DISCUSSIONS
I. Floristic Survey

Airborne pollen grains, the male gametophyte of angiospermic plants, are major factors causing upper tract respiratory allergic diseases. For this reason it was important to study, identify and evaluate the airborne pollen flora all over the country. The Indian subcontinent with its unique geographical position has a variety of climatic zones, viz., alpine, temperate, subtropical Himalayas, tropical Gangetic plain and coastal zones with an array of vegetational assemblages. The factor influencing the local airspora is mainly the composition of vegetation. For the present aerobiological survey, the sampling was performed in a farm situated in a suburban zone of Greater Calcutta Metropolis in the lower Gangetic plain in West Bengal. The floristic survey revealed the presence of 122 common and dominant angiospermic plant species (Table 1) with a varying habits, habitats, mode of pollination, flowering period, etc. In this study, along with the floristic survey, morphological configuration of relevant pollen taxa, is the core of aerobiological survey work.

II. Aerobiological Survey

The most important point to conduct an aerobiological survey is the selection of sampler. The sampling techniques work on the principles of gravity, impaction or suction. The gravitational samplers operate on the basis of collection by passive fall of the particulates on adhesive coated semi-horizontal surfaces. The examples are Durham sampler (Durham, 1946), vertical cylinder trap (Ramalingam, 1969), etc. These techniques, however, showed low efficacy, affected by meteorological factors like rainfall, wind speed etc., without allowing volumetric conversion of the pollen concentration.

Another type is wind impaction sampler with high sampling efficiency, largely used in the area with continuous power supply. In this case, the airborne particles are collected on rotating surfaces from the air. The examples are: rotorod (Perkins, 1957) and rotobar (Harrington et al., 1959) samplers. The airspora count in such cases are scarcely influenced by wind speed and other meteorological factors and can be used for intermittent, as well as in continuous airspora survey.

Currently the most commonly used samplers are the volumetric samplers, e.g., Hirst sampler (Hirst, 1952) and Burkard 7-day volumetric sampler (Burkard Manufacturing Co.) to record the periodicity of airborne pollen.

In this investigation, Burkard volumetric trap was used to study the seasonal and diurnal periodicities of airborne pollen grains of the study area. The vertical profile of
airborne pollen concentration of the recorded pollen taxa in their respective peak period was studied by rotorod samplers.

A. Seasonal Periodicities

In the two-year survey, 47 pollen types were recorded using Burkard sampler. There is an array of plant species growing in the study area though all the pollen types emerging from these plants were not found to be airborne. Some such plants were Adhatoda, Argemone, Artocarpus, Brya (Plate I:8), Calotropis, Canna, Cestrum, Heliotropium, Jasminum, Musa, Nicotiana, Oldenlandia, Oxalis, Passiflora, Petunia, Phlox, Raphanus, Rosa, Zizyphus, Malvaceae, Convolvulaceae (Plates III:8; IV:1), etc. The absence in air was probably due to their entomophilous nature. On the contrary, some members of Apiaceae (Plate VI:1), Asteraceae, Euphorbiaceae (Plate III:4), Fabaceae (Plates II:1 & 3; III:1; V:2; VIII:6, etc.), Scrophulariaceae, Solanaceae (Plate V:5) and other members like Bombax, Brassica (Plate VI:6), Delonix, Hygrophila (Plate III:7), Lagerstroemia, Lantana, Lawsonia, Mimusops (Plate IV:2), Moringa, Psidium (Plate V:4), etc. recorded their presence in the air despite their being entomophilous. The location of the sampler at a height of 0.5 meter could have been the cause of occurrence of some entomophilous herb pollen. The sampler was placed within a rice crop field, surrounded by mango and banana orchards and as a result, some pollen of Mangifera were recorded, whereas there was no trace of Musa pollen. Sometimes the pollen of Carica and Catharanthus were not found in the air for unaccounted reason in the air though these plants were flowering round the year.

