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MATHEMATICS, STRUCTURALISM AND BIOLOGY

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(Received: 1987–12–15)

A new approach is gaining ground in biology, one that has much in common with the structuralist tradition in other fields. It is very much in the spirit of an earlier view of biology and indeed of science in general. It is also, though this is not generally recognized, in the spirit of twentieth century physics. As in modern physics, however, it is not a question of ignoring all the progress that has been made within the former paradigm. On the contrary, the aim is to use it as a basis for setting out in a somewhat different direction. Complex phenomena do not generally lend themselves to reductionist analyses which seek explanation only in terms of detailed mechanisms, but a proper scientific discussion of structure must make full use of what we have already learned — by whatever means — about the processes that underly the phenomena we are trying to understand.

Keywords: Mathematics, structuralism, biology, Drosophila, catastrophes

INTRODUCTION

The term "structuralism" comes originally from linguistics, where it is associated with the ideas of Ferdinand de Saussure. The basic idea is that a language should be studied as a unity, rather than as a collection of words or phonemes. The analysis should be "synchronic", dealing with the

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interrelationships at a fixed time, rather than "diachronic", dealing with changes in individual units through time. A word is not necessarily to be identified with the same word used at a different time, since the two are parts of different structures, just like two apparently identical words in different contemporary languages.

Now even in linguistics the word "structuralism" can be used in more than one sense, and as it has been adopted in other fields it has acquired correspondingly more shades of meaning. The best known description is probably that given by Piaget /8/, the study of systems which concentrates on wholeness, on transformations and on regulation, but even this does not cover all structuralist work well enough to be acceptable as a definition.

It is clear, however, that the distinguishing feature of any structuralist approach is that phenomena are to be considered essentially as wholes and that it is the relations between the units, i.e. the structure, that is of primary concern. Thus Lyons /7/ writes that the central thesis of structuralist linguistics is that we cannot determine the units first and then inquire what the relationships are between them; the units can only be identified in terms of their relationships with other units. So whatever else may be involved in any particular version of the approach, the essence is the stress on wholeness and structure.

If we take this as defining structuralism, allowing for variations in detail in different fields and among different workers, we see the approach of Saussure and his successors as the modern form of a point of view which has existed for centuries, rather than as something which has appeared relatively recently and without warning. This gives us a better insight into some current developments in both mathematics and biology and indeed into structuralism itself. Besides, if we believe that transformation and regulation are crucial properties of systems, then we may expect the system of thought within which we hold this view to exhibit those properties as well.

The idea that the emphasis on structure is the core, and that properties such as regulation and transformation are more recent developments, is borne out by the common usage of the word "structure" in the English language. According to the Oxford English Dictionary, it first meant something static, like a building, but it later acquired a more dynamic connotation as well, as in an organism or a society.

STRUCTURALISM AND SCIENCE

In many cultures, and even in Western culture for much of the time, science has been, on the broader definition, structuralist. We do not find in Greek or mediaeval science the concentration on mechanistic accounts that we are now accustomed to. The aim was to show how phenomena fitted into the pattern of the universe. And because mathematics is the study of patterns, many of the accounts were in mathematical terms. The motion of heavenly bodies was supposed to be on spheres. Kepler tried to account for the distances between the successive planetary orbits in terms of quantities derived from the regular polyhedra. There was a great deal of numerology, from the four elements and the four humours to the intricate arguments of the Cabbalists, who made great play of the fact that in Hebrew the letters of the alphabet are also used as numbers.

But the justification for using mathematics was not at all the same one that we would give to-day. Spheres were chosen because they were seen as perfect forms, the regular polyhedra because there are only five of them (whereas there are infinitely many regular polygons). In both cases it was assumed that since these are special objects in mathematics they must also be special objects in science, mathematics itself being considered at the time to be a science.

Note, by the way, that this was essentially a synchronic structuralism; the heavenly bodies obviously moved but there was no idea of an evolution of the orbits themselves.

Everything changed with the Newtonian revolution. Explanations were now supposed to be in terms of mechanisms. Planets went around the sun in elliptical orbits not because conic sections are especially simple or important mathematically, but as a consequence of Newton's inverse square law of gravity. It is surely significant that gravity was held to be a force, just like a pull on a string, despite the objections of those who refused to accept the idea of a force that acts at a distance.

As structuralism was replaced by a reductionist mechanism, mathematics lost its primary place. It was still important, and indeed there was a tremendous development of the subject to meet the needs of science, but it was no longer a source of explanation in itself. Its role was to make more efficient the working out of the consequences of the laws of physics. One result of this was that much of the great progress that was made in mathematics was in those areas that are well suited to a reductionist approach.

The branch of the subject which gained the most was analysis, which is based on what happens in the neighbourhood of a single point. This of course increased the power of reductionism, and so even further contributed to the weakening of structuralism.

Biology too eventually moved away from the structuralist point of view. The rational morphology of the eighteenth century gave way to the Darwinism and cell theory of the nineteenth and the neo-Darwinism and molecular biology of the twentieth. Structure and form were no longer primary; they became secondary phenomena to be explained in terms of function through the mechanism of natural selection. Darwinism is not part of the Newtonian theory but it is part of the Newtonian paradigm, and indeed many biologists have seen physics as the model science to which their own should strive — though by this they mean the physics of the last century rather than the quite different paradigm of quantum mechanics and relativity.

Both these subjects have more in common with the pre-Newtonian structuralism than with the emphasis on mechanism and reduction that replaced it. For example, as a result of the development of general relativity, the "force of gravity", which many earlier workers objected to, has vanished from physics. The universe is now considered to be a four-dimensional curved space-time along whose geodesics move any particle moving under no forces — bearing in mind that gravity is now not considered to be a force.

Thus the planetary orbits again depend not on forces but on the structure of the universe. In turn, the structure of the space-time is essentially specified by its metric tensor, and the components of that tensor are determined by a set of partial differential equations, the "field equations". These are generally written in the form

$$R_{ab} - \frac{1}{2} R g_{ab} = -\kappa T_{ab} \quad .$$

Here the left hand side is a shorthand for a complicated combination of the metric tensor and its partial derivatives up to second order, while the right hand side is made up of terms representing the velocity, density and pressure of the matter in the universe.

To compute the orbit of a planet around the sun, we make the assumption that if the only massive body in the universe is a static sphere then the universe should be spherically symmetric and static. We solve the field equations subject to this condition, and then solve the geodesic

equations. At no time do we introduce the idea of a force, and the mass of the sun enters into the problem only as a constant of integration which is fixed by comparing a limiting case of the equations of radial geodesics with the Newtonian law of attraction.

Thus while there is still a connection between the material in the universe and the paths of particles, there is no causal relation in the sense that is familiar to us from Newtonian mechanics. The field equations are not derivable (as Laplace's and Poisson's equations are) from a hypothesis about the forces between individual masses. They are equations which have been arrived at by essentially heuristic arguments to describe the structure of space-time, and while they have been chosen to reflect certain ideas of what physics should be like and also to reduce to the classical equations in a suitable limit, they are essentially mathematical statements rather than translations into mathematics of physical ones.

Note how the whole idea of force as a fundamental concept is thus being eroded. In some cases it may still be appropriate, and in others it may serve as a convenient linear approximation, but just as we no longer think in terms of a gravitational force so it is too simplistic to see the interaction between an organism and its environment in terms of a single force, natural selection, which for all practical purposes may be considered to be exerted by the larger entity on the effectively passive smaller one.

BIOLOGY

To adopt a structuralist approach in biology is not to argue for a form of vitalism, or to deny that biological phenomena are ultimately due to physical and chemical interactions. But organisms are so complex that the reductionist programme is often quite impracticable; indeed one unfortunate consequence of the programme is that it leads biologists to concentrate their attention on those phenomena that can be explained by reductionist techniques rather than those which are intrinsically the most interesting (cf. Dawkins /2/, p. 24).

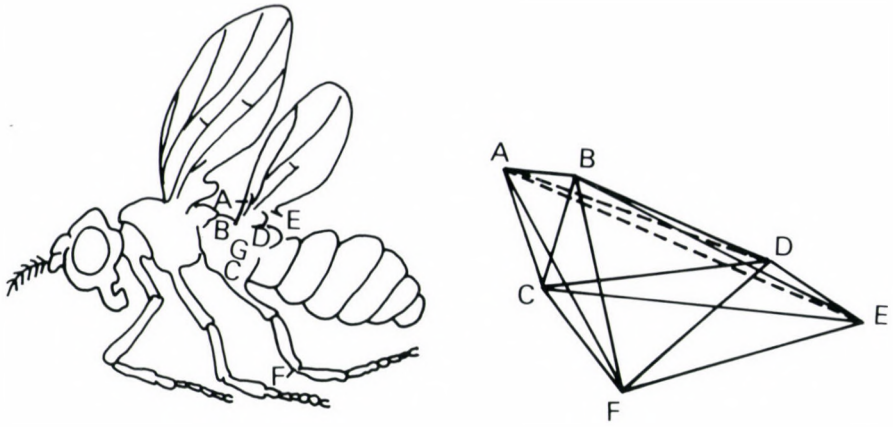
There are essentially two ways of applying structuralism in biology. One is biological: we seek wherever possible to use the fact that organisms are integrated wholes and develop as wholes. So instead of just analysis — looking for the mechanisms and the chemicals that underly a process — we also try to explain phenomena by showing how they fit into a larger pattern,

that given everything else that is going on, this is only to be expected. The other structuralism is mathematical. Having observed a phenomenon, we use mathematics to tell us either what else we may expect to observe or else to show how other apparently unconnected phenomena may in fact be different manifestations of the same process. The two approaches are not entirely separate, but it does seem useful to distinguish between them, at least some of the time.

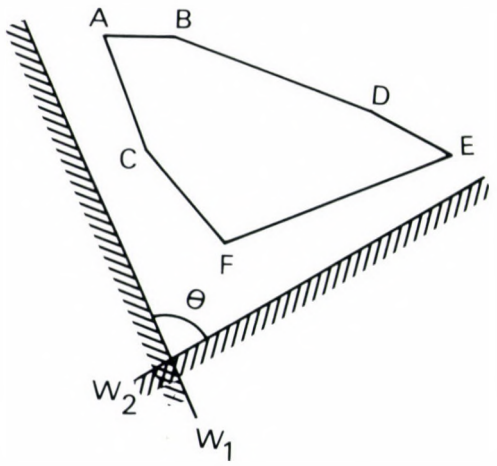
Examples of both techniques can be seen in recent investigations into the development of the fruit fly Drosophila melanogaster by Ho and her colleagues /4/, /5/. The aim has been to learn about normal development by studying what happens when it goes wrong. Most studies of this kind are done on mutants, such as the well known bithorax, in which what ought to be the metathoracic segment develops partly or entirely into the mesothoracic segment. If the transformation is complete, the result is a fruitfly with four wings instead of the usual two. The same effect can be achieved in genetically normal flies by exposing them to ether vapour at an early stage of development; this gives what are called phenocopies. Ho et. al have chosen to work with these because they involve a better defined perturbation of the system than mutations.

Now Drosophila development is comparatively simple, which is one of the reasons it is being so extensively studied. At an early stage the larva divides into segments, of which three will form the thorax, nine the abdomen, and the rest (and there seems to be some doubt about how many one should count) the head. Also comparatively early on (by comparison with most organisms) each segment appears to be divided into compartments, such that each cell within a given compartment will give rise to cells which will ultimately form a certain structure.

Figure 1 is a sketch of the mesothoracic segment in the embryo and the same segment in the adult fly. The letters indicate which parts of the so-called "imaginal disc" will give rise to which parts of the developed segment. One can draw a similar picture for the metathoracic segment, with the same compartments in the disc leading to structures in the developed segment, but the structures themselves will be slightly different. Instead of a wing there will be a haltere, a sort of balancing device, and the other parts look different enough to be distinguished as well under a microscope. The effect of either the mutations or the ether treatment is to cause some or all of the compartments on the metathoracic segment to develop not as they should but into the corresponding structures on the mesothoracic segment.



a



b

Fig. 1. The metathoracic segment of a bithorax phenocopy of Drosophila with compartments marked (a) on the adult (b) on the imaginal disc. Fig. 1b also indicates the orientation of the two plane waves referred to in the text

If all transformations were complete, the story would be less interesting, although we would still hope to learn something about the effect of the structure of the organism and its epigenetic (i.e. developmental) system on the possible variations. Usually, however, only some of the compartments within the segment are transformed, and we can gain additional information by noting which compartments tend to transform together and which do not.

The conventional explanation of the transformations is that the fate of a compartment is determined by a hierarchy of gene switches. A change in any gene will cause the transformation of all compartments below it in the hierarchy. This allows us to predict which compartments should transform together; for example on the generally accepted pattern of switches /3/ the co-transformations should be

ABCDEF ABDE CF AB DE A B D E.

In fact the pattern observed by Ho et. al was quite different, and can be shown not to be consistent with any model based on a hierarchy of gene switches. What is more, the transformations do not respect compartmental boundaries, as they ought to on the conventional model in which the compartments are specified as clonal units. The pattern is, however, consistent with simple physical contiguity: nearby compartments tend to transform, or not, together. And it is not too difficult to see that you can account for the pattern by assuming that two more or less plane waves pass through the region almost at right angles to each other. The idea is that the first wave sensitizes the cells and the second desensitizes them. Only if the ether is applied between the passage of the two waves is the cell diverted to the "wrong" state.

Not only does this model fit the pattern of co-transformations, it is also consistent with the observation that the transformations do not respect compartmental boundaries. On the other hand, it requires two waves passing through a region, and we have to explain where these waves could come from, and how they could be coordinated with the right sort of interval between them. Otherwise our explanation of the phenomenon is almost as complicated as the phenomenon itself, and we are not much further ahead.

Not enough is known about the processes that are going on in the embryo at this stage to allow us to make a convincing case for any par-

ticular mechanism to produce the waves. We can, however, still solve the problem, in the following way /11/:

We know that at an early stage cells are not yet determined; depending on environment and stimulus they can develop into different final forms. Later on, however, they are determined, and are certain (if they survive at all) to become bone, muscle, cartilage or whatever.

A simple way in which this transition can occur would be that there is some biochemical reaction within each cell which, once it is triggered, moves from one equilibrium state to another. If we also suppose, as seems reasonable, that the system can be diverted from its normal development only if it is perturbed while it is not at equilibrium, this explains why transformations occur only if the ether treatment is applied during a relatively short interval.

It might be that the reaction is initiated by direct genetic preprogramming with a sort of alarm clock in each cell, but this must be implausible. Much more likely is a signal which either passes through the region, or at least triggers the cells at one end which trigger those next to them and so on. In either case, there will be a wave of cells starting to become committed.The chemical reaction that does this will not be instantaneous, so there will be a second wave of cells actually becoming committed. The idea that changes of this kind happen in apparent waves is due to Zeeman /15/ though he did not envisage the origin of the second wave in quite the same way.

Now we know from catastrophe theory that the simplest mechanisms that will give us a transition from one equilibrium to another must have two "control variables", which here means two parameters of the reaction. How these affect the reaction depends on the model, but we can say in general that two cells which are disturbed from equilibrium at the same time will not reach the new equilibrium at the same time. It follows that the second wave will not follow directly behind the first; it can be slower, faster, in the opposite sense, or in a different direction altogether. So here we have the two non-parallel waves we need.

While this general argument is sufficient for our purposes and has the great advantage that the result does not depend on a particular choice of model, it may be helpful to see an example of an equation that will accomplish what is required. One that has been proposed in a somewhat different context is /6/:

$$dg/dt = S + g^2/(K+g^2) - Dg \quad .$$

Here g is an (unspecified) gene product, S is the concentration of some "signal substance" (also unspecified, but possibly calcium ions or cyclic ANP), K is the second parameter as mentioned above, and D is the rate of degradation of g. But while it may be reassuring to know that even a very simple equation can produce two waves at the sort of angle that is required (see /11/ for details), the whole argument goes through without any equations. We now know that if we observe a certain pattern of phenomena, here an apparent transition from one equilibrium to another, then consistent with this pattern — and the not unreasonable assumption that the mechanism that drives this is no more complicated than it has to be — we may expect to observe other phenomena, in this case the two waves.

Finally, we may ask what is the trigger that initiates the whole process. Here we apply a structural argument to the organism instead of to the mathematics. The process we have been describing occurs shortly after segmentation. Since segmentation is likely to involve waves moving longitudinally along the organism /1, 15/, and since that is the direction of the first of our two waves, it seems likely that there is a link.

