Application of phytoecdysteroids in sericulture

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Sericulture is an agro-based cottage industry, combining the features of rural agriculture and industry-based activities. Though India stands second in world raw silk production, the quality of raw silk is very low compared to other sericulturally advanced countries. Hence several technologies are being developed from soil to silk to improve this aspect. Labour-saving devices linked with improvement in quality of silk are the present needs of the industry. Utilization of bio-active chemicals during silkworm rearing is one of the means of increasing production of superior quality of silk. However, the application of phytoecdysones, influencing the growth and development in silkworms, to improve productivity is a new concept in Indian sericulture.

IT is established in insects that larval moulting is induced by the co-ordination of juvenile hormone, secreted by the corpora allata with the moulting hormone, secreted by the prothoracic glands and the metamorphosis is induced by the moulting hormone. The crystalline material of the moulting hormone secreted by the prothoracic gland was first isolated from *Bombyx* pupae and was named ecdysone[1]. Subsequently, another compound having moulting hormone activity was isolated and was called β-ecdysone by Karlson[2] and ecdysterone by Hoffmeister and Grutzmacher[3]. The extraction, quantification and determination of the chemical structure of insect hormones have greatly contributed towards understanding of functions and mechanisms of hormonal regulations in the development of the silkworm[4,5]. Apart from isolation of zoo-ecdysones from different life stages of insects, an array of compounds has been extracted from plants starting from Pteridophytes to Angiosperms. Because of their plant origin and moult-inducing effects, these compounds are called phytoecdysteroids, most of them having structural similarity to insect moulting hormones[6]. The exogenous administration of these substances is known to affect growth and development of silkworms[7,8]. At present, in some countries like China, Japan and South Korea, phytoecdysones are being used to improve productivity and quality of cocoons in sericulture[9,10]. The application of phytoecdysteroids is yet to be made practical in tropical sericulture as in India, though related studies are being carried out.

Occurrence of ecdysones in plants

Many plants contain a variety of chemicals with moulting hormone activity in insects. The ready isolation from plants in contrast to the extremely poor yield from insects and other sources makes it possible to produce large amounts of active substances for biological testing. Over 100 ecdysteroids have been structurally identified from a wide range of plant species[11]. An exhaustive list of the plants with ecdysteroid activity has been prepared by Bergamasco & Horn[6] and Horn & Bergamasco[12]. 20-hydroxyecdysone (20E)[13] is a widely-occurring ecdysone in plants[6]. The roots of *Cyanothis arachnoides* contain 2.9% of 20-hydroxyecdysone[14] (Table 1) and therefore this plant is a major source of moulting hormone in China. In India, *Achyranthes aspera*, *Trianthema portulacastrum*, *Gomphrena eclosoides*, *Silene* spp, *Sesuvium portulacastrum* and *Cassia tora* are some of the plants known to have phytoecdysteroids[15,16]. Some promising commercial sources are listed in Table 1.

In recent years, cell cultures are looked upon as an alternative means of production of ecdysteroids. Cell cultures from *Polypodium vulgare*[17] and *Serratula tinctoria*[18] were reported to yield lesser ecdysteroid contents when compared to plants. However, cell cultures of the fern, *Pteridium aquilinum* have produced ecdysteroids in higher quantities[19] and if the growth rate of these cultures are kept at optimum conditions, they promise to be a good source of phytoecdysteroids[20]. Attempts are also being made for the production of ecdysteroids by hairy root cultures of *Ajuga* and *Serratula* spp[18,20].

New phytoecdysteroids are still being identified, indicating the chemical diversity existing in plant systems[21–23]. Some have 24-alkyl phytosterol side chain and it is known that phytophagous insects remove this group to form cholesterol by an efficient dealkylating mechanism in the gut[24]. The pathway of conversion to cholesterol and biosynthesis of ecdysteroids from cholesterol are reviewed recently[25]. The elementary structural patterns characteristic for phytoecdysteroids with the average range of activity in insects, summarized by Slama *et al.*[26] are as follows: (i) an, α,β-unsaturated-6-keto group; (ii) *cis* fusion of the A/B rings; (iii) sterolic side chain, and (iv) multiple hydroxylic functions all over the sterolic molecule. The function of ecdysteroids in plants is yet to be clearly defined, though many

