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Do human platelets possess LDL-receptor specific for Apoprotein 'B' or cholesterol?

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The study, addressed to resolve the paradoxical interaction of human platelets with lipoproteins especially low-density liproprotein (LDL), revealed that: a) unlike human lymphocytes, platelets did not possess the conventional LDL receptor specific for Apoprotein 'B' moiety of LDL; b) platelets possessed a 69 kDa surface glycoprotein (containing cystein as well as mannose residues and having isoelectric point ≃ 5.0) which had the inherent ability to bind cholesterol moiety in the LDL particle. Based upon these observations, it is proposed that LDL may have two types of cellular receptors responsible for (i) cholesterol endocytosis through Apoprotein 'B' specific 160 kDa receptor and (ii) transmembrane signalling through cholesterol-specific 69 kDa receptor.

KEEPING in view the lipoprotein modulated increased platelet activity in both hypercholesterolemic animals and human subjects[1], various investigators explored the possibility that the platelets, like other cells, may possess a functional receptor specific to apoproteins. Although many workers demonstrated the occurrence of lipoprotein receptor on the platelet surface they failed to demonstrate the typical characteristics of low-density lipoprotein (LDL) receptor found in other cell types[1, 2]. This paradox got further strengthened by the observation that platelets derived either from normolipidemic subjects or from patients with familial hypercholesterolemia (FH), whether homozygous or heterozygous were shown to possess lipoprotein receptor[1]. Recently we have shown that exogenous cholesterol had the inherent capacity to induce initiation-promotion coupling of phospholipases 'D' and 'A_2' leading to the regulation of various second-messengers within human platelets[3–5]. Consequently it became in this context imperative to explore the possibility for the existence of another type of lipoprotein-receptor which had the affinity for cholesterol rather than apoprotein moiety in the LDL particle.

Gel-filtered platelets and lymphocytes from the blood of normal healthy donors were obtained by standard procedure[2, 6]. LDL fraction from fresh fasting plasma of normal healthy donors was isolated[2]. This LDL fraction as well as Apoprotein 'B' (Sigma) was radioiodinated with $Na^{125}I$ according to standard procedure[2]. The ^{125}I-LDL fraction was dialysed with Tyrode's buffer and centrifuged for five minutes at 11,000 rpm (6000 *g*) before use. Cholesterol (either labelled or unlabelled) was dissolved in ethanol and subsequently mixed with Tyrode's-albumin buffer (pH = 7.4) to obtain various concentrations of cholesterol. The final ethanol concentration in the incubation buffer never exceeded 0.5% and corresponding amounts of ethanol was added in the control samples.

In order to understand the interaction of cholesterol as well as LDL with human platelets, three types of experiments were initially designed: (i) Platelets at a final concentration of 10^8 cells/ml in Tyrode's-albumin buffer enriched with 2 mM EGTA + 2 mM EDTA were exposed to cholesterol (50 µg/ml final concentration) for a period of two hours at 25 °C; at the end of the incubation period, the platelet pellets were processed for the determination of total cholesterol, phospholipid and protein by standard procedures[7–9]. (ii) Cell pellets obtained from gel-filtered platelets and lymphocytes (cultured for 24 h in a serum-free medium to increase the LDL-receptor expression within these cells) were suspended in lysis buffer (10 mM Tris; 50 mM NaCl; 0.5% Triton X-100 and 0.1 M PMSF pH = 7.4) and protein pellets, obtained through chloroform-methanol precipitation method, were dissolved in electrophoresis buffer and subjected to 10% slab gel electrophoresis followed by western blotting. The blotted nitrocellulose was washed with buffer 'A' (10 mM Tris + 50 mM NaCl + 2 mM $CaCl_2$ pH = 8.0) and subsequently incubated with buffer 'A' enriched with 3% BSA for 2 h and followed by another incubation with buffer 'A' enriched with ^{125}I-Apoprotein 'B' for two hours. The nitrocellulose paper was washed thrice with buffer 'A' at 25°C and subsequently subjected to autoradiography as per the standard procedure. (iii) Platelets at a final concentration of 10^8 cells/ml were pre-exposed to Tyrode's-albumin buffer enriched with either chelating agents (2 mM EGTA + 2 mM EDTA) or chelating agents + 20 µM polycystein (Sigma) or chelating agents + cholesterol (50 µg/ml) for half an hour at 25°C and subsequently ^{125}I-LDL at a final concentration of 4 µg ml was added and again incubated for two hours at 25 °C. The results of these three types of experiments revealed (i) acquisition of cholesterol by platelets was highly selective and saturable without any significant change in platelet protein or phospholipid content (Figure 1*a*), (ii) human lymphocytes (known to possess LDL-receptors) had the ability to bind apoprotein, indicating the exi-

