI), $C_{23}H_{41}N_5O_5$, m.p. over 300° (decomp.), was produced. It was concluded that desthiomalformin contained two moles of alanine (Table 1) while half-cystine disappeared and the other constituents were unchanged. When malformin A was oxidized with performic acid for 4 hr, the main product (VII) consumed 2 moles of alkali by direct titration, indicating the presence of 2 moles of free sulfonic acid groups in the molecule. Oxidized malformin A shows maximum u.v. absorption at 271 mμ (ε, 358) only in alkali. Absorption was destroyed by warming for 25 min at 80° in $N/5$ NaOH.

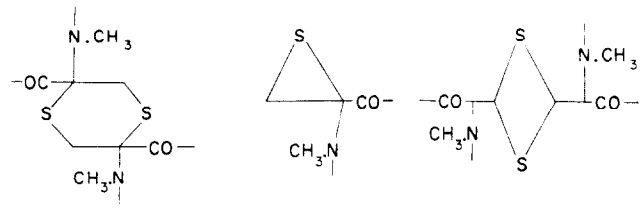


Cymerman and Willis[14] reported that cystine absorbs in the infra-red at 451 cm^{-1} and that other disulphides absorb in the region from 430–490 cm^{-1}. We have attempted to detect S–S absorption bands in this region in malformin A. Whereas cystine had an absorption band at 456 cm^{-1}, malformin A had no appreciable absoption and its spectrum was essentially the same as that of desthiomalformin A. NMR techniques were unsuccessful in providing additional information because of the poor solubility of malformin A in suitable organic solvents. The spectrum indicated only the presence of methyl groups. S–S bonds react selectively with sodium sulfite in aqueous solutions[15,16] producing free –SH groups which are estimated potentiometrically. Application of this method also failed because of the limited solubility of malformin A in the reaction solution. Because our experiments

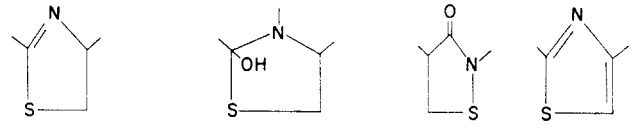
[14] J. Cymerman and J. B. Willis, *J. Chem. Soc.*, 1332 (1951).
[15] J. L. Bailey and R. D. Cole, *J. Biol. Chem.*, **234**, 1733 (1959).
[16] I. M. Kolthoff and W. Stricks, *J. Am. Chem. Soc.*, **72**, 1952 (1950).

have failed to demonstrate the presence of S–S bonds in the molecule we have investigated other structural possibilities.

Brockman[17] postulated a novel amino acid isomer, which occurred as a dithiane ring (X) in the antibiotic echinomycin. The possibility of other structures (XI, XII) were also discussed. These compounds were reported to be destroyed during acid hydrolysis, and methylamine appeared as a decomposition product from X.[18] These possibilities were excluded for malformin A because it liberates a 50 per cent yield of half-cystine after acid hydrolysis.



X XI XII



XIII XIV XV XVI

Lockhart *et al.*[19] and Weisiger *et al.*[20] have concluded that cysteine in bacitracin A was masked as a thiazoline (XIII), which could be detected by characteristic ultraviolet absorption at 270 mμ,[21] and was converted to free –SH groups by reducing with sodium cyanide or by mild hydrolysis in dilute HCl. In neutral solution malformin A had only end absorption (ε, 5400 at 220 mμ), indicating the absence of thiazoline rings in the molecule. It was also shown that cysteine, but not cystine, in peptidic compounds can be converted to a thiazoline in conc. HCl or HCl–MeOH.[21,22] We have also demonstrated this conversion using formylcysteine and reduced glutathione. Neither diformylcystine nor oxidized glutathione in conc. HCl had thiazoline-like u.v. absorption peaks. Malformin A was reduced by boiling with zinc in acetic acid. When the reduced compound (VIII) was suspended in conc. HCl and shaken for two days it showed max. u.v. absorption at 275 mμ (ε, 2700). Comparison of this absorption (Fig. 1) with that of related compounds treated similarly indicated the formation of a thiazoline ring in malformin A.

Thiazoline-containing malformin A (IX) was hydrolyzed and the amino acids were quantitatively determined by ion-exchange chromatography (Table 3). We obtained 0·16 moles of allo-*iso*leucine, whereas only a trace (?) was obtained when malformin A was hydrolyzed directly.[6] The appearance of allo-*iso*leucine from bacitracin after acid hydrolysis has been explained as a result of the double bond in a thiazoline ring (XVII) shifting to the α-carbon of *iso*leucine (XVIII) during hydrolysis, resulting in the racemization of L-*iso*-

[17] H. BROCKMAN, *Helv. Chim. Acta*, **42**, 305 (1959).
[18] H. BROCKMAN, *Helv. Chim. Acta*, **40**, 199, 205 (1957).
[19] I. M. LOCKHART, E. P. ABRAHAM, and G. G. NEWTON, *Biochem. J.*, **61**, 532 (1955).
[20] J. R. WEISIGER, W. HAUSMANN and L. C. CRAIG, *J. Am. Chem. Soc.*, **77**, 3123 (1955).
[21] R. E. BASFORD and J. M. HUENNEKENS, *J. Am. Chem. Soc.*, **77**, 3878 (1955).
[22] W. STOFFEL and L. C. CRAIG, *J. Am. Chem. Soc.*, **83**, 145 (1961).

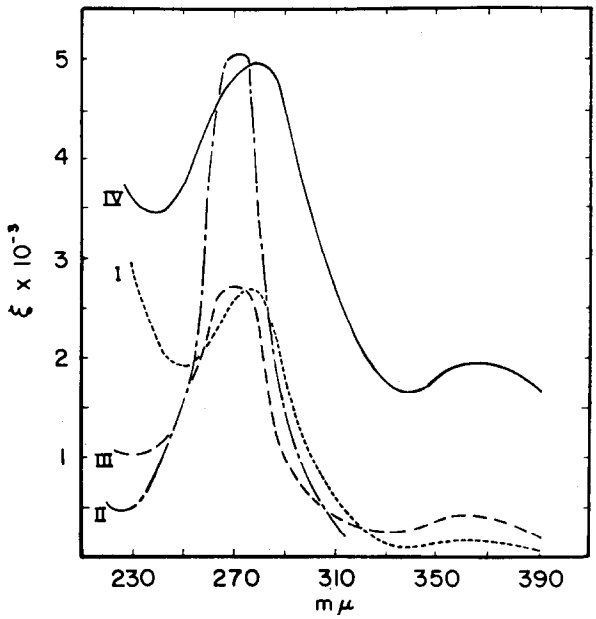


FIG. 1. ULTRAVIOLET ABSORPTION IN CONC. HCl BY REDUCED MALFORMIN A (I, $\varepsilon = 2700$ at 275 mμ), FORMYLCYSTEINE (II, $\varepsilon = 5030$ at 270 mμ), GLUTATHIONE (III, $\varepsilon = 2750$ at 268 mμ) AND CRUDE MALFORMIN A (IV, $\varepsilon = 4950$ at 275 mμ).

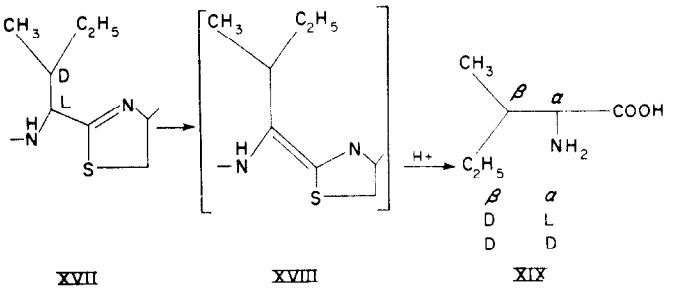
leucine (XIX, α, L) and production of D-allo-*iso*leucine (XIX, α, D).[23] On the basis of the molar extinction coefficient (ε, 2700), a thiazoline ring (IX) was not quantitatively produced from reduced malformin A (VIII). If the molar extinction coefficient of a thiazoline ring is taken as 5000 (bacitracin, $\varepsilon = 5000$, formylcysteine in conc. HCl, $\varepsilon = 5080$) about 27 per cent of reduced malformin A was converted to a thiazoline and 0·27 moles of thiazoline produced 0·16 moles of allo-*iso*leucine as a result of racemization.

TABLE 3. MOLAR RATIOS OF AMINO ACIDS IN THIAZOLINE-CONTAINING MALFORMIN A (IX), CRUDE MALFORMIN A PRETREATED WITH CONCENTRATED HCl, AND A NEW ISOMER OF MALFORMIN A

Amino acid	Molar ratios		
	Thiazoline-contg. Malformin A	Crude Malformin A pretreated with conc. HCl	New isomer of Malformin A
Leucine	1	1	1
*Iso*leucine	0·66	0·47	0·83
Allo-*iso*leucine	0·16	—	—
Valine	1·14	1·12	1·09
Half-cystine	1·52	0·58	1·47
Cysteic acid	0·09	0·25	0·06

When a sample of crude malformin A (i.e., prior to purification by alumina chromatography) was shaken in conc. HCl for two days, thiazoline-like u.v. absorption was observed (Fig. 1). No absorption occurred in neutral solution. These properties suggested the

[23] W. KONIGSBERG and L. C. CRAIG, *J. Am. Chem. Soc.*, **81**, 3452 (1959).