Among the recorded types (Table 2) the most abundant has originated from the Poaceae family. It has already been mentioned that the sampler was placed in a rice crop field which got reflected in the airborne pollen data. The other dominant pollen types came from Areca, Carica, Cheno-Amaranthaceae, Cyperaceae, Trema, etc. The seasonal periodicity (Fig. 1) reflected the respective flowering periods in almost all the cases. The variation in total pollen count (Fig. 4) showed two major peaks during September and April for both the years, which corroborated with the respective flowering period of wet and dry season of the rice crop and the surrounding vegetation.

Grass pollen as the most dominant type was reported by Chanda (1973), Battacharya et al. (1981) from other parts of West Bengal and Devadoss et al. (1997) from suburbs of Madras city. With regards to other countries, grasses were found to contribute 34% of total aeropollen load in Melbourne (Smart & Knox, 1979) in Australia, while in Europe the result varied from 12-27% in mediterranean region (Bosquet et al., 1984; D'Amato &
Lobefalo, 1989) and in The Netherlands (Spieksma et al., 1985).

The central Calcutta report showed the pollen peaks in May and September in 1985-86 and August-September in 1986-87, Trema being the dominant type (Banik & Chanda, 1992).

In the present survey, however, Trema pollen was the second dominant type. Chanda & Nandi (1971) did not find the presence of Trema pollen in the air of Calcutta, whereas Bhattacharya et al. (1981) recorded this pollen as an important constituent of aeropalynoflora in different parts of West Bengal. Again Banik & Chanda (1992) reported Trema to contribute 68-70% of the airborne pollen load of Calcutta. Such changes could have been caused by vegetational changes due to plantation and eradication programmes.

The third dominant type, i.e., Cyperaceae was also reported by Banik & Chanda (1992).

The other major pollen types like Areca, Carica, Cheno-Amaranthaceae, Cocos, etc., were reported to occur in other parts of India (Banik & Chanda, 1992; Oomachan et al., 1996; Sudha & Agashe, 1996).

B. Diurnal Periodicities

The diurnal periodicities of the recorded pollen types were classified into different categories following Gregory (1973) and Bhattacharya & Datta (1992). There are some differences from the results of previous workers. The pollen of Trema showed the trend of bimodal frequency at 8.00 h and 11.00 h (Fig. 2), whereas Banik (1990) reported morning pattern for the same pollen. Poaceae pollen showed bimodal frequency in this survey, whereas Cheno-Amaranthaceae and Cocos recorded early morning and forenoon pattern. These three types had shown mid-day pattern in the report by Avasthi & Agashe (1997). Banik's (1990) result did not show any specific pattern for Poaceae. In Spain Galán et al. (1991) reported the pollen of grasses and Cyperaceae to show the peak at 10.00 h and 12.00 h respectively instead of bimodal frequency recorded in the present study. Such periodicity variations may be due to change of time, place and climate which have a cumulative influence on flowering, pollination and pollen dispersal.

C. Vertical Profile of Pollen Concentration

The vertical profile of airborne pollen concentration was measured upto the height of
5 meter in the sampling site using rotorad samplers with same rotating speed mounted at different heights. The result was calculated during the respective peak days and periods of the pollen types. The correlation between the pollen concentration and sampling showed three major manifestations (Fig. 3) for the herbs, shrubs and trees respectively. Here Justicia, Lantana and Trema represented herb, shrub and tree respectively. The correlation co-efficient showed negative correlation for both herb and shrub with sampling heights which was significant at >2% level in t test. In case of tree, there was positive correlation significant at >0.1% level. This observation clearly indicates that the source height has a direct effect on the vertical frequency of pollen. For this reason, the herb and shrub pollen types were dominant at lower heights and the trees at a comparatively higher level.

III. Correlation of Variation of Total Airborne Pollen Load Due to Meteorological Factors

The climatic changes have a direct influence on the flowering, anthesis, dispersal and distribution of airborne pollen grains (Fig. 4). In this study, the variation of total pollen count is correlated (Table 4) with meteorological parameters like average temperature, relative humidity, rainfall and wind speed. The positive correlation of the pollen count with average temperature is not surprising, which was found to be significant only at <30% level. The average temperature showed little variation over the year. As a result not only the temperature, but also the other factors have a combined effect on the distribution of airborne pollen grains.

The pollen load showed negative correlation with relative humidity, which is probably due to delay in flowering to break the humidity threshold and attenuation of airborne pollen.

