A BALLOON EXPERIMENT TO DETECT MICROORGANISMS IN THE OUTER SPACE

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Abstract. The results of biological studies of a cryosampler flown with a balloon, in which air samples were collected at altitudes ranging from 20 to 41 km, well above the Tropopause over Hyderabad, are described. In the analysis carried out in Cardiff, voltage-sensitive dyes that could detect the presence of viable cells were used on these air-samples. Clumps of viable cells were found to be present in samples collected at all the altitudes. The images obtained from electron microscopy are consistent with the above finding. Reference is also made to another paper presented at this conference describing the identification of bacterial species in the sample carried out in Sheffield. Counter arguments are discussed against the criticism that the detected cells and microorganisms (in the samples collected above the local tropopause at 16 km) are due to terrestrial contamination.

1. Introduction

Though the phenomenon of life was speculated to be of universal origin since very early times, scientific investigations of producing life from non-life, starting from a constituent mixture of inorganic gases were attempted in the early part of the last century by Oparin (1953) and Haldane (1929). The Urey-Miller experiments

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of the mid-1950s showed how amino acids and nucleotides might form from a mixture of inorganic gases (Miller and Urey, 1959). Since then several experiments have demonstrated the production of hexaglycine under conditions similar to those prevailing in terrestrial hot springs (Imai et al., 1999) and the production of biologically relevant molecules like alcohols, quinones and ether from UV irradiation of polyaromatic hydrocarbons in water-ice (Bernstein et al., 1999). But what is important to recognise is the fact that while the production of chemical building blocks is a necessary condition, it is not sufficient. What is relevant is their involvement in the highly specific processes for the production of nucleic acids, enzymes and other proteins, membranes and organelles that are involved in life.

In the mid-1970s, Fred Hoyle and Chandra Wickramasinghe advocated the alternative scenario in which microbial life is considered to be of universal origin and brought to the Earth as panspermia from comets which visit the Earth's neighbourhood from time to time. They argued that life on Earth began with this input. Hoyle and Wickramasinghe (1980) have given theoretical arguments that life cannot originate in a small terrestrial pre-biotic pool; a cosmic origin is instead suggested. See also the article by Wickramasinghe (2002) in this volume.

If life is truly cosmic, then several questions arise: i) Is there evidence in favour of *'panspermia'* ? ii) Can microorganisms survive in the harsh extraterrestrial environments? iii) What mechanisms are available for transport of such microrganisms to the Earth? We consider these briefly.

Microorganisms expelled from any source into unshielded regions of interstellar space will firstly become deactivated and subsequently degraded by exposure to cosmic rays, UV and other intense electromagnetic radiation. This will lead to the production of free organic molecules and polymers. An impressive array of such molecules have been detected. The production of such a variety of interstellar organic molecules from wholly non-biological sources is highly unlikely, if not totally impossible.

Over the last decade it has been demonstrated by several laboratory experiments that the microorganisms can survive extreme conditions of temperature, pressure and even radiation (Hoyle and Wickramasinghe 2000). Further a carbonaceous coating of even a few microns thick provides essentially total shielding against UV radiation (Secker et al., 1994). So far as their transport to the Earth is concerned Hoyle and Wickramasinghe (2000) have been advocating a cometary transport of these microorganisms for more than two decades, adducing several lines of evidence in favour of this hypothesis. We will not go into details here but simply state that this hypothesis was one of the motivating factors for the present experiment.

2. ISRO Cryosampler Experiment

Though some attempts to detect direct evidence for extraterrestrial life-forms entering our upper atmosphere were made in the 1960s and 1970s by mainly NASA sup-

ported Balloon Programmes (see Bruch, 1967 for a summary) and a Soviet Rocket Experiment (Lysenko, 1979), no definite conclusions could be drawn due to the primitive nature of the sterilization procedures that were used. Actually some indications of extraterrestrially-derived microorganisms were claimed, but in view of the lack of sound techniques to conduct the experiments aseptically, it was impossible to rule out terrestrial contamination. In view of these difficulties, very stringent procedures to completely exclude terrestrial contamination had to be evolved. Such techniques became available in the late 1990s (Shyamlal et al., 1996). Biochemical, chemical and molecular biological studies to identify the collected microorganisms were also being developed (Smibert and Kreig, 1994). Furthermore extremely sensitive new dye-based detection methods for living organisms were developed in Cardiff (Lloyd and Hayes, 1995; Lopez-Amoros et al., 1995). So an experiment was proposed to collect direct evidence for extraterrestrial life in stratospheric balloon flights using an aseptic cryosampler and highly sensitive voltage-sensitive dyes (Narlikar et al., 1998).

