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Aflatoxin Contamination of Coconut Products

by

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SUMMARY No quantitative data exist in the local or world literature on the aflatoxin problem in coconut products.

We have examined local coconut products (poonac, oil and copra) in relation to the identity of contaminant fungi, the incidence of toxigenic strains, aflatoxin levels and the origin of aflatoxicity.

The pattern of isolates was similar to that described by earlier workers. A combination of toxigenicity test plates containing various media is recommended to identify the maximum number of potential producers of aflatoxin. Aflatoxin production was confined to strains of *Aspergillus flavus-parasiticus*.

Approximately half the number of samples tested had "medium" or "high" toxin levels and the attention of the coconut industry is drawn to this problem. In comparison with groundnut, the toxin levels detected, even in selected kernels, were very low ; the effects of the drying process with deposition of smoke on the kernels is suggested as a possible explanation.

Commercial poonac was found to be a very poor medium for aflatoxin production. The toxin detected in commercial samples is probably residual toxin derived from the initial contamination of the kernel.

INTRODUCTION

Toxic effects in man and animals resulting from the consumption of fungus infected foods have been recognised for several decades (Kraybill and Shapiro, 1969). However, it was only with the outbreak of disease in farm animals in Britain in 1960 (Allcroft, 1965) and in hatchery trout in the United States (Halver, 1965) that research in the mycotoxicoses received great impetus which resulted in the discovery of aflatoxins--the toxic and carcinogenic metabolites of *Aspergillus flavus*—and their significance in animal and human nutrition. The chemical nature, biological effects, methods of extraction and assay and their mode of action have been dealt with in several recent reviews (Goldblatt, 1966 and 1969; Wogan, 1966; Schoental, 1967).

Briefly, the aflatoxins have been shown to be toxic to a wide range of laboratory and farm animals including monkeys, cattle, pigs, ducks, turkeys, chicken, trout, salmon, rabbits, dogs, rats, guinea-pigs; to chick and duck embryos; to a variety of human and animal cells in culture and to plants. The toxic effects range from acute effects such as death with necrosis and nuclear abnormalities particularly in the liver of all susceptible species studied, while chronic effects include teratogeny and tumour formation. Indeed, in rats, aflatoxin, in terms of weight, has been recognised as the most potent hepatocarcinogen known. Chronic toxic effects of importance in farm practice include a fall in weight gain and milk yield (Allcroft, 1969). The nature and intensity of the toxic effects depend

on the dose, species, age and sex of the animal and the duration and route of administration. A fact of importance is that the acute toxic effects and carcinogenic response have been found to follow a single dose. Carnaghan (1967). World-wide investigations are in progress to determine whether any causal relationship exists between the consumption of aflatoxin-contaminated food and the incidence of disease in the human gastrointestinal tract including the liver(Oettle, 1964; Kraybill and Shapiro, 1969).

Apart from the hazards of direct consumption of aflatoxin in contaminated foods another possible route of ingestion could be through the milk of animals that have consumed aflatoxic feeds. Such animals have been shown to excrete a modified form of aflatoxin which retains the toxicity of the original aflatoxin (Allcroft and Carnaghan, 1963). A recent report (Robinson, 1967) attributes the development of biliary cirrhosis in infants to the consumption of the milk of mothers who had ingested preparations of aflatoxin contaminated groundnuts. It is however doubtful whether human intoxication could result from the consumption of meat from intoxicated animals.

Aflatoxins have been detected by both physico-chemical and biological methods in a wide range of human and animal foodstuffs including maize, wheat, sorghum, corn, cotton-seed, cocoa beans, palm kernel, soya flour, locust beans, barley, sesame, sweet potato, cowpeas, millet and casava. Quantitative data on the aflatoxin problem as far as coconut products are concerned, is however lacking (Goldblatt, 1968). Raymond(1966) mentions copra amongst the foods which have had detectable levels of aflatoxin but adds that in some of these assays the biological confirmation of toxicity and the quantitation of aflatoxin levels were not done. Even in the most recent review (Goldblatt, 1969) no data is available on the aflatoxin problem in relation to the coconut industry.

On account of the importance of coconut products as articles of food to the people of this country, as economically vital products and as a potential source of protein for human nutrition, we report here the results of a study of the following aspects of the aflatoxin problem :—

1. the identity of fungi contaminating coconut products and the incidence of aflatoxigenic strains amongst these isolates,

2. the degree of aflatoxin contamination of coconut products (copra, poonac and oil) assessed by both biological and fluorescence methods. We have shown earlier (Arseculeratne, de Silva, Wijesundera and Bandunatha, 1969) that fresh, grated coconut is an excellent medium for the production of aflatoxin and this finding also prompted the investigation as to whether the high aflatoxigenic capacity of the fresh kernel is also reflected in natural contamination.

The results described in this paper were derived from the examination of samples obtained from the mills and retail shops in the Kandy and Kurunegala districts and is therefore of restricted scope with regard to the country's coconut industry in general.

A comparative study of the methods applicable to aflatoxin assay of coconut products will be dealt with in a separate paper. Nathanael (1960) made a detailed study of the spoilage of copra by fungi and other agents and has outlined the economic aspects of such spoilage. The results of the present study complement those of Nathanael by presenting data on the toxicological aspects of fungal contamination and it is hoped that this preliminary data will draw the attention of the coconut industry to this problem to initiate a more comprehensive survey and to enforce remedial measures.