CATASTROPHE THEORY

When an aeroplane is flying at above the speed of sound, the sky can be divided into two parts. Ahead of the aeroplane we hear no sound at all whereas behind it we do hear sound, first loud and then dying away. The boundary between the two regions is the shock wave, observed as the familiar sonic boom, and it has the form of a cusp, or if drawn in three dimensions, a sort of cusped cone.

Now consider light falling onto the surface of a cup of coffee. Again there are two regions, one dark and one light, and a very bright boundary between them, called a caustic. The caustic too is cusp shaped.

The processes involved in these two examples are quite different, but it is no mere coincidence that they both produce cusps. Catastrophe theory /13/ (for an elementary introduction see /10/) tells us that the range of forms (and remember, forms are defined by boundaries) that can be produced by a very wide range of mechanisms is remarkably small; they can all be found in the famous list of seven elementary catastrophes. The range of mechanisms referred to includes almost anything that can be described by

any differential equation or set of differential equations that any biologist and most physicists would be likely to write down.

This gives the elementary catastrophes a very special status in explanation, just like spheres and dodecahedra and so on had in the past. There is, however, an important difference. We have not abandoned the idea of mechanism. On the contrary, the choice of these preferred forms, these archetypes if you like, has been made consistently with the idea that forms arise through the sorts of physical and chemical processes which modern science supposes. But we do not need to know the particular process which operates in any given situation; granted some quite weak hypotheses about what is going on, the rest follows mathematically. It is not magic; it is true because our results apply not to individual mechanisms but to whole classes.

Thus we are almost back to the days when mathematics was a science and objects that were special mathematically were believed to be special scientifically as well. It's how we decide what is "special" that has changed. We choose the seven elementary catastrophes instead of the five regular polyhedra for good scientific reasons related to the sort of analyses which have been carried out for many years. This is surely as it should be; one cannot imagine that the new structuralism and, if one may be so bold, the new preeminence of mathematics would have gained nothing from the centuries of successful mechanistics and reductionism.

These examples show how the structuralist approach is already being applied in biology. Catastrophe theory has featured prominently, which is not surprising as it is the most overtly structuralist of the recent advances in mathematics and was developed with biology specifically in mind, but it is by no means the only tool available. Many of the results of bifurcation theory, and indeed of topological dynamics in general, are relevant, as are the techniques of systems theory. The reaction-diffusion models first suggested by Turing /14/ make predictions which are largely independent of particular reaction schemes. It is also possible to carry out structuralist analysis using conventional mathematics, or directly within biology (see, e.g. /12/).

CONCLUSIONS

In mathematics and science as in other fields, structuralism tends to arouse controversy. This is hardly surprising, for anything which is a

new approach rather than just a new technique is likely to become the focus of a paradigm argument. Those with established positions in the old paradigm will naturally be inclined to defend it, and the radicals will have the disadvantage that their approach is still in the process of being developed. They are therefore bound to make mistakes and to present even their successes in a form less polished and less proof against attack than work done using techniques which have been refined for a century or more.

A common criticism of structuralist work, and particularly of applications of catastrophe theory, is that it provides at best a description, not an explanation, and amounts to nothing more than curve-fitting. Now it should be clear from the examples given above that this need not by any means be so, though it is not possible to defend every structuralist argument against the charge, any more than one can defend every application of any mathematical technique. Besides, any serious discussion of this point runs into the problem that it is not at all obvious what we really mean by the word "explanation". It was, after all, a physicist, Max Planck, who wrote, "As long as Natural Philosophy exists, its ultimate aim will be the correlating of various physical observations into a unified system and, where possible, into a single formula" /9/.

All the same, there is in structuralism a danger, not always avoided, of setting up empty formalisms and barren analogies. But there is also an important weakness in the mechanistic approach, which structuralism allows us to see clearly. Suppose it can be demonstrated on structuralist grounds that a certain phenomenon should occur. This implies that there is a large class of mechanisms each of which would produce the same result. Hence that a particular mechanistic account makes a prediction which accords with the observations is nowhere near as strong a verification as it is likely to be taken to be. So we are in danger of coming to wrong conclusions about mechanisms — quite apart from the tendency of some reductionists to be content with ad hoc equations with no theoretical justification whatsoever.

Consider the elementary derivation of Snell's law of refraction by calculating the least-time path from source to observer. Anyone who takes this seriously is left wondering how the photon knows what the shortest path is, especially before it sets out on the journey. Eventually, of course, one learns that it has nothing to do with photons trying to save time but is a matter of a stationary path length being necessary to avoid destructive interference. Now this situation is well understood, but an analogous problem arises in the application of game theory to animal

behaviour: it is very hard to distinguish between a genetically programmed game-theoretic solution to a problem and the possibility that an animal learns from experience — which is how computers typically find minimax solutions even where they exist.

The structuralist approach has a great deal to contribute to biology, as to the study of all complex systems. But the structuralism will be not that of the pre-Newtonian era, nor exactly that of Saussure. It will not be opposed to or ignorant of mechanism; on the contrary, what we know of mechanisms must be incorporated into our models. Moreover, while concentrating on the synchronic may have been a natural reaction to the reductionist diachronic approach that preceeded it, it is essential to include transformations in time. This is certainly true in biology, in which evolution is such an important feature. It is also true in linguistics: it may be wrong to track the change in one word as though it had nothing to do with the rest of the language, but that does not mean we should not be trying to understand how entire languages have been transformed over the centuries. We began with unchanging structures, we went on to changing particles, now the challenge is to cope with changing structures.

Mathematics, structuralism and biology are coming together again, but each of the three is different from what it was two millennia or even two centuries ago. There is no question of an attempt to return to an outmoded paradigm, neglecting all that has been learned in the meantime. Nor is it a matter of biology being subject to a sort of intellectual takeover bid by mathematics. Instead, both mathematicians and biologists are being challenged to learn how to take full advantage of the new connections that are being established between their disciplines. We must not miss the opportunity through a misunderstanding of what is on offer.

REFERENCES

1. Cooke, J., Zeeman, E.C. (1976) A clock and wavefront model for the control of the number of repeated structures during animal morphogenesis. J. Theor. Biol. 58, 455–476.
2. Dawkins, R. (1982) The Extended Phenotype. W.H. Freeman, Oxford.
3. Garcia-Bellido, A., Ripoll, R., Morata, G. (1973) Developmental compartmentalisation of the wing disk of Drosophila. Nature 245, 251–253.
4. Ho, M.W., Bolton, E., Saunders, P.T. (1983) The bithorax phenocopy and pattern formation. I. Spatiotemporal characteristics of the phenocopy response. Exp. Cell Biol. 51, 282–290.

5. Ho, M.W., Saunders, P.T., Bolton, E. (1983) The bithorax phenocopy and pattern formation. II. A model of prepattern formation. Exp. Cell Biol. 51, 291–299.

6. Lewis, J., Slack, J.M.W., Wolpert, L. (1977) Thresholds in development. J. Theor. Biol. 65, 579–590.

7. Lyons, J. (1973) Structuralism and linguistics. In: Robey, D. (ed.) Structuralism: an Introduction. Clarendon Press, Oxford.

8. Piaget, J. (1968) Le Structuralisme. Presses Universitaires de France, Paris.

9. Planck, M. (1925) A Survey of Physics (trans. R. Jones, D.H. Willams) Methuen, London.

10. Saunders, P.T. (1980) An Introduction to Catastrophe Theory. Cambridge University Press, Cambridge.

11. Saunders, P.T., Ho, M.W. (1985) Primary and secondary waves in prepattern formation. J. Theor Biol. 114, 491–504.

12. Sibatani, A., Sermonti, G. (eds) (1987) Proceedings of the International Workshop on Structuralism in Biology, Osaka. Rivista di Biologia – Biology Forum 80 (2).

13. Thom, R. (1972) Stabilité Structurelle et Morphogénèse. Benjamin, Reading.

14. Turing, A.M. (1952) The chemical basis of morphogenesis. Phil. Trans. R. Soc. London B 237, 37–72.

15. Zeeman, E.C. (1974) Primary and secondary waves in developmental biology. In: Levin, S. (ed.) Some Mathematical Questions in Biology VII. American Mathematical Society, Providence.

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MINIREVIEW

A UNIQUE CELL TYPE IN THE LUNG – THE CLARA CELL (THE NON-CILIATED BRONCHIOLAR EPITHELIAL CELL)

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(Received: 1987–12–27)

The non-ciliated bronchiolar epithelial cells (the Clara cells) are found most frequently in the distal conducting airways, but they are found throughout the tracheobronchial tree of different mammalian species. According to recent data, the main functions of the Clara cells can summarized as /1/, the secretion of certain components of the extracellular bronchiolar lining layer /2/, metabolism and detoxification of xenobiotics and other toxic compound /3/ and participation in the renewal process of the bronchiolar epithelium. The main goal of this paper is to collect and discuss some of the general features of Clara cells from a functional-morphological point of view, and their possible role in pathological alterations of the lung expecially in the pathogenesis of lung tumours originated from Clara cells.

Keywords: Clara cell – non-ciliated bronchiolar secretory cell – pulmonary surfactant – detoxification in the lung – lung carcinogenesis

INTRODUCTION

The non-ciliated bronchiolar epithelial cells were first noticed at the end of last century by Kölliker /23/. The light microscopic characterization of human and rabbit non-ciliated bronchiolar epithelial cell were given by Clara /6/ after whom the cells were named. The non-ciliated bronchiolar epithelial cells, usually called Clara cells, are found most

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frequently in the distal airways, but also troughout the bronchiolar tree of different mammalian species /14, 19, 39, 43/. Ultrastructural and cytochemical studies indicate that they are secretory cells having a high level of metabolic activity (see review 51).

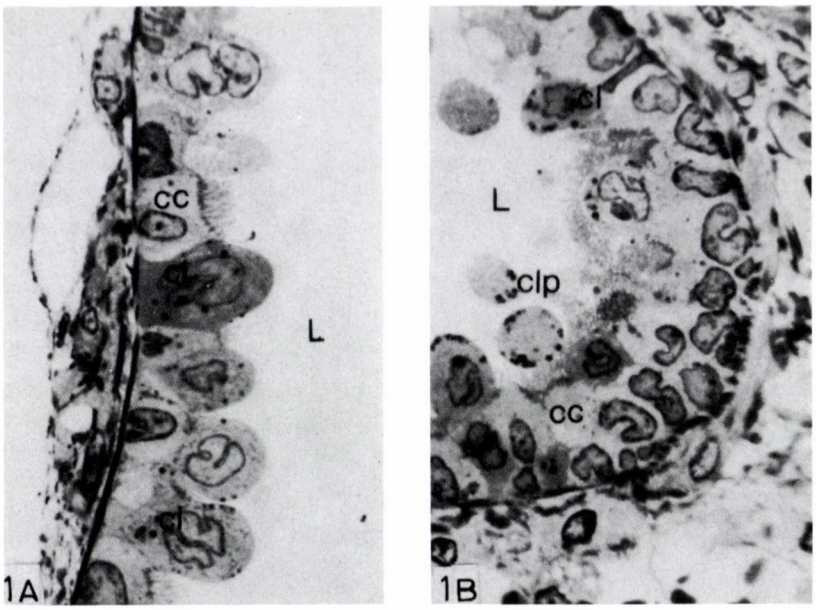
According to recent data, the main functions of the Clara cells can be outlined as /1/, the secretion of certain components of the extracellular bronchiolar lining layer /2/, metabolism and detoxication of xenobiotics and other toxic compounds due to their cytochrome P—450-dependent mixed-function oxidase system, and /3/ participation in the renewal process of the bronchiolar epithelium /29/. In spite of different light- and electron microscopical, cytochemical, immunological and toxicological experiments numerous details concerning the function of Clara cells are still controversial and open to debate.

The main goal of this review is to collect and discuss some of the general features of Clara cells from a functional-morphological point of view, and their possible role in the metabolism of different xenobiotics and in pathological alterations of the lung. The paper incorporates some of our findings regarding the chemical carcinogenic effect of 3-methylcholanthrene on the Clara cell.

Functional-morphology of the Clara cells

The Clara cells have a columnar shape, with cytoplasmic protrusions into terminal bronchioles and other airway lumina. By virtue of this structure, the Clara cells extend above the ciliated cells (Figs 1 and 2). Electron microscopic observations pointed out that the ultrastructural characteristics of Clara cells are quite similar in mammals. However, the quantitative appearance of the secretory granules, the rough- and smooth endoplasmic reticulum shows interspecies variations as described in comparative ultrastructural studies /35, 36/. Higher amounts of smooth endoplasmic reticulum and secretory granules are characteristic of Clara cells in the rat, rabbit and other animals but the presence of abundant secretory granules and rough endoplasmic reticulum have been identified in the human cell.

The membrane-bound secretory granules of the Clara cells are generally circular or oval in shape, and varied in size and electron density, although in some cases irregular or rodlike granules can be found /26, 35/. The secretory granules are present in the Clara cells of all mammalian species studied so far, except the cat and Macaca mulatta /36/.



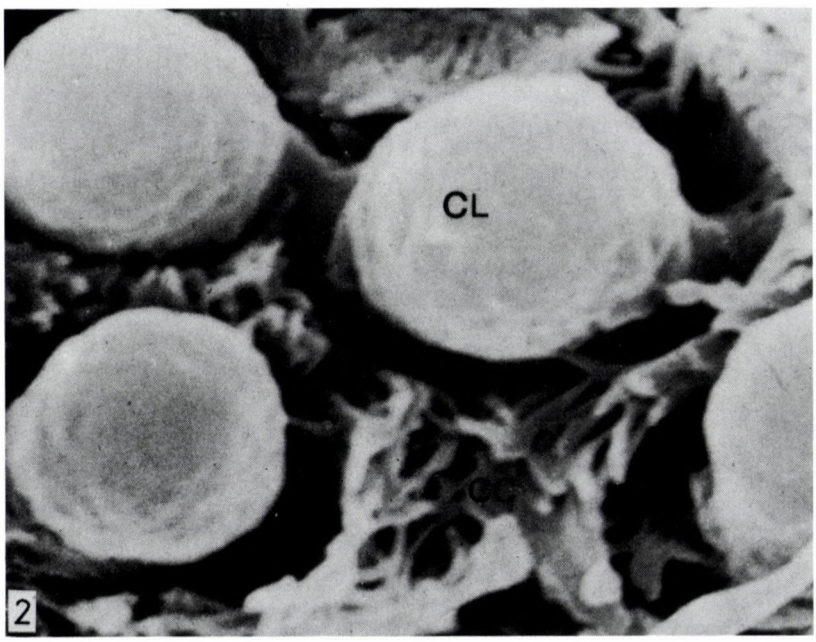
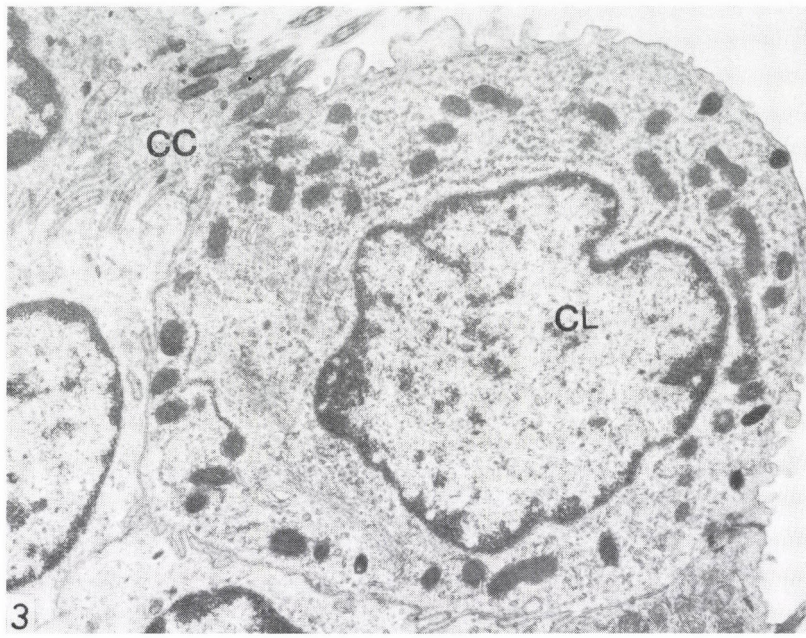


Fig. 1. Light micrographs of the terminal bronchiole of rat lung. The epithelium consists of Clara cells (non-ciliated bronchiolar epithelial cells) and ciliated cells (A). Detached Clara cell protrusions can be seen in the lumen (B). Semithin sections, toluidine-blue staining. CL = Clara cell, CC = ciliated cell, L = lumen of terminal bronchiole, Clp*= detached protrusion of Clara cell. x 1040