2.1. Collection of air samples using the cryosampler

The cryogenic sampler instrumentation comprised a 16-probe assembly. Each probe had a volume of 0.35 L and was made of high vacuum grade stainless steel. It was capable of holding a vacuum of 10^{-6} mb and pressure of 600 b. The temperature cycling ability of the probes was tested between -246°C and 140°C. To minimize contamination, the probes were machined from the above stainless steel stock, only the minimum required electron-beam welds were made, and the interior was electropolished. Just before the experiment, the probes and their manifold were cleaned with acetone and then four times with demineralised water. The assembly was then steam-baked and finally heated with infrared lamps to temperatures of 140°C. To prevent collection of any outgassed substances from the gondola, an intake tube of 2 m length formed a part of the payload ensemble and was sterilised as above. The probe mouth consisted of a metallic (Nupro) valve which was motor driven to open/close at a given altitude through ground command, using a telecommand-transmitter-receiver-decoder cryocontrol unit chain. During the flight, the probes remained immersed in liquid neon to create the cryopumping effect that allowed the ambient air samples to be collected on ground command.

2.2. First experiment

In order to test the feasibility of collection of air samples aseptically and to test the rDNA sequencing and other procedures for identification of the microorganisms, a preliminary analysis was conducted by S. Shivaji and G.S.N. Reddy at CCMB and by P.M. Bhargava at Anveshna, Hyderabad, of one of the probes having an air sample collected from the altitude range of 10–36 km. This sample was collected in a balloon flight launched from Hyderabad on April 29, 1999. All procedures were carried out under aseptic conditions using stringently sterilised equipment.

The sample air was passed first through a 0.45 micron and then through a 0.22 micron pore filter. The exiting air was passed through a calibrated flowmeter. Each filter was placed in a nutrient agar plate; no growth occurred at 25°C in seven days. The filter was then transferred to a blood agar plate and incubated at 25°C for 11 days, when six distinct colonies had grown from the 0.45 micron filter; none were obtained from the 0.22 micron filter. These colonies were sub-cultured and maintained on nutrient agar. The cultures have been deposited in the MTCC Type Culture Collection of the Institute of Microbial Technology, Chandigarh, India.

Based on the morphological, physiological and biochemical characteristics and additionally on the basis of the 16S rDNA sequencing the isolates were identified as *Pseudomonas stutzeri*. But, these isolates were distinct from all the earlier described strains of *P. Stutzeri* with respect to i) ability to grow well on lysine, ii) high percentage of C15:0 and C18:0 fatty acids, iii) presence of carotenoid pigments, and iv) the yellow pigment which was produced only at room temperature (25°C), and then only in the stationary phase. In addition it is noteworthy that *P Stutzeri* has so far not been described as an airborne organism, its natural habitat being soil and water. So it is tempting to speculate that this is evidence for an extraterrestrial bacterium. But the fact that the air samples were collected as low as 10 km altitude, where even debris from jet planes can provide terrestrial contamination, puts a question mark over the extraterrestrial nature of the detected organism. At the same time, the unique properties of the detected colonies on comparison with terrestrially known strains of *P Stutzeri* are remarkable. While the experiment demonstrated the capability of the techniques for collection of air samples and detection of microorganisms, it also showed the need for a more careful experiment, where samples are collected at heights well above the ones where normal terrestrial contamination is even remotely possible.

2.3. AIR SAMPLES COLLECTED BY THE CRYOSAMPLER BALLOON FLIGHT ON 21 JAN. 2001

The balloon carrying the cryosampler payload was launched on 21 January 2001 from Hyderabad. Air samples were collected at different heights above the local tropopause at 16 km. The lowest height was 19.8 Km and the highest at 41.06 km. Probes in this flight were in duplicate sets at each height. One set is being analysed in Cardiff and the other one at CCMB, Hyderabad. In this meeting the preliminary results from the probes being analysed in Cardiff are given, whose details are given in Table I.

We now describe the results of biological studies carried out in Cardiff.

TABLE I
Details of probes being analysed in Cardiff

Probe	Collection height range	Collected NTP volume (litres)
A	19.80–20.32 km	81
B	24.36–27.97 km	70.5
C	28.47–39.05 km	38.4
D	39.75–41.06 km	18.5

3. Detection of Viable Cells

All recovery procedures were conducted in a sterile system in a laminar-flow chamber. From each of the 4 probes, air was passed first through a 0.45 micron filter and then through 0.22 micron filter. Eight filters were thus derived. The probes were stored at $-70°$C before sample preparation, and likewise the derived membrane filters were stored at this temperature before isolates were obtained and tested.

