Phorbic Acid Biosynthesis in the Latex Vessel System of Euphorbia
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Abstract. Evidence is presented that phorbic acid is formed in the latex producing cell system, rather than in photosynthetic or chlorophyll-free tissues of Euphorbia resinifera Berg. When a branch of the plant was kept first in a 14CO2 atmosphere with 12 hr light-dark periods for 2 days and then left under natural conditions in the air outside for at least 2 to 3 days, radioactive phorbic acid was found in the latex. Phorbic acid synthesis appeared to be independent of the photosynthetic and respiratory activities of the plant.

Besides phorbic acid 2 other major radioactive compounds were recognized in the latex, a glycoside or oligosaccharide, and a lipid belonging to the group of triterpenoid compounds characteristic of the latex in several species of Euphorbia.

The crude drug Euphorbium, which consists mainly of dried latex from Euphorbia resinifera Berg, was studied by Nordal and Baerheim Svendsen (1) and found to contain a new lactonic acid. The structure and some of the physical and chemical properties of the acid was reported (2, 3, 4). Phorbic acid was found widely distributed within the Euphorbiaceae (3, 4) and there is evidence that the acid occurs also in other plant families (5).

The structure of phorbic acid, figure 1, and the fact that the acid appears in succulents where a typical succulent metabolism has been observed engendered further investigation of its biosynthesis and of some of the physiological aspects of its formation.

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\text{Phorbic acid} \quad \text{Monolactone} \quad \text{Dilactone}
\]

Fig. 1. Phorbic acid and products of its dehydration. The stereostructures of its asymmetric carbon atoms and of the spiro-dilactone are not yet assigned.

Materials and Methods

Experimental plants were heavily branched 30 cm specimens of the stem succulent Euphorbia resinifera Berg raised in a local nursery. Cutting or breaking of the thorns, or small incisions in the surface of the plant caused a vigorous flow of white latex. The rectangular section of the branches is divided into bark and wood (mainly pith) by means of a dark dotted line that can be observed with the naked eye. Microscopic examination of the cross section showed that only the outer part of the cortex contains chlorophyll, while the latex vessel system is concentrated in the phloem tissue in the inner part of the cortex. The dotted line that was observed with the naked eye between the cortex and the pith consists of small vascular bundles. The pith is made up from parenchymatic chlorophyll-free cells.

The fresh plant material was fixed with 95% boiling ethanol (10–20 volumes) and then extracted with boiling water (about 10 volumes). The combined extracts were evaporated in a N2 stream at 40 to 50°C to a volume approximating that of the original tissue.

The radioactive extracts were usually subjected to 2-dimensional paper chromatography and radioautography as the first method of investigation. Both Whatman No. 1 and No. 4 paper were used, and unless otherwise stated, water-saturated phenol water (100:39 w/w) was used as the first solvent and butanol-propionic acid-water (8:18:12 v/v) as the second. Further resolution of components or mixtures from these chromatograms was effected using thin layer chromatography, paper electrophoresis, and gas chromatography. Thin layer chromatography was mainly used for the resolution of phorbic acid and its lactones. For this purpose the plates (20 × 20 cm) were coated with silica gel-G, and acetic acid-n-pentanol (1:1) or n-butanol-acetic acid-water (12:3:5 v/v) served as developing solvent (6). Paper electrophoresis was carried out on the low voltage Research Specialties Company ap-
paratus at 600 v using 0.01 m (pH 6.0) citrate buffer. The gas chromatograph was a dual column Loenco Model 90A instrument equipped with thermal conductivity detectors. The instrument was also fitted with a high temperature ionization chamber with a Cary vibrating reed electrometer assembly which simultaneously recorded mass and radioactivity in the gas stream. Most of the analyses were carried out with a dual 1 and one-half feet × 0.25 inch column, containing silicone rubber (3 %) on Chromosorb W, HMDS treated.

Radioactivity of the samples and of chromatographic spots was usually measured using large diameter "pancake" type thin window G-M. detectors.