Table 1. Important flowering plants having convenient source of ecdysteroids

Name of the plant and family	Plant parts	Ecdysteroid isolated	Quantity present (g/kg)	References
Achyranthes aspera (Amaranthaceae)	Stem, leaves, roots, seeds	20E	0.04–0.25	15
Chenopodium album (Chenopodiaceae)	Whole plant	20E, polypodine B	0.36–1.57	27
Cyanothis arachnoides (Commelinaceae)	Whole plant, roots	20E	12.0–29.0	14
Gomphrena eclosiodes (Amaranthaceae)	Whole plant	20E	0.25	15
Pfaffia iresinoides (Amaranthaceae)	Roots	20E, polypodine B, pterosterone	3.1	23
Serratula tinctoria (Compositae)	Flowers, roots	20E, polypodine B	8.4–12.0	18
Sesuvium portulacastrum (Aizoceae)	Whole plant	20E	3.5	15
Vitex fishereii (Verbenaceae)	Root bark	20E, Vitexirone	50.0	22

endocrinologists suggest that these compounds play a qualitative defence mechanism in plants directed against non-adapted herbivores[17,27]. The report on the isolation of first ecdysteroid from mushrooms, viz. paxillosterone which shows selective action on Dipteran insects that are highly specialized mushroom herbivores[28], supports the theory of defensive role of ecdysteroids in plants.

Extraction and isolation of phytoecdysteroids

The usual and convenient methodology for extraction of ecdysteroids from plant materials is to homogenize the biological samples first in a mixture of an alcohol (methanol or 95% ethanol) and water[4]. Methanol extracts, if the pigments are removed by hexane partitioning, will be robust enough for bio-assays[29].

A common method for extraction of ecdysteroids from the dried plant materials used by CSIRO, Australia, is described by Horn and Bergamasco[12] which is given in Box 1.

Several techniques like reversed phase TLC, column chromatography, supercritical fluid chromatography followed by mass spectrometry have been followed[30,31] for the isolation of phytoecdysteroids. High pressure liquid chromatography is the most commonly used method for separation of ecdysteroids which has been noticeably improved in the recent years[11]. A major tool for measuring ecdysteroid concentration is the radioimmunoassay, which has been developed by several workers[32]. More recently, a competitive enzyme immunoassay (EIA) has been reported using 20-hydroxyecdysone-acetylcholinesterase derivative[33]. A simple and rapid microplate-based bioassay using ecdysteroid-responsive *Drosophila* cell line has been developed by Clement *et al.*[29].

Box 1. Flow diagram of the isolation of ecdysteroids from plants[12].

Dried and milled sample (10 g)

↓ Extracted with 96% ethanol (200 ml). Extract dried in vacuum and residue partitioned between hexane and 75% ethanol (10 ml of each phase). Hexane extract discarded.

Ethanol phase

↓ Concentrated in vacuum and partitioned between chloroform:ethanol:water (2 ml of each phase). Aqueous phase discarded.

Chloroform phase

↓ Concentrated to dryness and dissolved in ethyl acetate : ethanol (2:1) as a 5% solution and filtered through neutral alumina (10% H_2O, 2 g) and eluted with further solvent (25 ml). The total eluate was evaporated to dryness.

Crude ecdysteroids

Dissolved in chloroform:ethanol (2:1 to make a 5% solution w/v) and an aliquot (2 µl) transferred to a TLC plate.

Role of ecdysteroids in growth and development of silkworms

It is known that the prothoracic gland is the primary physiological source of ecdysone (E)[13] during most of the postembryonic development and 20E is formed from E by cytochrome P-450-dependent monoxygenase systems[24]. It was believed that E is a prohormone and is

converted to 20E which is the physiologically active form of moulting hormone as it is the predominant ecdysteroid found in the hemolymph during larval–larval and larval–pupal development[24]. But in the silkworm, both E and 20E are considered to play a significant role in the larval moulting process[34]. The interplay of juvenile hormone with ecdysteroids in hemolymph regulates the onset and timing of larval moulting cycles. In the final instar, there is a reduction of juvenile hormone in hemolymph at which stage a surge in the moulting hormone initiates the process of metamorphosis[35]. According to Sehnal and Akai[36], the baseline ecdysteroid level is maintained in each instar of silkworm larva for a brief period only, followed by a moult-inducing surge of hormone. But in the last larval instar, the baseline ecdysteroid level seems to be lower, though sustaining for a longer period than in the previous instars. Gu and Chow[35] observed that very low ecdysteroid titres during the early stage of the last larval instar may play an important role in initiating decreases in juvenile hormone titres as well as in directing metamorphosis. These authors suggest that low ecdysteroid titres during early stage are important developmental signals for corpora allata to cease juvenile hormone production as well as for larval–pupal transformation.

