of 2,5-di-*t*-butylhydroquinone diacetate and 109 g. (0.81 mole) of anhydrous aluminum chloride was divided into two portions. One portion was placed in a 1-liter beaker surrounded by an oil-bath maintained at 125–130°. The temperature of the periodically stirred mixture rose to 110° during 20 minutes, after which time the second portion was added, with stirring. After the addition was completed, the reaction mixture was heated at 115–120° (oil-bath 130°) for 0.75 hour. The reaction mixture was cooled rapidly by pouring it into a large enamel tray. The solid reaction residue was stirred with 400 cc. of concentrated hydrochloric acid and 1.0 liter of crushed ice. The ice–acid slurry was heated on the steam-cone until all of the lumps had melted to an oil, after which time the oil was solidified and removed by chilling the mixture in ice. The oil was stirred with 200 cc. of 20% sodium hydroxide. Acidification of the filtered alkaline solution yielded 4.8 g. of yellow solid, m.p. 195°, which, after recrystallization from 25 cc. of ethanol, gave 2.3 g. of pure acetylhydroquinone, m.p. 204–205°. The mixed melting point with an authentic sample of acetylhydroquinone was 204–205°. An additional 0.7 g. of crude acetylhydroquinone, m.p. 201–202°, was obtained from the aqueous mother liquors by further chilling the mixture to 0°. The total yield of acetylhydroquinone, m.p. 201–205°, was 3 g. (12.1%). All attempts to isolate other definite compounds from the tarry reaction product were unsuccessful.

Rearrangement of 2,5-Di-*t*-amylhydroquinone Diacetate with Aluminum Chloride.—When a mixture of 54.5 g. (0.16 mole) of 2,5-di-*t*-amylhydroquinone diacetate and 109 g. (0.81 mole) of anhydrous aluminum chloride was treated as described above for 2,5-di-*t*-butylhydroquinone diacetate, 3.3 g. of crude acetylhydroquinone, m.p. 190–192°, was obtained. Recrystallization gave 1.4 g., m.p. 202.5–203°, yield 5.5%.

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Coconut Milk Factor: The Growth-promoting Substances in Coconut Milk[1]

BY E. M. SHANTZ AND F. C. STEWARD

RECEIVED AUGUST 1, 1952

Coconut milk is the fluid endosperm that nourishes an immature embryo which later produces a spongy mass of cotyledonary tissue that eventually fills the central cavity of the seed. The dramatic ability of this fluid to foster rapid and random division of otherwise mature cells of higher plants has recently attracted attention. This communication will show that the growth-promoting qualities of coconut milk are due to a number of growth substances. At least three of these substances can be recognized as chemical entities. They have been isolated in small amounts, in crystalline form, and their general characteristics can be described. However, the actual number of such substances in coconut milk that give a distinct growth response when added separately and in low concentration to the nutrient medium may well be considerably larger.

When whole coconut milk is added to a basal medium containing mineral salts, sugar and vitamins it causes a striking increase in the growth by cell division of explants of certain tissues, notably carrot root phloem.[2] This response, under suitably controlled conditions, furnishes an assay method for the active substances[3] Whole coconut milk produces an optimum growth response when added to the culture medium at a level of about 15% by volume, which represents a concentration of about 10,000 p.p.m. on a dry weight basis.

The initial enrichment of the activity was made by treating whole coconut milk with an excess of mercuric acetate after dilution by an equal volume of ethanol. After filtration, the precipitate was suspended in water, treated with H_2S, and filtered to remove the precipitated sulfide. The filtrate was concentrated to a heavy sludge under reduced pressure and the sludge was twice extracted by agitation with 90% ethanol. Removal of the solvent left a dark heavy sirup equivalent to approximately 0.6% of the initial dry material of the coconut milk. This sirup showed optimum activity in the tissue culture growth test when added to the basal medium at a level of about 200 p.p.m.

