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

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In higher animals acetylcholine (ACh) is associated with the transmission of nerve impulses. It has also been found in the free-living protozoon *Paramoecium* (Bayer and Wense, 1936), in the bacterium *Lactobacillus plantarum* (Stephenson and Rowatt, 1947), in the parasite *Trypanosoma rhodesiense* (Bülbring, Lourie, and Pardoe, 1949), and in the gill plates of the mussel *Mytilus edulis* (Bülbring, Burn, and Shelley, 1953). In these lower organisms there is no transmission of nerve impulses, but, as ACh is associated with such motor activity as ciliary movement, Bülbring *et al.* (1953) suggested that ACh might act in these organisms as a local hormone. In plants ACh has so far been found only in ergot, *Claviceps purpurea* (Ewins, 1914), and in the nettles *Urtica urens* and *Urtica diocia* (Emmelin and Feldberg, 1947, 1949). The present communication reports another rich plant source of ACh—the seed and leaf of the Malayan Jack-fruit, *Artocarpus integra.*

METHODS

The Jack-fruit and its Seed.—The Jack-fruit is a compound fruit borne by a tree, *Artocarpus integra* (Fig. 1), belonging to a genus of some forty trees of the family *Urticaceae* found in South-east Asia. In Malay it is called "Nangka." According to Burkill (1935), it was originally found in India and then brought to Malaysia. The Jack-fruit tree is perennial and may bear fruits at any time of the year. The fruit, generally oblong, usually grows to an enormous size (Fig. 2A) and weighs about 40 lb. The external surface is like a beehive and is greenish-yellow. One Jack-fruit may contain forty to sixty small, fleshy, yellow units of edible fruit (Fig. 2B) with a mawkishly sweet and "mousy" taste, each of which has a prominent seed (Fig. 2C) weighing 5–10 g. The seeds are usually discarded, but after being boiled with salt they can be eaten as a vegetable. The seeds have two covering layers. The thicker external layer is brown when fresh but grey when dry; it is attached loosely to the inner layer, which is fawn-coloured and attached rather closely to the seed tissue, from which it can be removed only by scraping with a knife. The flesh of the fruit is claimed to be laxative, irritating, and indigestible when it is unripe. Nursing mothers are not supposed to eat it in Malaya, as it is stated to lead to secretion of a deleterious substance in the milk.

The Jack-fruit seeds used in the present experiments were from ripe fruits, except when specific correlations were attempted between the ACh content of the seed and the ripeness of the fruits. The seeds were separated from the flesh, washed with tap water, and used fresh

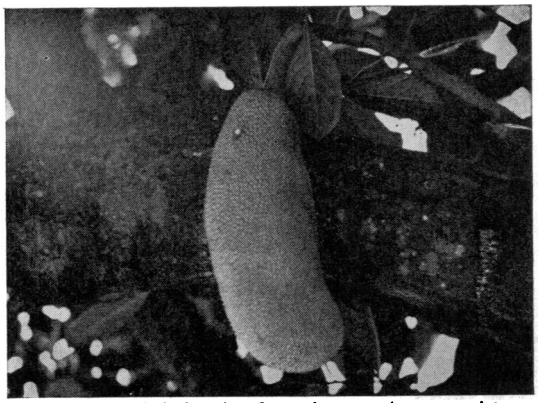

FIG. 1.—A Jack-fruit hanging from the tree *Artocarpus integra.*

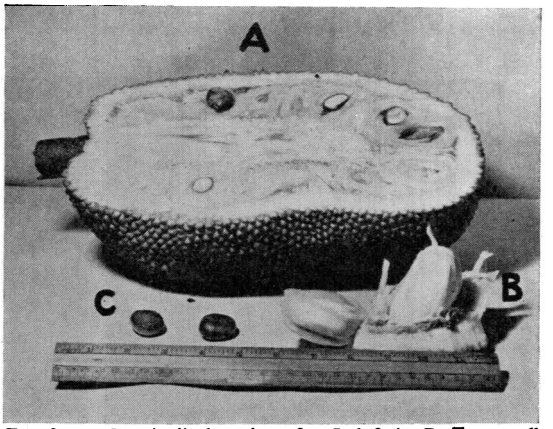

FIG. 2.—A, Longitudinal section of a Jack-fruit. B, Two small units of the fruit. C, Two seeds of the fruit.

or after storing in a refrigerator. In some experiments fresh leaves and newly cut stems of the fruit were also examined.