Since only the 0.45 micron filters are expected to have trapped microbial sized particles, these have been analysed. Approximately 4 mm^2 squares were aseptically cut from the filters and treated with fluorescent membrane potential sensitive dyes; either a cationic carbocyanine or an anionic oxonol dye was employed. Cationic dyes penetrate the cell membranes of viable cells, but not of dead cells, whereas anionic dyes penetrate the membranes only of non-viable cells. Any viable living cell present in a sample would therefore be expected to give rise to a fluorescent image when excited by light and could be located using an epifluorescence microscope. Light from laser Argon at the wavelength of 488 nm was used for this purpose. Depending on the optics employed for measurement, the images represent a single organism or a cluster. For details of the method see Lloyd and Hayes (1995).

Isolates treated with cyanine dye showed fluorescent images in the form of clumps of 0.3–1 micron sized organisms, the clumps themselves measuring 5–15 microns across. Higher resolution images will require deployment of a confocal microscope, but already the detection of viable cells by this technique (not found in the sterile controls) is beyond doubt.The use of anionic dyes revealed a comparable detection rate of dead or non-viable cells. In this report attention is focused only on the take-up of a cationic carbocyanine dye as an idicator of viable cells in the stratosphere.

Electron microscopy of aseptically isolated squares of membrane filters was performed next. The squares were mounted on 12 mm diameter sticky carbon tabs which in turn were mounted on 10 mm diameter aluminium tabs. The samples treated in this way were gold sputter-coated and imaged in a JOEL 5200 LV scanning electron microscope under a vacuum of 7 nanobar. The procedure adopted is suited to imaging bacteria because bacterial cell walls do not collapse or explode

under the conditions of observations. The structures were similar to the ones revealed by the epifluorescence microscope. Further analysis on the nature of the microorganism is in progress.

On each of the micropore filter isolates examined so far, measuring approximately 2 mm × 2 mm, a number N in the range of 1–3 microbial clumps were found. Since the air volume passing through each filter in A and B was about 80 litres at NTP (Table I) and the area of the entire filter is about 2000 mm^2, with N=3, the density of microbial clumps at 25 km is estimated as $[(3 \times 2000)/4]/80 = 18.75$ per litre at NTP. With an atmospheric pressure at 25 km of 0.025 bar, the estimated number density of microbial clumps at 25 km is 0.47 per litre. At a height of 40 km, 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 2000mm^2 of this filter being 18.5 litres (Table I) and the ambient air pressure being 0.0025 bar, the density of microbial clumps is about 0.068 per litre of air at 40 Km. With a clump comprised of about 100 bacterial cells the number density of viable cells at 40 Km, corresponding to this would be about 7 per litre.

Kasten (1968) had shown that if there is a steady infall of matter from outside the atmosphere, the number density $\mathcal{N}(h)$ of matter particles of any given kind would be inversely proportional to the terminal velocity $w(h)$ at that height. Using this formula, one gets an exponential drop in the number density with height:

$$\mathcal{N} \propto \exp(-ah), \tag{1}$$

where a can be determined from atmospheric parameters. Using the values available, one estimates that the number density of viable cells in steady state should follow a similar distribution and that the number density at height 41 km should be approximately one tenth of the number density at 25 km. The above calculation, given the experimental errors, does appear consistent with this hypothesis.

3.1. TERRESTRIAL CONTAMINATION

Considering the small number of about 7 living cells per litre of air at 41 km, as determined above, the main concern will be whether what has been found is of terrestrial contamination. As noted earlier, the possibility of contamination due to the instrumental collection process is completely ruled out by the stringent sterilization methods adopted. Hence the only possibility is the presence of terrestrial cells being carried aloft to such great heights by some extraordinarily rare events or spacecraft debris.

Under normal circumstances atmospheric mixing takes place only up to the tropopause, that acts as a barrier against the transport of waves responsible for mixing the terrestrial material with the upper atmosphere. However during some extraordinary events such as a very powerful volcanic eruption, it is possible for the material to reach large vertical heights. This was demonstrated by the volcanic eruption of Mt Pinatubo in Phillippines on June 15, 1991, which was estimated

to have injected nearly 20 million tonnes of SO_2 upto a maximum vertical height of 32 km (Grant et al., 1994 and references therein). The stratospheric mixing has been almost continuously monitored using several balloon flights (Deshler et al., 1992). It was shown that the gravitational settling to lower altitudes is rapid and after a few months, no significant mixing even in the lower stratosphere was taking place. Considering this direct evidence of no contamination of even as powerful a volcanic eruption as that of Mt Pinatubo, we can immediately rule out terrestrial contamination as an explanation of our results, as no volcanic eruption or any other extraordinary event took place even months prior to our experiment.

