HEXITOLS IN COCONUT MILK: THEIR ROLE IN NURTURE OF DIVIDING CELLS [1]

J. K. POLLARD, E. M. SHANTZ, & F. C. STEWARD

DEPARTMENT OF BOTANY, CORNELL UNIVERSITY, ITHACA, NEW YORK

Coconut milk, the liquid endosperm of the coconut (*Cocos nucifera* L.), has special interest because it will induce otherwise mature, non-growing cells to divide and to grow rapidly (3, 4). Similar properties reside in analogous morphological situations, such as the immature caryopsis of *Zea mays* and the liquid present in immature fruits of Juglans (18) or of Aesculus (12, 24, & also see 25). A nutritive relationship also exists between the female gametophyte, sometimes called endosperm, and the archegonia and embryos of the gymnosperm Ginkgo; extracts of this gametophyte will also induce cells of mature tissue of carrot root to resume active growth (18). Therefore, the fluids that nourish immature embryos seem especially able to induce growth in the mature cells even of some other plants than the ones in which they were laid down. This raises the question whether the behavior of the zygote is due to its special nature or to its nurture by the special fluid contents of the embryo sac, by the substances which are contained in the endosperm, and by other special nutritive organs.

Free cells obtained from mature carrot phloem may be cultured in media which contain coconut milk (21) and may grow and regenerate a complete and mature plant (20). In this respect the free cells imitate the zygote, and the coconut milk its normal nutritional supply. Moreover, as the cells grow and develop, they form structures which are strongly reminiscent of pro-embryonic development (15). Therefore, a full knowledge of the chemical constituents of coconut milk which cause these growth responses would have an important bearing upon many problems of cell growth and cell division. This knowledge would also have important implications for protein synthesis, which is stimulated in quantity and modified in kind during the induction of growth in carrot and potato cells (27).

Following the observations of Blakeslee and van Overbeek (28, 29), work upon the chemical constituents of coconut milk was pursued sporadically in different laboratories (for references see 25). In this laboratory, investigation has been in progress for some years. Clearly, the growth induction which is produced by the coconut milk, over and above the effects due to common nutrients and vitamins, is not a simple effect due to a single substance. On the contrary, it has been emphasized that no substance singly and independently controls cell division (see 25 & references there cited).

In part the effect of coconut milk is non-specific and is replaceable by casein hydrolysate, or by other sources of the reduced nitrogen compounds, from which the cells may synthesize protein more readily than they do from nitrate (13). Even whole coconut milk alone will not trigger the growth of some cells (e.g. potato tuber), for it needs to be supplemented by one of a large array of compounds which are now known to act synergistically with the coconut milk. The substance 2,4-dichlorophenoxyacetic acid (2,4-D), and many of its analogues with different ring configurations or different side chains, can also function in this manner (14, 16, see also 25). Several of the halogen-substituted phenylacetic acids (26) and certain α-substituted propionic acids (14) can also work along with the coconut milk. This paper now designates certain hexitols to be responsible for part of the effect for which coconut milk (or its morphological equivalent) has hitherto been regarded as a specific source.

Early work on the chemical fractionation of coconut milk and similar fluids recently has been reviewed (25). This work encountered the difficulty that, when purified, the isolated substances only expressed their activity in the presence of other sub-fractions from the coconut milk. While this statement still holds true, the work to be described permits the critical identification of at least three of the synergists which contribute to the total growth which is stimulated by whole coconut milk.

MATERIALS & METHODS

Coconut milk was fractionated on ion-exchange resins and by ordinary chemical means which are described below in the appropriate section. Assays for growth-promoting activity were made on standard (3 mg) carrot explants placed aseptically in a basal nutrient medium to which the materials to be tested were added. After 17 days, the final fresh weight was determined; this served as a measure of the relative activity of the materials being tested. Complete details of the method have been published (19).

RESULTS

Synthetic ion exchange resins were first applied to the fractionation of coconut milk to test whether or not organic phosphates, which could be bound to a suitable resin (Amberlite IR–45), contributed significantly to the growth induction stimulus. They did not; however, this led to the separation of whole coconut milk into two main parts by passing it over suitable resins. One part seemed to be ionic, usually called "active fraction", and the other part was either very weakly ionic or strictly neutral and is referred to as the "neutral fraction". Although these two fractions, separately, were usually weakly active in the carrot growth assay, they elicited activity which approached that of whole coconut milk when they were tested in appropriate combination. Subsequent experiments showed that the active fraction is also adsorbed by activated charcoal, from which it can be eluted with 50 % aqueous acetic acid followed by 5 % ammonium hydroxide. The chart of figure 1 shows the method which is now used for the separation of

Whole Coconut Milk, 1000 g (Promotes growth at 5000 ppm) ⟶ Pass through a column containing 50 g of activated charcoal ⟶ Filtrate, 998 g ↓ Pass successively over Dowex 50 and Amberlite IR-45

Elute with 50% (v/v) aqueous acetic acid ⟶ Eluate, 1.0 g

Wash with water ⟶ Discard

Elute with $\underline{N}$ NH$_4$OH ↓ Eluate, 0.15 g ⟶ Pooled ↓ Active Fraction (Promotes slight growth at 10 ppm)

Elute with NH$_4$OH (Dowex) and HCl (Amberlite) ↓ Inorganic salts 200 g (inactive) and amino acids, 20 g (act only as non-specific source of reduced nitrogen)

Filtrate, 850 g Neutral Fraction (Promotes slight growth at 250 ppm)