Preparation of the Seed Extract.—The two covering layers were removed. The seeds were immediately weighed, crushed in a mortar, and then macerated with acidified distilled water (1 ml. of N/3 HCl to 9 ml. of distilled water for each g. of seed), until the entire seed tissue was finely divided into granules. The supernatant fluid of the extract was centrifuged off and transferred into a graduated cylinder to which more acidified distilled water was added until the volume corresponded to 10 ml. for every g. of the peeled seed. The *p*H of this extract was about 3. For most of the pharmacological tests such a concentration of the extract was too high ; it was reduced to 1 to 2 mg./ml. by diluting it before use with Ringer, Locke or Tyrode solution. This also brought the *p*H of the extract to a level suitable for testing on the heart and other isolated tissues.

Pharmacological Tests.—The extracts were tested on the following conventional preparations : (1) on the guinea-pig ileum suspended in a bath of 8 ml. filled with Tyrode solution ; (2) on the virgin rat uterus suspended in a bath of 8 ml. filled with Locke solution containing only one-quarter of the normal amount of calcium chloride ; in both preparations the physiological solutions were aerated with a 95% O_2 and 5% CO_2 mixture and maintained at 37.5° C. ; (3) on the isolated toad heart perfused with Ringer solution through a Symes cannula ; (4) on the isolated toad rectus abdominis muscle suspended in a bath of 6 ml. containing eserinized Ringer solution at room temperature (about 30° C.) and aerated with air ; and (5) on the arterial blood pressure of cats anaesthetized with chloralose.

Estimation of Cholinesterase Activity and Chemical Precipitation of ACh.—Estimations of cholinesterase activity were made manometrically (Ammon, 1933 ; Augustinsson, 1948), or by incubating the seed extract at 37.5° C. in a bicarbonate Ringer solution containing ACh with and without eserine, and assaying the remaining ACh at the end of incubation on the toad rectus abdominis.

Chemical precipitation of the ACh from the seed extract was carried out according to the methods used by Dudley (1933), modified by Bülbring *et al.* (1953), to the step when the excess of barium was removed by adding sodium sulphate. The solution was then concentrated *in vacuo* and adjusted to *p*H 4 with HCl before it was assayed on the toad rectus for ACh. The standard acetylcholine chloride was freshly prepared each time from a stock solution (Hoffmann-La Roche glass ampoules).

Results

Pharmacological Actions of the Jack-fruit Seed

Isolated Guinea-pig Ileum.—Fig. 3A shows that the seeds contained a substance strongly stimulating the smooth muscle. The contraction caused by 0.25 mg. of the seed was greater than that produced by 0.1 μg. ACh. This stimulating action was lost when the seed extract was treated with NaOH (Fig. 3A, d and e). In an acid medium the stimulating substance was, however, very stable. When tested on an atropinized preparation, the stimulating action of the seed disappeared, even when the seed extract was added in a large dose (Fig. 3B, h).

Isolated Toad Rectus Abdominis.—Fig. 4A shows that 1 mg. of the seed caused a contraction of the muscle (at b) slightly bigger than that due to 0.5 μg. ACh (at a). The stimulating action of the seed was lost after the seed extract had been treated with NaOH. Further, (+)-tubocurarine added to the perfusion fluid rendered the muscle insensitive to ACh as well as to the seed extract (Fig. 4B). In concentrations of 1 and 5 μg./ml. of Ringer solution tubocurarine caused an identical degree of inhibition to the stimulating actions of 0.5 μg. ACh and 1 mg. of Jack-fruit seed.