The above extract was fractionated further by differential solubility in various solvents and by partition chromatography on cellulose. At this stage, fractions were obtained which, although active at much lower concentrations, failed to produce at any concentration a total response which approached that given by the addition of whole coconut milk. The full response, however, could be restored by recombination with certain other fractions which were known to contain, among other things, the bulk of the free amino acids present in the crude extract. A similar effect could be obtained by adding an enzymatic hydrolysate of casein to the basal medium at a level of 500 p.p.m., and to a somewhat lesser extent by the addition of pure amino acid mixtures. The addition of casein hydrolysate alone to the basal medium has but a relatively slight effect upon growth; a pronounced response is obtained only in combination with certain fractions from coconut milk. Extensive work has shown, however, that the degree of dependence upon casein hydrolysate varies somewhat among individual carrot roots from the same stock and to a slightly greater extent among roots from different stocks.

Following the discovery of this effect of added casein hydrolysate, various fractions of the coconut milk concentrate were re-examined to determine which ones, ineffective in themselves, became active when tested in the presence of casein hydrolysate. This was done by measuring the growth of aseptic carrot tissue explants in an otherwise synthetic medium. From fractions which proved to be active in this assay procedure small amounts of three substances have now been isolated which, when tested in the presence of casein hydrolysate, induce a rate of growth which approaches that obtained by the use of whole coconut milk.

Compound A.—This was obtained directly upon evaporation of the alcohol extract described above. The alcohol-soluble portion of the mercury-freed precipitate from 800 gallons of coconut milk was reduced to three liters of aqueous solution. This was filtered to remove a small amount of insoluble residue. Upon drying the filter paper a number of small white crystals could be seen and these were mechanically separated from extraneous material. The 78 mg. of crude crystals thus obtained were twice recrystallized from 2 ml. of hot absolute ethanol, giving a final yield of 56 mg. of fine white needles melting at 240.5° (uncor.). The maximum solubility of this compound in water at room temperature was approximately 40 mg./l. The ultraviolet absorption curve in absolute ethanol is shown in Fig. 1 and the infrared absorption curve in a Nujol mull is shown in Fig. 2. This material gave no color reaction with ninhydrin. *Anal.* C, 75.22; H, 6.93; N, 14.19. The growth response in the carrot tissue bioassay test is shown in Fig. 6.

Further Fractionation Procedure.—An amount of the crude concentrate equivalent to approximately 200 gallons of the original coconut milk was further enriched by several solvent fractionation procedures to yield 2.8 g. of material active in the growth assay at 20 p.p.m. This concentrate was chromatographed in *n*-butanol–acetic acid–water mixture on a column containing 800 g. of finely powdered cellulose and was divided into 300 fractions of 25 ml. each. Each fraction was examined for its ultraviolet absorption, fluorescence under ultraviolet, and intensity of its reaction, if any, with ninhydrin.

(1) This work commenced at the University of Rochester and has been continued at Cornell University. It has been supported by grants to one of us (F. C. S.) from the National Institute of Health. Access to the large bulk of coconut milk was made possible through the generous help of the Grasselli Chemicals Division of the du Pont Co. Mrs. Alice Peabody assisted with the growth assays by tissue culture methods.

(2) S. M Caplin and F. C. Steward, *Science* **108**, 655 (1948).

(3) (a) S. M. Caplin and F. C. Steward, *Nature*, **163**, 920 (1949); (b) F. C. Steward, S. M. Caplin and F. K. Millar, *Ann. Botany*, **16**, 57 (1952).

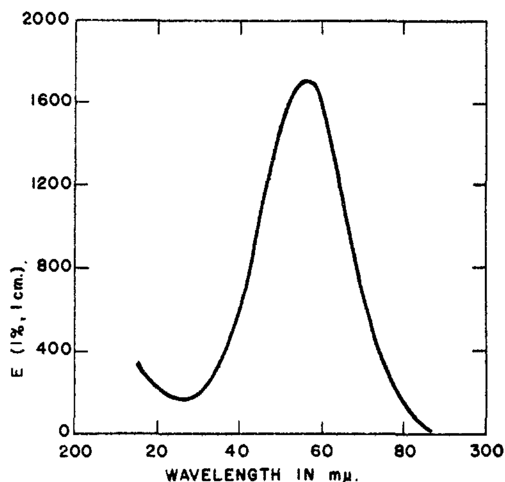

Fig. 1.—Ultraviolet absorption spectrum of compound A in absolute ethanol: E (1%, 1 cm.) at the maximum (256 mμ) = 1700.