Fig. 2. Scanning electron micrograph showing the surface of terminal bronchiole of rat lung. The cytoplasmic prostrusions of Clara cells are extended above the ciliated cells. CL = Clara cell, CC = ciliated cell. x 6000



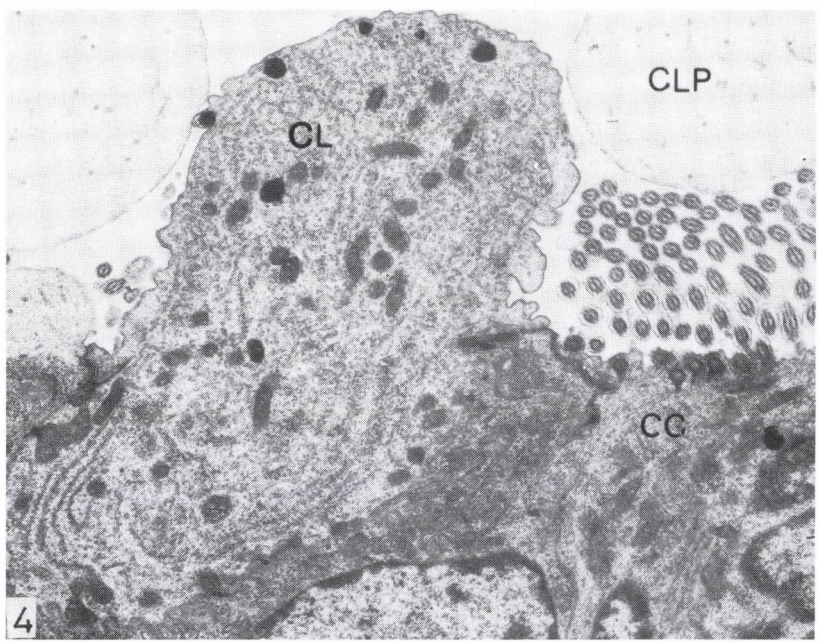


Fig. 3. Transmission electron micrograph of terminal bronchiole of rat lung showing Clara cell and neighbouring ciliated cells. The Clara cell shows abundant smooth endoplasmic reticulum in the apical part of the cytoplasm. Further explanation is in the text. CL = Clara cell, CC = ciliated cell. x 10 000

Fig. 4. Clara cell and ciliated cell of the terminal bronchiole of rat lung one week after 3-methylcholanthrene treatment. A large amount of smooth endoplasmic reticulum is observable in the apical part of cytoplasm. The bronchiolar lumen contains detached protrusions (blebs). CL = Clara cells, CC = ciliated cell, Clp = detached protrusion of Clara cell, L = lumen of terminal bronchiole. x 9900

The abundant content of smooth endoplasmic reticulum is highly developed in the apical part (protrusions or blebs) of the Clara cell cytoplasm, also cisternae of smooth endoplasmic reticulum contain different amounts of substance of low electrondensity (Fig. 3). Other cytoplasmic organelles (Golgi apparatus, mitochondria etc.) were detected in the Clara cells similar to other secretory cells. In some species extremely large mitochondria have been described /36/.

The nuclei are located in the basal region of the cells, and show indentations or invaginations in different degrees. In the nuclei small amounts of heterochromatin could be detected mainly as marginal chromatin (Fig. 3).

The luminal surface of Clara cells are covered with microvilli. On the surface of rat and mouse Clara cells apical blebs have been described /45/. These blebs may contain different cytoplasmic organelles (secretory granules, smooth endoplasmic reticulum, polysomes), but may be almost free of cytoplasmic organelles. These blebs are separated from the apical part of cytoplasm by a fine filament system. In some cases these apical blebs can be detached from Clara cell cytoplasm, and appear in the bronchiolar lumen, as seen with both light and electron microscopy (Figs 1B, 4, 6).

On the lateral surfaces of Clara cells, cytoplasmic interdigitations and desmosomes were observed (Fig. 3). These structural elements may have a function for bronchiolar epithelium such as fluid absorption, which is common to all species /36/.

Based on many observations and studies most authors agree that one of the main functions of Clara cells is secretion. At the same time the actual secretory mechanism is not completely understood. Both apocrine and merocrine secretory processes have been suggested in earlier and recent publications. According to results supporting the apocrine secretion, the apical cytoplasmic caps (protrusions or blebs) are extruded into the small airway lumen and their content is released as a secreted component of bronchiolar lining layer. The results of other examinations have demonstrated the merocrine secretory mechanism by exocytosis of secretory granules /25/. The question of the mode of secretion of Clara cells is still open, but most likely both apocrine and merocrine secretion mechanisms may exist in these cells depending on the physiological or pathological condition as suggested by an outstanding review of Widdicobe and Pack /50/ and other studies /45, 46, 47/.

Characterization of secretory granules and enzymes

The chemical composition of the Clara cell secretory granules have been characterized by cytochemical and autoradiographic methods. Cytochemical investigations have pointed out that the granules are PAS positive in most mammalian species /7, 36/. The autoradiographic results indicate ^{3}H-galactose incorporation into secretory granules /34/. The question still exists as to interspecies variations, because it was recently published that Clara cells of the rabbit lung do not react with light and electron microscopical cytochemical techniques for carbohydrates /38/. Cytochemical reactions for lipids or phospholipids are positive in the secretory granules /7/. The lipid synthesis and lipid content of secretory granules have been proved by isotope technique in mouse Clara cells /34/. The presence of protein in the secretory granules is evident on the basis of light- and electron microscopical cytochemistry; these findings are supported by ^{3}H-leucine incorporation /12, 34/. The majority of authors agree that Clara cells do not contain mucin granules.

Recently developed methods for the separation of Clara cells could be a powerful technique in the study of the chemical composition of its secretory granules located in the cytoplasm, or secreted into the small airway lumina /9, 33/. Moreover, these methods have opened up new prospects to study the activity of different enzymes in isolated Clara cells at biochemical, pathological and toxicological levels.

The enzyme content of Clara cells has also been investigated at a histochemical level by many authors. The end product of enzyme activity of these reactions were localized in the organelles at the apical portion of the cytoplasm and in the apical cell membrane (e.g. acid phosphatase /26, 51/, catalase /15/), peroxidase, phospholipid metabolizing enzymes, alkaline phosphatase /51/ enzymes for oxidative metabolism /1/ etc.

In the study of Clara cells, the development and application of immunological techniques have introduced some very important methodological possibilities.

The results of immunohistochemical and immunocytochemical methods are valuable contributions to data of classical enzyme histochemistry. For instance, the presence of the cytochrome P—450-monooxigenase enzyme system in the Clara cell was pointed out by using an immunohistochemical technique /39, 43, 44/. The results of these techniques indicate that there is a direct correlation between the amount of smooth endoplasmic reticulum and

the presence of the cytochrome P-450-monooxigenase system in the Clara cells /39/.

Also, immunocytochemical examinations have been used to localize the secreted protein of Clara cells in rat lung /28/. Light microscopic immunocytochemical results documented that the lumen of bronchioli contains proteins which are synthetized in the Clara cells. These secretory products were demonstrated to be present in the Clara cells and along the bronchiolar lining layer. Moreover, immunogold investigations, at the electron microscopic level, indicate that the major surfactant apoproteins are immunologically identical or closely related to the proteins synthesized and secreted by rat lung Clara cells. This type of protein is produced by type II pneumocytes, but this protein is complexed with phospholipids /49/.

Also the electron microscopic immunogold technique was used in a recently published experiment which pointed out the presence of 10 KD protein in the secretory granules of rat lung Clara cells. The 10 KD protein is one of the components of rat lung lavage, by this it can be concluded that this protein is a specific product of Clara cells /2/.

Metabolic activity and detoxication

Due to the large amount of smooth endoplasmic reticulum it has been suggested that the Clara cells exhibit a high metabolic activity because this organelle contain the microsomally bound cytochrome-P-450-dependent mixed-function oxidases /39/. It is generally accepted that this enzyme-system is responsible for the oxidative metabolism of different xenobiotics, including carcinogenic compounds /30/. Experimental data demonstrated that the Clara cells contain the highest concentration of cytochrome-P-450-dependent mixed-function oxidative enzymes, therefore the Clara cells are the main site of the xenobiotic metabolism. One of the most important experiments which proving this conclusion was performed with 4-ipomeanol. 4-ipomeanol a toxin of sweet potatoes (Ipomea batatas), produces selective damage in Clara cells, only the Clara cells were labelled by radioactive metabolite of this compound /3/. Due to its high level of xenobiotic metabolism, the Clara cell serves as an important place for detoxication in the lung. At the same time the highly reactive and toxic metabolites formed by enzymatic reactions lead to cell injury (degenerative changes, necrosis etc.), or chemical carcinogenesis /11, 50/. These changes play an important role from the viewpoint of toxicological and pathological studies

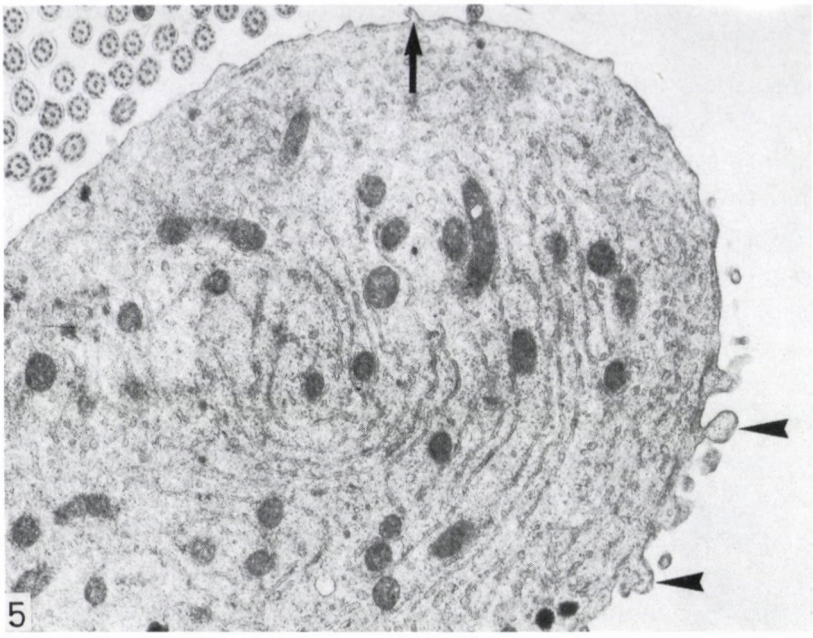
of the lung. Damage to Clara cells has been induced by carbon tetrachloride /5/, 3-methylfuran /4/, 2-methylnaphtalene /16/, 1,1 dichloroethylene /13/, 3-methylindole /48/, hexachloropentadiene /40/ etc. The nitrosoheptamethyleneimine /41/, ethyl-nitrosurea /21/, 4-nitroquinoline 1-oxide /17/ and other compounds were used in experimental pulmonary carcinogenesis connected with Clara cells. It is most likely that the hyperplasia and hypertrophy of smooth endoplasmic reticulum are one of the earliest and most important effects of xenobiotic-induced alterations in the Clara cells.

The effect of 3-methylcholanthrene, (which is a well known inducer of chemical carcinogenesis), was studied in the rat lung Clara cells by intratracheal administration in our experiments /22/. According to electron microscopical analysis the treatment with 3-methylcholanthrene resulted in enlargement of the smooth endoplasmic reticulum membrane system in the apical part of the Clara cell cytoplasm. The frequent occurrence of cytoplasmic protrusions, blebs were also characteristic (Figs 4, 5, 6).

The Clara cell related lung carcinomas

The observations on human Clara cell carcinomas were first published in 1966 /32/. Thereafter, in light- and electron microscopic case reports the possible role of Clara cells in the histogenesis of human bronchiolo-alveolar carcinomas has been described /18, 24, 31/. On the other hand different studies in the field of chemical carcinogenesis strongly suggest that the Clara cells may serve as the cell of origin of distal airway tumours. The main histologic types of lung tumours induced in these experiments are the adenomas, adenocarcinomas or mixed tumours /21, 41, 42/. It should be emphasized that the pathological behaviour of experimentally induced tumours, depending on the species, strain and chemical carcinogen used, can yield varied results. Based on the future development of immunohistochemical techniques we can expect more detailed information concerning both experimentally induced, and human Clara cell tumours.

From the point of veiw of malignant transformation of Clara cells the basic problem is the DNA repair capacity. It is well known that the DNA repair mechanism, which plays a central role in defense of the genome against carcinogen compounds or their metabolites is an important one. In the repair process the altered DNA nucleotides are eliminated by enzymatic removal. Recently an interesting experiment was published examining the DNA repair capacity of Clara cells, alveolar type II pneumocytes and macrophages



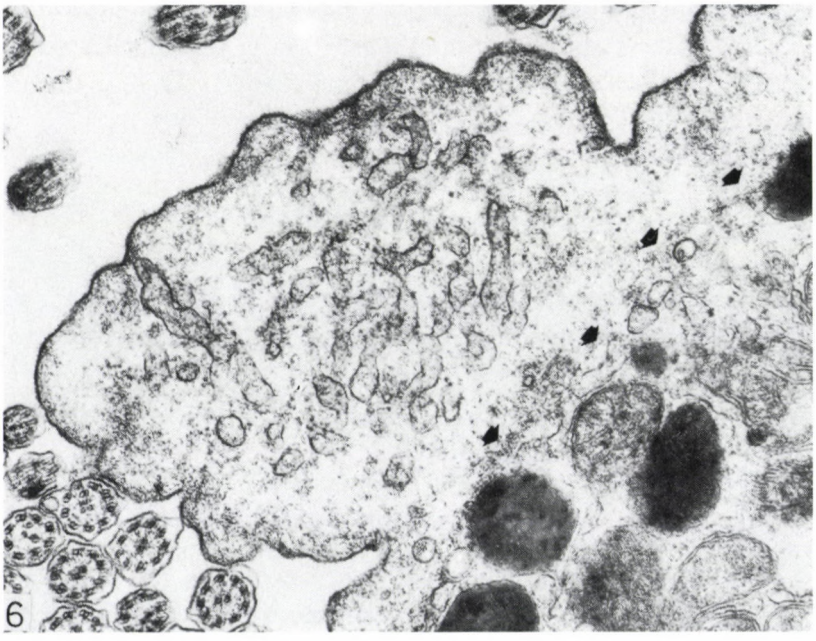


Fig. 5. Apical part of the Clara cell cytoplasm of rat lung 48 h after 3-methylcholanthrene treatment. Increased amount of smooth endoplasmic reticulum can be seen. Microvilli and protrusions are present on the cell. Arrowhead = microvillus, forming protrusion on the cell surface. x 13 200

Fig. 6. Cytoplasmic protrusion (bleb) of Clara cell 4 weeks after 3-methylcholanthrene treatment. The protrusion contains smooth endoplasmic reticulum containing an amorphous substance. There is a sharp border between the protrusion and the rest of cytoplasm (arrows)

isolated from rabbit lung /8/. The result of this experiment indicated that the level of DNA excision repair, as measured by unscheduled DNA repair synthesis, was lower than in the alveolar type II cells or in the macrophages (4–20-fold lower).

This observation on the DNA repair capacity and other similar results may have great importance from the viewpoint of malignant transformations in the lung. The Clara cells have been accepted as the main site of cytochrome-P–450-dependent mixed-function monooxigenase enzyme system; therefore, toxic or carcinogenic metabolites may form in high concentrations in their cytoplasm during the detoxication process of xenobiotics. These reactive derivates are known to interact with cellular DNA in the Clara cell genome causing different lesions. This interaction and modification are thought to be of critical importance due to the fact that modified DNA increases the infidelity of DNA synthesis and could subsequently results in an altered gene expression leading to cell transformation /27/. As a consequence of the low level of DNA repair capacity of the Clara cells it can be supposed that these cells are less capable to excise and repair the damaged DNA segments. These data suggest that low level capacity of DNA repair in conjunction with an increased metabolism of xenobiotics may be important factors which contribute to the pathogenesis of lung tumours originated from Clara cells.

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REFERENCES

1. Azzopardi, A., Thrulbeck, W.M. (1969) The histochemistry of the nonciliated bronchiolar epithelial cells. Am. Rev. Respir. Dis. 99, 516–525.
2. Bedetti, C.D., Singh, J., Singh, G., Katyal, S.L., Wong-Chong, M. (1987) Ultrastructural localization of rat Clara cell 10 KD secretory protein by the immunogold technique using polyclonal and monoclonal antibodies. J. Histochem. Cytochem. 35, 789–794.
3. Boyd, M.R. (1977) Evidence for the Clara cell as a site of cytochrome P-450-dependent mixed-function oxidase activity in lung. Nature (Lond.) 269, 213–215.

