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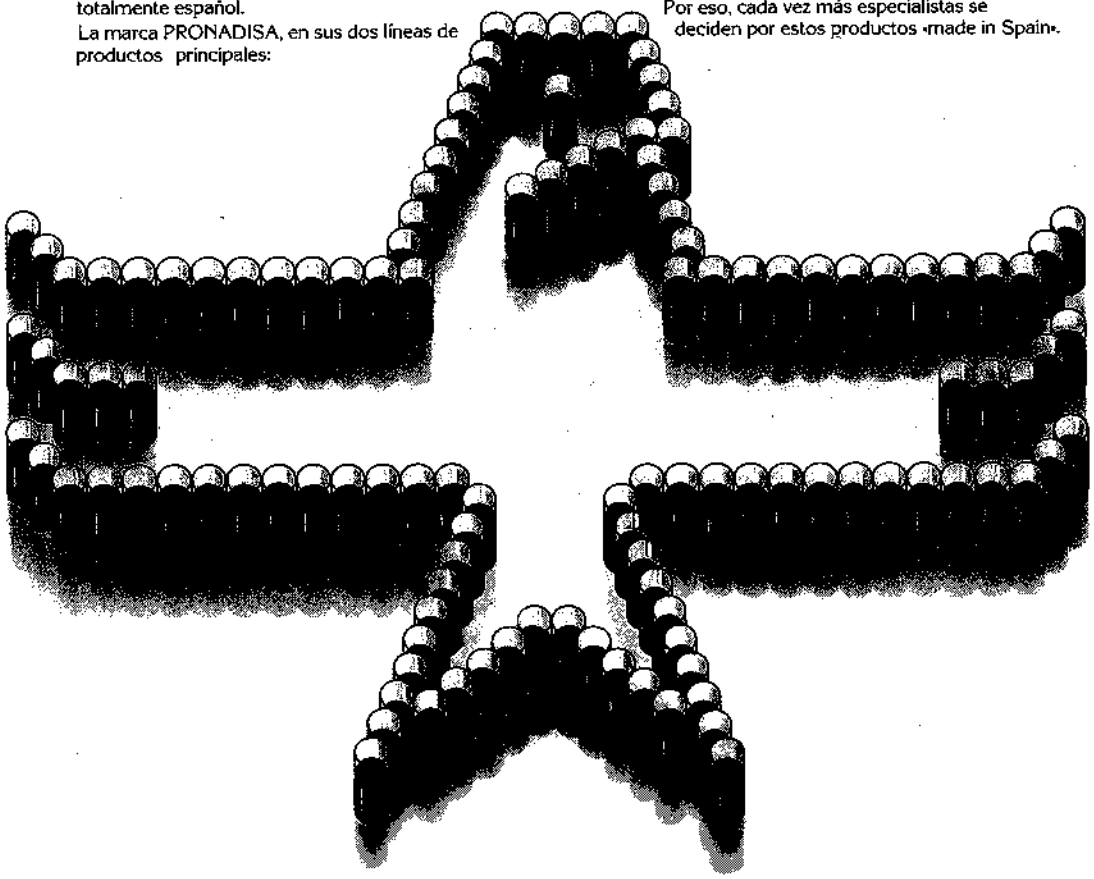

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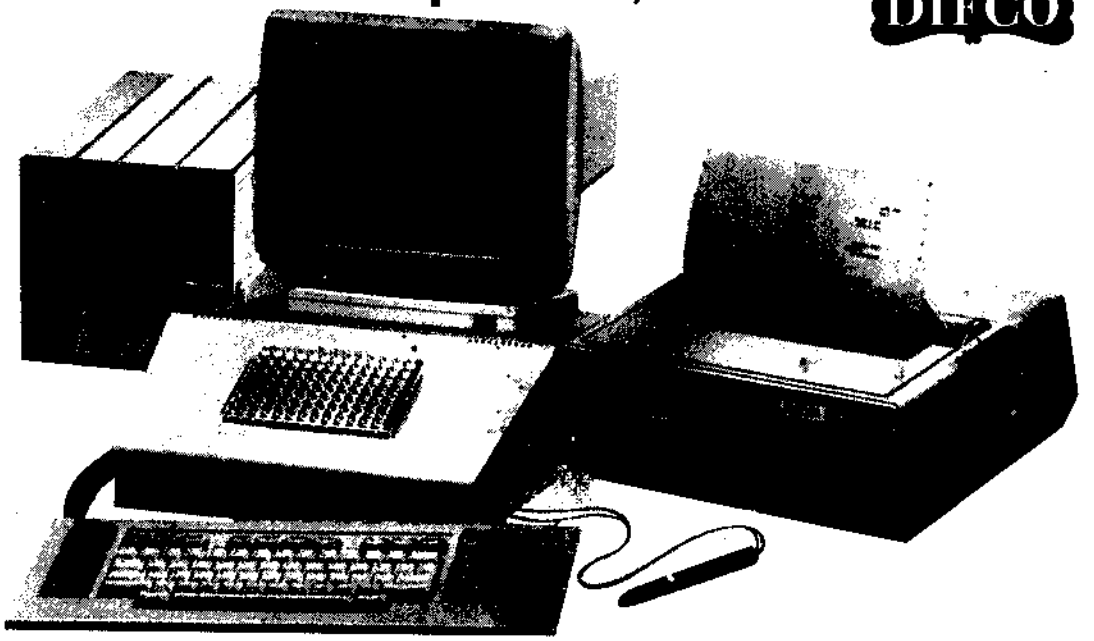

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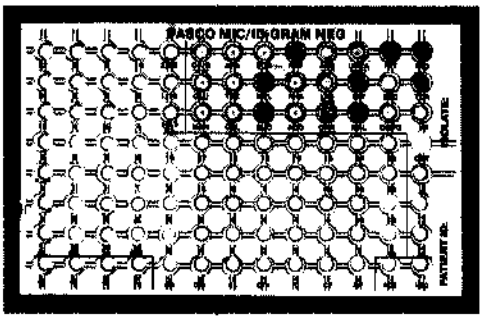

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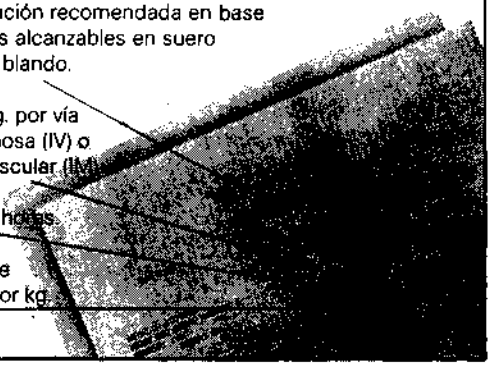

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MICROBIOLOGIA SEM 4 (1988), 141 - 148 **MINIREVIEW**

Self-regulation of the Earth as a living organism

James E. Lovelock

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(Received June 15, 1988)

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The living planet

The idea that the Earth is alive has probably been around ever since our species could talk about it. The first scientist to say so was James Hutton, who has often been called the father of geology. In 1785 he gave a lecture before the Royal Society of Edinburgh and said: «I consider the Earth to be a superorganism and that its proper study should be physiology.» He was a member of the Circulation Society, a scientific society that was inspired by the discoveries of physiology, like the circulation of the blood and the connection between oxygen and life. He applied these ideas to his view of the hydrological cycle and the movements of the nutritious elements of the Earth.

First of all, I will state why I think the Earth can be regarded as a living organism and why, therefore, physiology could be useful in understanding the oceans and the atmosphere. Afterwards, I will illustrate Gaia theory, or geophysiology, as it might be called, by the simulation of a model planet called Daisyworld. Finally, I will move on to some examples drawn mainly from marine science to illustrate the use of his approach to view evolution differently.

I suspect that many people view the idea that the Earth is alive as far fetched, if not absurd. They probably wonder why a sphere of rock, almost all of it incandescent and molten, could be alive.

I am grateful to the physicist Jerome Rothstein for suggesting a way to present this awkward idea more palatably. He compared the Earth to a giant redwood tree. It is alive, yet 99 per cent of it is dead wood. Like the Earth, it has only a skin of living tissue spread thinly at the surface.

When, twenty years ago, we first saw those pictures of the Earth from space, we had a vision of stunning beauty; that dappled white and blue sphere stirred us all, no matter that by now it is just a visual cliché.

It was the search for life on Mars that led to a return to Hutton's Earth. Being asked to think about ways of detecting life on Mars, it forced me to think about life on a planetary scale. The easiest, if not the only way to do it, was by examining the planet's atmosphere. On Mars, the atmosphere is the only available mobile medium. Therefore, were life to be present, it would be obliged to use the atmosphere as a transfer medium for raw materials and waste products. Such a use of it would render Mars distinguishably different from the near equilibrium state of a dead planet. This worked very well and showed that Mars was lifeless. But when in the imagination the same experimental approach was applied to the Earth atmosphere, it revealed a marvelous anomaly. The Earth is the only planet of the solar system to have an atmosphere that is extremely unstable in a chemical sense.

A look at the Earth with an infrared telescope from as far away as Pluto would give *prima facie* evidence that the Earth bears life in abundance. Moreover, the maintenance of this instability at a steady state for vast periods of time suggested a powerful regulatory process at work. The Earth shares with other living organisms this wonderful property of being able to control its chemical composition and keep cool when the environment outside is changing. The Earth has been able to keep its temperature constant since life began, in spite of a 25 per cent increase in the output of heat from the Sun. This fact has been due to the ability of living organisms to regulate the amount of carbon dioxide in the atmosphere by draining it from the air as the Sun warmed up. This is why at present carbon dioxide is about a thousand times less abundant than it was when life began.

The Gaia hypothesis

Fifteen years ago several evident facts led Lynn Margulis and me (4) to postulate the Gaia hypothesis. First of all, the intense disequilibrium of the atmosphere revealed the presence of life. Besides, the persistence of this unstable atmosphere at a steady state for periods much longer than the residence times of its gases revealed the presence of a controller, Gaia. The evolution of both living organisms and their physical and chemical environment are so closely coupled that they make up a single, indivisible process.

Gaia is easiest to see through the atmosphere. Even though similar disequilibria exist within the oceans, as Mike Whitfield pointed out in a review paper (5), the effects there are much diluted by the vast volumes of water and salt.

I wondered whether this new evidence, this new look at the Earth, could be explained by conventional science or whether it was actually necessary to put forward a new theory, even if it was an old one dressed up in new clothes. This is why now I will examine critically the three theories of the Earth that are considered by most scientists nowadays.

First, there is the theory that life evolved separately and independently of the Earth. It is still widely held and is the conventional wisdom expressed in most text books for students. This theory is quite wrong and is no more than an accident of the nineteenth century development of science. If the physics and chemistry of the Earth were evolving independent of life, and if organisms were simply adapting to the changes that took place, then there would be no reason to find any marked chemical disequilibria in the Earth's atmosphere. The Earth might have a different composition from the other planets, but there is simply no way for inorganic chemistry to lead to an atmosphere rich in reactive gases. There could be 21 % oxygen, but not if methane and hydrogen were present as well. There could be more than 90 % hydrogen and methane, as on the large outer planets, but not

in the presence of oxygen. This type of argument can be extended to other chemical substances and it always reveals the conventional wisdom of the text book to be in error.

In the last century, science has developed rapidly and has fragmented into a collection of near-independent professions. There were so many exciting discoveries about the world to be made that there were either little time or inclination to think about the total picture. To understand the world was like trying to assemble a jigsaw puzzle the size of the planet. The first thing to do was to collect the pieces into their categories, and it was all too easy to lose sight of the picture in that task.

The second theory, which is widely held by scientists, is the coevolutionist theory. It originated with the great Russian scientist Vernadsky, and is the basis of the modern science of biogeochemistry. It states that life and the environment interact, and it admits that gases like oxygen and methane are biological products. However, it recognizes neither the existence of a tight coupling between the organisms and their environment nor the active regulation of the chemical composition and climate of the Earth. Besides, and most importantly, it does not see the Earth as alive. Even though the coevolutionists recognize the folly of separating the Earth and life sciences, they either do not dare or do not want to take the full leap to Gaia. They are like the political centrists, preferring to stay in the middle of the road following the science of biogeochemistry or coevolution. In my opinion, this theory is also wrong. Nevertheless, it is more difficult to contradict than it was the first.

Before expounding why I think coevolution is inadequate to explain the facts of the Earth it is necessary to look at the viewpoint of biology.

Biologists among the readers may wonder why I have not mentioned the biological view of the Earth. I have not done so because biologists have in general assumed that the physical and chemical world evolves according to the rules laid down in the geology or the biogeochemistry department of their university, and the details of this material evolution need not be their concern. If the environment changes, then organismes can *adapt* to whatever change which takes place in their environment. Biologists had good reasons to ignore the physical and chemical world. If there were, and are, so many fascinating developments in evolutionary theory and molecular biology, why bother with controversies of other scientists? However, by accepting without thought the concept of adaptation, biologists and geologists both of them opted for ignorance rather than for understanding. If one accepts the idea that organisms adapt to their environment, it is all too easy to fail to see that the world is massively affected by the presence of life. The air we breathe, the oceans and the rocks are all either direct products of living organisms or else greatly modified by their presence. Therefore, organisms do not just «adapt» to a dead world determined by physics and chemistry alone. They live within a world which is the breath and bones of their ancestors, and which they are now sustaining.

The abundant evidence which exists of a tightly coupled interaction between life and the environment is fatal to the old conventional wisdom of a completely separate evolution of the Earth and life. It is also destructive to the coevolutionary argument. If organisms are adapting to a world whose material state is determined by the activities of their neighbors, changing the environment becomes part of the game. It would be absurd to suppose that organisms would avoid the act of changing their environment if, by doing so, they left more progeny. To believe in the loose uncoupled interaction of coevolution or biogeochemistry as a description of the Earth is to my mind as naive as to believe that a pair of naked teenagers who climbed into bed together would remain separate and engage in polite conversation rather than explore the joys of close coupling.

There are, I think, three main reasons why Gaia theory has not been taken seriously. First of all, it seems old fashioned, and goes right back to the Greeks and earlier. Therefore, it must be out of date. Moreover, I chose to present it somewhat poetically, and most scientists, as a result of a faulty education, have an instinctive distrust of anything of science not presented in their jargon. Finally, there was a lack of detail on how it worked. Most of us are unhappy about a theory if we cannot envisage its mechanism.

This led, indeed, to much more entertaining criticisms, mainly from two biologists, Richard Dawkins and Ford Doolittle. Dawkins, in his book «The Extended Phenotype», expounds at length, and with great fluency, that Gaia was impossible since planets do not reproduce and, therefore, there could be no natural selection of the most fit planet. The Canadian molecular biologist F. Doolittle rejected Gaia on the grounds that planetary self-regulation would need foresight and planning by the biota, and that there was no way for such a global altruism to evolve by natural selection. Symbiosis does occur, but always between closely coupled entities. Gaia would require the existence of a committee of the species that met annual to bargain next year's climate. Or a giant panglossian nanny who had looked after the Earth since life began.

These criticisms were absurd, and came from dogma. However, they were hard to answer. For a while I myself wondered whether Gaia was just an untestable notion like the anthropic principle. Something to be talked about rather than investigated. The feedback loops linking life with its environment are so numerous and so intricate, that I could see little hope of either quantifying or understanding them. Later on it occurred to me that I could, as always, in both art and science, make an abstraction of the essence of it. Those critics of Gaia could be answered by making a model that were entirely free of determinism.

Daisyworld

Think of a portrait artist who, with a few lines drawn on canvas, captures the likeness of the subject, and then spends months filling in the details. The same kind of abstraction can be done for the world by reducing the environment to a single variable, temperature, and the biota to a single species, daisies.

Imagine a planet like the Earth but with less ocean. It travels at the Earth's orbit around a star of the same mass and composition as our sun. This planet spins like the Earth, but its atmosphere has few clouds and no «greenhouse gases» to complicate its climate. In these circumstances it is easy to calculate the mean surface temperature from the balance between the radiation received from the star and the heat lost by radiation from the planet to space. This means that if you know the color, the albedo, of the planet you can calculate its temperature from the Stefan-Boltzman law.

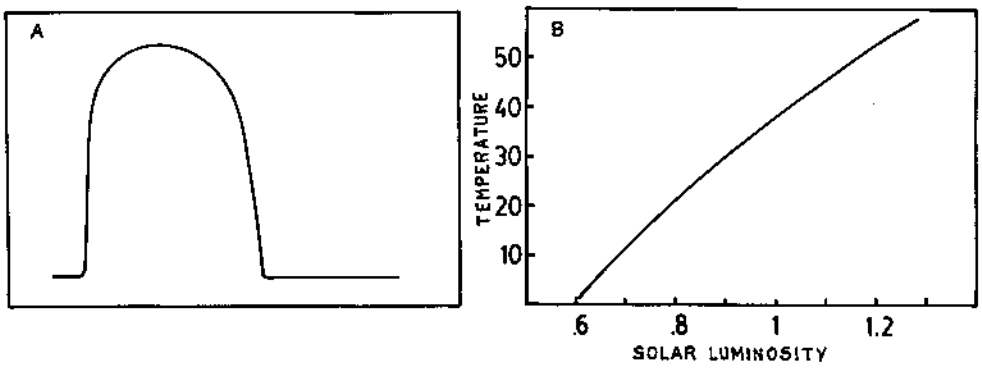

Fig. 1. Model of Daisyworld, with one only variable (temperature) and one only species (daisies). (A) Expected changes in the population of daisies as temperature increases. (B) The temperature of Daisyworld increases at its star grows more luminous. Beyond the point where it is too hot (40° C) daisies disappear.

This planet, Daisyworld, is seeded with daisies that begin to grow when the temperature is above 5° C and stop growing when it is above 40° C. They grow best at more comfortable temperatures in between. The mean temperature at Daisyworld's surface increases as its «sun» grows more lumi-

nous (Fig. 1 B). The expected population of daisies as the planet warms from cold to hot can be predicted (Fig. 1 A). This model is based on the conventional wisdom of physics and biology; the daisies can either respond or adapt to the physical environment, but do not alter it.

Now imagine that two species of daisies, dark and light in color, grow in Daisyworld. The seeds of both of them will germinate when temperature reaches 5° C. At the early stages dark daisies will grow better than light ones because they will absorb more sunlight and be warmer. They will also warm their surroundings and cause the temperature of the planet to rise rapidly. Now, the light-coloured daisies are at an advantage. In fact, they can remain cooler by reflecting sunlight, and, as they spread, they cool the planet (Fig. 2 A).

The competitive growth of the two species can keep the mean temperature of the planet's surface close to that most comfortable for daisies (Fig. 2 B).

Biologists may sceptically wonder what would happen if you had grey daisies. These flowers, in fact, would not need to spend energy making pigment. So, they would be at an advantage over both light and dark daisies. Would they eventually dominate the population of daisies and thus frustrate the temperature regulation of the planet?