Active Fraction + Neutral Fraction (Combination promotes much more growth; cf. fig. 2 and tables 2 and 3.)
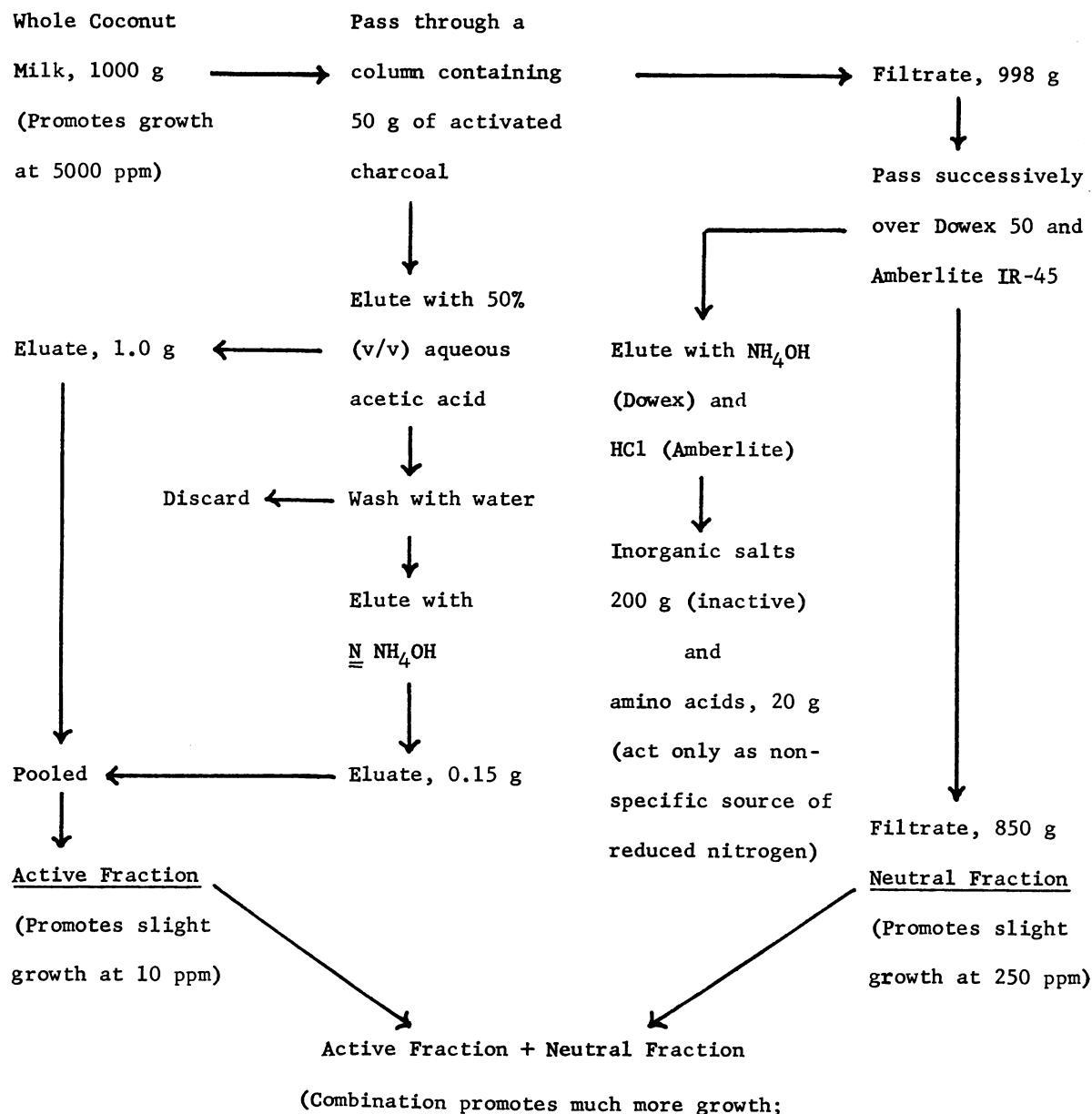
FIG. 1. Preparation of an active fraction and a neutral fraction from whole coconut milk. (Weights as shown indicate the approximate yields of dry matter from 20 liters of whole coconut milk, representing about 1,000 g of dry weight; activities at the concentration indicated refer to the standard carrot assay method.)

coconut milk into these principal fractions. The histograms of figure 2 show that active and neutral fractions supplement each other. When obtained in quantity, each could be used as a supplement to the basal medium when the growth-promoting components of the other were to be detected by the carrot growth assay. The present paper gives an account of the neutral fraction of coconut milk; this fraction is not retained when coconut milk concentrate is successively passed over the resins Dowex 50 and Amberlite IR-45 (see fig 1).

NEUTRAL, OR WEAKLY IONIC, COMPOUNDS OF COCONUT MILK. After the deonization procedures described above, the so-called neutral fraction was evaporated to a thick, dark colored syrup which represented 85 % of the dry weight of coconut milk, or 40 to 45 g/liter.

Sorbitol and *scyllo*-inositol (scyllitol) were crystallized, even before they were identified, from the syrup after it had been freed from reducing sugars by passage over a strongly basic resin (Dowex–1). *myo*-Inositol was identified chromatographically and then isolated by a modification of the lead acetate precipitation described by Haas and Hill (5). In table I are listed the criteria upon which these three hexitols were identified.

Subsequently, larger amounts of *scyllo*-inositol were readily crystallized from the crude neutral fraction by evaporating it to a heavy syrup, adding 2 volumes of methanol, and seeding the syrup with crystals of *scyllo*-inositol. After a week at room temperature, 8 g of crude *scyllo*-inositol were obtained in this way from the equivalent of 20 liters of coconut milk. A single recrystallization from water and methanol gave 4.5 g of pure *scyllo*-inositol.

The residue from this larger *scyllo*-inositol isolation was again evaporated to a heavy syrup and an equal volume of dimethylformamide was added. Large quantities of sorbitol crystallized after the mixture was placed in the refrigerator. In this way, 200 g of crude sorbitol, once recrystallized, were obtained from the equivalent of 20 liters of coconut milk. The yield of crystalline sorbitol was estimated at about half the total sorbitol present; therefore, the largest single constituent of coconut milk was one which had previously escaped identification.

The yield of *myo*-inositol was 50 mg from the equivalent of 3 liters of coconut milk. A bioassay, using an inositol-less mutant (no. 37401, kindly supplied by Prof. A. M. Srb) of *Neurospora crassa* (1). indicated that the *myo*-inositol content was approximately 0.25 % of the dry weight of coconut milk. *scyllo*-Inositol had no effect on this mutant. The approximate hexitol content of coconut milk is listed in table I.

