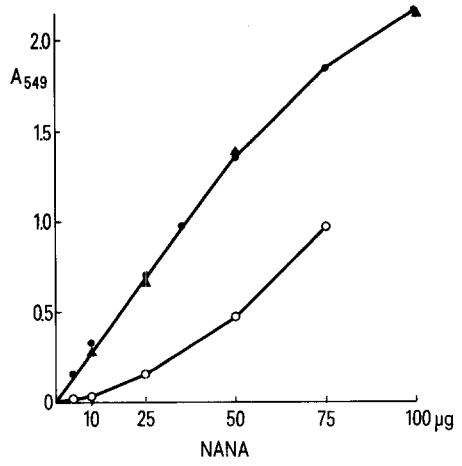

Fig. 1. Effect of hemoglobin on NANA determination. Closed circles, NANA in water; Open circles, NANA in hemolysate prepared by placing RBC (7.5%) in hypotonic solution; Triangles, NANA recovered after treatment of hemolysate with ethanol-chloroform as described above.

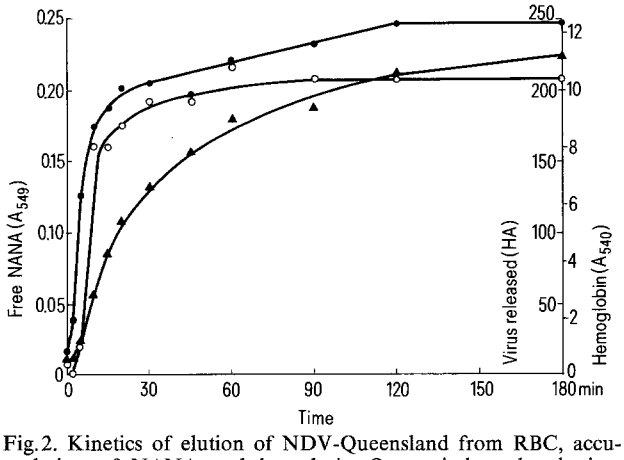

Fig. 2. Kinetics of elution of NDV-Queensland from RBC, accumulation of NANA and hemolysis. Open circles, eluted virus (HA); Closed circle, free NANA; Triangles, hemolysis.

nation test with 1% chicken erythrocytes. Hemoglobin was determined spectrophotometrically at 540 nm. NANA was determined by the Warren method[9] modified by Aminoff[10]. *Results and discussion.* In order to eliminate hemoglobin present in eluate, supernatant samples were deproteinized by the method of Tsuchihashi[11] modified for high concentrations of hemoglobin as follows. An aliquot of 0.24 ml of ehtanol-chloroform (2:1) was added to 0.5 ml supernatant. The protein was sedimented by centrifugation and alcohol-chloroform solvent was then removed by evaporation in vacuo and by heating at 70 °C. The validity of this method for sucessive determination of NANA was demonstrated by complete recovery of NANA determined after its mixing with hemolysates in various ratios (figure 1 and table). As can be seen, neither different concentrations of NANA (up to 100 µg, figure 1), nor different concentrations of hemoglobin (table) in NANA-hemolysate mixtures influenced the values of NANA determined after the deproteinization procedure. This method was then applied to the direct determination of NANA in the NDV-RBC system. It can be seen (figure 2) that the accumulation of NANA in the eluate can be reliably detected during the simultaneous processes of hemolysis and elution. The kinetics of NANA accumulation is correlated with the kinetics of elution. Thus, it is proposed that the detected NANA is accumulated in eluate as a result of in situ Nase action of eluting virus on NANA-containing RBC receptor substrate. This in situ action of the enzyme, contrary to its in vitro activity towards a soluble substrate, may be strongly dependent on steric conditions created between enzymatic sites (hemagglutinin-neuraminidase glycoprotein subunits) of attached virions and NANA-containing receptor sites of RBC. This would cause either facilitation or hindrance for the in situ Nase action. The actual outcome depends on the degree of congruence between viral and receptor sites and, hence, ultimately on the arrangement of the supercapsid viral subunits in the viral envelope. As the latter may be a particular NDV strain-specific property, corresponding studies using various NDV strains differing in their virulence are now in progress.

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A technique for sterile culture of fresh water sponges

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Summary. By treating the gemmules of freshwater sponges successively with hydrogen peroxide and with sodium hypochlorite in suitable concentrations, we obtained cultures that were free from any bacterial or fungal contaminant. This technique provides a useful tool for further studies on metabolic and antibiotic activities, and of the behaviour of isolated cells cultivated in artificial media.

Fresh-water sponges provide excellent models for the experimental study of cell differentiation and behaviour. Indeed, their organization bears more resemblance to a society of cells than to a stable tissue, and the few distinct cell-types are highly motile.

