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# The detection of living cells in stratospheric samples

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## ABSTRACT

Air samples collected aseptically over tropical India at various stratospheric altitudes ranging from 20 to 41 km using cryosampler assemblies carried on balloons flown from Hyderabad have shown evidence of living microbial cells. Unambiguous evidence of living cells came from examining micropore filters on which the samples were recovered with the use of voltage sensitive lipophilic dyes that could detect the presence of active cells. Clumps of viable cells were found at all altitudes using this technique, and this conclusion was found to be consistent with images obtained from electron microscopy. Since the 41 km sample was collected well above the local tropopause, a *prima facie* case for a space incidence of these microoraganisms is established. Further work on culturing, PCR analysis and isotopic analysis is in progress.

**Keywords:** Panspermia, cometary dust, stratosphere, microorganisms

## 1. INTRODUCTION

Two of the present authors (FH and NCW) have argued for over two decades that terrestrial life was introduced by comets, which serve both as amplifiers and distributers of life throughout the cosmos. Evidence for this point of view has grown steadily over the past few years[1,2]. This includes the discoveries of spectroscopic signatures in astronomical sources over a wide range of wavelengths that are consistent with biology, the presence of microbial life

on the Earth 4 billion years ago when the Earth suffered an epoch of intense cometary bombardment, and microbiological laboratory studies that have shown extreme survival properties of microorganisms. The contention is that if life was brought to Earth by comets 4 billion years ago, the process of cometary injection of living material must have continued to the present day and should therefore be testable. Hundreds of tonnes of cometary material enters the Earth's upper atmosphere on a daily basis [3], and some of this must contain biological material if the idea of cometary panspermia is to be sustained.

Studies of 5 interstellar dust particles analysed by mass spectrometers aboard the Stardust spacecraft showed heteroaromatic cross-polymers as high-impact break-up products[4] . This material has structural strengths similar to bacterial cell walls, and is exactly what one may expect from bacteria impacting the Stardust detectors[5] . Evidence for a bacterial component of cometary dust has also come from the recent discovery of a broad CH stretching feature in a persistent meteor train of the November 1999 Leonid meteor shower[6,7]. With such an impressive convergence of indirect evidence, what now remains is a direct unequivocal demonstration of viable bacteria entering the Earth's atmosphere from space. The present investigation is part of a programme of research to do precisely that.

## 2. EARLY BALLOON EXPERIMENTS

The earliest attempts to search for microorganisms in the upper atmosphere were made using balloons in the early 1960's. These experiments were conducted under the auspices of the US Space Agency NASA, presumably as a preparation for embarking on the Space Age and manned space flights. Although the techniques for conducting such experiments aseptically at the time were primitive, some dramatic indications of extraterrestrially derived microorganisms were obtained [8,9]. Viable cultures were recovered from air samples at 39 km and higher with an indication that they were introduced from outside. However, such a claim was difficult to defend in view of primitive nature of the sterilization procedures that were used. The NASA atmospheric biosampling program seems to have been terminated and not taken up again until the present time.

## 3. ISRO/IUCAA/TIFR/CARDIFF CRYOSAMPLER PROGRAMME

A balloon launched in the early hours onf 21 January 2001 from the National Scientific Balloon Facility of the Tata Institute of Fundamental Research at Hyderabad, India (lat 17° 28' 20", long 78° 34' 48") carried carried a scientific payload to sample the stratosphere under the most stringently aseptic conditions. The payload included a cryosampler manifold (Figure 1) with fully sterilised evacuated stainless steel probes each with a 0.35 litre capacity and capable of withstanding a pressure in the range $10^{-6}$ mb – 600b. The sterilisation methods used and the technical specifications of the instrumentation are described elsewhere[10]. The evacuated probes are cooled in liquid neon to produce the cryopump action with sterilized valves fitted to be opened at pre-determined heights through ground station telecommand.