Subsequent to the work described above, a search of the literature showed that a substance called cocositol was isolated from coconut leaves in 1907 and again from coconut milk by Müller (7); this was later shown to be *scyllo*-inositol (8).

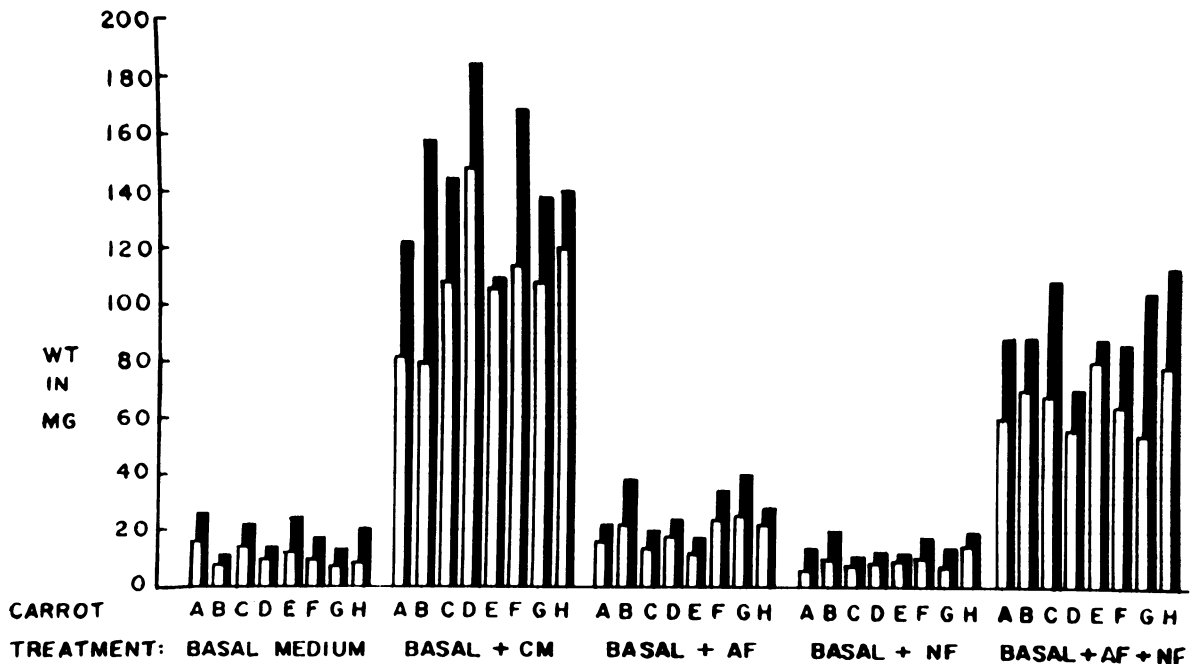INTERACTIONS OF SUBSTANCES OR CLASSES OF COMPOUNDS WHICH PRODUCE GROWTH RESPONSE DUE

FIG. 2. Growth response of explants from eight different carrot roots to active fraction (AF) and neutral fraction (NF), separately and in combination, grown in the presence (*solid bars*) and the absence (*white bars*) of casein hydrolysate, compared with their growth in basal medium alone and basal medium plus coconut milk (CM). Each bar represents the average final fresh weight of nine replicate cultures after a growth period of 17 days.

TABLE I

EVIDENCE FOR IDENTITY OF 3 HEXITOLS ISOLATED FROM COCONUT MILK

HEXITOL	APPROX. CONC IN COCONUT MILK	ANALYSIS			MELTING PT. (° C)
Sorbitol					
Isolated	15,000 ppm	C, 39.68;	H, 7.70;	O, 52.78	...
Authentic		C, 39.56;	H, 7.69;	O, 52.73	...
Sorbitol hexa-acetate					
Prepd. from isolated sample	...	C, 49.53;	H, 6.09		103
" " authentic sample	...	C, 49.76;	H, 5.96		103
scyllo-Inositol					
Isolated	500 ppm	C, 40.05;	H, 6.62;	O, 53.16	353
Authentic		C, 40.00;	H, 6.67;	O, 53.33	353
myo-Inositol					
Isolated	100 ppm	C, 39.95;	H, 6.77		227–32
Authentic		C, 40.00;	H, 6.67		227–32

In each instance, the isolated and authentic substances were chromatographically identical in several solvents.

TO COCONUT MILK. To reveal the individual and collective effects of various substances, or categories of substances which occur in coconut milk, the substances were supplied alone and in combination to standard carrot explants in factorially designed experiments. Relatively simple statistics permit the total growth which is observed in such an experiment to be partitioned into that growth which is due to lone action of each treatment and that which is due to interactions between the treatments.

The term "interaction", as used here, implies that two or more components work together to produce a response which is significantly different from the growth which would be predicted from the sum of their individual effects. If such a difference is positive, i.e., the growth produced by the combination of treatments is greater than that predicted from their single effects, then the term "synergism" is used to describe the interaction. If such a difference is negative, then it is referred to as "a negative interaction".

Active fraction, which contains the highly specific cell division factors per se, shows statistically significant interactions with A, casein hydrolysate, as a general amino acid source, B, auxin-like substances, including indoleacetic acid and such synthetic sub-

TABLE II

EFFECT OF INDOLEACETIC ACID (IAA), ACTIVE (AF), & NEUTRAL (NF) FRACTIONS FROM COCONUT MILK, SINGLY & IN COMBINATION, ON GROWTH OF CARROT EXPLANTS IN BASAL MEDIUM (BM) CONTAINING CASEIN HYDROLYSATE (CH)*