MATERIALS AND METHODS

Aflatoxin standards. Qualitative standards of aflatoxins B_1, G_1, B_2 and G_2 in chloroform and quantitative standards of aflatoxins B_1 and G_1 in chloroform were gifts from Dr. Leo A. Goldblatt of the US Department of Agriculture.

Aflatoxigenic strains. Strains NRRL 2999 and ATCC 15546 of *Aspergillus flavus* were used as highly toxigenic reference strains in studies on aflatoxin production.

Media. 'Difco' Czapek-Dox agar was used for the primary isolation and purification of isolates and for their slide culture. Commercial peanuts, screened under ultraviolet light to eliminate fluorescent kernels, and fresh, grated coconut were used for the preparation of homogenates for addition to modified Czapek-Dox medium in screening plates for aflatoxigenicity (de Vogel, van Rhee and Koelen-Smid, 1965). Grated coconut and crushed peanuts were also used. Grated coconut, crushed peanuts and commercial poonac, autoclaved at 14 lbf/in^2 for 10 min were used for studies on aflatoxin production, in flask cuture.

Coconut products. Samples from batches of copra, both uninfected and fungus infected, were obtained from mills, dealers and estates. Samples of bulked oil and poonac (coconut and "parings") were obtained from retail shops and from mills as soon as possible after processing.

Animals. Biological toxicity tests were done on Wistar' hooded and albino rats, weighing 100-200 g at the onset of the experiments and one day old Khaki-Campbell ducklings.

Processing of kernels. Selected kernels, naturally infected with aspergilli or penicillia or both, and uncontaminated kernels were crushed in stone mortars ('*sekku*') as done by rural folk for the extraction of oil. The ratio of oil to poonac was approximately 1 : 1 by weight in these samples; highly deteriorated kernels yielded only small amounts of oil. Some kernels were pulverised in stone mortars without oil expulsion. With large batches of oil and poonac, evidence of inhomogeneity with regard to contamination was obtained; inhomogeneity in relation to sampling will be discussed below, but the results presented were obtained in most instances with single samples.

Extraction of aflatoxin. Aflatoxin was extracted from oil by the method of Trager, Stoloff and Campbell (1964) or by the aqueous acetone method of Pons, Cucullu, Lee, Robertson, Franz and Goldblatt (1966). Commercial poonac and poonac from

selected kernels and fresh uninfected copra were extracted either by the methanol-homogenisor method, the methanol-Soxhlet method (Trager *et al.*, 1964) the water-chloroform method of Lee (1965) or by the aqueous acetone method of Pons *et al.*, (1966).

Assay procedure; chromatographic. Extracts in suitable dilutions in chloroform were spotted on 250μm thin layer silica gel ('Merck' Kieselgel G) plates which were run in the following solvents :—

methanol : chloroform 3 : 97 v/v
acetone : chloroform 1 : 9 v/v
methanol : chloroform : acetic acid 5 : 94.5 : 0.5 v/v.

Plates run in the first solvent were re-run in the third solvent. The use of several solvents would have reduced the misinterpretation of nonaflatoxin but fluorescent spots possessing a colour and Rf similar to those of aflatoxins. No information is available on such compounds in coconut products; they have however been reported in crude extracts of other substrates. Parallel spots of aflatoxin standards in at least three dilutions were run, for identification and quantitation of the unknown inocula. The plates were examined under ultraviolet ligth at 365nm.

Biological tests in ducklings. Extracts of commercial samples were fed to ducklings to obtain biological confirmation of toxicity; such confirmation was necessary since nonaflatoxin compounds could mimic aflatoxin in colour and Rf on thin layer plates. Use of concentrated extracts for feeding tests was preferred to feeding of commercial meal on account of the relatively low level of toxicity of these samples compared with that of selected samples and because the feeding of greater quantities of mouldy meal would have introduced interfering factors which are discussed below. Extracts were dissolved in propylene glycol, then diluted in distilled water 1:9 and fed to 1 day old ducklings in a calculated dose of 0.5 - 1.0 μg aflatoxin B_1 per day for 5 days. Control animals received a similar amount of 10% aqueous propylene glycol. The ducklings were fed their basal diet of corn meal with 10% skimmed milk powder and water *ad lib.*, and were killed on the 8th day. Their livers were fixed in 10% formol-saline and sections were stained with haematoxylin and eosin (H & E) . Biliary hyperplasia was taken as an index of aflatoxicity although no attempt was made to quantitate the toxin by this method on account of the absence of a precise dose-response relationship of this reaction.

Toxicity of samples was categorized as recommended by the Tropical Products Institute, London (Hiscocks, 1965) as follows :—

very high toxicity	over 1 part per million(ppm) aflatoxin B_1
high	0.25–1 ppm ,,
medium	0.05–0.25 ppm ,,
low or negative	below 0.05 ppm ,,

Biological tests with rats. Pulverised, selected kernels were tested for aflatoxicity by feeding to rats. Their normal diet of pellets was replaced by the pulverised toxic material which was fed in weighed amounts with water *ad lib.* Control rats received pulverised, uncontaminated kernels. The defects of such feeding trials are discussed below. The rats were killed at two week intervals upto 3 months of feeding and their viscera were fixed in 10% formol-saline. Paraffin sections were stained with H & E.