4. Boyd, M.R., Statham, Ch.N., Franklin, R.B., Mitchell, J.R. (1978) Pulmonary bronchiolar alkylation and necrosis by methylfuran, a naturally occurring potential atmospheric contaminant. Nature (Lond. 272, 270–271.

5. Boyd, M.R., Statham, Ch.N., Longo, N.S. (1980) The pulmonary Clara cell as a target for toxic chemicals requiring metabolic activation; studies with carbon tetrachloride. J. Pharmacol Exp. Ther. 212, 109–114.

6. Clara, M. (1937) Zur Histologie des Bronchoepithels, Z. Mikr. Anat. Forsch. 41, 321–347.

7. Cutz, E., Conen, P.E. (1971) Ultrastructure and Cytochemistry of Clara cells. Am. J. Pathol. 62, 127–141.

8. Deilhaug, T., Myrnes, B., Aune, T., Krokan, H., Haugen, A. (1985) Differential capacities for DNA repair in Clara cells, alveolar type II cells and macrophages of rabbit lung. Carcinogenesis 6, 661–663.

9. Devereux, T.R., Fouts, J.R. (1980) Isolation and identification of Clara cells from rabbit lung. In Vitro 16, 958–968.

10. Devereux, T.R., Serabjit-Singh, C.J., Slaughter, S.R., Wolf, R.W., Philipot, R.M., Fouts, J.R. (1983) Identification of cytochrome P-450 isozymes in nonciliated bronchiolar epithelial (Clara) and alveolar type II cells isolated from rabbit lung. Exp. Lung Res. 2, 221–230.

11. Dormans, J.A.M.A. (1983) The ultrastructure of various cell types in the lung of the rat: A survey. Exp. Path. 24, 15–33.

12. Ebert, R.V., Kronenburg, R.S., Teraccio, M.J. (1976) Study of the surface secretion of the bronchiole using radioautography. Am. Rev. Respir. Dis. 114, 567–573.

13. Forkert, P.G., Reynolds, E.S. (1982) 1,1-dichloroethylene-induced pulmonary injury. (1982) Exp. Lung Res. 3, 57–68.

14. Gail, D.B., Lenfant, C.J.M. (1983) Cells of the lung: biology and clinical implications. Am. Rev. Respir. Dis. 127, 366–387.

15. Goldenburg, H., Huttinger, M., Kolner, V., Kramar, R., Pavelka, M. (1978) Catalase positive particles from pig lung; biochemical preparations and morphological studies. Histochem. 56, 253–264.

16. Griffin, K.A., Johnson, C.B., Breger, R.K., Franklin, R.B. (1981) Pulmonary toxicity, hepatic and extrahepatic metabolism of 2-methylnaphtalene in mice. Toxicol. Applied Pharm. 61, 185–196.

17. Inayama, Y. (1986) Promoting action of glicerol in pulmonary tumorigenesis model using a single administration of 4-nitroquinoline 1-oxide. Jpn. J. Cancer Res. (Gann) 77, 345–350.

18. Jacques, J., Currie, W. (1977) Bronchiolo-alveolar carcinoma: Clara cell tumor? Cancer 40, 2171–2180.

19. Jefery, P.K., Reid, L. (1975) New observation of rat airway epithelium: a quantitative and electron microscopic study. J. Anat. 120, 295–320.

20. Jefery, P.K. (1987) The origin of secretions in the lower respiratory tract. Eur. J. Respir. Dis. 71. Suppl. 153, 34–42.

21. Kauffmann, S.L., Alexander, L., Stars, L. (1979) Histologic and ultrastructural features of the Clara cell adenoma of the mouse lung. Lab. Invest. 40, 708–716.

22. Komáromy, L., Reisz, S., Erhardt, P., Tigyi, A. (1984) Ultrastructural alterations of nonciliated bronchiolar (Clara) cells after 3-methylcholanthrene treatment. In: Electron Microscopy 1984. Vol. 3, 8th European Congress on Electron Microscopy, Budapest. 2249–2250.

23. Kölliker, A. (1881) Zur Kenntnis des Daues der Lunge des Menschens. Verh. Physic. Med. Ges. Würzburg 16, 1–24.

24. Kuhn, Ch. (1972) Fine structure of bronchiolo-alveolar cell carcinoma. Cancer 30, 1107–1118.

25. Kuhn, Ch., Callaway, L.A., Askin, F.B. (1974) The formation of granules in the bronchiolar Clara cells of the rat. J. Ultrastruct. Res. 49, 387–400.

26. Kuhn, Ch., Callaway, L.A. (1975) The formation of granules in the bronchiolar Clara cells of the rat. II Enzyme cytochemistry. J. Ultrastruct. Res. 53, 66–76.

27. Maher, V.M., McCormick, J.J. (1984) Role of DNA lesions and excision repair in carcinogen-induced mutagenesis and transformation in human cells. In: Greim, H., Jung, R., Kramer, M., Marquart, H., Oesch, F. (eds) Biochemical Basis of Chemical Carcinogenesis. Raven Press, New York.

28. Manabe, T., Ikeda, H., Moriya, T., Yamashita, K. (1987) Immunohistochemical localization of the secretory products of rat Clara cells. Anat. Rec. 217, 164–171.

29. Massaro, G.D. (1978) The Clara cell. Eur. J. Respir. Dis. 71. Suppl. 153, 52–58.

30. Matsuura, S., Fujii-Kuriyama, T., Tashiro, T. (1978) Immunoelectron microscope localization of cytochrome P-450 on microsomes and other membrane structures of rat hepatocytes. J. Cell Biol. 78, 503–519.

31. McDowell, E.M., McLaughlin, J.S., Merényi, D.K., Kieffer, R.F., Harris, C.C., Trump, B.F. (1978) The respiratory epithelium. V. Histogenesis of lung carcinomas in the human. J. Natl. Cancer Inst. 61, 587–606.

32. Montes, J., Adler, R.H., Brennan, J.C. (1966) Bronchiolar apocrine tumors. Am. Rev. Resp. Dis. 93; 946–950.

33. Patton, S.E., Gilmore, L.B., Jetten, A.M., Nettesheim, P., Hook, G.E.R. (1986) Biosynthesis and release of proteins by isolated pulmonary Clara cells. Exp. Lung Res. 11, 277–294.

34. Petrik, P., Collet, A.J. (1974) Quantitative electron microscopic autoradiography of in vivo incorporation of ^{3}H-leucine, ^{3}H-choline, ^{3}H-acetate and ^{3}H-galactose in nonciliated bronchiolar (Clara) cell of mice. Am. J. Anat. 139, 519–534.

35. Plopper, Ch.G., Mariassy, T., Hill, L.H. (1980) Ultrastructure of the nonciliated bronchiolar epithelial (Clara) cell of mammalian lung: I. A comparison of rabbit, guinea pig, rat, hamster and mouse. Exp. Lung Res. 1, 139–154.

36. Plopper, Ch.G. (1983) Comparative morphologic features of bronchiolar epithelial cells. The Clara cells. Am. Rev. Respir. Dis. 128, 337–41.

37. Plopper, Ch.G., Halsebo, J.E., Berger, W.J., Sonstegard, K.S., Nettesheim, P. (1983) Distribution of nonciliated bronciolar epithelial (Clara) cells in intra- and extrapulmonary airways of the rabbit. Exp. Lung. Res. 4, 79–98.

38. Plopper, Ch.G., St. George, J.A., Nishio, S.J., Etchison, J.R., Nettesheim, P. (1984) Carbohydrate cytochemistry of tracheobronchila airway epithelium of the rabbit. J. Histochem. Cytochem. 32, 209–218.

39. Plopper, C.G., Cranz, D.L., Kemp, L., Serabjit-Singh, C.J., Philipot, R.M. (1987) Immunohistochemical demonstration of cytochrom P-450 monooxigenase in Clara cells throughout the tracheobronchial airways of the rabbit. Exp. Lung Res. 13, 59–68.

40. Rand, G.M., Nees, P.O., Calo, C.J., Clarke, G.C., Edmondson, N.A. (1982) The Clara cell: An electron microscopy examination of the terminal bronchiolaes of rats and monkeys following inhalation of hexachlorocyclopentadiene J. Toxicol. Environ. Health. 10, 59–72.

41. Reznik-Schüller, H., Lijinksy, W. (1979) In vivo autoradiography and nitrosoheptamethyleneimine carcinogenesis in hamsters. Cancer Res. 39, 72–74.

42. Sato, T., Kauffman, S.L. (1980) A scanning electron microscopic study of the type II and Clara cell adenoma of the mouse lung. Lab. Invest. 43, 28–36.

43. Serabjit-Singh, C.J., Wolf, C.R., Philpot, R.M., Plopper, Ch.G. (1979) Cytochrome P-450: Localization in rabbit lung. Science 207, 1469–1470.

44. Serabjit-Singh, C.J., Wolf, C.R., Philpot, R.M. (1979) The rabbit pulmonary monooxigenase system: Immunochemical and biochemical characterization of enzyme components. J. Biol. Chem. 254, 9901–9907.

45. Smith. P.D., Heath, D., Moosavi, H. (1974) The Clara cells. Thorax 29, 147–163.

46. Stinson, S.F., Loosli, C.G. (1978) Ultrastructural evidence concerning the mode of secretion of electron-dense granules by Clara cells. J. Anat. 127, 291–298.

47. Stinson, S.F., Loosli, C.G. (1978) Ultrastructural evidence concerning the mode of secretion of electron-dense granules by Clara cells. J. Anat. 127, 291–298.

48. Turk, M.A., Breeze, R.G., Gallina, A.M. (1983) Pathologic changes in 3-methylindole-induced equine bronchiolitis. Am. J. Pathol. 110, 209–218.

49. Walker, S.R., Williams, M.C., Benson, B. (1986) Immunocytochemical localization of the major surfactant apoproteins in type II cells, Clara cells, and alveolar macrophages of rat lung. J. Histochem. Cytochem. 34, 1137–48.

50. Widdicobe, J.G., Pack, R.J. (1982) The Clara cell. Eur. J. Resp. Dis. 63, 202–220.

51. Yoneda, K. (1978) Ultrastructural localization of phospholipases in the Clara cell of rat bronchiole. Am. J. Pathol. 93, 745–752.

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REVERSAL OF ISOPROTERENOL-INDUCED CALORIGENIC ACTION IN THE HEART DURING MYOCARDIAL ISCHEMIA[+]

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With the aid of infrared thermography, a powerful new tool for studying the biological state of cardiac regulatory mechanisms, the reversal of the isoproterenol-induced calorigenic action in the in situ dog heart was demonstrated after establishing acute myocardial ischemia. The thermographic manifestations indicated a modified activity pattern of the cardiac (coronary) β-mechanism with a preserved sensitivity to its specific pharmacologic blockade. Results provide new evidence of the transformation (plasticity) of adrenoceptor qualities under pathologic circumstances.

Keywords: Isoproterenol — beta-adrenergic stimulation — myocardial ischemia — cardiac telethermography

INTRODUCTION

It is not uncommon to gain a generalized insight into biological problems from pathologic models which may represent paradigms for hidden regulatory aspects of trivial physiologic phenomena; in the present study we used this type of model to document the plasticity of cardiac adrenergic actions. Stability or convertibility of adrenoceptor-induced alterations

[+]Dedicated to our friends, Mathias Szentiványi (Budapest) and George Kunos (Montreal) on the twentieth anniversary of the discovery of what they called "adrenoceptor interconversion" — a fundamental principle that opened up new vistas and stimulated important research all over the world.

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(e.g. $\alpha \rightleftharpoons \beta$ shift) which is one of the paradigmatic examples of receptor plasticity) are hotly debated issues of adrenergic physiology. Although "conventional wisdom" favours a rigid classification /see e.g. 5/, unexpected transformations in the quality of cardiac and vascular adrenergic effects have been reported under the influence of environmental temperature /1, 6, 8, 9/, change in the ionic milieu /3/, modified thyroid state /6, 7/, neurally induced /12/ or toxic alterations /10/ of local metabolism, diabetes /4, 13/, and tissue ischemia /2/. At the same time these observations raise the question of whether or not the different, sometimes opposite, activity patterns of α and β adrenergic stimulation are always appropriate to specify adrenergic responses in relation to distinct subpopulations of receptors. Clearly some unequivocal measure has long been needed that incorporates the transformation of adrenergic activation (and not of blockade) as a potentiality in the intact organism. However, since phenomenological appearances of activator-induced cardiac actions are extremely diverse, it is very difficult to interpret in a straightforward manner whether or not any particular change torn from its context represents an essential or merely a compensatory alteration. Plasticity if any, in receptor qualities and/or in responses to receptor activation can be grasped best by an "overall" phenomenon which is common in every aspect of a given type of activation. In this study the calorigenic action of beta-adrenergic stimulation was considered such a generalized aspect, and it was analyzed with the new technique, computer-assisted cardiac telethermography developed recently in our laboratory /11/.

METHODS

Anaesthesia was induced in 16 mongrel dogs, weighing 16–28 kg, by i.v. injection of pentobarbital sodium (30 mg/kg). Artificial ventillation was maintained with room air. After transsternal thoracotomy a loose snare occluder was placed around the left anterior descending (LAD) coronary artery about 1/3 from its origin. Acute occlusion of the vessel caused severe ischemia in 24–28 per cent of the total centricular mass. For the administration of β-adrenergic activators (isoproterenol in most cases) a very thin indwelling needle with multiplex flexible connections was placed into the lumen distal to the snare loop; the β-adrenergic blocker oxprenolol was given i.v. Ventricular contractile force was recorded with two Walton-

Brodie strain gauges sewn to the free wall of the left and right ventricles, respectively. Arterial pressure was monitored with a Statham gauge, and heart rate was computed from pressure tracings.

Infrared telethermograms from the exposed cardiac surface were recorded photographically and analyzed mathematically according to the techniques described earlier in detail /11/. The sensitivity of the Thermovision equipment (AGA 750) was set to cover a range of 5 ^{o}C. For the topographical determination of the ischemically affected area, ice-cold methylene-blue (1–3 ml) was injected into the LAD to delineate, by comparing normal and thermographic photos, the size of the part of the cardiac image subserved to this vessel. Accordingly, computerized evaluation of the thermograms was performed for the two subunits: the unaffected (non-ischemic) and the affected (ischemic or potentially ischemic) zones. Knowing the total extension of these areas (number of thermographic samples, i.e. data points which was equivalent to about 5×10^4 data for each cardiac image), temperature profiles expressed in per cent distribution of isotherms in the relevant part (histograms), as well as a mean temperature characterizing the averaged heat emission from a particular region could be calculated by the computer program. Following LAD occlusion ischemic hearts were studied after a stabilization period of 1 h duration. Numerical values of both hemodynamic and thermographic data, recorded at the control state and at the height of adrenergic activation, are given as mean $\pm$S.E. Statistical calculations were made by using Student's *t*-test for paired and unpaired data.

RESULTS

Five dogs were employed to select the appropriate dose range of isoproterenol for detailed thermographic analysis, a time-consuming (and expensive) procedure not to be performed on a too large number of animals. By depicting the essence of this selection, Fig. 1 shows the dose-dependent relationship of inotropic responses after intracoronary isoproterenol administered in order to determine, in the control phase, the dose required for (supra)-maximal stimulation of the LAD-area, as well as an equivalent response elicited by the recirculating catecholamine in the adjacent right ventricular zone which was intended to be preserved intact. (It is known that the inotropic response-range of the latter area exceeds far that of the former one.) Following LAD occlusion the inotropic responses were sig-

nificantly reduced but clearly maintained in both areas by administering the 5 μg dose. Drug-induced blood pressure changes were negligible (< 10 mmHg) even during ischemia which, in itself, did not reduce resting mean pressure level (119±4 vs 118±7 mmHg), and the moderate tachycardiac actions (Fig. 1) also resembled each other in both experimental phases characterized by similar resting heart rates (161±12 vs 168±16 beats/min). In another series of six dogs similar principles were employed to select the adequate blocking range of oxperenolol given in increasing (cumulative) doses (0.125 – 1.0 mg/kg), and the 0.5 mg/kg dose was found to augment the threshold concentration of isoproterenol by nearly the second power (≃80 times).