In case of rainfall there was a mild positive correlation at <30% level of significance. It can be interpreted that though temperature cause water stress, temperature reduction due to rainfall triggers anthesis and flowering especially in the later half of dry seasons. Augspurger (1992) made detailed study on Hybanthus and emphasised on the role of rainfall in breaking of bud dormancy through release of draught stress.

The wind speed showed very weak but negative correlation with airborne pollen load. This may be due to atmospheric turbulence and long distance transport of airspora from
IV. Identification of Allergenic Pollen Grains

Air carries a vast array of pollen grains specially originated from wind pollinated plants. Pollen grains are known to cause respiratory troubles. In the two-year aerobiological survey a total of 47 pollen types were recorded to occur in air. Skin-prick tests performed on the allergic individuals of northern suburbs of Calcutta with relevant case history and 26 pollen types showed positive reaction (Table 5). SPT is a reliable in vivo test to study the allergenic potential (Olsen, 1996). It is known that all airborne pollen grains do not possess the potential to cause type I hypersensitivity. Here the maximum allergenic potentiality was shown by Saccharum, followed by Azadirachta, Phoenix, Borassus, Cyperus, Areca, Catharanthus, Cocos, Carica, etc. Grass pollen allergy is very common and in India 1180 species of grasses from 239 genera were recorded (Babu, 1977). Banik & Chanda (1992) reported optimum allergenicity of Saccharum, a grass member out of 22 allergenic pollen types from Calcutta. The same report placed the pollen of Catharanthus, Cocos and other grasses in important positions allergenically, unlike Borassus, Phoenix, Carica and Areca. This variation of response could be due to the constitutional differences in the patients.

Dominant pollen types like Trema, Tamarindus and Justicia, failed to cause +2/+3 reactions. On the contrary, the pollen of Azadirachta, Lantana, Delonix, etc., showed higher significance. Azadirachta pollen produced high allergenicity from Indian subcontinent (Karmakar & Chatterjee, 1994). Pollen of another cultivated grass, Oryza, also showed 16.80% allergenic response. Recently Tada et al. (1996) reported the production of transgenic rice plants deficient of a 14-16 kD allergen but could not decide whether hypoallergic rice pollen/seeds are tolerable to all patients allergic to rice.

Depending on the skin reaction results, eight pollen types were selected for further in vitro studies by ELISA (Table 6). The Poaceae members were already studied causing different aspects of allergic reactions (Singh & Knox, 1985; Kundu et al., 1988; Suphioglu et al., 1992; Sridhara et al., 1995). The pollen types selected were from Azadirachta, Phoenix, Borassus, Areca, Catharanthus, Cocos, Carica and Datura.

In ELISA, variable range of IgE levels were recorded in the patient sera with different levels of skin reactions (Table 6). The optimum count of P/N value was determined for Phoenix, followed by Borassus, Cocos, Azadirachta, etc. From the ELISA results,
Borassus and Phoenix pollen, the two important aeroallergens from our country, were selected for further studies on the basis of their major allergenic components.

V. Physico-chemical Analyses of Some Important Allergenic Pollen

The selected eight allergenic pollen types (Table 7) showed the total soluble protein content ranging from 12.25 - 42.41%, which was greater than the range (5-28.3%) recorded by Stanley & Linskens (1974). The range of total soluble carbohydrate was 5.2-26.00% and within the range (1.2% in Phoenix dactylis to 36.59% in Zea mays) given in the same report, where the percentage of total lipid ranged from 3.42-27.2% and was beyond the range (5-9%) shown by them.

In 11% SDS-PAGE of soluble protein of the mentioned eight pollen types, the overall profile (Fig. 5) showed a number of coomasie brilliant blue bound protein band of varying molecular weights.

VI. Identification of Major Allergenic Component/s of Two Important Allergenic Pollen Found in the Air of Madhyamgram

The two important allergenic pollen selected for the study to identify the major allergen were Borassus flabellifer and Phoenix sylvestris belonging to the family Arecaceae. The study of major allergenic component were performed by different clinico-immunological and physicochemical methods.