Fungal isolation. Poonac was inoculated on Czapek-Dox agar plates which were incubated at 30° C for 5-10 days. Cultures were purified on the same medium. Primary plates were examined on their reverse under ultraviolet light since it was observed that colonies of *A. flavus* showed purple-blue fluorescence restricted to small zones on the colony; subcultures from these areas retained their fluorescence production, on this medium. This fluorescence was taken as a provisional index of aflatoxigenicity since production of aflatoxin in Czapek-Dox broth has been recorded. Colony characteristics on Czapek-Dox medium at 5 and 10 days at 30° C were studied for identification of strains. Slide cultures were made on the same medium and were examined in lactophenol mounts at 5 and 10 days. Confirmation of the identity of some isolates was made by Dr David Park (Queens University of Belfast, N. Ireland.). Isolates were lyophilised in bovine serum as early as possible after purification.

Plate test for aflatoxigenicity. 'Hyflo supercel' plates contaninig modified Czapek-Dox medium with peanut homogenates (De Vogel *et al.*, 1965) and with coconut homogenates (Arseculeratne *et al.*, 1969) were used to screen isolates for aflatoxin production. Plates of Czapek-Dox medium used for the primary isolation and purification of cultures were examined on their reverse under UV light at 365nm and purple-blue fluorescence production was also taken as a provisional index of aflatoxigenicity.

Experimental inoculation of coconut products. 20 g amounts of freshly grated coconut or commercial poonac were autoclaved at 14 lbf/in^2 for 10 min, in Roux flasks or 500 ml Erlenmeyer flasks and were inoculated with a spore suspension of the test strain cultured on potato dextrose agar ('Difco') slopes, suspended in 0.1% Tween 80 in distilled water. Samples in comparative tests received equal volumes of inocula.

RESULTS

Fungal flora of copra. The identity of fungi isolated from copra has been described by Eyre (1932) and Nathanael (1960). We have ourselves isolated a similar pattern of fungi :—

aspergilli	other fungi
Aspergillus flavus	*Mucor sp*
A. wentii	*Penicillium* sp
A. glaucus	*Absidia* sp
A. fumigatus	*Candida* sp
A. niger	
A. nidulans	
A. parasiticus	

While a small proportion of infected kernels showed the presence of both aspergilli and penicillia, the majority of kernels were infected with either penicillia alone–which produced a blue matt of growth on almost the entire inner surface of the kernel which was often undamaged–or with aspergilli. The latter flora were prominent on kernels damaged by insects, rodents or splitting; in these kernels the focus of fungal colonisation was around the edges of the damaged area.

Incidence of toxigenic fungi. Out of a total of 173 strains of *A. flavus-parasiticus* tested, 75 strains (43%) were positive for the production of purple-blue fluorescence on one or more of the toxigenicity test media. Out of a total of 104 strains the incidence of positive reactions with each medium was as follows :—

medium	peanut	coconut	Czapek-Dox
% + strains	72	40	58

The distribution of positivity with medium or combination of media is shown below (Table I.)

TABLE I

The distribution of positivity for purple-blue fluorescence production by aspergilli from mouldy copra, on various media in the plate test

	plate or combination of plates						
	peanut (PN)	coconut (C)	Czapek-Dox (CD)	CD+C	PN+C	PN+CD	all 3 media
percentage number of strains (total 104) producing purple-blue fluorescence	16	11	16	4	15	26	9

A combination of all three media picked up the maximum number of positive strains. Some strains produced fluorescence on as early as even the 2nd day.

Identity of purple-blue fluorescence producing fungi. There were no fungi other than *A. flavus-parasiticus* which produced the purple-blue fluorescence characteristic of aflatoxigenic fungi, although fluorescence of other hues, especially deep green, was produced by *A. niger* and *A. nidulans.* A noteworthy observation was the patchy distribution of purple-blue fluorescence on and around the colony of some strains on primary isolation on Czapek-Dox medium. Subcultures from these fluorescent areas retained their fluorescence production on the toxigenicity test plates. It may therefore be a useful step to screen even primary isolates on Czapek-Dox plates without the 'hyflo' layer to pick up inocula from these areas which may result in a greater proportion of strains characterised as aflatoxigenic than when subcultures are obtained only from the rest of the colony.

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Aflatoxin production by strains which produced purple-blue fluorescence on 'hyflo' test plates. Seven strains selected at random from those which produced marked purple-blue fluorescence on peanut test plates were examined for aflatoxin production in coconut in flask culture, by thin layer chromatography. The results (Table 2) confirmed aflatoxin production by these strains.

