ISSN 0300—9254

JOURNAL OF THE NATIONAL SCIENCE COUNCIL OF SRI LANKA

VOLUME 4 No. 2 DECEMBER 1976

Journal of the National Science Council of Sri Lanka

PUBLICATION: One volume of two issues (June and December) is published annually by the National Science Council of Sri Lanka.

Subscription Annual subscription—Foreign $ 10.00 ; Local Rs. 25.00. Accepted on a calendar year basis. Rates include postage.

Single issues — Foreign $ 5.00; Local Rs. 12.50 each
Back issues — Foreign $ 4.00; Local Rs. 7.50 each
Rates include postage.

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Printed at Sri Lanka University Press, Katubedda, 15535 (8/77)

J. Natn. Sci. Coun. Sri Lanka. 1976 4(2) : 87—98

INVITATION CONTRIBUTION

X-rays and the Structure of Insulin*

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Medical history is to the observer full of events that produce sudden and spectacular changes : before, many die, and afterwards, and suddenly, they live. The isolation of insulin in the summer nearly 50 years ago was an event of this kind. Reading the account of the experiments of Banting and Best[3] in July and August 1921 and of the treatment of the first patients to be given insulin during the following winter is still a moving experience. Old and young who had "melted away" with diabetes, as Aretaeus described hundreds of years earlier, began again to live.

The isolation of insulin was made possible by the recognition that the hormone present in the β—islet cells of the pancreas was protein in nature and had to be protected from digestion by the proteolytic enzymes of the pancreas during extraction. It is, in fact, a rather tough protein, as proteins go, and this also helped to preserve its activity intact during the processes first used of acid alcohol extraction. Its purification was greatly helped in 1925 by its crystallization by J. J. Abel, Professor of Pharmacology at Johns Hopkins University.[1] For a little time his method of crystallization—dissolve in acetic acid and add alkali—seemed unreliable until Scott and Fisher (1934) in Toronto showed that Abel's crystals of insulin contained zinc, present also in the pancreas, and that zinc or some other similar metal had to be added to secure crystallization by his techniques.

Crystals and X-Rays

It was a sample of zinc insulin crystals, prepared for clinical use by Boots Pure Drug Company, that led to my own interest in insulin. The sample was given me by Professor Robert Robinson in 1935 because I was interested in protein crystals. This was the result of experiments carried out by J. D. Bernal at Cambridge the year before, passing X-rays through crystals of the enzyme pepsin. The crystals, 2 mm across, prepared by John Philpot in Uppsala, had been brought over to Cambridge in their mother-liquor. Bernal discovered that if they were kept wet when X-rays were passed through them they gave many diffraction spectra indicating that within the pepsin crystals there were very large molecules of definite size and regular arrangements. When the insulin sample came into my hands I looked up all that I could about it, grew large enough crystals by Scott's method—about 0.25 mm across—and found that they also gave good diffraction effects with X-rays.[6]

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The idea behind our experiments with crystals and X-rays was that we should be able to determine the chemical structures of complicated molecules from the relative positions in space of the atoms within them. Already in 1935 we knew in theory how we might do this. The diffraction spectra produced when X-rays are passed through crystals are the consequence of interference between wave trains scattered by the electrons in the atoms and can formally be recombined as in a lens to show the periodic atomic arrangements responsible for their appearance.

W. H. Bragg pointed out in 1915 that though we have no lens capable of recombining the X-ray waves, they could be recombined mathematically by the use of the relations discovered by Fourier, who represented periodic structures by adding together waves of known phase and amplitude. W. L. Bragg and others made the necessary measurements and calculations on simple crystals of known structure—sodium chloride, a silicate mineral, diopside, and hexachlorobenzene—and showed that the atoms appeared as discrete peaks of electron density. The chief experimental difficulty in extending the results to crystals of unknown structure involved the experimental determination of the phase relations of the scattered waves (readily calculated for crystals of known structure); the amplitudes were easily measured from the intensities of the diffraction spectra. However, in 1927, Cork had proved in a very limited experiment on alums how phases might be found. If the crystal studies could be modified by changing single atoms to heavier atoms in an isomorphous way—that is, without change of the structure as a whole—the difference in the intensities of the diffraction spectra could provide evidence on their phase relations. A process of direct structure analysis was therefore conceivable.

The first X-ray photographs of insulin crystals in 1935 defined the size of the unit rhombohedron—it contained a molecular weight of protein of the order of 36,000. Since the crystals had trigonal symmetry, this unit could be subdivided into three equivalent parts, each with a weight of about 12,000. One could calculate from the empirical analysis of the crystals that such a unit contained about 520 carbon, 130 nitrogen, 150 oxygen and 12 sulphur atoms, and that there were also present a large number of water molecules in the crystal. Before setting out to find the atomic positions in such a complicated crystal, it seemed desirable to try direct X-ray analysis on something simpler. Robertson and Woodward[12] showed that the method worked very beautifully with the phthalocyanines. We set out at the beginning of the war to try to find by this method the structure of penicillin.

Penicillin and Proteins

There were complications with penicillin, eventually solved by X-ray measurements involving crystals of sodium, potassium, and rubidium benzylpenicillin, of which the last two were isomorphous. The first three-dimensional map of the electron density that we calculated illustrates very well the character of our evidence. The

map is derived by evaluating the electron density from measurements and calculations at intervals throughout the body of the crystal unit—the figures are generally plotted on sheets perpendicular to one crystal axis. Contours at equal density intervals define the atomic peaks ; they may be drawn on glass or Perspex and the sheets stacked together to show the three-dimensional arrangement of the atoms. So one can see directly from the map in Fig. 1 that there is a four-membered β-lactam ring in penicillin fused to the five-membered sulphur-containing thiazolidine ring—results that seemed very surprising at the time they were first seen.[7]

Penicillin is still a very small molecule ($C_{16}\,H_{18}\,O_4\,N_2\,S$) compared with insulin, and further improvements in the development of electronic computers and in making large numbers of intensity measurements were desirable before an electron density map could be calculated for any protein. The most difficult problem, however, proved to be the preparation of an isomorphous series of heavy-atom-containing derivatives of a protein crystal, solved first in the case of haemoglobin and myoglobin by Perutz and Kendrew. For haemoglobin, chemical attachment of mercurials to *SH* groups in the molecules proved possible.[11] With myoglobin a more hazardous process of introducing heavy atoms into the spaces filled with solvent in the crystals was found to be successful.[18] In both cases three- dimensional electron density maps were calculated and found to be interpretable—given a good deal of information on the chemical structure of the two proteins studied.

The difficulties of interpreting the electron density maps of protein molecules even when the phase problem has been solved arise from their large size and the fact that in the crystals they form they are almost literally floating in solvent. As a result the X-ray spectra observable fade out at large angles to the X-ray beam and consequently the definition of individual atomic positions decreases. The electron density appears as streaks and peaks of various shapes representing atoms individually unresolved, and chemical information is desirable to help to interpret them.[9] Whereas with penicillin we found the chemical structure from the electron density map, with insulin the knowledge of the chemical structure obtained by Sanger played an essential part in helping us to interpret our map.

Insulin

Sanger's work on insulin defined the sequence of residues to be expected in the insulin we studied—pig insulin .[13] The formula shows their arrangement in the two chains A and B and permits us to count the atoms in the molecules exactly to compare with the first crystallographic counts we made. In the crystal it was clear from our early measurements that there were two Sanger molecules in the asymmetric unit. We chose pig insulin for our studies because it had been found to crystallize particularly well by Schlichtkrull in Copenhagen in the course of his work

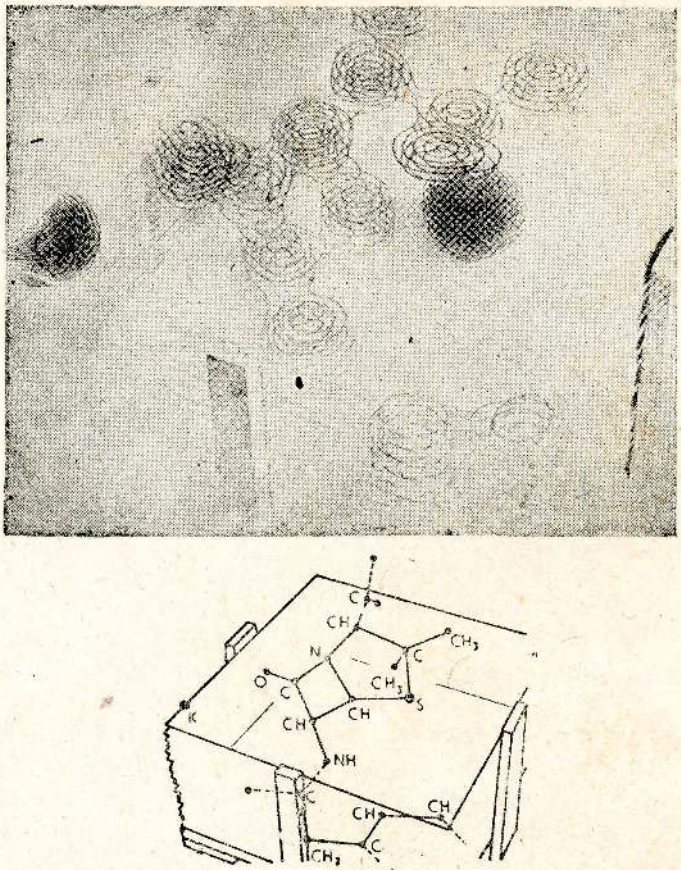
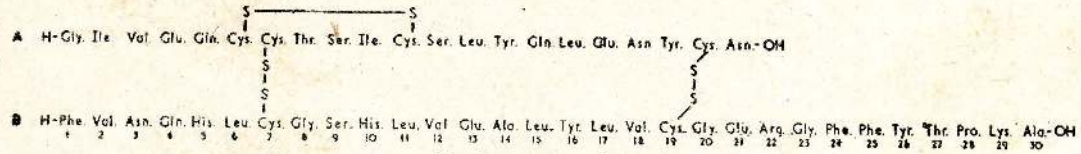


FIG. 1—Model showing the electron density map calculated over part of the crystal unit for potassium benzylpenicillin. As the diagram below indicates, it defines clearly the way in which the atoms are bonded together.

on slow-acting, partly crystalline insulin preparations for clinical use. Schlichtkrull[14] also found that the proportion of zinc in the crystals was two atoms for six Sanger molecules, and this figure defines the zinc atomic positions in our crystals as along the crystal threefold axis.

Our first idea for forming an isomorphous series of insulin crystals was naturally to introduce heavier atoms than zinc by crystallization ; apart from cadmium, not quite heavy enough for our purpose initially, this did not work. However, we did find it possible to replace zinc by lead in the crystals in a rather remarkable way. The crystals were left overnight standing in a dilute solution of a chelating agent, ethylenediamine tetra-acetic acid, which took the zinc out of the crystals. The following night they were left in dilute lead acetate solution. Lead ions occupied

both the old positions of zinc in the crystals and also other sites—one approximately between the two zinc positions, others that proved to be on the outskirts of the molecule. The substitution pattern was quite complicated. But by late 1968 we were able to make much more accurate measurements of the intensities of the X-ray diffraction effects,



The primary structure of pig insulin

and could interpret the small differences between substituted and unsubstituted crystals to place the heavy atoms within the crystals. Further experiments, leaking molecules and ions containing mercury and uranium into the crystals, also proved effective. By the summer of 1969 we had amplitudes measured and phases computed for some 2,000 independent X-ray spectra and so calculated a three-dimensional electron density map for insulin, drew it up on Mylar sheets at a scale of 1 cm to 1 Å, and looked at the answer.[2]

I used to say the evening that I developed the first X-ray photograph I took of insulin in 1935 was the most exciting moment of my life. But the Saturday afternoon in late July 1969, when we realized that the insulin electron density map was interpretable, runs that moment very close. As we expected, the positions of the zinc ions were marked by large electron density peaks but around them were three peaks with a shape that would fit with histidine, each attached to a strand of density, helical in form. We could trace, as in Fig. 2, other peaks attached to the helical chain which had the shape expected from the insulin sequence for the residues neighbouring on histidine B 10.

Arrangement in Space of Atoms in Molecules

From this point we began to trace the whole arrangement in space of the atoms in the molecules. There were occasional setbacks and false moves ; during the last 18 months we have built much more accurate models than our first, matching as precisely as we can the form of the electron density against atomic positions. There are still a few obscurities, involving particularly some of the polar residues on the outside of the molecule extending into the solution. But, within a little, we have recorded positions in three dimensions for the now exactly known numbers of atoms in the insulin molecules.[4,5]

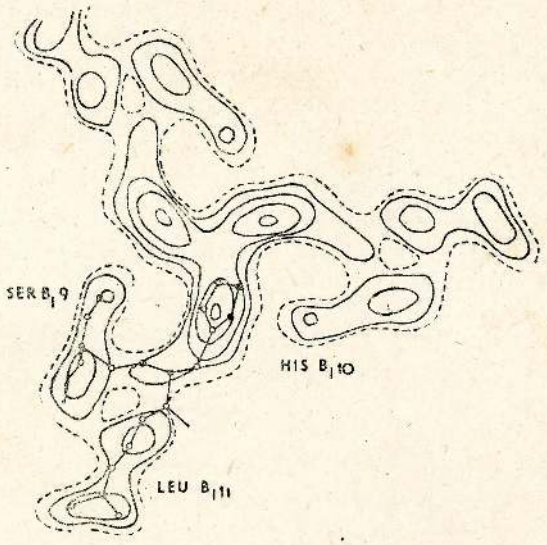


FIG. 2—Electron density contours in a plane perpendicular to the threefold axis, showing superimposed positions of the histidine, leucine, and serine residues. Filled circles— atoms close to section; open circles—atoms within 1 A of section

Owing to the complexity of the crystals studied we see two insulin molecules in our map which are very similar but not exactly identical. They are shown in Fig. 3. Each is a somewhat irregular object over the surface of which extend all the polar residues of the molecule and some non-polar residues. In both, the B chain follows the same general course. The first eight residues are extended ; the chain then passes into a rigid α helix between 9 and 19 ; from 19 to 21 it makes a U turn and again follows an extended form from 22 to 30. The A chain forms a small, compact loop resting within the solid framework of the B chain. There is an initial single turn of α helix followed by the loop closed by the 6-11 disulphide bridge and a later region of somewhat irregularly helical character between residues 12 and 19.

In the crystal, and probably also in many solutions, the two molecules together form a close dimeric unit. Fig. 4 illustrates their relative positions as we observe them. They are related approximately by a twofold axis of symmetry at the position shown. The interactions between them are both non-polar, as between the valine residues, B 14, or phenylalanine residues, B 24, along the lines of the twofold axis, and also polar ; particularly between the peptide groups B 24 and B 26 of neighbouring molecules, which lie anti-parallel to one another, there are hydrogen-bonded contacts within a β-pleated sheet, which closely combines the terminal parts of the B chains.

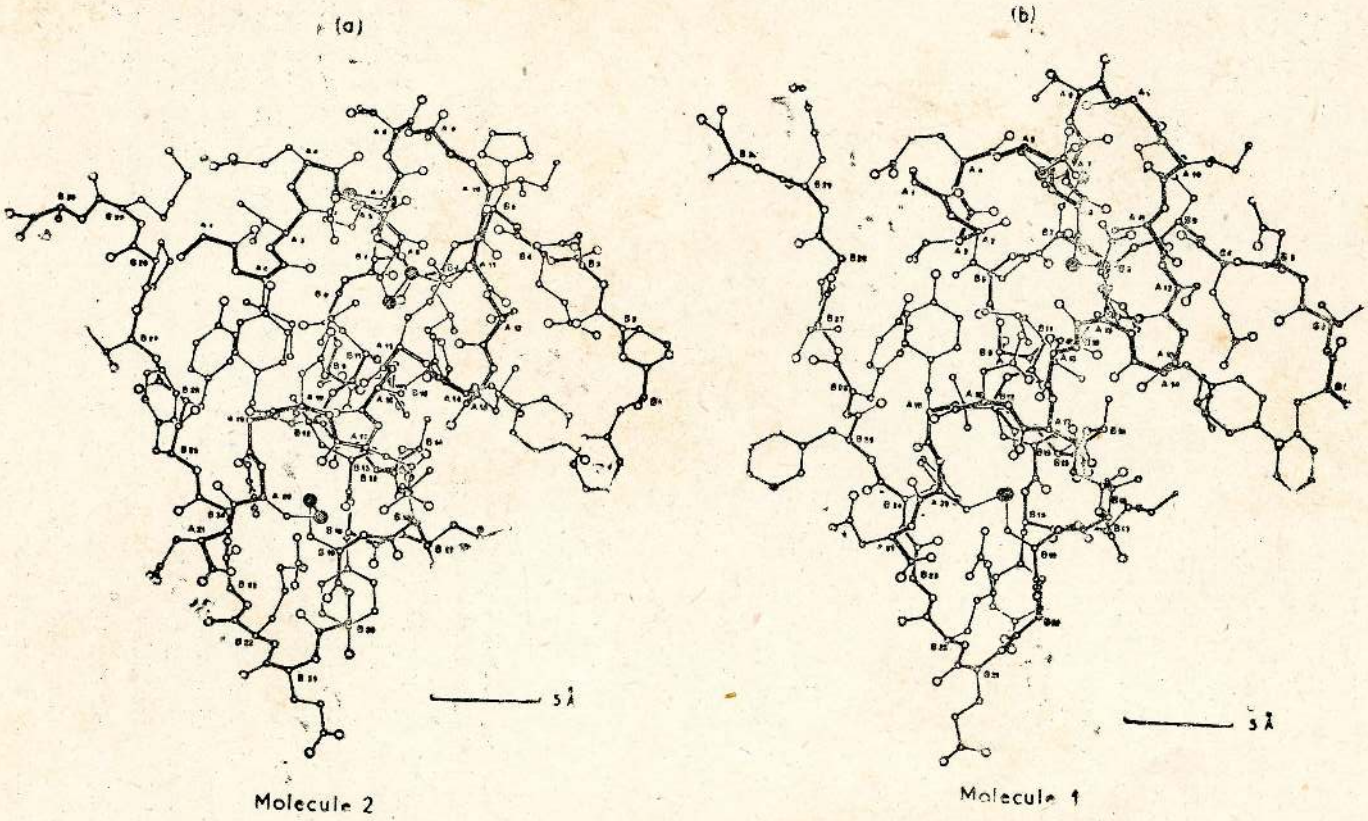


FIG 3—The positions of the atoms found in the two molecules of insulin of the crystal asymmetric unit, projected in equivalent directions normal to the threefold axis. Molecule II has been rotated through 180° from its position in Fig. 4, to compare better with molecule I.

Again in the crystal, and also in solution in the presence of zinc, the three dimers together are connected with two zinc ions to form a hexamer shown in Fig. 5. The main binding forces are the links between the histidine groups and the zinc ions but there are also close contacts, involving polar and non-polar residues, along the region where dimer meets dimer. The hexamer as a whole has a very smooth surface around its circumference, over which many of the polar residues in the molecule are extended. The ends of the dimers, however, project on the upper and lower surfaces of the hexamer as seen in projection in Fig. 4, locking in together when the hexamers pack in the crystal.

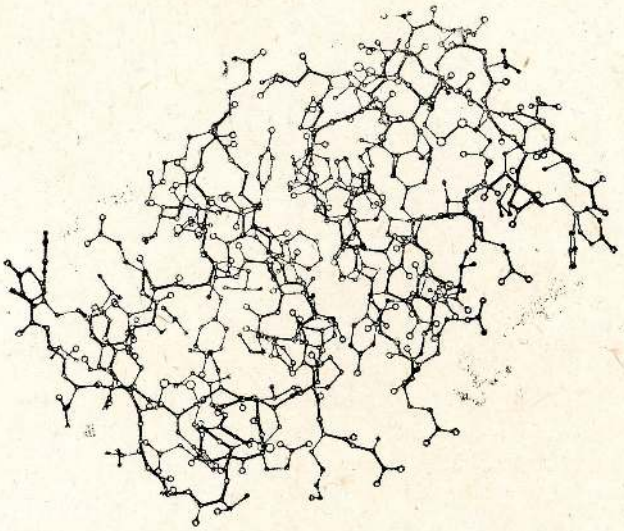


FIG 4—Projection of the atomic positions in the insulin dimer along the approximate twofold axis.

In projection along the threefold axis of the crystals, as in Fig. 5, the hexamer packing simulates close packing of spheres. The molecules make direct contacts with one another yet leaving between them solvent channels, 10 Å or more across, through which other smaller molecules and ions may diffuse (Fig. 6). And with the crystal structure as a whole we return once more to the pancreas. Many past observers have noticed small crystals in the β granules of the pancreas of different animals and particularly of the dog. With the electron microscope one can now see roughly spherical units packed within them. A particularly good example, taken of rat islet cells at the University of Sussex, shows lines across the granules representing the packing of particles 50 Å across (Fig. 7).[8] Almost certainly these are insulin hexamers formed around the zinc ions in the pancreas, since 50 Å is very nearly the diameter of the hexamers.

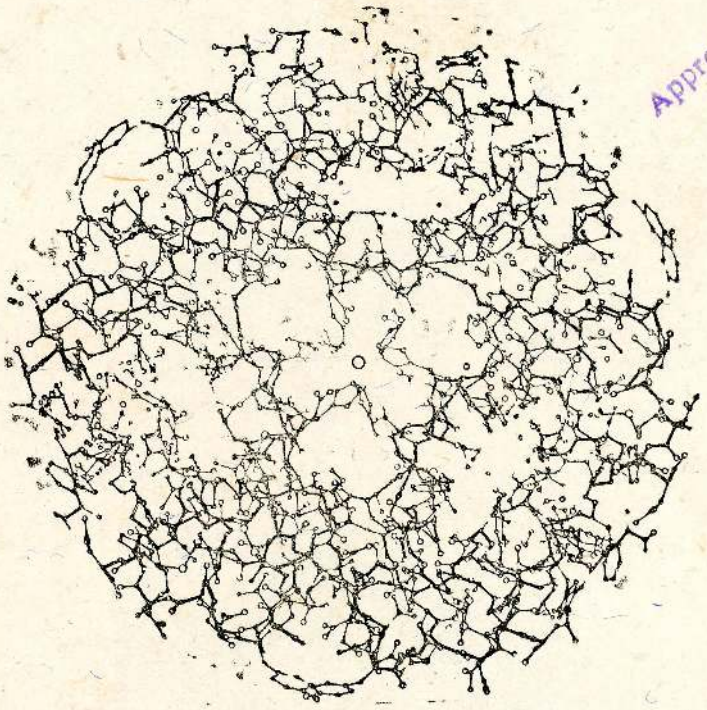


FIG. 5—Projection of the atomic positions in the insulin hexamer, parallel with the threefold axis.