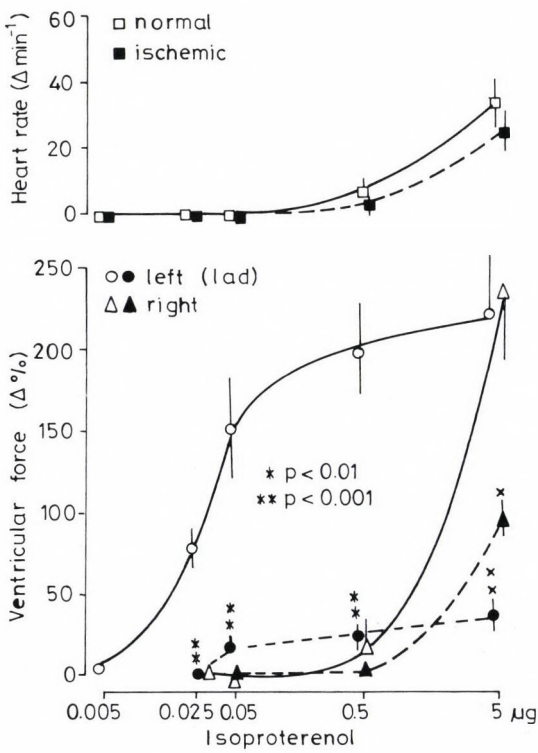

Fig. 1. Effect of LAD occlusion on isoproterenol-induced cardiac actions (n = 5). P-values refer to ischemic changes

Accordingly, in five dogs the 5 μg activator and the 0.5 mg/kg blocker doses were utilized for the thermographic analysis related to the principal question of the study. Fig. 2 illustrates a representative experiment, whereas Table 1 summarizes statistical evaluation. As shown, the well

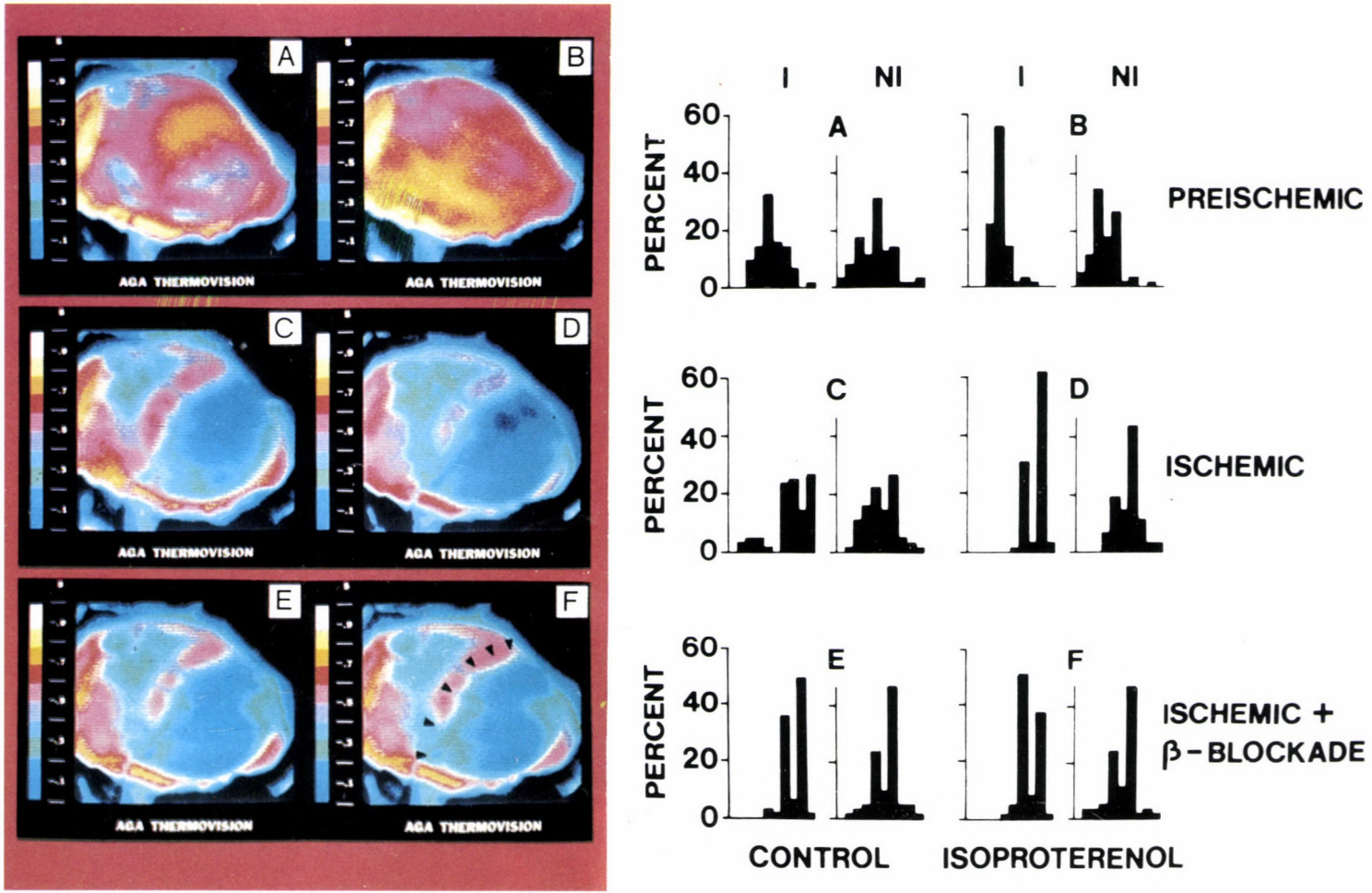

Fig. 2. Original thermograms and their computerized evaluation. A, C, E: control state, B, D, F: effect of isoproterenol (5 μg). Each color-shade represents a 0.5 ^{o}C isothermal range. From above downwards on the calibration scale (left margin): cooling. Arrowheads (F) denote the border of the ischemic area. Each column on histograms also represents 0.5 ^{o}C. Shift from left to right: cooling. I: ischemic area, NI: non-ischemic area

Table 1

Isoproterenol-induced epicardial temperature changes ($\Delta\,^{o}C$)$^{+}$

	A Pre-ischemic	B Ischemic	C β-blockade
Affected (LAD) area	+0.48 ± 0.05[a]	-0.15 ± 0.04[a]	+0.02 ± 0.05
		$p < 0.01$	$p < 0.05$
Non-ischemic area[b]	+0.36 ± 0.07[a]	-0.14 ± 0.08	+0.05 ± 0.03
		$p < 0.05$	$p \geq 0.05$

$^{+}$Mean values ± S.E., n = 5

[a]Statistically significant ($p < 0.05$) isoproterenol effect

[b]Differences between reactions of the two areas were non-significant ($p > 0.5$) in the three subsequent experimental phases (A, B, C)

known cardiac calorigenic action subsequent to β-adrenergic stimulation was completely reversed after establishing ischemia, which, in itself, created a temperature-gradient of 1.07 ± 0.13 °C between the two areas. It is interesting to note that the isoproterenol-induced cooling of the ischemic region did not surpass statistically the analogous reaction of the unaffected area. Thus, the phenomenon cannot be explained by the development of an epicardial coronary steal. In four experiments similar results were obtained with adrenaline or noradrenaline (5 μg each). Upon administering oxprenolol the negative calorigenic effects were blocked.

DISCUSSION

On the basis of our present data it would be premature to explain direct cause and exact mechanism of transformation of the adrenergic quality at the receptorial (or postreceptorial?) level. What the results do demonstrate is the possibility that a transformation predicted by the theory underlying Kunos and Szentiványi's discovery /9/ and adopted later by Ahlquist /1/ may occur in the intact organism. Regarded in the framework of the conventional classification of adrenergic receptors our observations are likely to cause confusion. However, if the scope of the interconversion hypothesis could be expanded into a general principle of receptor plasticity that allows the convergence of activation, it is possible to approach more satisfactorily these apparent controversies. As long as the subcellular mechanism of the effects obtained in the present study are unknown, it is reasonable to consider them phenomenologically determined and methodologically defined. The present experiments exemplified, therefore, in a simple and unequivocal manner merely a new technique by which such an action can be tested. One should bear in mind that all of the outcomes of the "normal" isoproterenol-induced cardiac effects (positive inotropic, chronotropic, direct metabolic, and β_2-coronary vasodilator actions) lead to an enhanced heat emission from the cardiac surface /11/. At the same time, the α-adrenoceptor activator phenylephrine has recently been shown to elicit, probably in connection with its coronary vasoconstrictor potency, a negative calorigenic effect /11/. In the present study the loss and reversal of the isoproterenol-induced generalized calorigenic effect which, in the first place, was probably also connected to the reversal of the vascular component in the β-action, suggests the restricted biological applicability of the rule concerning

receptor stability. In our ischemic model the β-adrenergic effect was found to be reversed but not insensitive to its specific blocking drug, oxprenolol. To what extent this type of reverse effect occurs in various biologic systems remains to be seen, but at least it serves to warn us that the stability-rule of activation easily fails under some circumstances. In other words, in order to understand the significance of these phenomena, perhaps elements of the biological variability are to be considered rather than those of the pharmacological subtleties involved in their classification.

REFERENCES

1. Ahlquist, R.P. (1973) Adrenergic receptors: a personal and practical view. Persp. Biol. Med. 17, 119–122.
2. Aviado, D.M., Juhász-Nagy, A. (1981) Alpha-adrenoceptors in ischemic canine heart blocked by phentolamine. Adv. Physiol. Sci. 27, 245–260.
3. Borda, L.J., Shuchleib, R., Henry, P.D. (1980) Hypoxic contraction of isolated canine coronary artery. Mediation by potassium-dependent exocytosis of norepinephrine. Circ. Res. 46, 870–879.
4. Cseuz, R., Wenger, T.L., Kunos, G., Szentiványi, M. (1973) Changes of adrenergic reaction pattern in experimental diabetes mellitus. Endocrinology 93, 752–755.
5. Grobecker, H., Philippu, A., Starke, K. (eds) (1986) New Aspects of the Role of Adrenoceptors in the Cardiovascular System. Springer-Verlag, Berlin.
6. Kunos, G. (1980) Reciprocal changes in α- and β-adrenoceptor reactivity – myth or reality? Trends Pharmacol. Sci. 1, 282–284.
7. Kunos, G. (1981) Modulation of adrenergic reactivity and adrenoceptors by thyroid hormones. In: Kunos, G. (ed.) Adrenoceptors and Catecholamine Actions. Vol. I, pp. 298–333, Wiley, New York
8. Kunos, G., Nickerson, M. (1976) Temperature-induced interconversion of alpha- and beta-adrenoceptors in the frog heart. J. Physiol. (London) 256, 23–40.
9. Kunos, G., Szentiványi, M. (1968) Evidence favouring the existence of a single adrenergic receptor. Nature 183, 1077–1078.
10. Matheny, J.L., Ahlquist, R.P. (1975) Metabolic inhibition and adrenoceptor interconversion. Arch. Int. Pharmacodyn. 218, 4–10.
11. Papp, L., Álló, G., Szabó, Z., Juhász-Nagy, A. (1985) Natural history of acute regional myocardial ischaemia revealed by infrared thermography in the canine heart. Acta Morph. Hung. 33, 123–142.
12. Szentiványi, M., Kunos, G., Juhász-Nagy, A. (1970) Modulator theory of adrenergic receptor mechanism: vessels of the dog hindlimb. Am. J. Physiol. 218, 869–875.
13. Szentiványi, M., Pék, L. (1973) Characteristic changes of vascular adrenergic reactions in diabetes mellitus. Nature. New Biol. 244, 276–277.

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BINDING OVERLAP AND INTERNALIZATION OF GONADOTROPIN AND THYROTROPIN IN NEONATAL RAT TESTICLE AND OVARY CELL CULTURES AND THE CHINESE HAMSTER OVARY (CHO) CELL LINE

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Colloidal-gold-labeled gonadotropin and colloidal-gold-labeled thyrotropin were bound by Chinese hamster ovary (CHO) and primary neonatal rat ovary and testicle cell cultures in the same sites and in the same quantities. The conditions of internalization and the intracellular fate of the bound gold-labeled hormones were also similar in every respect. Pretreatment with either hormone imprinted the cells also for the related hormone, as judged from the increased binding and internalizing capacity of the pretreated cells for either hormone, and from identical patterns of post-binding receptor aggregation.

Keywords: Hormone binding – cell culture – rat – testicle – ovary – electron microscopy

INTRODUCTION

The polypeptide hormones bind to membrane receptors. After binding, the receptor-hormone complexes aggregate in coated pits, which become invaginated, and the bound hormone enters the cell body along with the binding structures inside smooth-surfaced receptosomes. The hormone molecules so internalized become digested by the lysosomes, whereas the receptors are for the most part recycled to the plasma membrane /9, 10, 13–16/.

The receptors are generally regarded as specific binding structures, although they do not act as such in all conditions. In the perinatal

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period, when the receptors are still maturing, they may bind molecules structurally similar to but functionally different from the hormone, and may thereby promote the development of a hormone-like action. However, the interaction of the maturing receptor with an anadequate ligand leaves its mark in the developing binding structure (hormonal imprinting) for lifetime /1–4/.

Regardless of the specificity of their action, the hormones can be classified into well defined families. Related hormones are among others the gonadotropins (FSH, LH) and thyrotropin, which have a common alpha subunit; the specificity of their actions is associated with their beta subunits, which may themselves possess similar amino acid sequences /12/.

We investigated whether the mechanism of binding and intracellular fate of gonadotropins and TSH differed between neonatal gonadal cell cultures and Chinese hamster ovary (CHO) cells, i.e. whether the cells studied were able to differentiate between the related hormones acting differently in adulthood. Simultaneously we also examined the influence of hormone pretreatment (imprinting) on the binding of the hormones studied.

MATERIALS AND METHODS

Experiments in the CHO cell line

Monolayer cultures of the CHO K_1 cell line were used. The colloidal-gold-labeled hormones (FSH-LH, Pergonal, Human, Budapest – patent of Serono, Rome; TSH, Ambinon, Organon-Oss, Holland) were added to the cultures either immediately after outgrowth of the confluent monolayer, or 48 h after a 4-h treatment thereof with 10^{-5} M/ml FSH-LH or 10^{-6} M TSH. Exposure to both gold – hormone complexes lasted 10, 15, 30, 45 or 60 min. After hormone treatment the nutrient medium was decanted, the cells were rinsed thoroughly, scraped off the wall of the Falcon flasks, fixed in 1.25 per cent glutaraldehyde, and centrifuged at 2000 g for 10 min. The cell sediment was embedded in Araldite, the blocks were cut to ultra-thin sections and counter-stained with uranyl acetate for examination in a Philips 300 electron microscope.

Experiments in rat testicle and ovary cell cultures

Newborn CFY rats of both sexes were used as gonad donors. The primary testicle and ovary cell cultures were prepared in this laboratory.

The gonads were cleaned from attached tissues, minced, incubated in Ca^{2+}–Mg^{2+} free Tyrode solution for 20 min at 37 °C, and digested in 0.25 per cent trypsin solution for 3x10 min, also at 37 °C. The trypsinized cells were set up for culturing in Falcon flasks, in 10 per cent calf serum containing Parker medium, which was changed every day. The outgrowth of the confluent monolayer took 4 days in the testicle cell cultures and generally 6–8 days in the ovary cell cultures.

The monolayers were incubated in presence of 10^{-5}M FSH-LH or 10^{-6}M TSH for 4 h, washed thoroughly, returned to plain medium for 48 h, and exposed to colloidal-gold-labeled hormone for 10, 15, 30, 45 or 60 min. After hormone treatment the nutrient medium was decanted, the cells were scraped off the wall of the Falcon flasks, and were processed for electron microscopic examination as described above.

Preparation of colloidal-gold-labeled hormones

Colloidal gold was prepared by the method of Frens (8), and 5 ml of it was added to 0.3 ml FSH-LH stock solution (1 μg/ml) or 0.1 ml TSH stock solution (1 μg/ml). Five min later both systems were centrifuged at 20 000 *g* for 20 min, the supernatant was decanted, and the sediment was reconstituted in 0.5 ml phosphate buffer. The colloidal-gold-labeled hormone preparations were diluted tenfold in Parker medium for addition to the cultures. The control cultures were treated with colloidal gold alone in both series.

RESULTS

Light microscopic examination demonstrated three cell types each in stained preparations of the neonatal ovary and testicle cell cultures, such as small granulosa cells, large epithelium-like cells (ovogonia) and fibroblast-like cells in the former, and germ cells, Sertoli cells and Leydig's interstitial cells in the latter. However, we did not differentiate between the hormone binding capacities of the dissimilar cell types, for the applied electron microscopic techniques were not suitable for a reliable distinction.

There was no indication either of binding, or of internalization of the ligand in the control cultures treated with colloidal gold alone.