The impact of phytoecdysteroids on the larval growth and development of silkworm, *Bombyx mori* has been investigated by many workers. Kobayashi *et al.*[7] reported that ecdysterone and inokosterone isolated from the roots of *Achyranthes fauriei*, exhibited high moulting hormone (MH) activity in silkworms. The host plant of the silkworm, *Morus* spp. is also known to contain several phytoecdysteroids[37], so that the selection of leaves with optimal amounts of these compounds may be an important parameter in Japanese sericulture industry which utilizes powdered mulberry leaves for artificial diets[8]. The effects of 20E on *B. mori.* larvae vary according to the concentration of the compound, precise developmental stage of the insect exposed and the duration of exposure to the hormone[8]. It is reported that 10 μg of 20E injected on day 3 of IV instar promotes the larvae to moult while the same injection 24 hours later delays the moult to V instar[8]. Chou and Lu[14] observed that a single administration of moulting hormone to silkworm larvae at the end of V instar shortened their feeding period, while in the earlier stages the feeding period was prolonged. In the last instar, the ecdysteroid titre increases slightly before the gut purge and rises steeply until pupation. Therefore, oral administration of this hormone before spinning in silkworms apparently brings about an accelerated and synchronized spinning without affecting cocoon quality[14] (Box 2).

Under low doses of phytoecdysteroids, development of the silk gland is also accelerated as studied by Schnal and Akai[36]. The effect of 20E on the fibroin synthesis was first described by Shigematsu and Moriyama[38]. An increase or decrease of silk formation caused by a continuous supply of phytoecdysones is known to result in prolongation or shortening of the feeding period, accordingly. The amount of silk protein formed in the spinning period was also reported to increase with large amount of inokosterone[39]. An inhibition of fibroin synthesis was observed by Shigematsu and Moriyama[38] in 3-day-old V instar larvae, whereas a significant stimulation was found in 5-, 6- and 7-day old larvae receiving a critical concentration of 20E. They concluded that a stimulation of fibroin synthesis occurs by the addition of ecdysteroids only when a larva contains an insufficient amount of the steroid for maintaining synthesis at maximal level as in the case for 5- and 7-day old V instar larvae. A sudden rise in ecdysteroid titre apparently terminates feeding at the end of this instar and stimulates spinning which may be the result of accelerated effect on the development of the silk gland[36].

Box 2. Cocoon spinning in silkworms

The silkworm, *Bombyx mori*, a holometabolous insect, feeds only during the larval stage of its life cycle. The larva undergoes four moults and five instars before it starts spinning a cocoon and transforming itself into a pupa. Nutrition and synthesis of silk protein are the major functions in the larval stage. The pair of silk glands become very active during the fifth instar and at the end of the larval stage, most of the nutrients fed are utilized for silk synthesis. Cells in the silk gland have the capacity to synthesize enormous quantities of silk proteins during a very brief period. Fibroin, the main component of silk protein, is exclusively synthesized in the posterior part of the silk gland and sericin, the outer covering of silk filament is synthesized in the middle silk gland. The moulting hormone, mainly, 20-hydroxyecdysone (20E) is an important factor for the promotion and maintenance of fibroin synthesis. At the end of the larval period, when worms start spinning, they are picked by hand and put on mountages to facilitate cocoon formation. This process called mounting, requires more labour within a short period as all the worms in a batch have to be put on mountages at the correct time.

Application of phytoecdysteroids

The phytoecdysteroids such as inokosterone were discovered by Takemoto *et al.*[40] and attempts were made to use it for the control of growth and as an accelerator of larval maturation in silkworms[41]. The exogenous administration of phytoecdysones was found to increase the cocoon yield[7] and enhance the productivity of silkworm rearing with insignificant variations in the quality of cocoons[14,42,43]. Phytoecdysteroids, therefore, attained a distinct position as they can be commercially exploited to improve production in sericulture. In Japan, 20-hydroxyecdysone extracted from *Pfaffia iresinoides* was approved as agricultural chemical for sericulture in 1994

(ref. 10). In China, the moulting hormone, mainly extracted from the roots of *C. arachnoides*, is used by farmers to accelerate spinning which saves labour during the mounting process[9]. The exogenous administration can be used in various ways altering time and dosage depending upon the requirement to improve productivity in sericulture as mentioned below.

