Dissociation of sensory ganglia from the embryonic chick by pronase and other dispersing agents

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The gross effect of protein factors from mouse salivary glands and certain snake venoms on the growth, in tissue culture, of explants of sensory ganglia from chick embryos is well established (Levi-Montalcini, 1964, 1965; Angeletti, Calissano, Chen & Levi-Montalcini, 1967; Banks et al. 1968; Varon, Nomura & Shooter, 1967a, b). The effects of these substances, called nerve growth factors, on individual neurons in vitro have, however, been little investigated and no quantitative studies have been reported. In order to make such a study we required a method of making cell suspensions of high density from chick embryonic sensory ganglia: the target tissue normally used for the assay of nerve growth factors.

Several methods for obtaining dispersed cells from nervous tissue are recorded in the literature and, of these methods, micro-dissection of mammalian central nervous tissue (Hyden, 1959; Lowry, 1962; Hyden & Lange, 1965) appears to result in the least damage to cells. Suspensions of neurons suitable for hand collection and gradient centrifugation have also been prepared from mammalian central nervous tissue by a sieving technique (Roots & Johnston, 1964; Bocci, 1966; Satake & Abe, 1966; Rose 1967), but this method is only suitable for small numbers of cells. In addition, centrifugation of cells isolated by sieving caused readily discernible damage, particularly of embryonic tissue. Various chemical methods, convenient for routine work, involving the breakdown of intercellular binding material have been described. As the binding material is considered (Rinaldini, 1958) to be essentially collagogenous with elastic fibres embedded in a matrix containing metal ions, both proteases and sequestering agents have been used to effect cell dispersion. These methods give a number of relatively undamaged, separated cells. For example, neuroblasts

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from grasshopper have been dispersed by hyaluronidase (Armand & Tipton, 1954), chick neural retina by trypsin (Moscona & Moscona, 1966; Collins, 1966) and mouse brain by sodium tetraphenylboron (Rappaport & Howze, 1966a, b). Sensory ganglia from chick embryos have been dissociated by treatment with proteinase A (Nakai, 1956), by trypsin alone or in conjunction with EDTA (Levi-Montalcini & Angeletti, 1963; Utakoji & Hsu, 1965) and by pancreatin (Cohen, Nicol & Richter, 1964).

Cell suspensions from non-nervous tissues have been prepared with varying degrees of success both from adult and embryonic sources by enzymic methods, by change of pH (Townes & Holtfreter, 1955), by mechanical methods (Berry, 1962) and by treatment with sequestering agents (Anderson, 1953; Curtis & Greaves, 1965; Rappaport & Howze, 1966b). The use of sequestering agents sometimes causes extensive changes in ultrastructure and function (Harris & Leone, 1966; Friedmann & Epstein, 1967). Subcultures, too, may be affected (Rinaldini, 1959) since the agents may have inhibitory effects and are, in any case, difficult to remove from the suspension medium. It is generally felt that enzymic methods are preferable (Rinaldini, 1959; Mateyko & Kopac, 1963). The enzymes used include α-chymotrypsin (Weiss & Kapes, 1966), collagenase (Rinaldini, 1959; Hinz & Syverton, 1959), elastase (Rinaldini, 1959; Weiss & Kapes, 1966), hyaluronidase (Rinaldini, 1959), neuraminidase (Weiss & Kapes, 1966), papain (Mateyko & Kopac, 1963), trypsin (Weiss & Kapes, 1966; Moscona & Moscona, 1966, 1967), pronase (Mintz, 1962; Wilson & Lau, 1963; Gwatkin & Thomson, 1964; Sullivan & Schafer, 1966) and combinations of these proteases (Rinaldini, 1959; Yamada & Ambrose, 1966). The relative effectiveness of dissociating agents in producing cell suspensions from chick heart muscle (Rinaldini, 1959) and adult mouse tumor (Rappaport & Howze, 1966a, b) has been reported.

The preparation of cell suspensions from embryonic sensory ganglia by microdissection on a large scale presents difficulties. Consequently, the dissociating agents listed above have been screened for suitability. We now report a method of preparing cell suspensions from embryonic chick sensory ganglia, heart, brain and adrenal tissue.

