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THE DISTRIBUTION OF ACETYLCHOLINE IN THE MALAYAN JACK-FRUIT PLANT, *ARTOCARPUS INTEGRA*

BY

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The distribution of acetylcholine in the seeds and leaves of the Malayan Jack-fruit plant, *Artocarpus integra*, has been studied with the view to obtaining evidence for the site of its formation. The terminal growing leaves on the side branches had a very high concentration of acetylcholine (770 μg./g.), while the acetylcholine content of the other leaves on the same branch progressively decreased with age. The total amount of acetylcholine stored in the terminal growing leaves was only 42 μg., but in the second leaves which had grown nearly to their full size it was 540 μg. From the third leaves, the amount of acetylcholine stored gradually decreased. The midribs and the secondary veins of the leaves when combined had a higher concentration of acetylcholine than had the blades. The acetylcholine concentration of the pith of the stem was 4.2 times higher than that of the cortex-phloem layer while that of the xylem layer was the lowest; in the root the pith had a value only one-seventh of the cortex. The younger part of the pith and the cortex-phloem layers of the stem contained more acetylcholine than the older parts. These findings support the view that the acetylcholine is synthesized in the growing leaves. An unusual lenticel-like structure in the cortex layer of the root contained more acetylcholine than the surrounding tissue.

In a previous paper (Lin, 1955) it was reported that considerable amounts of acetylcholine (ACh) are present in the seeds and leaves of the Malayan Jack-fruit, *Artocarpus integra*, Merr. It was suggested that it would be of interest to find out in which part of the plant the ACh is formed. For locating the site of formation of other pharmacologically active substances in plants, a method of interspecific grafts involving members of different genera which have a different capacity for alkaloid production has been employed with some success by James (1953). For instance, James and Thewlis (1952) by studying grafts prepared from belladonna and *Datura innoxia* concluded that the root was the site of synthesis of the alkaloids hyoscine and hyoscyamine in these plants, and Evans and Partridge (1953) from a study of the distribution of alkaloids in grafts involving *Datura tatula* and *D. feroxia* found that the aerial parts of these plants were capable of synthesizing hysocyamine and meteloidine respectively. A similar method of approach could probably be employed for locating the site of formation of ACh in the Jack-fruit plant. However, since the concentration of ACh in the tissues is so high and its assay is possible even with as small an amount of material as 10 to 20 mg. of the pith of a stem or of the external cortex of the root, it was decided to measure the ACh concentration of representative portions of the whole plant.

METHODS

Only fresh tissues which had just been taken from the living Jack-fruit tree were used. The process of extraction of ACh from the various tissues of the plant and the procedure adopted for its identification were similar to those previously described (Lin, 1955). For the routine assay of ACh the isolated toad rectus abdominis muscle preparation was used. The values of ACh given refer to acetylcholine chloride.

RESULTS

Acetylcholine in the Leaves

Variation of ACh Concentration with Age.—The exact age of a leaf in a tree is not easy to determine unless its growth has been followed from the beginning. The relative age of the leaves grown on a side branch of a tree, however, is known to follow the order of the position of growth along the stem ; a lower leaf is older than the one above it and the top leaf is the youngest leaf of all those on the same branch. On this assumption the ACh concentration of each of a number of leaves was studied in relation to their age. For each experiment, a young side-branch of a Jack-fruit tree having 10 or 11 leaves was chosen. Immediately after the branch was cut down from the tree and brought to the laboratory, the leaves were removed from the stem one at a time, starting from the youngest leaf on the top to the lowest one at the bottom of the branch in the order of their position. Each leaf was at once cut into small pieces and ground in a mortar with some sand and with frequent additions of HCl-acidified water until a thorough extraction of the ACh had been obtained. The extract was centrifuged and the supernatant fluid assayed for ACh.

Fig. 1 shows the average results of three such experiments. Leaf No. 1 was the terminal growing leaf, which was still surrounded by the stipules, whilst the others, except the second leaf, were older and fully grown leaves, the age of which increased with their numbers in the table. In all experiments, the terminal growing leaves had the highest concentration of ACh and that of the other leaves decreased with their age. Fig. 1 also shows the total amount of ACh contained in each leaf. It is seen that the average amount stored in the second leaves is about 13 times more than that in the terminal growing leaves. This high value for ACh content is, however, confined to the second leaves, for that in all older leaves decreases.

ACh Concentration in the Terminal Growing Leaves.—A number of the terminal growing leaves were taken, each from a separate twig. After weighing, each leaf was extracted as in the previous experiments. The weights of these leaves are taken to represent the relative age of their growth, the heavier leaves being older. The results obtained showed a very high concentration of ACh in these terminal growing leaves, varying from 500 to 1,200 μg./g., with an average value of 770 μg./g. from 14 leaves. No correlation, however, was found to exist between the ACh concentration of each leaf and its weight.