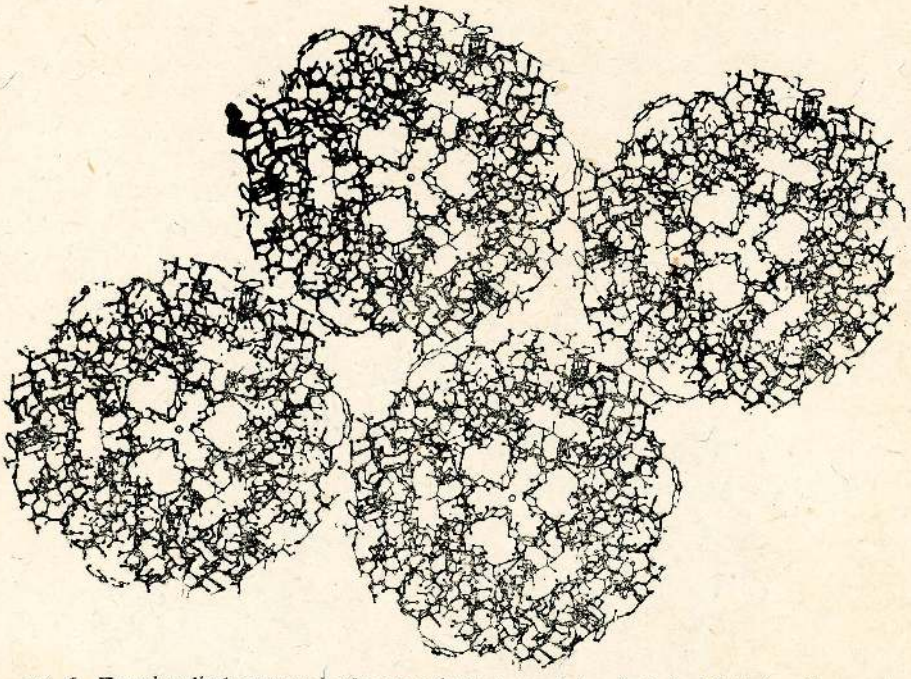


FIG 6—Four insulin hexamers in the crystal structure of rhombohedral 2 Zn insulin, seen projected along the threefold axis.

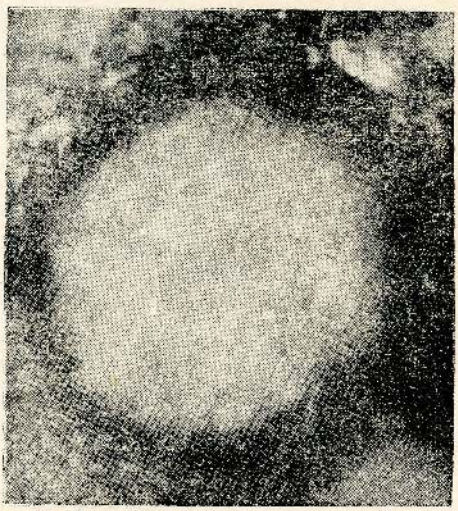


FIG 7—Negatively stained preparation showing an isolated beta granule. The lines across the granule represent the packing of particles 50 A across—almost certainly insulin hexamers. (Phosphotungstic acid X 200,000.) Reproduced, by permission, from Greider *et al.* (1969).

Clues in Amino-acid Distribution

It seems most likely that the formation of hexamers around zinc ions is a way of storing insulin which is found in many creatures, though not in all. From the islet cells the hormone is released for action ; at the dilutions at which it occurs in the body fluids it is very probably present as the dimer or monomer. We can already see certain clues in the amino-acid distribution in these structures that are suggestive in relation to the biological activity of insulin.

First, there are several observations made during the syntheses of insulin carried out in Aachen, Pittsburgh, Shanghai, and Peking. In each case, the syntheses were carried out by making separately the A and B chains, reducing them to the sulphydryl form, and leaving them in solution to unite in the correct order and shape. The A chain alone was observed to have some little activity, the B chain none. The reaction to produce insulin was rather inefficient ; clearly the organization of separate chains into the correct conformations, to make the correct internal links, is not at all automatic. In nature, indeed, a quite different course, via the single-chain precursor proinsulin, is adopted.

On the other hand, some biologically active insulin is formed by the chemical route ; it seems most likely that the B chain tends to fold in the specific form now observed and this may then support the A chain in a biologically active shape through the interaction of certain specific residues.

The nature of these specific residues is suggested by the study of different insulins and the changes that occur with species. The residues that so far are observed as unchanged include all the cystine residues, three glycines, and a number of leucine and isoleucine residues. These are concentrated in the core of the molecule and seem to be largely concerned with maintaining its correct three-dimensional form. Other residues, such as B 24 phenylalanine along the dimer twofold axis or the A chain residues, glycine A 1, glutamine A 5, tyrosine A 19, and asparagine A 21, might, on the other hand, constitute, in some part or other, an active surface ; removal of A 21 or the A1 amino group largely destroys activity. B 24 might well be opened for interaction with a membrane receptor by opening the dimer.

These clues do not yet tell us what it is that insulin does at the molecular level that affects glucose utilization and transport and protein synthesis and so our own continued well-being. But they may help us to devise new experiments so that in time we may understand how this remarkable molecule operates.

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J. Natn. Sci. Coun. Sri Lanka 1976 4(2) : 99—108

Studies on *Sesamia Inferens* Wlk. The shoot-borer pest of Sugar-cane in Sri Lanka.

1. Life cycle and aspects of biology

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(*Paper accepted :* 24 *August* 1976)

Abstract : The ecological studies on *Sesamia inferens* Wlk. as a pest of sugar-cane in Sri Lanka reveal that it prefers to lay eggs in the cane field grasses. Its collateral hosts are enumerated. The nature and scope of its damage at different stages of the host plant and the life cycle of the pest are traced. The natural enemies on the pest are recorded.

1. Introduction

Of the several species of insect pests recorded on sugar cane, the moth borers are the most serious group. *Sesamia inferens* Wlk. the shoot borer, is one of the important pests of this group in Sri Lanka. It is an oriental species occuring in Pakistan, India, South-east Asia, China, Taiwan, Philippines, Indonesia and Solomon Islands.[4]

The larva of the pest is harmful as it bores into the young cane shoots close to the ground level and causes the death of the host plant. The symptom of damage is the characteristic 'dead heart', where the central whorl dries up. The pest is also responsible for the secondary attack on millable cane stalks, causing loss of recoverable sugar.

Besides attacking sugar cane, *S. inferens* has been recorded as a serious pest of rice, maize, wheat, sorghum, kurakkan, finger millet and about 46 other graminaceous plants.[4,5]

2. Materials and Methods

The life cycle of the pest was investigated at the Sugar Cane Research Institute at Kantalai.

Out of a total of 4,250 late instar larvae collected at the field during the period August to November, 1974, about 563 were accidentally killed while examining the damaged shoots where they live. The remaining 3,687 were reared in cylindrical glass jars 10 inches tall and 8 inches in diameter, kept covered by drop-over wire mesh lids. About 25 larvae were placed in each jar containing finely pulverised sugar cane. This medium served as an ideal source of food. Around 2,825 larvae died as a result of parasitization that had already occured at the time of collection or escaped during rearings in the laboratory. The balance 862 larvae pupated over

the period. On pupation they were sorted out depending upon the sex and time of pupation. These were kept in petri-dishes. Of these 4 pupae died and about 858 emerged as adults, in a male to female ratio of 1 : 2. The emerging adults were transferred to bell jars containing grass shoots. Into each jar, one female and three to five males were introduced for mating trials. The grass shoots were examined daily to ascertain the egg laying. The eggs were laid underneath the leaf sheath. They were counted *in situ* and placed in separate bell jars to observe the incubation period.

3. Life Cycle

The adult female lays its eggs on the soil surface near the plant base[1] or in the leaf sheath of cane field grasses close to the cane plants. Details of the life cycle are furnished in Fig. I.

3.1 Egg

The eggs are laid in three to four rows or in clusters ranging from 50 to 150 at a time. A single adult female lays about 150 to 400 eggs in several egg masses. The newly emerged females when dissected have been found to contain as much as 489 eggs in their oviducts. Under laboratory conditions a larger proportion of unfertilized eggs were obtained.

The egg is creamy white in colour and semi-globular, being flattened on the dorsal surface. It is highly sculptured and measures 0.6 mm in diameter.

The incubation period shows some amount of variation during different months, and is influenced by climatic factors. This period lasts for 4 to 5 days during November (mean maximum temperature 90°F and relative humidity 96%) ; 7 to 8 days during January (mean maximum temperature 84°F and relative humidity 95%) ; 8 to 9 days during February (mean maximum temperature 80°F and relative humidity 93%).

By about the third day the eggs change to a light pinkish coloration. The developing embryo is seen on the fourth day as a pinkish streak slightly curled inside the shell. The larva with a prominent head could be made out on the sixth day. Unfertilized eggs turn dark, shrivel up and fail to develop.

3.2 Larva

Issac and Rao[2] described the larva of *S. inferens*. In laboratory rearing, the first instar larvae hatching off the eggs were counted. Each larva was placed in a two inch long grass shoot kept in a three inch specimen tube plugged with cotton wool. Several such tubes were set up depending on the number hatched. They were examined

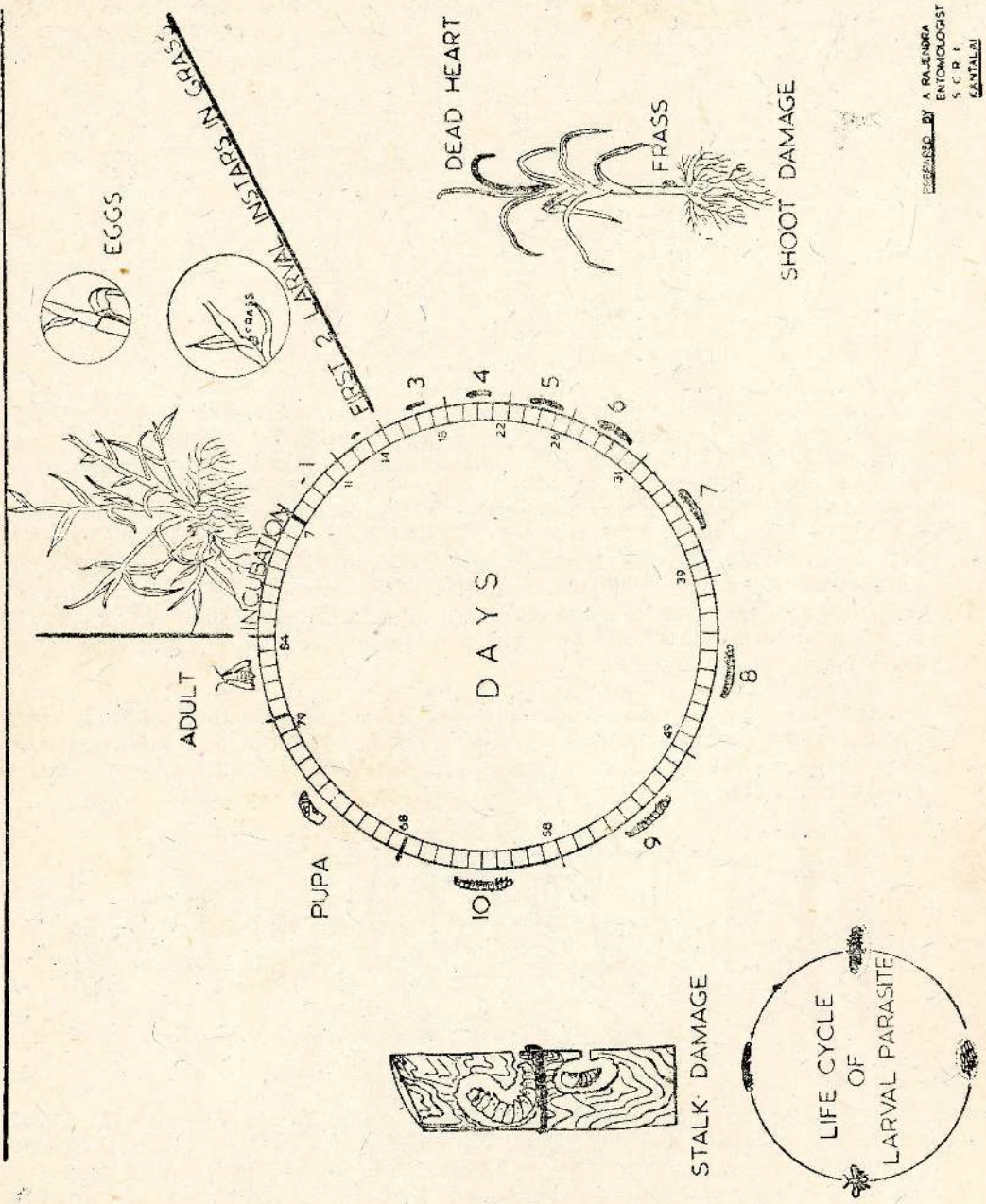

FIGURE 1. Life cycle of the shoot borer *Sesamia inferens*

daily to ascertain the duration of each instar. The fourth instar onwards were reared in pulverised cane kept in glass tubes. In this manner, the number of instars during the larval stage and the duration of each instar were recorded. (Table I).

TABLE 1—Details of Larval Development

Instar	Length of Larva mm.		Width at Broadest segment in (mm.)	Duration in days.
	Range	Average		
1	2.6 — 3.7	3.1	0.3	4
2	3.4 — 5.9	4.3	0.9	3
3	4.0 — 11.1	8.5	1.3	4
4	12.1 — 13.4	12.7	2.2	4
5	15.2 — 18.1	16.6	2.8	4
6	19.0 — 23.6	21.3	3.0	5
7	22.0 — 27.8	24.9	3.2	8
8	26.0 — 32.0	29.0	3.4	10

Jepson[3] found that the number of larval instars in *S. inferens* varied between five to eight but under local conditions some variation is recorded. Generally there are eight instars lasting 42 days but under unfavourable conditions as much as 10 instars were recorded lasting 61 days. The first four instars showed some regularity in their duration beyond which irregularity was observed.

In the cane fields, the first instar larva enters the grass shoot that is in proximity to the egg and lives within. Its presence in the grass can be easily made out by 'frass' coming out of the aperture made on entry. Should the grass shoot die, the larva abandons it and attacks fresh ones in the neighbourhood. Entry into cane takes place by about the third instar.

The size of the larva varies according to the season and availability of food. The first instar larva measures 2.6 mm to 3.7 mm in length. The final instar larva measures about 26 to 32 mm in length, is pinkish in colour and is popularly known as the Pink borer. (Fig. 2).

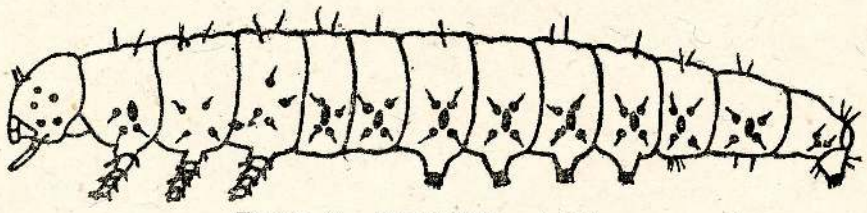

FIGURE 2. Larva of *Sesamia inferens*

3.3 Pupa

The pupa is brownish in colour and measures 15 mm to 17 mm (Fig. 3). The sex of the borer can easily be distinguished during this stage. Males are smaller with tapering abdomens and carry two small 'bumps' in front of the genitalia, while the females are larger with broad abdomens and devoid of any markings. Pupation is noted to take place in the leaf sheath from where adults emerge. The pupal stage lasts from 9 to 11 days, the norm being 11 days. No hibernation is recorded locally.

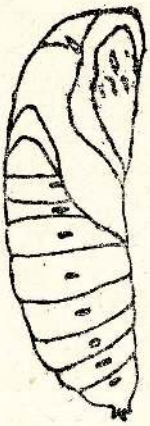

FIGURE 3. Pupa of *Sesamia inferens*

3.4 Adult

The adult (Fig. 4) is nocturnal in habit. It is brownish with wings off-white in colour. The female measures 14 mm to 16 mm in length with a wingspan of 28 mm, while the male is about 13 mm in length, has a tapering abdomen and wingspan of 25 mm. The sexes can thus be separated easily. The adults live for five to seven days, the norm being five days.

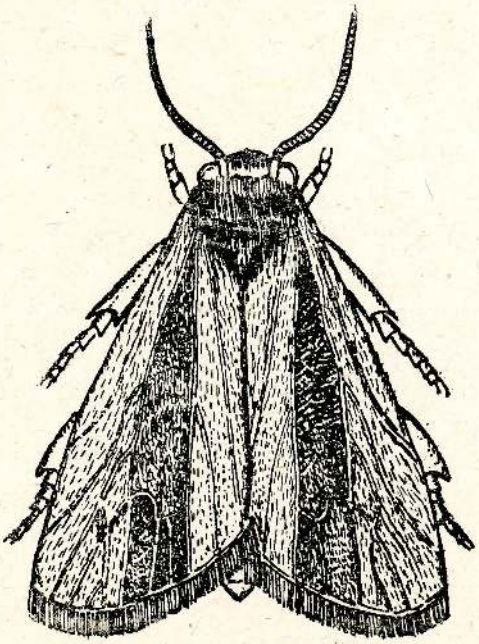

FIGURE 4. *Sesamia Inferens*

3.5 Emergence and Dispersion

The pupa which takes 9 to 11 days before adult emergence was closely watched every hour from the ninth day onwards. The pattern of emergence was studied from 626 pupae over a period of 100 days between 6th September and 12th December, 1974. Over 79% of the pupae emerged as adults after nightfall (between 7 p.m. and 6 a.m.). Although it was also noted to take place during daylight hours, most of the adults that emerged during this period showed deformed wings, were incapable of flight and died within a few hours. A peak of over 47% emergence took place between 8 p.m. and 12 midnight. About 17% emerged before this peak period between 4 p.m. and 8 p.m., and about 26% emerged after the peak period between 12 midnight and 6 a.m. (Fig. 5).

On emergence from the puparium, the adults are with rudimentary wings. The wing development is observed to take place in two stages. The first is the growth stage. Data obtained from 560 observations reveal that it takes about 16 minutes for the wings to grow from the wing buds (range : 5 minutes to 28 minutes) and is held in an upright position which is the second stage for a further period of about 13 minutes (range : 7 minutes to 25 minutes), before it is folded over the body to assume the characteristic position common to moths.

On completion of the wing development which takes about 30 minutes, the moth takes to its wings and disperses within the grasses in the boundary and the headlands of the neighbouring cane area, or, if the crop is mature, seeks grassy patches within. *S. inferens* females are capable of ovipositing immediately after dispersion. The egg-laying is noticed to take place in these grass associations from where the cycle is repeated. *S. inferens* is found throughout the year completing its cycle in about 65 days (8 instars) to 84 days (10 instars). Sugar cane is planted during the period March to August at Kantalai and the shoot stage of cane is available for the pest over a long period. The first brood occurs during May and June. The generations overlap one another. There are about four to five broods per year.

4. Host Plants

Though regarded primarily as a cane pest, *S. inferens* finds more congenial conditions for its egg laying in cane field weeds, where it spends the first two larval instars before attacking cane.

About 9 species of local cane field grasses are recorded as collateral hosts of the shoot borer. They include species such as *Eragrostis diplachnoides* steud., *Panicum repens* L., *Cyperus compressus* L., *Cyperus iria* L., *Digitaria ascendens* H.B.K. *Amaranthus viridis* L., *Echinochloa colonum* Link., *Brachiaria mutica* Stapf. and *Brachiaria distachya* Stapf.

5. Nature of Damage

S. inferens passes its early larval instars in cane field grasses before attacking cane. Damage to sugar cane takes place during the shoot and stalk stages.

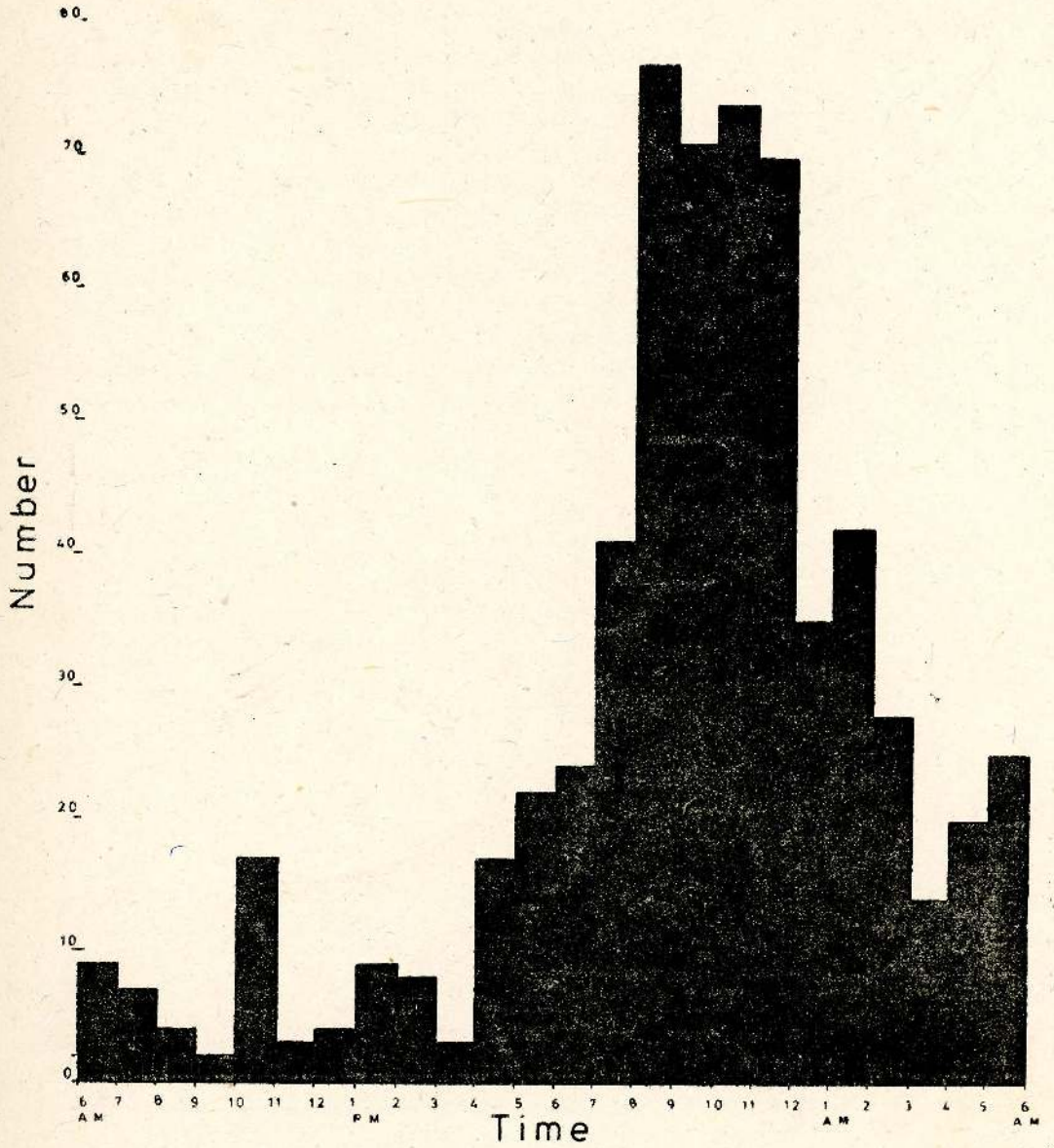

Fig.5. Sesamia inferens adult emergence

5.1 Shoot damage

Entry into the shoot stage of cane takes place by making a tiny hole in the lower portion of the shoot. It eats up the inner tissues of the plant and the 'frass' passes out through the aperture made on entry. This causes the central whorl of the shoot to dry up into what is known as the 'dead heart'.