TABLE 2

Patterns of aflatoxin B_1 and G_1 production by strains of *A. flavus-parasiticus* which produced purple-blue fluorescence on peanut 'hyflo' test plates

strain number	medium in toxigenicity test plate			TLC assay of aflatoxin production in flask culture* on coconut	
	Czapek-Dox	Peanut	Coconut	B_1	G_1
113	+	+	—	+	—
114	+	+	—	+	—
136	+	++	+	+	+
137	NT	++	+	+	+
209	—	+	+	+	—
214	+	+	+	+	—
218	++	+	—	+	+

NT = not tested

—, +, ++ = negative, positive and strongly positive for purple-blue fluorescence

* = serial cultures assayed at 2-3 day intervals for 2 weeks

There was no correlation between the production of purple-blue fluorescence or the intensity of such fluorescence on a given medium and the production of either aflatoxin B_1 or of G_1 on grated coconut. With the strains studied, peanut 'hyflo' plates appeared to be the most useful medium in revealing aflatoxin production although the coconut 'hyflo' plate was superior to the peanut medium with strains NRRL 2999 and ATCC 15546 with respect to the intensity of fluorescence produced. Inspite of appreciably greater yields of aflatoxin produced by the latter strains on fresh coconut, the coconut 'hyflo' medium was the least useful of the three plate media in screening wild strains.

Toxin levels in commercial poonac. Thirty eight samples from retail shops and 6 mills were examined; with retail samples information was available regarding their age since processing. Mill samples were obtained within 2-3 days of processing. The distribution of toxicity is shown in Table 3.

TABLE 3

Aflatoxin levels detected in 38 samples of commercial poonac

	grade of toxicity and level of aflatoxin					
	nil	<0.05 (low)	0.05—0.25 (medium)	0.25—1 (high)	over 1 (very high)	ppm aflatoxin B_1
number of samples	18	4	10	5	1*	

* confirmed by duckling bioassay

The maximum level recorded was in a sample of coconut (not parings) poonac which had 1.27 parts/10^6 B_1 and 0.38 parts/10^6 G_1.

Evidence of inhomogeneity of aflatoxin contamination. Replicate samples taken from various regions of heaps of commercial poonac each of which was milled as one batch showed a variation of aflatoxin content indicating inhomogeneity of contamination . For example :—

sample	aflatoxin content (ppm)	
	B_1	G_1
1	0.072	0.015
2	0.025	0.004
3	0.035	0.004
4	0.019	0.006
5	0.058	0.017

Such inhomogeneity raises problems in sampling, and will be discussed below.

Samples of both 'coconut' and 'parings' (*kuruttu*) poonac gave positive results. No conclusions could be drawn as to the greater toxicity of either type on account of the small number of samples examined.

Correlation of toxicity with the presence of toxigenic fungi. There was no correlation of toxicity with the presence of toxigenic fungi in the respective samples. Only 2 samples out of 20 toxic samples gave cultures of fungi, positive in the plate test. Conversely, samples which gave toxigenic isolates were non-toxic by chromatographic assay. This finding is discussed below in relation to the origin of toxicity in poonac.

Identity of toxin components in toxic samples. In the 38 samples examined, the distribution of components was as follows :—

	none	B_1 only	G_1 only	$B_1 + G_1$
number of samples	18	6	0	14

Toxin levels in commercial oil. Ten samples of bulked oil were tested; the levels detected are shown in Table 4.

TABLE 4

Aflatoxin levels detected in 10 samples of commercial coconut oil

	grade of toxicity and level of aflatoxin					
	nil	0.05 (low)	0.05—0.25 (medium)	0.025—1 (high)	over 1 (very high)	ppm aflatoxin B_1
number of samples	3	1	3	1	2*	

*one result confirmed by duckling bioassay

It is noteworthy that both 'very high' toxicity samples were obtained from the same mill. The distribution of positivity in respect of toxin components was as follows :—

	none	B_1 only	G_1 only	$B_1 + G_1$
number of samples	3	4	0	3

The colour of the oil samples ranged from pale straw to deep yellow and had no relation to the toxicity of the samples. Aflatoxins B_2 and G_2 were not observed in either poonac or oil from commercial sources.

Toxin levels in selected copra. Ten kernels with heavy growth of aspergilli were found to contain relatively low levels of aflatoxin B_1 with levels ranging from 0.05 ppm to 28 ppm with the majority of samples having below 1 part/10^6. The maximum level of G_1 recorded was 150 parts/10^6 in the sample which had 28 ppm B_1. Kernels infected with penicillia had negligible levels of aflatoxin. Mechanical (stone mill) extraction of oil from infected kernels produced a cake which had approximately the same level of aflatoxin as the expressed oil. Extraction of groudnut with solvents such as hexane in which aflatoxin is insoluble, has been recorded as producing nontoxic oil but leaving the original aflatoxin content in the residual cake (Goldblatt, 1966). A similar method of extraction may be applicable to copra for the production of nontoxic oil.

Aflatoxins B_2 and G_2 were not detected in these samples.

Biological tests for toxicity. Tests on rats with oil from selected copra. After 2 months feeding, the lesions seen were similar to the subacute reactions described in aflatoxin-fed rats (Butler, 1965) and consisted of nuclear pleomorphism, pyknotic nucleii, mononuclear cell infiltration with patchy periportal necrosis and hyperplasia of the bile ducts. After 3 months feeding, foci or microscopic nodules in the liver lobules of varying size were seen, in which were masses of hepatic cells with increased basophilia of the cytoplasm (Fig. 1). Such foci may represent precancerous tissue and have been described in the liver of aflatoxin treated trout (Halver, 1965).