**MATERIALS AND METHODS**

Glassware was sterilized by dry heat (120 °C). Solutions were either purchased sterile or were passed through Millipore filters (0.22 μm pore size) and stored at 4 °C before use. Gentle shaking in a tube vibrator (Towers, Widnes) is referred to as mechanical agitation. Pipetting operations were kept to a minimum and stirring was avoided since these procedures resulted in damaged separated cells.
Dissociation of sensory ganglia

Enzymes and other potential dispersing agents


Nerve growth factor (NGF)

NGF from Russell’s viper venom was prepared as previously described (Banks et al. 1968). The material was assayed on whole ganglia, which were cultured on collagen in hanging drops, for each experiment. Only samples which showed an activity of 3+ (see Banks et al.) at a concentration in the culture medium of less than $10^{-9} \text{g ml}^{-1}$ were used.

Dissociation conditions

Embryonic chick sensory ganglia (ca 75) from 7-, 8- or 9-day chick embryos were dissected from the lumbosacral and thoracic regions. (Pronase was also effective in dissociating ganglia from older embryos, e.g. 9–14 days.) The ganglia were placed in either Pannet and Compton’s saline or synthetic medium no. 199 (Glaxo Ltd., Greenford, Middx.) at room temperature, for immediate use. Dissociation was carried out by mechanical agitation at 37 °C in phosphate-buffered saline (Dulbecco & Vogt, 1954) at pH 7-2. The concentrations of dissociating agents, times of incubation and nature of the culture medium are noted in the results section. Enzyme solutions were made up immediately before use to prevent autolysis. Sodium tetraphenylboron was used as specified (Rappaport & Howze, 1966b) except that the suspension was agitated mechanically rather than by pipetting.

Assessment of dispersion

After gentle agitation for the specified periods, cells were separated by centrifugation (350 g, 5 min, room temperature) and the cell pellet was washed twice, for 2 min with 20 % cockerel serum (Flow Ltd., Irvine, Ayrshire), in buffered saline after protease treatment or with saline alone after treatment with non-enzymic reagents. Treatment with serum is said to inhibit proteases
(Gwatkin & Thomson, 1964) but pronase was not completely inhibited by 20 % cockerel serum. 50 % horse serum (Burroughs & Wellcome Ltd., London) was not completely effective either, but 20 % calf serum (Glaxo Ltd., Greenford, Middx.) which had been heated at 56 °C for 1 h prevented further action by pronase.

The washed cell pellet was suspended in tissue culture medium no. 199 (ca. 1 ml) and the cell density was determined using a modified Fuchs–Rosenthal haemocytometer. The best dissociating agents gave an initial cell density, in ca. 1 ml, of ca. $5 \times 10^5$ cells ml$^{-1}$.

Method of culture of dispersed cells

Glass rings (6 mm diameter, 4 mm high) were waxed on to coverslips coated with a dried film of reconstituted rat tail collagen. Cell suspensions (ca. 0.1 ml) were plated dropwise, from a Pasteur pipette, into the rings. Cockerel serum and buffered saline (with and without NGF) were added to give a composition of medium no. 199 : serum : saline of 2 : 1 : 1 by volume; (the amount of NGF added, was that which gave a 3 ± response in the hanging drop assay.) Typically, five pairs of rings were prepared for each cell suspension. The rings were placed in Petri dishes and maintained at 37 °C in the dark, for 24 h, in a water-saturated atmosphere of 5 % carbon dioxide in air. The medium was changed at 72 h intervals when cultures were maintained over a longer period to permit the development of a fibre network.

Fig. 1. Sensory cell culture prepared by a pronase dispersion of 8-day spinal ganglia. Culture grown for 48 h in a medium containing purified nerve growth factor. Methylene blue stain.
Dissociation of sensory ganglia

At the end of the incubation period, the rings were removed and the cultures were stained with methylene blue (0.01 % w/v in saline) for 30–40 min at 37 °C, fixed in ammonium molybdate solution (8 % w/v in glass distilled water) at 4 °C for 2–4 h and then washed with glass distilled water (10 min), rapidly dehydrated and mounted in Canada balsam for microscopic examination. Some cultures were stained with silver (Holmes, 1943), after fixation in a mixture of 10 % neutral formalin (5 %): glacial acetic acid (5 %): 80 % ethyl alcohol (90 %).