A 'dead heart' is invariably the result of the damage by a single larva. When the shoot dies before the completion of the larval period, the larva abandons it to attack new shoots. Investigations carried out daily over a period of 3 years from 1968 to 1971 by sampling a standard unit of 100 damaged shoots or stalks reveal peaks of infestation in the field depending upon the age of crop. Table 2 depicts the shoot damage at 4 months recorded in December 1969 wherein about 25% of the borer damaged shoots were found to contain live borers, from which it could be concluded that at least four young plants may be damaged by a single larva.

TABLE 2—Percentage Borers in Damaged Shoots and Stalks

Date	Shoots December 1969 (Age 4 months)	Stalks March 1970 (Age 7 months)
1	—	7
2	31	—
3	—	—
4	27	4
5	23	4
6	19	3
7	10	—
8	—	—
9	—	5
10	9	4
11	12	5
12	22	10
13	24	9
14	23	7
15	21	—
16	0	4
17	22	10
18	23	7
19	19	5
20	17	4
21	34	2
22	29	—
23	—	7
24	19	9
25	—	6
26	34	7
27	27	—
28	30	10
29	41	—
30	—	—
31	31	4
Average	24.08%	5.8%

5.2 Stalk damage

S. inferens is primarily a shoot borer but when the pest gets established in the young crop, the subsequent generations that breed in the neighbouring grasses feed on mature stalks. This may be regarded as a secondary damage. The larva causes damage to about two to four nodes per stalk eating up the inner tissues, which results in galleries ranging from one to four inches in length. In mature cane, about 6% to 10% of the damaged stalks contained borers in them so that a single borer may be responsible for the damage of 10 to 16 stalks. Table 2 reveals the number of borers expressed as a percentage of the damaged stalks as recorded in a seven month old crop in March 1970.

6. Natural Enemies

A few species of insects parasitic on the shoot borer have been recorded. They include :—

(*a*) Larval Parasites :—

Megaselia spp. (3)	Phoridae
Apanteles chilones Munakata	Braconidae
Apanteles flavipes Cam.	Braconidae

(*b*) Pupal Parasites :—

Tetrastichus israeli Mani and Kurian	Eulophidae

The larval parasite *Apanteles flavipes* Cam. is a prominent species which affords considerable control of the shoot borer (Fig. 1).

7. Discussion

S. inferens is restricted to Kantalai as a major pest of sugar cane. During the period 1964 to 1968 the occurrence was sporadic and this species showed the peak of larval incidence in December. This was so because cane was planted from June to August and the crop was in its shoot stage until December. With improved knowledge of cane culture, planting of cane is now carried out between March and August and this has resulted in the pest occuring throughout the year.

The pest makes its appearance in the newly planted areas by March and is at its peak by April/May. One of the conditions conducive for its rapid multiplication and spread is the presence of cane field weeds which act as collateral hosts. The pest completes its life cycle in 65 days to 84 days, depending upon environmental factors. Overlapping of generations is generally observed and there are about four to five broods per year.

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J. Natn. Sci. Coun. Sri Lanka 1976 4(2) : 109—119

A technique to investigate some aspects of the growth kinetics of bacterial colonies on agar media

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(*Paper accepted*: 15 *September* 1976)

Abstract : A simple theoretical model for the growth kinetics of a bacterial colony on agar medium is derived. An optical densitometry scanning technique for the study of the growth of micro-organisms in agar media is presented (the principle of the technique is discussed and an instrument employing the technique is described). The operational features of the instrument and some experimental data obtained with it is also presented. This experimental data was used to test the theoretical model derived. A few potential applications of the technique which can lead to new fields of investigation are mentioned.

1. Introduction

The optical densitometry technique for studying the growth of micro-organisms under varying environmental conditions is largely limited to liquid media. The application of this technique to solid media (e.g. growth of bacterial colonies on agar media) has not (to the knowledge of the authors) been reported.

The quantitative study of the growth of micro-organisms on solid media provides certain types of information that cannot be obtained from liquid media studies and hence could lead to new areas of investigation. In a liquid medium, for example, the cells are usually distributed homogeneously and the growth characteristics of the micro-organisms are the same in different parts of the culture with the result that only a gross picture emerges. On solid media, however, colonies of micro-organisms show characteristic morphological patterns, like the differential distribution of particle density within a bacterial colony and differing growth rates at different regions of the colony.

The techniques of studying colony growth on solid media which are presently available are mainly limited to observations (using microscopes) of shapes and sizes of colonies and their variations with time. The instrument described in this paper provides a quantitative method for the time variation studies of spatial particle density distributions within a colony as well as variations of shape and size of the colony under investigation.

2. Principle of the technique

The intensity of a light beam passing through a medium at a point where the particle density is N, is attenuated from I_0 to I according to the well known law in optics :—

$$I = I_0 \exp(-\alpha N), \quad \alpha = \text{a constant} \tag{1}$$

The fractional light absorption F is given by

$$F = \frac{I_0 - I}{I_0} = 1 - \exp(-\alpha N) \tag{2}$$

Hence

$$N = \frac{1}{\alpha} \ln \frac{1}{1 - F} \tag{3}$$

Where N is the partical density at the region under investigation at time t.

If the probability of cell division per unit time is $P(t)$ and the number of cells at time t is N and at time $t + dt$ is $N + dN$,

then $$P(t)\,dt = \frac{dN}{N} \tag{4}$$

(since the division of a single cell increases the population by one, i.e. its own disappearance and two new cells forming). Due to the environmental resistance to growth (e.g : reduction of nutrients, etc.) the probability of cell division could be expected to decrease as the colony grows and hence the probability of cell division would be both a function of the spatial position within the colony and also of time. Let us *assume* the time dependance to have the form of exponential decay,

$$P(t) = P(0)e^{-\lambda t} \tag{5}$$

Where λ is a constant depending on the nature of the bacteria, the medium and other environmental factors.

Eqns : (4) and (5) yield

$$\int_{N_s}^{N} \frac{dN}{N} = P(\mathrm{O}) \int_{\infty}^{t} e^{-\lambda t}\,dt$$

where Ns represents the saturation state of the population.

$$\ln \frac{N}{N_s} = -\frac{P(\mathrm{O})}{\lambda} e^{-\lambda t}$$

let $N_s - N = n$

$$\ln \frac{N}{N_s} = \ln \left(1 - \frac{n}{N_s}\right) = - \left\{\frac{n}{N_s}\right\} - \frac{1}{2}\left\{\frac{n}{N_s}\right\}^2 - \frac{1}{3}\left\{\frac{n}{N_s}\right\}^3 \ldots\ldots\ldots\ldots$$

Since n is always less than N_s and as saturation is approached

$$\frac{n}{N_s} << 1 \left(\text{as } t \to \infty, \quad N \to N_s\right)$$

$$\ln \frac{N}{N_s} \cong - \frac{n}{N_s} = \frac{N - N_s}{N_s}$$

$$\frac{N - N_s}{N_s} = - \frac{P(0)}{\lambda} e^{-\lambda t}$$

ie. $$N = N_s \left(1 - \frac{P(0)}{\lambda} e^{-\lambda t}\right) \qquad (6)$$

Equations (3) and (6) yield,

$$\lambda t = \ln \left\{ \frac{\ln \frac{1}{1 - F_s}}{\ln \frac{1 - F}{1 - F_s}} \right\} \qquad (7)$$

A plot of $\left\{ \frac{\ln \frac{1}{1 - F_s}}{\ln \frac{1 - F}{1 - F_s}} \right\}$ with respect to time t on semi-log

graph paper would yield a straight line whose slope with the t axis is λ.

The technique for measuring F for a bacterial colony growing on agar involved allowing a narrow beam of light (thickness of the beam $\lesssim 1/10$ diameter of the colony under investigation) to fall on a photodetector, after traversing through the point on the colony where the particle density was being estimated. When operating on the linear part of the photodetector characteristic, the photocurrent (i) is proportional to the light intensity (I)

and $$F = \frac{i_s - i}{i_s} \qquad (8)$$

In order to obtain the particle distribution within a colony, the colony was moved across so that the beam scanned along a diameter of the colony. The photodetector current was fed into a pen chart recorder which traced out the current corresponding to the spatial distribution of the particle density along the colony.

The study of the time variation of the spatial F distribution was achieved using an automatic periodic scanning device.

3. Instrument

Fig. 1 shows the densitometer cum automatic scanning system. A projection system is incorporated into the instrument so that the colonies that are being scanned could be traced out in two dimensions. In Fig. 2 details of the rotating disc and the glass cell for containing the culture medium are shown. Conventional petri-dishes were not used as their surfaces are not optically homogeneous.

Fig. 3 gives the electrical circuits for the illuminator and detector of the instrument to be operated automatically and with the minimum consumption of recorder chart paper.

4. Procedure

The glass cells to be used in the experiment were sterilised in an autoclave and sterile medium of 1.5% Oxide Nutrient Agar was poured into these cells to fill upto half their thickness. The medium was allowed to set and measured amounts from a very dilute suspension of *Escherichia coli* in phosphate buffer was inoculated with a micropipette onto a central spot on the surface of the medium.

The glass cell thus prepared was placed over an aperture on the rotating disc (Fig. 2) so that the light beam passed through the inoculation point. The surface the medium on which the bacterial suspension was inoculated faced downwards. This was a precaution against water vapour condensing on the lid of the glass cell.

The width of the light beam was adjusted to a fraction of a millimeter. The scanner was made to operate continuously for 48 hours. This represented a scan per each of the eight cells once every hour. At the end of this period, scanning was done for two hours every 12 hours for about two weeks, using an automatic switching device. A calibration spot on one of the glass cells (which did not change with time) was used for monitoring the constancy of the overall densitometer sensitivity.

5. Results

Some samples of chart recorder tracings made in the process of scanning the colony during the growth is shown in Fig. 4. Fig. 5 shows the time variation of the particle density at the centre of the colony and the diameter of the colony. Fig. 6 shows the plot as indicated in equation (8). For the particular example considered $\lambda = 0.17$ day^{-1}.

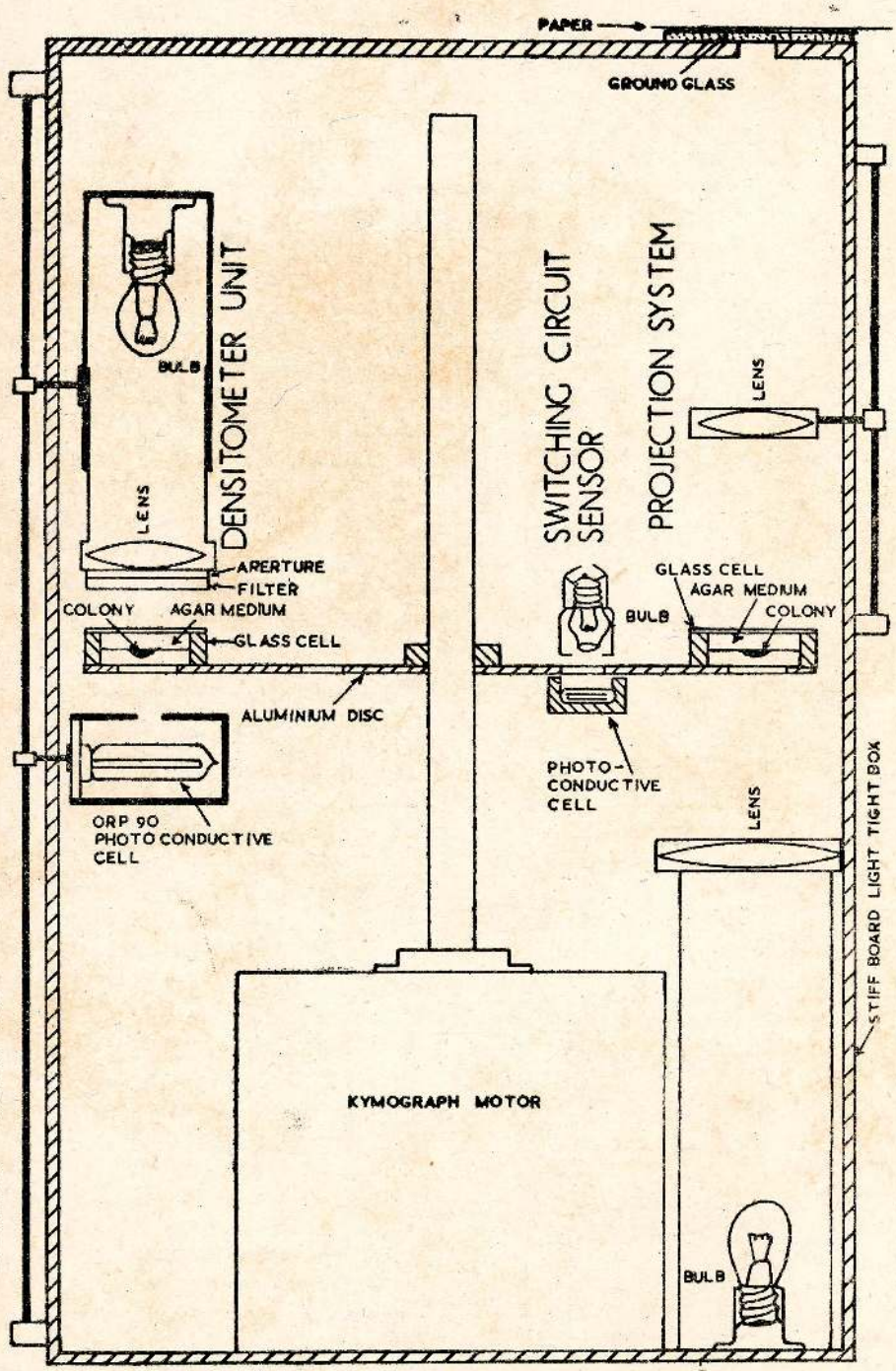

Fig.1 - Densitometer, scanning and projection system

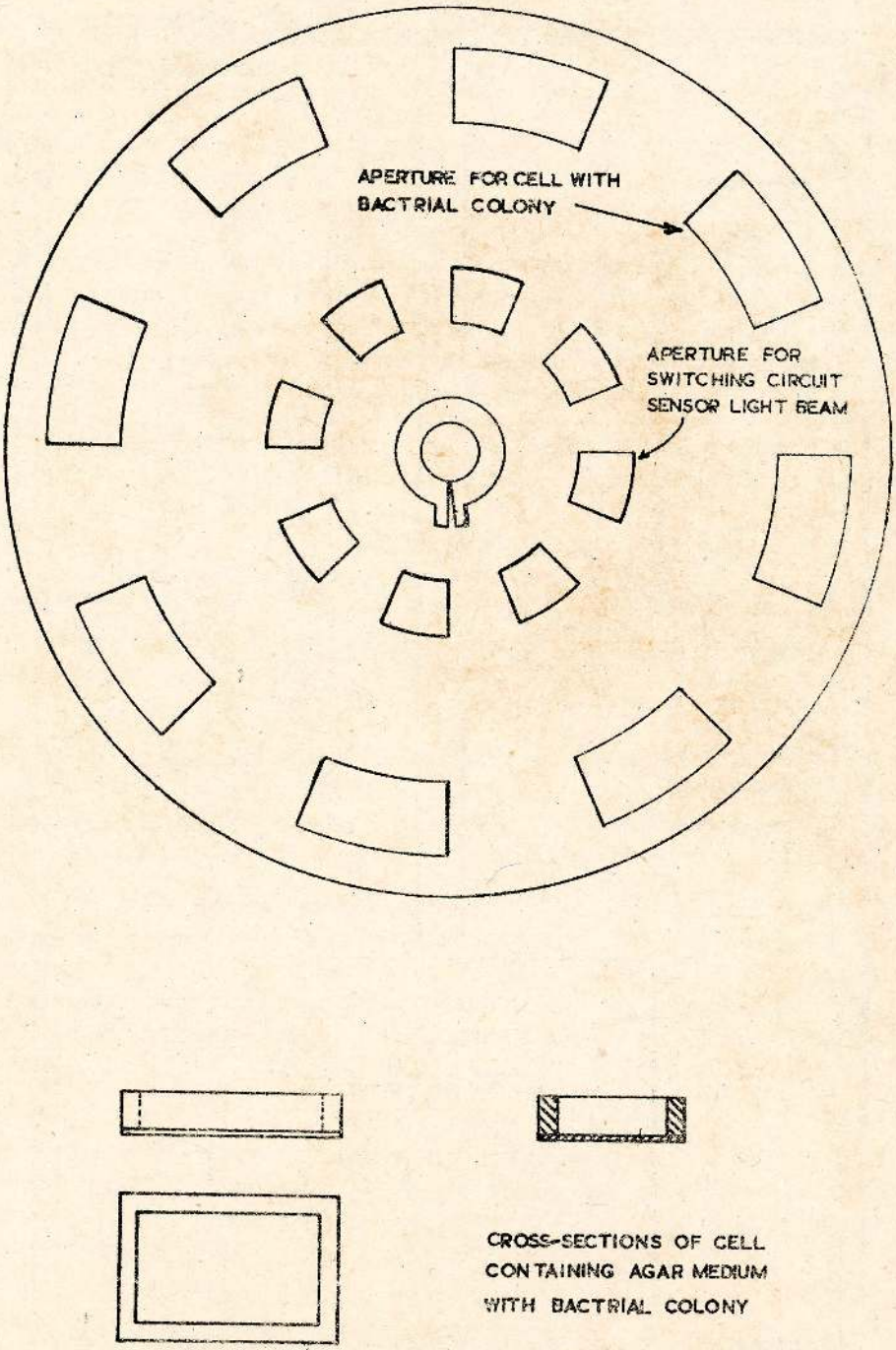

Fig. 2 - The scanner, rotating disc and a glass cell

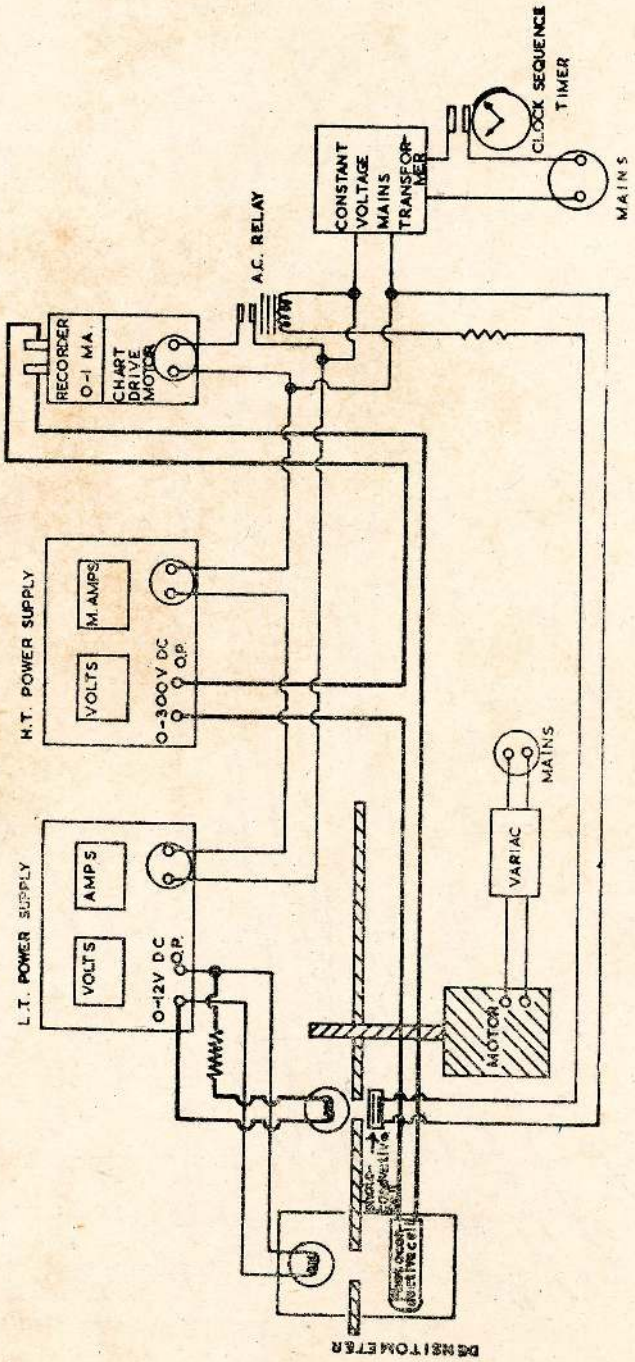

Fig. 3 - Electrical circuits for densitometer and the automatic scanning system

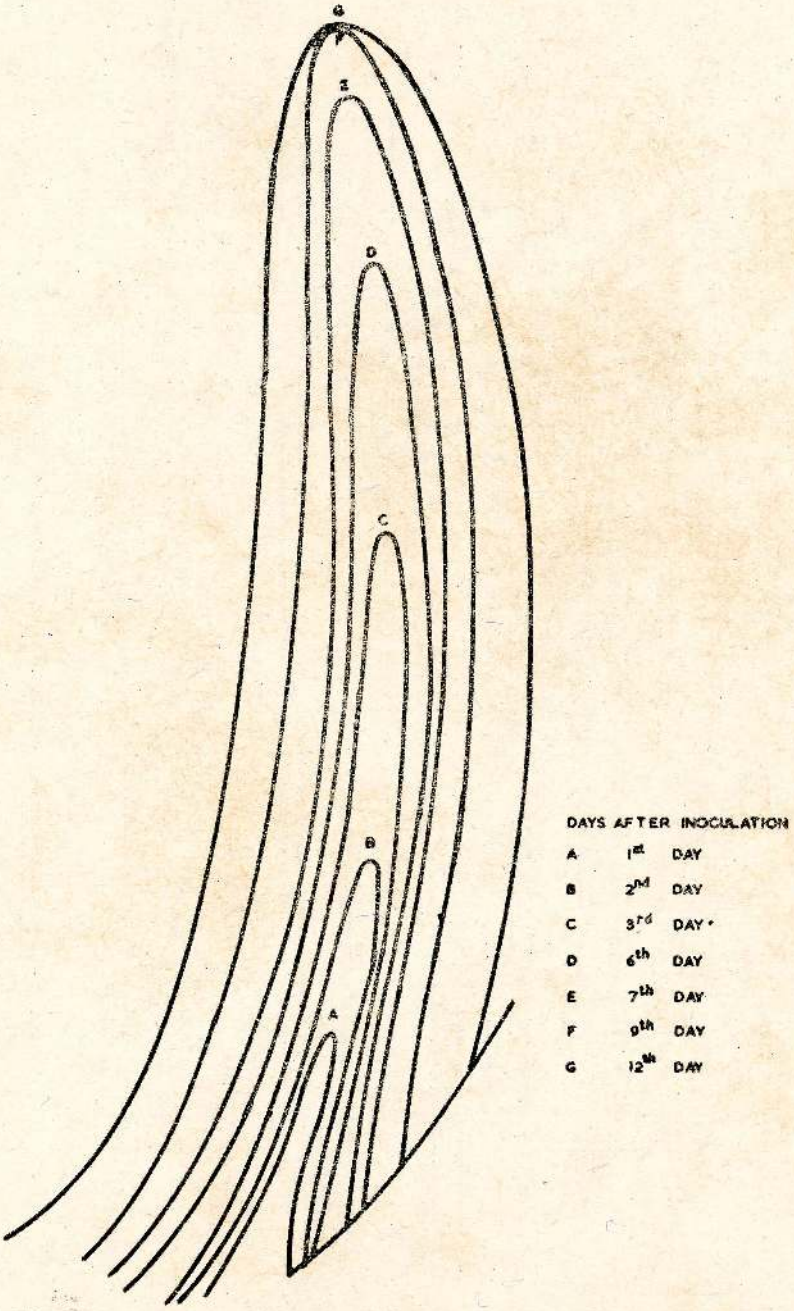

Fig. 4 – Pen chart recorder tracings on scanning
Note: Chart recorder pen introduces a curvature