In previous work[2] we have shown that it is possible to inhibit specifically the differentiation of 1 cell type, viz. the choanocytes by hatching the gemmules in the presence of hydroxyurea. Such sponges, devoid of any aquiferous system, are reduced to a closed dermal envelope stretched on

spicules like a tent. The floor of this 'tent' is strewn with a dense and almost homogeneous population of embryonic archaeocytes.

In an unpublished experiment, we observed that these archaeocytes, despite the treatment they had undergone, remained totipotent. When a sufficient number of them were taken from the abortive sponge and transferred to mineral medium without hydroxyurea, these cells would give rise partly by differentiation, within 3 days, to very small but fully organized and functioning sponges.

This observation led us to attempt to cultivate these archaeocytes in vitro in a medium that would support their multiplication. In the course of these experiments, 2 difficulties arose. One is that it seems to be quite impossible to maintain archaeocytes, in a nutrient medium without their differentiating into pinacocytes or scleroblasts. The other difficulty was the apparently trivial but nevertheless preliminary problem of the contamination of the cultures by bacteria. The aim of the present work is to track the sources of contamination, and to devise a simple and rapid technique to prevent it.

Material and methods. Live material and cultivation medium. The gemmules were collected in the preceding autumn from sponges (*Ephydatia fluviatilis* Vejd) cultivated in a pond near Brussels. They were stored until used at 0 °C. The mineral medium was prepared according to Rasmont[3] from previously heat-sterilized stock solutions, since heating of the complete mixture would cause carbonates and silicate to precipitate. When the medium contained hydroxyurea (1,31 mM; Nutritional Biochemicals) it was sterilized by filtration through Millipore filters (0,22 µm).

Chemicals used for sterilization of the gemmules. Hydrogen peroxide (Perhydrol 30% Merck) and sodium hypochlorite (technical grade; 13% active chlorine Belgolabo).

Experimental techniques. All manipulations were carried out in a laminar-flow cabinet. The gemmules were first carefully sorted under a binocular microscope so as to eliminate all the empty gemmule shells and other debris that might contribute to the contamination, and then washed 3 times with sterile mineral medium.

a) Treatment with the antiseptic agents. 3 kinds of antiseptic treatment have been used: - Hydrogen peroxide alone: diluted to 1/25th, acting for 5 min according to Rasmont[3]. - Sodium hypochlorite (1 or 2%) alone acting for 1, 2 or 4 min. - Treatment with both agents in sequence. The gemmules were ceaselessly agitated during the treatments; afterwards, they were thoroughly washed with 5 successive baths of sterile mineral medium.

b) Examination of the sterility of the gemmule shells. We used the techniques currently empolyed in tissue culture laboratories for the detection of infection. 3 batches of about 300 gemmules were incubated, each in 10 ml 'brain-heart infusion' (Oxoïd, 37 g/l distilled water) for bacterial contamination, 3 other batches in 10 ml 'Sabourand fluid medium' (Oxoïd, 30 g/l) for fungal contamination. We incubated the flasks at 20 °C, which is the temperature at which the sponges are normally grown in the laboratory. Each flask has been examined at 24-h intervals for 1 month. The results reported in the following table are those of the 30th day.

c) Investigation of the possible harmful effects of the treatment on the sponge development. After each kind of treatment, 3 batches of 50 gemmules were incubated in Petri dishes, with 10 ml sterile mineral medium. The mean percentages of hatching were calculated at 24-h intervals; the following table gives the results at the 5th day. Furthermore, we examined the cultures after 2 weeks, using binocular and phase contrast microscopy, to detect any delayed toxicity.

d) Control of the effect of the treatment on the gemmule shells. The shells of gemmules that had undergone hydro-

Effects of several chemical treatments on the bacteria and fungi carried by the gemmule shells and on the development of sponges

Chemicals			Controls of Infection		Toxicity	
Nature	Doses	Duration (min)	S	BHI	% hatching gemmules	% normal sponges
H_2O_2(A)	1/25	5	+	+	98	98
NaHC10 (B)	1%	1	+	+	97	97
(C)		2	+	+	98	95
(D)		4	+	+	89	89
(E)	2%	1	+	+	98	94
(F)		2	+	+	96	96
(G)		4	+	+	75	68
(H)	3%	1	+	+	72	50
(I)		2	−	+	53	19
(J)		4	−	−	17	0
H_2O_2 + NaHC10	1/25	5				
(K)	1%	1	+	+	96	94
(L)		2	+	+	97	97
(M)		4	−	+	91	86
(N)	2%	1	−	−	99	97
(O)		2	−	−	96	96
(P)		4	−	−	79	72

S, Sabourand fluid medium; BHI, brain heart infusion; +, infection.

gen peroxide treatment either alone or followed by a 2%, 2 min hypochlorite treatment, were observed with the scanning electron microscope or submitted to autoradiography. For scanning microscopy, the gemmules were fixed for 2 h with 1% osmium tetroxide in 0.025 M, pH 7.4 cacodylate buffer), washed dehydrated and dried at critical point and finally palladium and gold-coated. For autoradiography, the gemmules were incubated for 12,24 or 48 h after sterilization in a sterile mineral medium to which 20 µCi/ml[^{3}H]-leucine were added. The autoradiograms were made according to the technique described previously[4].