Not knowing that models of more than two species competing simultaneously are almost invariably unstable. I made a Daisyworld where between 3 and 20 different species of daisies of different colour grew competing for space on the planet (Fig. 3); therefore, regulating unconsciously their world. Grey daisies, which are the same colour as that of the bare planetary surface, were also included in these models.

I modelled temperature regulation by 10 species of daisies growing in competition. Temperature is regulated more accurately in this model than it was in the two-species model, and it is as stable (Fig. 3 C).

Daisyworld is just a model and it does not imply that the Earth regulates its temperature by growing daisies. The purpose of Daisyworld is to provide a reasonable explanation of how Gaia works and why foresight and planning are not required for planetary regulation.

Gaia answers the query

At the present stage of its development it matters little whether Gaia theory is right or wrong, for it is already providing a new and more productive view of the Earth and the other planets. If we take Gaia as real, there are two main aspects that it provokes us to consider:

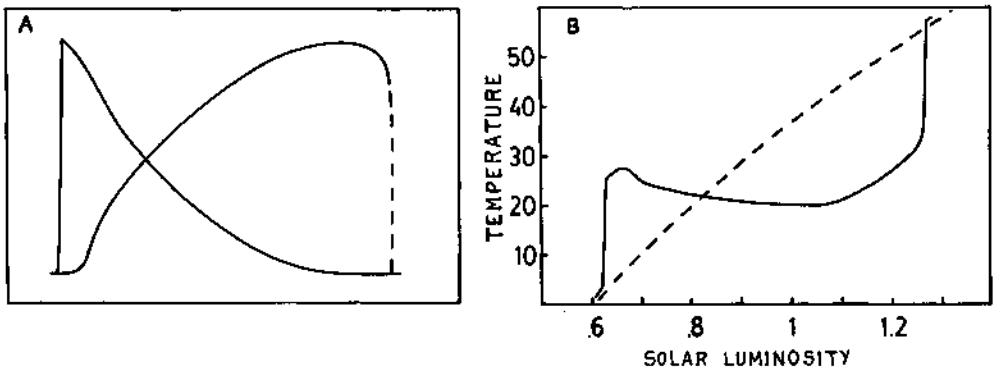

Fig. 2. Model of Daisyworld, with two species of daisies, light and dark in color. (A) Dark daisies will grow better at lower temperatures because they will absorb more sunlight and, thus, be warmer. As a result, the planet itself will be warmer. When the temperature of the planet has risen, light daisies, which will remain cooler by reflecting sunlight, will be at an advantage. (B) The competitive growth of daisies regulate the temperature of the planet's surface to that most suitable for the survival of both species.

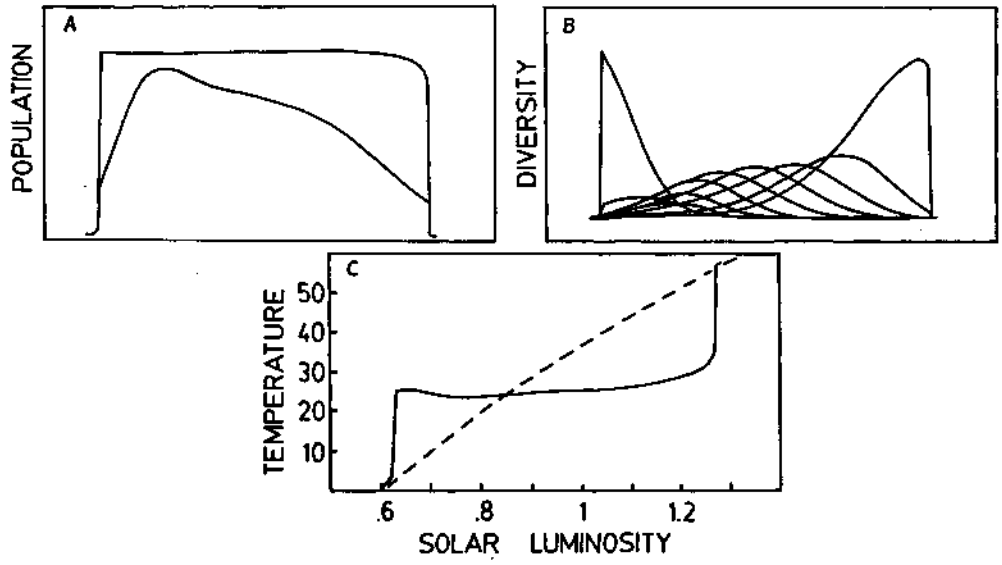

Fig. 3. Model of Daisyworld with ten species of daisies of different color growing on the planet. (A) illustrates the diversity of ecosystem and the total biomass. (B) shows the populations of each of the species, and (C) the increase in temperature of the planet's surface with daisies growing on it, and for the bare planet.

1. Life is a planetary scale phenomenon. There cannot be sparse life on a planet. Any more than there cannot be half an animal. Living organisms have to regulate their planet; otherwise, the ineluctable forces of physical and chemical evolution would soon render it uninhabitable.

2. Gaia theory adds to Darwin's great vision. It is no longer sufficient to consider the evolution of the species separately from the evolution of their environment. The two processes are tight coupled as a single indivisible proceeding. It is not enough to say that the organism that leaves the most progeny succeeds. Success comes only to the organism that also maintains a benign relationship with its physical environment.

Theories are judged useful in science if they make predictions that are useful or can be tested by experiment. At first sight it would seem that doing planet scale experiments is both expensive and reprehensible. However, they are certainly happening as a result of our own activities. Perhaps the largest of these experiments are the deforestation of the humid tropics and the burning of fossil fuels.

Gaia theory predicts consequences for these activities different from those of either conventional science or biogeochemistry. In particular, Gaia theory sees the Earth as a responsive living organism that tends, at first, to resist adverse environmental change and maintain homeostasis. However, if stressed beyond the limits of the current regulatory apparatus, it would jump to a new stable environment where many of the current range of species would be eliminated.

In spite of such excitements as the increasing storminess and the ozone hole over Antarctica, nothing much is yet happening to the environment that may suggest an imminent jump. Even so, these surprises should be looked on as indicators harbingers of larger non-linearities to come if man persists in his experiments with the Earth.

The plankton-climate connection

Last year, with some colleagues, I made a discovery about the Earth. That discovery was the fact that there is a connection between the algae that live in the surface of the waters of the ocean,

their production of sulfur gases, the clouds above the ocean and the climate of the Earth. Without Gaia, all these different interests would have been studied by separate scientists who rarely, if ever, talked to one another.

It all began because Gaia theory required the production of large quantities of special compounds able to transfer the elements sulfur and iodine from the sea, where they are abundant, to the land, where they are depleted. Curious about this, in 1971 I travelled aboard the R. V. Shackleton from Wales to Antarctica and back. I measured sulfur and iodine gases in the sea with my colleague Robert Maggs. Wherever the ship sailed the ocean surface contained a sulfur compound, dimethyl sulfide (DMS), and a iodine compound, methyl iodide. Their abundances in the sea were sufficient to allow a flux to the atmosphere enough to supply the needs of the land surfaces. It is important to point out that, had it not been for the predictive value of Gaia, both of these compounds would have remained as curiosities of algal biochemistry rather than considered as major carriers of the sulfur and iodine cycles of nature.

This finding and some lively discussions with my colleagues Andi Andrea, Robert Charlson and Steve Warren (1) led us to propose that the output of the sulfur gas dimethyl sulfide, produced by algae growing in the ocean surface, is the largest, if not the sole, source of the nuclei on which cloud droplets form over the oceans.

Cloud density, therefore, is linked with algal growth in the ocean surface. Robert Charlson has analyzed the phenomenon and calculated that its potential effect on global climate is comparable to that of the CO_2 greenhouse, if not greater. From the viewpoint of Gaia it is a very cheap way to manage the climate.

Andreae has found that the output of DMS from the ocean does not much depend upon productivity. As a matther of fact, it is highest over the desert regions, where the ocean is clear hot and deficient in nutrients. Yet, the output is highly species dependent. Patrick Holligan, an expert in algal ecosystems, told me that some species of phytoplankton, like coccolithophoridons, which are more abundant in tropical, oligotrophic waters, release copious quantities of DMS, whereas other, like diatoms, do not release none.

The most probable direct link between clouds and algal growth is a meterological one. The stirring effect of winds blowing over the ocean would stir the water and mix nutrients from the lower layers into the zone of algal growth. In fact, clouds and wind are well known by meteorologists to be closely linked.

This topic is developing rapidly, and direc measurements from satellites have already shown a direct connection between cloud albedo and the abundance of a sulphate aerosol in the atmosphere above the ocean. The DMS producing clouds by nucleating the water vapor could be acting like the white daisies of the Daisyworld model, keeping us cool, even though we do not understand the details of the climatic feedback.

Captain Cook reported the presence of this aerosol in his journals: «It is a dispensation of providence that wherever the ship sails in tropic waters there is a golden haze that protects the skins of the men and the rigging of the ship from the fierce rays of the sun» (2).

When thinking of the oceans, one must keep in mind that, while water may have been needed for the start of life, the persistence of water on the Earth is another of the consequences of the Gaia system. Venus and Mars had once abundant water, but having no life they could not keep it.

The effects of perturbing a living planet by pollution and species deletion are different from those of perturbing the non-living Earth of conventional wisdom. The consequences of adding greenhouse gases like carbon dioxide to the atmosphere and of cutting down the forests of the humid tropics are, therefore, likely to be different from those predicted by conventional models. The famous geochemist Wally Broecker has warned of non-linear responses, even from a conventional geochemical view. Nevertheless, if the Earth is actually a living planet, the surprises could be devastating for humans. Living systems tend to move suddenly from one stable state to another, like the

quantum jumps of physics. We can be asleep or awake. A child or an adult. We can be alive or dead.

The message from Gaia for human ecology

First and foremost, Gaia forces upon man a concern for the planet and its state of health, and offers an alternative to his near obsessive concern with the state of humanity.

Nevertheless, it is in man's own interest anyway to live well with the Earth. If man does not, Gaia will live on, but with a new biosphere that may not include humans. It is this thought that makes me disagree with many environmentalists, especially those who claim to be friends of the Earth, but in fact are friends of humanity. I recognize that we share the same objectives at heart. However, we differ in the priorities. To a friend of Gaia ozone depletion and Chernobyl are minor problems affecting mostly white people. The demolition of the forests of the humid tropics and the more and more increasing burden of greenhouse gases are real threats to Gaia and humanity alike.

As far as the health of the Earth is concerned, we are, in many ways, in the same state as the early physicians were towards the health of their patients. We have an impressive array of scientific equipment and expertise, but so did the nineteenth century physicians. There was microbiology and microscopes and the bacterial theory of disease. There was biochemistry and an understanding of many metabolic disorders. But there was almost nothing a physician could do apart from comforting and relieving pain, or doing simple surgery. It was not until after the second World War that high technology medicine came of age.

So it is with the Earth. Even though we have theories and equipment, there is little that science has to offer for the cure of the ills of Gaia. One thing, however, we can learn from the physician that is invaluable in our professional practice as planetary physicians: the hippocratic oath. Do nothing that would harm the patient.

In our enthusiasm or belief in some new nostrum we should take care that the cure is not worse than the disease.

The other and more optimistic message is that the evolution of Gaia seems to depend upon the activities of individual organisms. If these are favorable for the environment, they succeed. If not, they are doomed, but life goes on.

To me this means that it is more important to try to live in harmony with the Earth at a personal level than to expect any of the numerous human collectives or government agencies to take that responsability away from us.

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Partial purification and characterization of ADP sulfurylase from the purple sulfur bacterium *Thiocapsa roseopersicina*

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Summary

High activities of ADP and ATP sulfurylase were found in the soluble protein fraction of *Thiocapsa roseopersicina* strain 6311 (DSM 219). ADP sulfurylase was partially purified and characterized. It was a very labile soluble enzyme with a molecular weight of 250,000. The optimum pH was 7.5 and the optimal temperature 35° C. Under test conditions the apparent Km values were determined to be 0.33 mM for adenylylsulfate and 13 mM for phosphate.

Key words: ADP sulfurylase, sulfur metabolism, phototrophic sulfur bacteria, Thiocapsa roseopersicina.

Resumen

Se han detectado elevadas actividades de los enzimas ADP y ATP sulfurilasa en la fracción soluble proteica de la cepa 6311 de *Thiocapsa roseopersicina.* El enzima ADP sulfurilasa ha sido parcialmente purificado y caracterizado. Se trata de una proteína soluble y muy lábil cuyo peso molecular es 250.000. La temperatura y pH óptimos son 35° C y 7,5, respectivamente. Los valores de la Km aparentes fueron determinadas, resultando ser 0,33 mM para el adenililsulfato y 13 mM para el fosfato.

Introduction

Thiocapsa roseopersicina is a phototrophic purple sulfur bacterium capable of assimilatory sulfate reduction (19) with the exceptions of the strains BBS and SL in which growth is dependent of the presence of reduced sulfur compounds (13). In the dissimilatory sulfur metabolism of *Thiocapsa*

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Abbreviations: ADP, adenosin 5'-diphosphate; APS, adenosin 5'-phosphosulfate; ATP, adenosin 5'-triphosphate; DEAE, diethylaminoethyl; DSM, German Collection of Microorganisms, Braunschweig; PEP, phospho(enol)pyruvate; Tris, tris-(hydroxymethyl)aminomethane; U, international units, μmol/min.

reduced sulfur compounds such as sulfide, elemental sulfur or thiosulfate, serve as photosynthetic electron donors, being oxidized to sulfate via sulfite and adenylylsulfate (APS) (19).

The sulfate release step was first studied in 1968 when Thiele (18, 19) reported that *Thiocapsa roseopersicina* strain DSM 219 contained ADP sulfurylase (EC 2.7.7.5, ADP-sulfate adenylyltransferase) which, in connection with adenylate kinase would enable the organism to conserve APS energy by substrate phosphorylation. Ivanovskii and Petushkova (9) also investigated the ability of *T. roseopersicina* to synthezise ATP as a result of oxidation of sulfite during growth in the light. Until now APS reductase is the only enzyme involved in this pathway that has been purified and characterized from *T. roseopersicina* (21). In this work ADP sulfurylase has been partially purified and, in addition, evidence is presented for the presence of ATP sulfurylase (EC 2.7.7.4) in extracts of *T. roseopersicina*.

Materials and methods

Organism and culture

Thiocapsa roseopersicina strain 6311 (DSM 219) was grown photolithoautotrophically at 30° C and at a light intensity of 2000 lux in completely filled 1 l screw cap bottles. The medium used was that described by Pfennig and Trüper (15) containing 0.075 % sulfide. When the cells were free of intracellular sulfur globules the cultures were fed with a neutral sulfide solution (17). Cells in the exponential growth phase were harvested by centrifugation, washed once with 50 mM Tris-HCl buffer, pH 7.5, and kept frozen as packed cells.

Preparation of cell extracts

Packed cells were taken up in about once their volume of 50 mM Tris-HCl buffer, pH 7.5, homogenized and broken up by ultrasonic desintegration (30 s ml^{-1} at 4° C) in a Schoeller sonifier. The broken cell mass was then centrifuged at 25,000x*g* for 30 min at 4° C. The pellet, containing cell wall debris and elemental sulfur globules was discarded. The supernatant obtained, called «crude extract», was then subjected to further centrifugation at 140,00xg for 2 h in a Beckmann L5-65 ultracentrifuge, resulting in a clear supernatant (hereafter referred to as «soluble protein») and a pellet consisting of membranes.

The soluble protein was used in enzyme tests and for purification. The purification procedure is described in detail below.

Protein determination

The measurements of protein content in crude extract were carried out after Lowry *et al.* (11) using bovine serum albumin as a standard. The protein content of purified extracts was determined according to Bradford (3).

Enzyme assays

ADP sulfurylase: In a total volume of 1.0 ml the assay contained 100 mM Tris-HCl buffer, pH 7.6, 1 mM APS, 1 mM $MgCl_2 \times 6\ H_2O$, 0.12 mM NaNADH, 0.4 mM sodium phosphoenolpyruvate (Na-PEP), 50 mM potassium phosphate, 8 units (U) lactate dehydrogenase, 6 U pyruvate kinase and distilled water. The reaction was started with APS. The decrease in NADH, which is equivalent to ADP formation, was measured spectrophotometrically at 340 nm and 30° C. Specific activity was expressed as μmol ADP formed min^{-1} (mg $protein^{-1}$).

To determine the temperature optimum of ADP sulfurylase it was necessary to measure enzyme activity without auxiliary enzymes. In this case activity was measured in a test solution of 100 mM Tris-HCl buffer, pH 7.6, 1 mM APS, 10 mM $MgCl_2 \times 6\ H_2O$, 50 mM potassium phosphate, distilled water and cell free extract in a final volume of 1.0 ml. The assay was preincubated at different temperatures for five minutes and the reaction was started by addition of the enzyme. After 10 min ADP sulfurylase reaction was terminated by boiling for 2 min. Denatured protein was removed by centrifugation at 25,000xg for 10 min. An aliquot of supernatant was used for quantitative determination of generated ADP.

ATP sulfurylase was measured spectrophotometrically according to Cooper and Trüper (6).

Analytical procedures

ADP was determined in a coupled enzyme assay by the oxidation of NADH: The assay contained 100 mM Tris-HCl, pH 8.0, 0.4 mM Na-PEP, 2 mM $MgCl_2 \times 6\ H_2O$, 0.12 mM Na-NADH, 11 U lactate dehydrogenase, 10 U pyruvate kinase and up to 100 nmol ADP in the sample. The reaction was started with pyruvate kinase and the NADH oxidation was measured at 340 nm.

Molecular weight determination

Molecular weight of ADP sulfurylase was estimated by chromatography on Sephacryl S-300 with ferritin, catalase, aldolase, bovine serum albumin and ovalbumin as reference proteins (Boehringer Combithek II No. 104558). The column was equilibrated with 50 mM Tris-HCl buffer, pH 7.5.

Polyacrylamide gel electrophoresis

Non-denaturing polyacrylamide gel electrophoresis was carried out in 7.5 % acrylamide gel [gel system 1a in H. R. Maurer (14)], protein bands being located by staining with Coomassie-Blue R 250. Gels were scanned at 600 nm using a Beckmann Acta M IV recording spectrophotometer.

Chemicals and biochemicals

Standard chemicals (analytical grade) were obtained from Merck Darmstadt, FRG; nucleotides, auxiliary enzymes and combitheks from Boehringer, Mannheim, FRG; DEAE cellulose from Whatman Biochemicals Ltd, Maidstone, England; Sephadex G-25 and Sephacryl S-300 from Pharmacia, Uppsala, Sweden. Lyophilised adenylylsulfate, synthesized after Cooper and Trüper (5), modified by Imhoff (8), was kindly provided by Dr. A. Schug.

Results

Evidence for ADP and ATP sulfurylase

Crude extracts of *T. roseopersicina* were monitored for both enzyme activities. ADP sulfurylase activity was shown to be dependent on the growth phase. The highest activities occurred in the exponential phase of growth during which the specific activities of the enzymes in crude extracts were 190 mU/mg protein for ATP sulfurylase and 345 mU/mg protein for ADP sulfurylase (average values from seven measurements).