Characterization of dispersed cells

A considerable proportion of the cells resulting from the most successful dispersion methods were shown to be capable of sending out fibres in culture. The cells of interest usually contained two visible nucleoli and had a characteristic elliptical perikaryon (ca. 5 x 9 µ), which was hyperchromic under phase contrast. After staining with methylene blue, the cytoplasm became more intensely coloured than the nucleus while silver staining of cultured cells showed clearly defined neurofibrils. Fig. 1 shows a portion of a methylene blue stained culture.

Unsuccessful methods of dispersion and culture gave cells with spherical perikaryons and granulated cytoplasm and such cells were more uniformly stained with methylene blue. These cells produced stunted or completely retarded fibre growth after 24 h culture.

RESULTS

The results of various dispersion methods were assessed after culture of dispersed cells had been maintained for 24 h, with and without nerve growth factor in the culture medium. Cell density was scored as nil, low, medium or high, the proportion of neurons as 0, 1, 2 or 3 corresponding approximately to zero, 5, 15 and 25 % of the total cell population and the condition of neurons as 1, 2 or 3.

The dispersing agents which were relatively effective are listed in Table 1 with the relevant conditions and the results in terms of the density of cells and the proportion and condition of neurons.

Other reagents used without success are listed in Table 2.

Studies on pronase-dispersed cells

The optimum conditions for cell dispersion by pronase were found to be mechanical agitation and incubation with 0.01 % w/v pronase solution for 15 min at 37 °C either in Pannet and Compton’s saline or phosphate-buffered saline. In the resulting suspensions typically fewer than 10 % of cells were aggregated. In plated cell cultures which had been grown on collagen films for 4 h up to 30 % of the cells were nerve cells.
Table 1. Dispersion of cells of ganglia by different treatments

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Concen (w/v) %</th>
<th>Time (min)</th>
<th>NGF</th>
<th>Density</th>
<th>Proportion neurons</th>
<th>Condition</th>
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<tr>
<td>1</td>
<td>Trypsin</td>
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<td>2</td>
<td>α-Chymotrypsin</td>
<td>0.5</td>
<td>30</td>
<td>+</td>
<td>Med.</td>
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<td>3</td>
<td>Pancreatin</td>
<td>1</td>
<td>30</td>
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<td>0.15</td>
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<td>0.005</td>
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<td>Negligible</td>
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<td>4</td>
<td>Elastase</td>
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<td>5</td>
<td>Protease I</td>
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<td>30</td>
<td>+</td>
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<tr>
<td>6</td>
<td>Protease II</td>
<td>0.2</td>
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<td>+</td>
<td>Negligible</td>
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<td>7</td>
<td>Pronase</td>
<td>0.25</td>
<td>15</td>
<td>+</td>
<td>High</td>
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<td>0.2</td>
<td>10</td>
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Culture of pronase-dispersed cells on collagen

Microscopic examination (× 100 or × 400 final magnification) of cells grown on collagen showed that within 1 h of plating, a considerable proportion of neurons (elliptical in shape) had settled and attached to the substrate. Neurons, particularly from embryos which were more than 11 days old, sometimes possessed fibre stumps. Within 2 h of plating, some anchored neurons had started to develop fibres while appreciable fibre development was apparent at the end of a further hour. Microscopic examination, necessarily intermittent since the dispersed neurons were light sensitive, also indicated that both fibre outgrowth and withdrawal occurred and that some neurons, often with well-developed processes, rotated or migrated in the culture medium. Within 24 h
Dissociation of sensory ganglia

of plating, a fibre network had developed and cell aggregation was apparent. Most fibres were linked either to other fibres or to perikaryons but a few appeared to terminate on other types of cells or cell debris. A considerable number of neurons appeared to be bipolar. Supporting cells could be recognized from their characteristic morphology in cell cultures.

