The organisms used and their method of growth are listed in the Table. Suspensions were centrifuged, the deposit resuspended in water and an aliquot treated with alkaline glutaraldehyde for 2 h at 37 °C. The treated suspensions were then centrifuged, and the colour of the cell deposit examined visually. The results (Table) show that a red colour develops only with Gram-negative bacteria, although a yellow or light orange colour is also produced with vegetative and spore forms of various bacilli and with fattened cells of *Bacillus subtilis*.

In *E. coli*, the red colour is the result of combination of alkaline glutaraldehyde with the non-mucopeptide layers of the cell wall, probably the protein components, and is not caused by interaction with cytoplasmic constituents. The cell wall of *Proteus vulgaris* is similar in structure to that of *E. coli*[12], and if it is assumed that interaction of glutaraldehyde with cell wall protein is responsible for the red colour in all cases (this is also borne out by the finding that fattened cells of the Gram-positive organism, *B. subtilis*, give no increase in visual colour over unfattened cells) then this can be accounted for by the similarities in the structure and chemical composition of the walls of Gram-negative bacteria. In contrast to the large number of amino acids in these walls, the cell walls of Gram-positive cocci contain mucopeptide and teichoic acid[13], the mucopeptide consisting of some 5 main amino acids. It is, however, unlikely that the red colour observed when Gram-negative bacteria are treated with alkaline glutaraldehyde is contributing to their death, as a) cell death is apparent some time before the colour is visible, b) Gram-positive cocci, which do not show a red colour, are killed just as rapidly. The development of the red colour is thus concerned with differences in cell wall chemistry.

Résumé. La glutaraldéhyde produit une couleur rouge chez quelques genres de bactéries. Cette couleur est prise par la paroi cellulaire des organismes.

T. J. MUNTON and A. D. RUSSELL

Welsh School of Pharmacy, University of Wales Institute of Science and Technology, Cardiff (Wales, UK), 7 July 1970.

[1] R. E. PEPPER and V. L. CHANDLER, Appl. Microbiol. *11*, 384 (1963).
[2] A. A. STONEHILL, S. KROP and P. M. BORICK, Am. J. Hosp. Pharm. *20*, 458 (1963).
[3] P. M. BORICK, F. H. DONDERSHINE and V. L. CHANDLER, J. Pharm. Sci. *53*, 1273 (1964).
[4] P. W. ROSS, J. clin. Path. *19*, 318 (1966).
[5] S. D. RUBBO, J. F. GARDNER and R. L. WEBB, J. appl. Bact. *30*, 78 (1967).
[6] M. H. RITTENBURY and M. E. HENCH, Ann. Surg. *161*, 127 (1965).
[7] R. W. SNYDER and E. L. CHEATLE, Am. J. Hosp. Pharm. *22*, 321 (1965).
[8] V. LANE, J. D. MCKEEVER and M. FALLON, J. Irish med. Ass. *58*, 131 (1966).
[9] F. M. RICHARDS and J. R. KNOWLES, J. molec. Biol. *37*, 231 (1968).
[10] A. F. S. A. HABEEB and R. HIRAMOTO, Arch. Biochem. Biophys. *126*, 16 (1968).
[11] T. J. MUNTON and A. D. RUSSELL, J. appl. Bact., *33*, 410 (1970).
[12] R. E. BURGE and J. C. DRAPER, J. molec. Biol. *28*, 173, 189, 205 (1967).
[13] M. J. OSBORN, Ann. Rev. Biochem. *38*, 501 (1969).

PRO EXPERIMENTIS

Vital Staining of Neurosecretory Material with Acridine Orange in the Insect, *Periplaneta americana*

Neurosecretory materials vary in their chemical composition between different neurosecretory cells and no one staining technique has been found to stain all types of neurosecretory material[1]. In this paper it is shown that acridine orange can be used as a vital stain for all neurosecretory materials.

Vital staining of cells with acridine orange produces an orthochromatic green fluorescence in nuclei and sometimes a metachromatic red fluorescence in cytoplasmic granules. It is now generally accepted that green nuclear fluorescence is due to nucleic acids and that the red cytoplasmic granules are lysosomes[2–8], however, see AUSTIN and BISHOP[9] for an alternative interpretation. As well as staining with acridine orange, lysosomes show acid phosphatase activity amongst other hydrolytic enzymes[2–5].