Stability and storage

By daily assays it was shown that the ADP sulfurylase in crude extracts, when stored at 4° C, lost more than 50 % of its original activity during 2 days of storage. This loss of activity decreased with time and a residual activity of 30 % could still be found after 9 days of storage. When frozen (—18° C) the enzyme also lost about 50 % of its original activity in 2 days. The addition of 20 % glycerol to the crude extract as a stabilizer resulted in a loss of only 20 % of its original activity in 2 days and of only 25 % after 9 days of storage at 4° C. Fig. 1 summarizes the stability experiments.

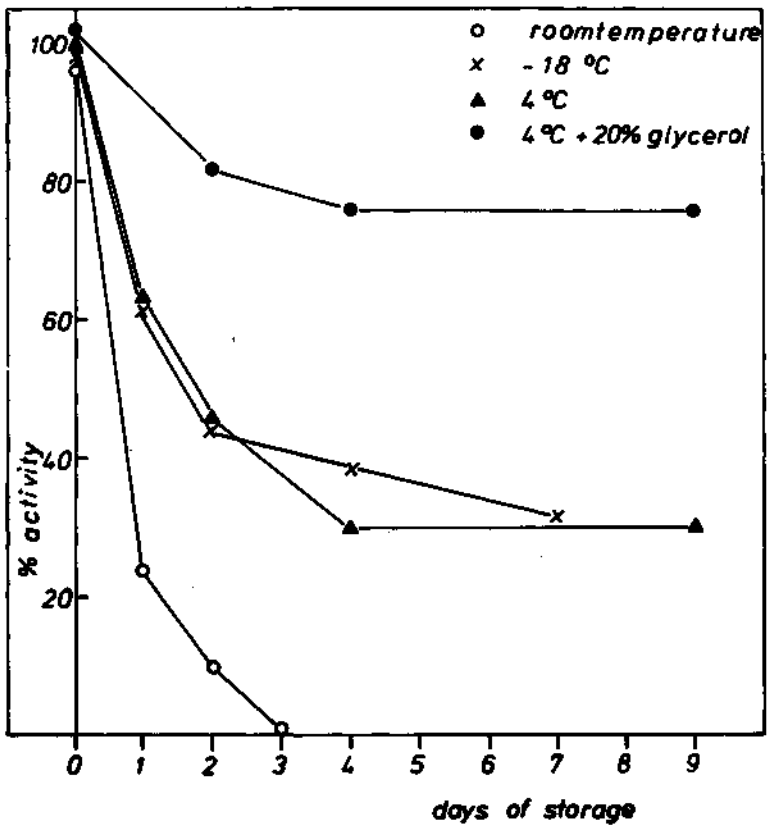

Fig. 1. Stability of ADP sulfurylase under different conditions of storage.

Purification of ADP sulfurylase from Thiocapsa roseopersicina

Several procedures of purification were tested, but the enzyme turned out to be very labile and proved difficult to purify. Neither changing of the disruption method nor application of various buffer systems could increase enzyme activity. Glycerol was a suitable stabilizing agent. Most attempts to purify ADP sulfurylase resulted in considerable loss of activity while the purification factor obtained was very low. For example ammonium sulfate precipitation was inapplicable. The fraction between 30 and 70 % saturation was found to contain 80 % of the recovered activity but the loss in total activity amounted to more than 80 % and the purification factor achieved was only 1.2.

For purification low molecular weight constituents were removed from the supernatant obtained after ultracentrifugation by gel filtration on Sephadex G-25 equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 20 % glycerol. The eluate was loaded on a diethylaminoethyl cellulose colums (Whatmann DE 52) with a volume of 40 ml equilibrated with 50 mM Tris-HCl, pH 7.5 containing 20 % glycerol. The flow rate employed was 30 ml/h. A linear KCl gradient (0-0.5 M in the before-mentioned buffer) was applied and ADP sulfurylase was eluted at a calculated KCl concentration of 120-150 mM. A representative elution pattern is shown in Fig. 2. The combined fractions

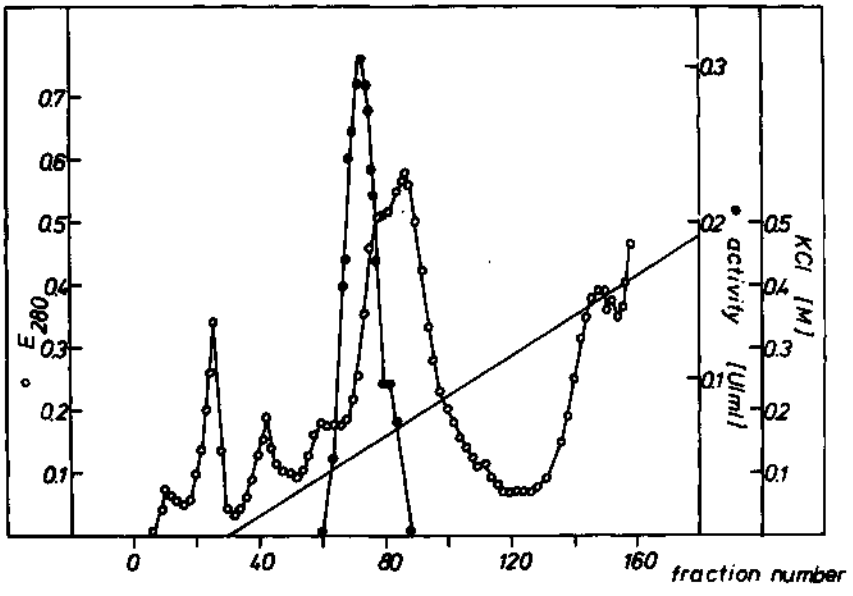

Fig. 2. Fractionation pattern on diethylaminoethyl cellulose (Whatman DE 52). Column proportions: diameter 1.5 cm, length 5.6 cm. Flow rate: 30 ml/h.

containing activity were concentrated by ultrafiltration in a Diaflo chamber (Amicon Corp.) using a PM 30 filter and a N_2 pressure of 2 bar. Characterization of the enzyme was done with this fraction. For further purification the concentrated enzyme solution was desalted by gel filtration on Sephadex G-25 equilibrated with 50 mM Tris-HCl, pH 7.5. The solution obtained was then passed through a Sephacryl S-300 column (1.5 cm × 90 cm, flow rate: 15 ml/h), equilibrated with 50 mM Tris-HCl buffer, pH 7.5. The gel filtration always resulted in two protein peaks (280 nm) of different height. Activity was only found in the first fractions of the second peak. The elution diagram of this last purification step which resulted in a 6.6-fold increase of specific activity is shown in Fig. 3.

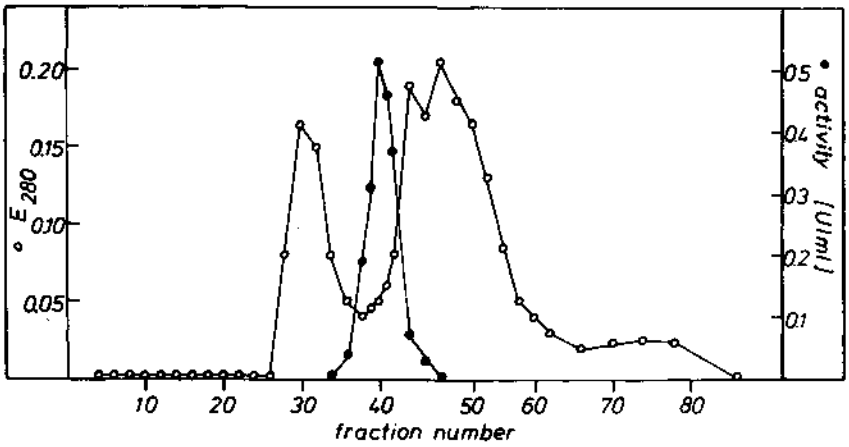

Fig. 3. Fractionation pattern on Sephacryl S-300. Column proportions: 1.5 × 90 cm. Flow rate: 15 ml/h.

To elucidate homogeneity of the enzyme preparation fractions containing the highest specific activity were combined and —after concentration by ultrafiltration— subjected to non-denaturing polyacrylamide gel electrophoresis. The partially purified preparation of ADP sulfurylase from *T. roseopersicina* migrated as one heavy and two rather weak bands (Fig. 4). This suggests a rather high degree of purification of the enzyme.

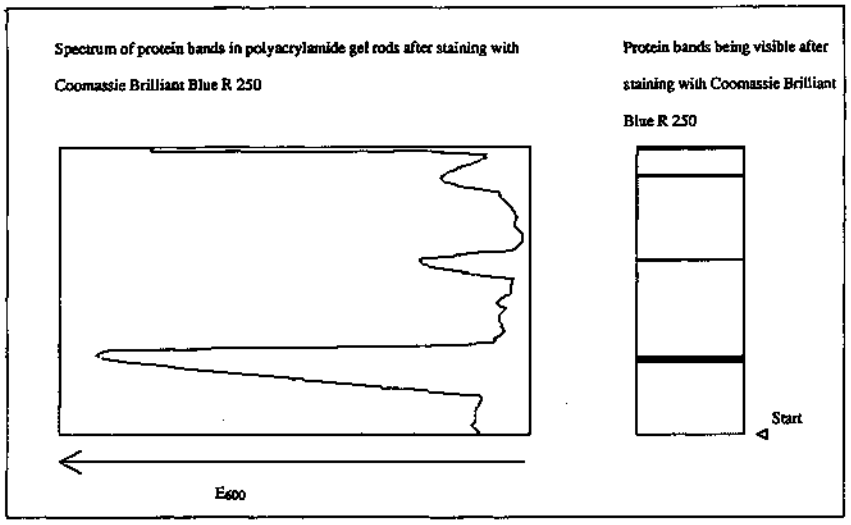

Fig. 4. Polyacrylamide gel electrophoresis of partially purified ADP sulfurylase.

A summary of the purification procedure is given in Table 1. An overall enrichment of about 19-fold was achieved.

TABLE 1
EFFICIENCY OF A TYPICAL PURIFICATION OF ADP SULFURYLASE FROM *THIOCAPSA ROSEOPERSICINA*

Fraction	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor
Crude extract	266.4	0.35	100	1
Ultracentrifugation	91.8	0.53	52.2	1.5
G-25 eluate	72.5	0.5	38.9	1.4
DE 52 eluate	3.1	1.0	4.9	2.9
Sephacryl S-300 eluate	0.3	6.6	2.1	18.8

Influence of pH and temperature on enzyme activity

Tests with Tris-HCl buffers at different pH values under otherwise constant conditions showed that the enzyme was active between pH 7.0 and 9.0 with the optimum at pH 7.5 (Fig. 5). These results are close to the pH optima reported for ADP sulfurylases from other bacteria.

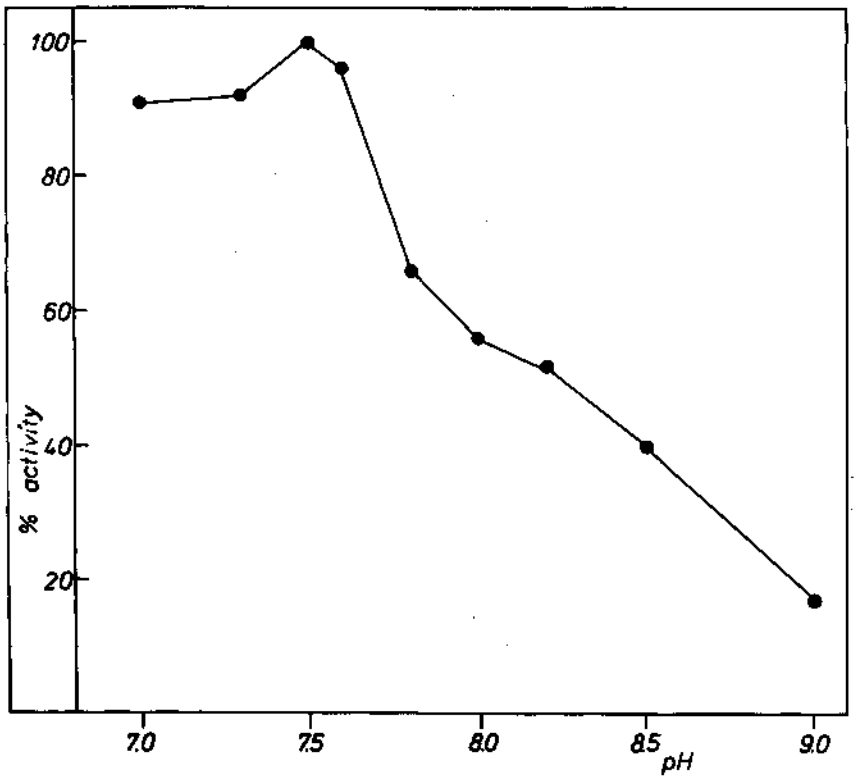

Fig. 5. Effect of pH on the activity of partially purified ADP sulfurylase. Tris buffer as described in materials and methods.

The temperature range was measured between 20 and 60° C preincubating the test solution for 5 min and then starting the reaction by addition of the enzyme. Activity was measured by determination of ADP after termination of the reaction. The enzyme showed a temperature optimum at 35° C (Fig. 6). At temperatures above 55° C the enzyme was denatured resulting in total inactivation.

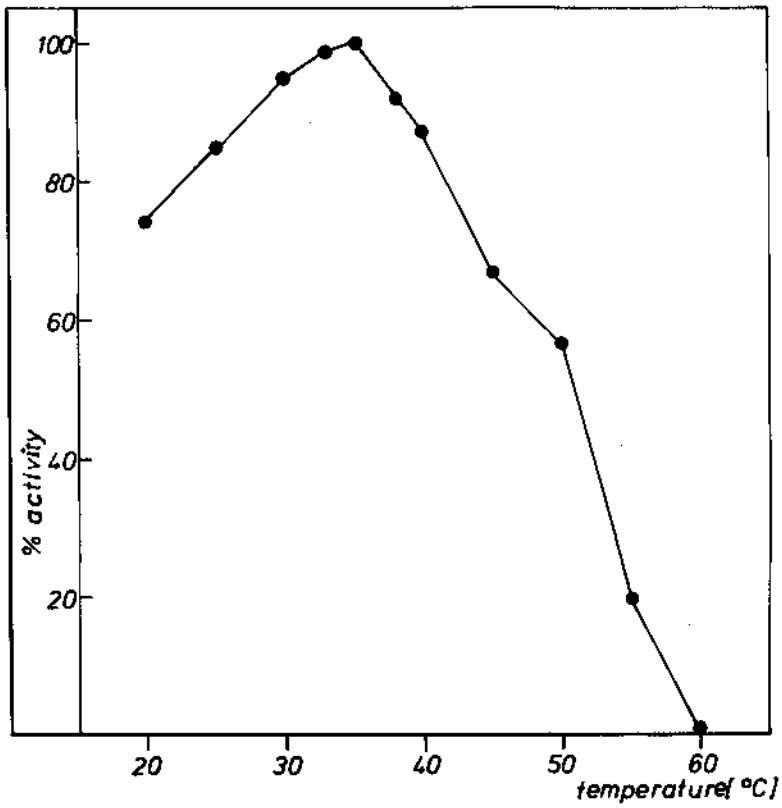

Fig. 6. Temperature optimum curve for ADP sulfurylase activity.

Influence of substrate concentration on enzyme activity

With the partially purified ADP sulfurylase from *Thiocapsa roseopersicina* activities were measured by individually changing the concentrations of the substrates. The appaent Km for adenylylsulfate (APS) was 0.33 mM (at 50 mM phosphate) and 13 mM for phosphate (at 1 mM APS). The enzyme reaction rate was constant at high APS and phosphate concentrations (Fig. 7 and Fig. 8).

Molecular weight of ADP sulfurylase

The molecular weight of the enzyme was estimated as described above by comparative gel filtration on a Sephacryl S-300 column. The enzyme protein had a molecular weight of 250,000 (Fig. 9). This value is somewhat higher than the molecular weights of other ADP sulfurylases described in literature (2, 7, 15, 22).

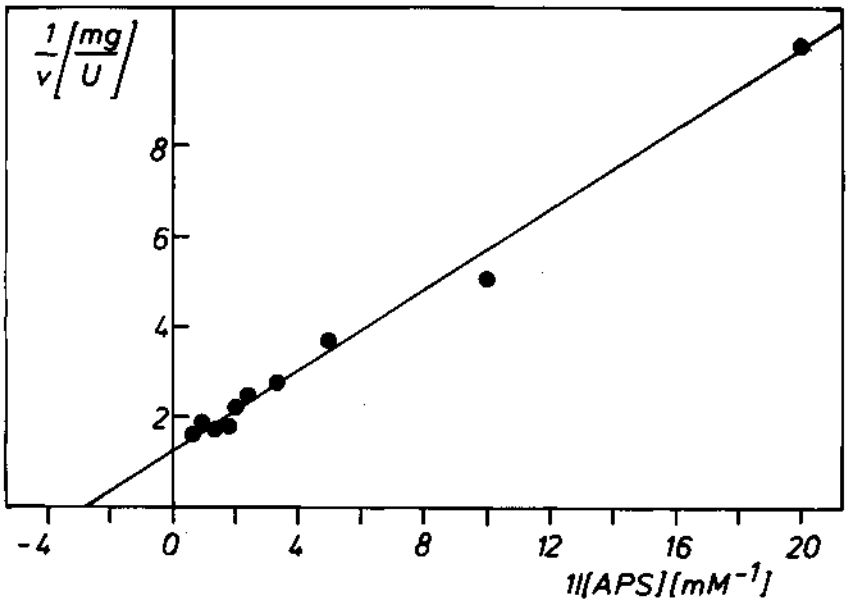

Fig. 7. Effect of APS concentration on ADP sulfurylase activity at a phosphate concentration of 50 mM. Results are presented as double reciprocal plot.

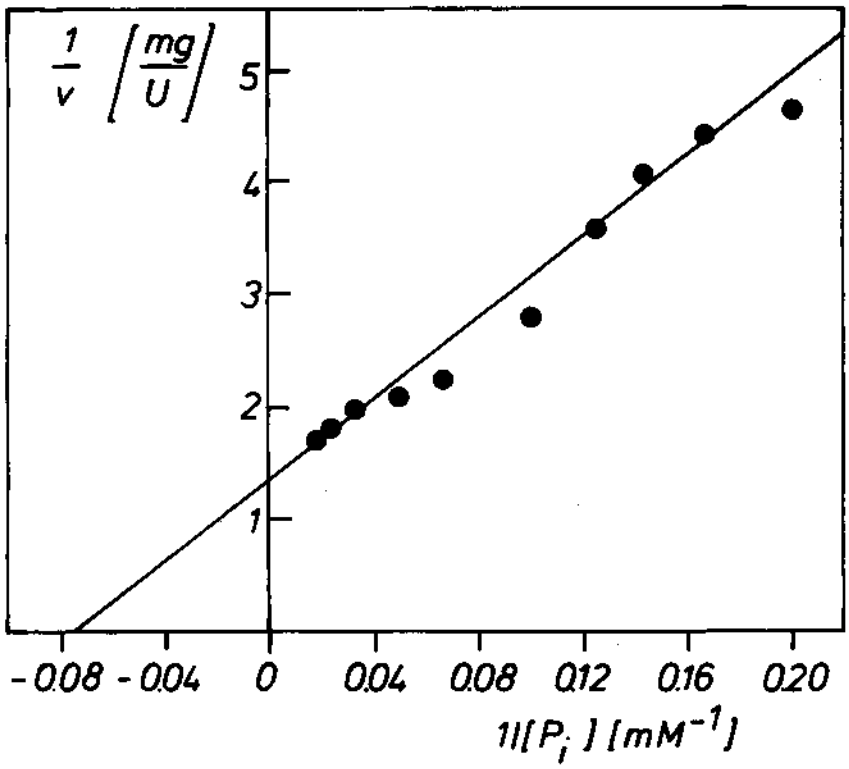

Fig. 8. Effect of phosphate concentration on ADP sulfurylase [(APS) = 1 mM]. Data are plotted according to the method of Lineweaver-Burk.