Hepatoma formation occurs in the trout as well as in rats after aflatoxin treatment. Nuclear enlargement and pleomorphism, condensation of chromatin in the periphery of the nucleus ('owl-eyed' nucleus) and binucleate cells were also common. In some areas, masses of enlarged hepatic cells ('megalocytes'- see Butler, 1965; Lancaster, 1968) with enlarged and double nuclei were also seen (Figs. 2, 3 and 4.)

Tests on rats with contaminated poonac from selected copra. Appearances similar to those produced by toxic oil were also seen in these animals and consisted of foci of necrosis with mononuclear cell infiltration in the periportal areas, double nucleated hepatic cells, enlarged, bizarre and pleomorphic nuclei and hyperplasia of the biliary epithelium (Fig. 5).

Similar appearances, though more marked were seen in rats starved for 1 week before feeding of the toxic diet.

In other regions, spherical microscopic foci of vacuolated hepatic cells were seen surrounded by a rim of hyperchromatic, non-vacuolated cells (Fig. 6). These abnormalities were most pronounced in rats fed on the toxic diet for a longer duration.

Tests in ducklings. Extracts from toxic commercial samples in the "very high' toxicity grades, when fed to ducklings produced biliary hyperplasia (Fig. 7), confirming the results of the chromatographic assay.

Growth of Aspergillus flavus and aflatoxin production on poonac.

Storage of poonac in mills and retail shops for variable periods, sometimes under adverse conditions permitting moistening, necessitated determination of the suitability of poonac as a medium for aflatoxin production. In addition, information was required as to whether the toxicity detected in commercial poonac was due to such infection during storage or whether it was residual toxin derived from the original copra infection. Table 5 shows the results of a test which suggests that poonac even when adequately moistened and infected with a highly toxigenic strain (NRRL 2999) was a relatively poor substrate for aflatoxin production.

TABLE 5

Aflatoxin production by *Aspergillus flavus* strain NRRL 2999, on poonac, freshly grated coconut and peanut.

substrate	aflatoxin B_1 content (ppm)		
	sterile	inoculated*	
		unmoistened	moistened
peanut	0.013	—	3.15 good growth and sporulation
coconut	0.008	0.009 no visible growth	19.8 good growth and sporulation
poonac	0.00	0.00	0.00 delayed growth, sterile myceliumtill late

*strain ATCC 15546 ; 26-28°C; 15 days incubation ; static

DISCUSSION

The factors which predispose to fungal spoilage of copra have been studied by Nathanael (1960) but we would emphasize from our own observations that contamination by aspergilli, in contrast to penicillia, is particularly common around areas which have been damaged mechanically or by insects, or rodents. Fungal spoilage is most evident to visual inspection when sporulation has occurred, but it is significant that detectable levels of aflatoxin could be present on groundnuts within 48 hr of harvesting (Hiscocks, 1965). Moreover, the optimum conditions for toxin production are not necessarily identical with those required for growth and sporulation and hence biologically significant levels of aflatoxin could be present without concomitant macroscopic evidence of fungal spoilage. These observations would emphasize the need for proper methods of processing and storage of copra.

Incidence of Aflatoxigenic strains. In an earlier study (Arseculeratne *et al.*, 1969) using highly toxigenic strains of *A. flavus* we found that a greater degree of purple-blue fluorescence was produced on 'hyflo' plates in which the peanut homogenate had been replaced by coconut homogenate. In the present study, however, we observed that there was no constant pattern of relative intensities of purple-blue fluorescence produced by freshly isolated wild strains of *A. flavus-parasiticus* on these toxigenicity plates, containing either peanut or coconut homogenate, or Czapek–Dox medium. Some strains produced greater intensity of fluorescence on peanut plates than on coconut plates. Evidently such strains, in spite of prolonged adaptation to growth on coconut in the field, nevertheless produced maximum fluorescence on peanut plates. The variation between different strains in respect of the intensities of fluorescence produced on these plates which contained various media suggests that the screening of isolates for toxigenicity, could advantageously be made on several

different media simultaneously. It has also been found (Arseculeratne and Bandunatha, 1969) that the content of aflatoxin content in serial cultures on coconut in flasks varies significantly with the duration of incubation and that serial assays are needed to obtain a true picture of the quantity and identity of the toxin components produced. In the plate test, however, the removal of the toxin by adsorption into the 'hyflo' layer may mask such variations and we have observed in plates, only a progressive increase followed by a slow decline of the fluorescence, even with strains which produced such cycles of variation of toxin content in flask culture.

