Purine-Like Substances from Coconut Endosperm and Their Effect on Senescence in Excised Cereal Leaves [1, 2]

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Introduction

The endosperm of young nuts of *Cocos nucifera* L. consists of a thin gelatinous layer of jelly and a liquid known as coconut water or milk. As the nuts ripen the jelly develops into a thick, firm, white meat. Steward and his collaborators consider that the specific growth-promoting activity of coconut water results from a synergistic interaction between an inactive neutral fraction, containing hexitols, and an active fraction (8, 11, 17). The latter contains several active substances, one of which is 1,3-diphenylurea (13) and some of which are purine-like (11, 12) though they do not resemble any known purines or pyrimidines. Although Mauney et al. (4) concentrated a growth-promoting fraction from coconut meat, pure substances were not isolated nor have kinetin or kinetin-like substances been isolated from coconut water (18).

In this paper we report the occurrence in extracts from the solid endosperm of young coconuts of purine-like substances which delay senescence in detached cereal leaves as do kinetin (7, 10) and benzimidazole (9). Our interest in this problem stems partly from our interest in the possible role of similar naturally-occurring substances in the formation of "green islands" at rust and mildew infections.

Materials and Methods

Freshly harvested coconuts were obtained by air freight from Barbados through the kind cooperation of Mr. A. Herbert. The jelly from 80 green nuts and meat from 80 mature nuts were homogenized separately with 500 to 1000 ml of cold (4°) absolute ethanol per nut in a large Waring blendor. The homogenates were filtered through filter paper by suction filtration to separate the debris and the alcohol extract was concentrated in vacuo at 35°. The concentrated extracts were filtered through celite and shaken 6 times with equivalent volumes of peroxide-free ether prepared as described by Larsen (3). The aqueous fractions were then treated with a slight excess of freshly prepared cuprous oxide and allowed to stand overnight at 4°. The white precipitates containing copper-purine complexes were filtered off, washed with distilled water and decomposed by treatment with hydrogen sulfide. The precipitated copper sulfide was removed by filtration and the filtrates were again concentrated in vacuo at 35° and filtered through celite.

The pH of each filtrate was adjusted to 10.5 with concentrated NH_4OH and each was then passed at 1.5 ml per minute through a column (1.3 × 25 cm) of Dowex 1 X-10 resin (200–400 mesh) in the formate form. The effluent liquids did not contain any ultraviolet absorbing material and were discarded. Each column was then eluted first with 200 to 600 ml of distilled water and then with 0.02 M HCOOH at a rate of 1.5 ml per minute. Aliquots of 20 ml were taken in a fraction collector. Each aliquot was screened for absorption at 260 mμ.

Results

Isolation of Purine-Like Substances. Substances absorbing strongly at 260 mμ were found in the formate eluate (120 ml) from the meat and in the water (60 ml) and formate (400 ml) eluates from the jelly. After concentration in vacuo at 35° about 12 mg of crystalline material separated out from each of the fractions from the jelly. These crystals were filtered off, washed in cold water and recrystallized from water. They were then washed in cold ethanol and dried in a desiccator. No crystals formed when the formate eluate of the meat was concentrated.

The crystals from the water eluate of the jelly were identified as adenosine and those from the formate eluate as adenine. The ultraviolet spectra of the isolated adenine ($E_{1\,cm}^{0.001\%}$ in 0.1 N HCl = 1.06 at 260 mμ) and adenosine ($E_{1\,cm}^{0.001\%}$ in 0.1 N HCl = 0.665 at 257 mμ) are shown in figure 1, A and B. Infrared spectra of the isolated and authentic compounds are shown in figure 2; they provide a satisfactory basis for the identification of adenine but leave room for some doubt about that of adenosine. The putative adenosine probably included smaller amounts of 2 other purines found in the water eluate of the jelly (table I). On treatment with picric acid a solution of the isolated adenine formed an insoluble picrate. The color reactions with the $AgNO_3$-bromo-