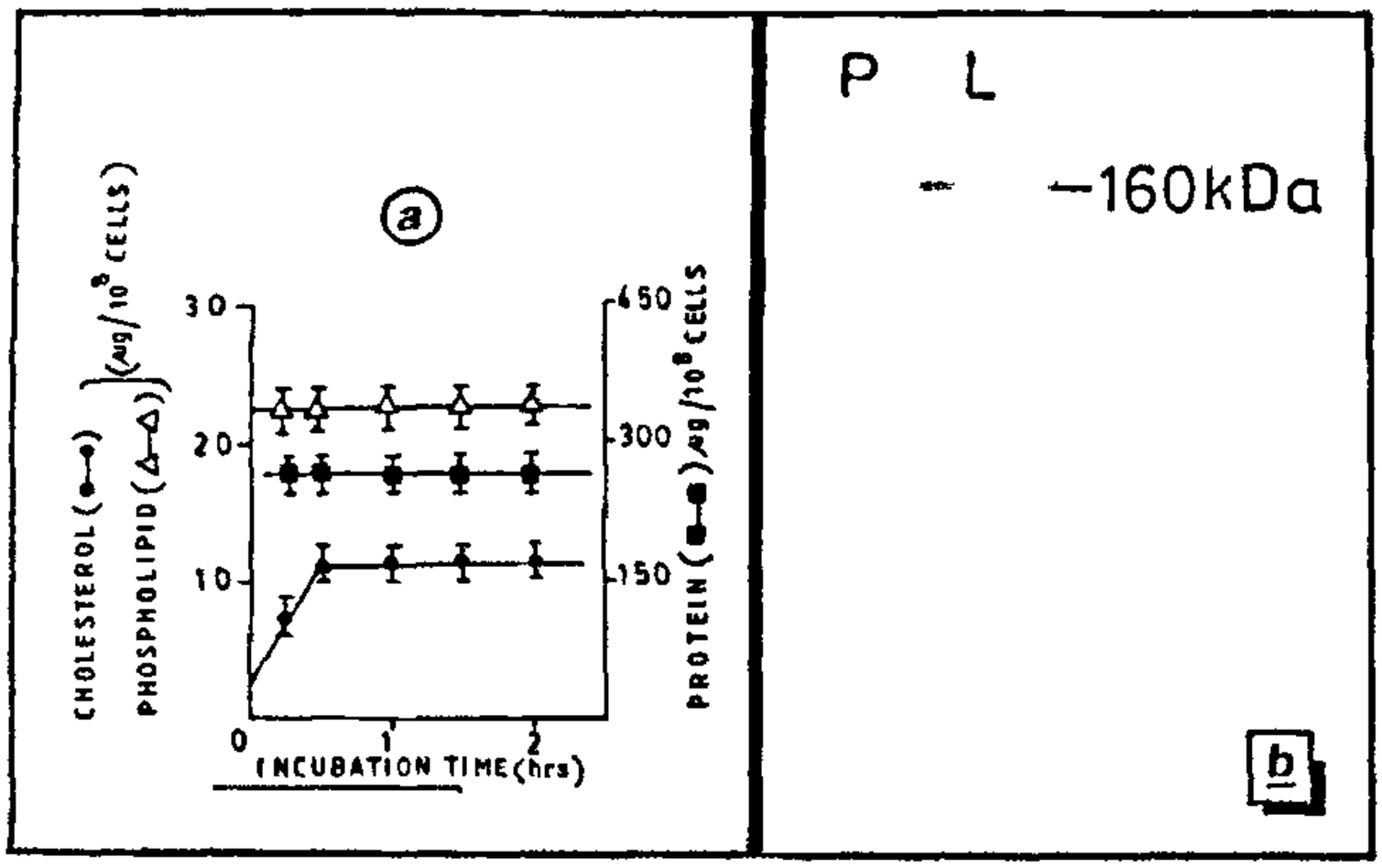

Figure 1. *a*, Effect of time course of cholesterol-binding to platelets upon their total protein, phospholipid and cholesterol content, *b*, Autoradiograph of the ^{125}I-Apoprotein 'B'-treated western-blotted protein bands from platelets (lane 'P') and lymphocytes (lane 'L') depicting the presence of 160 kDa LDL-receptor protein only in lymphocytes and not in platelets

Table 1. Effect of cholesterol and polycystein upon LDL-binding to human platelets

Pre-exposure	Additions	Total ^{125}I-LDL bound (ng/10^8 platelets)
EDTA (2 mM) + EGTA (2 mM)	^{125}I-LDL	1100 ± 20*
EDTA (2 mM) + EGTA (2 mM) + 20 μM Polycystein	^{125}I-LDL	49 ± 5 0
EDTA (2 mM) + EGTA (2 mM) + Cholesterol (50 μg/ml)	^{125}I-LDL	53 ± 2 0

^{125}I-LDL at a final concentration of 4 μg/ml was added to a platelet suspension (10^8 cells/ml) pre-exposed to Tyrode's-albumin buffer containing the indicated chelating agents as well as either cholesterol or polycystein. Binding was measured after two hours at 25 °C and the results are mean of triplicate determination

* Mean ± SD

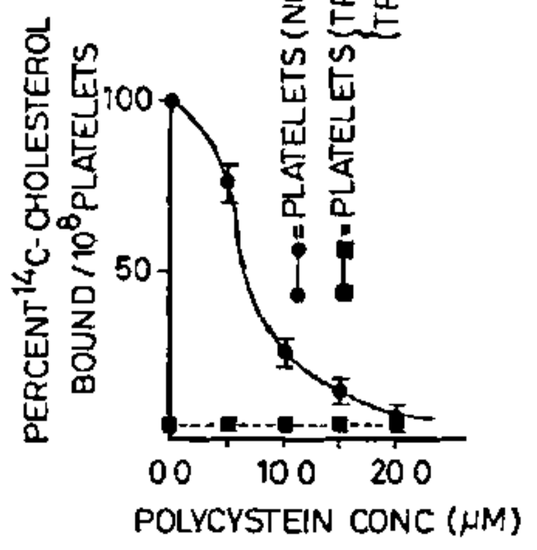

Figure 2. Effect of various concentrations of polycystein upon cholesterol-binding to platelets either untreated (normal) or pretreated with trypsin indicating that platelet surface protein was involved in the binding of cholesterol.