Grades of toxicity. According to the grades of toxicity classified by the Tropical Products Institute, several commercial samples of poonac and oil examined by us would fall into the medium toxic and very toxic categories. This would indicate that the aflatoxin problem with coconut products is a real one. The relatively low levels of aflatoxin detected even in selected kernels in the field contrasts with the very high levels we obtained with fresh grated coconut as culture medium. While one reason may be the poor toxigenicity of these wild strains, other factors in the kernel may also be involved; for example, the moisture level. Earlier, Arseculeratne *et al.*, (1969) had found that rehydrated desiccated coconut was also a good medium for aflatoxin production. An inhibitory effect of the smoking process on fungal growth and toxin production and the natural detoxification on storage may also be factors which might explain the low levels. The sparsity of growth of aspergilli except on areas of damage in which the inner parts (non-smoked) of the kernel is exposed, may also suggest some inhibitory action of the deposit of material as a result of the smoking process, on fungal growth. Groundnut kernels have been found to contain up to 10^5 ppb aflatoxin (Goldblatt, 1966): hence the acute toxic effects such as death which occurred in turkeys in Britain, will draw immediate attention to the intoxication. Copra and poonac, however, have relatively low levels of aflatoxin and therefore one could expect that the chronic effects of the consumption of aflatoxin-contaminated food would be more prevalent in this country and these effects may include reduction of weight gain and fertility rather than a high mortality in farm animals. This could perhaps be a reason for the fact that the aflatoxin problem has drawn little attention in this country.

Implications of aflatoxin contamination. Apart from the direct consumption of these toxic products by animals and man, the potential use of coconut as a source of supplementary protein in human nutrition makes the aflatoxin problem an even more urgent one. Work in other laboratories (Chelliah and Baptist, 1969) has been directed towards the extraction of protein from various vegetable sources including coconut. The promise of this material as a source of dietary protein emphasizes the need for the control of aflatoxin contamination of foods in this and other developing countries, especially in view of the following factors: (a) malnutrition increases the susceptibility to aflatoxin and this situation is likely to be found in precisely those countries where not only malnutrition needing dietary supplements exists but also because (b) our tropical environmental conditions are ideal for fungal growth and toxin accumulation on improperly processed or stored foods. In addition (c) the susceptibility to aflatoxin is greater, the younger the animal and it is therefore the young ill-nourished which are the

most vulnerable to aflatoxin. On account of this potential hazard, theWHO/ FAO/UNICEF has recommended a maximum permissible level of aflatoxin of 0.03 parts/10^6 in foods for nutritional purposes (Goldblatt, 1966). However, some authors would go so far as to suggest that any degree of detectable aflatoxin in foods would indicate that the food is unsuitable for consumption although the urgent needs of malnutrition itself will have to be weighed against the consumption of even minimally contaminated food. Butler (1965) states 'I would emphasise that the presence of traces of compounds such as the mycotoxins in a food warrants considerable attention as a health hazard to man.' Perhaps such caution is justifiable on account of the absence of data on the susceptibility of humans to aflatoxins, although in the rat it is the most potent hepatocarcinogen known.

Biological toxicity tests. The direct feeding of mouldy foodstuffs to animals has been used to detect and assay mycotoxicity. However, it may be preferable to use extracts of foodstuffs, added to regular non-toxic diets since the mould growth *per se* could not only reduce the nutritive value of the foods (Richardson, Wilkes, Godwin and Pierce, 1962) but also produce a wide range of toxic substances (Majumder, Narasimhan and Parpia, 1965). The malnutrition resulting from prolonged feeding of the nutritionally deficient food could aggravate the effects of the mycotoxin under study (Madhavan and Gopalan, 1965). A further disadvantage of the use of oil in feeding tests could be the development of disordered fat metabolism which might interfere with the interpretation of the abnormal histology of the intoxicated liver.

Origin of the toxicity in poonac. We have demonstrated that even when adequately moistened and infected with a highly toxigenic strain and cultivated under suitable conditions, poonac produced relatively low yields of aflatoxin when compared with yields from freshly grated coconut cultured under the same conditions. Similar results were obtained by Mayne, Pons, Franz and Goldblatt(1966) who showed that the by-products of cotton-seed processing were unsuitable media for fungal growth and aflatoxin production. Another observation which indicated that the aflatoxin detected in poonac is residual toxin and not toxin which was produced *de novo* in the poonac from contamination during storage, is that the aflatoxicity was not correlated with the presence of toxigenic strains in these samples. Conversely, the samples which had toxigenic strains were non-toxic by fluorescence assay which indicated that these toxigenic strains were not producing toxin in the samples in which the strains were found. This data would suggest that fungal contamination of poonac, stored even under conditions optimal for fungal growth will not accumulate significant levels of aflatoxin and that the aflatoxin found in such material is residual aflatoxin derived from the original, infected kernel from which the poonac was derived. While prevention of fungal spoilage by proper processing of kernels is of prime importance, the exclusion of infected kernels from the milling process would therefore also be an important step in the elimination of aflatoxin contamination of poonac and oil. Goldblatt's comments (Goldblatt, 1966) on groundnut are relevant -"If just one peanut with a million ppb of aflatoxin is admixed with 10,000 kernels that contain none, the whole lot will assay at the relatively high level of 100 ppb of aflatoxin."