TREATMENT	(A) SOURCE OF CARROT EXPLANTS								(B) EFFECTS CALCULATED FROM EXPLANTS FROM 8 SOURCES			
	510A	510B	510C	510D	510E	510G	510H	510J	MEAN GROWTH	OB-SERVED GROWTH INCRE-MENT	PRE-DICTED GROWTH INCRE-MENT	EFFECT DUE TO INTER-ACTION
1. Basal+CH	25.1	28.1	10.8	17.7	25.0	20.4	30.1	19.6	22.1	...	...	...
2. " " +IAA (0.5 ppm)	62.8	57.4	29.8	46.4	66.2	46.7	39.7	23.0	46.5	24.4	...	...
3. " " +AF (10 ppm)	18.8	33.5	17.6	61.8	26.6	16.2	23.1	36.7	29.3	7.2	...	...
4. " " +NF (250 ppm)	59.9	55.0	33.5	25.8	64.8	24.6	50.6	22.4	41.7	19.6	...	...
5. " " +AF+NF	59.6	81.1	62.9	84.3	90.5	77.9	99.1	116.2	84.0	61.9	26.8	+35.1**
6. " " +IAA+NF	81.5	72.2	56.4	47.8	88.0	44.5	52.0	36.5	59.9	37.8	44.0	− 6.2**
7. " " +IAA+AF	50.2	88.3	54.5	67.6	93.9	74.4	55.4	121.7	75.8	53.7	31.6	+22.1**
8. " " +IAA+AF+NF	63.8	112.4	56.2	68.2	111.6	86.6	89.3	163.8	94.0	71.9	102.2	−30.3**

* Data are mean final fresh weights (mg) per explant.

In Table IIB, whenever two components are present in the medium, the growth is predicted from the sum of their individual effects. When three additional components are present in the medium (no. 8), the predicted effect is the sum of the effects due to treatments 5, 6, and 7 minus that of treatments 2, 3, and 4. The interaction of the factors is measured by the difference between the observed and the predicted growth.

** Results significant at the 1 % level.

stances as 2,4-D, and C, with the neutral fraction or its individual components.

Table II shows the mean growth obtained in such an experiment which was replicated on explants from eight different carrots. The variable response shown by the explants from the different carrots is quite clear from this table; such variations from root to root have been previously described (23). Although the effects of each treatment on explants from particular carrot roots may be calculated from this table, the following discussion concerns the mean responses for all eight carrot roots as they are stated toward the right of table II. If the mean weight of the basal controls is subtracted from the mean weight obtained in each treatment, then the observed growth increment due to each treatment is derived (table II B).

The data in table II may be analyzed further as in table III. Table III shows, in summary form, the separate effect of each growth factor on the inter-

TABLE III

SYNERGISTIC INTERACTIONS BETWEEN GROWTH FACTORS: INFLUENCE OF A 3RD FACTOR ON INTERACTION BETWEEN 2 OTHERS

INTERACTION	INCREMENT IN mg fr wt DUE TO INTERACTION
AF & NF in absence of IAA	+ 35.1
AF & NF in presence of IAA	+ 4.7
AF & IAA in absence of NF	+ 22.1
AF & IAA in presence of NF	− 8.2
NF & IAA in absence of AF	− 6.2
NF & IAA in presence of AF	− 36.5

action between the other two which are concerned here. Thus, a clear synergism exists between active fraction and neutral fraction in the absence of indoleacetic acid, but if IAA is present, this synergism disappears. Similarly, active fraction and IAA are synergistic if neutral fraction is absent whereas, if neutral fraction is present, no such synergism can be demonstrated. The negative interaction between IAA and neutral fraction, predicted from the above observations, is confirmed by the data. Thus, the neutral fraction and IAA compete, i.e., they show a negative interaction which is accentuated in the presence of the active fraction.

The effect of the individual treatments as they act alone may be assessed from table II. A more complete statistical analysis of variance, not given here, established that the individual effects of active fraction, neutral fraction, and IAA were all highly significant over the whole experiment.

The complete interpretation of such interactions, synergistic or not, will depend on the eventual elucidation of the biochemical role that each component plays in the living system. The failure of IAA to elicit

the expected positive response in the presence of neutral fraction may indicate that, at least in part, these two growth-promoting substances overlap so that the requirement for one is reduced somewhat in the presence of the other. Alternatively, since both IAA and neutral fraction require active fraction for their best response, they may be in competition, at the biochemical level, for the substances which are contained in the active fraction, if all of these components are supplied together.

Although not detailed here, we tested the interaction of IAA and neutral fraction at four levels of active fraction on the growth of explants from four different carrots. If IAA and neutral fraction compete for active fraction, this should be most apparent as a significant negative interaction when the active fraction tends to be limiting. This did not occur. Therefore, the first alternative suggested above is still the preferred one, although it lacks biochemical explanation.

BIOLOGICAL ACTIVITY. The hexitols in coconut milk exert their effect on carrot explants when they are added to the basal medium (which contains salts, sucrose, & certain vitamins), particularly if the active fraction is also supplied. The latter elicits cell division when its active substances are present in the culture medium at concentrations of the order of a few parts per million; the hexitols, however, act at higher concentrations (generally of the order of 50 ppm). Thus a nutrient medium which contains 10 % by volume of whole coconut milk contains approximately 1,500 ppm of sorbitol, a concentration which our assays show are at least ten times greater than that which shows a response with carrot explants. Since these hexitols were added to a medium which was already well supplied with a general carbohydrate source (sucrose), their primary role is not as the source of carbon for the growing tissue. If sorbitol alone is the general source of carbon, the carrot explants cannot grow at all. Table IV contains the results of an experiment in which the growth stimulus was supplied by the active fraction from coconut milk (10 ppm) and by 100 ppm of myo-inositol. Of the additional carbon sources thus supplied, sucrose was the best, glucose substituted partially for sucrose, fructose alone was toxic, and sorbitol, mannitol, or dulcitol were without effect. Therefore, sorbitol is not a general carbon source for cultured carrot tissue.