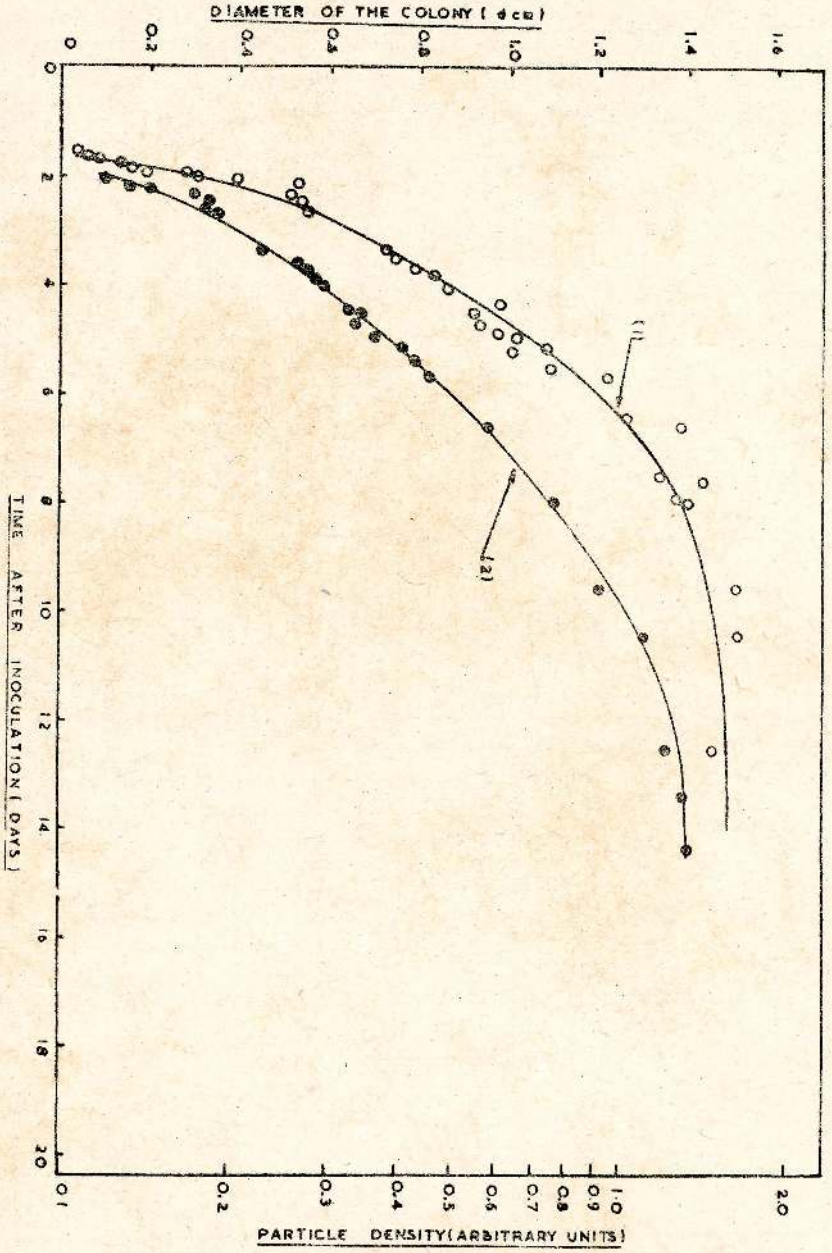

Fig. 5 1 CHANGE OF PARTICLE DENSITY (N) WITH TIME (t) NEAR THE CENTER OF THE COLONY.

2 CHANGE OF DIAMETER (d) OF THE COLONY WITH TIME (t)

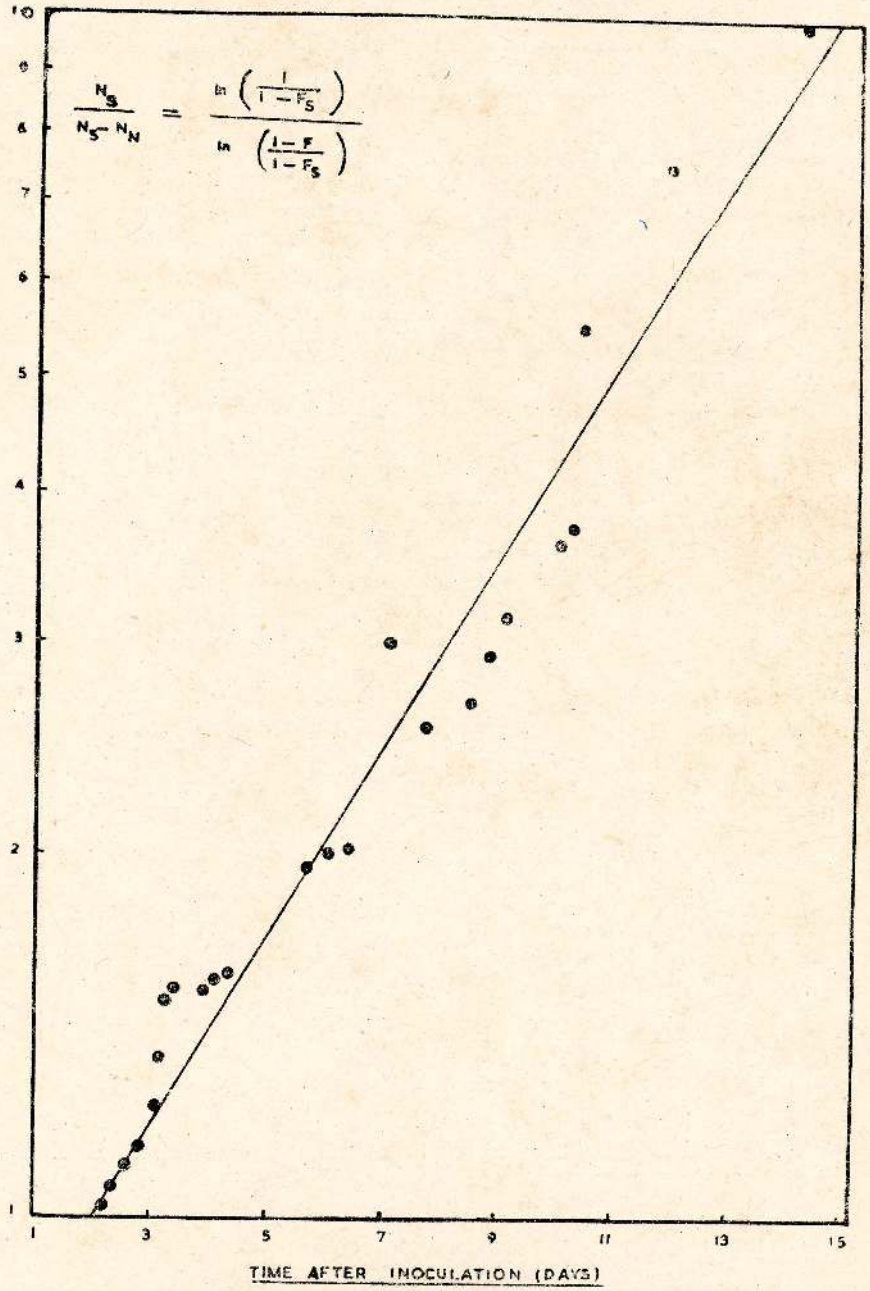

Fig. 6 CHANGE OF THE RECIPROCAL OF THE FRACTIONAL DEVIATION FROM SATURATION $\frac{N_S}{N_S - N}$ WITH TIME (t)

The paper is confined purely to a discussion of the basic technique. The instrument described is inexpensive but possesses a capacity for further sophistication if the problem investigated so demands. It could also be used without modification as an electrophoresis or chromatographic spot scanner and in other applications of densitometry.

The growth constant would be a useful quantitative index of growth. This index could be measured under varying environments and for different organisms.

This technique has potential application in a large variety of studies a few of which are the following :

(1) Kinetic studies of growth of bacterial and fungal colonies.

(2) Differentiation studies of fungal and bacterial colonies.

(3) Growth patterns of colonies in different types of media.

(4) Response of colony growth to different substances e.g. antibiotics, inhibitors, etc.

(5) Growth of bacteriophage plaques in bacterial cultures.

Acknowledgements

We wish to thank Mr. M.N. Kuruppu, Workshop Technician, Department of Physics, for the assistance rendered in constructing the instrument and preparing the diagrams for this paper and Mr. A. L. Amarawansa, Laboratory Technician, Department of Physics, for typing the manuscript.

J. Natn. Sci. Coun. Sri Lanka 1976 4(2) : 121—138

Sulphhydryl Group determination by Amperometric methods and its application to studies with Coconut Toddy

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(*Paper accepted : 22nd September* 1976)

Abstract : A comparative study of different methods of amperometric titration of the sulphhydryl group is described. The factors varied included: (1) the microelectrode, (2) the titrant, (3) pH and (4) voltage. The method was successfully applied to coconut toddy to determine both RSH and RSSR content. Studies with coconut toddy gave further support to the theory that cysteine provides the sulphur for H_2S formation in fermenting toddy and also indicated why some yeast strains do not produce H_2S during the fermentation of (sweet) toddy.

1. Introduction

The sulphhydryl content of biological material can be measured by several chemical and polarographic methods.[3,4,5,9,11]. Amperometric titrations have often been employed in the determination of sulphhydryl (SH) groups in organic compounds and have been found to be superior to chemical methods of determination. The accuracy and precision of the results from amperometric titrations as well as the convenience of the method depend on several factors such as :

(i) microelectrode system
(ii) titrant
(iii) reaction conditions (pH, voltage).

Although considerable work has been done in this field, a comparative study of the different methods (varying the above factors) have not been reported. Furthermore, unlike in this study, most of the reported work has been done using semi-automatic or automatic polarographs.

Sulphhydryl groups can be titrated against a titrant containing any one of the following ionic species.[11]

(*a*) Titrants involving mercury.

(*b*) Titrants involving silver.

(*a*) *Titrants involving mercury.* The reaction of Hg(II) species with mercaptans can either be monovalent or divalent with respect to mercury.

*These studies form a part of the M.Sc. thesis University of Sri Lanka, Colombo Campus, of M. K. G. S. Kalyananda.

$$RSH + Hg^{+2} \longrightarrow (RS)_2Hg \qquad \text{Divalent} \ldots\ldots (1)$$

$$RSH + Hg^{+2} \longrightarrow (RS)_2Hg_2 \qquad \text{Monovalent} \ldots\ldots (2)$$

In these studies it was found that the relative stability of these two mercaptans and hence the valency of mercury in this reaction is governed by several factors such as titrant and the pH of the medium. Selection of these conditions fixes the valency of mercury in the above reaction.

(*b*) *Titrants involving silver species.* The silver ion can react with mercaptans according to the following equation.

$$RSH + Ag^{+} \longrightarrow RSAg.$$

However, titrations carried out in ammonia-ammonium nitrate or in "tris" buffer have given abnormally high values (about 130% of the theoretical values) on titration with known quantities of cysteine hydrochloride ($HS\text{-}CH_2\text{-}CH(NH_2)COOH.HCl$) This is because the silver ion reacts not only with the sulphhydryl group, but also with some other site in the cysteine molecule.[11] This error can be minimised by carrying out the titration in the presence of small concentrations (0.1M) of sodium sulphite. However, the presence of SO_3^{2-} complicates the interpretation of the results if the medium contains disulphides (RSSR) as the latter react with sulphite ions according to the following reaction resulting in the formation of reduced sulphhydryl (RSH) which in turn reacts with Ag^{+}, forming RSAg :.

$$RSSR + SO_3^{-2} \longrightarrow RSSO_3^{-} + RS^{-} \ldots\ldots (3)$$

Furthermore, a sharp end point is obtained when mercury(II) compounds are used as the titrants, because (i) mercury(II) mercaptides are much more stable and considerably less dissociated than the corresponding silver complexes, (ii) although mercaptides of both mercury and silver can form complexes with excess metal ion the difference in stabilities between normal Hg(II) mercaptides and the complex with excess metal ion is much greater than the corresponding differences of the silver mercaptan system.[11]

In the different amperometric titrations described below, compounds containing Hg(II) species were used as the titrant. This paper describes a comparative study of the different methods of amperometric determination of the SH group using a home made polarograph and also introduces the application of a rotating, hanging, mercury-drop electrode in amperometric titrations of the sulphhydryl groups. The paper also deals with the application of this method in studies of the mechanismn of hydrogen sulphide formation in fermenting toddy.

2. Experimental

2.1 RSH determination

2.1.1 *Polarograph*

A simple polarograph was constructed using two rheostats, microammeter, avometer, saturated calomel electrode and a 2V battery of 120 Amperage. The electrical circuit of the polarograph employed is shown in figure 1.

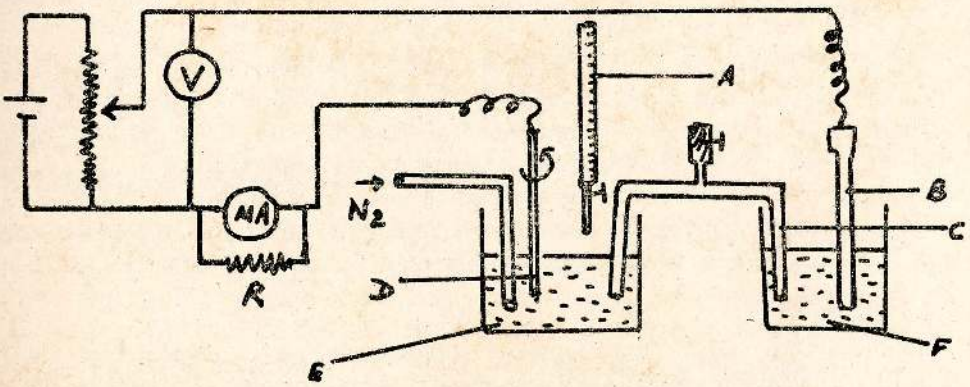

FIGURE 1—Diagrammatic representation of electrical circuit of the polarograph

A Microburette B Saturated calomel electrode
C Salt bridge (saturated solution of KCl with 3% Agar)
D Rotating hanging Hg drop electrode E Sample F Saturated KCl

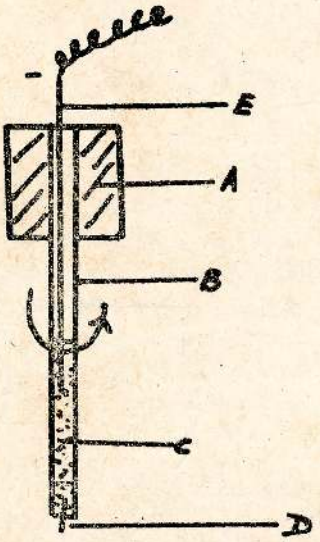

FIGURE 2—Rotating hanging Hg drop electrode

A Motor B Glass tube C Hg column D Gold plated Pt wire E Electrical connection

2.1.2 *Microelectrode system*

Two microelectrode systems were tried out.

(*a*) *Rotating Platinum Electrode.* A platinum wire (1 cm long, and 0.5 mm diameter) was sealed through the closed lower end of a glass tube (5 mm diameter) and electrical connections were made through a column of mercury.[11] It was fixed vertically to the axis of a motor. The effective length of the platinum electrode was 3 mm and the speed of rotation was 600 r.p.m.

(*b*) *Rotating hanging mercury drop electrode.* A platinum wire (1 cm long and 0.5 mm diameter) was sealed through the closed lower end of a glass tube so that 0.5 mm of the wire projected from the end of the rod. This projection was plated with a thin coating of gold and a drop of mercury, equivalent in size to one drop from a dropping mercury electrode, was attached to the gold plated wire. The electrical connection was made through a column of mercury.[3] (Figure 2). The electrode was fixed to a high speed mechanical motor rotating vertically (600 r.p.m.).

2.1.3 *Reagents*

Titrants : (*a*) 0.005M mercuric chloride solution (prepared using analytical grade $HgCl_2$) (*b*) 0.005M ethylmercury chloride (C_2H_5HgCl recrystallised) solution.

Samples : (*a*) 5×10^{-3}M cysteine solution.

(*b*) 5×10^{-3}M cysteine hydrochloride solution.

Buffers : (*a*) 0.02M acetate buffer pH 4.76

(*b*) 0.1M borax solution pH 9.5.

2.1.4 *The Amperometric Titration*

KCl (10 ml of 1M) was added to the sample (0—30 ml) in a clean beaker (150 ml). The solution was made upto 100 ml with buffer, and used as the titration cell in the circuit (fig. 1). This solution was de-aerated by a stream of nitrogen for 12 to 15 min. and the sulphhydryl group (SH) was titrated at a potential V with the titrant from the semimicroburette. The diffusion current corresponding to each titrant volume was recorded, and the endpoint determined from a plot of current and titrant volume (figures 3, 4 and 5). This titration was carried out under varying conditions, and after each addition of 1 ml of titrant a stream of nitrogen was passed through the sample for about 30 seconds.

2.1.5 *Experimental modification using toddy*

In the determination of RSH in toddy a known amount of toddy (40—80 ml) was taken into a 150 ml beaker and 1N NaOH was added to neutralise half of the total acid present in the toddy sample. The solution so obtained had a pH of about 4.75.

10 ml of 1M KCl was then added and the solution was made to 100 ml with acetate buffer (0.02M, pH 4.76). The rest of the procedure was the same as that described under section 2.1.4.

2.2. RSSR determination

The experimental procedure adopted in the determination of RSSR was the same as that described in section 2.1.4. except that the solution was made 0.1M with respect to sulphite prior to the titration, by adding the required quantity of solid Na_2SO_3 into the de-aerated medium. The final volume was adjusted to 100 ml with borax buffer. The addition of SO_3^{2-} converts all RSSR into RS^- and $RSSO_3^-$ according to equation (3).

2.3 RSH determination by ferricyanide method

Freshly prepared cysteine solution (0.20 mg/ml) was cooled in the water and phosphate buffer (0.2ml of 1M, pH, 6.7), sodium nitroprusside (0.1 ml of a 5% solution) and conc. NH_4OH (0.2 ml) were added. This resulted in a red colouration which was titrated rapidly with $K_3Fe(CN)_6$ (with continuous shaking). Standardisation of $K_3Fe(CN)_6$ was carried out with 3 different concentrations of cysteine solutions. Toddy (10 ml) was titrated with $K_3Fe(CN)_6$ using the same procedure.

2.4 H_2S determination

H_2S in fermented toddy was determined by the method described previously.[7]

2.5 Synthetic media

Czapex-Dox medium containing 15% glucose was used as the base to which was added the sulphur containing compound.

2.6 Materials and cultures

Sweet toddy suitable for these experiments was obtained by collecting the fresh sap of the coconut inflorescence in polythene bags. Yeast strains from the CISIR collection were used in this study and cultured as described previously.[6,7]

3. Results

3.1 RSH Determination

Results obtained under seven different methods (table 1) of amperometric titration are given in tables 2, 3, 4, 5, 6, 7 and 8 respectively. The relative advantages and disadvantages are described in section 4. The titration curves using these methods are given in figures 3, 4, and 5.

TABLE 1—Method used in RSH determination

Conditions	Micro-electrode system	Titrant	pH	Applied voltage	Other reagent added
Method 1	Rotating Pt electrode	$HgCl_2$	9.5	—0.2 V	—
Method 2	Rotating Pt electrode	$HgCl_2$	4.76	—0.45	—
Method 3	Rotating Pt electrode	EtHgCl	4.76	—0.45	—
Method 4	Rotating hanging Hg drop elec.	$HgCl_2$	4.76	—0.45	—
Method 5	Rotating hanging Hg drop elec.	EtHgCl	4.76	—0.45	—
Method 6	Rotating hanging Hg drop elec.	EtHgCl	9.5	—0.45	0.2M in Na_2SO_3
Method 7	Rotating hanging Hg drop elec.	EtHgCl	9.5	—0.70	0.2M in Na_2SO_3

See section 2 for details.

TABLE 2—Determination of cysteine by method 1.

Volume of 5×10^{-3}M cysteine (ml)	Observed end-point (ml)	Theoretical end-point (ml)
2.50	3.10	2.50
5.00	6.10	5.00
7.50	9.40	7.50
10.00	12.00	10.00

The general pattern of the titration curve under this condition is shown in fig. 3.

See Table 1 and section 2.1 for details.

TABLE 3—Determination of cysteine by method 2.

Volume of 5 × 10^{-3}M cysteine (ml)	Observed end-point (ml)	Theoretical end-point (ml)
10.00	5.00	5.00
20.00	10.20	10.00
30.00	15.00	15.00

The general pattern of the titration curve under these conditions is shown in fig. 3.

See Table 1 and section 2.1 for details.

TABLE 4—Determination of cysteine by method 3.

Volume of 5 × 10^{-3}M cysteine (ml)	Observed end-point (ml)	Theoretical end-point (ml)
5.00	2.30	2.5
10.00	4.90	5.0
15.00	7.30	7.5
20.00	10.10	10.0

The general pattern of the titration curve is shown fig. 3.

See Table 1 and section 2.1 for details.

TABLE 5—Determination of cysteine by method 4.

Volume of 5 × 10^{-5}M cysteine (ml)	Observed end-point (ml)	Theoretical end-point (ml)
5.00	2.50	2.50
10.00	5.00	5.00
15.00	7.20	7.50
20.00	10.00	10.00

The general pattern of the titration curve is shown in fig 4.

See Table 1 and section 2.1 for details.

TABLE 6—Determination of cysteine method 5.

Volume of 5 × 10^{-3}M cysteine (ml)	Observed end-point (ml)	Theoretical end-point (ml)
2.50	2.50	2.50
5.00	5.00	5.00
10.00	10.30	10.00
15.00	14.50	15.00

The general pattern of the titration curve is given in fig. 4.