XVII XVIII XIX

formation of a thiazoline or thiazoline-like chromophore and because such rings are not obtained from S–S forms in peptides, we considered that malformin A might contain a hydroxy-thiazolidine (XIV) which is easily converted to a thiazoline in conc. HCl. Under these conditions, however, purified malformin A showed no u.v. absorption. Absorption by crude samples of malformin A in conc. HCl was attributed to other substances (see later) and we discarded (XIV) as a possible structure.

The amino acid components of directly hydrolyzed crude malformin A were determined quantitatively by Dowex 50 resin chromatography. The results were the same as those obtained from malformin A. However, when the crude samples were pretreated in conc. HCl for 2 days, thus producing a u.v. absorption peak at 275 mμ, and then hydrolyzed, (1) the absorption in u.v. was still present and (2) the yield of *iso*leucine and half-cystine decreased (Table 3). Similarly, it was reported that thiazoline in bacitracin was converted in phosphate buffer (pH 7) to a thiazol derivative (XVI) stable to acid hydrolysis.[20] As a result, the yield of *iso*leucine and cystine decreased and u.v. absorption of the thiazol derivative was observed after complete acid hydrolysis. The absence of allo-*iso*leucine in hydrolysates of crude malformin A previously treated with conc. HCl suggested that the chromophore might be different from 2-thiazoline (IX) so that (1) L-*iso*leucine was not racemized to allo-*iso*leucine and (2) it was converted to a u.v. absorbing chromophore stable to hydrolysis. These data suggested further the possibility of an isomer of malformin A in the crude sample which differed in the structure surrounding the sulfur. Therefore, after elution of malformin A from columns of alumina we continued the elution with other solvent systems and were able to isolate another biologically active compound. Analysis of amino acids in this compound (Table 3) was similar to that of malformin A. Preliminary experiments showed that the u.v. absorption of this compound in conc. HCl was similar to that observed by crude malformin A and that the yields of *iso*leucine and half-cystine decreased after pretreatment for 2 days in conc. HCl and acid hydrolysis.

Malformin A was shown to contain 5 moles of active hydrogen which could be attributed to the presence of 5 peptide bonds. These data do not support (XIV) and (XV) as possible structures of malformin A. The dye, 2,6-dichlorophenol-indophenol, has been used for differential determination of masked thiols (i.e. S–S, thiazolidine, thiazoline, thiazol).[24] The reaction with malformin A was almost nil. The reaction velocity was of the same order as that given by S–S bonds ($<10^{-6}$ μ mole/ml per min). Thus, although we have been unable to confirm the presence of S–S bonds in malformin A, the possibility that they are present has not been excluded.

Experiments with malformin B also show that racemized cysteic acid is obtained after

[24] R. E. BASFORD and F. M. HUENNEKENS, *J. Am. Chem. Soc.*, 77, 3873 (1955).

performic acid oxidation and hydrolysis, but only D-alanine is obtained after desulfurization. In this respect, the nature of the sulfur in both malformin A and B appears to be similar. Further investigations on the structure of malformin B and other isomers, as well as synthesis of malformin A, may be helpful in determining the final structure of malformin A.

EXPERIMENTAL

Desthiomalformin A (VI)

Three hundred mg malformin A were dissolved in 500 ml methylcellosolve with gentle warming. Excess Raney Ni[25] was added, and the mixture was refluxed for 1·5 hr. Raney Ni was removed by filtration and the filtrate evaporated to dryness *in vacuo*. The residue was dissolved in a small quantity of methylcellosolve with warming, condensed until precipitation of (VI) began, and allowed to stand overnight. The precipitate was collected by filtration, washed with small aliquots of methylcellosolve, and dried over P_2O_5 (yield, 70 mg, m.p. over 300°). (Found: C, 59·03; H, 8·68; N, 14·95. $C_{23}H_{41}N_5O_5$ required: C, 58·10; H, 8·78; N, 14·98%).

Desthiomalformin A, 5·186 mg, was hydrolyzed with 6*N* HCl-acetic acid, reduced to dryness by distillation, and dissolved in 5 ml phosphate buffer (pH 3·42). 0·5 ml was used to determine amino acid components by Dowex 50 resin chromatography as described earlier.[6] The analysis showed that half-cystine was converted to alanine and the remainder of the molecule was unchanged (Table 1).

Oxidized malformin A (VII)

Four hundred mg malformin A was dissolved in 10% performic acid at 4° and allowed to stand at the same temp. for 4 hr. The solution was evaporated to dryness and the residue dried over KOH and P_2O_5. Twenty ml hot ethanol was added to 434 mg crude (VII), dissolving the major portion. The insoluble fraction (approx. 75 mg, m.p. over 300°) was separated by filtration and washed twice with 10 ml aliquots hot water. Three hundred and twenty mg of the ethonol soluble fraction were obtained (decomp. approx. 250°).

A quantity (20·4 mg) of the ethanol soluble compound were titrated directly with 0·1 *N* NaOH ($f = 1·004$) consuming 0·645 ml. On the basis of two free sulfonic acid groups per molecule, the molecular weight was calculated as 631 (calcd. for oxidized malformin A, 629). 14·7 mg of the ethanol insol. compound was titrated and consumed 0·218 ml of 0·1*N* NaOH. This suggested that one sulfonic acid group per molecule was present. On this basis the molecular weight was calculated as 674. The two compounds showed no difference in their amino acid content by paper chromatography. Neither had u.v. absorption peaks in neutral or acidic solution but showed reversible absorption in alkali as shown in Fig. 2. Absorption was gradually destroyed in *N*/5 NaOH and disappeared after warming for 25 min at 80°.

Reduced malformin A (VIII)

One hundred mg malformin A was dissolved in 100 ml glacial acetic acid. An excess of zinc dust was added and the mixture refluxed. Within 5 min. the mixture was positive for the nitroprusside test, reaching a max. in 15 min. The hot solution was filtered and evaporated to dryness. The residue was suspended in 20 ml water, stirred, and filtered. This process was repeated twice to remove zinc acetate. However, we were unable to

[25] R. MOZINGO, *Organic Synthesis*, **21**, 15 (1941).

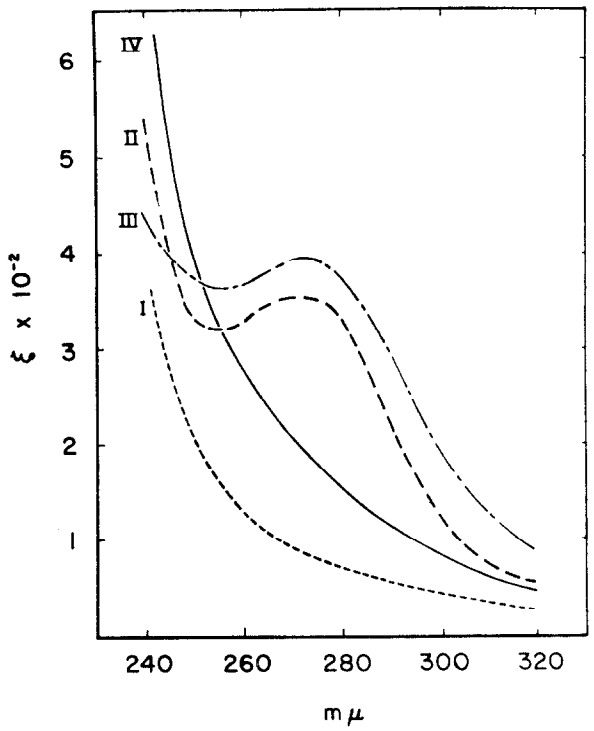


FIG. 2. ULTRAVIOLET ABSORPTION BY OXIDIZED MALFORMIN A IN WATER OR IN HCl (I), OXIDIZED MALFORMIN A (EtOH soluble fraction) IN N/5 NaOH (II, ε = 358 at 271 mμ), OXIDIZED MALFORMIN A (EtOH insoluble fraction) IN N/5 NaOH (III, ε = 395 at 273 mμ), AND (II) AFTER WARMING AT 80°C FOR 25 MIN (IV).

remove all traces by this method. Since (VIII) was insoluble in most organic solvents and soluble only in hot acetic acid or pyridine, further purification was difficult (yield, 95 mg).

Thiazoline-containing malformin A (IX)

Approx. 5 mg thiolmalformin A (VIII) was suspended in 5 ml conc. HCl and shaken for 2 days. Only a small portion was undissolved after this time. The solution was filtered before measuring u.v. absorption. Maximum absorption at 275 mμ indicated the presence of a thiazoline ring. The filtrate was hydrolyzed for 20 hr and the amino acids were determined by Dowex 50 resin chromatography. The yield of *iso*leucine decreased while allo-*iso*leucine increased (Table 3). Molar extinction coefficient (2700) was calcd. on the basis of the amount of leucine recovered.