Spray Reagents. For the detection of phorhic acid lactones on paper chromatograms, the hydroxamate test as modified by Krogh (6) for this purpose was used: Before spraying, the chromatograms or plates were heated at 100 to 105° for 1 hr in order to remove residual volatile acids from the developing solvents and to effect lactonization of phorhic acid. Then the chromatograms were sprayed, first with Solution A, and after drying with Solution B. Under these conditions phorhic acid lactones gave a pink color. Solution A was prepared by mixing equal volumes of a 7.0 % (w/v) methanolic solution of hydroxylamine hydrochloride and a 7.2 % (w/v) methanolic solution of KOH. The mixture was filtered before use. Solution B consisted of 1.0 g FeCl₃ dissolved in 100 ml of a 10 % solution of HCl in water.

A 0.1 % solution of bromophenol blue in methanol, adjusted to give a blue background and yellow acid spots was used as spray reagent for carboxylic acids. When this spray was to be applied, the chromatograms were developed with butanol-acetic acid-water (100:25:62) as the second solvent rather than with the usual butanol-propion acid-water, where the acid component was difficult to remove from the chromatograms before spraying. For the detection of amino acids, sugars, and alcohols, the well known spray reagents (ninhydrin, aniline oxalate, ammonical silver nitrate, etc.) combined with chromatography with authentic samples were used.

Dark Fixation of ¹⁴CO₂ by Green and Colorless Tissues. Experiment No. 1. Slices of about 4 × 10 × 15 mm of the green epidermal tissue and the chlorophyll-free xylem (or pith) tissue were exposed in a 50 ml glass-stoppered jar for 10 hr to ¹⁴CO₂, generated from 2 to 6 mg Ba¹⁴CO₃ (9 mg/me). Paper chromatography of the extracts showed that carboxylation had taken place in both samples, and that the dominant radioactive compounds were the same in both cases, namely malic and citric acids. Minor amounts of the normal amino acid products (aspartic, glutamic, asparagine, serine, glycine, and threonine) were also observed. No significant difference in the behavior of the chlorophyll containing and the chlorophyll-free tissue was noticed. Prolonged exposure (up to 70 hr) to ¹⁴CO₂ in the dark, of tissue slices or fresh branches of the plant, did not significantly alter the picture. In experiments involving longer time or strong illumination it was found that a desiccant (silica gel) prevented deterioration due to fungal infection.

Long Time Experiment in ¹⁴CO₂ Atmosphere With Alternate 12 Hr Light-Dark Periods. Experiment No. 2. A small fresh branch of the plant (11.54 g) was transferred to a 700 ml glass-stoppered jar. The plant rested on a 2 to 3 cm high base of aluminum foil intended to separate the plant from any condensed water. The foil also reflected the light back to the plant during the light experiment.

Generation of ¹⁴CO₂ was carried out in the following way: a 2 ml open vial with 15 mg of Ba¹⁴CO₃ was fixed with tape to the inside of the experimental flask. The jar with the plant was then turned over to the side so that the small vial rested in horizontal position. With a Pasteur pipette 2 to 3 drops of 50 % lactic acid was then transferred to the upper part of the horizontal vial. Then the container was closed with a greased stopper and raised with the result that the lactic acid flowed to the Ba¹⁴CO₃ and ¹⁴CO₂ was evolved.

The jar was first kept in the dark (18°) for 22 hr, whereafter it was kept at 12 hr alternate periods of light and dark. The light intensity outside the flask was about 5000 ft-c and the temperature in the room during the experiment was about 23°.

The first part of the experiment lasted for 7 days (including the first dark period). The flask was then carefully opened. Excess of ¹⁴CO₂ was trapped with a filter paper moistened with 1 N KOH, and 2.0 g of the lower part of the branch was excised for extraction and analysis (extract 1). The flask which contained about 1.5 ml of water inside, was washed and dried. The plant which during this first part of the experiment had lost about 4.0 g, appeared healthy except for a few small brown spots on the sides. It was put back to the jar, and the experiment was continued under the same conditions as before, this time in air containing ¹⁴CO₂ generated from 7.2 mg Ba¹⁴CO₃.