See Table 1 and section 2.1 for details.

TABLE 7—Determination of cysteine by method 6

Volume of 5×10^{-3}M cysteine (ml)	Observed end-point (ml)	Theoretical end-point (ml)
10.00	9.0	10.00
10.00	9.0	10.00
15.00	13.5	15.00
* 20.00	16.5	20.00
* 10.00	8.3	10.00

*Cysteine hydrochloride was used. The pattern of titration curve is given in fig. 5.

See Table 1 and section 2.1 for details.

TABLE 8—Determination of cysteine by method 7.

Volume of 5×10^{-3}M cysteine hydrochloride (ml)	Observed end-point (ml)	Theoretical end-point (ml)
10.00	10.00	10.00
20.00	20.30	20.00

The general pattern of the curve under these conditions is shown in fig 5, curve 7.

See Table 1 and section 2.1 for details.

TABLE 9—Determination of RSSR by modified methods 6 and 7

Conditions	Cystine (mg)	Observed end-point (ml)	Theoretical end-point (ml)
Method 6	12.00	11.10	10.00
Method 6	12.00	9.20	10.00
Method 6	6.00	5.00	5.00
Method 7	12.00	9.30	10.00
Method 7	6.00	5.00	5.00

See Table 1 and section 2.2 for details.

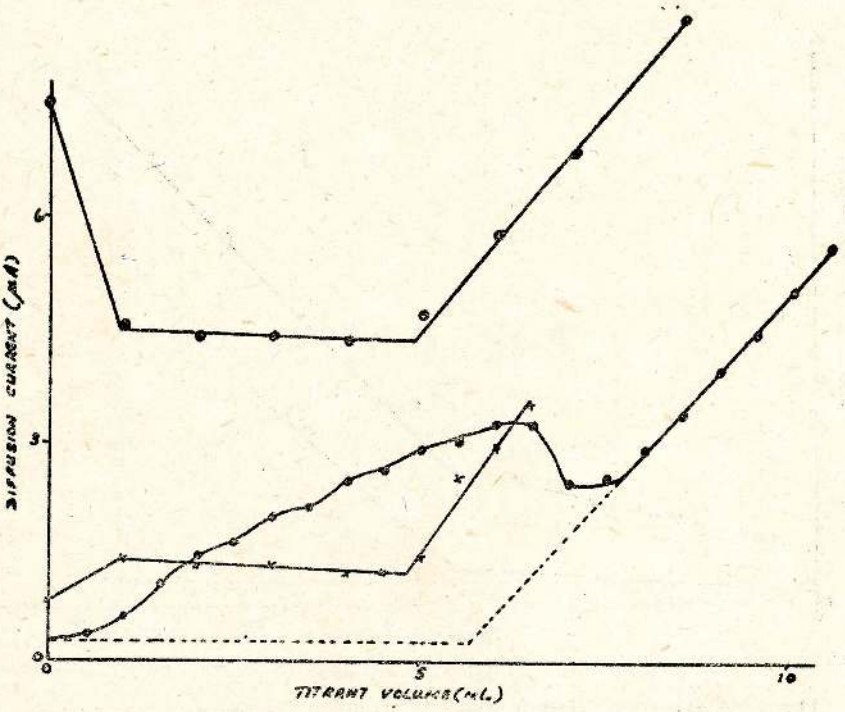

FIGURE 3—Titration curves for different methods of amperometric analysis

O — O, Method 1.
X — X, Method 2.
⊙ — ⊙, Method 3.

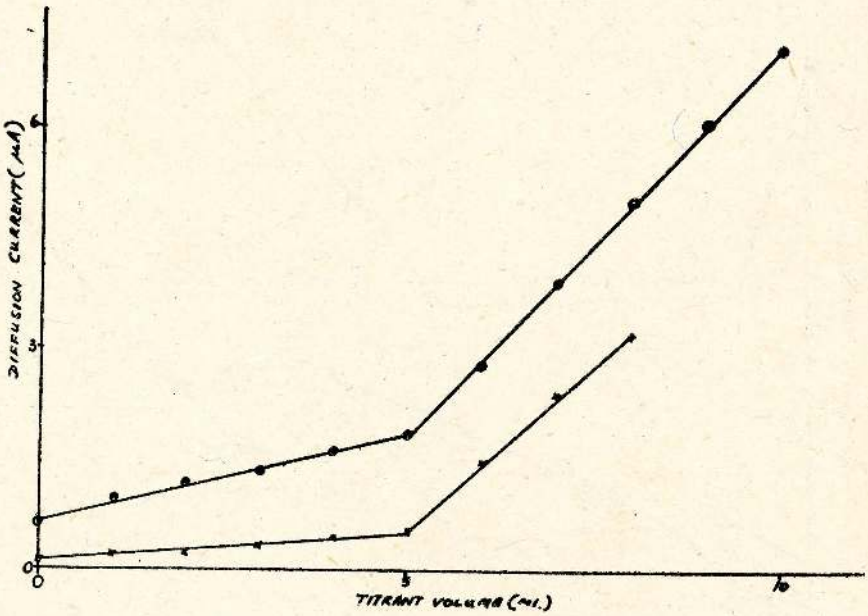

FIGURE 4—Titration curves for different methods of amperometric analysis

X — X, Method 4.
⊙–⊙, Method 5.

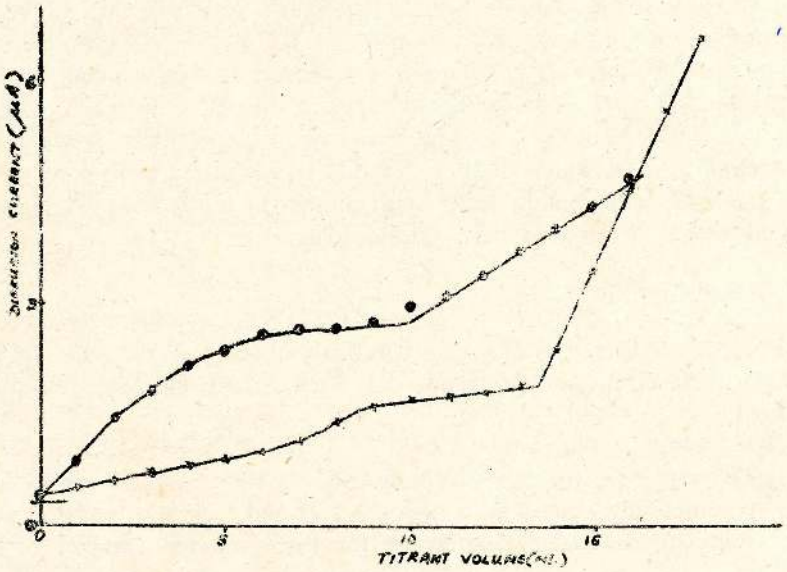

FIGURE 5—Titration curves for different methods of amperometric analysis

X—X, Method 6.
⊙–⊙, Method 7.

3.2 RSSR Determination

RSSR content was determined by the use of SO_3^{2-} as described in section 2.2 and discussed in section 4. Results are shown in table 9.

3.3 Application of method to toddy

3.3.1 *Preliminary Studies*

In using toddy the main problem was its low sulphhydryl content. In the determination of SH content in the above methods accurate results could be obtained only with a large quantity of toddy (40—80 ml). On the other hand when the volume of the sample (40—80 ml) was very large the procedure described under section 2.1.4. could not be used due to the difficulty of adjusting the pH of the medium (to 4.76) by the addition of acetate buffer. This problem was overcome by the method described under section 2.1.5 in which the pH of toddy was adjusted to 4.76 by neutralising half of the total acid (mainly acetic acid) present in toddy with 1N NaOH. The operation needs to be done with extreme care.

3.3.2 *Evidence for specificity*

As there can be no direct proof for the specificity of this method of determination of RSH content, several indirect methods were used. The evidence can be summarised as follows.

(*a*) Different amperometric methods used on toddy yielded the same result : e.g. methods 4 and 5 gave values of 1.21 and 1.21, 1.21 and 1.21, and 0.09 and 0.07 mM. SH respectively for three selected samples of toddy.

(*b*) Results using the amperometric method tallied with the $K_3Fe(CN)_6$ micro-titration[1,2] for SH group (using sodium nitroprusside as indicator).

(*c*) Analysis of the amperometric titration curve for toddy in the presence and absence of cysteine showed that the shift in end point corresponded to the amount of cysteine added. In addition there was no break in the titration curve until the new endpoint was reached.

(*d*) Analysis of the H_2S formed by fermentation of synthetic media showed that an initial cysteine content of about 1.6 mM produced about 0.13 mM H_2S. It was also found that the SH content of toddy (determined by the assay) that produced on fermentation the same quantity of H_2S was also of the order of 1.6 mM. In both sets of experiments the same strain of yeast was used.

3.3.3 *RSH and RSSR content of toddy*

The RSH content determined by method 5, showed that all samples of fresh sweet toddy analysed (over 25 samples), contained an SH content in the range of 1.4 to 1.9 mM, whereas the RSSR content of all these samples was negligible. However, on autoclaving sweet toddy, there was a dramatic decrease in RSH content and a corresponding increase in RSSR content. Further, on standing for a period of weeks the RSH content virtually declined to zero. Typical results showed that the —SH content of a sample of autoclaved toddy immediately after autoclaving and 7 days later was 0.6 mM and 0.45 mM respectively. At the latter stages RSSR content of autoclaved sweet toddy was sometimes as high as 0.9 mM (as SH).

When toddy was fermented with H_2S producing yeast strains (strains Nos. 2 and 20 of the CISIR yeast collection)[7,10] the final RSH content was generally zero although on one occasion a value of 0.09 mM was observed. RSSR content of fermented toddy on the other hand was high, generally in the range of 0.75 to 0.90 mM (as SH).

3.3.4 *Relationship between H_2S produced and RSH content*

Experiments carried out with autoclaved sweet toddy samples showed that variation of RSH content in these samples paralleled H_2S produced (table 10). Similar results were obtained using a synthetic medium under the same fermentation conditions (table 11).

TABLE 10—Effect of RSH content in autoclaved sweet toddy on H_2S produced

Sample	Initial RSH (mmoles/l)	H_2S produced (mmoles/l)
Tree No. 1	0.45	0.052
Tree No. 2	0.16	0.019
Tree No. 9	0.02	0.003
Tree No. 10	Nil	Nil

Aged autoclaved sweet toddy was analysed for SH content and fermented with a H_2S producing yeast strain (No. 20). H_2S was estimated after complete fermentation.

TABLE 11—H_2S formation in synthetic fermenting medium

Added cysteine (mmoles/l)	H_2S produced (mmoles/l)
0.3	0.015
0.6	0.043
0.9	0.110
1.2	0.097

To Czapex-Dox medium modified as in section 2.4 was added cysteine in the above quantities. The H_2S formed was determined after complete fermentation.

3.3.5 *Relationship between RSSR and H_2S formation*

Studies carried out with a synthetic medium (table 12) as well as those with toddy (table 13) led to the conclusion that cystine (RSSR) has no significant effect on H_2S formation during fermentation.

Samples with different initial RSH concentrations produced H_2S in quantities proportional to the initial RSH content. This was observed in the studies carried out on a synthetic medium (Czapek-Dox solution containing 15% sugar) using freshly grown yeast cultures 2 and 20.[7] The results showed that the RSH present initially was ultimately converted to RSSR and H_2S after fermentation. (Table 14) Further, it was found that the conversion of RSH to other forms (H_2S and RSSR) was quantitative, i.e. total sulphur in the form of H_2S, RSSR and RSH in the fermented product was often equal to the initial sulphur (present as RSH).

TABLE 12—Effect of RSSR on H_2S formation in a synthetic fermenting medium

Sample	Initial RSH (mmoles/l)	Initial RSSR (as SH) (mmoles/l)	H_2S produced (mmoles/l)
Synthetic medium (250ml)	Nil	Nil	Nil
Synthetic medium (250ml) + 11 mg cysteine	0.32	Nil	0.060
Synthetic medium (250ml) + 20 mg cystine	Nil	0.66	0.002

H_2S was determined after complete fermentation with H_2S producing strains (2 and 20).

TABLE 13—Effect of cystine (RSSR) on H_2S formation in fermenting toddy

Sample	Initial RSH (mmoles/l)	Initial RSSR (mmoles/l) (as SH)	H_2S produced (mmoles/l)
Autoclaved sweet toddy* (250ml)	Nil	0.76	Nil
Autoclaved sweet toddy (250ml) + 17mg cysteine	0.56	0.76	0.06
Autoclaved sweet toddy (250ml) + 30 mg cystine	Nil	1.78	Nil

Sweet toddy (aged and autoclaved) containing no —SH and 0.76 mmoles of RSSR was fermented after addition of cystine and cysteine and H_2S determined after complete fermentation.

*The sweet toddy sample used here was two weeks old.

3.3.6 *Results using attenuated yeast*

An interesting set of results was obtained when using attenuated yeast cultures. When yeast strains 2 and 20 (which are normally H_2S producing) were stored in a refrigerator at 5°C for about a week they no longer produced H_2S even though they could carry out fermentation efficiently. Study of a time-course of fermentation using these attenuated yeasts showed that even when fermentation was complete (at 36h) the SH content only fell from 1.65 mM to 1.50 mM and only traces of RSSR were formed ; H_2S was not detected. This showed that the yeast strains could not utilize RSH in toddy to any significant extent. It was also observed that the attenuated yeast could produce H_2S from added cysteine suggesting that the yeast differentiated between —SH in toddy and —SH of added cysteine. This failure to produce both H_2S and RSSR was of great interest because the same strain when not subjected to attenuation produced both H_2S and RSSR.

4. Discussion

(i) On comparing curves 1 to 7 it was found that all the titration curves were regular after the endpoint. However, the regularity of curves before the endpoint varied markedly, the rotating hanging mercury drop electrode (HMDE) being superior to the rotating platinum electrode for sulphhydryl determination. In using HMDE there were also other advantages such as (*a*) easy cleaning, (*b*) durability and (*c*) high sensitivity.

A sharp end point was not obtained only with method 1. Results using method 1 were precise to ± 1%[14] with a constant positive deviation of 27%. The end point of titrations obtained under conditions 2, 3 and 4 corresponded to the bivalent state of Hg whereas those obtained under methods 1, 5, 6 and 7 corresponded to the monovalent Hg.

The results of method 2 were precise to ± 1%. Although irregularities in the titration curve in method 2 were partly eliminated in method 3, a very high initial residual current was observed, which interfered with the estimation of low sulphhydryl concentrations (10^{-4} mg/l). However precision and accuracy remained unchanged at the ± 1% levels even under these conditions. The irregularities of method 3 were completely eliminated when the rotating platinum electrode was replaced by the rotating hanging mercury drop electrode and this is illustrated in the results obtained with methods 4 and 5 (fig 4); sensitivity was also increased under these conditions. This difference can be attributed to : (*a*) the high hydrogen overpotential and (*b*) the comparatively high cathodic range of mercury compared to platinum. Although results obtained were much more precise (± 1%) when method 4 was used instead of method 5, (which had a degree of precision of ± 2.5%), the latter method was more suitable for the estimation of small quantities of RSH. This was due to the fact that EtHgCl is monovalent when method 5 is used whereas $HgCl_2$ is divalent in method 4.

Of the two methods developed for the determination of RSSR, method 7 was more precise and accurate compared to method 6 ; a constant error of about 10% was observed in method 6. However, curve 6 of fig 5 reflects the superiority of method 6 over method 7 in the determination of small quantities of RSSR because of the low initial residual current of the former.

Method 5 was applied successfully in the determination of RSH in toddy. Sweet toddy was found to contain an SH content in the range of 1.4 to 1.9 mM. H_2S producing yeast strains (cultures No. 2 and 20) convert only a small fraction of this RSH into H_2S. However, the study demonstrates the involvement of the RSH group in the formation of H_2S confirming the conclusions of previous reports.[7,10] It is significant that this mechanism is very different from that of H_2S formation in beer which is brought about by the reduction of sulphite.[8,12,13]

Although evidence is by no means complete, we propose the following hypothesis: most of the cysteine (SH) in toddy is in the form of protein. In this state it will remain largely unoxidised unless the proteins are denatured (e.g. autoclaving as done in this study) or hydrolysed (as in the case of fermentation by yeast strain 2 and 20).

The lack of oxidation of RSH to RSSR in the experiment with attenuated yeast strains supports this. This is also supported by : (1) the absence of oxidation of SH in fresh toddy and (2) the differentiation between added cysteine and —SH in toddy by the attenuated yeast. That is, H_2S production in toddy can be visualized as a two-step process as given below :

	STAGE 1			STAGE 2	
Cysteine (bound in protein)	⟶ Hydrolysis	Cysteine (free) Cystine (RSSR)	(R—SH)	⟶ Cysteine de-sulphhydrase	H_2S

This two-step process involves the release of cysteine from protein in the first step which will explain the observation that H_2S and RSSR are always formed together or not at all (as in the case of attenuated yeast where both RSSR and H_2S were not formed).

This concept can account for the existence of non-H_2S producing strains of yeast (as shown in our earlier study).[7,10] This could be due to one of two reasons : (1) the lack of cysteine desulphhydrase as shown by yeast cultures No. 39 and 40 which cannot release H_2S from cysteine and (2) the lack of protein hydrolysing activity as suggested by attenuated cultures 2 and 20. More work is proceeding on our culture collection to screen strains of yeast for the absence of one or more of the necessary stages of H_2S production. A third possibility which is more remote is that RSH may be converted by some non-H_2S producers to a form which is not available for H_2S production.

Other results of ours have indicated that the effect of iron in increasing H_2S production may be due to an interference with the $2RSH \longleftrightarrow RSSR$ equilibrium. Work on this aspect is continuing.

Our previous study showed that H_2S formation in toddy can be controlled by (*a*) choice of strain of yeast and (*b*) addition of NH_4^+. This study provides a clue to other methods of reducing H_2S formation which could take the form of (1) promoting the conversion of cysteine to cystine and (2) inhibiting protein hydrolysing activity.

The described method of estimation of RSH and RSSR appears highly promising and should be of immense value in furthering current studies on the mechanism of H_2S formation in coconut toddy and other biological systems.

TABLE 14—Fermentation in synthetic medium

Sample	Initial* RSH (mmoles/l)	Residual RSH in fermented sample (mmoles/l)	RSSR in ≠ fermented sample as SH (mmoles/l)	H_2S produced (mmoles/l)
1	0.375	0	0.27	0.10
2	0.75	0	0.54	0.21
3	1.125	0	0.91	0.23
4	1.50	0.24	N.D.	0.31
5	1.875	0.48	N.D.	0.42

*Initial RSH is from added cysteine hydrochloride.

≠ Method 6 was employed in RSSR determination.

H_2S, RSSR and RSH were determined after complete fermentation.

N.D., Not determined as instrument broke down.

See section 2 for experimental details.

Acknowledgements

The authors wish to thank the Director, CISIR, for laboratory facilities and the National Science Council for a grant to Mr. M. K. G. S. Kalyananda. The authors also thank Miss Kanthi Jayaratne for secretarial assistance.

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Cyanide liberation from linamarin

III. Separation of the linamarases of manioc rind by DEAE-cellulose chromatography

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(*Paper accepted : 22nd September* 1976)

Abstract : Our previous studies showed that manioc rind contained two linamarin hydrolysing enzymes, linamarase A and B. Studies using DEAE—cellulose chromatography of rind extracts of eight cultivars of *Manihot esculenta* Crantz showed the presence of 4 to 6 cyanide liberating activity peaks, the major activity peaks being due to linamarase A and B in all but one case. Studies also showed that linamarase A could be a sub-unit of linamarase B. Some properties of linamarase C and D are also described.

1. Introduction

Multiple cyanogenic glucosidases have been reported in a few cyanogenetic plant species. Hughes[1] reported that the callus tissue of white clover stems contained two β glucosidases capable of hydrolysing linamarin, one specific and the other non-specific which differed widely in their Michaelis constants. Stevens and Strobel[5] isolated two linamarases from a psychrophilic basidiomycete. The β glucosidases, which were separated on a DEAE-cellulose column, showed a marked variation in their Michaelis constants, pH optima, temperature stability and energy of activation.

Our previous studies[4] showed that two cyanide liberating enzymes could be separated by DEAE-cellulose chromatography from rind extracts of *Manihot esculenta* Crantz (manioc). The initial aim of this study was to determine if these two enzymes were present in all common cultivars of manioc. The paper describes the linamarase activity profile of DEAE-cellulose column eluates of manioc rind extracts of 8 cultivars of manioc and compares some of the more important properties of linamarase A, B, C and D.

2. Experimental

2.1 Plant material

Manioc cultivars were obtained from the Central Agricultural Research Institute, Gannoruwa, by courtesy of Dr. (Mrs). C. Breckenridge.

2.2 Crude preparations of linamarase

These were prepared by acetone precipitation as described by Wood.[6]

* This paper forms a part of the Ph.D. Thesis, University of Sri Lanka [Colombo Campus] of Nirmala Pieris.

2.3 DEAE—cellulose chromatography

This was performed, by the procedure described previously[4], directly on the dialysed acetone precipitated fraction.

2.4 Assays

Absolute enzyme activity of fractions off the DEAE-cellulose column was measured as described previously,[4] cyanide being determined (in the presence of glucose) by the method of Wood.[2,3,6]

2.5 Properties of the enzymes

Temperature stability, temperature optimum and energy of activation were determined on linamarase C and D as described previously for linamarase A and B.[4] The effect of concentration on enzymic activity was tested by incubating linamarin (20 mM). 0.08M citrate buffer (pH 5.6) and linamarase in varying concentrations. Reaction time varied so that the same amount of cyanide would be liberated in all cases if an enzyme concentration effect was absent. Reaction volume was 0.43 ml and the reaction was carried out at room temperature (29°C).

3. Results

3.1 Studies on cultivars

Crude preparations of the rind of 10 cultivars of manioc were obtained by homogenisation in acetate buffer followed by acetone precipitation.[6] The cultivars used were MU-71, Selection 3, MU-18, MU-46, MU-51, JAVA, MU-22, MU-64, MU-10 and MU-44. The concentrated protein precipitate was dialysed against 0.01M acetate buffer, pH 5.5 and activity measured. Linamarase activity of cultivars MU-18 and MU-10 was very low and therefore further studies were done only with the other 8 cultivars. DEAE-cellulose chromatogaphy showed that there were 4 consistent linamarase activity peaks with varying degrees of activity at elution volumes of 104, 116, 128 and 166 ml respectively, which have been called linamarase D, A, B and C respectively. In addition, other minor peaks were observed. The four main activity peaks were present in all cultivars except the cultivar JAVA, where only the activity peaks of linamarase D and A were present (figure 1). The activity peaks most prevalent were those of linamarase A and B. It was found during the course of these studies that reproducibility observed with the DEAE-cellulose column used was very high.

3.2 Sub-unit structures of linamarase B

DEAE-cellulose chromatography of extracts of linamarase A and B in the presence of 4M urea showed that, whereas linamarase A maintained its position on the DEAE-cellulose elution pattern in the presence of urea, linamarase B split up into two peaks one each corresponding to linamarase A and B (figure 2). This showed that linamarase A was a modified form of linamarase B, probably an active sub-unit.