In the case of the CHO cells no difference could be observed between the binding relations of the gonadotropin-gold and TSH-gold complexes. After 10 min, the hormone-gold complex was intimately associated with the cell

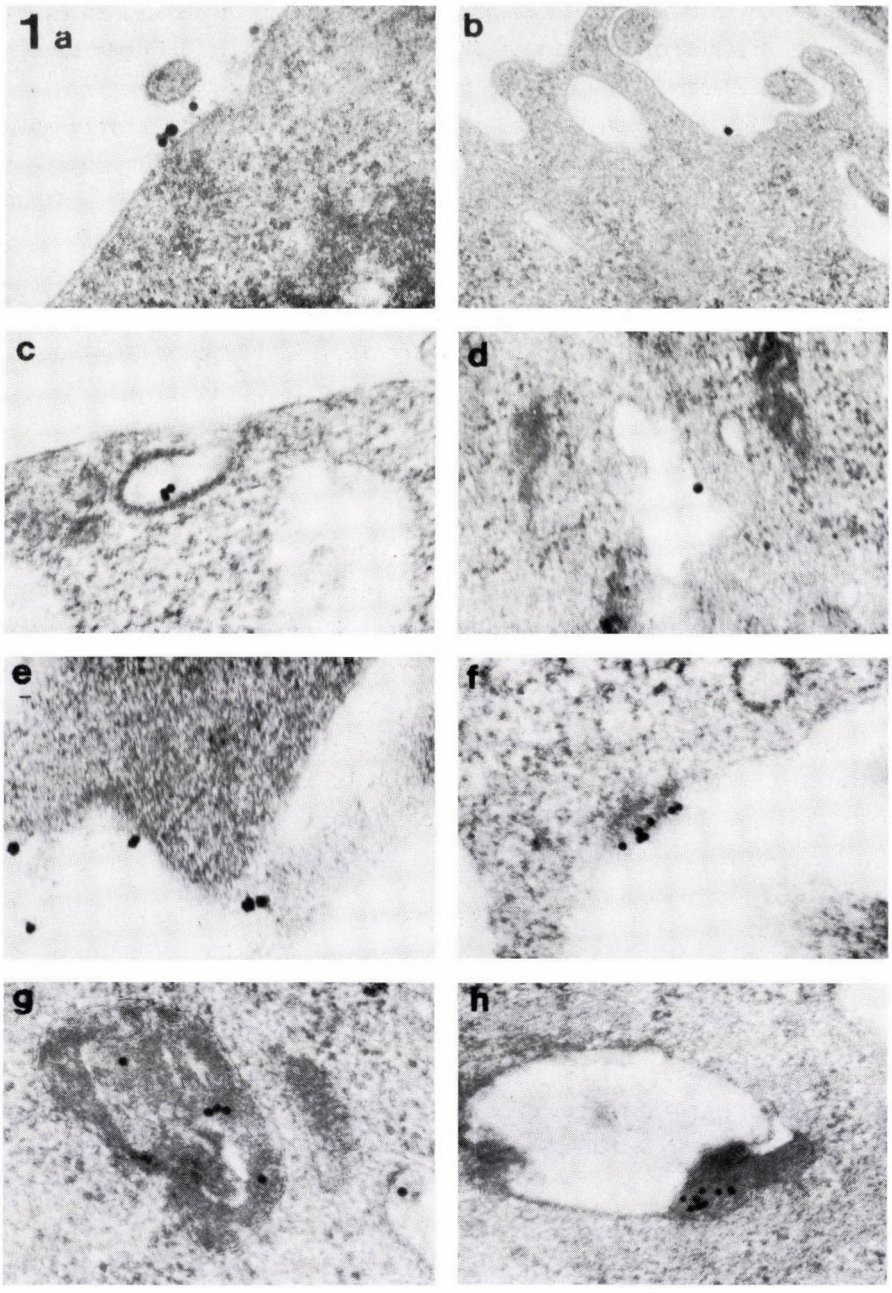

Fig. 1. Detection of the localization of colloidal-gold-labeled TSH in Chinese hamster ovary (CHO) cells (a,b) after 10 min (x 180 000; x 160 000), (c) 15 min (x 160 000), (d,e) 30 min (x 180 000; x 160 000), (f) 45 min (x 160 000) and (g,h) 60 min (x 160 000; x 140 000). The gold particles labeling the hormone initially associate with the cell surface, later appear in coated pits and, after internalization, inside lysosome

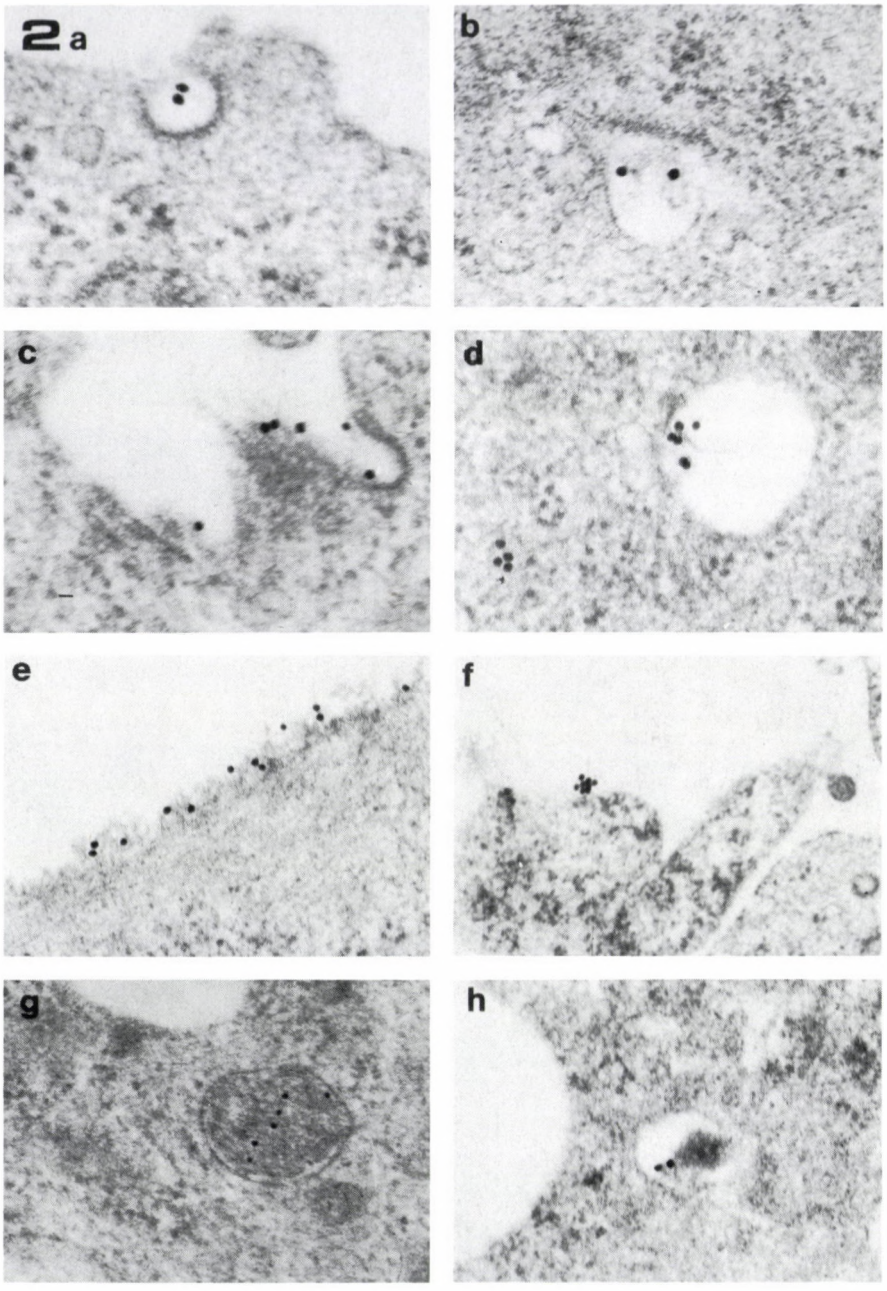

Fig. 2. Behaviour of cells treated with colloidal-gold-labeled FSH after pretreatment on the following schedules: (a,b) FSH for 10 min (x 180 000); (c) TSH; (d) FSH for 15 min (x 160 000); (e) TSH; (f) FSH for 30 min (x 140 000; x 120 000); (g) FSH for 45 min (x 120 000); (h) TSH for 60 min (x 140 000).
Note the higher intensity of binding, and earlier internalization of the colloidal-gold-labeled hormone relative to the not pretreated cells

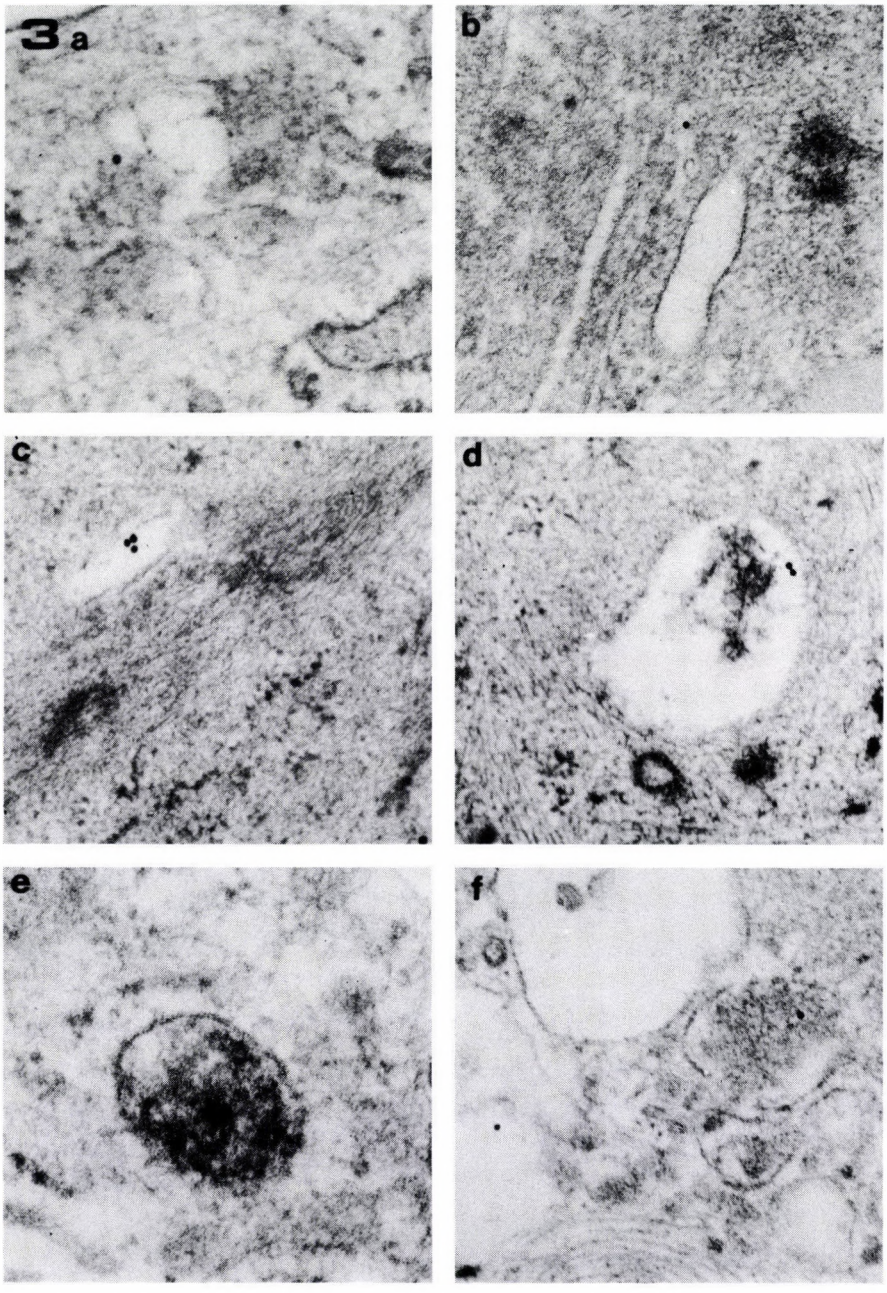

Fig. 3. Binding and internalization of colloidal-gold-labeled hormone in primary cultures of neonatal rat testicle and ovary cell cultures pre- and reexposed to plain and labeled hormone, respectively, on the following schedules: (a) FSH/TSH for 10 min (x 140 000); (b) TSH/FSH for 30 min (x 120 000); (c) FSH/FSH for 30 min (x 120 000); (d) FSH/FSH for 30 min (x 120 000); (e) FSH/TSH for 45 min (x 120 000); (f) FSH/FSH for 10 min (x 120 000)

surface, but no coated vesicles appeared yet. After 15 min, gold particles could be seen inside coated vesicles and also inside coated pits. After 30 min, a few gold particles appeared in intracellular localizations, and after 45 min also inside lysosomes. After 60 min, some lysosomes contained several gold particles, but the binding and internalization of these were still relatively scarce (Fig. 1).

The quantitative relations of binding were also similar in the pretreated CHO cell cultures, regardless of the type of gold-labeled hormone used for the second exposure. However, the intensity of binding increased over the not pretreated cultures, and coated pits were present already at 10 min. Internalized gold particles appeared inside smooth vesicles as soon as at 10–15 min. At 30 min, aggregations of bound gold could be seen on the membrane surface and in intracellular localizations as well. Groups of gold particles appeared inside lysosomes at 45 min, and also freely in the cytoplasm at 60 min (Fig. 2).

The testicle and ovary cells of neonatal rats, too, bound TSH-gold and FSH-gold to the same extent, irrespectively of the nature of the hormone used for pretreatment (Fig. 3). At 10 min the hormone-gold complex was for the most part associated with the cell surface, but a few internalized particles could already be seen. At 30 min the particles aggregated inside lysosomes and at 45 and 60 min in both intralysosomal localizations and inside internalized smooth vesicles, while their binding to the surface was still in progress.

DISCUSSION

Earlier investigations /11/ into the binding affinity of cultured testicular and ovarian cells for colloidal-gold-labeled TSH and gonadotropin demonstrated no quantitative difference between the binding relations of the two hormones. In the present study we examined the binding of colloidal-gold-labeled TSH and gonadotropin to CHO cells on direct exposure and after pretreatment (imprinting) with the hormone, to assess the impact of imprinting on binding and internalization. For comparison, we tested the binding relations of the gold-labeled hormones to primary cultures of testicular and ovarian cells derived from neonates, in which a preceding in vivo binding of TSH and gonadotropin could be excluded with certainty, and treatment with these in culture represented the primary exposure. This ex-

periment was necessary, for the CHO cells may have had interacted with the hormones studied in vivo, and therefore their response to these in culture might well have represented a revival of the "memory" of primary interaction rather than the issue of a genuine imprinting.

The experiments have shown that neither CHO cells, nor primary cultures of gonadal cells were able to differentiate between TSH and gonadotropin. The morphological evidence of this obtained in the present study, supports our earlier experimental observation /6, 7/ that TSH and gonadotropin equally exert a functional influence on the CHO cells, i.e. either is capable of imprinting these for the other. Evidence that imprinting had taken place emerged from the experimental facts that the preexposed cells showed a greater binding capacity, relative to those not preexposed an aggregated pattern of binding, earlier appearance of coated pits and earlier internalization of the hormone in greater amounts.

As to the pattern of binding and internalization, single receptors are involved if the cells have not yet interacted with the hormone or had interacted with it very long ago (CHO cells), whereas aggregation of the receptors — along with the bound hormone — in coated pits is predominant if primary interaction (imprinting) had already taken place. Intracellularly no gold-labeled hormone could be seen in coated vesicles, whereas it was abundantly present in smooth vesicles. These carry the hormone to the lysosomes which are, in all probability, responsible for its intracellular breakdown. Unlike earlier observations /5/, no gold particles could be seen inside the nucleus in the present study. This does not, however, exclude the possibility of the intranuclear penetration of gonadotropin and TSH in the mammalian testicle and ovary, since the dimensions of the colloidal gold particles used for labeling may have been too large for passage across the nuclear pores.

The present experimental observations support the conclusion that the primary interaction of the receptor with a structurally related, but functionally different hormone of the same family either in vivo or in culture does not endow the receptor with a specificity for the interacting active molecule, because the receptor is able to bind on second exposure another hormone of the same family, even to a greater degree than the hormone with which it had interacted for the first time.