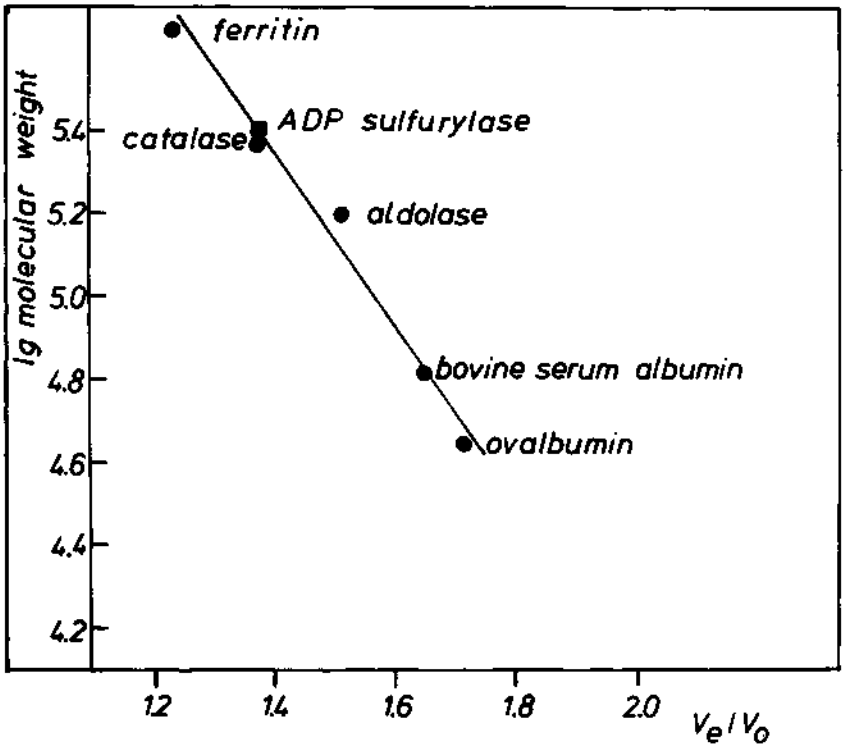

Fig. 9. Determination of the molecular weight of ADP sulfurylase.

Discussion

In agreement with Thiele (19) sulfate release in *Thiocapsa roseopersicina* strain 6311 is catalysed by ADP sulfurylase. In addition an ATP sulfurylase was also found in crude extracts of *T. roseopersicina*. Considering the fact that *T. roseopersicina* strain 6311 is able to grow with sulfate as the sole source of sulfur (18), Thiele (19) assumed as early as 1968 that in this organism during assimilatory sulfate reduction, sulfate is activated to APS by ATP sulfurylase and then reduced to sulfite by APS reductase. In 1981 Krasilnikova (10) confirmed that *T. roseopersicina* strain BBS contains ATP sulfurylase. Besides its function in assimilatory sulfate activation several authors have suggested a role of ATP sulfurylase in the oxidative dissimilatory sulfur metabolism: In crude extracts of *Thiobacillus thioparus* (12), *Thiobacillus denitrificans* (1, 16) and *Chromatium warmingii* (23) high activities of ATP sulfurylase have been measured besides ADP sulfurylase. In *Chlorobium limicola* sulfate release by APS cleavage only occurs via ATP sulfurylase, as the organism lacks ADP sulfurylase (2).

ADP sulfurylase activity in *T. roseopersicina* changes substantially with the growth phase. These results are in agreement with data from *Thiobacillus denitrificans* and *Chromatium warmingii* (23). On the other hand, ADP sulfurylase has been reported to be independent of the growth phase in *Thiobacillus denitrificans* (16) and *Thiobacillus ferrooxidans* (22).

ADP sulfurylase from *T. roseopersicina* strain 6311 showed a pH optimum very similar to those of other ADP sulfurylases. The enzyme activity was not very sensitive to pH in being active over a wide pH range.

With a molecular weight of 250,000 the enzyme molecule is bigger than ADP sulfurylase from baker's yeast (7), *Thiobacillus denitrificans* (16), *Chlorobium vibrioforme* (2) and several *Chromatium* species (4, 23) which show molecular weights in the range of 120,000 - 200,000.

The ADP sulfurylases of the above mentioned organisms (2, 4, 7, 16, 23) possess Km values of 10^{-4} M for APS and 10^{-3} M for phosphate. With 0.33 mM and 13 mM the respective Km values of ADP sulfurylase from *T. roseopersicina* are exactly in this range.

The purification factor of 18.8 for the partially purified ADP sulfurylase from *T. roseopersicina* is relatively low as compared with those obtained by other authors with other microorganisms. However Bias and Trüper (2) obtained a purification factor on only 15.8 for the *Chlorobium vibrioforme* enzyme. The comparably low enrichment factor is explained by the fact that normally enzymes of dissimilatory sulfur metabolism account for up to 2 or 3 percent of the total cellular soluble protein (21); this is substantiated for our enzyme by the rather high specific activity (0.345 U/mg protein) in the crude extract. In addition the lability of the enzyme is certainly responsible for the low yield during the purification procedure and probably has also contributed to a decrease in the enrichment factor.

Acknowledgments

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Minimun number of cells required for reconstitution of a foot-and-mouth disease virus-carrier cell culture

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Summary

A serial cell plating experiment has been designed to determine the minimun number of cells, isolated from a culture persistently infected with a virus, required to reconstruct the carrier state. For cell line C_1-BHK-Rc1, consisting of BHK-21 cells persistently infected with foot-and-mouth disease virus type C (isolate C-S8c1), more than 10^3 cells derived from one monolayer were needed to reinitiate a stable, FMDV-producing carrier culture. Thus, the FMDV-BHK-21 cell system cannot be explained merely as a sum of its individual components. The results suggest, instead, that the behaviour of C_1-BHK-Rc1 cultures is the result of complex interactions acting at the population level. Implications for viral persistence are discussed.

Key words: infection, persistence, BHK-21 cell, picornavirus.

Resumen

Se ha diseñado un experimento de sembrados sucesivos de células para determinar el mínimo número de células que, obtenidas a partir de un cultivo persistentemente infectado por un virus, se requieren para reconstruir el sistema portador. Para la línea celular C_1-BHK-Rc1, que consiste en células BHK-21 persistentemente infectadas con el virus de la fiebre aftosa (VFA) de tipo C (aislado C-S8c1), más de 10^3 células derivadas de una monocapa se necesitan para reiniciar un cultivo portador estable, productor de VFA. Por tanto, el comportamiento del sistema VFA-BHK-21 no se explica como mera adición de sus componentes individuales, sino que es resultado de interacciones complejas a nivel poblacional. Se discuten algunas implicaciones para la persistencia viral.

Introduction

Foot-and-mouth disease virus (FMDV) is a picornavirus that causes the most economically relevant disease of cattle, swine, and other artiodactyls (16). The virus can either cause an acute disease

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or establish an inapparent, persistent infection in ruminants (3, 19-21). Carrier animals represent an important problem for disease control since they constitute a reservoir of virus (2, 20) and promote its rapid genetic and antigenic variation (12). To facilitate the study of FMDV persistence we established BHK-21 cell lines persistently infected with FMDV (4, 6). One line, termed C_1-BHK-Rc1, was initiated by propagation of cloned BHK-21 c1 cells that survived a cytolitic inyection with plaque-purified FMDV C-S8c1 (4). Upon serial passage of C_1-BHK-Rc1 cells, two stages were distinguished: during the initial 60 to 120 passages, infectious FMDW was always present in the culture medium (4, 6). At later passages, no FMDV was detected, but viral RNA and antigens were found in the cells (4). The study of the carrier cultures at the FMDV-producing stage revealed several unexpected features. First, a coevolution of the BHK-21 cells and of the resident FMDV occurred that rendered the cells constitutively resistant to the virus and the latter, in turn, hypervirulent for the parental BHK-21c1 cells (6). A similar phenomenon has been described for reovirus (1) and the lymphotropic minute virus of mice (17). Second, analysis of 248 cells clones derived from C_1-BHK-Rc1 revelaled an extensive phenotypic heterogeneity as regards cell morphology, growth characteristics and resistance to FMDV (7). Several independent procedures suggested that the altered phenotypes were independent of the presence of FMDV, but were the result of stable heritable cellular modifications (5-7). Third, a clonal analysis of C_1-BHK-Rc1 at passage 19 showed thar only about 4 % of cells yielded infectious FMDV upon cloning, and these cells never developed into stable clones (7). Along with the cellular modifications. FMDW from the carrier cultures underwent continuous genetic variation (4, 6) and the viral population was genetically heterogeneous (7). Thus, C_1-BHK-Rc1 cultures may be described as an interaction between a quiasispecies distribution of viral genomes (9-11) and multiple cell variants (7). We have argued thar such dual heterogeneity endows the system with high biological flexibility and facilitates long-term persistence (7). The question then arises of how many different cells without added extracellular virus are needed to sustain a carrier culture producing infectious FMDV. In the present study we report a reconstruction experiment designed to determine the minimun number of cells, from a C_1-BHK-Rc1 cell monolayer needed to reinitiate a culture, that stably produces FMDV. The result indicates that a large number of cells, estimated al 10^3 to 10^4 are required for reconstitution of the carrier state. Implications of this observation for long-term viral persistence are discussed.

Materials and methods

Persistently infected cells

The origin of the persistently infected C_1-BHK-Rc1 cells and the procedures used for their culture have been described (4). A suffix indicates the passage number; for example, C_1-BHK-Rc1p20 is the FMDV-carrier BHK-21 cell culture, 20 passages after its establisment (4).

Reconstruction experiment

To reconstruct a FMDV-carrier culture, a C_1-BHK-Rc1p20 cell monolayer was washed with phosphate-buffered saline (PBS), treated with 0.06 % trypsin, 0.01 % EDTA (2 min. room temperature), and the detached cells were resuspended in 1 to 2 ml of Dulbecco modified Eagle's medium (DMEM) containing 5 % fetal calf serum (FCS). The cells were collected by centrifugation (2000xg, 2 min), resuspended in 0.2 M phosphate pH 6.0 to inactivate extracellular virions, centrifuged, and resuspended in 1 to 2 ml of DMEM-5 % FCS. They were counted in the haemocytometer, diluted in DMEM-5 % FCS and dispensed either in 2 cm^2-or 0.3 cm^2-multiwell plates to yield the desired average number of cells per well. The cells were cultured at 37° C in 5 % CO_2.

Detection of FMDV

Infections of BHK-21 cell monolayers in liquid culture and plaque-assays with FMDV were as described (8, 18). To test for FMDV production in reconstructed cell mixtures growing in microtiter plates, 100 μl aliquots of the culture medium from each well were transferred to 96 well microtiter plates containing preformed BHK-21 cell monolayers. Cytopathic effect (c. p. e) was monitored by visual inspection and staining with crystal violet, as described (4).

Results

Detection of FMDV in the culture medium of plated cells

A confluent C_1-BHK-Rclp20 cell monolayer (with about 10^6 cells, on a 50 mm petri dish) was producing infectious FMDV detected in the culture medium at titers of about 5×10^6 plaque-forming-units (pfu)/ml. To estimate the number of cells that were FMDV-producers, the monolayer was treated with trypsin, washed, and dispensed to multiwell plates as indicated in Materials and Methods. The results show that 20 h after plating, at least 10^2 cells were required to yield detectable FMDV (Table 1). Even with an average of 10^3 cells per well, FMDV was found in only 12 % of the wells. Plating of 10^4 cells increased this value to 75 %. To test if the FMDV-positive cultures maintained the ability to produce virus in a stable fashion, we carried out a serial cell plating experiment.

TABLE 1
DETECTION OF FMDV AFTER PLATING CELLS FROM CULTURE C_1-BHK-Rc1 p20

Cells/well	Total wells	FMDV-positive wells*
1×10^4	12	9
1×10^3	24	3
5×10^2	24	3
1×10^2	45	2
55×10^1	45	0
1×10^1	45	0
5	45	0

* The presence of FMDV in the culture medium was assayed 20 h after cell plating, using the procedure described in Materials and Methods.

About 10^4 cells are required for stable FMDV production

In this experiment, cells from series of 12 or 24 wells were passaged as shown in Table 2 A.In the series 1, 2 or 3, the average cell number plated in each passage was 1×10^4, 1×10^3 or 1×10^2 cells per well, respectively (rows 1 and 3 in Table 2 A). The cells were allowed to grow to confluence, and to reach the cell numbers given in rows 2 and 4 (Table 2 A).

Analysis of infectious FMDV in the culture medium was performed when cultures had reached confluence, and after one or several cycles of plating. The results, indicated in Table 2 B, show that while initally 33 % and 8 % of cultures with 1×10^3 and 1×10^2 cells, respectively, yielded infec-

tious FMDV, the system tended towards no viral production (Table 2 B, series 2 and 3). In contrast, a minimun cell number of about 10^4 ensured continued FMDV production (Table 2 B, series 1).

We conclude that the FMDV-carrier BHK-21 cell culture (4, 6) cannot be reconstructed with a limited number of cells, even when infectious FMDV is present initially. The system tends to become free of virus unless sufficiently large numbers of cells estimated in about 10^4 are co-cultured.

TABLE 2
SERIAL CELL PLATING EXPERIMENT

A. SCHEME OF THE TWO INITIAL PLATINGS WITH INDICATION OF NUMBER OF CELLS PER WELL (C/W)*

Platings and growth	Series 1	Series 2	Series 3
1. Initial Plating	1×10^4 c/w (12)	1×10^3 c/w (12)	1×10^2 c/w (24)
	↓	↓	↓
2. Growth to confluency	2×10^5 c/w (12)	1×10^4 c/w (12)	1×10^4 c/w (24)
	↓	↓	↓
3. Second Plating	1×10^4 c/w (12)	1×10^3 c/w (12)	1×10^2 c/w (24)
	↓	↓	↓
4. Growth to confluency	2×10^5 c/w (12)	1×10^4 c/w (12)	1×10^4 c/w (24)

B. ANALYSIS OF INFECTIOUS FMDV

Cell plating and growth cycles	Number of FMD-positive wells**		
	Series 1	Series 2	Series 3
1 → 4	12/12	4/12	1/12
1 → 4 Then 1	12/12	2/12	0/12
1 → 4 Twice	11/12	3/12	0/12
1 → 4 Three times	12/12	1/12	0/12

* The number of wells is given in parenthesis.

** The cell platings and growth cycles 1 → 4 correspond to steps 1 to 4 performed as indicated in part A of the table. Experiments summarized in the three columns involve successive platings of 1×10^4, 1×10^3 and 1×10^2 c/w, respectively, as indicated by their alignment with the corresponding columns (series 1, 2 and 3) in part A. The number of FMDV-positive cultures versus number of cultures (wells) analysed is indicated.

Discussion

The events that permit long-term persistence of viruses in cell culture are poorly understood (reviews in refs. 14, 22). The observation that FMD-carrier BHK-21 cultures evolve rapidly to produce a remarkable cell heterogeneity (6) in spite of having been initiated by a cloned BHK-21 cell population (4, 6) suggests that cell diversity could be an important element for long-term virus and cell survival (6). This proposal is reinforced by the fact that cell heterogeneity was noted as early as 17 passages after the establishment of persistence (7). The experiments reported here, while not providing that heterogeneity *per se* is a requirement for maintenance of the carrier state, strongly suggest that many phenotypically different cells must coexist in a culture to ensure long-term FMDV production. In spite of many attempts, we have been unable to isolate single cells that, upon cell division, could

maintain a FMDV-carrier state (7, and unpublished results). This is in contrast with the findings with other viral systems, such as human WISH cells persistently infected with the picornavirus echovirus 6 (13), in which viable, virus-producing cells could be isolated. Thus, the behaviour of C_1-BHK-Rc1 cultures is not embodied by individual cells, but it is only explained as the result of interactions among phenotypically different cells and viral particles. It is not clear at present whether cells may divide while carrying replicating FMDV [an event expected from steady-state persistent infections (15)] or if all infected cells lyse [as expected from a carrier cell system (15)]. Some previous observations with C_1-BHK-Rc1 cultures [(i) presence of viral antigens in most cells, (ii) 5 % to 70 % infected cells in C_1-BHK-Rc1p20, as measured in an infectious center assay on BHK-21 monolayers, (iii) undetectable interferon in the culture supernatants (6), and (iv) inability of the cells to be cured of FMDV by prolonged treatment with antibodies (4)] suggest the former type of mechanism. However, the difficulty to derive persistently infected culture from individual cells (7 and unpublished results) suggests a true carrier state. One interesting, as yet unproven, possibility is that although in a complete C_1-BHK-Rc1 culture all (or most) cells can duplicate while supporting FMDV replication (except for occasional cell lysis), isolation of an individual cell, away from the interaction with neighbouring cells, promotes some disequilibrium towards cell lysis. Experiments are now in progress to test this point.

The finding that a minimun of 10^3 to 10^4 individual cells are required to reconstruct a FMDV-producing culture explains that, occasionally, upon passaging of C_1-BHK-Rc1 spontaneous curing of FMDV may occur. In fact parallel serial passage of two sublines from C_1-BHK-Rc1 cells led to curing of one subline at passage 60, and to a continued viral production for more than 100 passages in the other subline (J. Diez and J. C. de la Torre, unpublished results). We now interpret such observations as meaning that on ocassion cell passage did not involve a cell number above the threshold value needed to ensure continued viral production. The results reported here open the possibility to explore if, in spite of not producing infectious FMDV, the negative cultures support a transient expression of viral antigens or harbor some defective RNA, as first observed in C_1-BHK-Rc1 cultures at their non-infectious FMDV-producing stage (4). Also, the serial plating experiment designed here will permit reconstructions with cells with particular phenotypes, such as the highly FMDV-resisant R-cells that are present in C_1-BHK-Rc1 cultures (Table 1 in ref. 7). Perhaps the stability of a carrier state can be modulated by the cellular composition of the cultures thus contributing to the frequently observed restriction of viruses to certain tissues and organs *in vivo*.