Probes carried on this flight were in duplicate sets. One set was to be analysed at the Centre for Cellular and Molecular Biology in Hyderabad, India, the other at Cardiff University, Wales, UK. We report in this paper preliminary results of analysis of the probes sent to Cardiff in March 2001. The details of the probes being analysed in Cardiff are listed below:

**Table 1**

Details of probes being analysed in Cardiff

| Probe | Collection height range | Collected NTP volume (litres) |
|---|---|---|
| A | 19.28-20.32km | 81 |
| B | 24.36-27.97km | 70.5 |
| C | 28.47-39.05km | 38.4 |
| D | 39.75-41.06km | 18.5 |

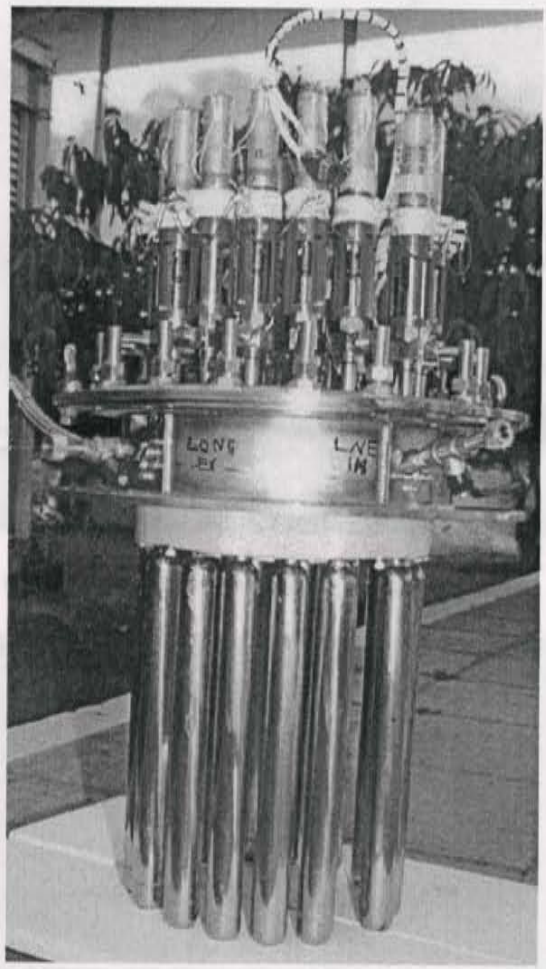


Figure 1. Cryosampler manifold in preparation for launch

From each of the 4 probes air was passed in a sterile system in a laminar flow chamber, first through a 0.45μ m micropore filter and then through a 0.22μ m filter. Eight filters were thus derived. The probes were stored at –70C before sample preparation, and likewise the derived membrane filters were stored at this temperature before isolates were obtained and tested.

## 4. PRELIMINARY RESULTS FROM CARDIFF SAMPLE ANALYSIS

We have so far analysed only the 0.45μ m micropore filters, which would be expected to have trapped microbial sized particles. Approximately 4 $mm^2$ squares were aseptically cut from the filters and treated with either a fluorescent cationic carbocyanine or an anionic oxonol dye sensitive to membrane potential.

Cationic dyes penetrate the cell membranes of viable cells, but not of dead cells, whilst anionic dyes penetrate the

membranes only of non-viable cells. The techniques of detecting viable cells is described by Lloyd and Hayes[11] and Lopez-Amoros et al[12]. Any viable living cells present in a sample would be expected to give rise to fluorescent spots when illuminated with ultraviolet light and could be identified under an epifluorescence microscope. Each such spot depending on resolution would represent either a single cell or a clump of cells.

Isolates treated with cyanine dye showed fluorescent spots in the form of clumps of 0.3-1μ m sized cells, the clumps themselves measuring 5-15micrometres across. An example of such a well-resolved clump in a sample from probe A is shown in Figure 2, and two less well-resolved fuzzy clumps from probe D is shown in Figure 3. Higher resolution images will require deployment of a confocal microscope which we propose to do, but already the detection of viable cells by this technique (not found in sterile controls) is beyond doubt. The use of anionic dyes revealed a comparable detection rate of dead or non-viable cells. In this preliminary report we focus attention only on the take-up of a cationic carbocyanine dye as an indicator of viable cells in the stratosphere.

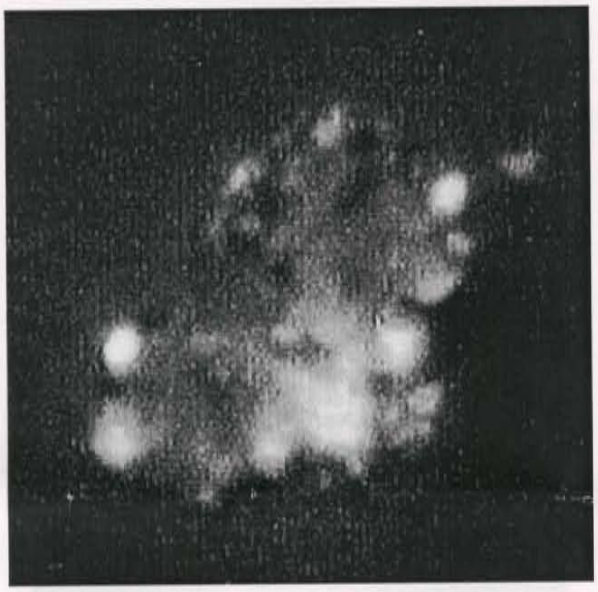


Figure 2. A cluster of viable cells flourescing in a cyanine dye from probe A (Scale of frame 27 x 26μ m)