A. Borassus flabellifer

Borassus flabellifer Linn., or palmra palm is a very common and economically important tree in India growing wildly or cultivated in West Bengal, Bihar and coastal areas of peninsular region (The Wealth of India, 1948). The prevalence of this pollen in air and its allergenicity was reported previously (Sathees et al., 1992; Chakraborty et al., 1996) but there was no further report about the allergens of this palm pollen. It has been recorded that in the peak months, i.e., in February-March, the relevant pollen contributed 7.32% and 9.12% of total pollen load in the air during the year 1994-95 and 1995-96 respectively.

The Fr. II derived from the first step (NH₄)₂SO₄ fractionation of whole extract of relevant pollen, was found to be maximum allergenic for both in vivo and in vitro tests (Fig.6 & Table 8). Similarly Fr.II A obtained from the second step (NH₄)₂SO₄ fractionation was evidenced as the highest allergenic fraction (Fig.7 & Table 8). The Fr.II A was then sub-
jected to gel filtration and resolved into two fractions (Fr.IIA1 & Fr.IIA2) respective to two peaks of elution profile (Fig. 8).

The analysis of the activity of Fr.IIA1 and Fr.IIA2 by ELISA inhibition test showed the degree of allergenicity. The dose-response curve (Fig.9) showed that Fr.IIA1 was almost parallel up to 10 μg concentration and beyond it was more active compared to Fr.IIA, revealing demonstrating Fr.IIA1 as a major allergenic component.

In case of polysaccharide rich Fr.IIA2, however, it was not possible to get the C50 value of inhibition to be compared with other fractions.

The Fr.IIA1 was found to be a single protein component in native condition, which was further found as a 90 kD component (Fig. 10) in SDS-PAGE. Further analyses by immunological techniques like rocket (Fig. 11) and two-dimensional immunoelectrophoresis (Fig. 12), its homogeneity was confirmed.

The determination of soluble protein and natural sugar in Fr.IIA1 suggested that the fraction contained a certain level of polysaccharides (Table 9). Therefore, the fact could not be ignored that there is a possibility of this 90 kD major allergen to be a glycoprotein as evidenced by earlier workers (Hoz et al., 1994; Fischer et al., 1996; Pike et al., 1997) as a very common character of pollen allergens.

B. Phoenix sylvestris

Date sugar palm, i.e., Phoenix sylvestris Roxb. occur throughout India up to an altitude of 1500 m both in wild and cultivated conditions, which is economically important to yield sugar and edible fruits (The Wealth of India, 1969). Phoenix sylvestris pollen were found to be airborne during January-April and was reported to be allergenic by Banik & Chanda (1992) and Chakraborty et al. (1995; 1996). In the present study, the relevant airborne pollen showed the peak count in February both in 1995 and 1996, contributing 15.97% and 14.86% of total aeropollen load respectively. In SPT, the pollen showed high percentage of positive reaction (Table 10). In vivo as well as in vitro tests, i.e., SPT and ELISA, established that Fr.II derived from (NH4)2SO4 precipitation was the most allergenic fraction of P. sylvestris (Table 10, Fig. 13). The Fr.II was further resolved into four fractions by gel filtration (Fig. 14) and in ELISA inhibition Fr.IIa was found to be the major allergenic fraction (Fig. 15). In native PAGE, the Fr.IIa showed the presence of a single component (Fig. 18A), which was found to be resolved into two subunits of 33 and 66 kD in SDS-PAGE (Fig. 18B). In immunoblotting, the whole extract showed the pres
ence of nine allergenic components, among them four were present in Fr.II (Fig. 17). Both the two subunits of Fr.IIa were found to be allergenic in further immunoblotting (Fig. 18C). In CIE, the Fr.IIa showed the presence of two antigenic components (Fig. 20). From this study, it may be concluded that the 33 kD and 66 kD protein components of Fr.II of Phoenix pollen are the major allergens present in the pollen extract.

In India, studies with the pollen of Amaranthus spinosus (Choudhary et al., 1990), Cocos nucifera (Jaggi et al., 1989) revealed the presence of a high molecular weight (128 and 116-200 kD respectively) glycoproteins as major allergens. However, with other pollen sources, e.g., Cynodon dactylon (Kundu et al., 1988), Parthenium hysterophorus (Gupta et al., 1996) low molecular weight proteins of 18-32 kD were found as major allergens. Similarly, Singh et al. (1995) reported a 90 kD major allergen from Brassica campestris pollen.