The second part of the experiment was stopped after 8 more days. The plant which was then entirely dried up (weight, 0.6 g), was extracted with warm water and ethanol, and the extracts combined and concentrated for analyses (extract 2).

Radioautograms of the 2 extracts, based on 2-dimensional paper chromatogram of the extracts, were very similar. As expected a wide variety of products from different groups of compounds such as carboxylic acids, amino acids and sugars were observed. Paper electrophoresis at pH 6 revealed much overlapping, especially of amino acids with the common monosaccharides and sucrose.

A dominant spot located between the normal positions of the glutamic acid and the glucose spot attracted much interest. Paper electrophoresis (pH 6) showed that only a minor part of the activity was amino acid, while about 80 % of the activity
migrated to the anode with nearly the same mobility as citric acid. Comparative analyses carried out later on with this compound and the radioactive lactones of phorbic acid isolated in experiment No. 4 from the fresh latex of the plant, showed that the substance in question is identical with one of the phorbic acid lactones, probably the dilactone.

Investigations of the Phorbic Acid Content in Latex and Plant Tissue. Experiment No. 2. The assay which was carried out involved gas chromatography of the methyl esters prepared from the carboxylic acid components of the samples. The investigation showed that the dominating carboxylic acid component of the latex was phorbic acid, while the same substance comprised only a negligible part of the carboxylic acid content of the whole plant (cf. fig 2). Isolation and esterification of the acids were carried out in the following way: A) The Latex Acids. The fresh tapped latex was mixed with 5 to 10 volumes of water and the mixture heated in the steam bath for about 10 min. Usually this resulted in a "curdling" of the latex emulsion so that a clear liquid was obtained upon centrifugation. Addition of alcohol, combined with a few drops of acetic acid, helped to break the (latex) emulsion.

The acids were precipitated from the clarified supernatant as lead salts which were then decomposed with H2S. The crude acid mixture was evaporated to dryness, the residue extracted with methanol and the soluble part of the extract again evaporated to dryness. About 20 mg of the clear syrupy residue was heated in at N2 stream at 100 to 105°C for about 10 min in order to lactonize the phorbic acid. The residue was taken up in 0.5 ml of methanol and esterified with an excess of diazomethane. The resulting mixture, which was partly solid, was evaporated in an N2 stream to a thick, turbid syrup. This was taken up in methanol, the extract was centrifuged and the clear supernatant was concentrated to a small volume (10-25 ml) for gas chromatography. B) The Acids of the Tissue. A fresh branch of the plant (about 50 g) from which the main part of the latex had been removed by incisions and cutting, was treated with 100 ml of water in a Waring Blender. The extract was cleared by centrifugation whereupon isolation of the acids and the preparation of the methyl esters were carried out in the same way as for the latex acids. Gas chromatograms of the ester mixtures are shown in figure 2.

Demonstration of Phorbic Acid Synthesis in the Latex Vessel System. Experiment No. 4. A fresh branch of the plant (15 g) was kept in an airtight flask (500 ml) with alternate 12 hr light-dark periods for 60 hr in a 14CO2 atmosphere generated from 12 mg Ba14CO3. The branch was removed and kept outside in the air under natural day-night rhythm for the rest of the experimental period (55 days, April-May). The first tapping was carried out 2 days after the branch was put outside, the second 6 days later. From now on the branch was tapped for latex after certain intervals (cf. table I). The latex was either applied directly on the paper for radiochromatography or it was first extracted with ether which very easily removed the "milky" or lipophilic part of the latex. In such cases the ether extract and the aqueous residue of the latex were chromatographed separately. A chromatogram from

![Fig. 2. Left: Gas chromatogram of the latex acids. Right: Gas chromatogram of the acids of the tissues.](image-url)
the first tapping showed a few radioactive components. Glucose was dominant, while the fructose and sucrose spots were very weak. These decreased rapidly in relative amount or disappeared in the following tappings.