Formylcysteine

Diformylcystine was prepared by the method of V. Du Vigneaud *et al.*[26] Fourteen g diformylcystine (m.p. 196–197°) was produced from 20 g of L-cystine. Diformylcystine was reduced to formylcysteine with zinc by the method of Pirie and Hele.[27] 2.4 g formylcysteine (m.p. 148°) was produced from 5 g diformylcystine.

[26] V. DU VIGNEAUD, R. DARFMANN and H. S. LORING, *J. Biol. Chem.*, **98**, 577 (1932).
[27] N. W. PIRIE and T. S. HELE, *Biochem. J.*, **27**, 1716 (1933).

Optical configuration of amino acids

Three hundred mg desthiomalformin (VI) was dissolved in 30 ml glacial acetic acid and an equal volume of conc. HCl was added. The solution was heated at 110° in an autoclave for 22 hr. The clear solution was evaporated to dryness in vacuo. Excess HCl was removed by repeated additions and evaporation of 10 ml aliquots of water. The residue, dried over KOH, was chromatographed by cellulose column (2·7×30 cm.) chromatography using butanol:acetic acid:H_2O = 4:1:5. Each fraction contained 3 ml. The tubes containing a particular amino acid were combined, dried *in vacuo*, and converted to the hydrochloride by addition of HCl and evaporation to dryness over NaOH in a desiccator. Because separation of leucine and *iso*leucine was incomplete they were combined. The residues were dissolved in 2 ml water, declorized with charcoal, dried over NaOH, and used to determine optical configuration.

A sample (93·6 mg) of a mixture of leucine and *iso*leucine was used. $[\alpha]_D^{25} = 9{\cdot}2°$ (C, 7·4% in 6*N* HCl) was calculated from the observed value of +0·679. This value agreed with the calculated value of a mixture of D-leucine (1 mole) and L-isoleucine (0·79 mole) (Table 4). The molar ratios of leucine (1·0) and *iso*leucine (0·79) were obtained by quantitative determination of the amino acids after separation by Dowex 50 resin chromatography. The optical configuration of leucine and *iso*leucine in the mixture was also determined by enzymatic methods. 0·96 mg was dissolved in 1·66 ml phosphate buffer (pH 7·2) and 500 μg of L-amino acid oxidase (Snake venom, Nutritional Biochemicals Corp.) in 1 ml of the same buffer was added. Controls consisted of authentic L-leucine and L-*iso*-leucine. The solutions were incubated at 35° for 3 hr. Forty μl were spotted on Whatman No. 1 paper, chromatographed using butanol:acetic acid:H_2O = 4:1:5, and the dried papers sprayed with 0·2% ninhydrin in acetone. *Iso*leucine disappeared while leucine was unaffected. These results support those obtained by measurement of optical rotation.

TABLE 4. CALCULATED [α] VALUES FOR MIXTURES OF OPTICAL ISOMERS OF LEUCINE AND *iso*LEUCINE

Leucine (1 mole)	*Iso*leucine (0·79 mole)	$[\alpha]_D^{25}$
L	L	+28·4°
L	D	−8·2°
D	L	+8·2°
D	D	−28·4°

40·8 mg of alanine hydrochloride was used to determine optical rotation. $[\alpha]_D^{25} = -10{\cdot}8°$ (C, 1·7% in 6 *N* HCl) was calculated from the observed value of −0·188°. This agreed with values for D-alanine (−14°) taken from the literature.

Valine was isolated from malformin A by the same procedure. However, the acid hydrolysates were cleared of HCl by passing through IR-4B(OH). $[\alpha]_D^{25} = +20{\cdot}8°$ (C, 1·0% in 6*N* HCl) was calculated from the observed value of +0·719. This indicated the presence of L-valine (literature value = +28·8°).

Cysteic acid was isolated from oxidized malformin A as above, and the optical rotation determined. Because $[\alpha]_D^{25} = -0{\cdot}9°$ (C, 5·1% in 0·1 *N* HCl) it was concluded that cysteic acid was almost completely racemized. Under similar conditions using authentic L-cysteic acid we obtained an optical rotation of +7·2° which agreed with literature values of +7·8°.

Partial hydrolysis of malformin A

Five hundred mg malformin A was dissolved in 120 ml glacial acetic acid, 20 ml conc. HCl was added, and the solution was heated at 100° for 1 hr. Partial hydrolysis products were separated by cellulose column chromatography using butanol:acetic acid:H_2O = 50:1:50 until valine began to appear in the eluate. At this time the solvent was changed to butanol:acetic acid:H_2O = 4:1:5. Thirty fractions were collected. Peptides P_2,P_3,P_4, and P_5 were contained in fractions 5,7,17–20, and 24–26, respectively. They were purified and isolated by multiple paper chromatography using butanol:acetic acid:H_2O = 50:1:50 (P_2, R_f = 0·90; P_3, R_f = 0·71) or butanol:acetic acid:H_2O = 4:1:5 (P_4, R_f = 0·10; P_5, R_f = 0·09). N-terminal amino acids were determined by using fluorodinitrobenzene reagent.[28]

Peptide P_4 was identified as cystinyl-valine as follows: DNP-P_4, isolated on paper chromatograms as a single spot, was extracted with ether, oxidized with performic acid for 4 hr, dried over KOH, and chromatographed using butanol satd. with 1 *N* NH_4OH. By this treatment DNP-P_4 was found to split into two yellow compounds, DNP-cysteic acid (R_f = 0·02) and a second yellow compound (R_f = 0·07). The latter was eluted with water, hydrolyzed with 6 *N* HCl at 120° for 20 hr, and chromatographed using two solvent systems. Free valine was identified using butanol:acetic acid:H_2O = 4:1:5 and DNP-cysteic acid using butanol satd. with 1 *N* NH_4OH. Similar methods were used to determine whether peptides P_2, P_3, and P_5 contained cystine or cysteine. Peptide P1 was obtained from malformin A, by methods similar to those described above, after hydrolysis in 0·1 *N* HCl–acetic acid for 5 hr at 110°. However, this method was discontinued when it was learned that partial hydrolysis in dil. acid and high temperature may cause peptide transformations.[29]

Oxidized malformin A (100 mg) was dissolved in 30 ml conc. HCl and held at 25° for 22 days. Desthiomalformin A (50 mg) was dissolved in 20 ml conc. HCl using butanol: acetic acid:H_2O = 4:1:5 to locate and isolate peptides. Methods used to determine N-terminal amino acids were similar to those described above.

Measurement of active hydrogen

Active hydrogen was measured by the method of Zerewitinoff.[30] One hundred mg malformin A was dissolved in 15 ml pyridine and Grignard reagent in 5 ml *iso*propylether was added with shaking. Methane release was measured volumetrically using glucose as a control. The results are given in Table 5.

TABLE 5. MEASUREMENT OF ACTIVE HYDROGEN IN MALFORMIN A

Compound	mg	CH_4 (ml)	No. Active H	Mean
Glucose	50·8	35·1	5·21	5·06
	50·0	33·4	4·90	
Malformin A	100·0	23·7	5·12	5·28
	100·0	25·2	5·44	

Isolation of new isomer of malformin A

1·8 g crude[6] malformin A was dissolved in ethyl acetate by refluxing three times with 100 ml aliquots of solvent. The solution was concentrated to approx. 100 ml and added

[28] F. SANGER, *Biochem. J.*, **39**, 507 (1945).
[29] F. SANGER and E. THOMPSON, *Biochem. Biophys. Acta*, **9**, 225 (1952).
[30] T. ZEREWITINOFF, *Z. anal. Chem.*, **50**, 683 (1911).

to a column of activated alumina (4×32 cm, Merck, reagent grade). The column was thoroughly washed with ethyl acetate and the solvent changed to 3 per cent ethanol in ethyl acetate which removed malformin A. Succeeding elutions consisted of approx. 1 l. each of ethyl acetate: abs. ethanol = 1:1 and ethyl acetate:90% ethanol = 1:1. In the last fraction a new isomer (approx. 50 mg) of malformin A appeared which had biological activity similar to that of malformin A. This new isomer was negative for nitroprusside and ninhydrin tests, showed no u.v. absorption peaks in methylcellosolve, and consisted of the same amino acids in approx. the same molar ratios as found in malformin A.

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CONTRIBUTION À L'ÉTUDE DES PRODUITS DU MÉTABOLISME D'*ASPERGILLUS VERSICOLOR* (VUILL.) TIRABOSCHI. II. FRACTION SOLUBLE DANS L'ÉTHER SULFURIQUE

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Résumé—L'analyse du résidu d'extraction du mycelium d'*Aspergillus versicolor* (Vuill.) Tiraboschi, souche 2172, par l'éther sulfurique au moyen de la chromatographie de partage sur papier, nous a permis de séparer cinq pigments de *Rf* très différents.