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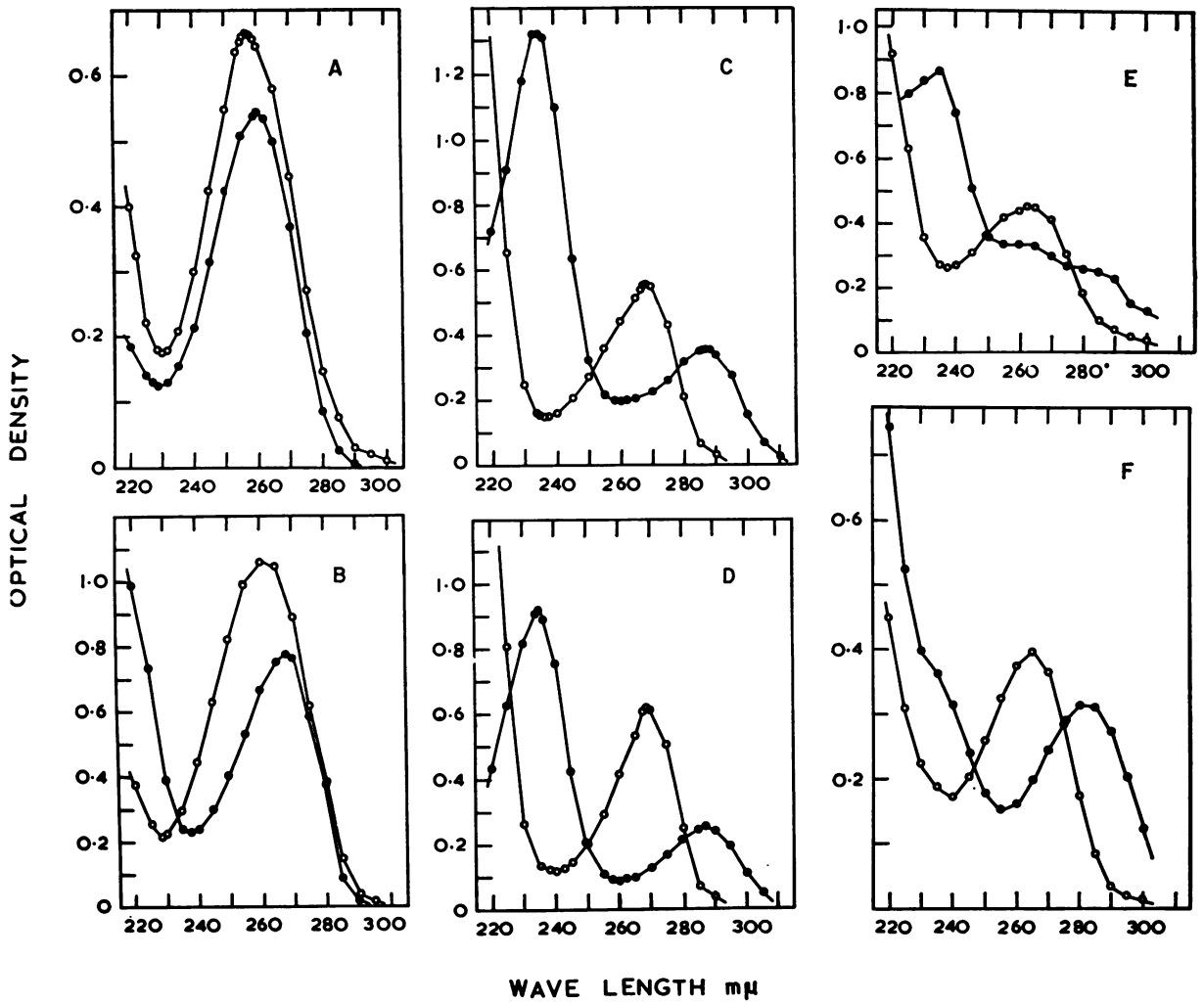

FIG. 1. UV spectra in 0.1 N HCl (open circles) and 0.1 N NaOH (solid circles) of substances from coconut endosperm. D (R_F 0.10) and A (R_F 0.44; adenosine) from water eluate of jelly; B (R_F 0.55; adenine) and C (R_F 0.67) from formate eluate of jelly. E (R_F 0.62) and F (R_F 0.30) from formate eluate of meat.

phenol blue reagent (16) and R_F values in N-butanol-acetic acid-water (4: 1: 5) of the isolated adenine and adenosine were similar to those of authentic samples of these compounds. Melting points were not determined.