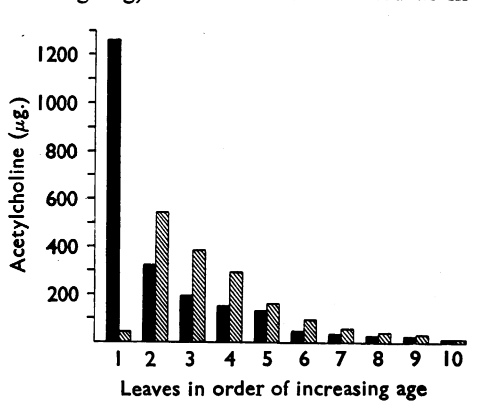

FIG. 1.—The variation of the ACh concentration in μg./g. (black) and of the total amount (hatched) of ACh in μg./leaf in each of 10 consecutive leaves on a stem of *A. integra*. The values plotted are the means of 3 experiments. The relative age of the leaves is represented by the number under each block.

ACh Concentration in the Veins and the Blade.—For each experiment two leaves, a relatively young one adjacent to the terminal growing leaf and an older one situated at the lower end of the same branch, were used. The midrib and the secondary veins of each leaf were separated from the blade and then combined to form one sample called the " veins." The veins and the blade were cut into small pieces and the ACh was extracted and assayed. The results of 5 experiments are given in the upper section of Table I. In order to see if the distribution of acetylcholine among the veins and the blade of the leaves is affected by light, a series of similar experiments was carried out with leaves collected in darkness at a different season of the year, together with some leaves collected in bright daylight during the same season. The results obtained are given in the middle and lower sections of Table I. The veins had a higher ACh concentration than the blade in every leaf, and the ACh concentration in the veins and the blade of the old leaves were much lower than that in the young leaves. The ratio of ACh concentration in the veins to that in the blades of

TABLE I

ACETYLCHOLINE CONCENTRATION IN THE VEINS AND BLADES OF LEAVES OF *A. INTEGRA* (μg./g.)

Season and Period of Day of Collection	Young Leaves			Old Leaves		
	Veins	Blades	Veins/Blades	Veins	Blades	Veins/Blades
Leaves collected in daylight from Nov. 30, 1954, to Jan. 13, 1955	300	140	2·2	120	29	4·1
	450	150	3·0	57	20	2·9
	500	470	1·1	30	8	3·8
	500	340	1·5	25	7	3·6
	780	680	1·2	50	19	2·7
Average			1·8			3·4
Leaves collected in daylight from April 15 to 24, 1956	160	60	2·7	15	5	3·0
	220	120	1·8	23	7	3·3
	130	70	1·9	17	4	4·2
	210	100	2·1	14	5	2·8
Average			2·1			3·3
Leaves collected in darkness from April 5 to 24, 1956	220	55	4·0	16	3	5·3
	230	85	2·7	5	1	5·0
	150	75	2·0	7	2	3·5
	140	40	3·5	9	3	3·0
	360	220	1·6	9	2	4·5
Average			2·8			4·2

the young leaves was lower than that of the old leaves. Neither light nor the process of photosynthesis had any great effect on the distribution of ACh in the leaves.

Acetylcholine in the Stem

ACh Concentration of Various Segments of a Stem.—A young side branch with 10 to 12 leaves was taken, the leaves were removed, and the stem, which had a diameter of about 8 mm., was divided into 6 to 8 segments. Each segment, after its weight had been taken and its length measured, was extracted for ACh and assayed. The results of 3 such experiments are depicted in Fig. 2, which

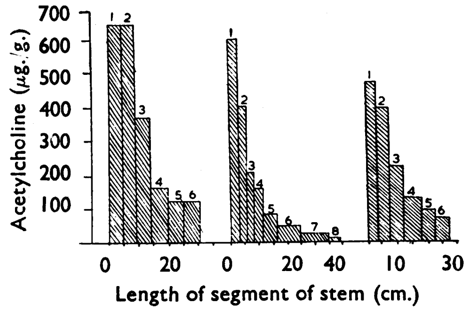

FIG. 2.—The ACh concentration of consecutive segments of three stems of *A. integra*. The relative age is represented by the numeral on each block; the lowest numeral represents the youngest leaf. The width of each block is proportional to the length of each segment tested (cm.).

shows that the uppermost or youngest segment of the stem always had the highest ACh concentration and that the concentration in the other segments decreased with age.