Detoxification. No data appears to exist on the detoxification of coconut products. Detoxification methods for groundnut products have been closely studied and the applicability of similar methods to poonac was shown us by the results (Table 6) of preliminary experiments in which the effects of moist heat (Coomes, Crowther, Fuell and Francis, 1966) and of dilute solutions of calcium chloride (Sreenivasamurthy, Jayaraman and Parpia, 1965) on contaminated poonac from selected kernels were studied :—

TABLE 6

Effect of moist heat and of calcium chloride on aflatoxin content of poonac from selected, contaminated kernels.

treatment	aflatoxin content (ppm) B_1	G_1
basal sample	5.60	1.68
	5.70	1.72
after stirring in 1% calcium chloride solution	0.73	0.11
	0·11	0.04
basal sample	0.104	
after autoclaving—1 h, 14 lbf/in²	0.036	
after autoclaving—3½ h, 14 lbf/in²	0.000	

Whilst it is possible to apply such methods to coconut poonac, an undesirable result may be the loss of quality or nutritive value of the detoxified meal (Goldblatt, 1966) which has been demonstrated of groundnut meal, in which the amount of available lysine was reduced after moist heat detoxification. In addition, the detoxification process may, in commercial practice, require elaborate equipment. It will therefore be far more economical, apart from being better practice, first, to reduce or eliminate spoilage of the original kernel by proper processing and storage and secondly, to exclude from admixture with uninfected kernels, the infected ones which might have escaped proper processing.

Preliminary observations (unpublished) indicate that the toxin levels of oil and poonac fall on storage at room temperature and hence we examined material direct from the mills soon after processing. Assays on the few samples which we examined suggest that the toxin levels of freshly prepared products are in fact higher than that of retail samples which have been stored for prolonged periods. While this observation needs confirmation, it raises questions as to the mechanism of the fall in toxin levels and it may perhaps be a useful factor in commercial practice and in field surveys.

Inhomogeneity of samples in respect of aflatoxin content raises the question of representative sampling. However the present commercial practices, which involve pooling and processing of batches of copra from different sources and varying degrees of mould

contamination and the bulk storage of the processed products, not only make adequate sampling difficult but also renders difficult, regulatory measures such as the exclusion of portions of material which show significant levels of toxicity.

Several millers with whom we have discussed the problem of fungal spoilage believe that while contamination of kernels with " yellow fungi" (aspergilli) is undesirable, the presence of " blue fungi " (penicillia) would indicate that the kernels have indeed been adequately dried. It has however to be pointed out that even the penicillia produce a wide range of toxic factors (Majumder *et al.*, 1965). An ancillary factor in the control of mycotoxin contamination in coconut products would therefore appear to be the education of the rural worker and miller in the implications, both economic and biological, of fungal spoilage.

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EXPLANATION OF PLATES

PLATE I

Fig. 1. Microscopic nodule (N) of hepatic cells with deeply basophilic cytoplasm in rat fed for 263 days with toxic oil from selected copra. Total dose of aflatoxin fed 7 μg aflatoxin B_1 (× 60. H & E.)

Fig. 2. Liver of rat fed toxic oil from selected copra (same animal as in Fig. 1) showing 'megalocyte' (M) and double nucleated cells. Normal liver tissue (N). (×60. H & E.)

Fig. 3. Megalocytes in liver of rat fed toxic oil from selected copra showing binucleate cells (arrow). Same section as in Fig. 2 (× 327. H & E.)

Fig. 4. Atypical enlarged hepatic cells (arrow) wth enlarged, vesicular and pleomorphic nuclei and prominent nucleoli in liver of rat fed toxic oil from selected copra for 100 days. (× 327. H & E.)

PLATE 2

Fig. 5. Portal tract of liver of rat fed toxic poonac from selected copra (total fed 18 μg aflatoxin B_1 and 6μg aflatoxin G_1) showing biliary hyperplasia (arrow) (× 60. H & E.)

Fig. 6. Focus of hepatic cells with cytoplasmic vacuolation (V) surrounded by a rim (arrow) of cells with enlarged nuclei in liver of rat fed toxic poonac from selected copra. (×. 60. H & E.)

Fig. 7. Liver of duckling fed extract of commercial coconut oil (aflatoxin content B_1 1.88 parts/10^6, G_1 1.88 parts/10^6) showing biliary hyperplasia (arrows). (×60. H & E.)