Table V shows the variable responses of explants from different carrot roots to the known components of the neutral fraction. Explants from carrot 498A responded to both myo-inositol and scyllo-inositol, the greater response being to the former; but in this group of explants no synergistic response between the inositols and (IAA+AF) was noted. This is, however, complicated by the antagonism between IAA and neutral fraction discussed earlier in relation to table II. With explants from root 498D the growth response was not to myo-inositol alone by to myo-inositol in the presence of (IAA+AF). Here the

TABLE IV

GROWTH OF CARROT EXPLANTS IN MEDIA WHICH CONTAIN VARIOUS CARBOHYDRATES AS SOURCE OF CARBON

CARBOHYDRATE SUPPLIED (1.5 %)	BASAL MEDIUM ONLY SOURCE OF CARROT EXPLANTS		BASAL MEDIUM PLUS ACTIVE FRACTION (10 ppm) & INOSITOL (100 ppm) SOURCE OF CARROT EXPLANTS	
	547A	547B	547A	547B
None	5.6	4.7	7.1	5.5
Sucrose	11.5	9.1	82.9	28.6
Sorbitol	6.1	4.5	5.8	5.0
Glucose	5.0	4.5	45.4	30.8
Fructose*	...	...	...	...
Mannitol	4.8	4.5	7.6	5.3
Dulcitol	5.6	4.9	5.2	5.1

* When fructose is supplied as the sole source of carbon, the medium is toxic after autoclaving. In a comparable experiment where the fructose was filter sterilized, the cultures grew to a final weight which was 70 % of that attained in the controls supplied with sucrose.

magnitude of the interactions was large enough to mask the negative interaction between IAA and the components of neutral fraction. Explants from root 498D did not respond to *scyllo*-inositol and, although the data are not given, explants from roots 498D and 498C did not respond to either of the inositols.

Table VI shows the growth responses of explants from 492B. *scyllo*-Inositol was effective only in the presence of casein hydrolysate and particularly so if active fraction was present as a synergist. Here the data are not complicated by the effect of IAA in the medium.

The results of an experiment designed to replace neutral fraction from coconut milk with a mixture of

TABLE VI

INTERACTING EFFECTS OF CASEIN HYDROLYSATE, ACTIVE FRACTION AT 10 ppm (AF) & SCYLLO-INOSITOL AT 25 ppm ON GROWTH OF CARROT EXPLANTS IN BASAL MEDIUM*

	BASAL MEDIUM		BASAL + CASEIN HYDROLYSATE	
	No AF	+ AF	No AF	+ AF
No *scyllo*-Inositol	9.7	12.7	15.9	29.4
+ *scyllo*-Inositol	9.9	12.6	18.1	48.4

* Data are mean final fresh weight in mg per explant; mean of nine replicates.

TABLE V

EFFECTS OF INOSITOLS & ACTIVE FRACTION WITH INDOLEACETIC ACID (AF+IAA) ON GROWTH OF CARROT EXPLANTS IN BASAL MEDIUM PLUS CASEIN HYDROLYSATE*

TREATMENT	CARROT 498A			CARROT 498B		
	FINAL WT	GROWTH INCRE-MENT	EFFECT DUE TO INTER-ACTION	FINAL WT	GROWTH INCRE-MENT	EFFECT DUE TO INTER-ACTION
Basal+casein hydrolysate	19.5	...	...	21.8	...	...
" " +scyllo-Inositol (25 ppm)	28.9	9.4***	...	21.9	0.1	...
" " +scyllo-Inositol (5 ppm)	27.6	8.1***	...	22.8	1.0	...
" " +myo-Inositol (25 ppm)	52.0	32.5***	...	20.1	− 0.7	...
" " +myo-Inositol (5 ppm)	43.3	23.8***	...	21.2	1.0	...
" " + (AF+IAA)**	49.1	29.6***	...	57.7	37.9	...
" " " +scyllo-Inositol (25 ppm)	68.1	48.6	+ 9.6	52.1	30.3	− 7.7
" " " +scyllo-Inositol (5 ppm)	57.9	38.4	+ 0.7	51.1	29.3	− 9.6
" " " +myo-Inositol (25 ppm)	65.4	45.6	−16.5***	70.2	48.4	+11.2***
" " " +myo-Inositol (5 ppm)	61.4	41.9	−11.5***	72.5	50.7	+11.8***
Basal+casein hydrolysate+10 % Coconut milk	124.0	104.5***		95.7	63.9***	

* All data are in mg mean final fresh weight per explant.
** AF used at 10 ppm, IAA at 0.5 ppm.
*** Indicates a result statistically significant at the 1 % level of probability.

carbohydrates are found in table VII. For carrot 510C the response to treatment with this synthetic neutral fraction at 500 ppm was greater than that shown to coconut milk neutral fraction at the same concentration. Explants from carrot 510H grew better on coconut milk neutral fraction, although they still responded to the synthetic mixture. The mixture of carbohydrates used above contained equal amounts of each of the three hexitols of coconut milk and of maltose, mannose, lactose, ribose, rhamnose, erythritol, turanose, trehalose, and glycerol. (In other experiments, all of these, save glycerol, have shown statistically significant stimulatory responses in the presence of the active fraction and inositols.)

In view of the carbohydrate nature of the neutral fraction, a survey was made of readily obtainable carbohydrates to see which were effective in this respect. Out of some 33 carbohydrates tested, those listed above were found to have some statistically significant effect in the presence of both inositols. Until there is actual evidence of the occurrence of these or any other effective carbohydrate in coconut milk, there is no reason to believe that the activity of natural neutral fraction owes anything to compounds other than the two inositols and sorbitol, which do occur in the coconut milk. However, other effective carbohydrates in the neutral fraction of coconut milk may yet be discovered.

Much still remains to be understood, particularly with respect to interactions between different sugars. However, table VII shows that the part of the total effect of coconut milk which is due to the neutral fraction may be replaced by a mixture of pure carbo-

hydrates, and this will, at least for certain groups of carrot explants, substitute for the natural product. In fact, there is no reason to assume that the composition of the neutral fraction of coconut milk is ideal for carrot cells, and, in time, a better mixture of carbohydrates for carrot cells may be developed than that which is found in coconut milk. For example, experiments have already shown that sorbitol is present in coconut milk at a concentration ten times greater than that at which it acts most effectively.