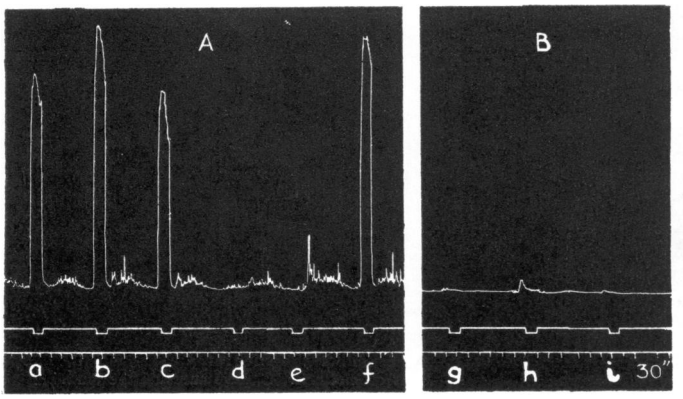

FIG. 3.—Contractions of guinea-pig ileum on the addition of the following substances to the bath: a, c, and f, 0.1 μg., and g, 2 μg. ACh; b, 0.25 mg., and h, 10 mg. of Jack-fruit seed; d, 0.25 mg., e, 20 mg., and i, 100 mg. of Jack-fruit seed treated with NaOH. Between A and B atropine sulphate 1.5×10^{-6} added to the bath.

Extracts of the flesh of the fruit caused no contraction of the rectus muscle.

The stimulating substance in the Jack-fruit seeds and in the leaves appears to be present in a freely soluble form, for when a thin slice of the seed or a small shred of the leaf was dropped into the toad's rectus muscle bath the muscle began to contract in about 5–10 sec.

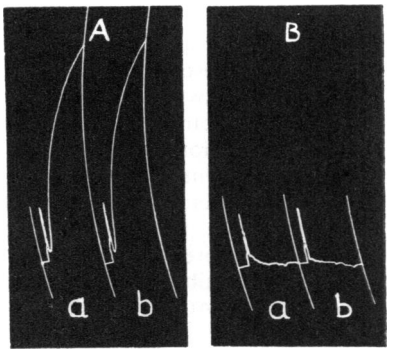

FIG. 4.—Contractions of toad rectus abdominis produced by 0.5 μg. ACh at a and by 1 mg. of Jack-fruit seed at b. Between A and B, 5 μg./ml. (+)-tubocurarine added to the bath.

Isolated Toad Heart.—Fig. 5 shows that on the isolated toad heart 0.4 mg. of the seed had a strong inhibitory effect which was abolished by atropine (Fig. 5A and B). When the seed extract was boiled with NaOH and then neutralized, it no longer exerted a cardio-inhibitory effect (Fig. 6B). On the contrary, it then had a stimulating action (Fig. 6B). This effect was also demonstrable with the untreated seed extract when tested on the atropinized heart. Extract corresponding to 2 mg. of the seed, which was five times bigger than that which had caused cardiac arrest before atropine (Fig. 5A), when injected into the Symes cannula after atropine caused strong, long-lasting stimulation of the heart (Fig. 5B). The Jack-fruit seed thus contains, in addition to the ACh-like substance, another pharmacologically active substance which has a stimulating action on the toad heart; its effect is normally masked by that of the ACh-like substance. The stimulating substance is also partly destroyed by the brief boiling in alkaline medium, because 50 mg. of the boiled seed was required to produce a stimulating effect (Fig. 6B), whereas 2 mg. of untreated seed produced a stronger response on the atropinized preparation (Fig. 5B). Repeated attempts to demonstrate the stimulating action of the Jack-fruit seed on the isolated perfused rabbit heart failed.

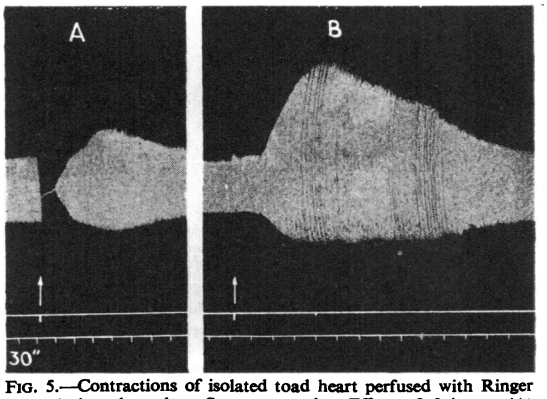

FIG. 5.—Contractions of isolated toad heart perfused with Ringer solution through a Symes cannula. Effect of 0.4 mg. (A) and of 2 mg. (B) of Jack-fruit seed. Between A and B 2 μg./ml. atropine sulphate added to the solution.