To promote uniformity in spinning

Approximately 10% of the total labour required for silkworm rearing is needed at the time of picking mature larvae for mounting. During inclement weather conditions or by poor rearing management, the duration of mounting between beginning to closure prolongs for 48 to 72 h. This leads to difficulties in mounting, management of labour, harvesting of cocoons and their marketing. By administering moulting hormone when the silkworms are about to spin, spinning will be more or less simultaneous, enabling improved efficiency in mounting and uniformity of pupation[9]. Ninagi and Maruyama[10] clearly showed that the administration at about one day after first feeding in V instar prolonged its duration while administration on and after the fourth day shortened the duration and therefore the time of administration is crucial to bring about the desirable results. The cocoon weights increased when 5 to 10 µg 20E was administered orally just before spinning[10]. While using artificial diet or automatic rearing machines, the use of phytoecdysteroids is obviously a boon to users as the time and labour can be saved because of the reduction in duration of mounting of spinning worms[44]. Other labour-saving methods like shoot feeding technique which is becoming popular in India, at present requires more labour at the time of spinning. The uniformity in spinning can save labour as free mounting and other easy mounting methods can be followed. It is reported that the application of phytoecdysteroids after 72nd hour of fifth instar will significantly accelerate the maturation of larvae of indigenous silkworm races without affecting the cocoon characters[16]. Similar observations were made in our laboratory using phytoecdysteroids during initiation of spinning[45], indicating reduction in mounting duration and insignificant differences in cocoon and shell weights in PM × NB4D2 silkworms (Figures 1 and 2).