Table 2. Unsuccessful treatments

<table>
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<tr>
<th>Reagent</th>
<th>Conditions</th>
<th>Comments</th>
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<tr>
<td>Hyaluronidase</td>
<td>0·5 % w/v, 30 min</td>
<td>No effect</td>
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<tr>
<td>Pepsin</td>
<td>0·5 % w/v, 30 min, pH 7·2</td>
<td>No effect</td>
</tr>
<tr>
<td>EDTA</td>
<td>0·001 % w/v, in Ca-Mg-free saline, pH 8 (NH₄Cl)</td>
<td>No effect</td>
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<tr>
<td>Papain</td>
<td>0·2 % w/v, 0·02 % cysteine hydrochloride, 0·05 %</td>
<td>Partially or totally disintegrated</td>
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<td></td>
<td>w/v, pH 7·2</td>
<td>the ganglia but no intact cells</td>
</tr>
<tr>
<td>Sodium tetraphenyboron</td>
<td>0·1 %, 10–12 min, 20–37°C</td>
<td>Destruction of ganglia</td>
</tr>
<tr>
<td>Collagenase</td>
<td>0·05 or 0·01 % w/v, in buffered saline 20–25 min</td>
<td>Cell debris plus fibroblasts pro-</td>
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<tr>
<td>Melittin</td>
<td>0·1 % w/v, 10–30 min</td>
<td>No effect</td>
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</table>

Culture of pronase-dispersed cells on plasma clot

Fibre growth is more readily assessed in supporting than in liquid media. Culture of cell suspensions in plasma clot resulted in an average fibre growth of ca. 70 μ, compared with an average outgrowth of ca. 300 μ from whole ganglia, in hanging drop cultures, under the same conditions. In the presence of nerve-growth factor, the fibre growth in plasma clot averaged ca. 100 μ after 24 h culture with some exceptional growths to ca. 400 μ. In addition, culture in plasma clots in the presence of nerve-growth factor resulted in the development of processes by a higher proportion of the neurons present. About 90 % of all neurons in cultures on plasma clot were unipolar.

Removal of cultured cells from collagen and glass surfaces

Trypsin (0·03 %, 10 min, 37 °C) is commonly used to remove cultured cells from collagen and glass surfaces. Pronase (0·001 %) was also found to be highly effective; 5 min gentle agitation of sensory cell cultures grown on collagen and glass supports yielded suspensions of single cells.

Dissociation of other tissues by pronase

Under the optimum conditions described for treatment of embryonic sensory ganglia, pronase was found to be effective in giving single cell suspensions from heart and brain tissue (cut into 1 mm cubes) of 7–16 day chick embryos and from
adrenal tissue of 13–16-day-old embryos. More concentrated pronase (0.05 % w/v) was effective in the dissociation of spinal cord from 7–16-day-old embryos. The cell suspensions were not examined in detail, but neurons were readily discernible in the suspensions from central nervous tissue and adrenals while morphologically characteristic cells were seen in the suspensions from heart tissue. Little debris was found in these experiments.

Sympathetic spinal ganglia, pooled from six 7–13-day embryos, when treated with pronase (0.2 % w/v for 35 min) did not give true single cell suspensions. The cells tended to clump but individual cells behaved in tissue culture in a manner similar to that observed in the culture of cells from sensory ganglia.

DISCUSSION

The successful use of a pronase as a dissociating agent has been reported for a number of tissues including chick embryo muscle (Wilson & Lau, 1963), whole chick embryo (Gwatkin & Thomson, 1964), adult and embryonic mouse tissue (Mintz, 1962; Gwatkin and Thomson, 1964), various epidermal tissues (Sullivan & Schäfer, 1966) and Hela cells (Niitsu & Handa, 1965). On the basis of these results, pronase has come to be regarded as a better dissociating agent than trypsin for these tissues. However, a report that this same enzyme causes severe damage to neurons from ox brain (Bocci, 1966) confirms the fact that the choice of dissociating agent and the optimal conditions for dissociation depends critically on the particular tissue being studied.

In our hands, pronase has been used successfully to obtain suspensions of single cells from chick embryo sensory ganglia, heart, brain, spinal cord and adrenal tissue and, with somewhat less success, from sympathetic spinal ganglia.