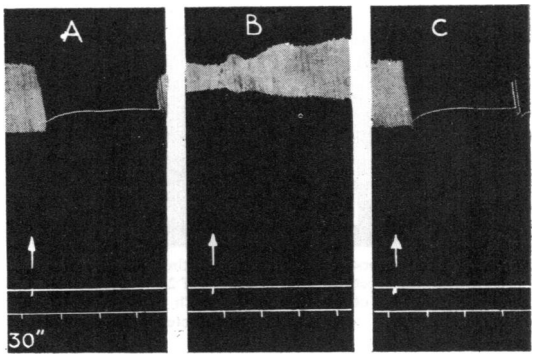

FIG. 6.—Contractions of isolated toad heart perfused with Ringer solution through a Symes cannula. A shows effect of 0.4 mg. Jack-fruit seed, B of 50 mg. NaOH-treated Jack-fruit seed, and C of 0.3 μg. ACh.

Cat's Arterial Blood Pressure.—Fig. 7A shows that the intravenous injection of extract corresponding to 0.5 mg. of the Jack-fruit seed caused a fall in the arterial blood pressure similar to that produced by 0.2 μg. ACh. This depressor effect was increased about fivefold after eserine (0.25 mg. intravenously) and abolished by atropine (Fig. 7B). An extract of the Jack-fruit seed treated with alkali no longer lowered the blood pressure, even when 50 mg. was injected. There was no indication of a stimulating action on the cat heart, either after atropine or when large doses of alkali-treated extract were injected.

Isolated Virgin Rat Uterus.—Fig. 8 shows that extract corresponding to 1.6 mg. of the Jack-fruit seed (b) caused contraction of the uterus almost exactly equal to that caused by 0.4 μg. ACh (a). As expected, 10 μg. of histamine caused slight relaxation of the rat uterus (Fig. 8c), whereas a thin slice of unextracted Jack-fruit seed added to the

bath produced strong contraction (Fig. 8d). These results, together with the observation made on the atropinized guinea-pig ileum, indicate that no histamine is present in the Jack-fruit seed.

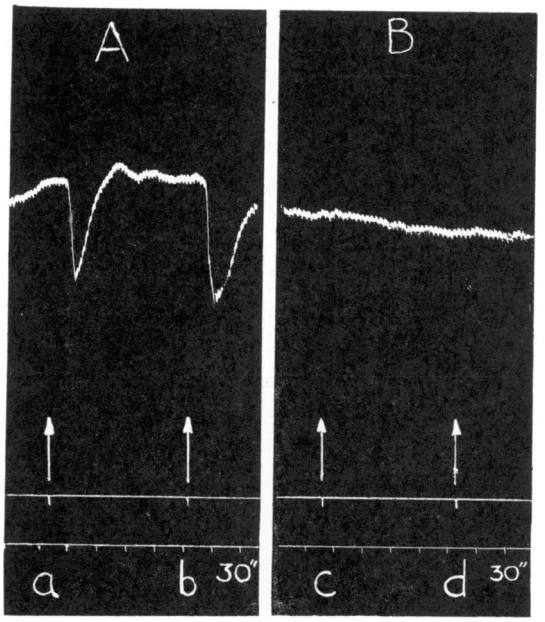

FIG. 7.—Arterial blood pressure of cat anaesthetized with chloralose. Intravenous injections of 0.5 mg. and of 2 mg. of Jack-fruit seed at a and d, and of 0.2 μg. and 1 μg. ACh at b and c. Between A and B, 2 mg. atropine sulphate injected intravenously.

Assay of Jack-fruit Seed Extracts by Different Methods.—It is generally accepted that, if the unknown substance in an extract gives the same value when assayed against the same standard solution by different biological preparations, it can be taken as evidence that the unknown substance is the same as the standard. Three separate extracts of Jack-fruit seeds were therefore prepared and tested against a standard ACh solution on various preparations. The values obtained are shown in Table I, and they can be said to be identical for each method within the limit of experimental error. This strongly supports the claim that the depressor and muscle stimulating substance in the Jack-fruit seed is ACh. The activity will therefore be referred to as being due to ACh.