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Transfection of MDCK cells with influenza virus ribonucleoprotein complexes

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Summary

Influenza virus ribonucleoprotein complexes isolated from MDCK-infected cells have been used to optimize trensfection conditions of MDCK cells. Ribonucleoprotein complex-mediated infection was strictly dependent on pretreatment of the cell cultures, resistant to mild NP40 treatment and sensitive to RNase treatment. Under optimal conditions, up to 10^4 plaque forming units per μg of viral RNA could be obtained.

Key words: RNA virus, gene transfer, infectivity.

Resumen

Se han usado complejos de ribonucleoproteína obtenidos de células MDCK infectadas por virus de la gripe para buscar las condiciones experimentales idóneas para la transfección de células MDCK. La transfección fue dependiente del adecuado tratamiento previo de los cultivos celulares y resultó sensible a RNasa y resistente al tratamiento suave con NP40. Bajo condiciones óptimas, se obtuvieron hasta 10^4 unidades formadoras de placa por μg de RNA viral.

Introduction

In the last decades, methods have been developed for DNA-mediated gene transfer to somatic cells in culture, including adsorption to DEAE-dextran (7), co-precipitation with calcium phospahte (5), fusion to bacterial protoplasts (18) or liposomes (20), microinjection (4) or electroporation (15). These techniques have proven to be a very powerful tool to study gene expression and its regulation, among other biological phenomena.

In the case of viruses, the transfer of the complete genome to susceptible cells allows the production of progeny virus if the expression of the genome does not require the concomitant entry of any virus-specific protein. This is the case for SV40 (7) or Adenovirus (12), although in the latter, infectivity is greatly enhanced by the presence of the terminal protein (2). The RNAs of viruses like poliovirus or FMDV are also infectious (6, 23). In addition, cloned DNA copies of poliovirus RNA have been shown to be infectious (16, 19), allowing the use of *in vitro* recombination and site directed mutagenesis to study the virus infection cycle and the pathogenicity of the infection *in vivo*.

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Influenza A viruses are Orthomixoviruses containing eight segments of single-stranded RNA of negative polarity. Each segment encodes a single viral protein except the two smaller ones that contain the genetic information for two viral genes [for a review see (8)]. The infectivity of influenza virus RNA would then require the formation of a complex with the nucleoprotein and each of the polymerase proteins PB1, PB2 and PA (RNP-complex), as well as the presence of each of the viral segments. Therefore, efficient conditions for RNP-complex mediated infection would be of great help to test functional reconstitution of RNP-complexes with viral RNA syntehsized *in vitro* from cloned DNA copies or to assay functionality of the products of influenza virus *in vitro* replication systems.

Using RNP-complexes obtained from infected cells we describe conditions for transfection of MDCK cells that yield up to 10^4 pfu per μg of viral RNA.

Materials and Methods

Biological materials

The MDCK cell line was obtained from the American Type culture Collection and maintained by subculturing twice a week in Dulbecco's modified Eagle's medium supplemented with 5 % fetal bovine serum. A frozen stock was prepared at passage 53 and the cells were used for not more than 10 passages thereafter. Influenza virus strain A/Victoria/3/75 was obtained from Centro Nacional de Microbiología, Virología e Inmunología Sanitarias (Majadahonda, Madrid) and cloned by repeated plaque isolation.

Preparation of ribonucleoprotein complexes

The conditions for the isolation of RNP-complexes were as described (3). MDCK cells were infected at a multiplicity of infection of 5-10 pfu/cell, as described (13). At 6-8 hours postinfection, cells were washed with cold PBS, scrapped off the plates and collected by centrifugation for 5 min at 1000 g and 0° C

Cellular pellets were solubilized in 10 mM Tris-HCl-10 mM KCl-1.5 mM $MgCl_2$-5 mM 2 ME-0.5 % NP40 (HB-0.5 % NP40) (1.0 ml for 10^7 cells), for 15 min at 0° C, vortexed and centrifuged for 5 min at 1000 g. Nuclear pellets were washed with the same buffer (0.5 ml per 10^7 cells) and the supernatants were pooled. After centrifugation for 10 min at 16,000 g and 0° C, the microsomes of the cytoplasmic fraction were pelleted for 1 h at 100,000 g and 0° C, resuspended in HB containing 44 % glycerol and stored at –80° C. To estimate the amount of virus-specific RNA present in the microsomal pellets, total RNA was extracted as described by Chelley and Anderson (1) and applied to nylon filters (21). To prepare a specific probe, the internal EcoRI-Pst I fragment of cloned RNA segment 8 (14) was subcloned into pGEM4 vector (Promega Biotec) and a positive polarity riboprobe was synthesized as described (11). The dot-blots included known amounts of unlabelled riboprobe of negative polarity to be used as internal standards. Quantitations were done on microdensitometer readings of ten-fold dilutions of each sample or standard.

Transfection assay

The experiments leading to the optimized conditions described below are detailed under Results. Confluent monolayers of MDCK cells grown on 35 mm dishes were washed twice with PBS-G (100 μg/ml autoclaved gelatine in PBS) and incubated for 30 min at room temperature with 1 ml of a so-

lution containing 300 μg/ml DEAE-dextran (M. W. 5.10^5 Da) and 0.5 % DMSO in PBS-G. After removal of this solution, the RNP-complexes were diluted in 100 μl of PBS-G (usually 10^{-2} dilution factor) and allowed to adsorb to the cells for 1 h at room temperature. The cells were then washed twice with PBS-G and 2 ml of plaquing medium were added. Virus plaques were scored after incubation for three days at 37° C.

Infectivity assays

The conditions for plaque-assay were as described. For hemadsorption assay, infected or transfected cell cultures were washed with PBS and incubated with a suspension of freshly washed guinea pig erythrocytes (0.5 % in PBS), for 20 min at O° C. After washing with cold PBS, the rosettes were counted under a low-magnification microscope.

Results

Characterization of RNP-complex mediated infectivity

Different batches of RNP-complex prepared as described under Materials and Methods showed variation in their infectivity when directly determined by plaque-assay. Some had no detectable infectious virus while other had titers up to 4×10^5 pfu/ml. This variation did not correlate with virus multiplicity or with the amount of NP40 used to lyse the infected cells. In spite of this variation, when the transfection procedure was followed, an increased infectivity was always observed. To be certain that the observed infectious units were due to RNP-complexes and not to infectious virus, those preparations with any residual infectivity were further treated with NP40 as indicated in Table 1. While treatment with NP40 at increasing temperature abolished the infectivity as determined by

TABLE 1
TREATMENT OF RNP-COMPLEXES WITH NP40

Temperature (° C)	Transfection[a]				Infection[b]			
	Time (min)							
	0	10	30	60	0	10	30	60
0	> 600	52 ± 3[c]	32 ± 6	55 ± 22	> 400	4 ± 2	6 ± 1	16 ± 1
20	> 600	23 ± 9	12 ± 13	50 ± 6	> 400	0	0	0
37	> 600	17 ± 6	10 ± 4	8 ± 1	> 400	0	0	0

[a] One μl aliquots of RNA-complex preparation (equivalent to extract derived from 2.10^5 infected cells) were used to transfect MDCK cells as described under Materials and Methods after treatment for the indicated times and temperatures with 0.05 % NP40 in HB-44 % glycerol.

[b] One μl aliquots of the same RNP-complex preparation referred to above were used for direct plaque-assay after NP40 treatment.

[c] Number of plaques per μl. Average of 3 determinations.

directed plaque-assay (Table 1, treatment at 20° C), transfection-mediated infectivity was always retained, although at reduces levels. To further confirm that the infectious units detected were RNP-complex mediated, their sensitivity to RNAse was studied. As shown in Table 2, RNAse treatment of RNP-complexes abolished their ability to transfect MDCK cells. As expected, identical treatment

of infectious virus did not alter their infectivity. Therefore, RNP-complex mediated infection is characterized as NP40-resistant, RNase sensitive and strictly dependent on transfection conditions, as described below.

TABLE 2
TREATMENT OF RNP-COMPLEXES WITH RNAse

Assay	RNP-complexes		Virus	
	–RNAse	+RNAse[a]	–RNAse	+RNAse[a]
Plaque-assay	0	0	150 ± 20[b]	115 ± 12
Transfection	19 ± 2	0	95 ± 25	91 ± 2

[a] RNP complexes or infectious virus were treated for 30 min at room temperature with 25 μg of pancreatic RNAse.
[b] The numbers represent the average and standard deviation of 2 determinations.

Optimization of transfection conditions

Following the original report of Rochovansky and Hirst (17), the effect of treatment of the cells with DEAE-dextran was studied. As shown in Fig. 1, there is a dose response of the infectivity with

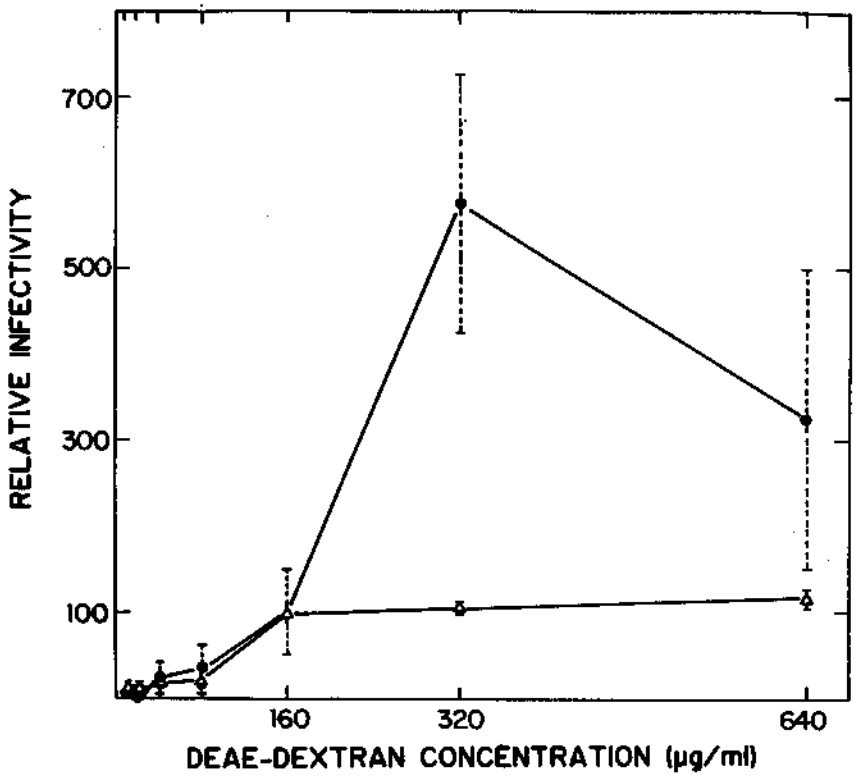

Fig. 1. Effect of DEAE-dextran concentration on RNP-mediated infectivity. Samples of RNP-complex were allowed to adsorb to MDCK monolayers treated with DEAE-dextran at the concentrations indicated and the infectivity revealed by plaqueassay, as described under Materials and Methods. Two independent experiments are shown and the results are normalized to the values obtained for the dose of 160 μg/ml [96 pfu for experiment 1 (Δ) and 12 pfu for experiment 2 (●)]. The values are the average and standard deviation of 2 and 4 determinations, respectively.

increasing concentrations of DEAE-dextran. The observed maxima corresponded to treatment with 300-600 μg/ml DEAE-dextran and a concentration of 300 μg/ml was chosen to avoid excess toxicity of the drug at higher concentrations. These experiments were performed with a DEAE-dextran of molecular weight 5.10^5 dalton which proved to be five times more efficient than that of 2.10^6 dalton (data not shown). A slight increase in infectivity was also observed by including 0.5 % DMSO in the treatment mixture (data not shown).

The kinetics of treatment was next studied and the results are shown in Fig. 2. A maximum in relative infectivity was obtained after aproximately 30 min and therefore this treatment time was chosen for further assays. When the kinetics of adsorption of RNP-complexes to treated cell cultures

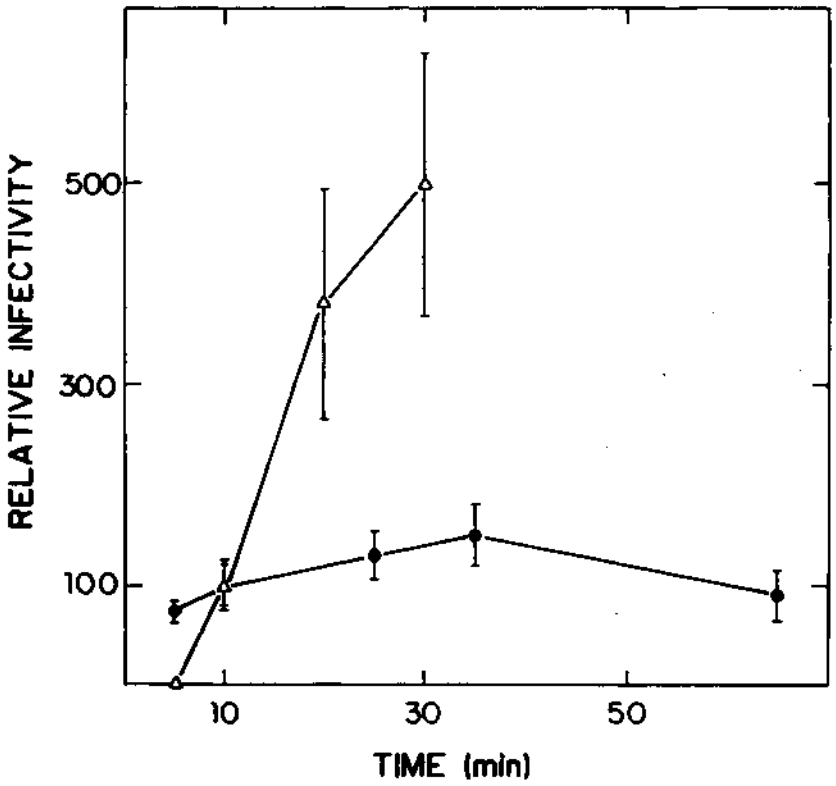

Fig. 2. Effect of treatment time on RNP-mediated infectivity. Monolayer cultures of MDCK cells were treated with 300 μg/ml DEAE-dextran and 0.5 % DMSO for the times indicated as described under Materials and Methods. Identical samples of an RNP-complex preparation were then allowed to adsorb and the cultures processed for plaque-assay. Two independent experiments are shown. The results are normalized to the values obtained for the 10 min time-point [6 pfu for experiment 1 (Δ) and 15 pfu for experiment 2 (●)]. The values are the average and standard deviation of 4 and 3 determinations, respectively.

was assayed, the results presented in Fig. 3 were obtained. Since an optimun was observed for 60 min, this adsorption time was selected.

Every optima for the parameters studied were checked under optimal conditions for the rest of them.

To estimate the specific infectivity mediated by RNP-complexes, total infectivity was determined under optimal conditions and compared to total viral specific RNA found in the RNP-

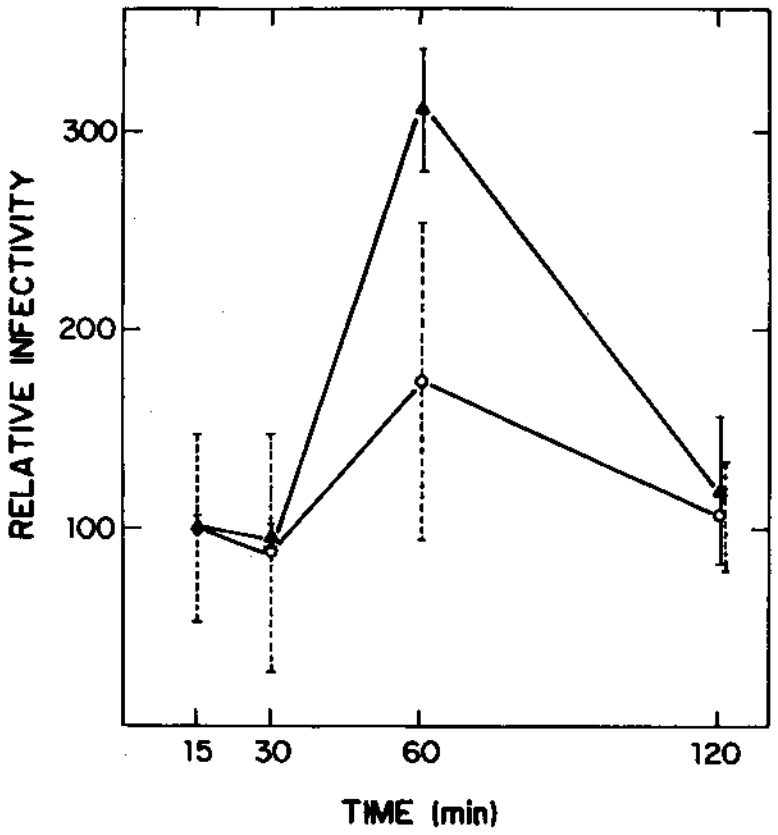

Fig. 3. Kinetics of RNA-complex adsorption. MDCK cell cultures were treated with DEAE-dextran and DMSO as described under Materials and Methods. Identical samples of an RNP-complex preparation were allowed to adsorb for the times indicated and their infectivity determined by plaque-assay. Two independent experiment are shown. The results are normalized to the values obtained for the 30 min time-point [2 pfu for experiment 1 (○) and 13 pfu for experiment 2 (▲)]. The values are the average and standard deviation of 2 determinations.

complexes. The values obtained ranged between 2.10^2 and 2.10^4 pfu/μg, depending on the RNP-complex preparation. Attempts to increase further the specific infectivity by either DMSO or glycerol shock (9) or by chloroquine treatment (10) were unsuccesful.

RNP-complex concentration dependence of infectivity

The RNP-complex dose-response curve of infectivity may lead to valuable information regarding the number of separate physical particles that should interact with a cell to yield an infectious unit. The results of such an experiment are presented in Fig. 4, when the infectious units are quantitated by either plaque-assay or hemadsorption. In neither case is an exponential curve obtained. Rather, a linear relationship is found, although a saturation effect is apparent when the plaque-assay technique is used. These results could indicate that the interaction of a single particle is sufficient to infect a MDCK recipient cell.