REFERENCES

1. Csaba, G. (1980) Phylogeny and ontogeny of hormone receptors: the selection theory of receptor formation and hormonal imprinting. Biol. Rev. (Cambridge) 55, 47–63.
2. Csaba, G. (1981) Ontogeny and phylogeny of hormone receptors. Karger, Basel–New York.
3. Csaba, G. (1984) The present state of the phylogeny and ontogeny of hormone receptors. Horm. Metab. Res. 16, 329–335.
4. Csaba, G. (1986) Receptor ontogeny and hormonal imprinting. Experientia 42, 750–759.
5. Csaba, G., Sudár, F., Robenek, H. (1986) Internalization and localization of membrane associated DNA in the Tetrahymena. Acta Histochem. 79, 175–180.
6. Csaba, G., Török, O. (1983) Impact of FSH-TSH overlap on the growth of Chinese hamster ovary (CHO) cell culture. Acta Biol. Hung. 34, 433–434.
7. Csaba, G., Török, O. (1987) Impact of gonadotropin (FSH-LH) – thyrotropin (TSH) overlap on the growth of Chinese hamster ovary (CHO) cell culture after single and repeated treatment. Cell. Biol. Internat. Rep. 11, 495.
8. Frens, G. (1972) Controlled nucleation for the regulation of particle size in monodisperse gold suspension. Nature Phys. Sci. 241, 20–22.
9. Gorden, P., Carpentier, J.-L., Obberghen, E., van Barazzone, P., Roth, J., Orci, L. (1979) Insulin-induced receptor loss in the cultured human lymphocytes: qualitative morphological perturbations in the cell and plasma membrane. J. Cell. Sci. 39, 77–88.
10. Gorden, P., Carpentier, J.-L., Freychet, P., Orci, L. (1980) Morphological probes of polypeptide hormone receptor interaction. J. Histochem. Cytochem. 28, 811–817.
11. Gruszczynska, M., Török, O., Sudár, F., Csaba, G. (1987) Binding, overlap and internalization of colloidal-gold-labeled gonadotropin and thyrotropin in testicle or ovary cell cultures. Z. mikr.-anat Frosch. 101, 281–287.
12. Kahn, L.D., Shifrin, S. (1982) Receptor structure and function: an explanatory approach using the thyrotropin receptor as a vehicle. In: L.D. Kahn (ed.) Hormone receptors. Wiley et Sons, Chicester, New York, Singapore, pp. 1–42.
13. Khan, M.N., Savoire, S., Khan, R.J., Bergeron, J.J.M., Posner, B.I. (1985) Insulin and insulin receptor uptake in to rat liver. Chloroquine action on receptor recycling. Diabetes 34, 1025–1030.
14. King, A.C., Cuatrecases, P. (1981) Peptide hormone-induced receptor mobility, aggregation and internalization. N. Eng. J. Med. 305, 77–88.
15. Pastan, I., Willingham, M.C. (1983) Receptor-mediated endocytosis: coated pits, receptosomes and the Golgi. TIBS 8, 250–254.
16. Steinmann, R.M., Melmann, I.S., Muller, W.A., Cohn, Z.A. (1983) Endocytosis and the recycling of plasma membrane. J. Cell. Biol. 96, 1–27.

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AGGRAVATION OF CHOLESTEROL INDUCED HYPERLIPIDEMIA BY CHRONIC VITAMIN C DEFICIENCY: EXPERIMENTAL STUDY IN GUINEA PIGS

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Chronic vitamin C deficiency was induced in guinea pigs by restricting their vitamin C intake to 0.5 mg daily. This was just sufficient to prevent rapidly fatal scurvy and 55 per cent of the animals survived. In 16 weeks their serum ascorbic acid (SAA) fell to 0.16 ± 0.06 mg/dl as compared to 0.73 ± 0.11 in control animals receiving 5 mg vitamin C daily. There was a marked increase in serum cholesterol, LDL-cholesterol, VLDL-cholesterol, triglycerides and total lipids. HDL-cholesterol was, however, decreased resulting in a shift of the LDL/HDL ratio from 1.13 ± 0.16 in the control to 5.91 ± 1.70 in the low vitamin C group. Cholesterol feeding (100 mg/day) by itself lowered the SAA significantly, besides producing hyperlipidemia. When the vitamin C intake was reduced to only 0.5 mg/day, the effects of cholesterol feeding were exaggerated; the magnitude of hyperlipidemia was now significantly greater than with simple cholesterol feeding. The LDL/HDL ratio rose to 19.02 ± 3.32 from 1.13 ± 0.16 in the normal guinea pigs. Chronic vitamin C deficiency seems to affect the blood lipid profile unfavourably which could promote atherogenesis.

Keywords: Vitamin C, SAA, chronic scurvy, experimental hyperlipidemia, blood lipids

INTRODUCTION

Several reports in the last two decades have shown that vitamin C administration, about 5 to 20 times above the physiological requirement, can lower blood cholesterol in man /1–4/ and animals /5–7/. Conversely,

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elevated blood lipids and atherogenic changes in the aorta have been observed during acute vitamin C deprivation in guinea pigs /8, 9/. However, acute scurvy is a complex metabolic state in which the effect of haemorrhages, anorexia, marked weight loss and associated other nutritional deficiencies complicate the picture. It does not simulate the chronic, mild or latent vitamin C deficiency clinically encountered today. It is likely that the changes in blood lipids would be different in severe, rapidly fatal scurvy and a mild prolonged hypovitaminosis. This fact, as well as differences in the age and sex /10/, initial cholesterol level /11/ etc. of the subjects could account for some of the discrepancies reported in the vitamin C — blood lipid relationship.

We have now investigated the blood lipid changes in male guinea pigs kept on a controlled vitamin C intake to simulate a chronic deficiency state, with serum ascorbic acid (SAA) levels less than one-third of the control. Some of these animals have also been challenged with a high cholesterol intake to throw further light on the role of vitamin C in blood lipid regulation.

METHODS

Seventy male guinea pigs (weight 200—300 g) were kept on a vitamin C free diet /12/. They were separately given 5 mg vitamin C (L-ascorbic acid, Roche) daily by mouth to ensure a controlled and optimal vitamin C intake, assuming the average daily requirement for guinea pigs to be 16 mg/kg body weight /13/. They also received 1800 IU of vitamin A and 140 IU of vitamin D (0.2 ml of Adexolin, Glaxo) twice a week. After two weeks of acclimatization, sixty vigorous animals were picked up and randomly divided into four groups:

A.	Control	...	Vitamin C free diet + 5 mg vitamin C daily.
B.	Low vitamin C	...	Vitamin C free diet + 0.5 mg vitamin C daily.
C.	High cholesterol	...	Vitamin C free diet + 5 mg vitamin C + 100 mg cholesterol daily.
D.	High cholesterol + low vitamin C	...	Vitamin C free diet + 0.5 mg vitamin C + 100 mg cholesterol.

All the animals continued to receive vitamin A and D as before. The weight of the animals and their general condition was recorded every week.

At the end of four months of treatment the animals were sacrificed after an over-night fast. Blood was collected and following estimations made: Serum ascorbic acid /14/, total cholesterol /15/, HDL-cholesterol /16, 17/, triglycerides /18/, phospholipids /19/ and total lipids /20/. The LDL-cholesterol was determined indirectly according to Friedwald et al. /21/ by subtracting HDL-cholesterol and VLDL-cholesterol (estimated as 1/5 of triglycerides) from total cholesterol.

The data was analyzed using Student's t-test.

RESULTS

During the four months of follow-up 2 of the control guinea pigs died due to unexplained reasons, without any signs of infection or injury. The mortality was obviously higher (46.6%) in the two chronic vitamin C deficient groups B and D. It is well known that guinea pigs are highly sensitive to vitamin C deficiency and it is not easy to maintain them in a state of hypovitaminosis C over a prolonged period. Similarly, 5 animals died in the cholesterol fed group (Group C).

At the end of the experiment all the surviving animals were found to have gained weight, but to a much lesser extent in the two low vitamin C groups receiving only 0.5 mg vitamin C daily as compared to the control who were on 5 mg daily (Fig. 1).

Table 1 shows tha SAA levels after 4 months of different feeding schedules. Vitamin C restriction lowered the SAA level significantly ($P < 0.001$), as seen in Groups B and D. It had fallen to almost one-fifth of the mean control value. It was interesting to note that cholesterol feeding by itself also significantly ($P < 0.001$) lowered the SAA level, despite the normal 5.0 mg daily vitamin C intake.

Table 2 summarizes the lipid profile of the four groups at the end of the experiment. Vitamin C restriction produced a marked increase in serum total cholesterol, LDL-cholesterol, triglycerides and total lipids ($P < 0.001$). On the other hand, HDL-cholesterol recorded a significant fall, markedly increasing the LDL-cholesterol/HDL-cholesterol ratio.

Cholesterol feeding (Group C) also presented an essentially similar change in the lipid profile as vitamin C restriction. The magnitude of the

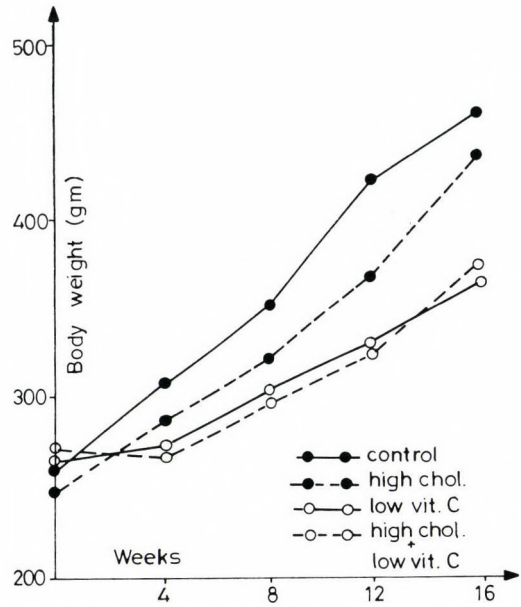

Fig. 1. Changes in body weight of guinea pigs kept on different dietar regimes for 16 weeks. The body weight (g) increased from 260.0 ± 13.1 to 460.5 ± 11.6 in the control group; from 221.0 - 12.9 to 408.5 ± 12.8 in the high cholesterol group; from 261.5 ± 20.1 to 368.0 ± 15.7 in low vitamin C group; and from 270.5 ± 20.1 to 375.2 ± 13.6 in high cholesterol + low vitamin C group. The values are mean ± S.E.

Table 1

SAA of guinea pigs on different dietary regimes for 16 weeks

Group	No.of animals surviving out of 15	SAA (mg/dl) mean ± SD
A. Control	13	0.73 ± 0.11
B. Low vitamin C	8	0.16 ± 0.06
C. High cholesterol	10	0.50 ± 0.08
D. High cholesterol + low vitamin C	8	0.10 ± 0.04

Statistical analysis: Group A with Group B ... P 0.001
Group A with Group C ... P 0.001
Group C with Group D ... P 0.001

Table 2

Serum lipid profile of guinea pigs (values mean ± SD)

	Group A Control	Group B Low vitamin C	Group C High cholesterol	Group D High cholesterol + low vitamin C
Total cholesterol (mg/dl)	91.3 ± 10.6	200.4 ± 33.7***	353.9 ± 27.2***	404.6 ± 42.8**
HDL-cholesterol (mg/dl)	37.6 ± 5.3	26.9 ± 4.8***	24.2 ± 5.3***	19.5 ± 3.3*
LDL-cholesterol (mg/dl)	42.5 ± 6.8	153.2 ± 25.0***	304.3 ± 30.8***	357.5 ± 44.8*
VLDL-cholesterol (mg/dl)	11.1 ± 1.9	20.2 ± 1.0***	25.3 ± 1.7***	27.3 ± 2.2
Triglycerides (mg/dl)	55.8 ± 10.0	101.2 ± 5.4***	126.9 ± 8.7***	136.9 ± 14.4
Phospholipids (mg/dl)	64.0 ± 10.2	96.6 ± 11.5***	122.3 ± 11.8***	126.6 ± 19.2
Total lipids (mg/dl)	249.5 ± 25.7	487.8 ± 30.2***	662.0 ± 64.6***	717.2 ± 51.8
C/P ratio	1.43 ± 0.14	2.07 ± 0.18***	2.89 ± 0.20	3.19 ± 0.57
LDL-chol/HDL-chol ratio	1.13 ± 0.16	5.91 ± 1.70***	13.30 ± 3.84***	19.02 ± 3.32*

The low vitamin C (B) and high cholesterol (C) groups have been compared with control (Group A). High cholesterol + low vitamin C Group (D) has been compared with high cholesterol group (C). *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; rest not significant with $P > 0.05$

change in blood lipids was further enhanced in Group D where cholesterol feeding was combined with vitamin C restriction.

DISCUSSION

Guinea pigs, like man, do not synthesize vitamin C and depend solely on its availability in the diet. In the present study 5 mg vitamin C daily has kept the animals healthy and active with normal gain in body weight. On the other hand, a daily intake of only 0.5 mg just sufficed to prevent acute fulminating scurvy. The animals still gained weight, but to a much lesser extent (Fig. 1). The deficiency state is also reflected in the SAA level which dropped to 0.16 $\pm$ 0.06 mg/dl from 0.73 $\pm$ 0.11 in the control group (Table 1). In acute full blown scurvy SAA falls to undetectable levels and such guinea pigs do not survive for more than 3–4 weeks /22, 23/. In the present study, a minimum daily supplement of 0.5 mg vitamin C allowed 16 out of 30 animals to survive in a state of chronic hypovitaminosis C without any intercurrent infection. This permitted a sufficiently long follow-up of 16 weeks for the assessment of blood lipid changes. It must be emphasized that the animals which had died during this period have already been excluded and, therefore, there is no possibility of any other factor influencing the blood lipids, except the vitamin C deficiency.

Prolonged vitamin C deficiency has produced an overall hyperlipidemia with total cholesterol, VLDL-cholesterol, triglycerides and total lipids almost double the control values. But perhaps the most striking change is in relation to HDL and LDL. HDL-cholesterol moderately decreased while LDL-cholesterol registered a sharp rise from 42.5 $\pm$ 6.8 to 153.2 $\pm$ 25.0 mg/dl. Both these changes have affected the LDL/HDL ratio unfavourably. In other words, the increase in blood cholesterol induced by vitamin C deficiency is mostly in the LDL fraction and this relative preponderance of LDL + VLDL over HDL is known to promote atherogenesis /25, 26, 27/. The cholesterol/phospholipid (C/P) ratio has also gone up (Table 2). Normally, plasma phospholipids stabilize the colloidal dispersion of cholesterol and, thereby, prevent its deposition in the arterial wall. This increase in C/P ratio would, therefore, add to the risk of atherogenesis /28/. A similar increase in total cholesterol and triglycerides has been reported earlier in scorbutic guinea pigs /8, 9, 29, 30/.

The hyperlipidemic effect of vitamin C deficiency has been further

exaggerated by a high cholesterol diet. Total cholesterol and LDL-cholesterol are significantly higher in cholesterol fed animals when they receive only 0.5 mg vitamin C daily instead of the normal 5 mg. The LDL/HDL ratio is shifted further towards an unfavourable limit, reaching a value of 19.0 as compared to 1.1 in the control. Thus, the potentially atherogenic effect of a high cholesterol diet is being augmented by vitamin C deficiency.

Interestingly enough, cholesterol feeding alone has significantly lowered the SAA despite a normal vitamin C intake. This shows that the vitamin C – blood lipid relationship operates in both directions. Ginter /31/ has also found that prolonged cholesterol feeding depletes tissue ascorbic acid and increases the body's demand for vitamin C. Although not directly involved in lipid metabolism, ascorbic acid indirectly influences it in many ways. For example, it is known to promote 7 alpha-hydroxylation of cholesterol to bile acids /32/, and enhance the activity of lipoprotein lipase /8, 30/. It can, therefore, be surmised that chronic vitamin C deficiency compromises the body's ability to eliminate or metabolize excess endogenous and exogenous cholesterol. Consequently, hyperlipidemia results.

To what extent hyperlipidemia in human subjects is associated with a latent vitamin C deficiency is difficult to state at present. But this would be a pertinent nutritional question in many parts of the world where either fat intake is excessively high or vitamin C intake excessively low.

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REFERENCES

1. Spittle, C.R. (1971) Atherosclerosis and vitamin C. Lancet 2, 1280–1281.
2. Ginter, E. (1972) Atherosclerosis and vitamin C. Lancet 1:1233.
3. Ginter, E. (1976) Vitamin C and plasma lipids. New Engl. J. Med. 294, 559.
4. Kothari, L.K., Jain, K. (1977) Effect of vitamin C administration on blood cholesterol level in man. Acta Biol. Acad. Sci. Hung. 28, 111–114.
5. Nambisan, B., Kurup, P.A. (1975) Ascorbic acid and Glycosaminoglycan and lipid metabolism in guinea pigs fed normal and atherogenic diet. Atherosclerosis 22, 447–461.