NEUTRAL FRACTION FROM OTHER SOURCES OF GROWTH-PROMOTING ACTIVITY. The composition of the neutral fraction from other good sources of the growth-promoting activity toward carrot explants may differ from that which is present in coconut milk. For example, an investigation for hexitols in the liquid from Aesculus fruits produced a somewhat surprising result. When 116 g of this fluid, which had already been through charcoal and freed of certain inorganic substances (equivalent to 145 g dry wt of the whole Aesculus fluid) was de-ionized with Dowex 50 and Amberlite IR-45, only 52 g of organic, neutral fraction remained. This was concentrated to 100 ml; 200 ml of methanol were added, as in the process for preparing scyllo-inositol from coconut milk, and crystals appeared instantly. A yield of 14 g of hexitol was thus obtained; however, the melting point did not correspond to scyllo-inositol but rather to myo-inositol. This product was then identified by comparing the melting points of both the isolate and its acetate with authentic myo-inositol and its acetate. The isolate, the myo-inositol, and a mixture

TABLE VII

COMPARISON OF EFFECTS OF NEUTRAL FRACTION FROM COCONUT MILK, & OF SORBITOL, WITH MIXTURE* OF HEXITOLS & SUGARS ON GROWTH OF CARROT EXPLANTS IN BASAL MEDIUM PLUS CASEIN HYDROLYSATE WITH & WITHOUT ACTIVE FRACTION

TREATMENT	SOURCE OF CARROT TISSUE					
	CARROT 510C			CARROT 510H		
	FINAL WT	GROWTH INCRE-MENT	EFFECT DUE TO INTER-ACTION	FINAL WT	GROWTH INCRE-MENT	EFFECT DUE TO INTER-ACTION
Basal + casein hydrolysate	14.5	...	...	28.8	...	...
" " + neutral fraction (500 ppm)	21.6	7.1†	...	41.0	12.2†	...
" " + mixed hexitols & sugars*	22.4	7.9†	...	30.6	1.8	...
" " + active fraction (10 ppm)	21.4	6.9†	...	24.2	− 4.6	...
" " " + neutral fraction	49.8	35.3	+21.3†	63.7	34.9	+27.3†
" " " + mixed hexitols & sugars	54.6	41.1	+26.3†	39.8	11.0	+13.8†
Basal + casein hydrolysate + sorbitol (100 ppm)	15.9	1.4	...	28.8	0.0	...
" " " + active fraction (10 ppm)	20.8	6.3	− 2.0**	29.5	1.0	+ 5.6** ***
Basal + casein hydrolysate + 10 % coconut milk	59.8	45.3†	...	116.0	87.2†	...

* The mixture consisted of equal parts of the three hexitols in coconut milk plus eight sugars to which carrot tissue had previously shown a significant positive response. The final concentration of the mixture was 500 ppm.
** These results are typical of many. They show for certain carrot roots a small, but significant, positive response to sorbitol in the presence of active fraction, but this is lacking in tissue from other roots.
*** Indicates a result statistically significant at the 5 % level of probability.
† Indicates a result statistically significant at the 1 % level of probability.

of the two all melted in the range 227 to 232° C. Also, the acetate of the isolate, the acetate of authentic *myo*-inositol, and a mixture of the two acetates all melted in the range 220 to 221° C.

At this time neither sorbitol nor *scyllo*-inositol has actually been demonstrated to be present in the Aesculus fluid by its isolation, although either or both may well be present. In contrast to coconut milk, where sorbitol is the predominant hexitol, the liquid of Aesculus contains *myo*-inositol predominantly. In fact, *myo*-inositol represents at least 10 % of the total dry weight, or roughly 25 % of the neutral fraction from Aesculus.

myo-Inositol was also isolated from 15 g of the neutral fraction from *Zea mays*. Following the process which was used to isolate this inositol from Aesculus, 100 mg of crude *myo*-inositol (recrystallized to give 34 mg of pure *myo*-inositol with appropriate melting & mixed melting points) were obtained. Consequently, *myo*-inositol in greater or lesser amounts is present in and is characteristic of three natural sources of the growth promoting activity in question, namely coconut (Cocos), corn (Zea) and horsechestnut (Aesculus). In the last of these sources, *myo*-inositol is present in very large amounts for a substance which is commonly thought to act catalytically or as a vitamin.

DISCUSSION

When the fully autonomous growth of cambium cells gives place to the almost completely quiescent mature phloem cells, losses of metabolic competence occur. These losses may occur at various points which may be relieved in different strains by either exogenous supplies of indoleacetic acid or of the different hexitols, which may be supplied separately or in combination. Indeed, one now can visualize that carrot cell clones with a specific requirement for a given inositol, or for sorbitol, could be established. In fact, as this work progresses, the use of selected clonal strains of cells, cultivated by the methods which have been described (21), promises to be instructive and to permit the complex needed to induce growth to be partitioned into components for the assay of which special strains will be available.

CHEMICAL COMPLEX REQUIRED FOR GROWTH INDUCTION. To unleash the totipotency which is now seen to reside in even mature phloem parenchyma cells of the carrot root, an array of chemical regulants or stimuli is required. What E. W. Sinnott once referred to as the "primitive built-in goal of growth", which resides in the fertilized egg, is evoked by the nurture which it receives while in the ovule. Whole coconut milk is a substitute for this special nurture, as seen by its successful application to embryo culture, which was first practiced by van Overbeek and Blakeslee (28, 29).

Failure of any of the living cells of the plant body to maintain indefinite growth by cell division can be ascribed to loss of the stimuli in question, to superimposition of an inhibitor in a system of sensitive, poised, chemical regulatory controls, or to simple loss, through irreversible differentiation, of their original capacity to respond to the chemical regulators.

Whereas the inherent capacity of cells to grow may be determined by their genetic constitution, its ultimate expression at any given time is certainly subject to regulation by factors of a non-genetic, or epigenetic, nature in the sense of Waddington (30). For example, both naturally-occurring (17) and exogenously-supplied (22) inhibitors may sensitively inhibit growth of carrot phloem explants that would otherwise be stimulated by coconut milk. In the first case cited, the growth inhibition which is due to the natural product hydroxy-L-proline may be alleviated by simply supplying sufficient L-proline in the growth medium. Identical examples for other growth systems could be cited.