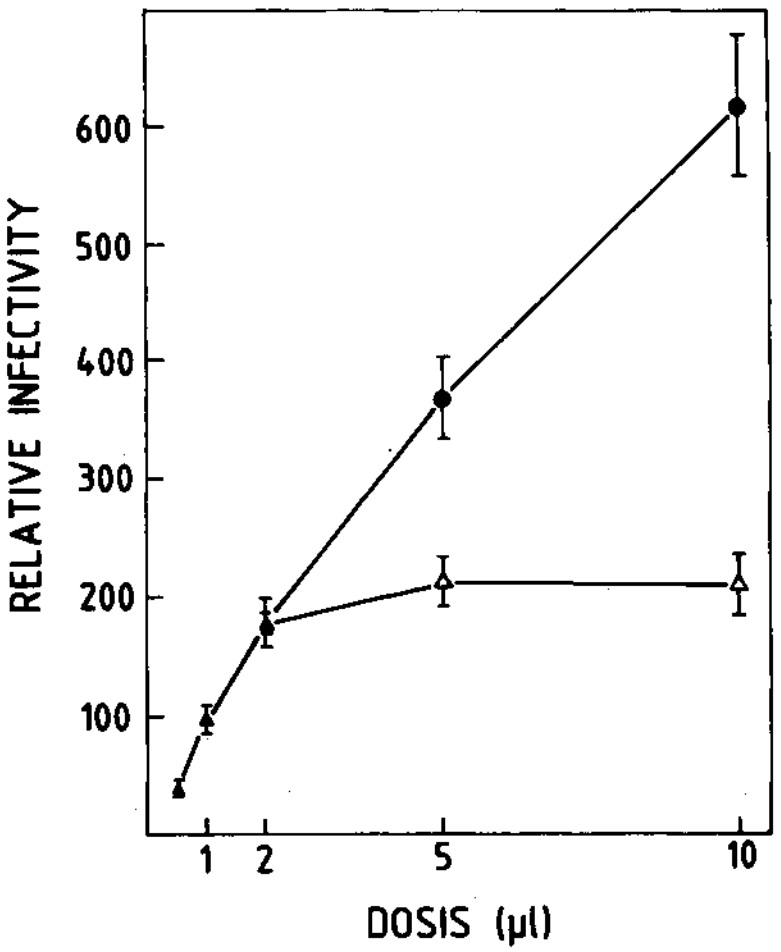

Fig. 4. Dose-effect on infectivity. Increasing doses of RNP-complex were used to transfect MDCK cell cultures as described under Materials and Methods. In experiment 1 (Δ), the infectivity was determined by plaque assay, while in experiment 2 (●) it was determined by hemadsorption. The results are normalized to the values obtained for the 1 µl dose (203 pfu for experiment 1 and 1066 rosettes for experiment 2). The values are average and standard deviations of 3 and 2 determinations, respectively.

Discussion

In order to interpret correctly the results obtained in a RNP-complex mediated transfection it is vital to exclude that the infectious units observed may be due to infectious virus. The nature of the residual infectivity detected in some RNP-complex preparation is not clear, but its removal by NP40 treatment (Table 1) suggests it could be due to some persistent inoculum virus or, most probably, subviral particles in the final stages of morphogenesis. This possibility is further suggested by its partial sensitivity to RNAses (data not shown). In any cases, true RNP-complex mediated infection (i.e. infection not receptor-mediated but dependent on pretreatment of the cells with DEAE-dextran) was shown to be RNAse-sensitive, in distinction to virus mediated infection (Table 2). Furthermore, a negative control of plain plaque-assay of RNP-complex was included in every experiment to be certain that the infectivity observed was not receptor-mediated.

The results of dose-response of RNP-complex on infectivity suggest that a single particle is ca-

pable of initiating the infection. It could then be concluded that the RNP-complexes used in transfections contain all viral RNA segments. Whether these complexes are the result of an artifactual aggregation in the preparation procedure or reflect an *in vivo* relevant structure is at present unknown.

The RNA of some positive-strand RNA viruses has been synthesized in vitro from full-length DNA clones using bacteriophage promoters and shown to be infectious upon transfection (24, 25). This possibility opens the way to incorporate into infectious virus directed mutations produced *in vitro* in cloned DNA versions. In the case of influenza virus this reverse genetics approach is hampered by the negative polarity of the genomic RNA and its segmented nature. The results presented in this report fulfill the requirement for an efficient transfer of RNP-complexes to the susceptible cell, although the functional reconstitution of the complexes from RNA transcribed from cloned DNA still remains unsolved.

Acknoledgments

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MICROBIOLOGIA SEM 4 (1988), 177 - 179 **NOTES**

Evaluation of four methods for the detection of streptococcal group A antigen directly from throat swabs

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Summary

We have compared the sensitivity, specificity and reproducibility of four rapid tests for the detection of group A beta-hemolytic streptococci antigen directly from a throat swab. The four methods were very specific, all of them offered reproductibility and surpassed conventional culture in speed and simplicity.

Key words: Rapid detection group A Streptococci.

Resumen

Se ha comparado la sensibilidad, especificidad y reproductividad de cuatro técnicas rápidas para la detección del antígeno del estreptococo ß-hemolítico grupo A directamente del frotis faríngeo. Los cuatro métodos han resultado ser muy específicos, todos ofrecen reproductibilidad y tienen como ventajas principales en relación con el cultivo convencional su rapidez y simplicidad.

For many years, the diagnosis of streptococcal pharyngitis was made by standard culture techniques; unfortunately conventional culture and identification of the organism requires 24 to 48 hours. Early identification of group A Streptococci in throat swabs is essential for selecting patients with pharyngitis to receive penicillin, in order to prevent suppurative complications and acute rheumatic fever.

Rapid tests have been recently introduced that detect the group A streptococcal antigen on throat swabs specimens (1, 2, 3).

The purpose of this study was to compare the sensitivity, specificity and reproducibility of four rapid tests for the detection of group A beta-hemolytic streptococci antigen directly from a throat swab, under controlled conditions.

The four rapid methods evaluated were:

1. Directigen Rapid Group A Strep test (Becton Dickinson) is a latex card agglutination test for the qualitative detection of group A streptococcal antigen directly from throat swabs (4, 5); the specific antigen is obtained by chemical extraction from throat swabs specimens containing Group A *Streptococcus*.

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The Directigen Rapid Group A Strep test can be completed in approximately seven minutes after collection of the specimen.

2. Direct Antigen Identification Strep A test (Difco), «DAI Strep A test», is a method designed to detect group A streptococcal antigen directly from throat swabs by using an enzymatic extraction followed by latex agglutination (8). This procedure may take 45 min to perform.
3. Test Pack Strep A (Abbott) is an enzyme immunoassay with chemical extraction procedure for the rapid detection and confirmation of group A streptococci from throat specimens (6); this test requires only seven minutes of processing.
4. Phadirect Strep A (Pharmacia Diagnostics) is based on chemical extraction procedure and the coagglutination technique which allows rapid identification of Group A *Streptococcus* using a simple slide technique (7). The test takes five min to perform.

The study was performed as follows: Four or five colonies of Group A streptococci were suspended in sterile physiologic saline and adjusted to the turbidity of the 0.5 McFarland standard (10^8 CFU/ml) and were then further diluted 1/10, 1/20, 1/30, 1/40, 1/50, 1/60, 1/70, 1/80, 1/90, 1/100, 1/200, 1/300. Swabs (made of dacron) were then placed in each dilution of the bacterium during five seconds and processed by each of the four procedures in a manner similar to the processing of the clinical throat swabs; this was repeated three times.

Additionally the four methods were performed on the swabs suspended in approximately 10^7 to 10^8 CFU/ml of the following representative species of normal oropharyngeal flora:

Streptococcus agalactiae, Streptococcus pneumoniae, Group D *Streptococcus, Streptococcus viridans* group, *Staphylococcus aureus* and *Branhamella catharrhalis.*

The Fig. 1 shows the sensitivities of the four methods.

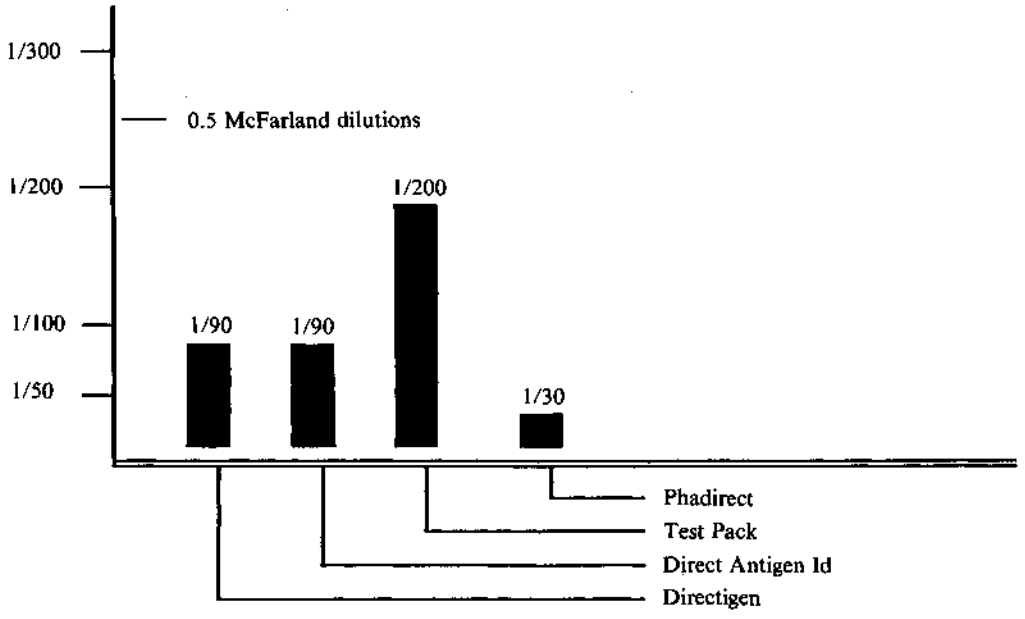

Fig. 1. Sensitivity of the four tests.

In this comparative study the sensitivity of the two latex agglutination tests (Phadirect and Direct Antigen Identification) was similar, both were positive at 1/90 dilution, while the coagglutination technique (Phadebact) only detected until 1/30 dilution, and the enzyme immunoassay test (Test Pack) achieved the highest sensitivity (positive at 1/200 dilution).

The four antigen detection systems for Group A streptococci were very specific, none of the other organisms tested were positive. All of them offered reproducibility and surpassed conventional culture in speed and simplicity.

Detection of Group A streptococci by culture requires 24 to 48 h. The advantages of the rapid group A beta-hemolytic streptococci antigen detection tests are the speed, simplicity and specificity (1, 2, 3). The rapid results allow the physician to treat promptly and appropiately, thereby preventing any sequelae due to group A beta-hemolytic streptococcal infection, and also preventing the overuse of antimicrobial agents (5); besides the early diagnosis may limit the risk of cross infection both in the family and in hospital.

The results of this study suggest that the four methods we evaluated are easy to perform and suitable for use in a clinical laboratory.

Under the same conditions, the Test-Pack system shows the highest sensitivity. This method is rapid (may take only seven minutes to perform), simple to use, very easy for interpretation and therefore attractive both for a microbiology laboratory and a physician's office.

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NOTES

Characterization of an *Aeromonas hydrophila* strain isolated on a septicemic out-break in a fish-farm of Spain

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Summary

An *Aeromonas hydrophila* strain (AH-3) was isolated from a septicemic out-break on a gold-fish farm near Barcelona (Spain). On the bases of their virulence and surface characteristics was classified as moderate to weakly virulent.

Key words: Aeromonas hydrophila, *virulence, lipopolysaccharide.*

Resumen

La cepa de *Aeromonas hydrophila* AH-3 fue aislada de un brote septicémico en una psicofactoría cerca de Barcelona dedicada al cultivo de carpa dorada (*Carassius auratus).* En base a su virulencia y características superficiales se ha clasificado este aislado como de virulencia intermedia.

Aeromonas hydrophila is an opportunistic as well as primary pathogen of a variety of aquatic and terrestrial animals including humans (6). On the bases of their virulence, *A. hidrophila* strains had been classified in three different groups: the first one with strains like TF7, highly virulent in mice (7) or fish (10) that showed a lipopolysaccharide (LPS) with O-polysaccharide chains of homologous length with an average of 10 repeat units (4); the second one with strains like Ba5, moderate to weakly virulent in mice and fish (7, 10) that showed a LPS with O-polysaccharide chains of highly heterologous length (4); and the third one with strains like ATCC7966 no virulent in mice or fish (7, 10) that showed very heterogeneous forms of LPS.

Strain AH-3 was isolated from kidney, skin and blood of ill gold-fish from a fish-farm near Barcelona during an out-break in 1986. Strain AH-3 was the ethiological agent of the gold-fish illness because when we reinoculated healthy fish with this strain identical illness was produced, and we recovered the same strain from kidney, skin and blood of ill or recently dead gold-fish. We also used a spontaneous Sm^r mutant of strain AH-3 and we recovered this strain using streptomycin as the selective agent. The strain AH-3 was classified as *Aeromonas hydrophila* according to the Bergey's Manual os Systematic Bacteriology (IX ed., vol. 1).

A. hydrophila strains were usually cultured on triptic soy broth (TSB). TSA and TSA soft-agar were obtained by adding 1.5 % and 0.6 % agar to TSB, respectively. Bacteriophages 2, 4, 11, 14, 18,

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22, 24, 45, 55, 60, 66, 69, 77, 145 and 157 from *A. hydrophila* had been previously described (15). *A. hydrophila* strains AH-3 and Ba5 are sensitive to bacteriophages 18, 24, 69 and 145; and both strains are resistant to bacteriophages 2, 4, 11, 14, 22, 55, 60, 66, 77 and 157 when assayed by spot test. *A. hydrophila* strain TF7 was sensitive to bacteriophage 55 and resistant to all the other ones. Also, toxin activities recovered in the cell-free supenatant of *A. hydrophila* strain AH-3 as well as other *A. hydrophila* strains (TF7 and Ba5) are shown in Table 1. The supernatant produced haemolysin, dermonecrotic facto and a small amount of enterotoxin.

TABLE 1
TOXIC ACTIVITIES FOUND IN THE CELL-FREE SUPERNATANT OF SOME *A. HYDROPHILA* STRAINS

	N.° of units[a]		
Strains	Haemolysin[b]	Enterotoxin[c]	Dermonecrotic factor[d]
AH-3	160	8	100
Ba5	160	8	100
TF7	8	1	100

a Last dilution of the cell-free supernatant showing positive activity.
b Haemolysin was determined using sheep red blood cells (11).
c Enterotoxin was determined by the suckling mouse test (3).
d Dermonecrotic factor was determined by the rabbit skin test (12).

We studied the average lethal dose (LD_{50}) for some *A. hydrophila* strains in fish and mice. In fish we used rainbow-trout (12-18 g) and gold-fish (10-15 g) in 20 l static tanks at 17° C and 20° C, respectively; and also in sea-bass (*Dicentrarchus labrax)* mantained in 20 l static tanks at 18° C on salty water. Fish (4 per test sample dilution) were injected intraperitoneally with 0.1 ml of the test samples. Mortality was recorded up to two weeks; all the mortalities occurred within 2-5 days. In mice (6 to 10 weeks old albino female mice, 4 per test sample dilution) we injected intraperitoneally 0.1 ml of the test samples. Mortality was recorded up to 7 days; all the mortalities occurred within 1-3 days. The LD_{50} was calculated by the method of Reed and Munchen (13). Strain AH-3 showed a LD_{50} of $10^{5.5}$ in rainbow-trout, gold-fish or sea-bass; and a LD_{50} of 10^{7} in mice. Both LD_{50} allow to consider the AH-3 strain as moderate to weakly virulent (7, 10).

Because of the similarity between strain AH-3 and Ba5 in bacteriophage sensitivity, toxin production and virulence assayed by the LD_{50} we decided to investigate the surface characteristies of these *A. hydrophila* strains. Cell envelopes were prepared by French pressure cell lysis at 16,000 Pa of whole cells or were disrupted by ultrasonication (4 cycles of 1 min at 90 watt and 4° C), followed by the removal of unbroken cells at 10,000 x *g* for 10 min and by sedimentation of the membrane fraction at 100,000 x *g*, as previously described (14). Cytoplasmatic membranes were solubilized twice with sodium-lauryl-sarcosinate (SLS), and the outer-membrane (OM) fraction was sedimented twice at 100,000 x *g* for 2 h. OM proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by a modification (1) of the Laemmli procedure (8). Protein gels were routinely stained with Coomassie blue. Protein concentrations were determined by the Lowry procedure (9) using bovine serum albumin as the standard. LPS was purified by the method of Westphal and Jann (17). Purified LPS was analyzed by SDS-PAGE and silver stained bt the method of Tsai and Frasch (16).

Fig. 1 shows the OM protein and the LPS profiles of *A. hydrophila* strains AH-3, Ba5, TF7 and ATCC7966. It may be deduced the great similarity between AH-3 and Ba5 strains on the bases of

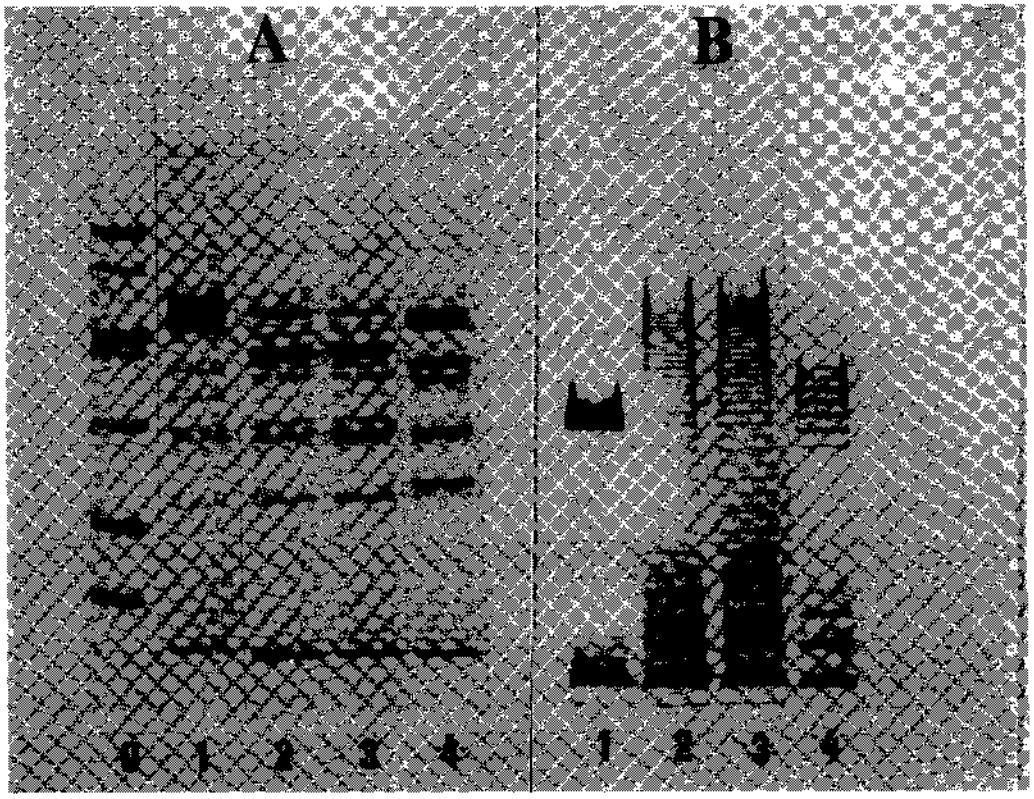

Fig. 1. SDS-PAGE of OM proteins (A) and LPS (B) from *A. hydrophila* strains. OM proteins were obtained as sodium lauryl sarcosinate-insoluble material (5). Purified LPS was assayed by the method of Tsai and Frasch (16). A) Lanes: 0, molecular size standards (14.0, 20.1, 30.0, 43.0, 67.0 and 94.0 Kilodaltons) from Pharmacia Fine Chemicals; 1, strain TF7; 2, strain Ba5; 3, strain AH-3, and 4, strain ATCC7966. B) Lanes: 1, strain TF7; 2, strain Ba5; 3, strain AH-3, and 4, strain ATCC7966.

the both characteristics, besides the heterogenicity on the OM protein profile among *A. hydrophila* strains previously described (2).