Materials and methods. Adult and nymphal forms of the cockroach, *Periplaneta americana* from laboratory colonies were used in this study. The following stains were obtained from Chroma-Gesellschaft; acridine orange, acridine yellow, coriphosphine, acriflavine, phosphin 3R, euchrysin 3RX, and euchrysin 2G. All are acridine derivatives. They were dissolved in 0.9% NaCl at a concentration of 0.1 mg/ml.

Pieces of nervous tissue were dissected from the insect under saline and placed in a drop of stain solution on a microscope slide. The tissue was stained for 1 min and then moved to a drop of saline further along the slide. A coverslip was added and excess saline removed. The whole mount was then viewed with blue light from a Wild microscope fluorescence system.

For acid phosphatase localization, tissue was fixed in formol-calcium over-night. The Gömöri lead method and the simultaneous coupling azo dye method using naphthol AS-TR and hexazotized pararosaniline were used[10]. Incubations without substrate were used as controls.

The stains were analyzed by thin-layer chromatography using Kieselgel and *n*-butanol:ammonia:ethanol:water (16:0.15:5:5) as developing solvent[11]. The chromatograms were viewed with long-wave UV-light.

Results. Differentiation of neurosecretory cells and 'ordinary' nerve cells was obtained with acridine orange, euchrysine 3RX, and coriphosphine, however the latter showed only weak differentiating ability. Negative results were obtained with the other stains. Neurosecretory material appeared as red fluorescing granules while the cytoplasm of 'ordinary' nerve cells and axons was a uniform weakly fluorescent green. Nuclei of glial and nervous tissue showed a strong green fluorescence with distinct nucleoli. The cytoplasm of glial cells was not stained. The red fluorescence was not stable under continuous irradiation and faded after 5 min. The nuclear

staining retained its colour for up to 15 min before turning yellow. After 20 min irradiation the tissue took on a general red tint which has been taken as indicative of cell death[6].

Suitable tissue for comparison of neurosecretory and 'ordinary' axons were the lateral cardiac nerves in which the 2 types of axons are fairly well partitioned into 2 bundles[12]. After staining, the more lateral bundle of neurosecretory axons was found to contain dense masses of red granules while the 'ordinary' axons were a weakly fluorescent green. The neurosecretory axons of the lateral cardiac nerves cannot be stained by the classical neurosecretory stains, but at the ultrastructural level they have been shown to contain elementary neurosecretory granules[12].

In the head, neurosecretory cells in the pars intercerebralis could be distinguished from neighbouring cells because their cytoplasm was almost completely filled with red granules while 'ordinary' nerve cells contained only a few large red granules which were probably lysosomes. Also, the axons of these cells contained considerable numbers of small red granules. The nerves to the corpus cardiacum showed red granules in some axons and only weak green general staining in others. The corpus cardiacum itself possessed scattered red granular accumulations among the intrinsic cells whose nuclei stained green. This distribution of neurosecretory material agrees with electron microscope studies made by SCHARRER[13]. Nerves leading to, around, through, and away from the corpus allatum all exhibit large accumulations of red granules. Those axons passing through the corpus allatum often had a varicose arrangement of red granules. Again, this distribution agrees with electron microscope studies made by SCHARRER[14]. Also, the median nerves of the ventral nerve cord exhibit red granules in some of the axons proximal and distal to the neurohaemal organs[15,16].

Since it was thought that neurosecretory material in other animals may give a similar reaction with acridine orange, the following representative systems which contain neurosecretory material were investigated; the eyestalk and pericardial organ of the crab, *Paragrapsus gaimardii*, the retrocerebral complex of *Calliphora stygia*, the heart of *Helix aspersa*, and the cerebral ganglion of the earthworm, *Megascolides polynephricus*. In each case neurosecretory cells and/or axons could be distinguished from 'ordinary' nerve cells and axons.

Acridine orange and euchrysine 3RX gave similar chromatograms which showed 6 (possibly 7) components with the slowest moving spot being the major constituent. The chromatogram of coriphosphine showed a fairly strong spot with an Rf value and fluorescent colour similar to the major component of acridine orange, but the other spots were all different in Rf value and/or fluorescent colour. Acid phosphatase was not localized in either type of axon in the lateral cardiac nerves by either of the methods used.