An elongated spot, $R_F$ 0.10, 0.30 (phenol-water, butanol-propionic acid-water), that later was identified as a mixture of phorbic acid and its lactones, was already observed on the chromatograms from the first tapping. During the following tappings this elongated spot or area (referred to as the phorbic acid area) increased quickly in relative amount (table 1), and showed a clear tendency to divide into 3 spots, $R_F$ values 0.03, 0.16, and 0.30, representing the acid, the mono- and the dilactone respectively (cf. figs 1 and 3). This tendency could be observed more clearly when the 3 spots (the phorbic acid area) were excised and rerun in phenol-water solvent, or better, if the extract of the spots was deionized (with Dowex 50-H+) before the second run. In that case 3 elongated spots with a sharp front and strong tailing resulted. The $R_F$ values of the spots, measured at their front edges, were 0.23, 0.40, and 0.56.

The 3 spots within the phorbic acid area gave nearly the same $R_F$ values, 0.32, 0.33, and 0.35 when rechromatographed in the butanol-propionic acid-water solvent. They showed only a slight tendency to split up during the chromatographic process. This indicates that a lactonization of phorbic acid and the phorbic acid monolactone ($R_F$ 0.33 and 0.16 in the phenol-water solvent) had taken place during drying of the chromatogram from the first run and in the subsequent chromatographing in the butanol-propionic acid-water solvent. The higher acidity of the second solvent strongly promoted lactonization.

An extensive but diffuse spot or area appeared at the chromatographic front referred to as the lipid area (fig 3). It increased in relative intensity during subsequent latex tappings (table 1). It was absent in chromatograms of the ether-extracted latex.

Ether extraction of the latex prior to chromatography did not effect other spots or areas except a residual spot of lipid air-oxidized during drying of the first solvent (cf. fig 3).

In the tappings of latex made 15 to 20 days after the start of the experiment, a third spot ($R_F$ 0.80, 0.40) gradually increased in intensity (cf. fig 3). It showed glycosidic properties (see below) and was a major radioactive compound of the latex at the end of the experiment. Table 1 shows the relative amounts of radioactivity found in the main compounds or chromatographic areas during the first 45 days. Phorbic acid increased gradually during the first 24 days, thereafter it decreased slowly.

Investigation of the Radioactive Compounds of the Latex. Phorbic Acid. The total phorbic acid area from 6 chromatograms (cf. fig 3) was eluted with water and concentrated in N$_2$ stream to about 0.5 ml. This preparation which represented about 100,000 cpm, as counted on paper with the end-window G.-M. detector, will be referred to as phorbic acid-$^{14}$C solution. Phorbic acid-$^{14}$C solution, corresponding to 3370 cpm, mixed with 100 $\mu$g phorbic acid dilactone in 10 $\mu$l of water, was dried on a small piece of filter paper (4 $\times$ 13 mm) and applied to the moist paper electrophoresis sheet. A reference sample of radioactive malic acid (about 2000 cpm) applied in the same way. Radiography revealed that the main part of the activity of the phorbic acid-$^{14}$C was found in a double spot located about 8 cm from the origin while the rest of the activity was found in a stripe that started out about 2 cm beyond the double spot and stretched out for about 8 cm towards the anode. The radioactive malic acid spot was located about 17 cm from the origin. If this mobility is referred to a $M_m$ = 1.0, the $M_m$ value of the double spot will be $M_m$ 0.45. (Under

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**Figure 3.** Radioautogram of a 2-dimensional chromatogram derived from fresh latex of Euphorbia resinifera, treated as described under experiment No. 4 and collected 47 days after start of the experiment.
the same conditions the $M_m$ value of citric acid was found to be 0.44).

After drying and heating of the chromatogram at 105° for 1 hr, the hydroxamate test produced a pink spot that matched the double spot of the chromatogram.