The similarity between strains AH-3 and Ba5 was clearly demonstrated by immunological methods. We obtained anti-LPS serum from adult New Zealand white rabbits previously injected with 50 μg of purified LPS from strain AH-3 in Freund complete adjuvant, followed by two succesive injections at 2-week intervals. After 2 weeks, the animals were bled, and serum was collected. We assayed the antibody levels of this immune rabbit serum with a solid-phase enzyme-linked immunosorbent assay (ELISA) using 1.0 μg of purified LPS or 10^5 cells as antigen. Table 2 shows the OD_{405} of an ELISA using antiserum against purified LPS from strain AH-3 and using as antigens purified LPS or 10^5 cells of both *A. Hydrophila* strains. Antibodies developed against purified LPS from strain AH-3 were able to cross-react with purified LPS or 10^5 cells of strain Ba5 with similar values as for strain AH-3.

The similarity between strains AH-3 and Ba5 was clearly demostrated by their phage-sensitivity pattern, toxin production, their virulence and their cross-reactivity by immunological methods. No similarity in phage-sensitivity pattern, toxin production, virulence or immunological cross-reactivity was observed between strain AH-3 and strain TF7. The virulence of strain AH-3 seems to be independent of the type of fish (rainbow-trout, gold-fish or sea-bass). For this reason we want to point out the economical importance of this *A. hydrophila* group of strains belonging to the moderate to

TABLE 2
ELISA USING DIFFERENT ANTIGENS AND ANTI-LPS SERUM[a] OBTAINED USING PURIFIED LPS OF STRAIN AH-3 AS ANTIGEN

Antigen	OD_{405}
LPS of *A. hydrophila* AH-3 (0.5 μg)	1.275 ± 0.07
LPS of *A. hydrophila Ba5* (0.5 μg)	1.268 ± 0.08
10^5 cells of *A. hydrophila* AH-3	0.635 ± 0.05
10^5 cells of *A. hydrophila* Ba5	0.620 ± 0.05

[a] *The anti-LPS serum was used at 10^{-4} dilution.*

weakly virulence categorie. Work is in progress in order to study the pathogenic mechanism of these *A. hydrophila* strains.

Aknowledgments

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MICROBIOLOGIA SEM 4 (1988), 185 - 189 **NOTES**

The annual cycle of zooplankton-associated *Vibrio cholerae* and related vibrios in Albufera lake and its coastal surrounding waters (Valencia, Spain)

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Summary

Most probable numbers of zooplankton-associated *Vibrio* spp. and *V. cholerae* were determined in Albufera lake, Spain, and in its coastal receiving waters throughout a year. Highest counts of 10^5 bacterial cells/g of plankton were associated to high temperatures and were also related to the kind of water. All isolates were non-01 serovars, and most belonged to Heiberg groups I and II.

Key words: Vibrio *spp.*, Vibrio cholerae, *zooplankton, waters.*

Resumen

Durante un ciclo anual, en el lago de la Albufera y aguas costeras sometidas a su influencia, se han determinado los niveles de *Vibrio* spp. y *Vibrio cholerae* asociados a zooplancton. Los recuentos máximos han alcanzado 10^5 bacterias por gramo de zooplancton durante la época cálida. La temperatura y las características del agua han influido significativamente sobre el número de vibrios aislados. Todas las cepas han correspondido a la serovariedad no 01, quedando la mayoría incluidas en los grupos I y II de Heiberg.

V. cholerae is presently considered a natural member of brakish and estuarine waters (4, 8, 13, 16, 19), and has been detected in absence of cholera outbreaks in many parts of the world (2, 3, 4, 5, 6, 8, 13, 16, 19). Its association with chitinous members of the zooplankton communities, especially copepods, has also been reported (9, 10, 11, 14, 20), and might contribute to the survival or dispersion of this organism in the aquatic environment.

In certain geographical areas, periodical outbreaks of cholera occur after an increase of the zooplankton populations (11). The region studied by us, Albufera lake and its surroundings, south of Valencia, corresponds to the area where the last two cholera invasions of the nineteen century started. Interestingly, they began always in spring or early summer, and reached their maximun in September (1). Albufera lake is a hypereutrophic shallow coastal lagoon with very high levels of phyto- and zooplankton. The latter is represented mainly by the copepod *Acantocyclops vernalis*, and the rotifers *Brachionus angularis, B. leydigi, Polyarthra platyptera* and *Synchaeta tremula* (18). Zoo-

(*) Corresponding author.

plankton densities reach their maximum spring or early summer, simultaneous to the occurrence of the highest annual chlorophyl concentrations, and both decrease during the autumn, with annual minima in November (18). The lake is connected with the sea through three channels provided with gates used to regulate the water level in the lake for fishing and agricultural purposes.

In the twentieth century, and during the seventh pandemic, several other sporadic cholera outbreaks, including a case of septicaemia by a non-01 strain, were reported in different regions of Spain (1, 7, 15). More recently, in summer 1984 and in autumn 1987, a few cases of confirmed cholera have been detected in Barcelona and Valencia respectively, probably caused by water-contaminated food. In previous studies, high numbers of *Vibrio* spp. and *V. cholerae* have been recovered from the lake and from coastal waters influenced by the lake discharges (6). Since the presence and annual fluctuations of this species, both free-living and attached to higher organisms, represent a vital information to develop an understanding of the epidemiology of cholera, we have conducted a study on zooplankton-associated *V. cholerae* and related vibrios in this same environment throughout an annual cycle.

TABLE 1
CHARACTERISTICS OF THE WATERS STUDIED

	Sampling event									
	1	2	3	4	5	6	7	8	9	10
Station of Puchol										
Date	1/11	2/1	2/24	5/3	6/1	6/21	7/26	9/6	9/27	12/7
Temperature °C										
lake (site 1)	15	13	15	20	23	28	30.5	25.5	23	10.5
sea (site 2)	13	12	12	18	22	25	29	25	24	14.5
pH										
lake (site 1)	8.5	9.1	9.2	8.2	9.1	9.0	8.7	8.9	8.7	8.2
sea (site 2)	8.0	8.3	7.9	8.0	8.2	7.9	7.9	8.3	8.0	8.0
Salinity %										
lake (site 1)	ND	0.56	0.64	0.7	ND	1.4	1.43	0.57	0.58	0.34
sea (site 2)	ND	21.3	21.8	32.7	11.9	30.4	21.9	12.0	34.8	28.5
Number of gates opened	1	3	1	0	4	0	0	6	3	0
Station of Perello										
Date	1/25	2/8	5/17	6/14	7/7	8/2	9/13	9/29	11/23	12/13
Temperature °C										
lake (site 3)	10	12.5	20	25.5	29	27	26.5	23	14	11.5
sea (site 4)	11	13.5	19	24	27.5	28	25.5	22.5	16	13
pH										
lake (site 3)	8.3	8.0	7.8	8.3	7.4	7.9	7.4	8.1	7.9	7.9
sea (site 4)	8.1	7.9	7.4	7.9	7.4	7.9	7.5	8.3	8.0	7.9
Salinity %										
lake (site 3)	0.47	0.6	0.8	ND	1.37	ND	2.5	0.81	0.27	0.24
sea (site 4)	8.27	28.1	ND	ND	12.8	13.5	14.0	22.0	30.2	27.5
Number of gates opened	1	1	0	0	1	1	2	2	0	0

ND: non determined.

Zooplankton was collected at four sampling stations, two corresponded to lake water, and the other two corresponded to sea water sampled approximately 50 m away from the mouth of the communication channels between the lake and the Mediterranean sea. Temperature, salinity and pH were determined in all stations. Zooplankton was collected by plankton tow provided with a 63 to 250 μm mesh net, and towed at approximately 2 knots speed. Samples were retained in sterile glass bottles containing surface water from each site. In the laboratory, the samples were filtered through a 63 μm filter to determine the wet weight. The gauze containing the plankton was then placed in a 500 ml flask with 100 ml of the four-salts solution of Kaneko and Colwell (12). To remove surface bacteria, the flask were vigorously shaken of five minutes. The solution was then filtered through a new sterile gauze to retain the plankton, and exoskeletons were then prepared for scanning electron microscopy (SEM) to confirm the removal of the attached bacteria by comparison to fresh zooplankton. The filtrate containing the bacteria was inoculated into MPN tubes and flasks with single strength alkaline peptone water. The isolation and identification of *Vibrio* spp. and *V. cholerae* was performed following previously described procedures (6). Heiberg group, use of chitin as sole carbon source, and serological profile were also determined as described (6).

Table 1 indicates the characteristics of the water analyzed, as well as the position of the gates that regulate the amount of water released into the sea (the lake is surrounded by rice fields that have to be flooded during certain months, and the gates help to maintain the adequate level of water in them). Water temperatures ranged from 10° C in January to 30.5° C in July. The pH values, between 7.4 and 9.2, always more alkaline in the lake samples, were favourable for the survival of vibrios, which were more abundant during the warm season (Fig. 1). In these monhs, and especially for the lake samples, most vibrios selected from the TCBS plates were identified as *V. cholerae*. Highest MPN's recorded were 10^5/g of zooplankton (Fig. 1). During the winter, all counts decreased significantly, but *V. cholerae* could still be isolated. As can be observed in Table 1 and Fig. 1, from the two sea sites analyzed in our study, the one more influenced by the lake discharges, with lower salinities (between 8.27 and 30.2 per mille), consistently yielded higher counts of *V. cholerae* than the sea site which received less amount of lake water (between 12 and 34.89 per mille). These findings are in accordance with the analysis of variance, which revealed two significant effects: temperature and kind of water analyzed. These same parameters had been significant for the free-living vibrios (6).

All 343 *V. cholerae* strains identified along the study were non-01, and most of them were included in Heiberg groups I (43 %) and II (46 %). A small number belonged to groups III, V and VI. Chitin as sole carbon source was used by 60 % of the *V. cholerae* isolates, a higher value than the one obtained for the water ones (6). The rest of biochemical characteristics, as well as the evolution along the year, was similar to the previously reported for the free-living isolates (6).

The favourable environmental characteristics during most of the year, together with the high zooplankton densities in the lake water, seem to enhance the presence of zooplankton-associated vibrios in the lake, and, consequently, in the sea site more influenced by the lake discharges. In typically endemic areas of cholera, such as Bangladesh, the epidemics occur at approximately the same time each year, for example when environmental conditions cause an increase of the zooplankton populations (11). Bearing in mind the historical and the present circumstances of the area studied by us, this coincidence seems to exist in our zone as well, although the incidence of cholera or «cholera-like» cases in developed countries is very low. Nevertheless, the recent cases reported, suggest the existence of new endemic foci and stress the importance of drastic improvements in environmental conditions all over the world.

The results obtained in the present study and in a previous one (6) confirm that *V. cholerae* exists unattached in the water column as free-living form, as well as attached to zooplankton members in the waters studied. This could indicate a potential source of infection for humans from the water itself, the copepods or possibly through the ingestion of higher organisms that concentrate the bacteria.

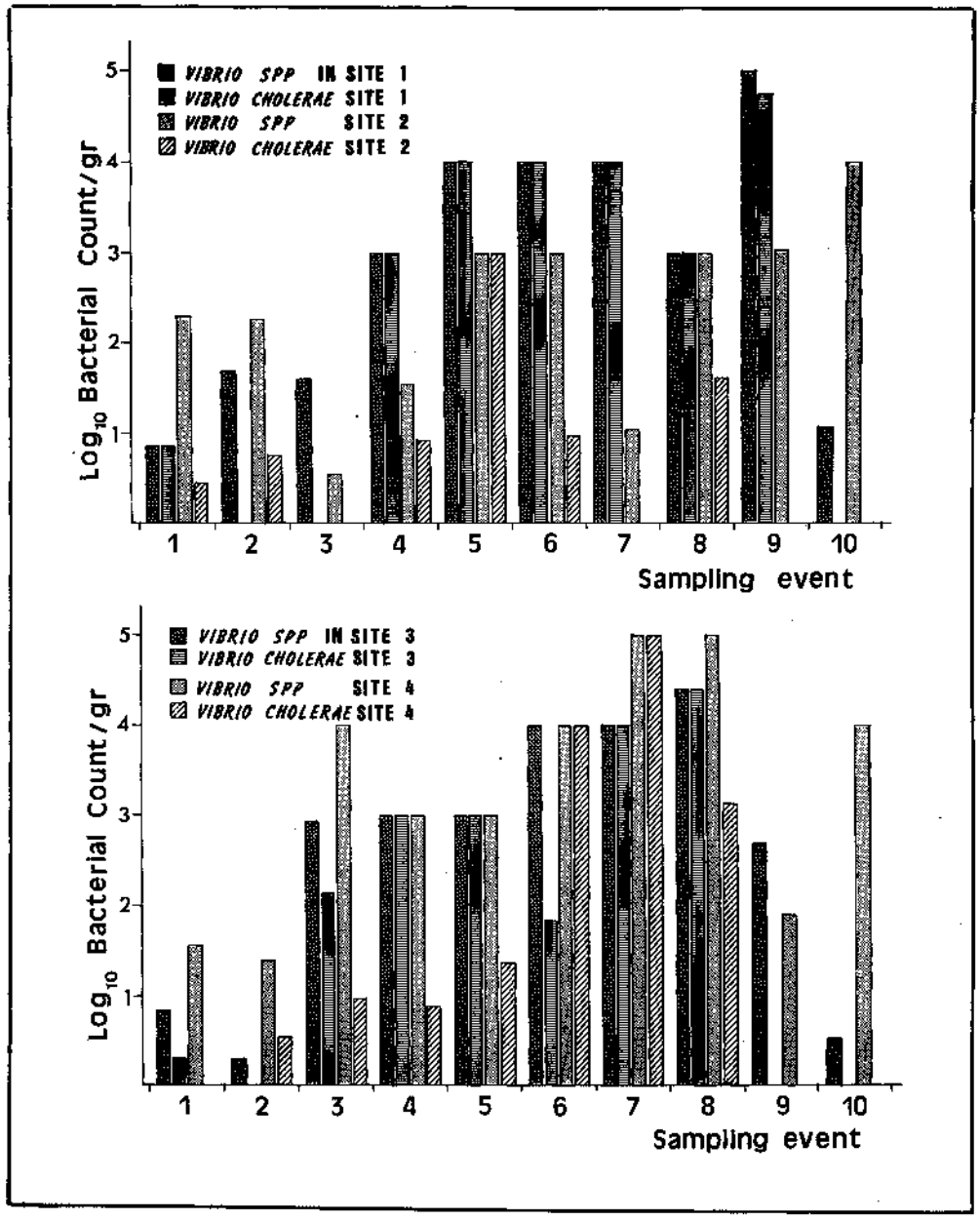

Fig. 1. Most Probable Numbers of zooplankton-associated *Vibrio* spp. and *Vibrio cholerae* along an annual cycle.

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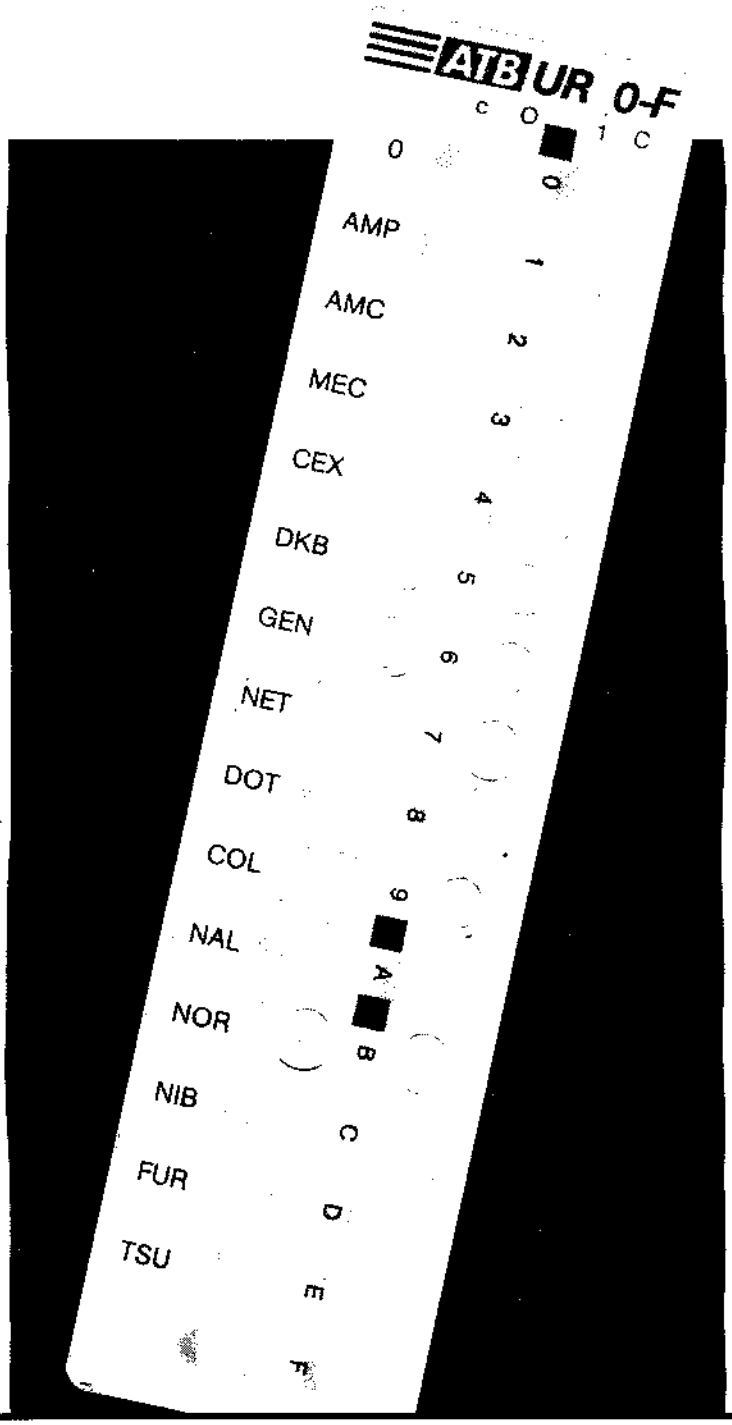