stence of 160 kDa LDL-receptor band whereas platelet did not possess the receptor-protein having affinity for Apoprotein 'B' (Figure 1*b*); (iii) LDL binding to platelets was maximum in absence of divalent ions and this binding was significantly reduced in these cells when pre-exposed to either cholesterol or polycystein (Table 1). These results when coupled together showed that unlike lymphocytes, the platelet–LDL interaction was not mediated through Apoprotein 'B' as well as cholesterol and polycystein could somehow regulate the platelet–LDL interaction. In order to resolve the role of cholesterol as well as polycystein in the platelet–LDL interaction, the gel-filtered platelets (10^8 cells/ml) were (i) exposed to ^{14}C-cholesterol in Tyrode's-albumin buffer enriched with polycystein (0.20 μM final concentration) at 25 °C for two hours; (ii) pretreated with 0.2% Trypsin in PBS-EDTA buffer for five minutes at 37°C washed twice with Tyrode's-albumin buffer containing trypsin inhibitor and subsequently exposed to ^{14}C-cholesterol in Tyrode's-albumin buffer enriched with polycystein (0.20 μM) at 25 °C for two hours. At the end of incubation period, the platelets were isolated by gradient-centrifugation method[2] and subsequently analysed by measuring the amount of bound ^{14}C-cholesterol to platelets. The results of this set of experiments (Figure 2) revealed (i) binding of ^{14}C-cholesterol to platelets was inhibited by polycystein in a dose-dependent fashion, (ii) platelets pretreated with trypsin exhibited neither cholesterol binding nor any effect of polycystein upon such binding (Figure 2). These results indicated that platelet surface-protein was involved in

the interaction of platelets with cholesterol and this surface protein had probably cystein residues involved in some kind of interaction with cholesterol.