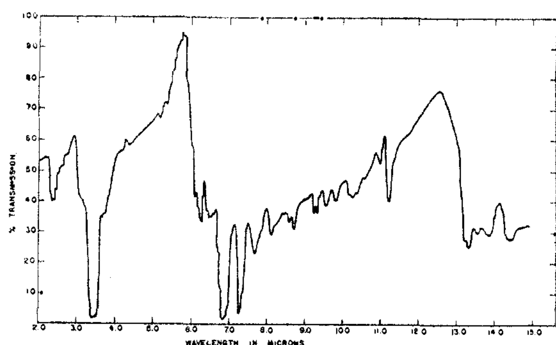

Fig. 2.—Infrared spectrum of compound A in a Nujol mull. The strong absorption bands at 3.45 and 7.26 μ and part of the band at 6.90 μ are due to the Nujol.

About 50% of the material preceded the first strong ninhydrin reacting region. Strong biological activity was found distributed throughout these early fractions, and further fractionation of material from this region indicates that there are undoubtedly several active substances, not yet obtained pure but identifiable by differences in ultraviolet absorption or, in at least one instance, by an intense blue fluorescence.

From the fastest-moving ninhydrin-reacting region, crystalline phenylalanine was obtained and its identity established by paper chromatography. From a later region of fractions, showing both a strong ninhydrin test and ultraviolet absorption at 275 mμ, crystalline tyrosine was obtained. Between the phenylalanine and tyrosine regions there were fractions which absorbed strongly at 260–275 mμ but did not react with ninhydrin. From these fractions B and C were isolated.

Compound B.—Crystalline compound B was obtained in very small amount from the fractions immediately preceding the tyrosine upon evaporating to a small volume. The crystals were filtered, washed with alcohol, redissolved in water, and ultimately recrystallized from 0.3 ml. of absolute alcohol One and two-tenths milligrams of small white needles were obtained after filtering, washing and drying. From the adjoining fractions an additional 0.7 mg. was obtained. The distinctive ultraviolet absorption curve of this compound with its pronounced shift between acid and alkaline solution (Fig. 3) made it possible to determine that very little of this substance remained in the mother liquors. The small yield precluded any extensive chemical tests but two separate growth assays, one of which is shown in Fig. 6, demonstrated that this substance possessed definite growth-promoting power at very low concentrations.

Compound C.—This substance was obtained from the fractions immediately following phenylalanine in the cellu-

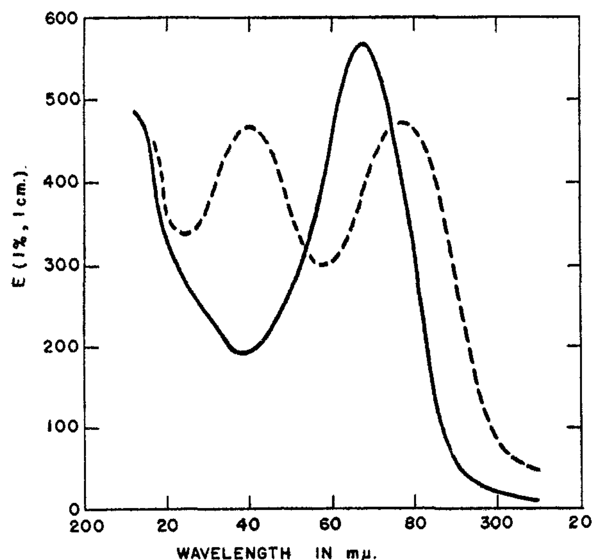

Fig. 3.—Ultraviolet absorption curve of compound B in 0.001 N HCl (solid line) and in 0.001 N NaOH solution (dotted line). The values of E (1%, 1 cm.) at the maxima are 568 at 267 mμ in acid solution, and 470 at 240 mμ and 475 at 277 mμ in alkaline solution.