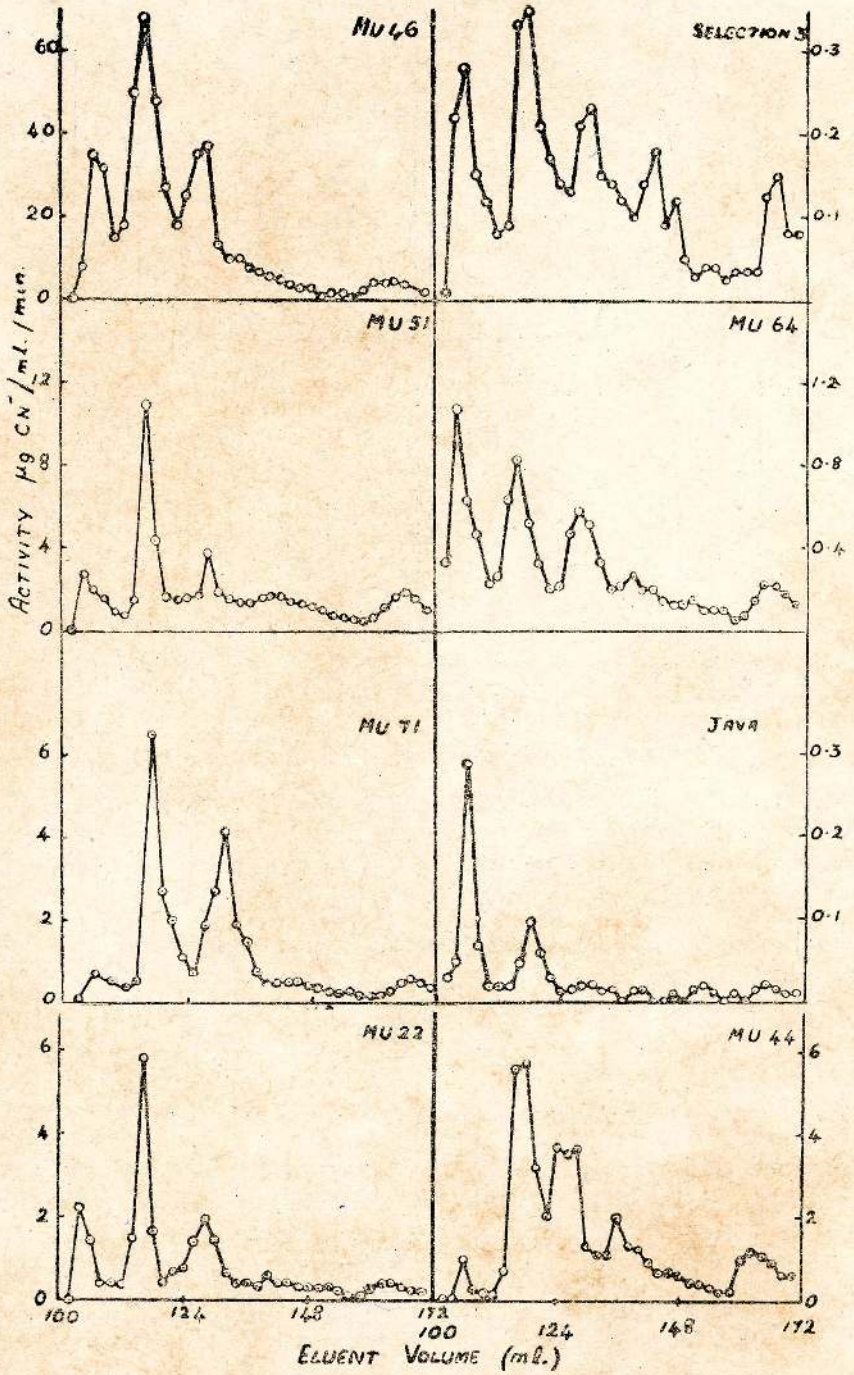

FIGURE 1—*DEAE-cellulose chromatography of rind extracts of various cultivars.*

Bed Volume, 34 ml; Rate of Elution 25 ml/h. Eluents used were as follows: 0.01M Acetate buffer pH 5.5 containing (*a*) no NaCl added (30ml) (*b*) 0.1M NaCl (15ml) (*c*) 0.15M NaCl (15ml) (*d*) 0.2M NaCl (15ml) (*e*) 0.25M NaCl (15ml) (*f*) 0.3M NaCl (30ml) (*g*) 0.4M NaCl (30ml). Symbols at top right hand corner of each inset refer to the name of cultivar.

The eluent volumes of linamarase A, B, C and D were 116, 128, 166 and 104 ml respectively.

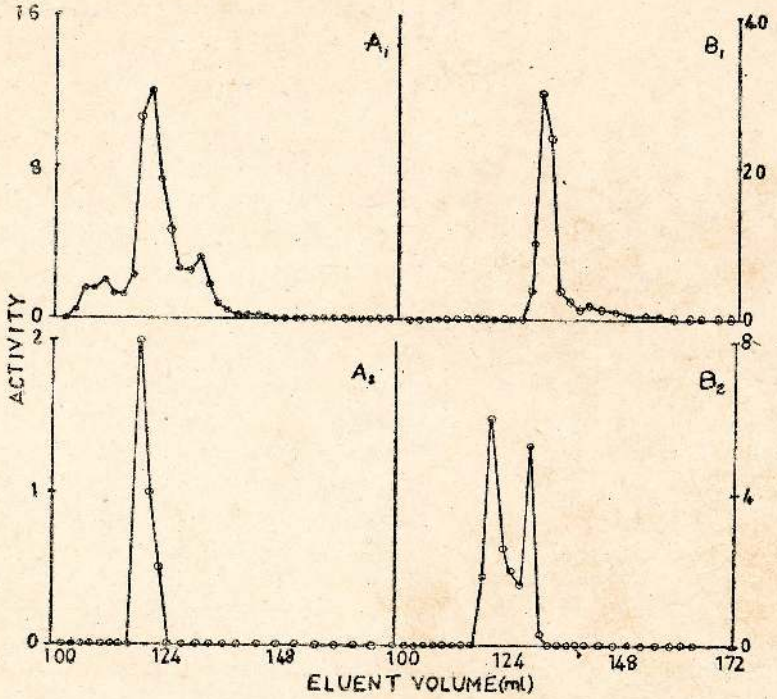

FIGURE 2—*Effect of 4M urea on linamarase A and B.*

A_1—Peak of MU 46 linamarase A, (see figure 1) rechromatographed on a DEAE-cellulose column.

A_2—Peak of A_1 rechromatographed on DEAE-cellulose after exposure to 4M urea.

B_1—Purified linamarase B rechromatographed on DEAE-cellulose.

B_2—Peak of B, rechromatographed on DEAE-cellulose after exposure to 4M urea.

Other details as in figure 1.

3.3 Some properties of linamarase C and D

The properties of linamarase A and B have been described in a previous communition.[4] Similar studies on linamarase C and D showed that like linamarase A and B, linamarase D had a disproportionate increase in activity with increased enzyme concentration. The opposite was found to be true for linamarase C which showed negative deviations (table 1). A time course of cyanide liberation of these two enzymes is shown in figure 3.

TABLE 1—Effect of enzyme concentration on activity of linamarase C and D.

Linamarase	Volume of extract (ml)	Cyanide released (umoles/min)	Activity (umoles/ml/min)
C	0.1	0.05	0.50
	0.2	0.098	0.49
	0.4	0.130	0.33
D	0.1	0.044	0.44
	0.2	0.098	0.49
	0.4	0.210	0.53

Concentration of protein in extract for enzyme D was 0.065 mg/ml. The corresponding value for enzyme C for this table of results is not available as the sample was lost in a laboratory accident.

For experimental details see section 2.

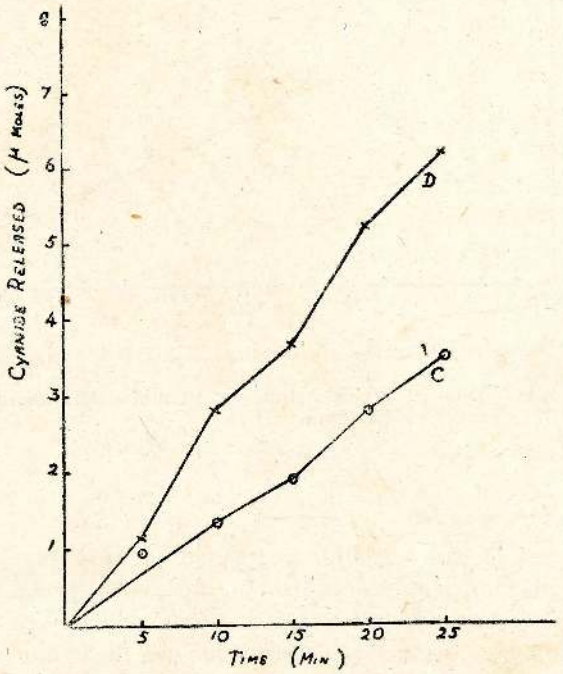

FIGURE 3—*Time course of initial reaction of linamarase C and D with linamarin*

The reaction mixture contained linamarin (25 mM) citrate buffer(0.08M, pH 5.6) and linamarase in a total volume of 0.37ml for each experimental point. The reaction which was carried out at room temperature was terminated by addition of Na_2CO_3—picric acid reaction mixture.[6]

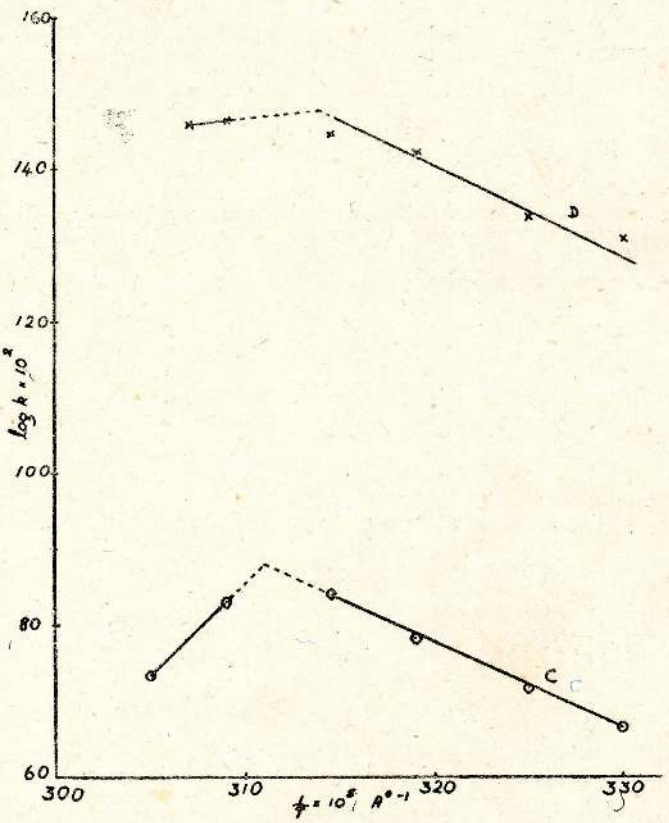

FIGURE 4—*Arrhenius plots of the activity of linamarase C and D at various temperatures.*

Experimental details as in figure 3. Reaction time was 10 min. and linamarase activity for C and D was 0.05 and 0.025 μmoles cyanide/min.

Linamarase C and D had activation energies of 5.2 Cal/mole and 5.4 Cal/mole respectively; Arrhenius plots are shown in figure 4. Temperature optima of linamarase C and D were found to be 45°C and 55°C respectively, while temperature stability studies, in which the enzymes were incubated for 10 min. in a thermostated water bath prior to reaction with linamarin, showed that the two enzymes were 50% deactivated at 55°C and 66°C respectively, (figure 5).[4]

Table 2 gives a summary of some properties of linamarase C and D together with data obtained with linamarase A and B which were reported earlier.[4]

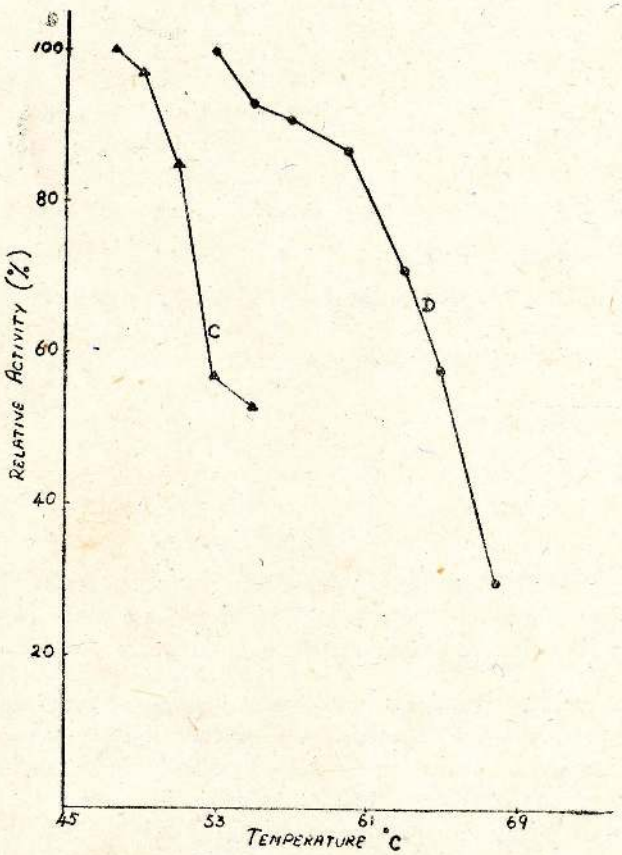

FIGURE 5—*Temperature stability of linamarase C and D.*

The enzymes were held for 10 min. in a thermostated water bath and then reacted at room temperature with linamarin as in figure 3. Reaction time was 20 min. and 10 min. for linamarase C and D respectively.

TABLE 2—Summary of properties of linamarase A, B, C and D.

Property	Linamarase			
	A*	B*	C	D
1. Position on DEAE-cellulose column	116	128	166	104
2. Migration to negative electrode on electrophoresis at pH 5.9 (mm)	23	08	—	—
3. Effect of enzyme concentration (deviation)	+ive	+ive	—ive	+ive
4. Michaelis Constant (K_m)	2.2	2.2—2.5	—	—
5. pH optimum	6—6.6	6.2	—	—
6. E Activation (K Cals/mole)	5.7	3.3	5.2	5.4
7. Temperature of 50% deactivation (°C)	60	62	55	66
8. Temperature optimum (to nearest 5°C)	45	60	45	55

*Published previously.[4]

—, No experimental point.

4. Discussion

Our previous communication[4] clearly demonstrated the existence of 2 linamarases in the extracts of manioc rind and the initial aim of these studies was to determine the distribution of these two enzymes in various cultivars of manioc.

Investigations revealed that multiple forms of linamarase activity were present in the rind of these cultivars. The enzymes were present in different proporuons in the extracts of each cultivar. However, the absolute activity of each enzyme cannot be attributed much significance because of : (1) the possibility of selective loss of enzymes during the acetone purification stage and (2) the possibility that the ratios of the different enzymes could vary depending on factors such as environment and maturity of the tubers (on which no studies have been done). Nevertheless, these studies confirm the presence of several cyanide liberating enzymes which appear to vary in proportion from cultivar to cultivar.

A significant feature was that linamarase A and B represented the bulk of the activity in all cultivars. This fact together with the conversion of linamarase B to linamarase A in the presence of urea suggested that linamarase B is the major cyanoglucosidase of manioc rind while linamarase A is an artifact of acetone precipitation. However, this has to be confirmed since one line of evidence does not support this

conclusion. Namely, the formation of linamarase A from linamarase B does not result in an abnormal degree of loss of activity which leads to the conclusion that linamarase A (the sub-unit or modification) must be as active as the linamarase B (the enzyme *in vivo*). However, the temperature of deactivation of linamarase B is very comparable to temperature of loss of enzymic activity in the tissue (72°C) as reported by Joachim and Pandittasekere[2] giving some support to the theory that linamarase B is the form in which the enzyme is found in nature.

The presence of activity peaks C and D show that there are other proteins (in manioc rind) capable of hydrolysing linamarin. In one instance (MU 46) the activity of linamarase D was too large to be due to a non-specific β glucosidase and therefore the enzyme appears to be a true linamarase. Linamarase C is present only in small quantities in all cultivars and is probably a non-specific β glucosidase. In addition, DEAE column eluates have shown the presence of minor activity peaks.

From these studies it is concluded that multiple forms of linamarin hydrolysing enzymes are present in manioc rind. The significance of this situation is not understood but it will explain why cyanoglucosidase negative cultivars of manioc have not been demonstrated.

Acknowledgements

The authors thank the Director, CISIR, for facilities provided, Mr. E. E. JeyaRaj, Head, Industrial Microbiology Section, CISIR, for encouragement, Dr. (Mrs.). C. Breckenridge for plant material and Miss K. Jayaratne for secretarial assistance.

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J. Natn. Sci. Coun. Sri Lanka 1976 4(2) : 149—155

Absolute convergence factors for integrals

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(*Paper accepted : 11th October* 1976.)

Abstract : The absolute convergence factor problem for integrals of finding conditions necessary and sufficient that $f(x)g(x) = 0(x^{p+q}) \mid C, r \mid$ whenever $f(x) = 0(x^p) \mid C, k \mid$ where r, k are non-negative integers such that $r \geqslant k$, p, q are real and $p + q \leqslant -1$, is considered here. It is shown that the conditions on the convergence factor g restrict it to being either zero or a polynomial.

1. Introduction

Tyler (1) has considered the following absolute convergence factor problem for sequences : If r, k are non-negative integers, what conditions are necessary and sufficient that the sequence $\{ \epsilon_n \}$ be such that $s_n \epsilon_n$ be limitable $\mid C, r \mid$ whenever s_n is limitable $\mid C, k \mid$? The method used by Tyler can be adapted to find the conditions on the sequence $\{ \epsilon_n \}$ in the more general problem : What conditions are necessary and sufficient that $s_n \epsilon_n = 0(n^{p+q}) \mid C, r \mid$ whenever $s_n = 0(n^p) \mid C, k \mid$, where p is real, $p + q > -1$? Tyler's method cannot be used to discuss the case $p + q \leqslant -1$, and this problem has not been considered before.

The author has considered the integral analogue of this problem in the more general case when p is real and q is real ; viz: What conditions are necessary and sufficient that $f(x)g(x) = 0(x^{p+q}) \mid C, r \mid$ whenever $f(x) = 0(x^p) \mid C, k \mid$ where p, q are real, $r \geqslant k$? The conditions on the convergence factor g in the case $p + q \leqslant -1$ turn out to be of character quite different to those in the case $p + q > -1$. It is shown that in the case $p + q \leqslant -1$, $f(x) = 0(x^p) \mid C, k \mid$ can imply $f(x)g(x) = 0(x^{p+q}) \mid C, r \mid$ only when g is zero or belongs to a restricted class of functions. The conditions in the case $p + q > -1$ do not restrict g to such a narrow class.

Leaving out the trivial case $r = k = 0$, it can be shown that if $p + q > -1$, the conditions on g are the following :

(I) If $k = 0$, $r \geqslant 1$, the only condition is that $g \in G_1$; i.e.

$$\int_1^x t^{-q} g(t)\, dt = 0(x) \text{ as } x \to \infty.$$

(II) If $r \geqslant k \geqslant 1$, then $g \in G_2$; i.e.

(i) $g(x) = 0(x^q)$ as $x \to \infty$.

(ii) $g^{k-1}(x) = 0(x^{q+1-k})$ as $x \to \infty$.

The conditions in I and II turn out to be necessary even in the case $p + q \leqslant -1$, but a stronger result is proved here. It is shown that it is necessary that g belongs to classes smaller than G_1 and G_2 in the cases $k = 0$ and $k \geqslant 1$ respectively.

This paper deals with the case $p + q \leqslant -1$ in detail. The case $p + q > -1$ will be dealt with in a subsequent paper.

2. Preliminary Definitions

All functions considered here are real and defined on $[1, \infty)$. If f is Lebesgue integrable locally (i.e. in every finite subinterval of $[1, \infty)$), write $f \in L$. In what follows, take $f \in L$, and if $k = 0$, take g to be bounded and measurable locally, and if $k \geqslant 1$, take g^{k-1} to be absolutely continuous locally, g^m denoting m – th derivative of g, g^0 denoting g.

Let r, k be non-negative integers such that $k \geqslant 0$, $r \geqslant k$.

Let $f_m(x)$ be the m – th integral of $f(x)$; i.e. $I_m f(x) = f_m(x) = \int_1^x f_{m-1}(t)\,dt$ for $m \geqslant 1$, $I_0 f(x) = f_0(x) = f(x)$.

Then, $f_m(x) = \int_1^x \frac{(x-t)^{m-1}}{(m-1)!} f(t)\,dt$ for $m \geqslant 1$.

If p is real, write $f(x) = 0(x^p) \mid C, k \mid$ if $x^{-p-k} f_k(x) \in BV[1, \infty)$ and is $0_R(1)$ as $x \to \infty$. In this paper, take p to be real, $p + q \leqslant -1$.

Theorem. (i) If $k = 0$, $r \geqslant 1$, a necessary and sufficient condition that $f(x) g(x) = 0(x^{p+q}) \mid C, r \mid$ whenever $f(x) = 0(x^p) \mid C, k \mid$ is that

(a) $g(x) = 0$ for almost all $x \geqslant 1$.

(ii) If $r \geqslant k \geqslant 1$, let M be the greatest integer such that $M < p + q + k + 1$; $M \leqslant q$, except in the case $r = k$, $p + q + k + 1 \leqslant 0$, $q \geqslant 0$, when M is taken to be zero. If M turns out to be negative, this is taken to mean $g(x) = 0$. Then, a necessary and sufficient condition that $f(x) g(x) = 0(x^{p+q}) \mid C, r \mid$ whenever $f(x) = 0(x^p) \mid C, k \mid$ is that

(*b*) $g(x)$ is a polynomial in x of degree not exceeding M.

Note. If $M \geqslant 1$, then (ii) means that $g(x)$ is a polynomial of degree less than or equal to M, and if $M \leqslant 0$, then $g(x)$ is constant.

3. Subsidiary Results

Lemma 1. Let N be a positive integer, $X > 1$, $\theta \in L[1, X]$. Then, a necessary condition that $\int_1^x \theta(t)\, s(t)\, dt = 0$ whenever $s^N \in L$ and $s(X) = s^1(X) = \ldots = s^{N-1}(X) = 0$ is that $\theta(t) = 0$ p.p in $[1, X]$.