Phorbic acid-14C solution corresponding to 1200 cpm was deionized with Dowex 50×H+ and mixed with 40 μg of phorbic acid dilactone in 4 μl of water. The plates were heated at 100° for 3 min to cause partial lactonization of phorbic acid. They were then developed in ascending chromatography with n-butanol-acetic acid-water (12:3:5) as a solvent. The experiment was stopped when the solvent was about 2 cm from the edge of the plate. A radioautograph showed an 8 cm long trail that stretched out from the origin of the chromatogram towards the solvent front, followed by 2 small spots 0.5 cm and 1.0 cm apart. The plate was heated at 105° for 30 min, cooled, and sprayed with the hydroxamate reagents. This gave 2 small pink spots and a faint trail. The spots and the trail coincided with the spots and the trail of the radioautograph.

Phorbic acid-14C solution, 25,000 cpm, was deionized (Dowex 50×H+) and evaporated to dryness in a N2 stream. The residue was purified, esterified as in experiment 3, and concentrated to 5 μl. A 1.5 μl aliquot, mixed with 0.1 μl of the monomethyl ester of phorbic acid dilactone, gave the gas chromatogram shown in figure 4. In the figure the slight difference in the positions of the peaks (of phorbic acid) showing mass and radioactivity resulted from offset of the 2 recording pens. This taken into account, the curves correspond exactly. The 2 peaks in dotted lines, figure 4, show the location of malic acid dimethyl ester and citric acid trimethyl esters chromatographed under the same conditions. The experiment shows that the complex referred to as the "phorbic acid area", represents a mixture of phorbic acid and its lactones which upon lactonization and esterification is converted into the mono-
methyl ester of the dilactone, the compound recorded on the gas chromatogram.

The Lipid Area. The "lipid area" (cf. fig 3) was cut out and extracted with chloroform-methanol (2:1). The extract was subjected to the usual decacylation procedure (7), but only a slight hydrolysis took place. The extract also resisted strong acid and basic hydrolysis (1 N HCl or 0.2 m methanolic KOH for 3 hr at 100° in a sealed tube). This indicates that the main constituents of the lipid area are related to or identical with the triterpenoid compounds (euphol, euphorbol, etc.), that have been isolated from the latex of several euphorbiaceous plants including *Euphorbia resinifera* Berg (8, 9, 10).

The "Glycoside" Spot. Paper chromatography, paper electrophoresis and acid hydrolysis showed that the third dominating radioactive compound of the latex was a neutral substance that behaved like a glycoside or an oligosaccharide. It was hydrolyzed by heating in 2 N HCl for 3 hr at 100° in a sealed tube. A preliminary investigation of the products of hydrolysis indicated that the compound had been split into a monosaccharide (glucose) and a second radioactive compound with chromatographic coordinates like glycerol or rhamnose. The compound will be subjected to a closer investigation.

Results

By exposing slices of fresh branches of *Euphorbia resinifera* to 14CO2 in the dark or in the light it was found that phorbic acid was not readily biosynthesized by the plant. Even biosynthetic experiments with 14CO2 carried out over 15 to 20 days gave no appreciable amount of radioactive phorbic acid in the total extract of the plant. In subsequent experiments with 14C-labeled tissues it was discovered that the main part of the phorbic acid content of the plant is located in the latex vessel system and not in the parenchymal tissues. We were led to the conclusion that phorbic acid is a secondary metabolic product, formed in the specialized latex-producing vessels of the plant from precursors derived from the surrounding tissue.

To test this hypothesis, a fresh branch of the plant was allowed to assimilate 14CO2 for 2 days and then continue to grow outside under natural conditions. The amount of 14C lost by respiration was surprisingly small. Fresh latex from the branch was tapped at selected intervals and subjected to radiochromatographic analysis. The first tapping was carried out 4 and one-half days after the start of the experiment. It was found that the plant could restore the latex reservoir in the branch in 2 to 3 days. The latex was tapped in such a way as to avoid release of sap from the assimilating tissue which, under the conditions, always contained radioactive malic and citric acids, amino acids, and sugars.