Nous avons désigné ces cinq pigments par les lettres *A*, *B*, *C*, *D* et *E*; nous constatons que les pigments *A*, *B*, *C* et *D* sont visibles en lumière naturelle tandis que le pigment *E* n'apparait qu'en lumière ultra violette. Les pigments *A*, *B*, *C* et *D* réagissent avec la solution méthanolique d'acétate magnésique (Sol. 5 pour cent), la solution benzénique de pipéridine (Sol. 50 pour cent) et l'ammoniaque; le pigment *E* ne donne aucune réaction avec ces réactifs.

Nous pensons que les pigments *A*, *B*, *C* et *D* sont, en fonction de ces réactions, des substances de nature anthraquinoniques; le pigment *E*, de nature toute différente, s'est révélé être, après examen, la stérigmatocystine de l'Ecole anglaise et japonaise.

Abstract—Ether extracts of the mycelium of *Aspergillus versicolor* (Vuill.) Tiraboschi, Strain 2172, have been analysed by paper chromatography and shown to give five spots, four of which are visible in daylight and react with $Mg(Ac)_2$ piperidine and ammonia. According to these positive reactions and their u.v. spectra, it is suggested that these four compounds are anthraquinones. This is in contrast to earlier English and Japanese workers who only separated one such compound. The fifth compound is only visible in u.v. light and does not react with the anthraquinone reagents.

DANS un article antérieur, nous avons, en collaboration avec Dury,[1] analysé les constituants principaux du mycelium d'*Aspergillus versicolor* (Vuill.) Tiraboschi, souche 2172, solubles dans l'éther de pétrole léger (d. 0,640) quand cet organisme est cultivé pendant 24 jours à l'obscurité, à 25° en milieu de Czapek–Dox, formule II. Dans le présent article, nous faisons part des résultats obtenus par analyse de la fraction mycélienne soluble dans l'éther sulfurique, après extraction par l'éther de pétrole léger, et culture dans les mêmes conditions.

La première étude des produits du métabolisme de cet organisme date de 1953 époque à laquelle Abou Zeid[2] isole deux métabolites cristallisés et colorés; l'un dénommé *A* fond à 240–241°, l'autre dénommé *B* fond à 233–234°. En 1954, Hammady[3] isole également ces deux substances: *A* par l'éther de pétrole, *B* par l'éther sulfurique. La substance *A* s'identifie à la stérigmatocystine de Hatsuda *et al.*[4,5]; elle cristallise en aiguilles jaunes. La substance *B*, extraite par l'éther sulfurique, forme des aiguilles orangées à P_F 233–34°. Jusqu'à présent sa nature exacte n'est pas définie; l'école japonaise[4,5] lui donne le nom de versicolorine. Birkinshaw et Hammady[6] ne considèrent cependant pas que le produit *B* qu'ils isolent par l'éther sulfurique s'identifie à la versicolorine. Ainsi donc, deux écoles reconnaissent dans l'éther sulfurique une substance colorée mais ne s'accordent pas sur son identité.

[1] J. L. RAMAUT et P. DURY, *Rev. Fermentations et ind. aliment.* **14**, No. 6. (1959).
[2] M. M. M. ABOU ZEID, Ph.D. Thesis, London University (1953).
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[4] Y. HATSUDA et S. KUYAMA, *J. Agr. Chem. Soc. Japan.* **28**, 299 (1954); *ibid*, **29**, 14 (1954).
[5] Y. HATSUDA, S. KUYAMA et S. TERASHIMA, *J. Agr. Chem. Soc. Japan.* **28**, 992 (1954); *ibid*, **29**, 11 (1955).
[6] J. H. BIRKINSHAW et I. M. M. HAMMADY, *Biochem. J.* **65**, 162 (1957).

CULTURE—EXTRACTION—ISOLEMENT

(*a*) Partant d'une souche revivifiée sur moût de bière, nous réalisons une suspension de conidies avec laquelle nous inoculons des boîtes à pénicilline contenant 2 l. de milieu de Czapek–Dox formule II (glucose 50 g; $NaNO_3$ 2 g; KH_2PO_4 1 g; $MgSO_47H_2O$ 0,5 g; KCl 0,5 g; $FeSO_47H_2O$ 0,01 g; eau distillée à 1000 ml). Incubation à 25°, obscurité, 20–24 jours.

(*b*) Après ce temps, on sépare mycélium et filtrat, dessèche le mycélium sous vide à 45°; le mycélium sec est broyé et successivement extrait au Soxhlet par l'éther de pétrole léger, l'éther sulfurique puis l'acétone.[4,6]

(*c*) Le résidu sec de l'extraction par l'éther sulfurique est repris par l'éther de pétrole léger; après agitation, on observe une solution pétroléique rouge, une suspension de particules très fines et un insoluble compact abandonné au fond du récipient. On filtre et recueille la suspension qui est une matière rouge que l'on dénomme fraction *a*. L'insoluble compact est ensuite repris par l'éther sulfurique; on sépare une solution éthero sulfurique rouge et un insoluble rouge foncé dénommé fraction *b*. La fraction *b* traitée ensuite par l'acétate d'éthyle permet la séparation d'un insoluble de couleur beige dénommé fraction *c* et d'une solution rouge.

ANALYSE CHROMATOGRAPHIQUE

Les différentes fractions *a*, *b* et *c*, ainsi que l'extrait sulfurique brut ont été chromatographiées dans les conditions suivantes: Papier S et S. 2043 *b*-Mgl, méthode descendante, température 23–25°, atmosphère saturée en vapeur d'éther de pétrole léger; phase: éther

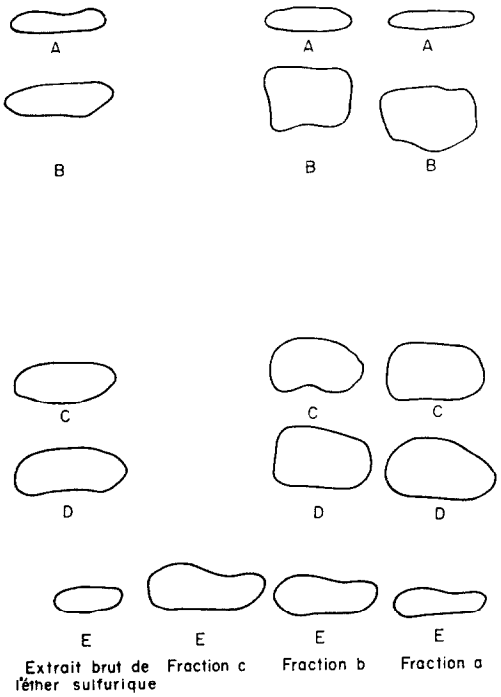

FIG. 1. CHROMATOGRAMME 1. ÉTUDE DES SUBSTANCES SOLUBLES DANS L'ÉTHER SULFURIQUE MYCELIUM D'*Aspergillus versicolor* (VUILL.) T. CULTURE ÂGÉE DE 24 JOURS.
Chromatographie descendante (4-2-1-1). Papier S et S. 2043 b. Mgl. Révélateurs–Ammoniaque—solution benzénique de pipéridine (1-1)—solution méthanolique 0,5% d'acétate magnésique.

de pétrole léger–toluol–xylol–méthanol (4 : 2 : 1 : 1). Les diverses fractions en solution dans l'éther sulfurique ou l'acétone sont appliquées sur le papier sous forme de bandes et non de tâches.

RÉSULTATS

L'examen du chromatogramme (Fig. 1) montre que l'extrait brut obtenu de l'éther sulfurique, et les fractions *a* et *b*, donnent à la chromatographie 5 bandes absolument distinctes dont les 4 premières *A*, *B*, *C*, *D*, sont naturellement colorées sur le papier, la 5ème, *E* apparaissant en rose en lumière u.v. La chromatographie de la fraction *c*, cependant, donne une seule bande visible seulement en u.v. par sa couleur rose; il s'agit donc de la bande *E*.

L'aspersion du chromatogramme par la solution méthanolique à 0,5 pour cent d'acétate magnésique entraîne une légère coloration orangée des bandes *A*, *B*, *C*, *D*; la bande *E* ne réagit pas. L'aspersion par la solution 50 pour cent de pipéridine dans le benzène donne une coloration violacée avec les bandes *A*, *B*, *C*, *D*, la bande *E* ne réagit pas. Les vapeurs d'ammoniac entraînent également une légère coloration des bandes *A*, *B*, *C*, *D*. L'ensemble de ces réactions milite en faveur de substances anthraquinoniques, excepté pour la bande *E* (fraction *c*) dont la nature semble toute différente.