The investigation of coconut milk as a source of these chemical regulators has now progressed to the point where it may be briefly summarized. In addition to the usual inorganic nutrients, main sources of carbon, and common vitamins, this complex of additional requirements for growth may be divided into three component parts as follows:

I. NITROGENOUS COMPONENT. This consists of reduced nitrogen compounds in the form of amino acids and their amides. The requirement for this component is customarily met by adding enzymic casein hydrolysate, individual amino acids, or even urea or ammonia to the nutrient medium. These substances supply the greater need of growing carrot tissue for organic nitrogen compounds than that which can be met from nitrate alone.

II. NEUTRAL COMPONENT. This component consists of a variety of carbohydrates, which are not required primarily as sources of carbon. They do, however, permit the cells to respond to other growth-inducing compounds. While all the substances in this category may not yet be completely characterized, *myo*-inositol, *scyllo*-inositol, and sorbitol are prominent.

III. ACTIVE COMPONENT. These substances interact with the other parts of the system, and they include representatives of the two main classes of known growth regulators for plant cells. The auxins are best known for their effect on cell enlargement, and they include the naturally-occurring substances like indoleacetic acid, or such synthetic substances as 2,4-dichlorophenoxyacetic acid. By contrast, the class of regulators which specifically stimulate cell division and which are found in that fraction of coconut milk which has been designated "active fraction" is still not completely known. Equivalent active fractions are readily obtainable from immature corn (*Zea mays*) extracts or from the fluid from young Aesculus fruits.

Hitherto, an obstacle has been that substances in

this class, even when isolated from relatively large amounts of material, have been obtained in too small amount for satisfactory purification and identification. Now, however, in the secure knowledge that the constituents of coconut milk (which comprise two of the three main components of coconut milk) are sufficiently defined to be furnished directly, a massive attack upon the constituents of the active component may be made.

The prominence given here to the hexitols as constituents of coconut milk is suggestive from the following points of view. First, inositols, free and as phosphates, have long been known to be prominent in endosperms. Second, Braun (2) found that *myo*-inositol, along with an auxin-like compound, was required for the growth in culture of tissue of crown gall origin. A different type of proliferative growth, namely the development of nodules on legumes, is also enhanced by the presence of *myo*-inositol in the culture medium. This was demonstrated in root cultures by Raggio, Raggio, and Burris (10). Finally, there has developed the empirical use of coconut milk as an extender for the fluid in which bull sperm are maintained for artificial insemination purposes (9). The unexpected effect of coconut milk in the latter case serves as a reminder that seminal fluid always appears to contain *myo*-inositol and sorbitol; the latter compound occupies a key role in the respiration of the sperm (6).

The exact role of the hexitols in the carrot tissue remains to be determined. The solution of these latter problems awaits the outcome of experiments with C^{14}-labelled hexitols which are now in progress.

Recommendations are continually being made concerning the composition of so-called synthetic media for the growth of plant tissue in culture; a particular example is the medium suggested by Reinert (11) for the growth of carrot tissue. In this example, the growth in question is the continued growth of long established, or habituated, tissue in contrast to the induction of growth in the hitherto quiescent cells of carrot phloem, which is most in question in this paper. Despite the many constituents of this supplemented basal medium of White, as suggested by Reinert (11), even the habituated or cloned carrot cultures grew better in the presence of coconut milk. However, many of the main components of coconut milk are in fact represented in the medium suggested by Reinert, as follows: The neutral component is partially represented by inositol, in Reinert's section B; the nitrogenous component by a long list of 18 amino acids, which in our work are replaced by casein hydrolysate or by certain simple forms of reduced nitrogen (13); the more biologically active component of coconut milk is partially represented by the added auxin (IAA or 2,4-D). Thus the medium recommended by Reinert contains substances which may, at least in part, play the role of the three main components of coconut milk, whose interactions have been studied in this paper, but the active fraction which specifically promotes cell division is con-

spicuously lacking, and the possibility of overlapping —or even opposed effects—between IAA and the constituents of neutral fraction (e.g. inositol) was not appreciated. Therefore, the Reinert medium, though apparently defined, is incomplete for maximum growth of carrot tissue. To the extent that the Reinert medium adds so large a number of substances (36 substances) both organic and inorganic, many of which may be dispensable and from which it is difficult to exclude all impurities, it does not define the growth requirements of the tissue as strictly as may seem to be the case.

SUMMARY

The role of liquid endosperms, particularly coconut milk, depends on a delicately balanced complex of interacting components. These include a source of reduced nitrogen which may be replaced by casein hydrolysate, a source of cell division factors supplied as a concentrate which may be prepared by adsorption from coconut milk on activated charcoal and subsequent elution, and a neutral fraction which is prepared by de-ionization of whole coconut milk. The growth data obtained by the use of the carrot tissue culture system indicate that the active fraction, which is required in concentrations of the order of a few parts per million, acts synergistically with the neutral fraction and with indoleacetic acid. Neutral fraction and IAA, acting together, usually produced less growth than that which was to be expected if their effects were additive. This is interpreted to mean that, in at least part of the growth response, neutral fraction and IAA produce overlapping or competing effects.

myo-Inositol, *scyllo*-inositol, and sorbitol have been isolated in crystalline form from coconut milk neutral fraction. They have been critically identified and shown to contribute most of the growth-promoting potential of this fraction. All the evidence assigns to *myo*-inositol the key role in this fraction, with lesser effects being due to sorbitol, to *scyllo*-inositol, and possibly to compounds in the neutral fraction which still remain to be identified. In addition, *myo*-inositol has been isolated from corn in the milk stage and from the fluid from Aesculus fruits, in which it represents 10 % of the dry weight.

Of the various components of coconut milk which promote the growth of carrot tissue explants, only the active fraction complex remains to be more completely identified.

Parks at Miami, Fla.; and the evaporation was carried out with the help of the Minute Maid Corp. at Orlando, Fla. All this help is gratefully acknowledged.

Valued assistance with the aseptic cultures used in the assay procedures was rendered first by Miss Kathryn Mears and later by Mrs. Marion O. Mapes. This help also is acknowledged.