TABLE I

ACETYLCHOLINE CONTENT OF JACK-FRUIT SEED ASSAYED BY DIFFERENT METHODS

Extract No.	Preparation	ACh Content in μg./g.
1	Cat B.P.	350
	Toad rectus	310
	„ heart	350
2	Toad rectus	430
	Guinea-pig ileum ..	440
3	Toad rectus	500
	Guinea-pig ileum ..	550

Precipitation of ACh from Jack-fruit Seed Extract

Eight Jack-fruit seeds with a total weight of 48.2 g. were crushed in a glass mortar into small pieces and macerated with acidified water until they had been converted to a pulp-like mixture. The extract was then squeezed through cheesecloth, to remove the insoluble material, and the filtrate was centrifuged twice to remove sediments. One ml. of the supernatant fluid was diluted with acidified Ringer's solution (0.4 ml. N/3 HCl to 100 ml. of Ringer's solution) and, when assayed for ACh on a toad rectus abdominis muscle, it gave a value of 720 μg./g. From this value the total amount of ACh present in the seed extract was computed. The results of the two experiments in which the yields of ACh precipitated from the seed extract together with that precipitated from a standard ACh solution are presented in Table II. It shows that an appreciable amount of ACh could be recovered by precipitation from the Jack-fruit seed extract. The yield values of 38.3% and 27.9% appear to be rather low, but the substance precipitated was undoubtedly ACh, as shown by its pharmacological actions and alkaline instability.

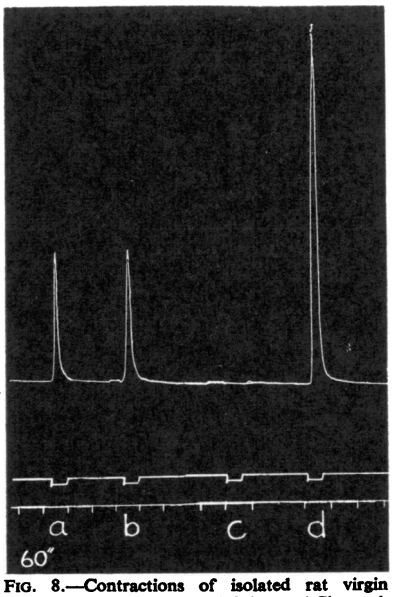

FIG. 8.—Contractions of isolated rat virgin uterus. At a effect of 0.4 μg. ACh, at b of 1.6 mg. Jack-fruit seed, at c of 10 μg. histamine, and at d of a thin slice of Jack-fruit seed dropped into the bath.

TABLE II

RESULTS OF CHEMICAL PRECIPITATION OF ACETYLCHOLINE FROM JACK-FRUIT SEEDS

Expt.	Amount of Jack-fruit Seed Used	ACh Content μg./g. By Assay	Total ACh	ACh Recovered By Assay	Yield
1	—	—	Std. ACh 5·0 mg.	2·375 mg.	47·5%
2	6·52 g. (one seed)	450	2·95 mg.	1·120 mg.	38·3%
3	48·20 g. (six seeds)	720	30·70 mg.	8·58 mg.	27·9%

ACh Content of the Jack-fruit Seed, Leaf, and Stem

The amounts of the active substance present in the seed and other tissues of the Jack-fruit tree were assayed against a standard ACh chloride solution on the isolated toad rectus abdominis. Forty-one estimations were made on the seeds of ripe and unripe fruit collected during a period of nine months, and the results obtained gave ACh content values varying from 300 to 1,000 μg./g. of fresh seed, with an average value of 564 μg./g.

A comparison was made of the extraction of ACh from seeds by acidified distilled water, 5% trichloracetic acid and 95% ethyl alcohol, but a higher value of ACh was always obtained from the seeds with acidified distilled water than with the other two solvents. There was no correlation between the ACh content and the size of the Jack-fruit seed or the ripeness of the fruits. The ACh content of the seeds was not affected by storage for a period of one month or more in the refrigerator. Drying of the seeds, however, resulted in a rapid fall of the ACh content. For example, the average ACh content of three seeds from the same fruit was 600 μg./g. when fresh and 100 μg./g. after being dried in a container surrounded by hot water for 72 hr.