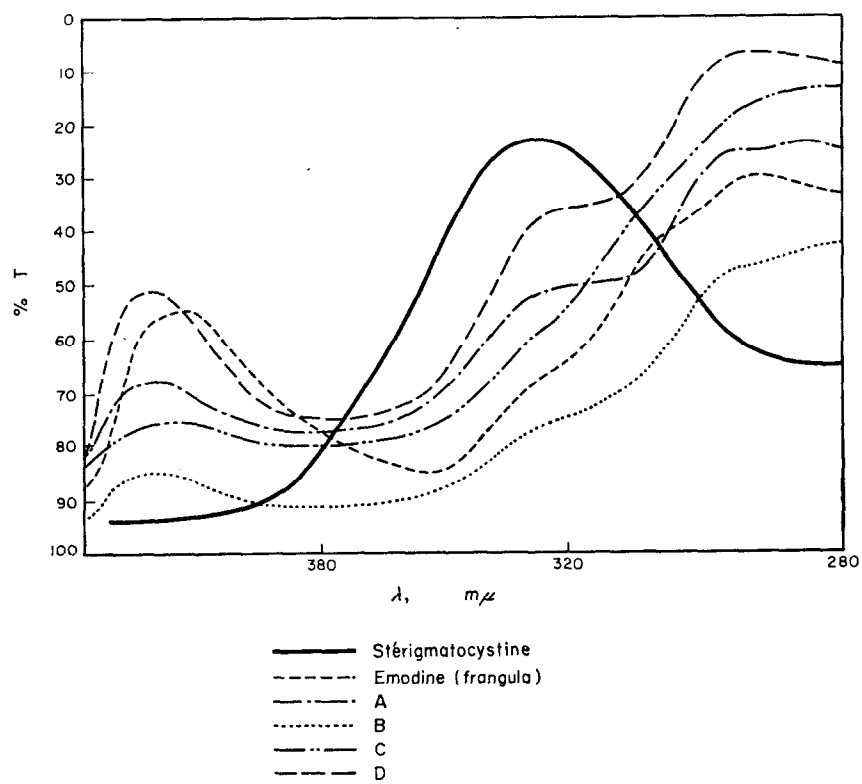

FIG. 2. COURBES D'ABSORPTION DES DIFFERÉNTES BANDES.

Ainsi donc l'extrait brut obtenu de l'éther sulfurique renferme au moins 4 si ce n'est 5 substances de *Rf* différents alors que l'école anglaise tout comme l'école japonaise ne font etat que d'une substance dénommée *B* par les Anglais, versicolorine par les Japonais.

Nous avons ensuite séparé les diverses bandes et nous les avons éluées par l'alcool absolu en vue d'en faire un examen spectrophotométrique rapide. La Fig. 2 rassemble les courbes relatives aux différentes bandes éluées. Nous avons ajouté sur la figure, la courbe de l'émodine (*Frangula*) en solution alcoolique car cette anthraquinone est très répandue dans le règne végétal. Les courbes d'absorption indiquent que les corps isolés correspondants aux bandes *A*, *B*, *C*, *D*, sont très voisins, tandis que la courbe relative à la bande *E* (fraction *c*) a une allure toute différente. Nous pouvons déjà préciser que la fraction *c* (bande *E*), aprés quelques examens, semble s'identifier à la Stérigmatocystine décrite par l'école japonaise et anglaise (un minimum à 290 mμ et un maximum à 325 mμ).

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HYDROXYLYSINE FROM ALFALFA ROOTS

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Abstract—Hydroxylysine has been found in extracts of the free amino acids from alfalfa root tissue. Its identification was based on chromatography on ion exchange columns with the isolated peak being in the same position as the synthetic amino acid. This peak increased in size upon co-chromatography with hydroxylysine. The 570/440 mμ absorbance ratio of the coloured products formed with ninhydrin was the same as the synthetic hydroxylysine but greatly different from many other amino acids.

INTRODUCTION

A SURVEY showing the discovery of new amino acids reveals a dramatic rise during the last 10–20 years. This increase is the result of the extensive use of both chromatographic procedures and the highly sensitive ninhydrin color reaction. One of the amino acids which has been reported in animal tissues but has not been reported to exist free in plants is hydroxylysine. It is the purpose of this paper to report the identification of this uncommon amino acid in extracts of legume roots where it accumulates as the free amino acid at certain stages of growth in the fall and winter.

Van Slyke and Hiller[1] in 1921 reported the occurrence of a base of unknown constitution in the lysine fraction of a gelatin hydrolysate. Schryver *et al.*[2] isolated from isinglass a new base from which they prepared a tribenzoyl derivative and suggested that it was a hydroxyderivative of lysine. In 1938, Van Slyke *et al.*[3] isolated hydroxylysine as the picrate from acid hydrolysates of gelatin. Later Van Slyke *et al.*[4] analyzed the electrometric titration curve of the new base and showed that the position of the hydroxyl group was either in the δ- or ε-positions by the liberation of one molecule of ammonia and formaldehyde upon periodate cleavage.[5]

Martin and Synge[6] applied an acetylation–benzoylation procedure to the lysine fractions of gelatin and isinglass hydrolysates and isolated in each case a picrate corresponding to the description given by Van Slyke *et al.*[3] Using the periodate liberation of ammonia, Van Slyke *et al.*[7] presented data indicating that hydroxylysine existed in demonstrable amounts in gelatin and isinglass. Of all the many plant proteins examined, they found only 5 that gave hydroxylysine (ammonia liberation) ranging from 0·10–0·54 per cent. Rees[8] found hydroxylysine only in gelatin and collagen but in no other proteins.

Desnuelle and Antonin[9] described a quantitative method for the determination of hydroxylysine in proteins based on the separation of hydroxylysine from serine by iono-

[1] D. D. VAN SLYKE and A. HILLER, *Proc. Nat. Acad. Sci.*, **7**, 185 (1921).
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[3] D. D. VAN SLYKE, A. HILLER, R. T. DILLON and D. A. MACFADYEN, *Proc. Soc. Exp. Biol. Med.*, **38**, 548 (1938).
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[8] M. W. REES, *Biochem. J.*, **40**, 632 (1946).
[9] P. DESNUELLE and S. ANTONIN, *Biochim. Biophys. Acta.*, **1**, 50 (1947).

phoresis, and the colorimetric microdetermination of formol formed by the oxidation of the amino acid with periodic acid. They reported that of the 22 proteins or protein mixtures, only collagen and gelatin contained hydroxylysine in amounts greater than 1 per cent.

Heathcote[10] and Sheehan and Bolhofer[11] have pointed out the difficulties in separating lysine from hydroxylysine and suggested newer isolation methods including use of ion-exchange resins. In 1950, Sheehan and Bolhofer[12] showed the structure of hydroxylysine to be α, ε-diamino-δ-hydroxycaproic acid by conversion to methyl α, ε-diphthalimido-δ-keto-DL-caproate, which was prepared for comparison from glutamic acid by an unambiguous synthesis. Fones[13] synthesized four isomers of this hydroxy acid; the diastereoisomers were separated by Hamilton and Anderson[14] using Amberlite IR 120 cation exchange resin.

In addition to gelatin, isinglass, and collagen, this amino acid has been reported to occur as a phosphatide of *Mycobacterium phlei*, in which the linkage is through the ε-amino and δ-hydroxy groups.[15] Evidence also is given for its occurrence in calf embryo in a phosphorylated form.[16] Piez[17] reported an allo form of hydroxylysine to exist in the collagen of human dentine.

Hydroxylysine has been shown to inhibit the growth in tissue cultures of carrot phloem.[18] This inhibition was antagonized by lysine and the results are interpreted in terms of the competitive effects of hydroxylysine on protein synthesis. An inhibitory action on protein synthesis or growth in certain tumor cells by δ-hydroxylysine has been reported.[19]

RESULTS AND DISCUSSION

Lyophilized alfalfa (*Medicago sativa* L., variety Vernal) roots harvested in December were extracted with acetone–water, the proteins removed with picric acid[20] and the free

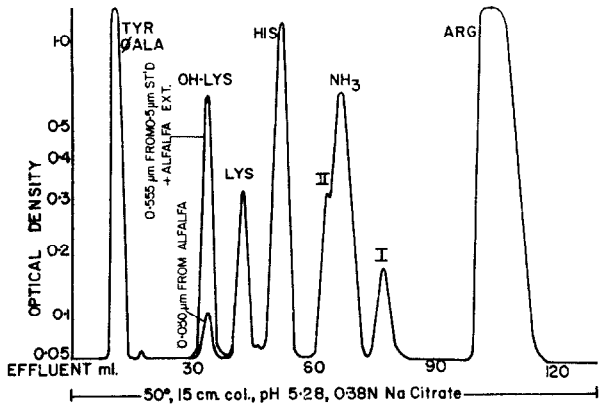

FIG. 1. AN ELUTOGRAM OF A VERNAL ALFALFA ROOT EXTRACT SHOWING THE BASIC FREE AMINO ACIDS AND THE CO-CHROMATOGRAPHY WITH 0·50 μM OF SYNTHETIC HYDROXYLYSINE.

[10] J. G. HEATHCOTE, *Biochem. J.*, **42**, 305 (1948).
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[19] M. RABINOWITZ, *Biochim. Biophys. Acta.*, **28**, 308 (1958).
[20] W. H. STEIN and S. MOORE, *J. Biol. Chem.*, **211**, 915 (1954).

amino acids in the extracts separated by ion exchange chromatography[21] as previously described.[22,23] It was observed that a new component appeared immediately before lysine on the 15 cm column. This basic ninhydrin reacting component was easily detectible in the December harvest of the alfalfa roots. Because of its greater occurrence when the plant had gained winter hardiness, it was considered important to establish its identity.