The cell suspensions from sensory ganglia could be cultured, and maintained for at least 7 days, on collagen films in a medium containing 25 % fresh cockerel serum (at the high cell densities used). Well-developed fibre networks were observed within 24 h of plating, both with and without the addition of NGF if the culture medium was supplemented with 25 % cockerel serum or 10 % horse or calf serum. However, although large numbers of sensory nerve cells survive to regenerate a network of neurites when cultured under these especially favourable conditions, without the addition of purified NGF, it must be remembered that NGF is present in the serum which constitutes a large part of the culture medium.

Further, the similarity between dissociated cell cultures treated with NGF and their controls is apparent only at the favourably high cell densities recorded. At lower cell densities appreciably fewer nerve cells survive to regenerate nerve fibres in untreated control cultures. Omission of serum from the culture medium leads to a marked irreproducibility in the resulting cultures. These cultures may be maintained for at least 5 days in a medium 199 containing NGF and nerve cells survive to regenerate the extensive network of nerve fibres described above.
Appreciable numbers of supporting cells also survive, although markedly fewer than when serum is included in the medium. Sensory cell cultures plated in medium 199 in buffered saline alone typically show massive degeneration within 72 h but small numbers of neurons can survive at least 5 days. (The role played by NGF in dissociated sensory nerve cell cultures has been investigated in some detail and the results of these experiments will be published later.) The high quality of cultures prepared by pronase dispersion and grown under our conditions without the addition of NGF might be contrasted with previous reports (Levi-Montalcini & Angeletti, 1963; Cohen, Nicol & Richter, 1964; Utakoji & Hsu, 1965) that cell suspensions obtained by the dissociation of embryonic sensory ganglia by other enzymes only developed fibre networks when cultured in a liquid medium to which NGF had been added. Even in culture media containing NGF, fibre networks were developed more slowly from trypsin-dispersed cells than from the pronase-dispersed cells described in the present work. The effect of trypsin as a dissociating agent has been largely confirmed in the present work, the cell suspensions from embryonic sensory ganglia being cultured reproducibly only in the presence of NGF in addition to serum. Without NGF in culture medium, trypsin-dispersed cell cultures progressively deteriorated over a period of 24 h although occasionally excellent fibre networks were established within this period of culture.

These results are consistent with the view that the dissociation of chick embryo sensory ganglia by pronase gives cells which are less damaged than those obtained by carrying out the dissociation with trypsin.

Most proteases tested in the present work gave cell suspensions from sensory ganglia of varying degrees of viability. Hyaluronidase and pepsin had little effect on the same tissue while collagenase and papain were too destructive. Sodium tetraphenylboron was also found to be too destructive and EDTA was without effect.

**SUMMARY**

1. The effectiveness of a number of proteolytic enzymes and metal chelating agents in promoting the dissociation of chick embryo sensory ganglia has been examined.

2. The enzyme pronase was found to be the most satisfactory dispersing agent.

3. Sensory nerve-cell cultures prepared by a pronase dissociation can be maintained on collagen supports in a serum supplemented-liquid medium for at least 7 days, during which time an extensive network of nerve fibres develops.

4. The nerve-growth factor requirement for the maintenance of nerve-cell viability and the regeneration of nerve fibres is briefly discussed.

5. Conditions under which pronase treatment gives single cell suspensions from chick embryo heart, brain, adrenal tissues and spinal cord are described.
RÉSUMÉ

Dissociation de ganglions sensitifs d'embryons de poulet par la pronase et autres agents de dispersion

1. L'efficacité d'un certain nombre d'enzymes protéolytiques et d'agents chélateurs pour dissocier les ganglions sensitifs d'embryons de poulet a été examinée.

2. L'enzyme pronase s'est montrée l'agent de dispersion le plus satisfaisant.

3. Des cultures de cellules nerveuses sensibles préparées par une dissociation à la pronase peuvent être maintenues sur des supports de collagène dans un milieu liquide contenant du sérum pendant au moins 7 jours, période pendant laquelle se développe un important réseau de fibres nerveuses.

4. L'exigence d'un facteur de croissance nerveuse pour le maintien de la viabilité de la cellule nerveuse et pour la régénération de fibres nerveuses est brièvement discutée.

5. Les conditions dans lesquelles un traitement à la pronase donne des suspensions de cellules isolées en provenance de coeur d'embryon de poulet, de cerveau, de tissu surrénal et de moelle épinière sont décrites.

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