method, 2 cm^3 of the tested fluid were placed in a small dish 3 cm in diameter which was then covered with the membrane. The dish was inverted over a glass tube of the same diameter confining the bugs.

In all cases, different stages of the mealy bugs were isolated from a standard culture maintained on potato sprouts at 30 °C. 10 replicates, each with 10 individuals, were made for each test. The insecticide to be tested was a dimethoate compound (Roger) 0,0-dimethyl-S (N-methyl carbamyl methyl) phosphorodithioate. The survival of adult insects was first determined on water and sucrose, and in screening the insecticide, the mortality of different insect stages was determined after 24 h access feeding time. Moribund individuals were considered dead. The mortality was corrected according to Abbott's formula. Prophylactic skin was also tried and proved to be efficient in feeding as well.

Results. The mealy bugs successfully ingest liquids accessible via artificial membranes of stretched Parafilm or prophylactic skin. Starved adults lived for 2–4 days and those fed on a water diet lived for 4–6 days. Adults fed on 10% sucrose survived for 7–9 days and they even laid eggs in small numbers.

In screening the toxicity of the dimethoate Roger through artificial feeding of *P. citri*, the EC50 were found to be 23, 50, 95 and 120 ppm for the 1st, 2nd, 3rd and adult stages respectively. The probability that the mortality was due to refusal to feed on the diet, or to a fumigant action, was ruled out, since the bugs can survive starved for 2–4 days and also the percent insects survival was high when they were kept in a small tube enclosed within a bigger one with a high concentration of the insecticide. This was also reported for aphids (MITTLER and PENNEL[2]).

The aforementioned method can be used as a standard test for screening insecticides, chemosterilants, derivatives produced in plants from the insecticide originally applied as well as chemicals in feeding behavioural studies of mealy bugs in general.

Zusammenfassung. Verschiedene Methoden zur Toxizitätsbestimmung von Insektiziden bei künstlicher Ernährung von *Planococcus citri* Risso mit Zuckerdiät und durch eine Parafilmmembran oder eine prophylaktische Haut werden beschrieben.

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Heart Cells in Culture: A Simple Method for Increasing the Proportion of Myoblasts

Cell cultures derived from trypsinized hearts of young rats, hamsters, or chick embryos contain a mixture of 2 kinds of cell: muscle cells and mesenchymal cells[1], with an initially high percentage of the latter[2–4]. These mesenchymal cells multiply more quickly than the myoblasts and pervade all the free space[2].

Methods so far reported for elimination of the mesenchymal cells[3,5–7] are not convenient when plates of confluent beating myoblasts are routinely needed in large numbers for electrophysiological, biochemical and electron microscopical investigations[8]. Therefore, we have tried to find whether the speed of attachment to the dish surface would be different for myoblasts and mesenchymal cells, and if so, whether this difference could be used to increase the proportion of myoblasts in culture.

We found that, following trypsinization of the hearts, most of the mesenchymal cells attach to the dishes before the myoblasts. The technique described here is based on this difference.

Although the elimination of the mesenchymal cells was never complete, the present technique gave better results than those achieved by modifying other culturing conditions such as temperature, centrifugation parameters or use of antimitotics.

Materials and methods. Preparation of cell suspension. Whole hearts of 2- to 3-day-old rats (not over 30 at a time) were cut into about 8 pieces and washed twice for 5 min in cold phosphate buffer solution (PBS)[9] with gentle stirring. The fragments of cardiac tissue were then subjected to 9 successive 8 min periods of trypsin digestion at 37 °C, again with gentle stirring (Difco Trypsin 1:250 – 0.25% in phosphate buffer without Ca^{++} and Mg^{++}), which generally sufficed to disaggregate them almost completely.

The first 3 supernatants were discarded because they mainly contained mesenchymal cells and debris. Each of the following supernatants was poured into half its volume of precooled medium in a centrifuge tube (usually 3 supernatants per tube) and kept at about 5 °C in iced water. (Medium was: MEM powder – GIBCO – plus 0.06% yeast extract and 0.25% lactalbumine hydrolysate, 10% calf serum, $NaHCO_3$ 14 m*M*, penicilline 400 U/ml and streptomycine 200 µg/ml.)

Once digestion of the tissue was complete, the cell suspensions were centrifuged for 3 min at 670 × *g*. The pellets of cells were resuspended in medium and the suspensions pooled into a beaker kept at 5 °C. To minimize pipetting and to prevent clumping of the cells the following procedure was used: about 20 ml of medium was added to each pellet and the tubes were gently shaken; as soon as part of the cells were resuspended 5 ml of the suspension was sucked off and transferred into the beaker and fresh medium added to the tube. This process was repeated until the pellets had been completely resuspended; at that time, the beaker contained the

[1] Fibroblasts as well as endothelial cells are referred to as 'mesenchymal cells'.

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required amount of cell suspension for preparing the plates, and only a few clumps were left in the tubes.

The cell suspension was then distributed in large plastic Petri dishes (Falcon or Greiner – 10 cm diameter) in aliquots of 10 ml per dish (from 5.5×10^5 to 1×10^6 cells/ml).