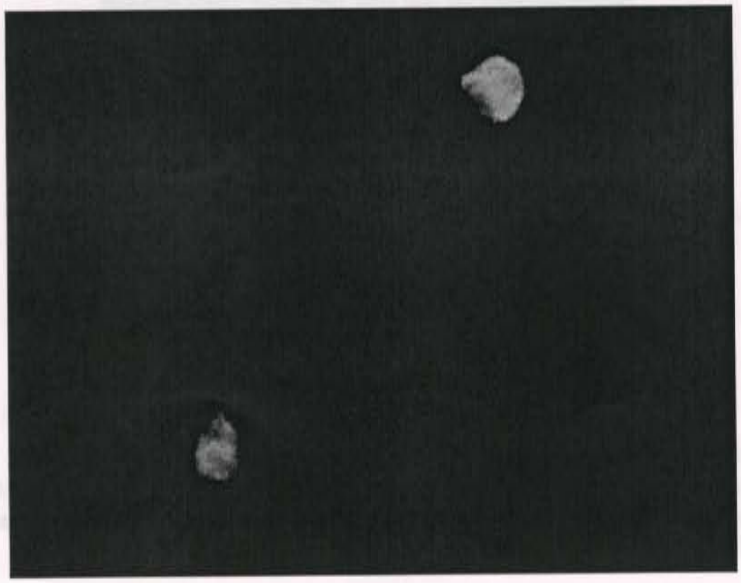


Figure 3. Fuzzy clusters of viable cells flourescing in a cyanine dye from probe D (Scale of frame 116 x 91μ m)

We next performed electron microscopy on aseptically isolated squares of membrane filters. The squares were mounted on 12mm diameter sticky carbon tabs which in turn were mounted on 10mm diameter aluminium tabs. The

samples treated in this way were gold sputter-coated and imaged in a JOEL5200LV scanning electron microscope under a vacuum of 7 x $10^{-9}$ bar. The procedure adopted is suited to imaging bacteria because bacterial cell walls would not collapse or explode under the observing conditions. Examples of putative bacterial clumps from isolates from probe B are shown in Figure 4. The similarity of the structures in Figure 2 and the upper frame of Figure 4 is clearly evident.