On the positive side, as mentioned above, the observation of the depth profile of the organisms matches that predicted for extraterrestrial cells injected at the top of our atmosphere, using the method of Kasten (1968). Furthermore, the same calculation yields the clearance of any transient injection (e.g. of space debris) through gravitational settling in a time of order of months. Hence it stretches credulity to maintain that the cells detected in our experiment are of terrestrial origin.

Milton Wainwright at Sheffield university (who is reporting his findings in another paper in this meeting) has claimed to have isolated two species of bacteria, *Bacillus simplex* and *Staphylococcus albus* from the samples at 41 km. Details of this finding may be seen in his paper (Wainwright, 2002) in this volume.

4. Concluding Remarks

Viable living cells have been detected using the cationic cyanine dyes at all heights ranging from 21 km to 41 km. Terrestrial contamination is ruled out because of the sample collection at altitudes well above the tropopause during a time when there were no extraordinary terrestrial events like volcanic eruption etc. Furthermore the use of stringent procedures for sterilization rules out the contamination due to the instruments or balloon. With an average falling speed for 3 micron sized clumps at 40 km of about 0.3 cm/s (Kasten, 1968), the infall rate of clumps with a number density of 0.068/litre over the entire Earth 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 3×10^{-14} g in a clump, a daily mass input of about a third of a tonne of biomaterial is deduced. Although these estimates are very tentative, they serve the purpose of illustrating the amount of infall matter involved, if the results of this investigation are accepted, and a *prima facie* case for a space incidence of bacteria into the Earth is seen to be established.

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References

Bernstein, M.P., Stanford, S.A., Allamandola, L.J., Gilette, J.S., Clement, S.J. and Zare, R.N.: 1999, *Science* **283**, 1135.
Bruch, C.W.: 1967, in: P.H. Gregory and J.L. Montieth (eds.), *Airborne Microbes Symposium of the Society for Microbiology*, **17**, p. 345, Cambridge University Press).
Deshler, T., Hofmann, D.J., Johnson, B.J. and Rozier, W.R.: 1992, *Geophys. Res. Lett.* **19**, 199.
Grant, W.B. et al.: 1994, *J. Geophys. Res.* **99**, 8197.
Haldane, J.B.S.: 1929, *The Origin of Life*, Chatto and Windys.
Hoyle, F. and Wickramasinghe, N.C.: 1980, *Evolution from Space*, J.M. Dent & Sons.
Hoyle, F. and Wickramasinghe, N.C.: 2000, *Astrophys. Sp. Sci.* **268**, 1.
Imai, E., Honda, H., Hatori, K., Brack, A. and Matsumo, K.: 1999, *Science* **283**, 831.
Kasten, F.: 1968, *J. Appl. Meteorology* **7**, 944.
Lloyd, D. and Hayes, A.J.: 1995, *FEMS Microbiology Lett.* **133**, 1.
Lopez-Amoros, R., Mason, D.J. and Lloyd, D.: 1995, *J. Microbiol. Meth.* **22**, 165.
Lysenko, S.V.: 1979, *Mikrobiologia* **48**, 1066.
Miller, S.L. and Urey, H.C.: 1959, *Science* **130**, 245.
Narlikar, J.V. et al.: 1998, *Proc. SPIE Conf. On Instruments, Methods and Mission for Astrobiology* **3441**, 301.
Oparin, A.I.: 1953, *The Origin of Life*, translated by S.Margulis, Dover.
Secker, J., Wesson, P.S. and Lepock, J.R.: 1994, *Astrophys. Sp. Sci.* **329**, 1.
Shyamlal et al.: 1996, *Ind. J. Radio Sp. Phys.* **25**, 1.
Smibert, R.M. and Kreis, N.R.: 1994, in: P. Gerherdt, R.G.E. Murray, W.R. Wood and N.R. Kreig (eds.), *Methods for General and Molecular Bacteriology*, Am. Soc. Microbiol., p. 603.
Wainwright, M.: 2003, Reference to article in this volume.
Wickramasinghe, N.C.: 2003, Reference to article in this volume.