Instead of counting the cells it was found quicker and more convenient to prepare a number of dishes equal to $^2/_3$ the number of hearts initially trypsinized. The cells were incubated at 37 °C in a mixture of air $+CO_2$ to maintain the pH at 7.4 ± 0.05.

Selection procedure. The cells were incubated in the large Petri dishes for 3 h. During this period most of the mesenchymal cells attached to, and spread out on the dish surface, while the myoblasts remained spherical and floating. At the end of this 3-h-period, the dishes were shaken and the supernatants, containing the myoblasts, were briskly poured into a beaker. The plates were rinsed once with medium (5 ml per 10 plates). The cell suspension was then distributed into new Petri dishes, care being taken to inoculate a large number of cells per dish (e.g. from 3 to 5×10^6 cells per 6 cm diameter dish). Routinely, as many 6 cm dishes as hearts initially excised, were filled with 7 ml of the cell suspension. The cultures were then incubated for 24 h, after which time they were washed 3 times with PBS to remove dead cells, and given fresh medium. A confluent monolayer of beating cells was obtained after 2–3 days in culture.

Cell ratios. In order to evaluate the proportion of mesenchymal cells in the cultures, at any time following the first 24 h incubation, the cells were rinsed twice with PBS, fixed, stained and counted directly in the Petri dishes.

Fixation: Bouin 30 min, followed by several washings with luke-warm water until the complete disappearance of picric acid stain.

Staining: a) Mayer's haemalum for 45 min, then 8 sec differentiation in alcohol-acid, followed by several washes in luke-warm water until nuclei stained deep blue. b) Eosin (Siegfried 0.25%) – 15 min, followed by a single quick rinse with cold water. c) A small amount of liquified glycerol-jelly (not too warm) was run over the cell monolayer to ensure, after gelification, a very thin protective layer.

Appearance of the cells (Figure 1). The myofibrils as well as the numerous large mitochondria of the myoblasts stained red, while no red inclusions showed in the pale blue cytoplasm of the mesenchymal cells. Nuclei of myoblasts were spherical, and usually displayed a single nucleolus (sometimes 2 or 3). The nuclei of the mesenchymal cells were larger, of a paler blue, and contained several nucleoli. Thus it was easy to differentiate the two kinds of cells.

Cell counts. The percentages presented (Figure 2) were computed on basis of cell counts of several fields for each culture (to a total of 1000 cells per culture, i.e. per dish) using partially occluded eyepieces to demarcate the fields, objective 40, and filter BG 38-Schott.

Results (Figure 2). Although mesenchymal cells were never completely eliminated, their number could be markedly reduced as compared to that which occurred without the selection treatment. Furthermore, as shown by the graph, the skill of the operator handling the cells played a role in the selection. Variations due to undetectable modifications may also be encountered between different batches of cultures. In order to obtain a high proportion of beating cells after the selective treatment, it is important to inoculate the plates with a large number of cells for the following reasons: a) part of the myoblasts do not attach and die, as already shown by MARK and STRASSER[2], and b) the mesenchymal cells tend to pervade all the free space. The use of very young rats is also important to ensure a good yield of myoblasts.

Cultures of cardiac mesenchymal cells. When such cultures were desired, the cells from trypsin fractions 2 and 3 were kept, pooled and plated. After $1^1/_2$ h of incubation or less, the supernatants (containing the myoblasts) were

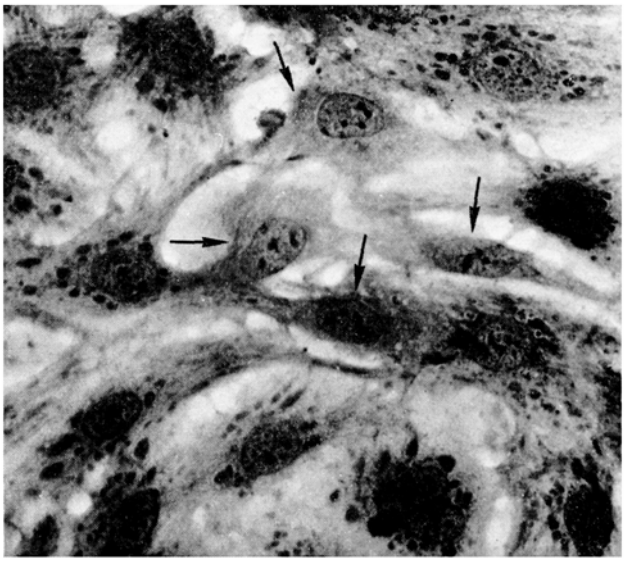

Fig. 1. Differential staining of myoblasts and mesenchymal cells. Four mesenchymal cells (arrows) are visible in this field. ×624.