Results. Action of the antiseptic agents on the microorganisms carried by the gemmule shells and on the viability of the gemmules. The results of the different treatments are summarized in the table. It is clear from these results that hydrogen peroxide, used alone, is not sufficient to kill all the bacteria and fungi carried by the shells (line A). The same is true for hypochlorite used alone (lines B to H) except for the highest concentration (3%) and longest duration (4 min) at which it is harmful to the sponges (lines I and J). From the lines N and O of the table, it can be seen that the combination of 2 treatments, each of which on its own is harmless to the sponges and insufficient to kill the bacteria and fungi, is quite effective in sterilizing the shells. When the treatment with 2% hypochlorite is limited to 1–2 min, though a slowing down of the hatching can occur there is no decrease in the maximum percentage of hatching nor of normal development.

It is well known now that many species of marine sponges harbour huge numbers of symbiotic bacteria. Though nothing of this kind has been reported yet about fresh-water sponges, it is not wholly impossible that bacteria could be trapped in the germinal core of a gemmule and liberated when the sponge hatches. To test this eventuality, we proceeded to test the bacterial and fungal sterility of media in which sponges have hatched from gemmules.

Taking in to account that it was in hydroxyurea treated sponges that we had been faced with the most serious infection problems, we tested the supernatants of both normal and hydroxyurea treated cultures.

Gemmules were treated for 5 min with the hydrogen peroxide solution, washed, then treated for 2 min with 2% hypochlorite, and finally washed with sterile mineral medium. 3 batches of 100 were incubated at 20 °C in Petri dishes with 10 ml sterile mineral medium, 3 other batches in mineral medium containing 100 µg/ml hydroxyurea.

After 2 weeks' incubation, when the sponges had fully developed either their normal organization or their typical hydroxyurea-inhibited structure, 2 ml supernatant were taken from the dishes and incubated with brain-heart infusion or with Sabourand medium, in the same way as the unhatched gemmules had been. After 1 month's incubation, all these test cultures remained clear. As a comparison, the same test was performed on the supernatants of cultures from gemmules that had been treated with hydrogen peroxide alone; in the latter case, bacteria were found from the 3rd day on.

Discussion. When gemmules are gathered from a sponge grown in the open, their shells bear a large variety of microorganisms, spores and eggs. When these gemmules are incubated at 20 °C in mineral medium, even after having been thoroughly washed with water, the cultures almost always contain various protozoa and mould mycelia and quite often nematodes or even rotifers and gastrotricha.

The techniques described by Rasmont[3], including the cleansing (erroneouly termed 'sterilizing') with hydrogen peroxide are clearly efficient in getting rid of all these eukaryotic contaminants but not of the bacteria.

When the gemmules are grown into normally functioning sponges, however, bacterial contamination stays within very low limits. Unpublished tests run in our laboratory showed that the concentration of live bacteria in the medium of a 25 ml Petri dish containing a population of sponges hatched from 120 gemmules was in the order of magnitude of 100/ml. This is not surprising if one bears in mind that bacteria can be in vitro[3], and are in ecological conditions[5,6], a suitable diet for sponges.

It is quite possible that in Petri dish cultures prepared along Rasmont's technique, the multiplication of bacteria on the organic products of the sponges, and the capture of bacteria by the sponges result in a relatively stable but small 'standing crop' of bacteria.

If this is true, it is not surprising that cultures of hydroxyurea inhibited sponges, devoid of choanocytes and thus of any capacity for filtration, harbour thriving populations of bacteria. These problems of contamination become intolerable once you start working with media containing organic nutrients, as we did in our attempts to cultivate archaeocytes.

By treating the gemmules successively with hydrogen peroxide and with sodium hypochlorite in suitable concentrations, we succeeded in freeing them completely from bacterial and fungal contaminants.

Korotkova et al.[7] also obtained sterile sponge cultures by treating their gemmules for long periods successively with hydrogen peroxide, and 'Iodinol'. Our 1st intention was to try and utilize their techniques; unfortunately, 'Iodinol' is a preparation of the USSR pharmacopeia and we were not able to ascertain its chemical composition. Our experiments also lead to the conclusion that in gemmules, only the shell surface is septic.

The 'spontaneous' bacterial sterility of the core of the gemmules and, probably, of the inner layers of its shell may be ascribed to one or more of the following reasons: – The intense phagocytotic activity of the cells that take part in the building of the gemmule[8,9]. – The action of antimicrobial substances, that have been found in marine sponges[10–12] but not yet in fresh-water species[13]. – The activity of nucleases, found by Harrison to be located in the gemmules[14] and to which he ascribes this hypothetical function.

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