LITERATURE CITED

1. BARRATT, R. W., D. NEWMEYER, D. D. PERKINS, & L. GARNJOBST. 1954. Map construction in *Neurospora crassa*. Adv. in Gen. 6: 1–93.
2. BRAUN, A. C. 1958. A physiological basis for autonomous growth of the crown gall tumor cells. Proc. Nat. Acad. Sci. 44: 344–9.
3. CAPLIN, S. M. & F. C. STEWARD. 1948. Effect of coconut milk on the growth of explants from carrot root. Science 108: 655–7.
4. CAPLIN, S. M. & F. C. STEWARD. 1949. A technique for the controlled growth of excised plant tissue in liquid media under aseptic conditions. Nature 163: 920–5.
5. HAAS, P. & T. G. HILL. 1922. An Introduction to the Chemistry of Plant Products. P. 389. Longmans, London.
6. KING, T. E. & T. MANN. 1959. Sorbitol metabolism in spermatazoa. Proc. Royal Soc. (London) Ser. B, 151: 226–243.
7. MÜLLER, H. 1907. Cocositol (cocosite) a constituent of the leaves of *Cocos nucifera* and *Cocos plumosa*. Proc. Chem. Soc. 23: 219.
8. MÜLLER, H. 1907. Inositol & some of its isomerides. J. Chem. Soc. 101: 2383–2410.
9. NORMAN, C., C. E. JOHNSON, I. D. PORTERFIELD, & R. S. DUNBAR, JR. 1958. Effect of pH on the life span & metabolism of bovine spermatazoa kept at room temperature. J. Dairy Sci. 41: 1803–1812.
10. RAGGIO, N., M. RAGGIO, & R. H. BURRIS. 1959. Enhancement by inositol of the nodulation of isolated bean roots. Science 129: 211–212.
11. REINERT, J. 1959. Uber die kontrolle der morphogenese und die induktion von adventivembryonen an gewebkulturen aus karrotten. Planta 53: 318–333.
12. SHANTZ, E. M. & F. C. STEWARD. 1956. The general nature of some nitrogen free growth-promoting substances from Aesculus and Cocos. Plant Physiol. suppl 30: xxxv.
13. SHANTZ, E. M. & F. C. STEWARD. 1959. Investigations on growth & metabolism of plant cells. VII. Sources of nitrogen for tissue cultures under optimal conditions for their growth. Ann. Botan. 23: 371–390.
14. SHANTZ, E. M., F. C. STEWARD, M. S. SMITH, & R. L. WAIN. 1955. Investigations of growth & metabolism of plant cells. VI. Growth of potato tuber in culture: The synergistic action of coconut milk & some synthetic growth-regulating compounds. Ann. Botan. 19: 49–58.
15. STEWARD, F. C. 1958. Growth & organized development of cultured cells. III. Interpretations of the growth from free cells to carrot plants. Am. J. Botan. 45: 709–713.
16. STEWARD, F. C. & S. M. CAPLIN. 1951. A tissue culture from potato tubers: the synergistic action of 2,4-D & coconut milk. Science 113: 518–20.
17. STEWARD, F. C. & S. M. CAPLIN. 1952. Investigations of growth & metabolism of plant cells. III. Evidence for growth inhibition in certain mature tissues. Ann. Botan. 16: 477–489.
18. STEWARD, F. C. & S. M. CAPLIN. 1952. Investigations on growth & metabolism of plant cells. IV. Evidence on the role of the coconut milk factor in development. Ann. Botan. 16: 491–504.
19. STEWARD, F. C., S. M. CAPLIN, & F. K. MILLAR. 1952. Investigations on growth & metabolism of plant cells. I. New techniques for the investigation of metabolism, nutrition, & growth in undifferentiated cells. Ann. Botan. 16: 58–77.
20. STEWARD, F. C., M. O. MAPES, & KATHRYN MEARS. 1958. Growth & development of cultured cells. II. Organization in cultures grown from freely suspended cells. Am. J. Botan. 45: 705–713.
21. STEWARD, F. C., M. O. MAPES, & JOAN SMITH. 1958. Growth & organized development of cultured cells. I. Growth & division of freely suspended cells. Am. J. Botan. 45: 693–703.
22. STEWARD, F. C., J. K. POLLARD, A. A. PATCHETT, & B. WITKOP. 1958. The effects of selected nitrogen compounds on the growth of plant tissue cultures. Biochim. Biophys. Acta 28: 309–317.
23. STEWARD, F. C. & E. M. SHANTZ. 1954. The growth of carrot tissue explants & its relation to the growth factors in coconut milk. II. The growth-promoting properties of coconut milk for plant tissue cultures. Annee Biol. 30: 399–410.
24. STEWARD, F. C. & E. M. SHANTZ. 1956. The chemical induction of growth in plant tissue cultures. In: The Chemistry & Mode of Action of Plant Growth Substances. R. L. Wain, & F. Wightman, eds. 165–186. Butterworth Scientific Publications, Ltd., London.
25. STEWARD, F. C. & E. M. SHANTZ. 1959. The chemical regulation of growth (Some substances & extracts which induce growth & morphogenesis). Ann. Rev. Plant Physiol. 10: 379–404.
26. STEWARD, F. C., E. M. SHANTZ, KATHRYN MEARS, & JOAN SMITH. 1956. The induction of growth in potato tissue: Substances which act synergistically with coconut milk. Plant Physiol. suppl. 31: xxxviii.
27. STEWARD, F. C., J. F. THOMPSON, & J. K. POLLARD. 1958. Contrasts in the nitrogenous composition of rapidly growing & non-growing plant tissues. J. Exp. Botan. 9: 1–10.
28. VAN OVERBEEK, J. 1942. Hormonal control of embryo & seedling. Cold Spring Harbor Symposia Quant. Biol. 10: 126–134.
29. VAN OVERBEEK, J., M. E. CONKLIN, & A. F. BLAKESLEE. 1941. Factors in coconut milk essential for growth & development of very young Datura embryos. Science 94: 350–1.
30. WADDINGTON, C. M. 1957. The Strategy of the Genes: A Discussion of Some Aspects of Theoretical Biology, MacMillan, New York.