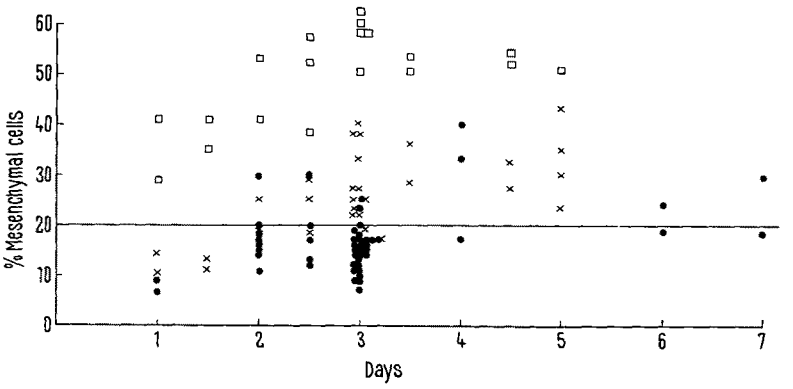

Fig. 2. Percentage of mesenchymal cells found in samples of several batches of cultures. Cultures treated for myoblast selection: ● and ×, two groups of operators; □, non-treated cultures. Less than 20% mesenchymal cells are found in 3-day-old treated cultures handled by skilful operators compared to more than 50% found in nontreated cultures.

discarded, and the plates carefully rinsed once or twice with medium before being returned with fresh medium, to the incubator. Large plates used in the selection procedure for myoblast cultures also constituted a valuable source of pure cultures of mesenchymal cells, if care was taken to rinse them before returning them to the incubator, in order to remove all floating myoblasts[10].

Résumé. La plupart des cellules mésenchymateuses présentes dans une suspension cellulaire obtenue par trypsination de cœurs de rats nouveau-nés s'attachent au récipient de culture avant les myoblastes. Cette propriété est utilisée pour obtenir des cultures confluentes contenant une proportion élevée de myoblastes actifs.

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Quantitation of Fibrinolytic Agents Released in Tissue Culture

The endothelium of certain vessels contains a potent fibrinolytic agent[1,2] that is believed to be continuously presented to the bloodstream to break down fibrin deposits and thereby prevent thrombosis[3,4].

Tissue cultures have been used to investigate the mechanism of this release. Supernatants of cultures of renal and of some other tissues[5,6] show fibrinolytic activity on fibrin plates, according to ASTRUP and MÜLLERTZ[7]. But we have found the results of such assays during tissue culture to be less uniform. This may perhaps be explained by variation of the rate of release of the fibrinolytic agent and by the unknown amount of the agent inevitably inactivated at the temperature (37 °C) prevailing during culture.

The method described below was first developed to avoid the error due to possible fluctuation in the rate of release of this activity; and secondly, to avoid the loss due to denaturation and thereby to get a better idea of the amount of the agent released during a certain period. We cultured human tissues in the presence of, but not in direct contact with, a standard fibrin clot. The amount of fibrin digested was then calculated directly from the degradation products accumulated in the culture medium.

Lung, liver, bone marrow and kidneys from normally developed 16–20-week-old foetuses were obtained at legal abortion by hysterotomy. We used foetal tissue so that we could culture it in a defined synthetic medium. Explants from the organs were cultured as organ cultures on gelatine-sponge (Spongostan, Ferrosan, Malmö) in Leighton tubes containing 1 ml of Parker 199 (Statens Bakteriologiska Laboratorium, Stockholm) synthetic medium and a preformed clot obtained by adding 1 ml of human fibrinogen (Fibrinogen Kabi, 1% in distilled water) to 0.02 ml of thrombin (Topostasin Roche, 75 NIH U/ml saline). The explants were not in contact with the standard clot (Figure). Every 24 h after the beginning of culture, a small volume (0.06 ml) of medium was aspirated and assayed quantitatively for fibrin degradation products, according to an immunochemical method[8]. At the end of the culture period (4 days), the explants were examined histologically as well as histochemically for activators of fibrinolysis by a modified[9] fibrin slide technique[2]. We also determined the inhibitors of urokinase, the α_2-macroglobulin and the total antitrypsin activity[10].

Fibrin degradation products regularly appeared in increasing amounts in the medium of lungs, kidneys and bone marrow (Table). They were practically absent in the culture medium of liver and controls, i.e. gelatin sponge alone. Conventional histological sections showed good survival of the explants. The fibrin slide technique revealed fibrinolytic activity located around small blood vessels in the explants from all the organs except those of the liver. Such activity was also demonstrable in explants cultured for up to 2 weeks. No inhibitors of

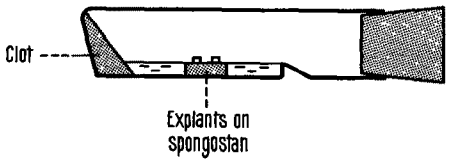

Diagram of culture system.

Illustrative example of fibrinolytic split products in the culture medium (mg/100 ml)

Day	1	2	3	4
Control 1	0	0	0	traces
Control 2	0	0	0	1
Liver	0	0	0	1.5
Lung	3.5	5	100	145
Bone marrow	2.5	9.5	24	67
Kidney	3.5	40	350	450

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