After partial removal of adenosine and adenine by crystallization, the residual water and formate eluates from the jelly and the formate eluate from the meat were applied as bands to Whatman No. 3 MM paper and chromatographed with N-butanol: acetic acid: water (4: 1: 5) as descending solvent. After chromatography the purines were located by dipping a guide-strip in the $AgNO_3$-bromo-phenol reagent. The results are summarized in table I. Three purines including adenosine (R_F 0.44) were detected in the water eluate from the jelly; 6, including adenine (R_F 0.51–0.55) were detected in each of the formate eluates from jelly and meat. Larger amounts of all these substances were found in the jelly than in the meat. Appropriate sections of the chromatograms were eluted with distilled water and the utlraviolet spectra of the eluates were determined both in 0.1 N HCl and in 0.1 N NaOH. Concentration and rechromatography of the eluates from individual segments of the original chromatograms gave the same results.

It is apparent from the results in table I that all the unknown purines from the jelly had very similar spectra. Typical examples are shown in figure 1, C and D. With the exception of one compound (E, fig 1) all the unknown purines in the formate eluate of the meat had spectra similar to that for the compound shown in figure 1, F. The spectra of all the purines were shifted when the pH was changed from the acid to the alkaline range (table I).

Effects on Senescence of Cereal Leaves. All the substances listed by R_F values in table I were tested for their capacity to delay senescence and the loss of