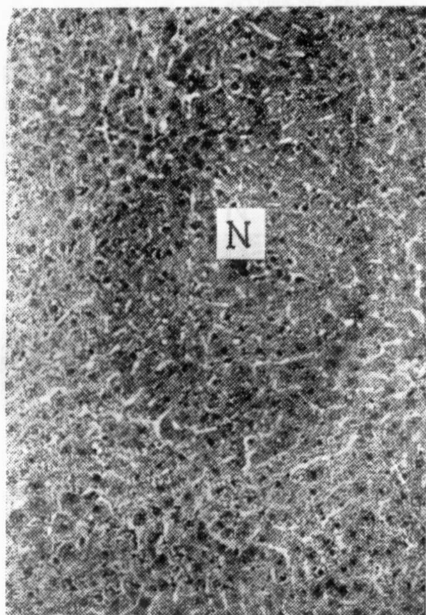

FIG. 1

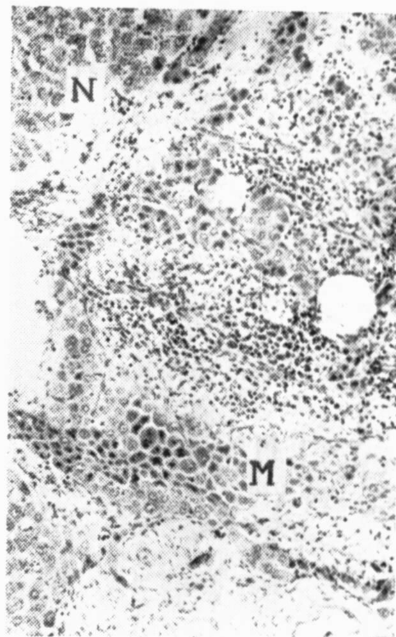

FIG. 2

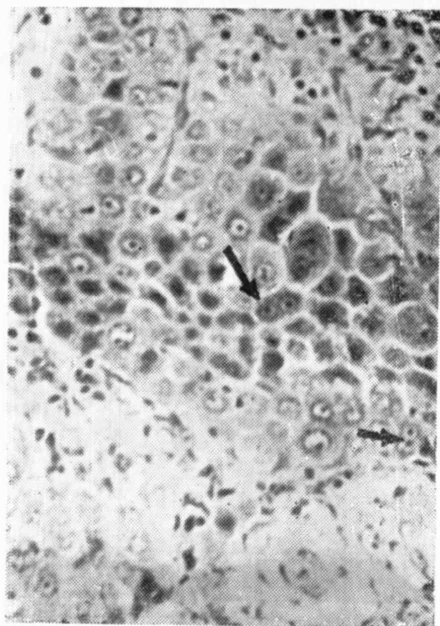

FIG. 3

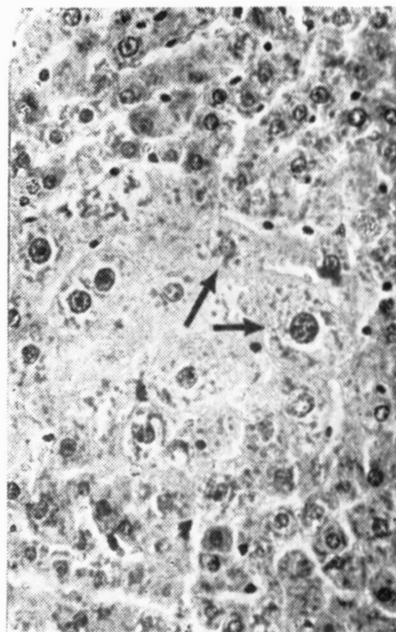

FIG. 4

Ceylon J. med. Sci., **20**, (No. 2, Dec.) 1971, *pp. 60–75.*

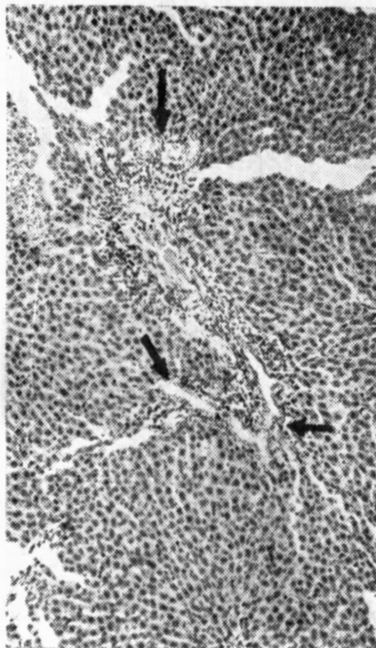

FIG. 5

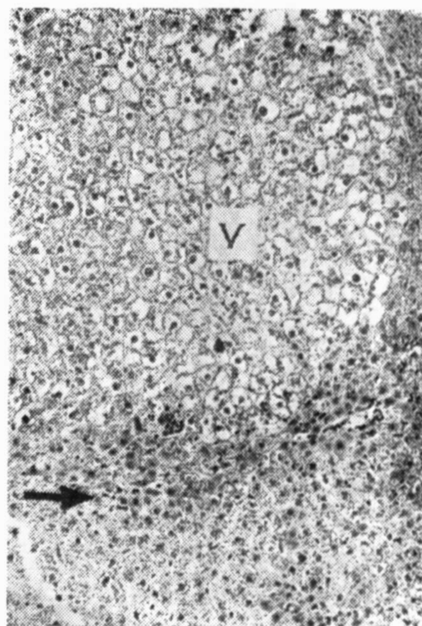

FIG. 6

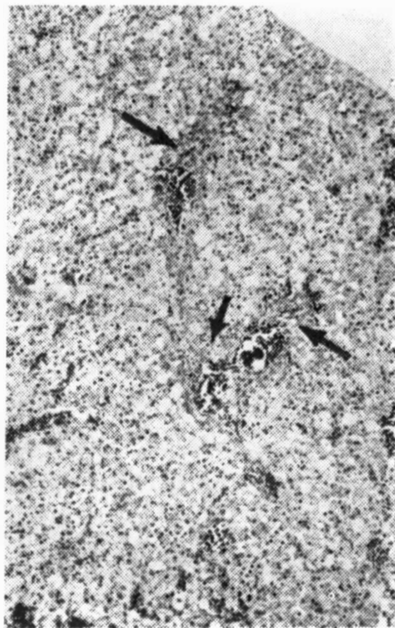

FIG. 7