Several uncommon amino acids, which were considered as possibilities for the unknown component, were selected and run on the 50 cm column using a pH 4·26 citrate buffer at 50°. An analysis from the alfalfa root extract was made on the same column. When the position of the standard synthetic hydroxylysine on the elutogram was recognized as being the same as the unknown component, a run was made on the short column. Figure 1 shows the relationship of this isolated component (labelled OH-Lys) with the other basic amino acids. This eluation behavior gave good evidence for believing that the unknown peak was indeed hydroxylysine.

The short column was used in preference to the 50 cm column because of the reduced time it required to make a run and the sharpness of the peaks produced. Using the same solution of ninhydrin as the color developing reagent, two runs were made using (a) 2 ml of the alfalfa root extract, and (b) 2 ml of the extract plus 0·5 μmoles of standard, synthetic hydroxylysine. The resulting peaks from the elutograms were integrated after the manner described by Spackman *et al.*[21] Integration for color values of the isolated peak showed that it contained 0·050 μmoles of hydroxylysine based on the color values for synthetic hydroxylysine. As will be seen in Fig. 1, when 0·50 μmoles of the synthetic acid was co-chromatographed with the alfalfa extract, only this one peak increased in size. The peak then represented 0·555 μmoles of hydroxylysine. This shows that within the experimental error in integrating the peaks, the theoretical increase in peak height calculated from the amount of the synthetic acid added and the amount of hydroxylysine in the extract was obtained.

Another identifying characteristic of amino acids is the absorbance ratio of 570 mμ–440 mμ of the colored products formed upon reaction with ninhydrin. This ratio was compared between the synthetic hydroxylysine, and the unknown peak labelled OH-Lys, as well as with arginine. The unknown peak from alfalfa root extracts and that from synthetic hydroxylysine gave the same 570/440 mμ absorbance ratio of 3·25. Arginine gave a ratio of 5·40. The 570/440 mμ ratio for lysine is very close to that of hydroxylysine but that of other amino acids was generally quite different.

It should be pointed out that this new peak which was thus identified by chromatography as hydroxylysine was found in larger amounts in the December sample. There were five or six components that were detected in the alfalfa root extracts prepared from the tissue collected in December which could not be seen in the August sample. This may suggest that hydroxylysine and perhaps other uncommon amino acids accumulate in the root tissue in the fall. The biological significance of hydroxylysine in the plant is not known.

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SOME EFFECTS OF CHLORAMPHENICOL ON ISOLATED WHEAT ROOTS

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Abstract—Chloramphenicol inhibits glucose uptake of isolated wheat roots. The kinetics and other factors of this inhibition have been studied. After a short lag-period during equilibration, inhibition of permeability increases up to 90 per cent after 3 hr. The inhibiting effect of chloramphenicol on the uptake of solutes by plants seems to be a general phenomenon.

Protein synthesis is also blocked, either directly or perhaps by the effect on permeability. If it is assumed that, as with bacteria, the primary effect of chloramphenicol is an inhibition of protein synthesis, then its effect on the permeability of plant cells could be taken as evidence that active uptake is dependant on such synthesis.

INTRODUCTION

IN THE course of experiments on glucose incorporation in the cell wall polysaccharides of wheat roots, we became interested in the possibility of blocking protein synthesis in cells without interfering with the intermediary metabolism of glucose. In this respect chloramphenicol (CPh) seemed to us a promising cell poison, this antibiotic being well known as a specific inhibitor of protein synthesis in bacteria.[1]

In agreement with this, Webster has shown that CPh inhibits the incorporation of D-alanine in the isolated ribosomes of pea seedlings.[2] However, the inhibition by CPh of *in vivo* plant protein synthesis (or turn-over or both) is difficult to measure reliably because it has to be estimated in short term experiments where the total protein shows only a small variation.[3-5]

On the other hand, we found that the permeability of roots to glucose is greatly affected by CPh. In this paper we shall deal primarily with the evidence and conclusions to be drawn from this inhibition which also possibly affects the uptake of other metabolites.

RESULTS

Glucose uptake by wheat root tips in presence of chloramphenicol

D-Glucose penetrates quite rapidly into root cells,* where it is actively metabolized.[6,7] After incubation of roots in glucose-U-^{14}C, the radioactivity contained in the hot 50% ethanol-extractible pool of metabolites (*Pool*) can be measured and give, in short time experiments, a good estimate of the total uptake of glucose. In effect, the radioactivity incorporated into the ethanol-insoluble extracted roots, although not negligible, is only a small fraction of the *Pool* radioactivity. This is especially valid with roots cultured for at

* In the following the abreviation "root" is used for "isolated wheat root tips"; see Experimental section.

[1] F. E. HAHN, J. E. HAYES, C. L. WISSEMAN, H. E. HOPPS and J. E. SMADEL, *Antibiotics and Chemotherapy* **6**, 531 (1956).
[2] G. C. WEBSTER, *J. Biol. Chem.* **229**, 535 (1957).
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least 30–48 hr without a carbon source (O-roots). In O-roots incorporation in polysaccharides of ^{14}C from glucose is reduced to 1–2 per cent of the *Pool* uptake,[7] the radioactivity of other insoluble components being of a smaller order of magnitude. Therefore it seemed preferable to use O-roots to study the effect of CPh on glucose uptake. The total radioactivity taken up from radioactive sugars in the *Pool* of glucose or sucrose-grown roots (G-roots) is quantitatively the same, and radioactive chromatograms, obtained from *Pools* of O- or G-roots, have shown that in both, most of the sugar-derived intermediary metabolic compounds could be found in comparable amounts, although some differences occur in the qualitative and quantitative patterns of the two *Pools*.[7]

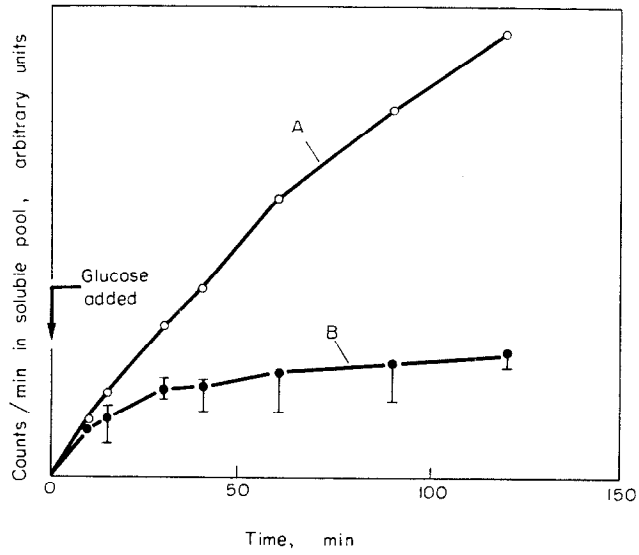

FIG. 1. RADIOACTIVITY COUNTS IN POOLS AFTER INCUBATION OF *O-roots* WITH LABELLED GLUCOSE. *Roots:* Cappelle wheat grown 42 hr in mineral medium. *Radioactive incubations:* 10 roots in 20 ml of 20 m*M* glucose, 12 mμc ^{14}C. Curve A, glucose. Curve B, glucose+10 m*M* CPh. *Vertical Strokes:* give the limits of spread of the results, in per cent of the control.

Figure 1 A shows the amount of radioactivity found in the *Pool* for O-roots as a function of incubation time with glucose-U-^{14}C. By addition of 10 m*M* CPh to the incubation medium, glucose uptake was strongly inhibited after the first 10 min. The values of the radioactivity in the *Pool* are given by Fig. 1 B, when roots were incubated with glucose-U-^{14}C and CPh. In all these latter experiments the curve reached a plateau after 30 min whereas the initial slopes with and without CPh were very similar.

Relationship between concentration of chloramphenicol and inhibition of glucose uptake by roots

O-roots were first cut and washed as described in the Experimental section; each lot of 10 roots was then incubated for 30 min in 5 ml of mineral medium supplemented with 50 m*M* unlabelled glucose and CPh at the same concentration used during the radioactive incubation that followed. Roots were then washed rapidly with cold water, drained and transferred into glucose-U-^{14}C for 30 min in the presence of CPh as indicated in Table 1.

The inhibitory effect on glucose uptake increases with CPh concentration. In the Line-

weaver and Burk plot[8] of the inverse of the radioactivity taken up versus concentration of inhibitor, the curve obtained is not linear: this is not surprising on account of the fact that the initial velocities of glucose uptake are equal, whether the roots were inhibited or not (Fig. 1).

TABLE 1. INHIBITION OF GLUCOSE UPTAKE IN ROOTS* BY INCREASED CONCENTRATIONS OF CPH

Chloramphenicol (m*M*)	0	1	3	10
Radioactivity in *Pool* (arbitrary units)	652	631	567	215
Per cent inhibition	0	3	24·5	67

* Roots: Cappelle wheat.