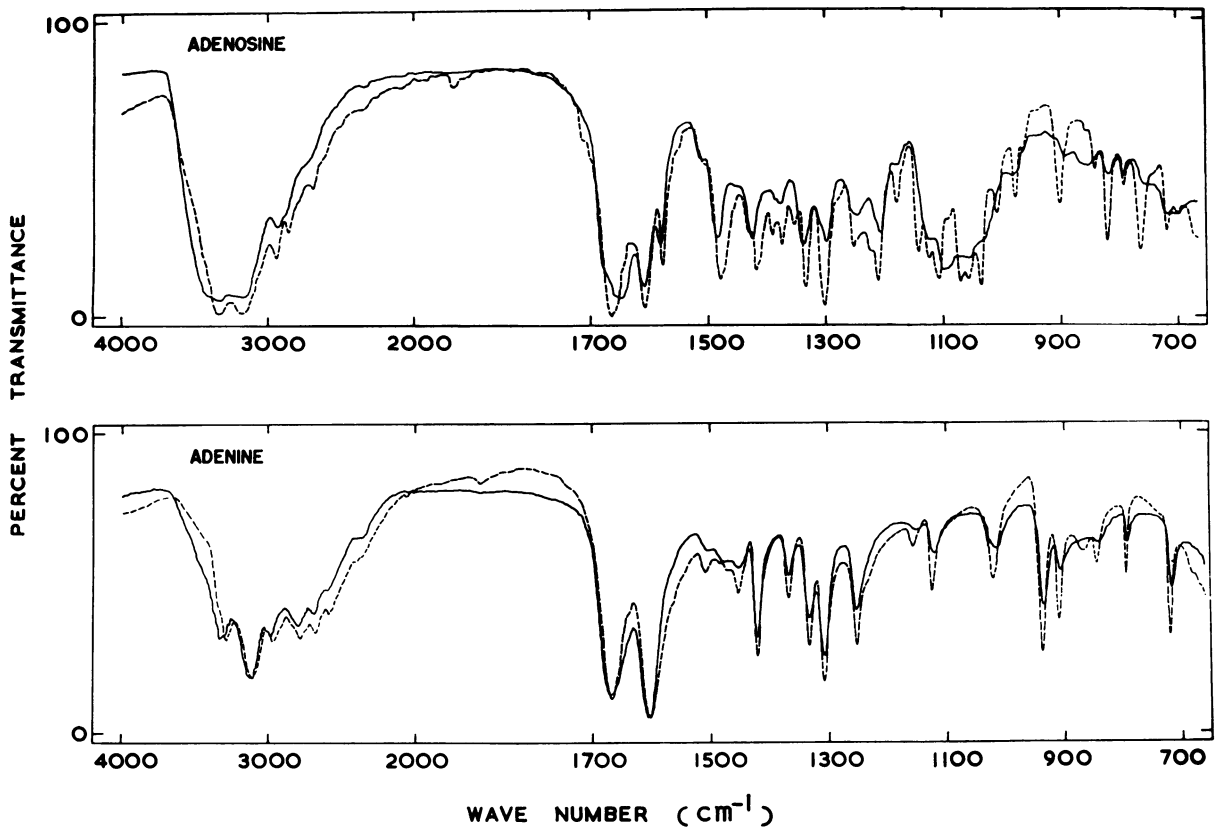

FIG. 2. Infrared spectra (KBr disk) of authentic (dotted lines) and putative (solid lines) adenine and adenosine.

Table I. *The Color Reactions and Ultraviolet Absorption Characteristics of Compounds Detected in Coconut Jelly and Meat*

Coconut endosperm	Fraction	R_F value*	Activity in preserving chlorophyll	Color with $AgNO_3$-Bromophenol blue	In 0.1 N HCl Min	In 0.1 N HCl Max	In 0.1 N NaOH Min	In 0.1 N NaOH Max
Jelly	Water eluate	0.10**	++++	Blue	240	269	260	235,287
		0.44***	Inactive	Deep blue	230	257	228	260
		0.56	+	Blue	237	269,211	258	235,286
	0.02 M HCOOH eluate	0.05	++++	Blue	236	269	260	235,287
		0.17	++++	Blue	235	263	265	235,287
		0.21	++++	Blue	235	268	264	235,286
		0.33	++++	Blue	237	269,211	260	235,285
		0.55†	Inactive	Deep blue	228	260	237	268
		0.67**	+	Blue	236	269,211	260	235,287
Meat	0.02 M HCOOH eluate	0.05	++++	Deep blue	245	265	265	278
		0.16	++++	Deep blue	237	260	260	280
		0.30**	++++	Blue	240	265	255	280
		0.39	++++	Blue	237	263	255	270
		0.51†	Inactive	Deep blue	230	260	245	270
		0.62**	Inactive	Blue	237	263	255	235,260

* In N-butanol: acetic acid: water (4: 1: 5).
** Ultraviolet spectra presented in fig. 1.
*** Identified as adenosine.
† Identified as adenine.

chlorophyll in detached first seedling leaves of Little Club wheat. About 10 leaves (12 days old; fully expanded) were floated on 20 ml of solution having an optical density of about 0.1 at 260 mμ in petri dishes kept under fluorescent light of about 500 ft-c for 12 hours daily. Leaves floated on water served as controls.

Solutions of adenosine, adenine and the eluate from the zone centered on R_F 0.62 of the chromatogram of the meat were all inactive and leaves floated on them turned completely yellow in 6 or 7 days, as on water. The eluates from R_F 0.56 and R_F 0.67 of the chromatograms of the jelly extract delayed yellowing slightly. All the other purine eluates from chromatograms of both jelly and meat extracts were highly active and the leaves tested stayed green for 3 weeks or longer. In some cases, chlorophyll contents were determined using the methods described by Smith and Benitez (17) and the absorption coefficients of Comar and Zscheile (2). The results, given in table II, speak for themselves. Three weeks after excision the chloroplasts in the control leaves were represented only by clumped brownish-yellow droplets which were visible in free-hand sections of the fresh leaves but which were usually destroyed by fixation. At this time the chloroplasts in purine leaves were normal in color and appearance, but were only about two-thirds the size of those in newly detached leaves. Typical control and purine leaves were fixed in formalin-acetic acid, sectioned at 12μ and stained with tannic acid, safranin and orange G (14). Even when the chloroplasts had become very small, the nuclei in the mesophyll cells of leaves floated on water were often normal in size and appearance.