In Saudi Arabia, Harfi et al. (1992) recorded five major allergenic components of date palm, i.e. Phoenix dactylifera, in the range of 12-67 kD. From Spain Blanco et al. (1995) reported the pollen of Phoenix canariensis as an important allergenic pollen. The present study on Phoenix sylvestris pollen allergen supplied more data to enrich the knowledge of Phoenix pollen grains.

VII. Allergenic and Antigenic Relationship Among Four Dominant Airborne Palm Pollen from Madhyamgram

Different in vivo and in vitro studies reveal that pollen allergic patients are rarely sensitive to a single pollen type. It is frequently seen that the patients show sensitivity to a number of pollen taxa even when some of the species have not been encountered at all (Pham et al., 1994). The presence of cross-reactive or shared allergens have been reported from different pollen types taxonomically related (Fernandez et al., 1993; Sridhara et al., 1995) and also unrelated (Pham & Baldo, 1995). Many investigations were conducted on different cross-reactive pollen types belonging to Poaceae (Baldo et al., 1982), Oleaceae (Obispo et al., 1993), Chenopodiales (Wurtzen et al., 1995), Asteraceae (Fernandez et al., 1993), etc., but not on palm pollen grains. In the present investigation four palm pollen types were considered, namely, Areca catechu L. (areca palm), Borassus flabellifer L. (palmyra palm), Cocos nucifera L. (coconut palm) and Phoenix sylvestris Roxb. (date sugar palm), all of which occurred in the air Madhyamgram and were referred to be allergenic. Out of these four, Borassus and Phoenix were seasonal and other two perennial.
In a random survey of 551 patients undergoing skin-prick tests for allergy diagnosis in the Institute of Child Health, Calcutta, 241 (55.89%) patients were found to be positive to one or more palm pollen extracts tested. Among them, 70 patients were selected for repeat test with the same (Fig. 21). Although the Areca pollen responded to highest percentage (48.5%) and Borassus the least (38.5%), the difference was not so marked. The overall skin-prick test result showed (Table 5) that Phoenix was the most reactive among the four palm pollen, but here Areca showed higher sensitivity in the study population. This may be due to the fact that the particular survey on cross reactivity was conducted in the period of March-May, 1995, when high concentration of Areca pollen was recorded from the air. The majority of the individuals suffering from palm pollen allergy reacted to more than one palm pollen extract (Fig. 21). This may be due to the presence of some common allergenic proteins. ELISA was performed to evaluate the allergenic cross reactivity (Fig. 22) among them. Analysis of the positive ELISA values for one palm pollen extract in comparison to the other three, showed elevated IgE levels to more than one allergen in many patients. The positive values of ELISA to one extract was taken as 100% and were compared with the values of the other three extracts.

The result of dot blotting analysis (Fig. 23), which is a simple and reliable technique, indicated the presence of cross-reacting allergens in the relevant palm pollen extracts.

ELISA inhibition was performed in order to quantitate the extent of cross reactivity using pooled sera and serial dilutions of the four extracts (Table 11 & Fig. 25). Although the inhibitory capacity of the extracts was found to be different depending on the serum pool and the solid phase allergens used, the nature of curves were very much similar. This may be due to the presence of some common allergenic proteins in the pollen extracts.

Further to confirm the presence of cross reacting antigens, antibodies were raised in rabbits against Phoenix and Borassus extracts (Fig. 24). Phoenix antigen showed a maximum number of precipitin rockets with homologous antisera, whereas the number of same were less in the other three antigens. In IgG specific ELISA Inhibition (Fig. 26), with anti-Pheonix antibody, serial dilutions of Areca, Borassus and Cocos were able to inhibit its binding to solid phase Phoenix antigen. Similar results were obtained with anti-Borassus antibody. These findings with the palm pollen allergens further enrich the general thinking of the presence of common allergenic and antigenic components in the pollen grains of taxonomically closely related plant species. Isolation of the cross reactive components and elucidation of allergenic epitopes commonly binding to them may
be helpful in designing peptide based vaccine in the treatment of palm pollen induced respiratory allergy. The present study may be termed as a priming effort of further analyses of palm pollen allergens yet to be studied in detail.