Proof. Assume that the conclusion is false. Then,

$$\int_1^x |\theta(t)|\, dt = 5K, \text{ where } K > 0. \tag{1}$$

There exists a step function $u(t)$ with a finite number of steps in $[1, X]$ such that $\int_1^x |\theta(t) - u(t)|\, dt < K$. (2)

(1) and (2) give $\int_1^x |u(t)|\, dt > 4K$. (3)

Since $u \in L[1, X]$ there exists $m^1 > 0$ such that, for any measurable subset e of $[1, X]$ with $m\, e < m^1$, we have $\int_e |u(t)|\, dt < K$. (4)

Clearly, it is possible to select a finite set of pairwise disjoint intervals in $[1, X]$ not containing any discontinuity of $u(t)$, such that their union H satisfies $m\{[1, X] - H\} < m^1$.

Thus (4) gives $\int_1^x |u(t)|\, dt - \int_H |u(t)|\, dt < K$ (5)

Now, there exists s such that $s^N \in L$, $s(X) = s^1(X) = \ldots s^{N-1}(X) = 0$

and $s(t) = \operatorname{sgn} u(t)$ in H, (6)

$|s(t)| \leqslant 1$ in $[1, X] - H$. (7)

From (7), (2), (6), (5) and (3) used in that order, it follows that

$\int_1^x \theta(t)\, s(t)\, dt > K$, which is a contradiction, and hence the lemma.

Lemma 2. A necessary condition that $\int_1^x \theta(t)\, s(t)\, dt = 0$, where $X > 1$, $\theta \in L(1, X)$ whenever $s \in L$ and $s(X) = 0$ is that $\theta(t) = 0$ p.p in $[1, X]$.

Proof. Proceedings as in Lemma 1, we see that there exists a step function $u(t)$ such that $\int_1^x |u(t)|\, dt > 4K$.

Clearly, there exists s such that $s \in L$, $s(X) = 0$ and $s(t) = \operatorname{sgn} u(t)$ in $[1, X)$.

Then, $\int_1^x \theta(t)\, s(t)\, dt > 3K > 0$, and hence the lemma.

Lemma 3. If $\phi \in BV[1, \infty)$ and $\delta > 0$, then $t^{-\delta} \int_1^t u^{\delta-1} \phi(u)\, du \in BV[1, \infty)$. See (2), Lemma 3 .

4. Proof of the Theorem

When $r \geq k \geq 1$, repeated partial integration gives

$$I_r f(x)\, g(x) = \int_1^x \frac{(x-t)^{r-1}}{(r-1)!} f(t)\, g(t)\, dt$$

$$= (-1)^k \int_1^x f_k(t)\, (D_t)^k\, G_r(x, t)\, dt + \delta_{rk} f_k(x)\, g(x), \tag{8}$$

where $D_t = \frac{\partial}{\partial t}$, $G_r(x,t) = \frac{(x-t)^{r-1}}{(r-1)!}\, g(t)$ and δ_{rk} is the Krönecker delta.

Formula (8) also holds when $k = 0$, $r \geq 1$.

We want conditions necessary and sufficient that $x^{-p-q-r} I_r f(x)\, g(x) \in BV[1, \infty)$ and is $0(1)$ as $x \to \infty$ whenever $x^{-p-k} f_k(x) \in BV[1, \infty)$ and is $0(1)$ as $x \to \infty$. (9)

Necessity. By (9), it is necessary that $x^{-p-q-r} I_r f(x)\, g(x) = 0$ (1) whenever $f \in L$ and $f_k(x) = 0$ for all $x \geqslant X$, where X is fixed, arbitrary and greater than 1, $k \geqslant 0$. (10)

For such $f, f_1(x) = f_2(x) = \ldots = f_{k-1}(x) = 0$ for all $x \geqslant X$ when $k \geqslant 1$,

and hence (8) gives $I_r f(x)\, g(x) = (-1)^k \int_1^x f_k(t)\, (D_t)^k G_r(x, t)\, dt$

for all $x \geqslant X$. (11)

Hence $I_r fg = \sum_{m=1}^{r} c_m x^{r-m} \int_1^x f_k(t)\, (d/dt)^k [\, t^{m-1} g(t)\,]\, dt,$ (12)

where $c_m = \dfrac{(-1)^{k+m-1}}{(r-1)!} \binom{r-1}{m-1}$.

Formula (12) holds for $k = 0$ too.

Thus (10) and (12) give : $\sum_{m=1}^{r} c_m x^{-p-q-m} \int_1^x f_k(t)\, D^k [\, t^{m-1} g(t)\,]\, dt = 0$ (1)

whenever $f \in L$ and $f_k(x) = 0$ for all $x \geqslant X$. (13)

Let n be the integer such that $-n-1 < p+q \leqslant -n$ if $p+q > -r$, and $n = r$ if $p+q \leqslant -r$.

Then, $x^{-p-q-m} \to +\infty$ as $x \to \infty$ for $m = 1, 2, \ldots n$, and hence each coefficient of x^{-p-q-m} should be zero for $m = 1, 2, \ldots n$ in the series in (13).

Thus $\int_1^x f_k(t)\, D^k [\, t^{m-1} g(t)\,]\, dt = 0$ whenever $f \in L$ and $f_k(x) = 0$ for

all $x \geqslant X$, $m = 1, 2, \ldots n$. (14)

Write $N = k$, $s(t) = f_k(t)$, $\theta(t) = D^k [\, t^{m-1} g(t)\,]$ when $k \geqslant 1$, and $s(t) = f(t)$, $\theta(t) = t^{m-1} g(t)$ when $k = 0$.

Then by (14), $\int_1^x \theta(t)\, s(t)\, dt = 0$ whenever $s^N \in L$, $s(X) = s^1(X) =$

$\ldots = s^{N-1}(X) = 0$ when $k \geqslant 1$, and $\int_1^x \theta(t)\, s(t)\, dt = 0$ whenever

$s \in L$, $s(X) = 0$ when $k = 0$.

By lemmas 1 and 2 it follows that $\theta(t) = 0$ p.p in $[1, X]$. (15)

If $k = 0$, this gives $g(t) = 0$ p.p in $[1, X]$, and since X is arbitrary, it follows that $g(t) = 0$ for almost all $t \geq 1$, which is (a).

If $k \geq 1$, by (15), $D^k [t^{m-1} g(t)] = 0$ p.p. in $[1, X]$, for $m = 1.2...n$, and hence $g(t)$, $tg(t)$,$...t^{n-1} g(t)$ are polynomials in t of degree at most $k - 1$. (16)

Thus $g(t)$ is a polynomial of degree at most $k - n$. (17)

If $n = r$, $r \geq k + 1$, (16) implies that $g(t) = 0$. (18)

If $n = r = k$, (16) implies that $g(t) =$ a constant. (19)

If $n < r$, then $-n-1 < p + q$ and hence $k - n < p + q + k + 1$.

Thus $g(t)$ is a polynomial of degree less than $p + q + k + 1$ if $p + q + k + 1 \geq 1$, by (17), and since it is also necessary that $g(t) = 0(t^q)$, (since $g \in G_2$), the required conclusion follows in the case $p + q + k + 1 > 0$.

If $r = k$, $p + q + k + 1 \leq 0$, $q \geq 0$, then $p + q + r = p + q + k < 0$ and hence $n = r$. Thus (19) implies that $g(t) =$ constant.

If $r = k$, $p + q + k + 1 \leq 0$ $q < 0$, again $g(t) =$ constant A, but $f(x) = 0(x^p)$ $|C, k|$ can imply $Af(x) = 0(x^{p+q})$ $|C, k|$ only if $A = 0$, i.e. $g(t) = 0$.

If $p + q + k + 1 \leq 0$, $r \geq k + 1$, by (18) it follows that $g(t) = 0$, which is the required conclusion, since $M < 0$ in this case.

This completes the necessity in (ii).

Sufficiency The sufficiency in (i) is trivial.

(ii) Omit the trivial case when $g(t) = 0$, and consider the case when $g(t)$ is a polynomial in t of degree at most M, where $M \leq q$, $M < p + q + k + 1$, $M \geq 0$.

Let $P^{(m)}(x)$ denote a polynomial in x of degree at most m, possibly different at each occurrence.

Then, $g(t) = P^{(M)}(t)$, and substituting for $g(t)$ and regrouping terms,

we get $(D_t)^k G_r(x,t) = \sum_{i=0}^{M+r-1-k} t^i P^{(M+r-1-k-i)}(x)$.

Hence (8) gives $I_r f(x)\, g(x) =$

$$\sum_{i=0}^{M+r-1-k} P^{(M+r-1-k-1)}(x) \int_1^x t^i f_k(t)\, dt + \delta_{rk} f_k(x)\, P^{(M)}(x). \qquad (20)$$

$$= \sum_{i=0}^{M+r-1-k} P^{(M+r-1-k-i)}(x)\,[0(x^{p+k+i+1}) + 0(\log x)] + 0(x^{p+k+M}).$$

$= 0(x^{p+M+r}) + 0(x^{M+r-1-k}\log x) + 0(x^{p+k+M}) = 0(x^{p+q+r})$, since $M + r - 1 - k < p + q + r$, $M \leqslant q$, $k \leqslant r$, except in the case $r = k$, $M = 0$, in which case $I_k f(x)\, g(x) = A f_k(x) = 0(x^{p+k}) = 0(x^{p+q+k})$. (21)

Now, since $M \leqslant q$, $t^{-p-k} f_k(t) \in BV[1,\infty)$, it follows that $t^{M-p-q-k} f_k(t) \in BV[1,\infty)$, and taking $\delta = p + q + k + 1 - M + i > 0$, $\phi(t) = t^{M-p-q-k} f_k(t)$ in Lemma 3, we see that $x^{M-p-q-k-1-i} \int_1^x t^i f_k(t)\, dt \in BV[1,\infty)$.

Hence from (20), it follows that $x^{-p-q-r} I_r f(x)\, g(x) \in BV[1,\infty)$.

This completes the sufficiency.

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J. Natn. Sci. Coun. Sri Lanka 1976 4(2) : 157—162

The Diophantine Equation $(y(y+1))^2 = 3x(x+1)$

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(*Paper accepted : 12th October* 1976).

Abstract : It is shown by using the properties of quadratic reciprocity that the only solution in positive integers of the Diophantine equation

$$(y(y+1))^2 = 3x(x+1)$$

is $x = 3$, $y = 2$.

The Equations

On putting $2x + 1 = V$ and $2y + 1 = Y$, the equation of the title reduces to

$$(Y^2 - 1)^2 = 12(V^2 - 1),$$

which implies that 6 divides $Y^2 - 1$.

Now, if we put $Y^2 - 1 = 6U$, the last equation becomes

$$V^2 - 3U^2 = 1. \tag{1}$$

The general solution of the equation (1) is given by

$$U_n = \frac{\alpha^n - \beta^n}{2\sqrt{3}}, \quad V_n = \frac{\alpha^n + \beta^n}{2} \tag{2}$$

where

$$\alpha = 2 + \sqrt{3}, \quad \beta = 2 - \sqrt{3}.$$

Hence, we must have

$$Y^2 = 6U_n + 1. \tag{3}$$

We obtain easily from (2) the following relations :

$$U_{m+n} = V_m U_n + V_n U_m, \tag{4}$$
$$V_{m+n} = V_m V_n + 3U_m U_n, \tag{5}$$
$$U_{-n} = -U_n, \; V_{-n} = V_n, \tag{6}$$
$$U_{2n} = 2U_n V_n, \tag{7}$$
$$V_{2n} = V^2_n + 3U^2_n = 1 + 6U^2_n = 2V^2_n - 1, \tag{8}$$
$$U_{3n} = U_n(4V^2_n - 1), \tag{9}$$
$$V_{3n} = V_n(4V_n{}^2 - 3), \tag{10}$$
$$U_{5n} = U_n(16V_n{}^4 - 12V^2_n + 1), \tag{11}$$
$$V_{5n} = V_n f(V_n) = V_n h(U_n), \tag{12}$$

where $f(V) = 16V^4 - 20V^2 + 5$

and $h(U) = 144U^4 + 36U^2 + 1.$

The following congruences hold :

$$U_{n+2r} \equiv -U_n \pmod{V_r}, \tag{13}$$

$$U_{n+2r} \equiv U_n \pmod{U_r} \tag{14}$$

We need the following results which can be easily established by induction :

(i) U_n is odd or even according as n is odd or even ;

(ii) $U_2 \equiv 4 \pmod{13}$, and if $k = 2^t$ with t an integer > 1, $U_k \equiv \pm 4 \pmod{13}$, according as t is even or odd ; (15)

(iii) If $k = 2^t$, then $V_k \equiv 2, 7, 5, 3, -6 \pmod{23}$ when $t \equiv 0, 1, 2, 3, 4 \pmod{5}$ respectively. (16)

We also note that since $Y = 2y + 1$, Y is odd and so if U_n satisfies (3), then U_n must be even.

Hence, n must be necessarily even for (3) to hold.

We require the following table of values :

n	U_n	V_n
0	0	1
1	1	2
2	4	7
3	15	26
4	56	97
5	209	362
6	780	1351
7	2911	5042
8	10864	18817

The proof is now accomplished in five stages :

(a) (3) is impossible if $n \equiv 4 \pmod{10}$.

For, using (14) we find that

$$\begin{aligned} U_n &\equiv U_4 \pmod{U_5} \\ &\equiv 56 \pmod{209} \\ &\equiv 56 \pmod{11}, \text{ since } 11/209 \\ &\equiv 1 \pmod{11}. \end{aligned}$$

Thus we find that

$$6U_n + 1 \equiv 7 \pmod{11}$$

and since $(7/11) = -1$, (3) is impossible.

(*b*) (3) is impossible if $n \equiv 6 \pmod{10}$.

For, using (14) we find that

$$\begin{aligned} U_n &\equiv U_6 \pmod{U_5} \\ &\equiv 780 \pmod{209} \\ &\equiv 780 \pmod{11} \\ &\equiv -1 \pmod{11}. \end{aligned}$$

Thus we find that

$$6U_n + 1 \equiv -5 \pmod{11}$$

and since $(-5/11) = 1$, (3) is impossible.

(*c*) (3) is impossible if $n \equiv 8 \pmod{10}$.

For, using (14) we find that

$$\begin{aligned} U_n &\equiv U_8 \pmod{U_5} \\ &\equiv 10864 \pmod{209} \\ &\equiv 10864 \pmod{11} \\ &\equiv 7 \pmod{11}. \end{aligned}$$

Thus we find that

$$\begin{aligned} 6U_n + 1 &\equiv 43 \pmod{11} \\ &\equiv -1 \pmod{11} \end{aligned}$$

and since $(-1/11) = +1$, (3) is impossible.

(*d*) (3) is impossible if $n \equiv 0 \pmod{10}$, $n \neq 0$, that is, if $n = 5k(2m + 1)$ where $k = 2^t$ with t an integer ≥ 1 and m is an integer.

For, using (13) we find that

$$\begin{aligned} U_n &\equiv \pm U_{5k} \pmod{V_{5k}} \\ &\equiv \pm U_k (16V^4_k - 12V^2_k + 1) \pmod{f(V_k)} \\ &\equiv \pm 4U_k (2V^2_k - 1) \pmod{f(V_k)} \end{aligned}$$

Whence, $6U_n + 1 \equiv \pm 24U_k (2V^2_k - 1) + 1 \pmod{f(V_k)}$

Now,

$$\left(\frac{1 \pm 24U_k(2V^2_k - 1)}{f(V_k)}\right) = \left(\frac{16V^4_k - 20V^2_k + 6 \pm 24U_k(2V^2_k - 1)}{f(V_k)}\right)$$

$$= \left(\frac{2}{f(V_k)}\right)\left(\frac{2V^2_k - 1}{f(V_k)}\right)\left(\frac{4V^2_k - 3 \pm 12U_k}{f(V_k)}\right)$$

$$= \left(\frac{12U^2_k + 1 \pm 12U_k}{h(U_k)}\right)$$

$$= \left(\frac{h(U_k)}{12U^2_k \pm 12U_k + 1}\right)$$

$$= \left(\frac{12 \cdot 13\ U^2_k}{12U^2_k \pm 12U_k + 1}\right)$$

$$= \left(\frac{13}{12U^2_k \pm 12U_k + 1}\right)$$

$$= \left(\frac{12U^2_k \pm 12U_k + 1}{13}\right)$$

$$= \left(\frac{-U^2_k \mp U_k + 1}{13}\right)$$

$$= \left(\frac{-16 \mp 4 + 1}{13}\right) \text{ by (15)}$$

$$= \left(\frac{2}{13}\right) = -1$$

and so (3) is impossible.

(*e*) (3) is impossible if $n \equiv 2 \pmod{10}$, $n \neq 2$, that is, $n = 2 + 10km$, where $k = 2^t$ with t an integer $\geqslant 0$ and m is an odd integer.

For, using (13) we find that

$$U_n = -U_2 \equiv -4 \pmod{V_{5k}}.$$

Thus,

$$6U_n + 1 \equiv -23 \pmod{V_k f(V_k)},$$

and so (3) would imply

$$\left(\frac{-23}{V_k}\right) = \left(\frac{-23}{f(V_k)}\right) = +1. \tag{17}$$

But it is easily seen that

$$V_2 \equiv 3 \pmod 4,$$
$$V_k \equiv 1 \pmod 4 \text{ for } t \geqslant 2,$$

and $f(V_k) \equiv 1 \pmod 4$ for $t \geqslant 0$,

so, using quadratic reciprocity, (17) implies

$$\left(\frac{V_k}{23}\right) = +1 \text{ for } t \geqslant 1 \tag{18}$$

and $$\left(\frac{f(V_k)}{23}\right) = +1 \text{ for } t \geqslant 0. \tag{19}$$

Now by (16) the residues of V_k modulo 23 and so $f(V_k)$ modulo 23 are periodic with respect to t, and the length of the period is 5. The following table gives these residues and the signs of their Legendre symbols $(V_k/23)$ and $(f(V_k)/23)$; we see that (18) or (19) is impossible.

t	0	1	2	3	4	5	6
$V_k \bmod 23$		7	5	3	17	2	7
$f(V_k) \bmod 23$	20	20	6	17	11	20	20
$(V_k/23)$							
$(f(V_k)/23)$	−	−	+	−	−	−	−

We have now two further cases $n = 0, 2$ to consider.
When $n = 0$, we find $x = 0, y = 0$, a trivial solution.
When $n = 2$, $U_2 = 4$, $V_2 = 7$.
Thus we get $x = 3$, $y = 2$.

Hence, $x = 3, y = 2$ is the only solution of the given equation in positive integers. Now we can write down the complete solution in integers; it consists of the four trivial pairs of solutions obtained by equating both sides of the equation of the title to zero and the four pairs given by the following table :

x	y
3	2
3	−3
−4	2
−4	−3

Acknowledgement

The author wishes to dedicate the article to the memory of the late Professor P. Kanagasabapathy.

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J. Natn. Sci. Coun. Sri Lanka 1976 **4** (2) : 163—166

SHORT COMMUNICATION

Alkaloids of the *Catharanthus* Species of Sri Lanka

Some preliminary studies†

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(*Paper accepted*: 24*th May* 1976)

Catharanthus roseus (L) G. Don (family—Apocynaceae, *Sinhala*—Mini-mal, *Tamil*—Patti-poo, *English*—Madagascar Periwinkle) incorrectly called *Vinca rosea* is a perennial herb or subshrub endemic to Madagascar, and now widely cultivated and naturalised in many tropical countries. In Sri Lanka it is a common weed in the dry and in the wet zones at low elevations, especially on sandy soil in the coastal areas.[1] Three different forms of *C. roseus* have been reported;[4] one with white flowers and pale green stems, another form with rose pink flowers and brown stems, and a third one with pink centered white flowers and pale green stems. *C. pusillus* (Murr.) G. Don is an annual herb indigenous to Sri Lanka and India. In Sri Lanka it is a rather rare weed of cultivated land and has been recorded exclusively from Batticaloa and Jaffna districts.[5]

Catharanthus species are well known for their pharmaceutically important alkaloids.[3] Vincaleukoblastine also called vinblastine (VLB) (I), a major alkaloid of the *Catharanthus* species has been used in the treatment of leukemia in children.[7] A minor alkaloid, leurocristine also known as vincristine (LCR) (II) from these species is more potent than VLB and fetches about US$ 400,000 per kg. in the world market.[2] Ajmalicine (AJM) (III) and vincamine (VIC) (IV) are two other useful alkaloids reported from these species.[2] Although the chemical transformation of VLB to LCR is known, *Catharanthus* species still remain the major source of these valuable alkaloids.[2] Thus, the whole plant of *C. roseus*, at present, is exported by local agents to drug manufacturers in Europe for the extraction of these alkaloids. This practise has two obvious disadvantages. Uprooting the whole plant as it is done now would probably lead to total extinction of this species from Sri Lanka.

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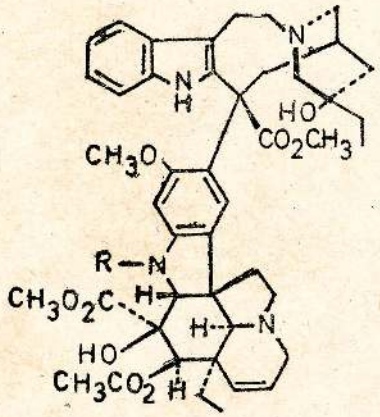
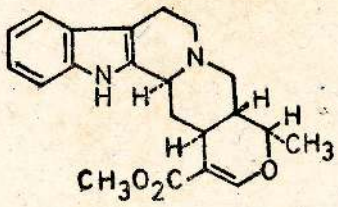
†A part of this work was presented at the 31st annual sessions of the Sri Lanka Association for the Advancement of Science, 1975.

The high freight charges paid by the local agents for sending the dried plant material to recipient countries is another disadvantage. Awareness of these prompted us to initiate work on these species with the hope of obtaining better returns from this non-traditional export. Our preliminary studies reported herein constitute an assessment of the cost of extraction of the total alkaloidal mixture using locally available solvents. A comparison of the alkaloid yields and compositions of *C. roseus* (all three forms) and those of *C. pusillus* has also been made.

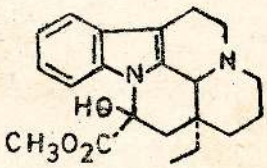


(I) R = CH_3 ; VLB

(II) R = CHO ; LCR



(III) ; AJM



(IV) ; VIC

Different parts of the plants were subjected to separate small scale extractions with chloroform and methanol. TLC examination of the crude extracts thus obtained revealed that the pharmaceutically useful alkaloid VLB could be effectively extracted with either solvent. Methanol was preferred for large scale extractions as it is cheaper. However, for the extraction of the less polar alkaloids such as AJM, chloroform was found to be a better solvent. The leaves of the three forms of *C. roseus* had a similar alkaloid composition except that the form with rose pink flowers had a comparatively lower VLB content. The alkaloid constituents of the stems and roots of these three forms were again found to be similar. But the relative amounts of some of the alkaloids present were different. The roots of all three forms of *C. roseus* were rich in AJM and VLB, whilst their stems were relatively poor in AJM. VIC was found to be abundant in the stems of the variety with pink centered white flowers. TLC

examination of the alkaloid extract of the different parts of *C. pusillus* collected in Batticaloa showed their alkaloid constitutents to be similar. The total number of alkaloids in *C. pusillus* was found to be less than that in *C. roseus*. Therefore, further processing of the alkaloid mixture from *C. pusillus* would prove to be less difficult. However, the rareness of this species made us restrict our large scale work to the readily available *C. roseus*. The method adopted for extraction is outlined in the figure.