Glucose uptake after pre-incubation with CPh

In the previous experiments, CPh was added 30 min before the incubation with radioactive sugar. The addition of the inhibitor before the radioactive uptake began did not change significantly the inhibition rate of glucose uptake (Table 2), at least in short time experiments. The results of Table 2 were obtained from experiments carried out as described above except for incubation periods and medium as specified.

TABLE 2. EFFECT OF ADDITION OF CHLORAMPHENICOL BEFORE INCUBATION WITH LABELLED GLUCOSE ON ROOT UPTAKE*

Preincubation (1 hr)	Incubation†	Radioactivity in Pool (arbitrary units) 15 min	60 min
Mineral med.	Glucose-U-^{14}C	121	402
Mineral med.	Glucose-U-^{14}C +CPh 10^{-2} *M*	73	143
Mineral med‡ +CPh 10 m*M*	Glucose-U-^{14}C +CPh 10^{-2} *M*	68·6	141·5

* Florence-Aurore.
† Glucose-U-^{14}C 20 m*M*, 10μl for 10 roots.
‡ CPh added 30 min before the end of the preincubation period.

Preincubation with glucose

The glucose uptake measured by the *Pool* radioactivity was not significantly reduced by a pre-incubation with unlabelled glucose. But such pre-incubation increased the inhibition of uptake by CPh, as shown in Table 3, where experimental conditions were as described above except for the pre-incubation medium. There appears, at present, no satisfactory explanation for this fact which has been consistantly observed with O-roots.

Chromatographic map of the chloramphenicol inhibited Pool

Assuming that an essential metabolic reaction of glucose was blocked or strongly reduced, it was of interest to compare the *Pool* from inhibited roots with that of the control by chromatographic analysis (Fig. 2). The main points observed were: (*a*) The major components of the intermediary metabolism of glucose were found both in the absence and

[8] H. LINEWEAVER and D. BURK, *J. Am. Chem. Soc.* **56**, 658 (1954).

TABLE 3. EFFECT OF ADDITION OF UNLABELLED GLUCOSE BEFORE INCUBATION ON UPTAKE OF ROOTS*

Preincubated with	Incubated† with	Radioactivity in Pool 15 min	60 min
Water (60 min)	Glucose-U-^{14}C	102	250
Glucose‡ (120 min)	Glucose-U-^{14}C	100	252
Water	Glucose-U-^{14}C + CPh 10^{-2} *M*	73·4	121
Glucose‡ (30 min)	Glucose-U-^{14}C + CPh 10^{-2} *M*	65	70·5
Glucose‡ (90 min)	Glucose-U-^{14}C + CPh 10^{-2} *M*	54	75·0

* Roots—Florence-Aurore.
† Glucose-U-^{14}C 20 m*M*, 10 μl for 10 roots.
‡ 50 m*M*.

in presence of CPh, namely sugar-phosphates, sucrose, fructose, glucose and sugar-nucleotides. (*b*) In the CPh inhibited *Pool*, no glutamate and much less glutamine were visible than in the control. On the other hand, alanine did accumulate more into the inhibited *Pool* and there were other changes in the amino acids pattern.

After a long incubation time (180 min, Fig. 3) with CPh, a general slow down of metabolic reactions was observed in absolute as well as in relative values on a glucose uptake basis. Nevertheless, amongst other weaker spots, glucose, sucrose and alanine still appeared in major amounts on the chromatogram of the CPh inhibited pool. It is difficult to comment on such long term experiments where the effect of CPh is more generalized.

In conclusion, as shown by the chromatograms obtained from short time experiments (Fig. 2), it would seem that the most significant inhibiting effect of CPh is related to the amino acids metabolism. But one should be aware of the fact that lack of radioactivity in a spot does mean that none of the compound is present, but that its turn-over is slow or non-existent. On the other hand, an increase in radioactivity of a spot, compared to the control, would be a sign that the formation of the compound was less affected than its utilization.

These experiments agree with the results of Balogh *et al.*[5] who, using a different analytical method, found a significant increase in the alanine pool of wheat roots, in presence of CPh.

Effect of CPh on respiration of roots

Calo and Varner,[9] and Sutcliffe[10] have shown with potato-, carrot- and beet-tissue slices that CPh does not inhibit respiration as measured by the oxygen consumption of tissue, but their experiments were carried out with resting tissue.

With roots in active growing condition, we found similar results, at least with short time measurements. Table 4 shows that respiration is not significantly modified by CPh during the first hour. During the next hour it decreases slowly with reference to the control. The figures recorded in Table 4 refer to measurements with a Warburg apparatus with G-roots (Cappelle Wheat) which had been treated as described in the Experimental section.

Thus, there is no reason to conclude that the decrease in glucose uptake is related to inhibition of respiration.

[9] N. CALO and S. E. VARNER, *Plant Physiol.* **32,** 46 (1957).
[10] S. F. SUTCLIFFE, *Nature* **188,** 297 (1960).

Effect of CPh on protein synthesis in roots

On account of the small rate of variation of the total protein nitrogen in roots, we measured the incorporation of ^{35}S into root proteins where it is found in S-containing amino acids. It seemed reasonable to assume that the overall ratio of S-amino acids to the total protein content was not modified in a short time experiment. It should be mentioned that the ^{35}S technique, as any tracer incorporation, does not permit distinction between net synthesis and protein turn-over, if any.

TABLE 4. RESPIRATION OF ROOTS IN PRESENCE OF CHLORAMPHENICOL

CPh concn. m*M*	0–60 min	Q_{O_2}*	120–180 min
0	25		24·5
0·5	27·5		29·5
1	25·5		25·5
2	23		19·5
5	21·5		18·5
10	24		20·5

* Measured on 20 root tips (Capelle) of 7 mm length.

In these experiments Cappelle wheat roots were cultured for 40 hr in mineral medium without carbon source. They were then incubated for 5 hr in the glucose-supplemented medium, each lot in presence of CPh at the indicated concentration. During the last 270 min or 200 min before the end of the incubation, $^{35}SO_4^=$ was added to the medium, in tracer amounts. The incubated roots were then washed free of external medium, and ^{35}S incorporated into proteins was measured (Table 5). It can be seen that CPh inhibition approached 100 per cent for a 10 m*M* concentration.

TABLE 5. THE EFFECT OF CHLORAMPHENICOL ON THE RADIOACTIVITY INCORPORATED INTO PROTEINS AFTER INCUBATION OF ROOTS* WITH ^{35}S-SULPHATE

I		II	
CPh concentration m*M*	Radioactivity† 270 min	CPh concentration m*M*	Radioactivity† 200 min
0	127	0	366
0·1	124	0·5	290
0·3	140	1	254
1	121	2	182
3	65	5	86
10	5	10	5

* Cappelle.
† Arbitrary units.

The Lineweaver and Burk plot of $1/^{35}S$ versus the inhibitor concentration gives linear curves with a good approximation: the calculated Ki is $\backsimeq$1·9 m*M*.

Effect of CPh on sulphate uptake

It was necessary to know whether the inhibition effect of CPh on protein biosynthesis was direct or not. Sutcliffe had observed the inhibition of the uptake of other anions in plant tissues[10] and this observation led us to the study of $^{35}SO_4$ uptake in the *Pool*.

In the same experiment, ^{35}S radioactivity was measured in both the hot TCA extracted pool and the protein fraction (Table 6). The experiments were run as previously, using wheat Florence Aurore.

It is clear from Table 6 that ^{35}S uptake, as analysed in the pool, was strongly inhibited by CPh. Inhibition of protein synthesis was again observed and the ratio of protein incorporated ^{35}S to the *Pool* ^{35}S was smaller in presence of CPh.

TABLE 6. THE EFFECT OF CHLORAMPHENICOL ON THE UPTAKE OF RADIOACTIVE SULPHUR INTO THE SOLUBLE POOL AND INCORPORATION INTO THE PROTEINS OF ROOTS*

Roots incubated with:	Period of incub. (min)	*Pool* ($\times 10^3$)	Radioactivities of protein	Ratio Protein $\times 10^3$ / *Pool*
Medium	30	17·3	48	2·8
Medium+CPh†	30	14·6	33	2·2
Medium	60	24·7	139	5·7
Medium+CPh†	60	17·55	65	3·7
Medium	120	80·5	757	9·4
Medium+CPh†	120	23·9	113	4·7

* Florence–Aurore. † 10 m*M*.

The result of this experiment proved that sulphate uptake is inhibited by CPh. Although this last phenomenon is not evidence against the idea that protein synthesis is the primary site of inhibition, it was not conclusive in this respect, since the soluble ^{35}S *Pools* were different.

DISCUSSION

The foregoing experiments, when considered with Sutcliffe's evidence,[10] show that the inhibition by CPh of the uptake of metabolites and ions by plant cells could be a universal phenomenon. These were investigated, taking advantage of the fact that CPh strongly and rapidly inhibit glucose uptake.