The leaves of the Jack-fruit tree had an average water content of 60%, and five separate determinations gave an average ACh value of 60 μg./g. (1.6–120 μg./g.). On two occasions the midribs of several Jack-fruit leaves combined were extracted and found to have an ACh content of 250 and 350 μg./g. respectively. A short length of woody stem bearing the leaves was found to contain 8 μg./g. of ACh.

The seeds of another fruit, borne by a tree closely related to the Jack-fruit tree called *Artocarpus champeden* (or "Chempedak" in Malay), contained an average amount of only 3.0 μg./g. of ACh in eight different estimations.

Absence of Cholinesterase in the Jack-fruit Seed

In six experiments Jack-fruit seed extract was incubated with ACh as follows: 2 ml. of a concentrated seed extract (250 mg./ml.) together with 1 ml. of a 0.4% ACh solution plus 1 ml. of bicarbonate Ringer solution were incubated in a small conical flask on a water bath at 37.5° C. and shaken at a rate of 75/min. Both the seed extract and the ACh solution were prepared in a bicarbonate Ringer solution so that the *p*H of the medium was maintained at 7.4. Usually, two sets of such duplicated samples were set up—one with 0.1 ml. of a 0.5% solution of eserine salicylate and the other without eserine. In addition, one set of duplicated samples was set up, in which the enzyme preparation had been boiled. At the end of 1 hr. incubation 2 ml. of N/3 HCl was added to each sample, which was then boiled in order to stop any enzyme action. The amount of ACh remaining in each sample was assayed on the toad rectus abdominis preparation. Out of six experiments carried out in this manner, two indicated some enzymic hydrolysis of the added ACh, but the other four showed no sign of cholinesterase activity. Similar experiments carried out with a rat brain extract as the source of the enzyme, however, always gave positive results.

Since these results were not conclusive, the more precise manometric method of estimating cholinesterase activity, by measuring the rate of CO_2 release from a bicarbonate Ringer solution in a Warburg microrespirometer, was employed. In these experiments the reaction mixture in each manometer vessel had a total volume of 2.1 ml., consisting of 1.6 ml. of a 0.4% ACh solution in the main compartment and 0.4 ml. of the Jack-fruit seed extract plus 0.1 ml. of bicarbonate Ringer solution in the side arm. When the effect of eserine on the cholinesterase activity was to be studied, the bicarbonate Ringer was replaced by the same volume of a 0.5% eserine salicylate solution. The vessels and manometers were filled with a 95% N_2 and 5% CO_2 mixture and constantly shaken in a water bath at 37.5° C. at a rate of 90/min. When a simultaneous test was carried out with the Jack-fruit seed extract and a rat brain extract, the vessels containing the brain extract gave an average net manometer reading increase of 24.5 mm./hr., whereas the two vessels containing Jack-fruit seed extract gave an average value of only 3 mm./hr., which was due entirely to non-enzymic hydrolysis of ACh.

In order to test if the ACh in the Jack-fruit seed could be hydrolysed enzymically by cholinesterase from another source, such as rat brain, six samples, each containing 1.6 ml. of a concentrated Jack-fruit seed extract (400 mg./ml.) in a manometer vessel, were set up and incubated as above. To

two of these samples, which served as control, 0.5 ml. of bicarbonate Ringer solution was added. To each of the other four, 0.4 ml. of rat brain extract was added, and 0.1 ml. of a 0.5% eserine salicylate solution was further added to the last two samples which contained both Jack-fruit seed and brain extracts. An example of the results obtained is shown in Table III.