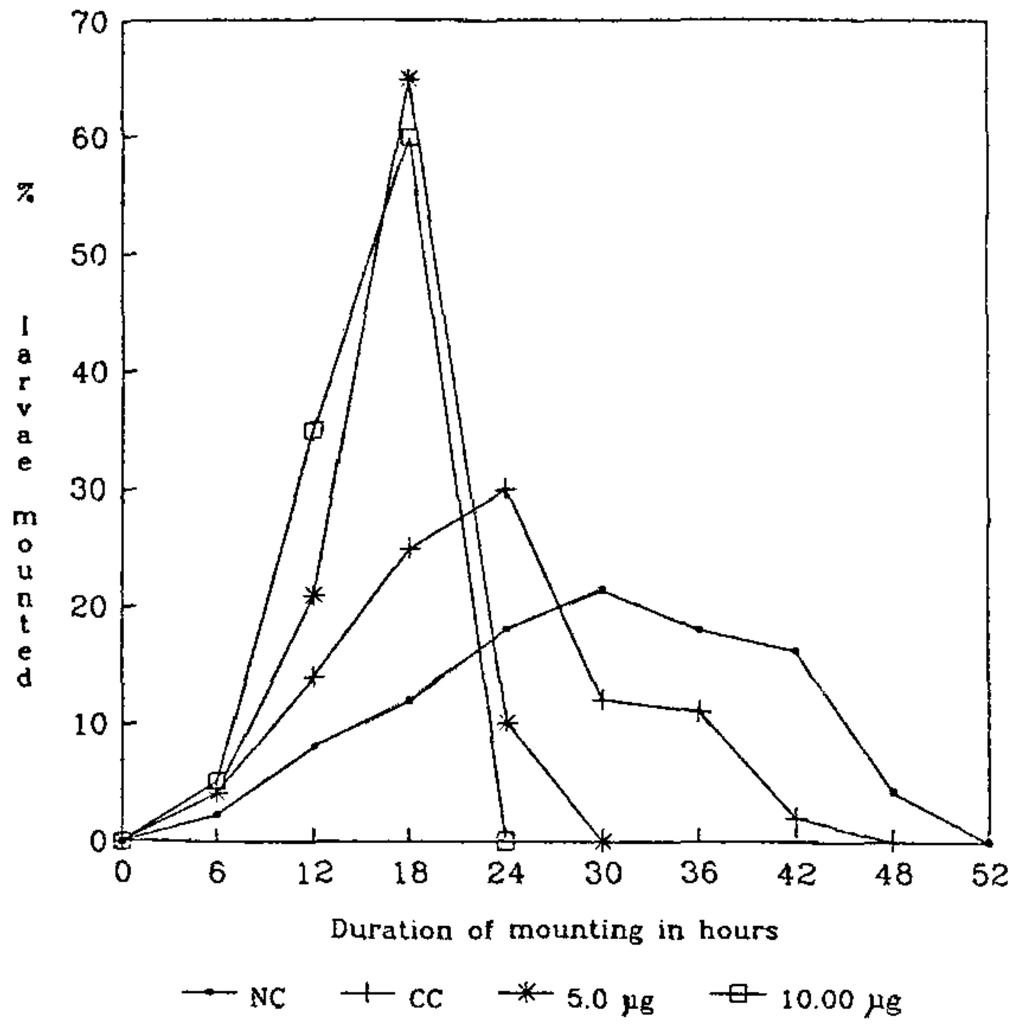

Figure 1. Effect of phytoecdysone treatment on duration of mounting in silkworm (Race – PM × NB4D2).

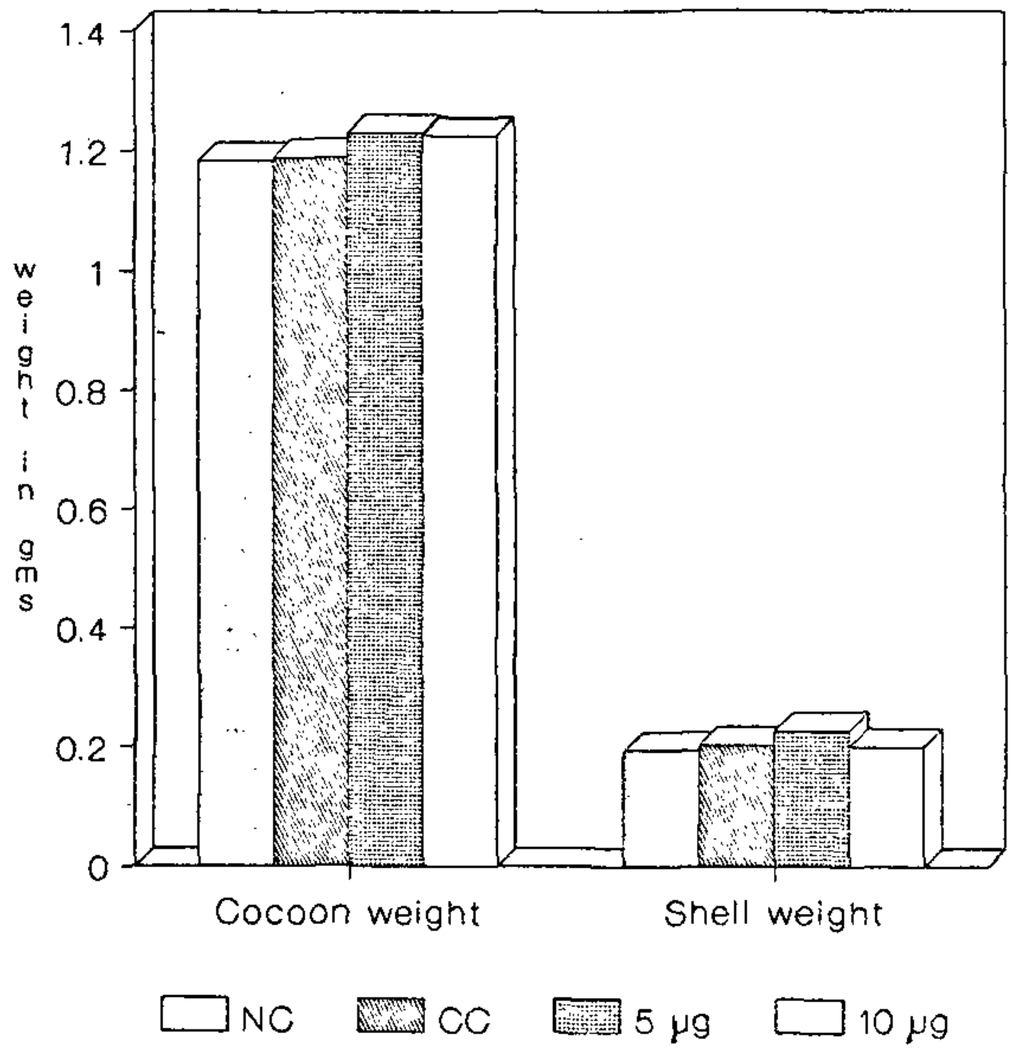

Figure 2. Effect of phytoecdysone treatment on cocoon characters of silkworm (Race – PM × NB4D2).