Distribution of ACh in the Different Layers of Tissue of the Stem.—A transverse section of the young stem shows the cork, outer cortex, inner cortex, phloem, xylem and the pith which forms a conspicuous central cylinder. Microscopic examination showed that the pith cells contained an abundance of latex. In studying the distribution of ACh among these tissues, it was found practical to separate them into three layers: an outer or cortex-phloem layer which consisted of the outer and inner cortices without the cork but with part of the phloem which came off with the inner cortex, a middle layer which consisted mainly of the xylem, and the pith. A segment of young stem 5 to 8 mm. in diameter and about 30 mm. in length was used. It was first split into two halves from which the cortex-phloem layer was peeled off. The pith in the centre of each half was dissected out so as not to include the middle layer. In order to eliminate the possibility that some pith cells might still be attached to the inner side of the xylem and that part of the phloem tissue might have remained on the outer surface of the xylem, both the inner and the outer surfaces of the xylem layer were scraped until smooth. These three layers of tissue were then extracted separately for ACh. The results obtained from the examination of 8 different samples of Jack-fruit stem are given in Table II. The first 5 samples were taken from 5 different side branches

TABLE II

ACETYLCHOLINE CONCENTRATION OF DIFFERENT LAYERS OF TISSUE OF THE STEM OF *A. INTEGRA* (µg./g.)

Cortex-Phloem	Xylem	Pith	Pith/ Cortex-Phloem
25	8	64	2·6
85	120	600	7·1
53	16	130	2·4
65	24	330	5·1
160	70	320	2·9
130	36	410	3·2
50	12	360	7·2
35	8	130	3·3
Average 75	37	290	4·2

while the last 3 samples were from one branch. It shows that in every sample of the stem examined the pith has the highest and the xylem the lowest ACh concentration.

ACh Concentration of the Pith of Different Parts of Stem.—A young side branch of about 22 cm. in length was taken, and, after all the leaves on the stem had been removed, the stem was divided into 11 segments. Six alternate segments were used, while the others were discarded. The pith of each of these segments was dissected out and assayed for ACh. Table II gives the results, which

TABLE III

ACETYLCHOLINE CONCENTRATION IN THE PITH AND THE CORTEX-PHLOEM LAYER IN DIFFERENT PARTS OF THE STEM OF *A. INTEGRA*. THE VALUES (µg./g.) ARE THE MEANS OF THREE EXPERIMENTS AND WERE OBTAINED FROM DIFFERENT STEMS

Order of Segment by Age	Pith	Cortex-Phloem
1	130	190
2	110	130
3	78	70
4	75	42
5	57	41
6	45	32
7	—	31

show that the pith of the younger part of the stem has a higher ACh concentration than the older part.

ACh Concentration of the Cortex-Phloem Layer of Different Parts of Stem.—A young side branch of about 40 cm. in length and 8 mm.

in diameter was taken, and, after removing the leaves, the stem was divided into a number of segments. The two end segments and five other alternate segments in the intermediate region were used. The cortex-phloem layer of each segment was peeled off and then assayed for ACh. The results are given in Table III. The ACh concentration of the cortex-phloem layer of the stem also decreased with the age of the stem.

Acetylcholine in the Root

Presence of ACh in the Rootlets.—Nine different samples of rootlets varying from 1 to 2 mm. in diameter were taken from the side roots of a Jack-fruit tree on different dates. Each sample, after it had been cleaned with water and dried in air for a few minutes, was extracted for ACh. A mean value of ACh of 89 μg./g. was obtained (range, 65 to 120 μg./g.).

Distribution of ACh in the Tissues of the Root.—The external cork layer of the root dries up quickly and tends to break away from the outer cortex soon after collection. The cork and the outer cortex are thin, papery and crimson in colour and can be separated from the underlying tissues. There are a number of yellow spots or lenticel-like structures, each of about 1 to 3 mm. in diameter over the surface of the outer cortex as shown in Fig. 3. The number of yellow spots/unit area of the root surface varies from one part of the root to another, but as many as 7 spots have been counted within 1 sq. cm. on one root. They were seen to grow in the inner cortex layer. Such yellow spots are not normally seen in the roots of other plants. A transverse section of a spot showing a great number of loose "packing" cells alternating with bands of firm "closing" cells is shown in Fig. 4. These structures resemble

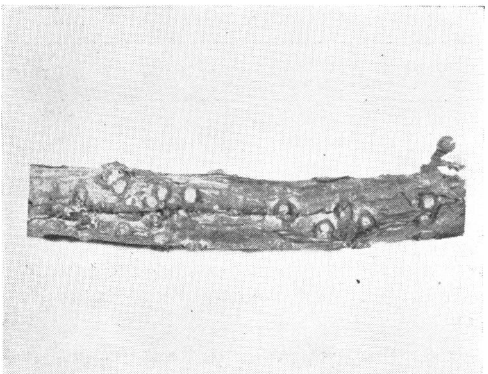

FIG. 3.—External appearance of the root of *A. integra* showing the yellow spots. About actual size.