Keeping in view that platelet surface protein having affinity for cholesterol was involved in LDL–platelet interaction, an attempt was made to isolate this protein as well as determine its nature. In this particular set of experiments the platelets (10^8 cells/ml) were exposed to Tyrode's-albumin buffer containing cholesterol (50 µg/ml) and chelating agents (EGTA 2 mM each) for half an hour at 25°C. The platelet pellets obtained were processed for digitonin precipitation as per the standard method[10]. The protein–cholesterol–digitonide precipitates were washed several times with PBS containing chelating agents (buffer-'B') and subsequently washed with buffer 'B' enriched with 3 M NaSCN. This fraction was dialysed overnight against buffer 'B' and dialysed protein fraction was then applied to Con 'A'-sepharose column pre-equilibrated with buffer 'B'. The column was eluted with 10 ml of buffer 'B' containing 50 mM mannose. This eluted fraction was subsequently analysed by electrophoretic chromatographic system as well as 10–15% SDS-PAGE phast system using silver staining. The results showed a single protein of about 69 kDa molecular weight (Figure 3 *a*, *c*) and could be isolated through this procedure by reproducible manner. Further, in order to verify whether the purified protein had the capacity to bind cholesterol, the 69 kDa fraction was also subjected to isolectric focusing using phast IEF, gel (3.5–9.0 pH) and subsequently the gel was western-blotted to nitrocellulose paper which was washed with buffer 'C' (10 mM Tris + 50 mM NaCl pH = 8). The washed nitrocellulose was first incubated with buffer 'C' containing BSA for two hours and subsequently followed by another incubation with buffer 'C' enriched with ^{14}C-cholesterol (3 µM) at 25 °C and thereafter subjected to auto-radiography. The results (Figure 3*b*) revealed that the 69 kDa glycoprotein (having mannose as well as cystein-rich residues) has pI ≃ 5.0 and inherent capacity to bind cholesterol.