![Fig. 4. Gas chromatogram of phorbic acid dilactone monomethylester (smooth line) and the corresponding 14C-labeled compound (ragged line). The 2 peaks plotted with dotted lines show the location of malic acid dimethyl ester and citric acid trimethyl esters chromatographed under the same conditions.](image-url)
Phorbic acid appeared in the latex from the first tapping as a relatively weak elongated spot near the origin, R<sub>f</sub> 0.10–0.30 (phenol-water; butanol-propionic acid-water). In time the 14C content of the phorbic acid spot became more and more dominant. It exhibited a characteristic tendency to form several chromatographic components as a result of lactonization. For this reason it is perhaps more correct to refer to the “phorbic acid areas” than to a phorbic acid spot, in describing its chromatographic coordinates (cf. figs 1 and 3 and the experimental part of the paper). The readiness with which the lactones of phorbic acid open and close makes it difficult to recognize this acid in chromatograms of biological materials.

As may be noted in figure 3 and table I a rather large part of the latex radioactivity was located in the lipid area of the chromatogram. It consisted mainly of nonsaponifiable compounds, probably identical with the type of triterpenic compounds that make up a significant part of the latex from *Euphorbia resinifera* and other euphorbiaceous plants (8, 9, 10).

A third dominant, radioactive spot, R<sub>f</sub> 0.80–0.40 (phenol-water; butanol-propionic acid-water), recorded in the latex, was located between the phorbic acid and the lipid areas. A preliminary investigation revealed that this compound has glycosidic properties. It will be referred to below as “the radioactive glycoside” or “the glycoside spot” (cf. fig 3). After 40 days, the radioactivity in these 3 areas comprised about 80% of the total activity of the fresh latex sample applied at the origin of the chromatogram. In contrast, chromatograms of the total extract of a branch after only 6 days metabolism revealed at least 25 components. There exists a significant difference in the composition of the latex and the rest of the plant and it appears that phorbic acid is actually a secondary metabolic product synthesized in the specialized latex producing vessels of the *Euphorbiaceae*.

**Discussion**

The initial objective of the present investigations was to study the possible role of phorbic acid in the diurnal fluctuations of the organic acid content which has been observed in many succulents. It was presumed that the high concentration of phorbic acid in *Euphorbium* in the stem succulent *Euphorbium resinifera* was located in the assimilating tissue and therefore closely associated with CO<sub>2</sub> fixation. It was found that phorbic acid is not readily formed during CO<sub>2</sub> fixation in the dark or in the light as are male, citric, and other acids typical of “succulent metabolism” but is produced separately in the latex vessel system. Its precursors are not yet known but must be derived from the surrounding tissues. Mentzer (11) suggested that phorbic acid is formed by a mechanism related to the tricarboxylic acid cycle by a condensation reaction depicted in figure 5. It now seems that the acid is formed by enzymes located in the latex; experiments where fresh latex and labeled precursors of the type indicated above are involved should provide an answer to the question. Latexes of several plants contain enzymes involved in caoutchouc synthesis from its precursors, including certain carbohydrates (12, 13). It therefore seems reasonable that the latex of *Euphorbium resinifera* might contain enzymes directly involved in the biosynthesis of phorbic acid. Nothing is yet known of its biological function. From its chemical structure and behavior it is reasonable to consider that it plays a role in water and pH regulating mechanisms of the plant. Phorbic acid lactones open and close very readily; cyclization proceeds upon simple drying of the acid at ordinary temperature and is reversed by adding water to the lactonized forms of the compound. Lowering of the pH of an aqueous solution of the acid, a process which normally takes place in the succulents during the night, may also cause a partial lactonization and, accordingly, a “deacidification” of the compound. Screening tests for lactonic acids conducted in connection with this investigation indicate their wide distribution among succulents. The presence of labile lactonic acids in living tissue could markedly enhance their buffering capacity. With its ability to form a dilactone, phorbic acid could be an important powerful buffering agent and physiological factor in cells where it comprises a significant part of the total acid content as in the latex vessel system of *Euphorbium resinifera*.

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**Literature Cited**


