HISTOCHEMISTRY

Acid Mucopolysaccharides in the Cuticle of the Gizzard of Earthworms

A HISTOCHEMICAL examination of the cuticle of the gizzard of earthworms has recently been made on *Eisenia foetida* S. by Van Gansen-Semal[1], who suggested the presence of a non-fibrillar elastin. We have undertaken the investigation recorded here on a different species, *Lumbricus terrestris* L.

The results of the diverse techniques were as follows:

(1) *Tests for lipids*	
Sudan black *B*	(−)
Sudan III and IV	(−)
Nile blue sulphate	(−)
(2) *Tests for proteins*	
Ninhydrin–Schiff method	(−)
Alloxan–Schiff method	(−)
Millon reaction (Bensley-Gersh)	(−)
Coupled tetrazonium reaction	(−)
Barrnett and Seligman reaction (DDD)	(−)
DDD following thioglycollic acid treatment	(−)
Performic acid–Schiff reaction for SS groups	(−)
(3) *Tests for elastic tissue*	
Resorcin method (Weigert)	(−)
Orcein in acid (hydrochloric or nitric) alcohol	(−)
Aldehyde–fuchsin (Gabe) without previous oxidation	(+)
Aldehyde–fuchsin following methylation	(−)
Aldehyde–fuchsin following methylation and demethylation	(+)
(4) *Tests for glycogen and mucopolysaccharides*	
Best's carmine method	(−)
Periodic acid–Schiff (PAS)	(+)
PAS following ptyalin extraction	(+)
PAS following acetylation	(−)
PAS following acetylation and saponification	(+)
Toluidine blue metachromasia	(+)
Alcian blue 8 *GS*, *p*H 3	(+)
Alcian blue following methylation	(−)
Dialysed iron method (Hale)	(+)
Dialysed iron method following methylation	(−)
Schulze reaction (chlor-zinc-iodine)	(−)
Testis hyaluronidase extraction	(−)
Bacterial hyaluronidase extraction	(−)
(5) *Tests for inorganic constituents*	
Perl's reaction	(−)
Von Kòssa method	(−)

The details concerning the various techniques used have been described elsewhere[2].

These results exclude the presence of lipids, free amino radicals, tyrosine, tryptophan, histidine, SH and SS groups and of elastic tissue. The staining with aldehyde–fuchsin is attributable to sulphate ester groups and not to elastic fibres since the staining disappears after methylation[3] and reappears after demethylation.

The presence of mucopolysaccharides is established by the positive periodic acid–Schiff reaction, its persistence after ptyalin extraction, its disappearance after acetylation and its reappearance after saponification.

The acid character of these mucopolysaccharides or of certain among them, if several varieties exist in the cuticle, is demonstrated by the metachromasia of toluidine blue, the positive staining by Alcian blue and the positive Hale reaction. Alcian blue staining and its control by methylation are considered by Lison[3] as one of the best methods for the demonstration of acid mucopolysaccharides.

Under certain conditions, chitin can also be stained by the periodic acid–Schiff, Alcian blue and the Hale reactions[4], but its presence here appears to be eliminated by the metachromasia of toluidine blue and the negative Schulze reaction.

The testicular and bacterial hyaluronidases have no action on these acid mucopolysaccharides. Therefore, this excludes the presence of hyaluronic acid, keratosulphates and of chondroitin sulphates *A* and *C* (ref. 5).

Finally, we may conclude from the negative results for inorganic constituents that, in spite of its hardness, the cuticle of the gizzard of *Lumbricus terrestris* L. is not coated with mineral salts.

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Culture of Replicate Monolayers of Fibroblasts under Identical Conditions

IN cell culture investigations, the degree of 'conditioning' of the medium during growth is frequently of crucial importance[1,2]. With the usual techniques of monolayer cell culture which use multiple Petri dishes, Leighton tubes, French square and prescription bottles, etc., small variations in *p*H, temperature, cell inoculum size, degree of attachment and growth of cells, volume of medium delivered, degree of evaporation of medium, etc., are compounded so that the degree of 'conditioning' frequently varies from preparation to preparation.

An obvious solution to this problem is to grow all the monolayers required for any given experiment in the same container, so that they are exposed to a common pool of medium. This can readily be accomplished by grouping a series of coverslips in a vertical array. Containers of various capacities can be improvised from glass or plastic (preferably polypropylene, which can be autoclaved). Aluminium coverslip racks are readily constructed and can be coated with polypropylene. Stainless steel coverslip racks are commercially available (Lipshaw, Detroit). The technique which I have most commonly used, however, involves the use of replicate monolayers grown on 22-mm square coverslips in Columbia staining jars. These commercially available glass jars (A. H. Thomas and Co., Philadelphia) are designed to accommodate four coverslips, but we have regularly grown 8 coverslip monolayers in a jar (4 back-to-back pairs) without difficulty. The jars can be provided with screw caps lined with white rubber.

My experience is limited to established diploid fibroblast strains derived from skin, usually new-born foreskins, established by either explant or trypsinization techniques. The cells were grown in Waymouth's medium or in modifications of this medium to be described in detail in a subsequent publication. The modified media were designed to permit more rigorous *p*H control and to limit growth rate for certain experiments. They usually contained *tris* (hydroxymethyl) aminomethane-hydrochloric acid buffers and decreased amounts of bicarbonate and amino-acids. Calf serum was added to the media to give a final concentration of approximately 10 per cent.

Stock cultures, maintained in 6-oz. prescription bottles, were collected with 0·05 per cent Difco trypsin and 0·54 mM disodium ethylenediamine tetraacetic acid in a *tris*-buffered (*p*H 7·4) balanced salt solution and plated at concentrations of 20–100,000 cells/ml. after dilution by media. Two or 2·5 ml. of the cell suspensions were plated in 35-mm plastic Petri dishes (Falcon blue label, Los Angeles) which had previously been loaded with cover slips. A 'pre-pipetter' (custom made sizes are available through Aloe Scientific, St. Louis) was found quite useful for efficiently and accurately delivering replicate samples. When it was necessary to prepare numer-