In another series of experiments, it was found that neither the coconut purines (optical density circa 0.1) nor kinetin (1–40 μg/ml) had any effect in delaying senescence or maintaining turgor in the albino leaves of a variegated barley (1), which contain only 1.2 μg chlorophyll per gram fresh weight (15), either in the presence or absence of sucrose (0.1–2 % w/v).

Discussion

Miller (5) has isolated a kinetin-like factor from kernels of *Zea mays* in the milk stage. Substances which behave like kinetin in delaying senescence of detached cereal leaves also occur in the endosperm of the coconut. It does not seem likely that these substances could have been formed during the procedures used to isolate them from the alcohol extract, but this possibility must be kept in mind. Even if they were, they are of considerable interest. The concentrations of the various purine-like substances in the extract of the meat were much lower than those in the extract from the jelly. Since lower concentrations and consequently the presence of a higher proportion of impurities derived from the chromatographic paper may have combined to obliterate the peak at 235 mμ for the substances from the meat (table I), it is difficult to be certain whether or not the meat substances are all different from those in the jelly.

In 0.1 N NaOH the spectra of the jelly substances differ from those of kinetin and the maize substance (5) in the appearance of a new peak at 235 mμ and in the shift of the maximum to 287 mμ and the minimum to 260 mμ. Kinetin and the maize substance do not exhibit such a shift when the pH is raised, though they both show a shoulder at 280 to 285 mμ. (5) The spectrum of compound B of Shantz and Steward (12), obtained from coconut water, has a minimum at 260 mμ and maxima at 240 and 277 mμ in alkaline solution and, therefore, shows some resemblance to the spectra of the jelly substances.

Mothes and Engelbrecht (6) have shown that kinetin induces the localized accumulation of amino acids in leaf tissue, but that this does not occur in leaves kept in the dark for a long period of time or in albino leaves. They suggested that ATP derived from photosynthetic phosphorylation was necessary for kinetin-induced accumulation to occur. In agreement with this, neither kinetin nor the coconut substances had any effect in delaying the senescence of albino leaves. This suggests the possibility that kinetin and the active substances from coconuts act at centers within or at the surface of the chloroplasts and it would be of extreme interest to know whether they play any role in the maintenance of membrane structure.

We have been careful not to imply that activity in the senescence test employed in this paper is necessarily correlated with growth-promoting activity in the carrot tissue culture assay (11, 12, 13, 18) i.e. with

Table II. *The Chlorophyll Content of Little Club Wheat Leaves after Fourteen days Floating on Water and Coconut Endosperm Fractions*

Coconut endosperm	Chromatogram segment	Chlorophyll A*	Chlorophyll B*
Jelly	Water eluate, R_F 0.1	1.260	0.480
	H_2O Control	0.217	0.064
	Formate eluate, R_F 0.06	0.870	0.384
	H_2O Control	0.049	0.029
Meat	Formate eluate, R_F 0.05	0.778	0.393
	H_2O Control	0.071	0.036

* Expressed as milligram chlorophyll/g fr wt of leaves.

kinin activity. In view of their general resemblance to kinetin and to some of the substances from coconuts first described by Steward's group, it would be surprising if some of the coconut substances described in this paper were not, in fact, kinins. Finally, it should be noted that activity in the carrot assay does not necessarily imply activity in the senescence test.

Summary

Adenine, a substance which was probably adenosine, and several unidentified purine-like substances were isolated by ion-exchange and paper chromatography from alcohol extracts of the solid endosperm of young coconuts. Ultraviolet spectra of the unidentified substances were determined in 0.1 N HCl and 0.1 N NaOH. Five unidentified substances found in the jelly of young nuts and 4 found in the meat of mature nuts were active in delaying senesecence and the loss of chlorophyll in detached wheat leaves. Neither these active substances nor kinetin had any effect in delaying senescence in detached leaves of an albino barley. It is suggested that kinetin and the coconut substances act at centers in or on the chloroplasts.

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