TABLE III

MANOMETER READINGS SHOWING HYDROLYSIS OF THE ACETYLCHOLINE IN THE JACK-FRUIT SEED BY THE CHOLINESTERASE FROM RAT BRAIN

Manometers No. ..	1	2	3	4	5	6
Jack-fruit seed extract, 1·6 mg.	+	+	+	+	+	+
Bicarbonate Ringer in ml.	0·5	0·5	0·1	0·1	0	0
Rat brain extract, 0·4 ml.	0	0	+	+	+	+
Eserine salicylate, 0·1 ml.	0	0	0	0	+	+
Manometer readings: net increase in mm./hr. at 37·5° C.	1·5	3·0	13·4	14·0	1·0	2·0

The readings of manometers 1 and 2, the vessels of which contained no rat brain, were practically the same as those of manometers 5 and 6, in the vessels of which the cholinesterase from rat brain was inhibited by eserine. This means that hydrolysis of ACh in the Jack-fruit seed was not the result of enzymic action. The readings of manometers 3 and 4, however, gave an average increase of 13.7 mm./hr., which showed that the Jack-fruit seed contained a substance which could be hydrolysed enzymically by rat brain cholinesterase but not if this enzyme was inhibited by eserine as in manometers 5 and 6. Besides demonstrating the absence of cholinesterase in the Jack-fruit seed, this further supports the conclusion that the main active substance in Jack-fruit is ACh.

DISCUSSION

With pharmacological methods it could be shown that the seeds and leaves of the Jack-fruit tree, *Artocarpus integra*, contain considerable amounts of ACh. This finding was substantiated by the fact that the ACh in the seed could be chemically precipitated and hydrolysed by cholinesterase from rat brain.

The high ACh equivalent of over 500 μg./g. in the seed and of 60 μg./g. in the leaves of the Jack-fruit is surprising. Such high values of ACh are not found in animal tissues, but an even higher concentration was found in the nettle hair fluid (Emmelin *et al.*, 1947). Since the total weight of the leaves of one Jack-fruit tree may amount to more than 250 kg., the leaves alone must contain about 15 g. of ACh. Another 5 g. of ACh may be estimated to be present in the seeds, making a total of 20 g. in one tree. When taken by mouth, the seeds would probably be pharmacologically active; and this may be the reason why the seeds are only eaten after they are cooked with salt—a procedure which would hydrolyse the ACh.

The Jack-fruit seed differs from the nettle plant in three respects (Emmelin *et al.*, 1947): (1) it contains no histamine, which the nettle plant contains in considerable quantity; (2) it contains, besides ACh, no other smooth-muscle-stimulating substance, whereas the nettle plant does; (3) it contains a toad-heart-stimulating substance, the nature of which is as yet unknown. It seems to be fairly stable in alkaline medium, but the fact that it lacks a general stimulating action on hearts of higher animals speaks against it being a sympathomimetic amine or a cardiac glycoside.

The absence of cholinesterase in the Jack-fruit seed is perhaps not surprising, because, if it were present, such great quantities of ACh could not be stored there. Cholinesterase is also absent in the nettle plant (Emmelin *et al.*, 1947). It must be recalled that in the animal kingdom, where ACh transmits nerve impulses, its release is intermittent and cholinesterase is indispensable to ensure that the ACh acts only for very brief periods. Even in the lower organisms, the need for cholinesterase to prevent the accumulation of ACh in the vicinity of the cilia is still apparent (Bülbring *et al.*, 1953). In plants the function of ACh is still not clear. But in the nettle plant it might serve a defence purpose by causing a burning pain in the skin in association with histamine (Emmelin *et al.*, 1947). The function of ACh in the Jack-fruit seed is at present unknown. Obviously the ACh in the seed is the result of a slow process of synthesis and accumulation; probably the seeds of the Jack-fruit form but a depot of ACh synthesized somewhere else in the plant. This raises the question: where is the ACh formed, in the leaves or in the root? It also suggests that the Jack-fruit tree may offer excellent material for studying the biological synthesis of ACh in plants.

SUMMARY

1. Pharmacological tests, comparative assays and chemical precipitation have established that the seed and the leaves of the Jack-fruit tree, *Artocarpus integra*, contain considerable amounts of acetylcholine (ACh).

2. The average values of ACh were 564 μg./g. of seed, 300 μg./g. of midribs of leaves and 60 μg./g. of whole leaves.

3. In contrast to the high ACh content of Jack-fruit seed, the seed of the fruit of a closely allied species, *Artocarpus champeden*, contained only 3 μg. ACh/g.

4. A heat- and alkali-stable toad-heart-stimulating substance was also found in Jack-fruit seed.

5. No cholinesterase activity was found in Jack-fruit seed.

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