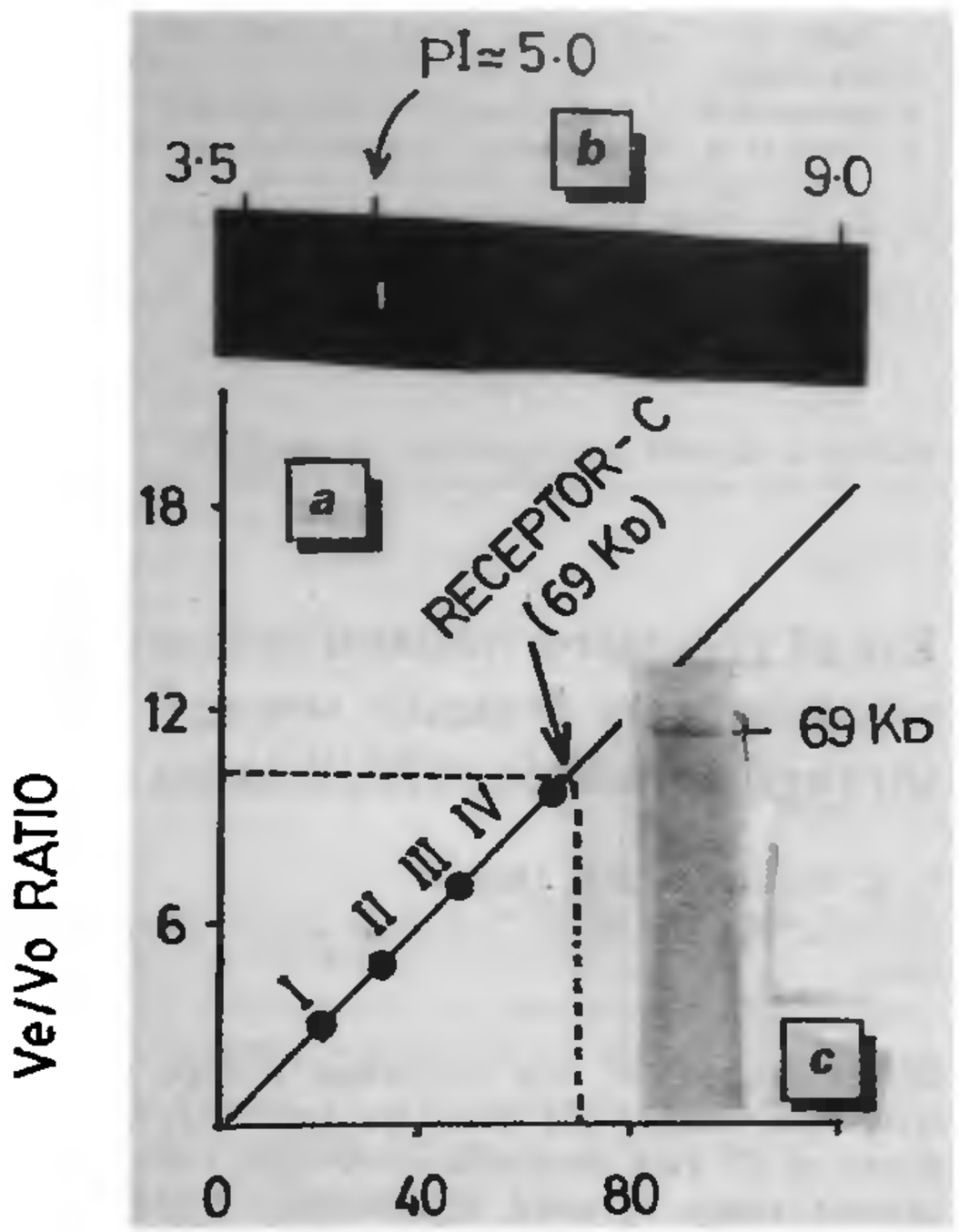

Figure 3. Electrophoretic characteristics of the platelet protein fraction obtained through extraction of protein-cholesterol-digitonide precipitate with 3 M NaSCN followed by dialysis and Con 'A'-sepharose affinity elution of this dialysed protein fraction *a*, analysed by electrophoretic chromatography precalibrated by standard protein markers I–IV Trypsin inhibitor, carbonic anhydrase, egg albumin and bovine albumin, depicting molecular weight of 69 kDa, V_e / V_o depict ratio of elution volume to void volume *b*. Autoradiograph of the ^{14}C-cholesterol-treated western-blotted 69 kDa protein fraction from IEF phast gel showing pI ≃ 5.0. *c*. analysed by 10–15% phast gel followed by silver staining, indicating again a single band of about 69 kDa

In conclusion, the results reported here not only showed unambiguously that human platelets did not possess the 160 kDa LDL-receptor having high affinity for Apoprotein 'B' but also they possessed a 69 kDa surface glycoprotein which had the capacity to bind cholesterol and could also bind the cholesterol moiety present in the LDL particles. However, more data are required in order to ascertain whether 69 kDa glycoprotein qualifies to be called as a novel LDL-receptor having affinity for cholesterol. However, there are various paradoxical findings which support this proposition. For example, the binding of ^{3}H-cholesterol from LDL by LDL-receptor-positive and receptor-negative human fibroblasts was found to be identical[11] and platelets derived from normolipidemic subjects or from patients with familial hypercholesterolemia, whether homozygous or heterozygous were shown to possess lipoprotein receptors[1]. Consequently, it is tempting to speculate that cells may have two types of receptors responsible for cholesterol endocytosis (160 kDa receptor specific for Apoprotein 'B' in LDL particle) and transmembrane signalling (69 kDa receptor specific for cholesterol in LDL particle). Studies addressed to this possibility are being carried out in our laboratory at present.