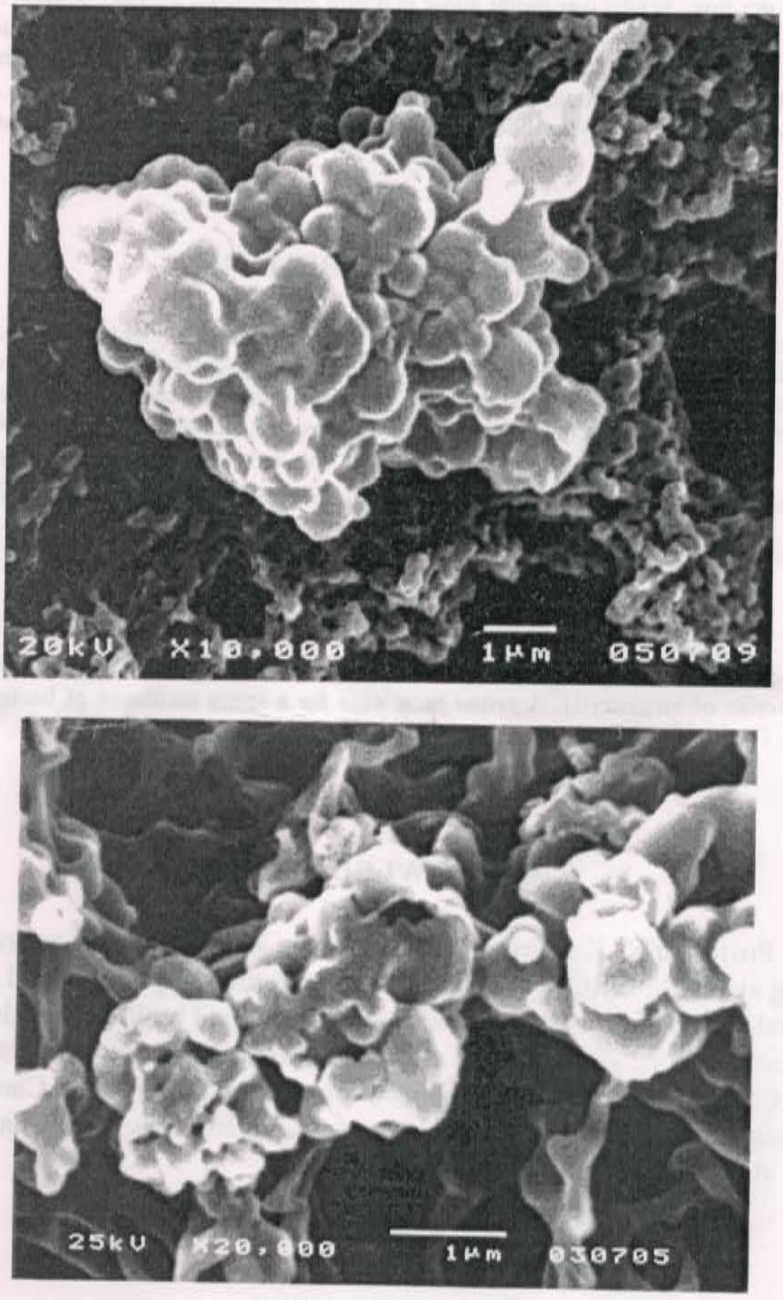



Figure 4. Electron micrograph showing clumps of putative microorganisms from probe B

On each of the micropore isolates examined so far, measuring approximately 2mm x 2mm, a number N in the range 1 - 3 of microbial clumps were found. Since the air volume passing through each filter in A and B was about 80 lit NTP (Table 1) and the area of the entire filter is ~2000 $mm^2$ , with N=3, the density of microbial clumps at 25 km is estimated as [(3x2000)/4]/80 = 18.75 per litre at NTP. With an atmospheric pressure at 25 km of 0.025bar the estimated number density of microbial clumps at 25km is 0.47 per litre.

At a height of 40km (sample D) the average number of clumps per 4$mm^2$ of membrane filter may provisionally be taken as N=1 subject to further analysis. The NTP equivalent volume of air that passed through 2000$mm^2$ of this filter being 18.5 litres (Table 1) and the ambient air pressure being 0.0025bar we can estimate a density of clumps of about 0.068 per litre of air at 40km. On the basis of the *infall* of micron sized clumps in a standard atmosphere, the steady-

state number density of particles at 40km is expected to be one tenth of that at 25 km[10], which is consistent with what is observed, within the accuracy of the measurements completed so far. (Note: The mass fraction of cells in the air at 41 km would in fact be *higher* than at 25 km by a factor of approximately 1.4.)

With a clump comprised of ~ 100 bacterial cells the number density of viable cells at 40 km, according to the above estimate, would be ~ 7 per litre. Whilst one might argue that rare atmospheric events could on occasion loft microbes to such heights, or that spacecraft debris could transiently contribute to a population of cells, such processes would be woefully inadequate to maintain microbial densities of the order implied by our data. A transient injection of cell clumps of radius 3 – 10 μ m at 40 km would be cleared through gravitational settling in a time of order months[13]. No exceptional events were recorded globally in the run-up to our sample collection date of 21 January 2001.

## 5. CONCLUDING REMARKS

The electron microscopy could be equivocal in regard to the detection of viable cells, but the epifluorescence microscopy leaves little doubt that clumps of viable cells with sizes appropriate to bacteria are present at all heights ranging from 24-41km. Since the local tropopause over the launch site was estimated at 16km, the isolates are all above the level at which any terrestrial contamination can be expected, particularly so at the 41km altitude. With an average falling speed for 3 micron sized clumps at 40km of about 0.3 cm/s (Kasten[13]), the infall rate of clumps typified in Figures 2 and 4 (assuming a number density of 0.068 per litre) over the entire Earth, area $5 \times 10^{18}$ $cm^2$ would be

$$(0.068 \times 10^{-3}) \times (0.3) \times (5 \times 10^{18}) \text{ per second.}$$

Assuming an average of 100 individual bacterial cells each of mass $3 \times 10^{-14}$ g in a clump we obtain a daily mass input of about a third of a tonne of biomaterial. A *prima facie* case for a space incidence of bacteria onto the Earth may have been established. Further work on culturing and PCR analysis is in progress.

## 6. ACKNOWLEDGEMENT

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