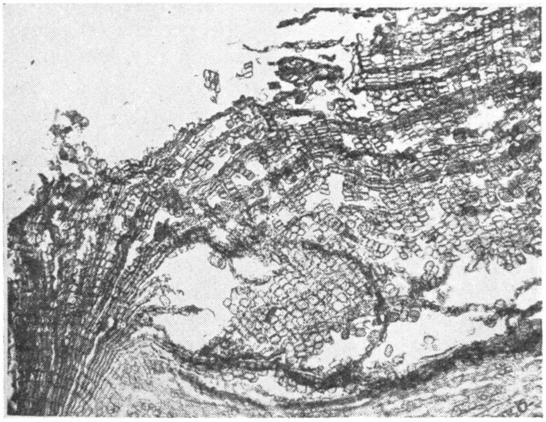

FIG. 4.—Transverse section of a yellow spot in the root of *A. integra*. Magnification, ×160.

those of the lenticels present in the stems of some plants. The phloem layer in the root is much thicker than it is in the stem and, hence, it can be separated from the inner cortex layer quite easily. The pith in the root is less extensive than in the stem.

The root-cork contained no ACh, or traces only. The other layers of the root tissue contained an appreciable amount of ACh, as given in Table IV.

TABLE IV

ACETYLCHOLINE CONCENTRATION IN DIFFERENT LAYERS OF TISSUE OF THE ROOT OF *A. INTEGRA* (μg./g.)

	Outer Cortex	Inner Cortex	Phloem	Xylem	Pith	Pith/ Inner Cortex
	70	400	85	30	52	0·13
	120	250	45	36	38	0·15
	140	190	28	25	26	0·14
Average	110	280	53	31	39	0·14

The distribution of ACh in the root differed, however, from that in the stem. The ACh concentration in the pith of the stem (Table II) was 4.2 times greater than that in the cortex-phloem layer, whereas it was only one-seventh of that in the inner cortex layer of the root.

ACh in the Yellow Spots.—A short section of a side root about 10 mm. in diameter was taken and used immediately. The cork layer was removed and discarded and both the outer and inner cortex layers were separated and removed from the underlying tissues. Each layer was then divided into 2 portions, one containing all the yellow spots and the other containing the surrounding tissues. These four different portions of tissue were assayed for ACh. The results obtained are given

TABLE V

ACETYLCHOLINE CONCENTRATION IN DIFFERENT PARTS OF THE CORTEX OF THE ROOT OF *A. INTEGRA*. YS=THE YELLOW SPOTS (μg./g.)

	Outer Cortex			Inner Cortex		
	Without YS (I)	With YS (II)	$\frac{II}{I}$	Without YS (I)	With YS (II)	$\frac{II}{I}$
	60	220	3·7	50	460	9·2
	60	170	2·8	26	73	2·8
	15	35	2·3	20	110	5·5
	54	70	1·3	11	15	1·4
	60	240	4·0	70	600	8·6
Average	50	150	2·8	35	250	5·5

in Table V. It shows that, of both the outer and inner cortex layers of the root, the yellow spots have a much higher ACh concentration than the surrounding tissues.

DISCUSSION

The origin and function of substances in plants which have highly specific actions in animals are both a puzzle and a challenge. A study of the distribution of ACh in different tissues of *A. integra* throws some light on the site of formation of at least one pharmacologically important drug. The finding that young leaves and stems contain a much higher concentration of ACh than the corresponding older tissues suggests that ACh synthesis is confined to the period of growth.

The veins of the leaves have a higher concentration of ACh than the blade, and the ratio of the concentration in the veins to that in the blade is lower in the younger leaves. This might indicate that ACh is formed in the blades of the growing leaves and is then transported by the veins to the stem and roots.

In the stem, the pith has a higher concentration of ACh than the other layers of tissue ; it probably acts as a storage tissue. In the roots, on the other hand, the concentration of ACh is low in the pith and high in the inner cortex. The cortex contains some unusual yellow spots, in which ACh is concentrated. It is therefore possible that these yellow spots either synthesize ACh or absorb it from bacteria in the soil. On this hypothesis, ACh would be supplied by the roots to the actively growing leaves and stem by a diffusion process. In this case, however, it might be expected that the concentration gradient in the stem would be the opposite of that found, and the ACh concentration of the younger and more peripheral part of the stem would be lower than that of the older parts. This argument, together with the fact that there is no definite evidence for the synthesis of ACh by bacteria in the soil, or by the cells of the yellow spots, suggests that these spots are best regarded, like the pith in the stem, as storage structures. The growing leaves, then, are the most probable site of synthesis of ACh, but the question of how it is formed there still remains to be answered.

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