Unstained whole mounts of lateral cardiac nerves were viewed under dark field illumination. Neurosecretory material exhibited its characteristic blue colour in the form of granules or dense accumulations. The distribution of neurosecretory axons revealed by this method was similar to the distribution shown by vital staining with acridine orange.

Discussion. The metachromatic red fluorescence of neurosecretory material when stained with acridine orange allows a rapid method for identifying neurosecretory cells and axons. All the known sites of neurosecretory material in the insect nervous system give positive results by this method whereas previously different staining techniques and electron microscopy have been necessary to show the presence of all types of neurosecretory material. A similar staining method[17] has been used on the nerves in the mesentery of the frog, *Rana temporaria* and the cerebral ganglion of *Lumbricus terrestris* and it was found that the nerve cell bodies and axons contain red granules. Some of the granules have a varicose arrangement in the axons. This, together with the positive reaction in the neurosecretory cells and axons of the other animals used in this study, would indicate that all neurosecretory granules have some common feature which makes them stain with acridine orange. Whether this a particular chemical compound or a particular type of membrane structure which combines with acridine orange is not known. However, the entities responsible possess a highly orientated array of negative binding sites which are spaced at a distance which allows interaction between the adjacent acridine orange molecules[11].

The absence of acid phosphatase in the neurosecretory axons would suggest that it is not the presence of lysosomes which produce the metachromatic staining, but that the neurosecretory material itself takes up this stain. Whether this similarity in staining is due to similar compounds or membranes is unresolved, but both lysosomes and neurosecretory granules contain biologically active substances in a latent form[1,5].

The thin layer chromatographic analysis of the 3 stains which gave positive results indicate that acridine orange and euchrysine 3RX are the same, or very similar, products and that coriphosphine is contaminated with acridine orange. It is this which gives metachromatic staining of neurosecretory material[18].

Résumé. On expose une méthode rapide de la coloration vitale à l'acridine orange des produits de neurosécrétion dans le tissu nerveux des insectes. Irradiés avec la lumière bleue, tout les types connus de produits de neurosécrétions entrent en fluorescence en prenant une teinte métachromique rouge.

T. M. BEATTIE

University of Tasmania, Department of Zoology, Hobart (Tasmania 7001, Australia), 4 May 1970.

1 M. GABE, *Neurosecretion* (Pergamon Press, Oxford, London 1966).
2 A. C. ALLISON and M. R. YOUNG, Life Sci. *3*, 1407 (1964).
3 P. P. H. DE BRUYN, R. S. FARR, H. BANKS and F. W. MORTHLAND, Expl. Cell Res. *4*, 174 (1953).
4 H. KOENIG, J. Cell Biol. *19*, 87A (1963).
5 A. B. NOVIKOFF, in *The Neuron* (Ed. H. HYDEN; Elsevier Publishers Co., Amsterdam 1967), p. 319.
6 E. ROBBINS and P. I. MARCUS, J. Cell Biol. *18*, 237 (1963).
7 E. ROBBINS, P. I. MARCUS and N. K. GONATAS, J. Cell Biol. *21* 49 (1964).
8 A. V. ZELENIN, Nature *212*, 425 (1966).
9 C. R. AUSTIN and M. W. BISHOP, Expl. Cell Res. *17*, 35 (1959).
10 T. BARKA and P. J. ANDERSON, *Histochemistry: Theory, Practice and Bibliography* (Harper and Row, New York 1963).
11 F. H. KASTEN, Int. Rev. Cytol. *21*, 141 (1967).
12 B. JOHNSON, J. Insect Physiol. *12*, 645 (1966).
13 B. SCHARRER, Z. Zellforsch. *60*, 761 (1963).
14 B. SCHARRER, Z. Zellforsch. *62*, 125 (1964).
15 N. DE BESSE, C. r. Acad. Sci., Paris *263*, 404 (1966).
16 J. BRADY and S. H. P. MADDRELL, Z. Zellforsch. *76*, 389 (1967).
17 K. ZEIGERS and H. HARDERS, Z. Zellforsch. *36*, 62 (1951).
18 I am indebted to Prof. B. JOHNSON for helpful suggestions and criticisms. This study was supported by a University of Tasmania Research Grant and the author has received financial support from I.C.I.A.N.Z. and University of Tasmania Post-Graduate Scholarships.