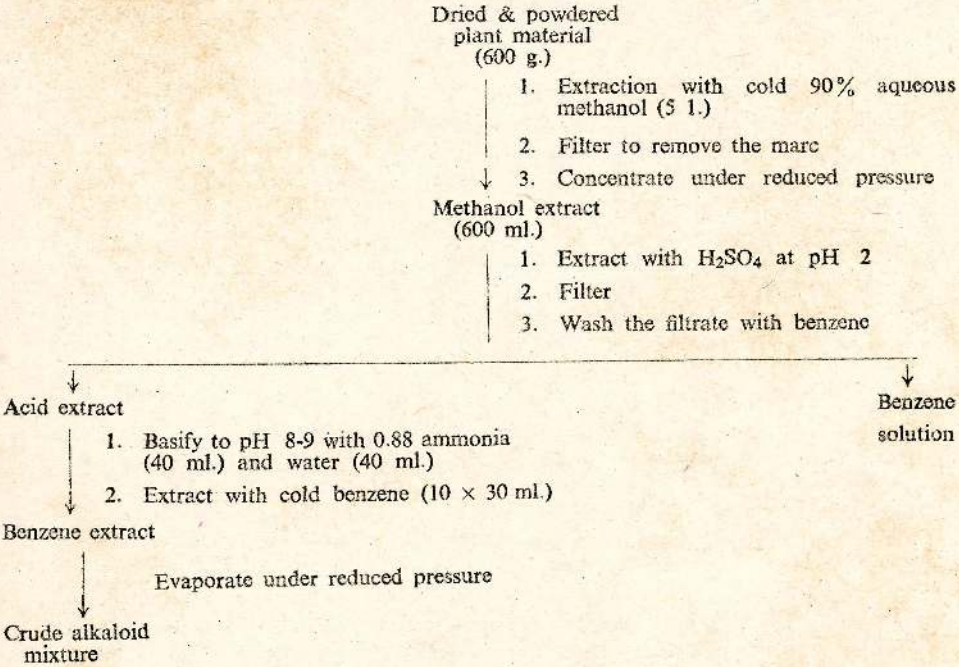


FIGURE. A typical extraction procedure of the crude alkaloid mixture from *Catharanthus* species

In one experiment 600 g. of dried leaves of the pink flowered variety yielded 2.8 g. of the crude alkaloid mixture. When 1 kg. of the stems and roots were extracted in a similar manner only 1.75 g. of the alkaloid mixture was obtained. Assessment of the cost of extraction (only for the chemicals) using locally available solvents is presented in the Table.

TABLE

Results of the large scale extractions of *C. roseus*.

Plant part(s) used	Weight	Weight of extract (% yield)	Cost of extraction	Cost/gm. of the extract
Leaves	600 g.	2.8 g. (0.47)	Rs. 1.45	Rs. 0.52
stems & roots	1 kg.	1.75 g. (0.18)	Rs. 1.92	Rs. 1.12

Our preliminary work on *C. roseus* has led to two important findings. Firstly, it is not necessary to uproot the whole plant in order to extract the pharmaceutically useful alkaloids. Instead, the plant can be cultivated and the leaves harvested at periodic intervals as a ratoon crop. However, field trials are needed before making any conclusions, especially to find out whether the alkaloid content diminishes over a period of time. Secondly, the export of the crude alkaloidal extract which could be obtained with locally available solvents would bring about a large reduction in the cost of freight.

We have extended this study to the isolation of the individual alkaloids present in the crude mixture with locally available solvents and facilities and the results of this work will be reported elsewhere.

Acknowledgement

Professor N. R. Farnsworth of the Department of Pharmacognosy and Pharmacology, University of Illinois at the Medical Centre, Chicago, is thanked for providing the authentic samples of VLB, VIC, and AJM.

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ලිපිවල සාරාංශ – සිංහල පරිවර්තන

සේසමියා ඉන්ෆරන්ස් WLK (ලෙපිඩොප්ටෙරා: නොක්ටුයිඩේ) නම් ලංකාවේ උක්ගසේ ප්‍රරෝහගුල්ලාගෙන් ඇතිවන පළිබෝධය.

උගේ ජීවන චක්‍රය හා ජීවවිද්‍යාත්මක ලක්ෂණ.

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ශ්‍රී ලංකාවේ උක්වගාවට පළිබෝධයක්ව පවතින සේසමියා ඉන්ෆරන්ස් W. L. K. නම් ගුල්ලා ගැන කරන ලද ජීවී පරිස්ථිති විද්‍යාත්මක අධ්‍යයනවලින් පෙනී යන්නේ ඌ උක් වගා බිම්වල වැවෙන තණවර්ගවල බීත්තර දැමීමට මහත් රුචියක් දක්වන බවයි. මේ කීටයා බෝ වීමට ආධාරවන සමපාර්ශ්වික ශාකයන් ගැන විස්තර කෙරේ.

උගෙන් ධාරක ශාකයට (උක්ගසේ) විවිධ අවස්ථාවලදී ඇතිවන හානියේ ප්‍රමාණය සහ එහි ස්වභාවය ගැනද පළිබෝධකයාගේ ජීවන චක්‍රය ගැනද පරීක්ෂණයක් පවත්වා ඇත. මේ පළිබෝධකයාගේ ස්වභාවික සතුරන් ගැනද වාර්තා කොට ඇත.

ඒගාර් මාධ්‍යවල වැඩෙන බැක්ටීරියා ගණවාසයන්ගේ වර්ධන වාලක ශක්තිය පිළිබඳ ලක්ෂණ කීපයක් විමර්ශනය කිරීමේ තාක්ෂණ ක්‍රමයක්.

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ඒගාර් මාධ්‍යයක් තුළ වැඩෙන බැක්ටීරියා ගණවාසයක වර්ධන වාලක ශක්තිය හඳුනා ගැනීමට ආධාර වන සරල සෛද්ධාන්තික ආදර්ශයක් අත්හද බලා ඇත. ඒගාර් මාධ්‍යවල ක්ෂුද්‍ර ජීවීන්ගේ වර්ධනය හැදෑරීම සඳහා ප්‍රකාශ ඝනත්වමිතික පරිලෝකන ක්‍රමයක් ඉදිරිපත්කොට ඇත. (මේ තාක්ෂණ ක්‍රමයේ මූලධර්මය සාකච්ඡාවට භාජනය කරන අතරම එම ක්‍රමය සඳහා භාවිත කරන උපකරණය පිළිබඳවද විස්තර කොට ඇත.) මේ උපකරණය ක්‍රියා කරවන ආකාරය හා ඒ මඟින් ලබාගත් පරීක්ෂණාත්මක දත්ත කීපයක් ද මේ ලිපියෙන් ඉදිරිපත් කොට ඇත. උත්පාදනය කරන ලද සෛද්ධාන්තික ආදර්ශය පරීක්ෂා කිරීම පිණිස මෙම පරීක්ෂණාත්මක දත්ත උපයෝගී කර ගෙන තිබේ.

අලුත් විමර්ශන ක්ෂේත්‍රයන් කරා ගෙන යා හැකි මේ තාක්ෂණයට අදාළ තවත් ප්‍රයෝජන කීපයක් ගැනද මෙහි සඳහන් කොට ඇත.

ඇම්පෙරෝමිතික ක්‍රම අනුසාරයෙන් සල්ප්හයිඩ්‍රිල් ගණය නිර්ණය කිරීම හා පොල් රා වලින් කරණ පරීක්ෂණ සඳහා එය යොදා ගත හැකි ආකාරය.

කල්යනන්ද, එම්. කේ. ජී. එස්., ජෑන්ස්, ඊ. ආර් සහ ජයරාජ්, ඊ. ඊ.

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සල්ප්හයිඩ්‍රිල් ගණයේ ඇම්පෙරෝමිතික අනුමාපනයේ විවිධ ක්‍රම පිළිබඳව තුලනාත්මක අධ්‍යයනයක් මෙහි විස්තර කොට ඇත. පරීක්ෂණයේදී වෙනස් වූ කරුණු වශයෙන්: (1) මයික්‍රෝ ඉලෙක්ට්‍රෝඩය, (2) අනුමාපකය, (3) P. H. ක්‍රියාකාරිත්වය සහ (4) වෝල්ටීයතාවද දක්වා ඇත. RSH හා RSSR ප්‍රමාණය නිර්ණය කිරීම සඳහා පොල් රා සමඟ පරීක්ෂණ පැවැත්වීමට මේ ක්‍රමය සාර්ථක ලෙස යොදා ගත හැකි විය. 7, 10. රා පැසවීමේදී H_2S සෑදීම සඳහා අවශ්‍ය සල්ෆර් ලබාදීමට සිස්ටීන්උපකාර වේය යන මතය, මෙසේ පොල්රා යොදාගෙන කළ අධ්‍යයනය මගින් නවත් සනාථ විය. එසේම මීරා පැසීමේදී ඇතැම් වර්ගවලට අයත් යීස්ට් වලින් H_2S උත්පාදනය නොවීමේ හේතුවද මේ පරීක්ෂණ මගින් හෙළිවිය.

ලිනමරින්වලින් සයනයිඩ් මුදහැරීම.
III. DEAE - සෙලුලෝස් වර්ණ ලේඛ ක්‍රමය මගින් මංසෙක්කා ලෙල්ලේ ලිනමරේස් වර්ග වෙන්කොට ගැනීම.

පීරිස්, නිර්මලා සහ ජෑන්ස්, ඊ. ආර්.

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ලිනමරේස් 'ඒ' සහ 'බී' වශයෙන් හැඳින්වුණ ලිනමරින් විවිජේදනය කරන එක්සයිම වර්ග දෙකක් මංසෙක්කා ලෙල්ලේ ඇති බව අප විසින් මීට පෙර කරන ලද පරීක්ෂණ වලින් පෙන්වා දෙන ලදී. මැනිහොට් එස්කියුලෙන්ටා ක්‍රාන්ට්ස් (මංසෙක්කා) වගා වර්ග අටක පොත්තේ නිස්සාරකයන් ගැන D EAE— සෙලුලෝස් වර්ණාලේඛය මගින් කරන ලද පරීක්ෂණවලින් පෙනී ගියේ සයනයිඩ් මුදහැරීමේ ක්‍රියාකාරිත්වය පිළිබඳ උච්ච අවස්ථා 4 සිට 6 දක්වා ඇති බවය. ඉහත සඳහන් වර්ග අටෙන් එකක හැර අනික් ඒවායෙහි ප්‍රධාන උච්ච අවස්ථා ඇතිවූයේ ලිනමරේස් ''ඒ'' යහ ''බී'' පැවති නිසාය. ලිනමරේස් ''බී'' වර්ගය ලිනමරේස් ''ඒ'' හි උපාංගයක් වන බවද, පරීක්ෂණවලින් හෙළිවිය. ලිනමරේස් ''සී'' සහ ''ඩී'' වල ලක්ෂණ කීපයක් ද මෙහි විස්තර කොට ඇත.

අනුකල සඳහා නිරපේක්ෂ අභිසාරිතා සාධක.

යෝගවන්දන්, සී.

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$r \geqslant k$ වන පරිදි r,k යන මේවා සෘණ නොවන අනුකල වශයෙන් පවතිද්දී $f(x) = e(x^p)/c, k$ ලෙස පවතින අවස්ථාවලදී $f(x)\, g(x) = e(x^{p+q})/c, r$ ලෙස පැවතීමට අනිවාර්ය හා ප්‍රමාණවත් අවශ්‍යතාවන්, අනුකල සඳහා වෙවීමේ නිරපේක්ෂ අභිසාරිතා සාධක ගැටලුව ගැන මෙහි දී සලකා බැලේ: මෙහි p,q යන මේවා තාත්වික වන අතර $p + q \leqslant -1$ ද වේ.

g අභිසාරිතා සාධකය කෙරේ බලපාන අවශ්‍යතාව ශූන්‍යතා මාත්‍රයකට හෝ බහුපද මාත්‍රයකට හෝ සීමාවෙන බවද මේ ලිපියෙන් දක්වා ඇත.

$(y(y+1))^2 = 3x(x+1)$ යන ඩයෝපැන්ටනීය සමීකරණය.

බාලසුන්දරම්, හරිමාලාදේවි.

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$(y(y+1))^2 = 3x(x+1)$ යන ඩයෝපැන්ටනීය සමීකරණයේ ධන පූර්ණ සංඛ්‍යාවල එකම විසඳුම $x = 3, y = z$ බව, වර්ගජ පරස්පරතා ගුණයන්ගේ මාර්ගයෙන් විසඳා මෙහි දක්වා ඇත.

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இந்த இதழின் கட்டுரைகளின் சுருக்கங்கள்

சேசமியா இன்பெறன்ஸ் WLK (லெப்பிடொப்ரெரு: நொக்குய்டே) எனப்படுகின்ற இலங்கையின் கரும்புப் பயிர்த் தண்டு துளைப்பான் பீடை.

அதன் வாழ்க்கைச் சக்கரமும் உயிரியற் பண்புகளும்

இராசேந்திரா, ஏ.

J. Natn. Sci. Coun. Sri Lanka 1976 4(2):

இலங்கையின் கரும்புச்செய்கைக்கு ஒரு பீடையாகவுள்ள சேசமியா இன்பெறன்ஸ் WLK எனப்படும் தண்டுதுளைப்பான் பற்றிய சூழலியற் கல்விகளால் அறியப்படுவது யாதெனில் அது கரும்பு வயற் புற்களில் முட்டையிடுவதற்குப் பெருவிருப்பம் காட்டுகிறது என்பதே. இப்புழுவின் பெருக்கத்திற்குத் துணைபுரியும் ஓம்பித் தாவரங்களைப் பற்றி இக்கட்டுரையில் விளக்கப்பட்டுள்ளது.

அது காரணமாக ஓம்பித்தாவரத்திற்கு (கரும்புப்பயிருக்கு) பல்வேறு தருணங்களில் ஏற்படும் சேதத்தின் அளவும் அதன் தன்மையும், இப்பீடைக் கிருமியின் வாழ்க்கைச் சக்கரம் ஆகியவற்றைப் பற்றியவொரு சோதனை நடாத்தப்பட்டுள்ளது. இப்பீடைக் கிருமியின் இயற்கையான எதிரிகளைப் பற்றியும் விளக்கப்பட்டுள்ளது.

ஏகார் ஊடகங்களில் வளரும் பற்றீரியாச் சமுதாயங்களின் வளர்ச்சி இயக்கம் பற்றிய சில சிறப்பியல்புகள் ஆய்வுக்குட்படுத்தப்படும் தொழினுட்ப முறை.

பர்ணாந்து பீ. சி. பீ; நந்ததாசா, எச். ஜீ; பவுலிஸ், டீ. வீ. என். எம்.

J. Natn. Sci. Coun. Sri Lanka 1976 **4** *(2)*:

ஏகார் ஊடகத்தில் வளர்கின்ற ஒரு பற்றீரியா சமுதாயத்தின் வளர்ச்சி இயக்கத்தினை அறிவதற்குத் துணை புரிகின்ற எளிய அறிமுறைக்குரிய மாதிரியுரு வொன்று ஆய்ந்து பெறப்பட்டுள்ளது. ஏகார் ஊடகங்களில் நுண்ணுயிரிகளின் வளர்ச்சியை ஆய்தல் பொருட்டு, ஒளியியல் அடர்த்திமானி முறைசார் வரிசையாகப்பார்க்கும் தொழினுட்பமுறையொன்று சமர்ப்பிக்கப்பட்டுள்ளது. (இந்தத் தொழினுட்பத்தின் அடிப்படைக் கொள்கையைப் பற்றி ஆராய்வதோடு அம்முறைக்கெனப் பயன்படுத்தப்படுகின்ற கருவியைப் பற்றியும் கூறப்பட்டுள்ளது). இக்கருவியை இயக்குகின்ற முறையும் அதன் வழி பெறப்பட்ட ஆய்வுமுறைசார் தரவுகள் சிலவும் இக்கட்டுரையில் தரப்பட்டுள்ளன. நிருமாணிக்கப்பட்டுள்ள அறிமுறைக்குரிய மாதிரியுருவினைச் செவ்வைப் பார்ப்பதற்கும் இந்த ஆய்வுத்தரவுகள் உபயோகிக்கப்பட்டுள்ளன.

புதிய ஆய்வுத்துறைகளுக்கு உதவக்கூடிய இத்தொழினுட்பஞ்சார் ஏனைய சில பயன்பாடுகளும் இதில் விளக்கப்பட்டுள்ளன.

அம்பியர்மானி முறைகளைத் தழுவி சல்பிஹைட்டிரில் தொகுதி சார் துணிபும் தென்னங்கள்ளை உபயோகித்துச் செய்கின்ற ஆய்வுகளுக்கு அதனைப் பயன்படுத்தலும். கல்யானந்தா, எம். கே. ஜீ. எஸ்; ஜான்ஸ், ஈ. ஆர்; ஜயராஜ், ஈ. ஈ.

J. Natn. Sci. Coun. Sri Lanka 1976 4(2):

சல்பிஹைட்டிரில் தொகுதி சார் அம்பியர்மானி முறையான நியமிப்பின் பல்வேறு முறைகளைப் பற்றிய ஒப்புநோக்கற் கல்வியே இங்கு விளக்கப் பட்டுள்ளது. ஆய்வின் போது வித்தியாசப்பட்டவைகளாக,

(1) மைக்ரோ இலெக்ரோடு

(2) நியமிப்பி (3) PH தொழிற்பாடு,

(4) உவோற்றளவு என்பவற்றை எடுத்துக்காட்டலாம். RSH உம் RSSR அளவினையும் துணிவதன்பொருட்டு தென்னங்கள்ளினைக் கொண்டு பரிசோதனைகளை நடாத்துவதற்கு இம் முறையை வெற்றிகரமாக பயன் படுத்திக் கொள்ள முடிந்தது. 7. 10 ஆகிய கள்ளினை நொதிக்கும்போது H_2S உண்டாதலுக்குத் தேவைப்படுகின்ற கந்தகத்தைப் பெற்றுத்தர சிஸ்ரீன் துணை புரிகிறது என்னும் கோட்பாடு இங்ஙனம் தென்னங்கள்ளை உபயோகித்து செய்த பரிசோதனையால் மேலும் உறுதிப்படுத்தப்பட்டது. அதே போன்று பதனீர் புளிக்குமிடத்து சிலவகைகளுக்குரிய மதுவங்களால் H_2S உண்டாகாத காரணமும் இப்பரிசோதனைகளால் அறியப்படலாயிற்று.

''லினமறின்'' இடமிருந்து சயனேட்டு விடுதலை செய்தல்.

III DEAE—செல்லுலோசு நிறப்பதிவியல் முறை மூலம் மரவள்ளிக் கிழங்குத் தோலின் லினமறேஸ் இனங்களைப் பிரித்தெடுப்பு.

பீறிஸ், நிர்மலா; ஜான்ஸ், ஈ, ஆர்.

J. Natn. Sci. Coun. Sri Lanka 1976 4(2):

லினமறேஸ் ''A'' ''B'' எனப்படும் லினமறின் பகுத்தும் நொதியங்கள் இரண்டு வகைகள் மரவள்ளிக் கிழங்குப் புறணியில் உளவென்பது நாம் இதற்கு முன் செய்த பரிசோதனைகளின் மூலம் நிரூபிக்கப்பட்டது. மனிகொற் எஸ்குயுலென்ரு கிருன்ற்சு (மரவள்ளிக் கிழங்கு) இன் எட்டு வகையான வளர்ப்பினங்களின் புறனிப்பிழிச் சாற்றினை DEAF— செலுலோசு நிறப்பதிவியல் மூலம் செய்யப்பட்ட பரிசோதனைகளின்படி, சயனேட்டு விடுவிக்கும் தொழிற்பாடுபற்றிய உச்சநிலைகள் 4 முதல் 6 வரையாக உள்ளன வென்பது அறியப்பட்டது. மேற்கூறிய எட்டு இனங்களுள் ஒன்றைத் தவிர்ந்த ஏனையவற்றில் முக்கிய உச்சநிலைகள் தோன்றுவதற்கு லினமறேஸ் ''A'' வும் ''B'' உம் காரணமாக விருந்தன. லினமறேஸ் ''B'' என்பது லினமறேஸ் A வின் துணை உறுப்பு என்ற உண்மையும் இப் பரிசோதனைகளால் அறியப்பட்டது. லினமறேஸ் C வினதும் D வினதும் சில சிறப்பியல்புகளைப் பற்றியும் இங்கு விளக்கப்பட்டுள்ளது.

தொகையீடுகளுக்கான தனி ஒருங்கற் காரணிகள்.
யோகசந்திரன், சி.

J. Natn. Sci. Coun. Sri Lanka 1976 **4**(2):

$P \geqslant k$ ஆகுமாறு r, k என்பன மறையில்லா நிறையெண்களாக இருக்க $f(x = e(x^p)/ c, k$ ஆக இருக்கும்போதெல்லாம் $f(x)\, g(x) = e(x^{p+p}) / c, r$ ஆக இருப்பதற்கு வேண்டிய போதிய நிபந்தனைகளைத் தொகையீடுகளுக்குக் காணும் தனி ஒருங்கற் காரணிப் பிரசினம் இங்கு எடுத்து நோக்கப் பட்டுள்ளது: இங்கு p, q ஆகியன மெய்யும், $p+q \leq -1$ உம் ஆகும்.

g ஒருங்கற் காரணி மீதான நிபந்தனை பூச்சியமாகவோ பல்லுறுப்பியாக வோ ஓர் எல்லைக்குட்பட்டுள்ளதென்பதும் இங்கு காட்டப்பட்டுள்ளது.

$(y\ (y+1))^2 = 3x\ (x+1)$ என்னும் தியோபந்தசு சமன்பாடு.
பாலசுந்தரம், ஹரிமாலாதேவி

J. Natn. Sci. Coun. Sri Lanka 1976 (**4**)2:

இருபடி நிகர்மாற்று உடைமைகளின் மூலம் $(y(y+1)^2 = 3x(x+1)$ என்னும் தியோபந்தசு சமன்பாட்டின் நேர் நிறையெண்களுக்குரிய ஒரே தீர்வு $x = 3, y = 2$ என்பதே என்று இக்கட்டுரையால் காட்டப்பட்டுள்ளது.

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JOURNAL OF THE NATIONAL SCIENCE COUNCIL OF SRI LANKA

The National Science Council of Sri Lanka,
Colombo.

1976
VOLUME 4

Published by

The National Science Council of Sri Lanka, 47/5, Maitland Place, Colombo 7, Sri Lanka.

ISSN 0300 — 9254

Printed at

Sri Lanka University Press, Katubedda.

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Vol. 4 No. 1 June 1976

Journal of the
National Science Council
of Sri Lanka

Vol. 4 No. 2 December 1976

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Published by
The National Science Council of Sri Lanka
and Printed at Sri Lanka University Press, Katubedda.