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Use of vegetated wetland to remove nitrogen from domestic sewage through ammonia volatilization

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Direct disposal of raw municipal sewage for crop irrigation without any prior treatment revealed evidence of its rich ammonium-nitrogen content. The present study revealed significantly higher (× 2.8 times) nitrogen loss through ammonia volatilization in the vegetated wetland (flux, 113 mg ammonia/m² surface/day) because of higher flood water ammonium-N concentration compared to the wetland area without any macrophytes (flux, 40 mg ammonia/m² surface/day). The amount of ammonia loss and nitrate-N status in sewage irrigated soil without any crop was 1.5 times higher (flux, 89) than the soil with the crop (flux 57). Absence of rhizospheric factor and nitrate-N uptake enhanced both the processes at the former site. The overall study pointed that vegetated aquatic system provides a better treatment avenue for nitrogen removal.

LAND application of sewage is desirable because it decreases the hazard of environmental pollution and related health problems[1–3], creates an alternative water source since the effluent is suitable for irrigation[4] and increases economic benefits due to its high nutrient content which minimize the need for fertilization. It is essential to remove excess nitrogen from sewage before it is discharged to water bodies, as it can cause eutrophication and increase the cost of potable water treatment. Ammonia volatilization has received considerable attention in recent years because it is a major route of nitrogen loss from the plant–soil–water environment. The present investigation was undertaken to evaluate the extent of ammonia loss in: (i) wetland body containing sewage for land disposal, and (ii) sewage irrigated agricultural field, in order to find out an effective way by which the excess nitrogen of the sewage can be reduced before the reuse of the effluent.

Ammonia loss measurements *in situ* were done during Summer 1991 (May, mean min. temp. 29°C and mean max. temp. 41°C) at the Sewage Farm of Ujjain city (23°N, 74°E). The Farm covers a land area of about 2000 ha and receives untreated municipal wastewater for crop irrigation by intensive network of open and a few closed drains. Within the Farm, ammonia loss measurements were made in a shallow earthen wetland (size 2 ha, depth 120 cm) aquatic ecosystem. At times when the sewage irrigation is not required in the rest of the farm, municipal wastewater is stored into this wetland for supplemental irrigation of crops. It is a perennial aquatic body and has three major zones: (i) a dense green mantle of the free floating plant, *Lemna minor* (Duckweed) in 2/3rd area, (ii) *Typha angustifolia* (Cattail) completely covering the peripheral margins of the pond, the roots of which are as deep as one metre, and (iii) pond water surface without any macrophyte.

In this aquatic system, ammonia volatilization was measured in static acid traps, where ammonia volatilized was trapped in dilute boric acid (0.33 N). The traps consisted of 30 cm lengths of PVC pipe (i.d. = 15 cm). The lower end was inserted into water/soil and the upper end was closed by a 5 cm piece of PVC (i.d. = 16 cm) which contained two layers of fine nylon mesh (size 100 µm). Between these two layers, a glass-wool pad of 3 cm thickness (weighing 40 g) was sandwiched, soaked with 250 ml of 0.33 N boric acid and sealed by thermocole disc (5 cm thickness) to make the whole fabrication air-tight. The upper end of ammonia-absorbing unit was easily replaceable by the fresh ones for subsequent measurements. The traps were floated in the water by a tight-fitted thermocole at the base. Ammonia loss was monitored everyday for five days at two sites in three replicates: (i) free-floating *Lemna* species (15 cm water column), (ii) 'root bed' zone of *Typha* covered marginal area (15 cm water column depth for the set). Ammonia trapped in the pads was quantitatively extracted in the laboratory by fresh washings by boric acid and simultaneously squeezing to a total 250 ml extractant (since some amount of boric acid was lost between the set installation and set-dismantling for the record of observations). Ammonia concentration was measured by Orion ion analyser in a 50 ml aliquot using an ammonium electrode. Each day, the measurement sets were moved to a new site in the vicinity of the same area supporting the *Lemma/Typha* plants of almost identical age. Thus in total 15 sites were measured with 3 replicates each day during the five-days period.

In the terrestrial system ammonia loss was measured by placing the acid traps on surface of the sewage-irrigated soil at two sites – (i) soil supporting a crop (one-month-old bottle gourd, *Lagenaria vulgaris*), and (ii) sewage irrigated bare soil. The ammonia trapping and measurement method was the same as described above.