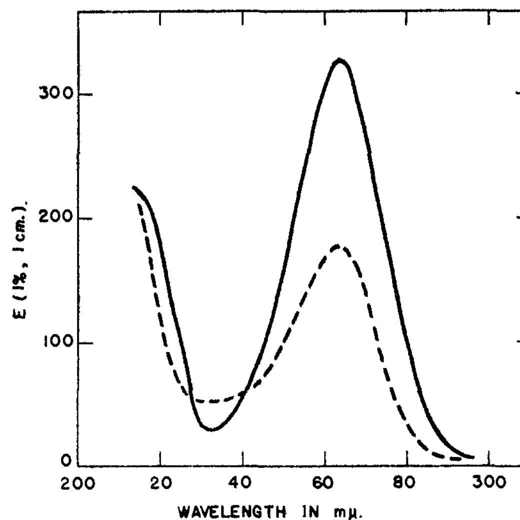

Fig. 4.—Ultraviolet absorption curve of compound C in 0.001 N HCl (solid line) and in 0.001 N NaOH (dotted line). The values of E (1%, 1 cm.) at the maximum (263 mμ) are 326 in acid solution and 177 in alkaline solution.

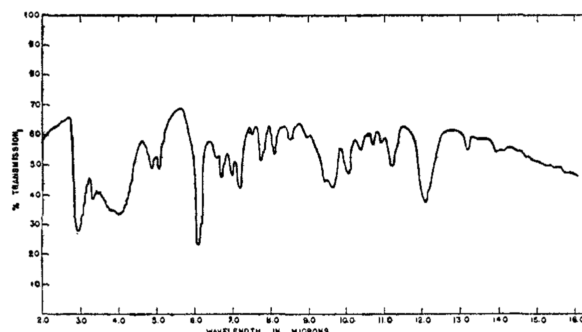

Fig. 5.—Infrared spectrum of compound C in the form of a thin semi-crystalline glass.