To shorten the larval duration

During silkworm rearing, shortfall of mulberry leaf is often encountered mostly due to improper planning of rearing schedule or due to unfavourable weather for the growth of mulberry plants. Under these circumstances, the administration of phytoecdysteroids after the middle stage of fifth instar on 4th or 5th day at higher doses will result in shortening of larval duration[9]. It will be a boon to the farmers as it shortens the larval duration by 2–3 days though the cocoon yield is reduced to a little extent[9]. Hence, this method can only be employed to avert total crop losses in extreme conditions.

To prevent or reduce non-cocooning silkworms

The occurrence of non-cocooning silkworms, due to high rearing temperature or feeding of tender leaves can be brought down to 9.6% by phytoecdysteroid application[9]. By the application of high dosage of juvenile hormones, which are used to prolong the larval duration for higher cocoon yield, sometimes non-cocooning silkworms might appear, in which case, use of ecdysteroids will promote spinning[46]. Thus a combination of juvenile and moulting hormones if administered at the proper dosage and time may prove to be more beneficial by increasing productivity in sericulture.

To increase cocoon filament yield

Administration of MH, at earlier stages of fifth instar is reported to increase cocoon filament yield because of the increase in cocoon shell ratio[9], as moulting hormone is known to stimulate silk synthesis[36].

To reduce crop loss due to diseases

Mounting hormone can also be administered for shortening the larval duration and reducing crop losses caused by diseases. The mode and time of administration is similar to that when the larval duration has to be reduced due to leaf shortage. By inducing early spinning, the contamination and spread of the disease can be checked avoiding total crop loss[9].

Summary

Generally, plants produce numerous secondary metabolites out of which insect moulting hormones form a major group of chemicals. It has been clearly established that most of the common phytoecdysteroids with 20-hydroxyecdysone-like activity affect insect growth and development on ingestion. There are many types of ecdysteroids in plants, the most common being 20-hydroxyecdysone (20E), the amount of which varies among plant species. Because of their abundant occurrence in plants, phytoecdysteroids form an important and cheaper source for commercial application in sericulture. Their moulting hormone activity induces different responses in silkworms which can be manipulated for maximum benefit like early and uniform spinning behaviour, increase in silk yield, enhancing productivity and reducing crop losses. The most advantageous use of phytoecdysteroids is the induction of uniform spinning behaviour which will not only make the management of mounting easier but because of harvesting and marketing at appropriate time, the cocoon quality will be improved. With the labour becoming more scarce even in developing countries, any labour saving method improves productivity and with the added advantage of the improvement in quality of cocoons, this method will be a boon to rearers. Hence it can be concluded that the application of phytoecdysteroids in sericulture under tropical conditions has a promising future.

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RESEARCH ARTICLE

Calcutta's industrial pollution: Groundwater arsenic contamination in a residential area and sufferings of people due to industrial effluent discharge – An eight-year study report

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An industry was producing 20–30 tons of Parin Green [Copper acetoarsenite ($Cu(CH_3COO)_23Cu(AsO_2)$] per year and was discharging most of the effluent without proper treatment in an open land just outside the boundary of the factory. Due to the high porosity of the soil, arsenic percolated and contaminated the underground aquifer. More than 7000 people living around the discharge point, were exposed to arsenic contaminated water. Primary investigations and follow-up studies in the area, carried out for the last 8 years, have revealed that some of the distant tubewells which were earlier free from arsenic, are getting contaminated now. In May 1997, a preliminary analysis of arsenic in the urine, hair and nails of some of the people drinking contaminated water from CMC deep tubewells indicated a higher arsenic concentration than in the normal population.

INSTANCES of arsenic contamination in the world are mainly of two categories: (a) natural groundwater contamination; and (b) the contamination of water, air and the environment caused by the use of arsenical pesticides, industrial activities, mining and smelting operations.