**VIII. Airborne Pollen of *Borassus flabellifer*: Quantification of Allergen and Antigen**

Investigations pertaining aerobiological survey of pollen in India have been carried out in different biozones to get informations about the daily occurrence of pollen grains including allergenically significant ones for pollen season forecasting. The point to note is that, the essential information for the pollinosis patients is not the quantity of pollen, but the amount of allergen or at least antigen they bear. Some results of airborne pollen antigens and allergens have been reported which were concerned mainly with aero-allergen/antigen exposures of the pollen of ragweed (Agarwal et al., 1984), Japanese cedar (Takahashi et al., 1993), grasses (Takahashi et al., 1993) and birch (Ekebom et al., 1998). This newly emerged approach has been proved to be useful and significant to understand the extent of exposure in pollinosis patients.

In a country like India with a rich and diverse vegetation, this technique will be very helpful to minimize the tedious and time consuming laboratory procedure. The result here presents a report on an aeroallergen and antigen recorded dealing with *Borassus flabellifer* L. (palmyra palm) pollen probably for the first time from India.

The identification of pollen antigen spots was performed by immunoblotting with anti-*Borassus* rabbit IgG where recognizable spots were recorded. In case of immunoblotting with pollinosis patient sera, no detectable IgE spots were found. This was also reported by Takahashi et al. (1991). Therefore, the detection was performed by using more sensitive chemiluminescent immunoblotting.

The objectives of this study were as follows:

1. Recording of allergens and antigens originating from palmyra palm pollen trapped on the adhesive tapes of Burkard sampler for quantification of actual aeroallergen exposure during the pollen season.

2. To compare and correlate the occurrence of total airborne allergenic pollen with those of its allergens and antigens detected by immunoblotting.

3. To examine the possibility of complementation of routine airborne pollen counts by direct evaluation of allergens/antigens in air.
The results obtained showed that the pollen of *Borassus flabellifer* have been proved to be a dominant aeroallergen during its flowering period. The presence of airborne pollen grains showed positive correlation (Table 13) with allergen, which, however, was not significant \((p > 0.1)\) to a marked high level. In case of antigen occurrence, the pollen count showed strong positive correlation \((p > 0.001)\). In case of allergen and antigen also, there was positive correlation, which was again not highly significant \((p > 0.1)\) on peak days, but were significant in a comparatively higher level \((p > 0.02)\) in the days of lower concentration. From the results, it can be concluded that the protein particles having antigenicity were not always allergenically active.

To summarise:

The difference of pollen count and number of allergen/antigen spots (Fig. 29 & 30) the variation of spot sizes (Figs. 27 & 28) in both the cases were indicative of the fact that the air allowed transportation not only of pollen, but also of smaller antigenic or allergenic particles. Airborne pollen grains as larger particles \((15 - 50 \mu m\) in size) are trapped in the upper airways and only reach the bronchi in minute amounts. Nevertheless, a fair proportion of pollen allergic patients experience asthma in the pollen season. This is probably because they are exposed to pollen allergen in particles much smaller in size than intact pollen grains. Probably the grains after coming in contact with the moisture, dew or raindrop, release its soluble protein to water which, in turn, become airborne in the form of very small particles, which later become airborne, inhaled and deposited in lower respiratory tract (Mygind *et al.*, 1996). The existence of smaller airborne allergenic pollen particles had already been reported for ragweed, birch, grasses and Japanese cedar pollen (Agarwal *et al.*, 1984; Takahashi *et al.*, 1991; 1995; Rantio-Lehtimaki *et al.*, 1994; Spieksma *et al.*, 1995). The larger spots originated probably as a result of merging of a number of spots and consequent diffusion during the transfer for immunoblotting (Takahashi *et al.*, 1993).

The study, however, had some limitations of allergen/antigen spot counting with absolute accuracy due to size variation \((approximately 5-200 \mu m\) or more) and sometimes due to overlapping. Nevertheless, such a demonstrative view of allergen or antigen occurrence in air obtained through such investigations could not have been done otherwise.