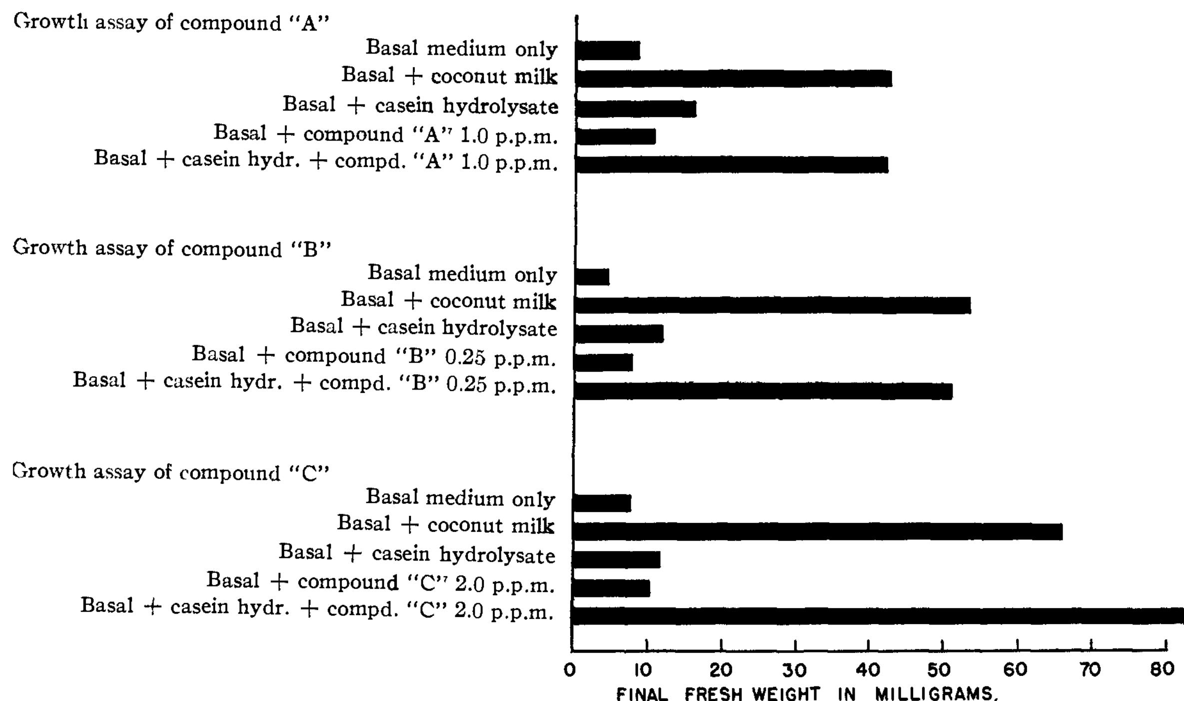

Fig. 6.—Tissue culture growth assays of compounds A, B and C. Each bar represents the average weight of 4 carrot phloem explants after a 14 day test period. Original weight of each explant = 2.6 mg. These tests were not done concurrently and therefore each of the three groups depicts the growth of explants from a different carrot root.

lose column partition chromatography procedure. These fractions (210 mg.) were dissolved in 50 ml. of hot acetone, the volume reduced to 10 ml. and the solution stored for 2 days at −20°. Clusters of prismatic white crystals were obtained which were filtered, washed with cold acetone, recrystallized twice at room temperature from 5 ml. of hot acetone, washed, and dried under vacuum giving a final yield of 19.9 mg.

When determining the melting point it was observed that the material sublimed at 200–210° and condensed in a crystalline state on the cool portions of the capillary. By ultraviolet and infrared absorption, the sublimed material was found to be apparently unchanged and its biological activity in the growth test also withstood this procedure.

The ultraviolet absorption curve of compound C in acid and alkaline solution is shown in Fig. 4. In alkaline solution the absorption maximum at 263 mμ is markedly depressed but unchanged in position. The infrared absorption curve is shown in Fig. 5.

An elementary analysis (single determinations only) showed C, 56.42; H, 8.11; N, 7.67. The growth promoting power of this substance in the carrot phloem explant tissue culture test is shown in Fig. 6.

In summary, the growth-promoting qualities of coconut milk are due in part to a substance or group of substances replaceable by casein hydrolysate. Over and above this, however, there are distinct substances, not contained in casein hydrolysate, which do not appear to be identical with other known vitamin-like compounds. Three such substances have been isolated in crystalline form and the almost certain occurrence of several others has been detected through the use of a carrot tissue culture bioassay procedure. The coconut milk growth factor (C.M.F.) is, therefore, not a single substance but a number of substances, possibly closely related, the identity of which still remains unknown. In view of their dramatic ability to incite random cell division in plant tissues, the isolation of these substances in greater quantity is now being undertaken and their nature and interactions with casein hydrolysate investigated further.

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The Composition of "Cycloheptanol" Produced by the Demianov Rearrangement

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RECEIVED AUGUST 14, 1952

The Demianov rearrangement of hexahydrobenzylamine with nitrous acid has been used as an example[1] of facile ring-expansion, and the product of the reaction has been and is being used as an intermediate in synthesis with the explicit assumption that it is pure cycloheptanol, free from other substances of similar boiling point.[2] We have found that the product consists surely of four and perhaps six, components, of which cycloheptanol constitutes not more than 65%. The other components are cyclohexylcarbinol, 1-methylcyclohexanol and the acetates of one or all of these alcohols. The sequestering of much of the product as acetate is presumably responsible for the failure of Ruzicka and Brugger to detect the cyclohexylcarbinol by reaction with phthalic anhydride.[2]

We have prepared ester-free "cycloheptanol" using sodium dihydrogen phosphate instead of acetic acid; its infrared absorption spectrum (Fig. 1, C) differs from that (Fig. 1, B) of the product ob-

(1) For example, see R. C. Fuson in H. Gilman's "Treatise on Organic Chemistry," Vol. I, John Wiley and Sons, New York, N. Y., 1943, p. 97.

(2) L. Ruzicka and W. Brugger, *Helv. Chim. Acta*, **9**, 399 (1926).