The inhibition of $^{35}SO_4^=$ uptake seems to parallel that of glucose although the observed lag phase lasted longer, in agreement with the results of Sutcliffe who, operating with resting tissue, observed even longer lag periods in the inhibition of ion-uptake. In wheat roots experiments, two phases could be detected. The first, which lasts 5–10 min, is insensitive to CPh and might correspond to a passive equilibration of the *Pool* with the external medium. The second which is CPh sensitive, might be an active, energy requiring, phase. The hypothesis of a specific inhibiting activity of CPh on protein biosynthesis led us to consider whether this uptake inhibition had anything to do with deficiency of protein synthesis, as postulated by Steward and Preston.[11]

Unfortunately it is not an easily demonstrable relationship. It is a fact that CPh blocks protein synthesis in plants: as previously mentioned, Webster obtained a 75 per cent inhibition of the incorporation of D-alanine in isolated ribosomes from pea seedlings. With intact cells the difficulty arises from the fact that the uptake of tracer substance itself is blocked, as shown for $^{35}SO_4^=$, and it can be asked whether the inhibition constant we calculated effectively corresponds to inhibition of protein synthesis or of uptake.

In a recently published paper, Balogh *et al.*[5] found little or no inhibition by CPh of the uptake of various compounds by wheat roots (bromide, phosphate, glycine, methionine). These findings fit with ours or Sutcliffe's[10] without contradiction since Balogh *et al.* used

[11] F. C. STEWARD and C. PRESTON, *Plant Physiol.* **16**, 85 and 481 (1941).

CPh concentrations of the order of 1m*M*, i.e. near the limit of any measurable effect (e.g. Table 5).

The high concentration of the antibiotic necessary to obtain the maximum inhibition is quite remarkable. Accordingly, even if the primary impact of CPh on plant cells results in a block of protein synthesis, the lower inhibiting concentration is much higher than that observed in bacteria (10–100 μM).

Rönnike[12] only observed a 50 per cent inhibition of the growth of tomato roots with CPh solutions as concentrated as 3 m*M*. We found a similar result for the incoporation of ^{35}S into protein (Table 5), (with the previously mentioned restrictions on the validity of the method). If our experiments, showing that pre-incubation with CPh does not increase its inhibiting effect (Table 2), are taken in account, it seems that the rate of uptake of CPh itself is not responsible for the relative resistance of plant cells to the antibiotic. These facts seem to indicate a different mechanism of inhibition of plant and bacterial growth.

Nevertheless the accumulation of a high concentration of some amino acids and the lack of turn-over of others such as glutamic acid observed on our chromatograms (Fig. 2), and, on the other hand, the inhibition of uptake of materials as various as glucose, sulphate or sodium, has led us to think that the block of protein synthesis by CPh might well be the primary effect and which is in fact the explanation for the inhibition of uptake. It should be mentioned finally that, at a concentration of 0·1 m*M*, CPh slows down the growth of isolated wheat roots cultured in synthetic medium. This concentration is practically without effect on the uptake of glucose and ions. This has already been recorded by Sutcliffe[10] with reference to CPh activity on carrot tissue cultures.

It could therefore be concluded that this antibiotic may specifically inhibit the synthesis of some essential plant proteins or that, apart from the obvious inhibition of uptake, it could act on an as yet unknown metabolic pathway.

EXPERIMENTAL

Plant material

Two varieties of wheat were selected (a) *Triticum sativum* Lmk Cappelle (Et. F. Desprez, Cappelle par Templeuve, Nord, France) 1959, (b) *T.s.* Florence-Aurore (C.O.S.E.M., 6, rue H. Thameur, Tunis, Tunisie) 1961.

Neither variety had been treated by any pesticide or other chemical before use. The second variety was used in some of the later experiments, because it was found impossible to obtain uniformly aseptic root cultures from native wheats which were heavily contaminated. Most of the experiments carried out with the Cappelle variety were later duplicated with the Florence-Aurore.

Cultures

The preparation of wheat roots, isolated from seedlings which had been germinated over 48 hr at 21–22°, has been described previously.[7] The isolated roots were cultured in mineral medium[7] with NH_4NO_3 replaced by KNO_3 20 m*M*. The medium was either supplemented with D-glucose 50 m*M*, or used without carbon source, as indicated in text or legends.

The cultures in liquid medium were run in flasks at 22°, with a mechanical shaking (frequency 80 osc/min) as previously described. Cultures were kept aseptic and contaminated flasks discarded. The volume of medium was 20 ml for 40 root tips, in 150 ml Erlenmeyer flasks. The cultures were kept in the dark.

[12] F. RÖNNIKE, *Physiol. Plantarum*, **11**, 421 (1958).

Radioactive tracers

Uniformly ^{14}C labelled D-glucose (U-^{14}C) was obtained from the Commissariat à l'Energie Atomique, France; specific radioactivity ca. 50 $\mu c/\mu$Mol. The radioactive sugar was conveniently diluted for experiments with pure unlabelled D-glucose.

^{35}S labelled sulphate was obtained from the same source in the form of carrier free H_2SO_4. Since the culture medium contained sulphate at a 50 μM concentration, the introduction of the carrier free labelled sulphate at tracer concentration did not significantly affect the medium composition.

Radioactive incubation

Incubations in presence of labelled glucose were carried as follows: cultured wheat roots were drained free of medium and rinsed with water on a sintered glass filter. They were then cut at 7 mm from the apex. The tips (referred to as "roots" in text) were then washed 1 hr in mineral medium (20 ml for 40 roots), unless otherwise indicated in text. After draining, the washed roots were put side by side on a dry microscope slide in a wet petri dish at 22°. At zero time the roots were covered with a drop of ^{14}C-glucose, of known volume, measured from a micropipette fitted to an Agla microsyringe (Burroughs, Welcome and Co. Ltd., London) and kept in the covered petri dish throughout the incubation time. Unless otherwise specified in text, the concentration of glucose-U-^{14}C was 20 mM. At the end of the chosen period of incubation, roots were washed free of the external radioactive medium with cold water during 3 min.

The soluble metabolic pool (*Pool*) was extracted by 50% (v/v) boiling ethanol, 3×2 ml vol. per 10 roots. An aliquot of the extract was plated on an aluminium planchet, dried under infra-red light and counted at negligible thickness with a gas-flow, thin-window Geiger–Muller tube using Tracerlab integrating and recording equipment. Radioactivity was estimated from at least 1,000 counts.

Incubations in presence of ^{35}S sulphate were run in culture flasks, the tracer was added to glucose-supplemented normal medium in which roots were introduced after cutting and washing as previously described. At the end of the incubation period the roots were washed free of medium and measurements of radioactivities were carried out on the washed roots in the following way: (*a*) roots were treated with 3×2 ml of 5% trichloroacetic acid (TCA) at 100°. The hot TCA soluble fraction was centrifuged off and its radioactivity measured at negligible thickness as described above. (*b*) The residue from the hot TCA extraction was washed with water until neutral and mineralized by the Dohlman[13] method. An aliquot of the extract in 0·046 N H_2SO_4 was plated on a lead planchet where the acid used as an ^{35}S carrier is converted to lead sulphate. The radioactivity was then estimated at infinite thickness with the same counting equipment.

To avoid the difficulty of removing the TCA which interfered with counting, a slightly different method was finally used for measuring ^{35}S in the *Pool*. After washing, the incubated roots were first extracted by hot 50% ethanol as described for the incubations in presence of glucose. Thereafter the roots were treated with TCA, and ^{35}S incorporated into proteins measured as before. The amounts of ^{35}S radioactivity obtained by first extracting the incubated roots either by hot TCA or by 50% ethanol were found to be equal. Therefore the ^{35}S soluble *Pool* could be most conveniently measured on the ethanolic extract prior to the hot TCA treatment.

[13] C. H. DOHLMAN, *Arkiv. Kemi* **11**, 255 (1957).

Respiration measurements

The respiration of roots was estimated using 30 hr cultured G-roots by the conventional direct Warburg method, at 27°, each vessel containing 2 ml of standard mineral medium supplemented with 50 m*M* glucose. Before each test, roots were washed 1 hr at 22° in water. Glucose was added routinely for the manometric observations, but G-roots show little respiratory changes whether or not incubated with glucose.

Chromatography

From roots incubated in labelled glucose, two-dimensional chromatography of the extracted *Pool* was realized according to Benson *et al.*[14] on unwashed Arches 304 paper, in the following solvent systems: (1) Phenol–3·6 m*M* EDTA in water 72 : 28 (w/w). (2) *n*-Butanol–propionic acid–3·6 m*M* EDTA in water 100 : 50 : 70 (v/v). The development time was (1) : 33 hr. (2) : 24 hr at 22°. Chromatograms were revealed by autoradiography with Kodirex double-surfaced negatives.

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[14] A. A. Benson, J. A. Bassham, M. Calvin, T. C. Goodale, V. A. Haas and W. Stepka, *J. Am. Chem. Soc.* **72**, 1710 (1950).

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