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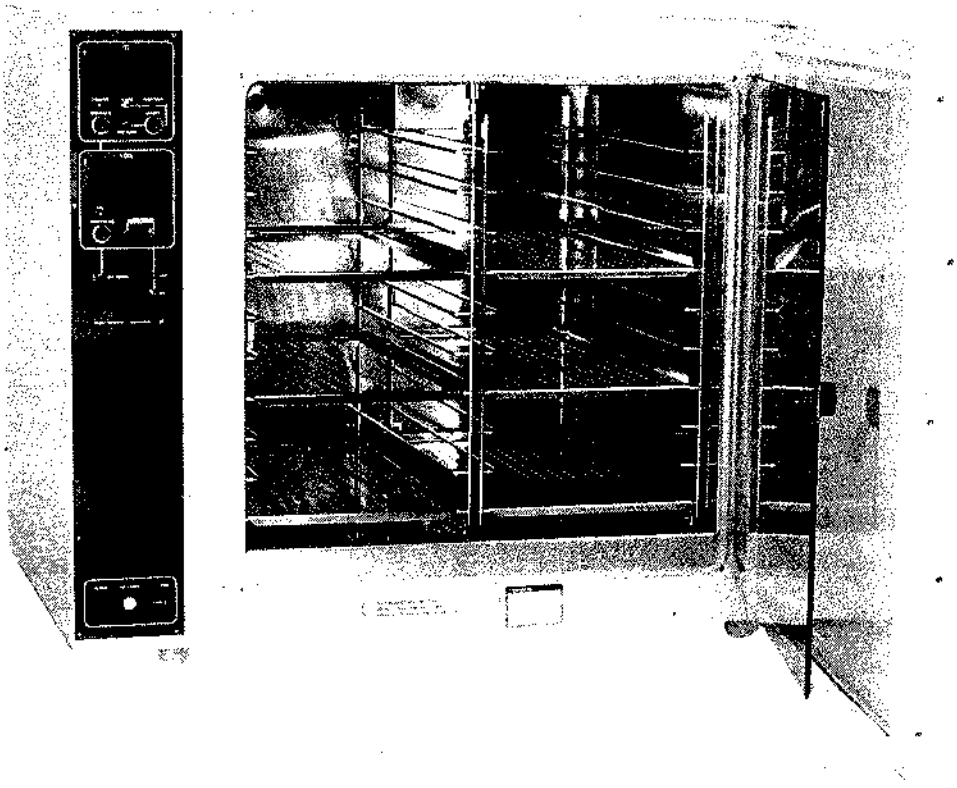

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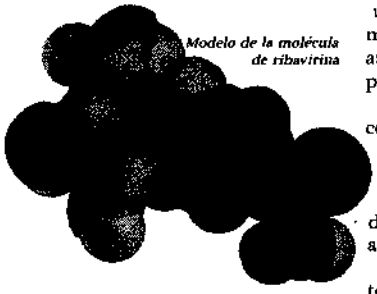


Modelo de la molécula de ribavirina

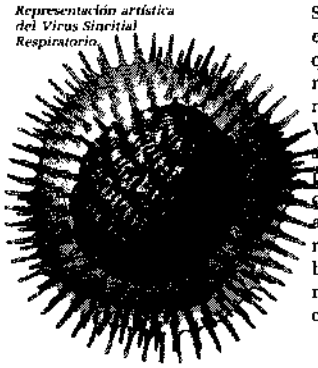
INDICACIONES

Tratamiento hospitalario de las infecciones graves del tracto respiratorio inferior, causadas por el virus sincitial respiratorio (VSR), en pacientes de alto riesgo (niños y lactantes con enfermedad pulmonar crónica o con cardiopatías congénitas o en prematuros).

El tratamiento es efectivo cuando se inicia en los tres primeros días de una infección del tracto respiratorio inferior causada por VSR.

No se recomienda el uso de ribavirina en aerosol si no hay identificación del agente causa.

Representación artística del Virus Sincitial Respiratorio.



POSOLOGIA

La ribavirina debe ser administrada exclusivamente en forma de aerosol con un generador de aerosol de partículas pequeñas especial (ver NORMAS PARA LA CORRECTA ADMINISTRACION).

La administración se realiza con una concentración final con 20 mg/ml, previa dilución con agua estéril.

El tratamiento se realiza durante 12-18 horas al día, con un mínimo de tres días y un máximo de siete días de tratamiento. La duración real dependerá de la respuesta clínica.

Usando la concentración recomendada, el flujo necesario para administrar los 300 ml de preparado durante 12 horas, la concentración media del aerosol es de 190 µg/L de aire.

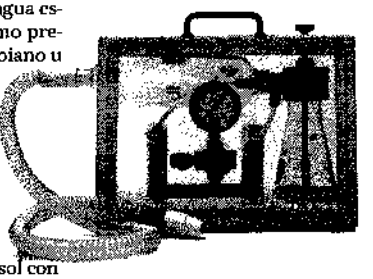
Es importante que el agua estéril para la disolución no presente ningún antimicrobiano u otra sustancia o medicación. Así como no debe emplearse mediante otro tipo de generador de aerosol. En cualquier caso pueden producirse anomalías en la formación del aerosol con peligro para el paciente.

NORMAS PARA LA CORRECTA ADMINISTRACION

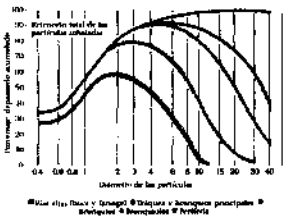
VIRAZID® liofilizado debe ser administrado exclusivamente en forma de aerosol. Sólo debe emplearse mediante el generador de aerosol de pequeñas partículas SPAG®, o aparato similar que proporcione el mismo tamaño de partícula. VIRAZID® liofilizado se presenta en viales de 100 ml de capacidad conteniendo 6 gramos de ribavirina. Usando técnica aséptica, debe disolverse en el mismo vial con 100 ml de agua bidestilada estéril sin aditivos ni antimicrobianos. Esta solución debe transferirse al reservorio instalado a este fin en el SPAG®, una vez en él, se debe añadir agua bidestilada estéril hasta un volumen total de 300 ml. La concentración obtenida es de 20 mg/ml. La solución debe ser transparente y libre de partículas de producto en suspensión.

Una vez preparada la solución de 300 ml de ribavirina para administración en aerosol debe usarse de forma inmediata. En caso de que no se vaya a utilizar inmediatamente, esta solución puede guardarse en condiciones estériles y a temperatura ambiente (20 °C-30 °C) durante 24 horas. Debe desecharse cualquier cantidad que pasadas 24 horas de su reconstitución, permanezca en el reservorio del SPAG®.

SISTEMA EXCLUSIVO DE ADMINISTRACION DE AEROSOL



Efecto del tamaño de la partícula del aerosol sobre el grado de retención del fármaco en el tracto respiratorio.



ADVERTENCIA

El aerosol de ribavirina no debe usarse en niños que requieran ventilación mecánica asistida, ya que la precipitación del fármaco en el equipo de respiración (tubos y válvulas) puede interferir con una ventilación del paciente segura y efectiva.

Si el fármaco se administ
en pacientes con ventilació
mecánica es imprescindib
controlar cada hora la posib
precipitación y condensació
excesiva en la intubación. Igu
mente, cada 1-4 horas se car
biarán rutinariamente los f
tros y válvulas.

CONTRAINDICACIONES

Embarazadas o mujeres q
puedan estar embarazadas d
rante la exposición al fármac

No se recomienda el uso
ribavirina en aerosol een m
dres durante la lactancia, del
do a que las infecciones p
VSR en esta población es aut
limitado.

PRECAUCIONES

Los pacientes con infeccion
del tracto respiratorio inferi
causadas por VSR requier
una perfecta monitorizació
atención de su estado respira
rio y de los fluidos corporal

Los estudios «in vitro» e
vivo» que se han realizado c
ribavirina han manifestado
tividad carcinogenética y m
tuagénica, así como lesion
testiculares en ratas, si bien
experimentación ha sido p
vía oral y de forma prolonga

INTERACCIONES

A la solución final de riba
rina para nebulizar no se de
añadir ninguna sustancia, f
maco o aditivo.

La ribavirina combinada
vitro» con la zidovudina (AZ
antagoniza la actividad an
viral.

EFECTOS SECUNDARIOS

Pulmonares: empeoramie
de la función respiratoria, n
monía bacteriana, neumotó
apnea y dependencia al res
rador.

Cardiovasculares: parada
díaca, hipotensión y toxici
digitálica.

Hematológicos: por vía or
i.v. la ribavirina no se acum
en los eritrocitos, niveland

:FICAZ EN EL TRATAMIENTO :CIONES ESPIRATORIO (VSR)

Virazid®
(Ribavirina)
liofilizado para administración en aerosol

ios 4 días. Esta acumulación sminuye gradualmente con ıa vida media aparente de 40 as. Estas vías de administra-ón se han asociado con una ıemia hemolítica dosis-ependiente, que remite redu-endo la administración. En s pacientes tratados con riba-rina nebulizada en aerosol,) se han reportado casos de ıemia. Con la administración ı aerosol se han citado casos : reticulocitosis.

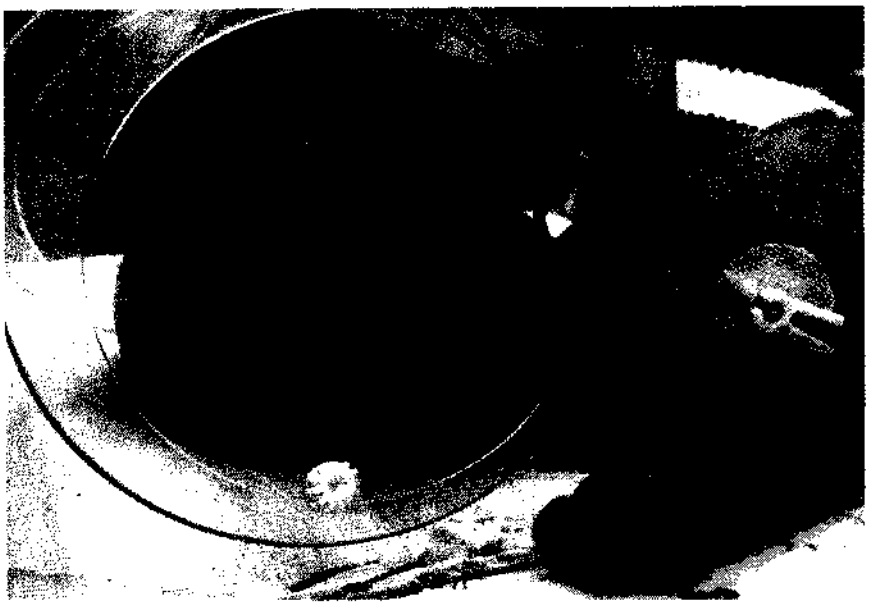
'TOXICACION Y SU RATAMIENTO

No se ha comunicado ningún so de intoxicación con la ad-inistración en aerosol, ni exis- experiencia clínica sobre in-xicación por sobredosis de bavicina en aerosol.

ONDICIONES E CONSERVACION

La ribavirina liofilizada pue-: almacenarse en lugar seco, temperatura ambiente (entre 5° y 25 °C), evitando la expo-ción directa al sol u otras ıentes de calor.

Una vez preparada la solu-ón final de 300 ml (20 mg/ml) ;ta conserva sus propiedades, se mantiene a temperatura nbiente (entre 20° y 30 °C) y ı condiciones estériles, duran-24 horas.



RESENTACION

Cajas conteniendo uno tres viales.

79351 VIRAZID
IOFILIZADO 1 VIAL
ON 6 GR.
iofilizado Aerosol.
aja con 1.
1.601, (1) imp. inc.

79369 VIRAZID
IOFILIZADO 3 VIALES
ON 6 GR.
iofilizado Aerosol.
aja con 3.
24.804, (1) imp. incl.

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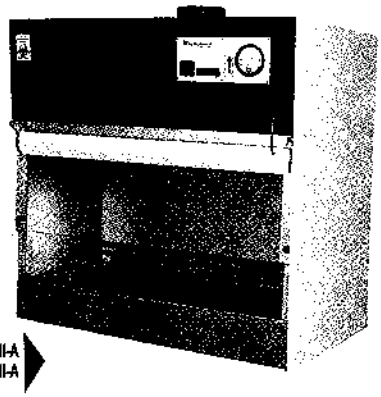
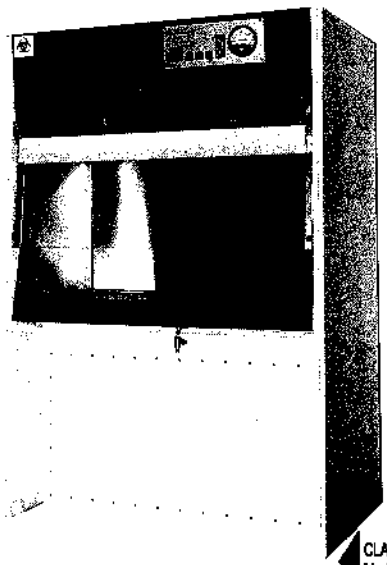
Cabinas de seguridad biológica «Biohazard»



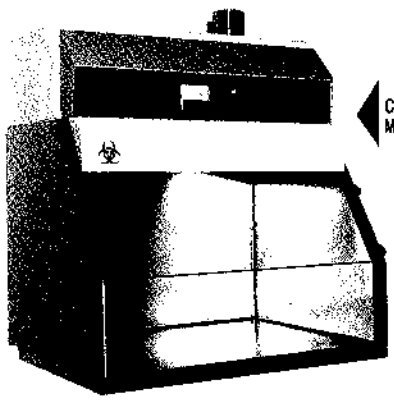
CLASE II-A
Mod. BIO-II-A

PROTECCION TOTAL DEL OPERADOR (ZONA TRABAJO EN DEPRESION), DEL AMBIENTE (FILTRO HEPA EN LA EXPULSION DEL AIRE), Y DEL PRODUCTO.

EL FACTOR DE PROTECCION DE ESTAS CABINAS, CUMPLE LAS ESPECIFICACIONES EXIGIDAS POR LA NORMA BRITISH STANDARD BS 5726, LA N.S.F. STANDARD 49 Y LAS G.L.P. PARA CABINAS **BIOHAZARD**.



CLASE II-B
Mod. BIO-II-B



CLASE I
Mod. DEP-I

INDICADAS PARA MANIPULACIONES BACTERIOLOGICAS DE MICROORGANISMOS PATOGENOS.

telstar s.a.

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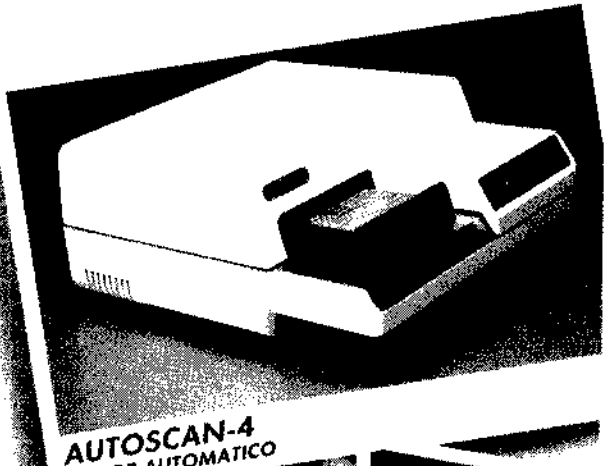
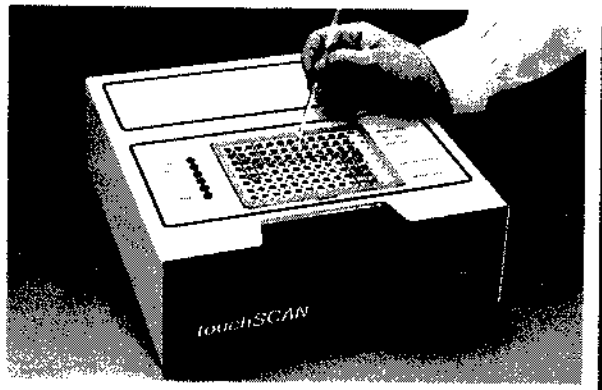
CRUFAC

INSTRUMENTACION



AUTOSCAN-4
LECTOR AUTOMATICO



TOUCH SCAN
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D.M.S.

DOS OPCIONES DE AUTOMATIZACIÓN

NUEVO SISTEMA DE PROCESO
DE DATOS (DMS) CONECTADO AL LECTOR
MAYOR CAPACIDAD DE MEMORIA
NUEVO SISTEMA DE BUSQUEDA DE DATOS
POR MULTIPLES PARAMETROS
BASE DE DATOS CON MAS DE
350 MICROORGANISMOS

NUEVOS PANELES

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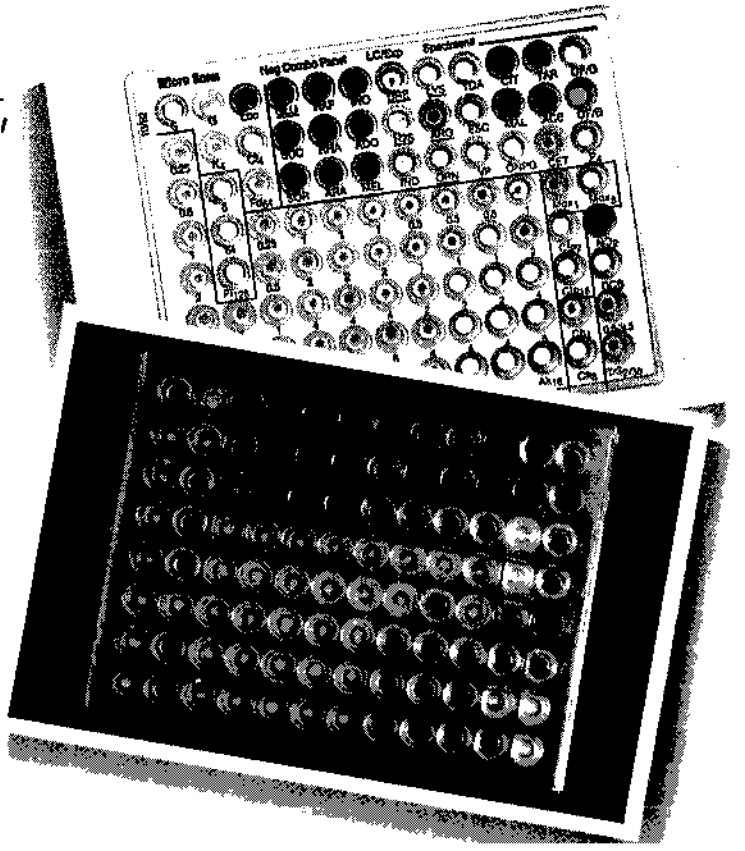
- GRAM-NEGATIVOS
- GRAM-NEGATIVOS ORINA
- GRAM-POSITIVOS

PANELES DE C.M.I. SOLO PARA:

- GRAM-NEGATIVOS
- GRAM-NEGATIVOS-ORINA
- GRAM-POSITIVOS

PANELES COMBO DE IDENTIFICACION Y SENSIBILIDAD (BREAKPOINT). PARA:

- GRAM-NEGATIVO
- GRAM-POSITIVO



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