6. Odumosu, A., Wilson, C.W.M. (1979) Hypocholesterolemic effect of vitamin C, Clofibrate and diosgenine in male guinea pig. Brit. J. Pharmacol. 67:456–457.

7. Sharma, P., Pramod, J., Sharma, P.K., Kothari, L.K. (1988) Effect of vitamin C on serum and aortic lipid profile of guinea pigs on normal and atherogenic diet. Ind. J. Med. Res. 87, 283–289.

8. Fujinami, T., Okado, K., Senda, K., Sugimura, M., Kishikawa, M. (1971) Experimental atherosclerosis with ascorbic acid deficiency. Jap. Circul. J. 35, 1559–1565.

9. Yokota, F., Igarashi, Y., Suzue, R. (1981) Hyperlipidemia in guinea pigs induced by ascorbic acid deficiency. Atherosclerosis 38, 249–254.

10. Pramod, J., Sharma, P., Kothari, L.K. (1986) Effect of age and sex on serum and leucocyte ascorbic acid levels in normal human subjects. Vitaminologia 2, 93–99.

11. Ginter, E. (1979) Pretreatment serum cholesterol and response to ascorbic acid. Lancet 2, 958.

12. Banerjee, S. (1945) Relation of scurvy to the adrenaline content of adrenal gland of guinea pigs. J. Biol. Chem. 159, 327–331.

13. Reid, M.E., Bieri, J.G. (1972) Nutrient requirement of guinea pig. In: Nutrient Requirements of Domestic Animals, No. 10, 2nd revised edition, National Academy of Sciences – National Research Council (Washington DC), 9–19.

14. Natelson, S. (1971) Determination of ascorbic acid by using 2,4-Dinitrophenylhydrazine. Techniques of clinical chemistry. Charles C Thomas Publisher, Springield, Illinois, USA, 162–165.

15. Abell, L.L., Levy, B.B., Brodie, B.B., Kendall, F.E. (1952) A simple method for the estimation of total cholesterol in serum and demonstration of its specificity. J. Biol. Chem. 195, 357–366.

16. Burnstein, M., Scholnick, H.R., Morfin, R. (1970) Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanion. J. Lipid. Res. 11, 583–595.

17. Lopes-Virella, M.F., Stone, R., Ellis, S., Colwell, J.A. (1977) Cholesterol determination in high density lipoproteins separated by three different methods. Clin. Chem. 23, 882–884.

18. Gottfried, P., Rosenberg, B. (1973) Improved manual spectrophotometric procedure for determination of serum triglycerides. Clin. Chem. 20, 1077–1078.

19. Zelversmit, D.B., Davis, A.K. (1950) Microdetermination of plasma phospholipids by trichloroacetic acid precipitation. J. Lab. Clin. Med., 35, 155–160.

20. Jacob, S.L., Henry, R.J. (1974) Determination of total lipids. In: Clinical Chemistry, Principle and Techniques. Bioscience, Harper and Row Publisher, London, pp. 1427–1428.

21. Friedwald, W.T., Levy, R.I., Fredrickson, D.S. (1971) Estimation of the concentration of low density lipoprotein cholesterol in plasma without use of the preparative ultracentrifuge. Clin. Chem. 18, 499–502.

22. Barnes, M.J., Constable, B.J., Impey, S.G., Kodicek, E. (1973) Mortality rate in male and female guinea pigs on scorbutogenic diet. Nature (Lond.) 242, 522–523.

23. Hughes, R.E., Hurley, R.J., Jones P.R. (1971) The retention of ascorbic acid by guinea pig tissues. Brit. J. Nutr. 26, 433–438.

24. Ginter, E., Bobek, P., Ovecka, M. (1968) Model of chronic hypovitaminosis C in guinea pigs. Intern. J. Vitamin Res. 38, 104–113.

25. Gordon, T., Castelli, W.P., Hjortland, M.C., Kannel, W.B., Dawber, T.R. (1977) High density lipoprotein as a protective factor against coronary artery disease. The Framingham Study. Am. J. Med. 62, 707–714.

26. Castelli, W.P., Doyle, H.T., Gordon, T., Hames, C.G., Hjortland, M.C., Hulley, S.B., Kagan, A., Zukel, W.J. (1977) HDL-cholesterol and other lipoprotein in coronary heart disease. The cooperative lipoprotein phenotyping study. Circulation 55, 767–72.

27. Grundy, S.M. (1983) Atherosclerosis: Pathology, Pathogenesis and Role of Risk Factors. Vol. 29. Year Book Medical Publishers INC. Chicago, London pp. 31–35.

28. Oliver, M.F., Boyd, G.S. (1953) The plasma lipids in coronary artery disease. Brit. Heart. J. 15, 387–392.

29. Ginter, E., Ondreicka, R., Bobek, P., Simko, V. (1969) The influence of chronic vitamin C deficiency on fatty acid composition of blood serum, liver triglycerides and cholesterol esters in guinea pig. J. Nutr. 99, 261–66.

30. Bobek, P., Ginter, E. (1978) Serum triglycerides and post-heparin lipolytic activity in guinea pigs with latent vitamin C deficiency. Experientia 34, 1554–55.

31. Ginter, E. (1970) Effect of dietary cholesterol on vitamin C metabolism in laboratory animals. Acta Med. Sci. Hung. 27, 23–29.

32. Ginter, E. (1973) Cholesterol: Vitamin C controls its transformation to bile acids. Science 179, 702–704.

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HISTOCHEMICAL DETECTION OF HEMP TRICHOMES AND THEIR CORRELATION WITH THE THC CONTENT

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Authors investigated some fiber and some hashish hemp sorts concerning their trichomes on the leaves. Histochemical reactions were developed using the Fast Blue Salt (FBB) reagent applied so far only thin layer chromatography. Glandular hairs were found giving positive reactions due to cannabinoids contained by the cells. The electron microscopic features were studied and the cannabinoid content was measured with GC. A correlation was found between the number of typical glandular hairs and cannabinoid content.

Keywords: Hemp, Cannabis sativa THC, trichomes, detection

INTRODUCTION

In the last decade many hashish type and fiber type hemp sorts were investigated for ecological and genetical purposes by several authors including ourselves /2, 10/. During this work we turned our attention on the anatomical features, and have found, that the well known and described cannabis trichomes /1, 3, 7/ are characteristic only for the petals. The leaves are covered with covering trichoms, and other kind of glandular hairs, which are present also on the petals. As the trichomes are suitable

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Abbreviations: THC = tetrahydrocannabinol psychotopic compound; GC = gaschromatographic method; OPTLC = overpressure thin layer chromatography

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markers for identification /5, 7/, we compared the different hemp sorts based on their trichom character, and measured parallelly cannabinoid content.

METHODS

Sorts of hemp

Hashish sorts given by UNO Narcotics Laboratory, Geneva 1976 were:

1. Mexico sort UNC 347
2. Thailand sort UNC 255.

   Fiber sorts, given by Agrobotanical Research Institute (Dr. I. Bócsa) Kompolt (Hungary)
3. Kompolti Hungarian sort
4. Chinese unisex sort
5. Spanish sort

Histochemistry

Cross sections were made and epidermis skins immersed into the reagent for 10 min. After this procedure the samples were rinsed and covered with glass. The detection was made on 10 parallels by light microscope (Zeiss Jena type) at two different magnifications (40x and 120x).

For detection of cannabinoid in the trichomes Fast Blue B=FBB was applied. This reagent was used so far only for the detection of cannabinoids in thin layer chromatography (TLC). A 2% solution of the reagent in 70% alcohol stains the cannabinoids red.

The typical Labiatae-type glandular hairs were observed with a transmission electron microscope (TESLA), magnification was 200–800x.

Electron microscopic investigations were done according to Sárkány /6/.

For gas chromatographic (GC) analysis 2–10 ml of the extraction solvent, containing 4-androstene-3,17-dione as internal standard at a concentration of 2 g/l was used /8, 10/.

For OPLC analysis a Chrompres 10 and a Chrompres 25 system obtained from Labor MIM (Budapest, Hungary) were used /4/.

RESULTS

It has been stated that the occurrence of trichomes on the abaxial leaf epidermis is variable and characteristic for the species.

On the abaxial surface of the leaf of the so called non-hashish hemp (C. sativa convar. sativa) containing lower levels of THC, a great number of long, slender covering hairs can be found (Fig. 1). In the Spanish and Kompolti sorts short retort hairs develop having a basally oval shape and their apex ends are in a short peak. Their characteristic feature is, that they are surrounded by plasm-rich, parenchymatic epithelial cells (Figs 2, 3, 4).

All these hairs give a positive Toluidine blue reaction, but not with the FBB reagent. It means that they do not contain THC and the FBB stained cannabinoids. On the other hand, certain glandular hairs, which are well-marked at the hashish hemp species give positive (red colour) reaction with FBB (Echtbluesaltz) (Figs 5, 6, 7), and a black reaction with Toluidine blue (Fig. 8). This glandular hairs on the leaf are manifold. There are little glandular hairs having one-cell stalk but multicellular head; multicellular /2—3/ stalk and head with 8 cells arranged (like the Labiatae hairs).

These hairs can be found mainly on Mexican and Thailandian species (see Table 1) (Fig. 9).

Data suggest that the anatomical and histochemical analysis of the abaxial epidermis of the leaf gives information about the chemical character of the examined hemp. The reagent used for histochemistry is a characteristic one which can develop cannabinoids on the OPLC plate. The colours of neutral and acidic components are similar to each other and to the colour of painted glandular hairs (Fig. 10).

Electron microscopically we could detect the secretion of cannabinoids in the Golgi apparatus, and during the process the vesicles grew larger forming vacuoles. In the young glandular hairs there were numerous small vacuoles present fusing subsequently to form a big center vacuole (Figs 11, 12, 13).

The process of resinification was observable, and parallelly an increase in density, forming more and more resins in the vacuoles was noticed. In young states the chloroplasts were detectable showing the connection between assimilation and cannabinoid biosynthetic processes (Fig. 14).

The mitochondria are clearly visible in the earlier stage, but later they get deformed and disappear from the glandular hairs (Fig. 15).

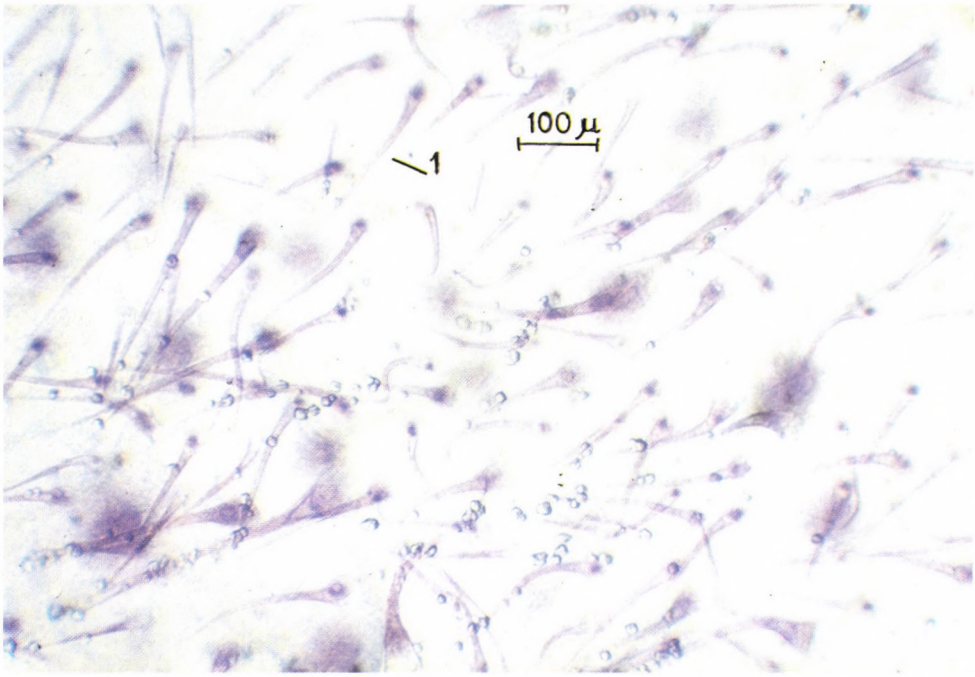

Fig. 1. The Kompolti fiber sort with low THC content. Dominant are the simple covering trichomes /1/

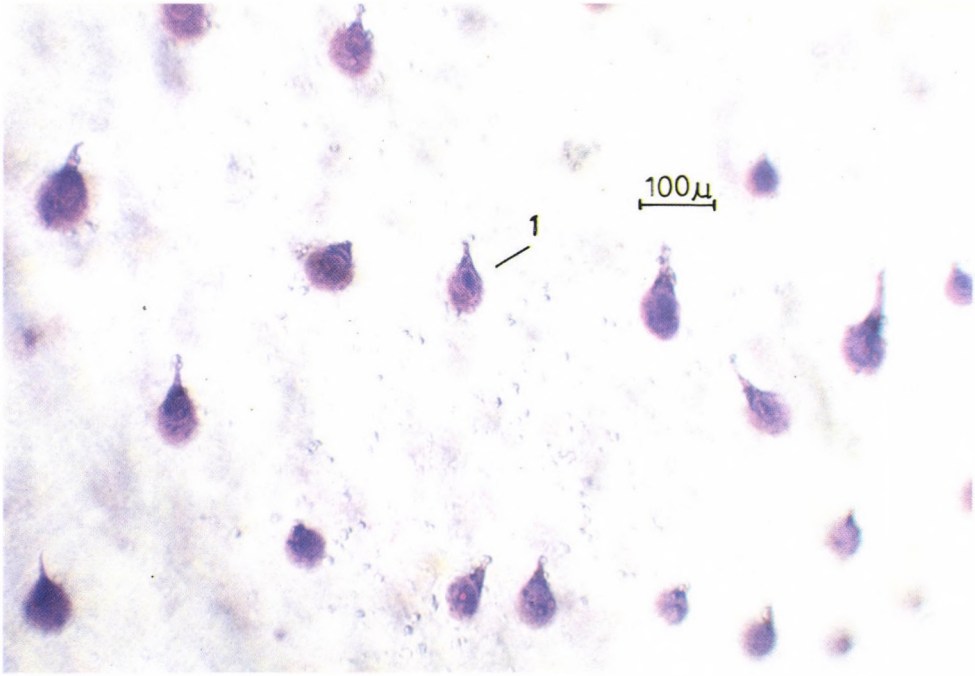

Fig. 2. On the lower surface other non-hashish — so-called cystolith containing trichomes /1/ are present

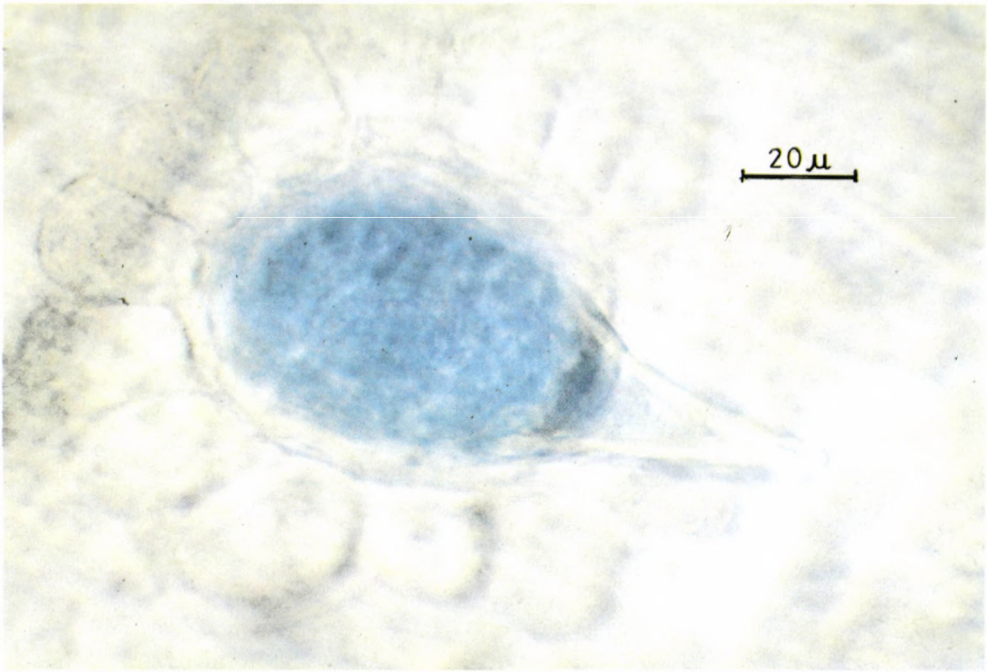

Fig. 3. The same hemp sort. Cystolith trichome x 100

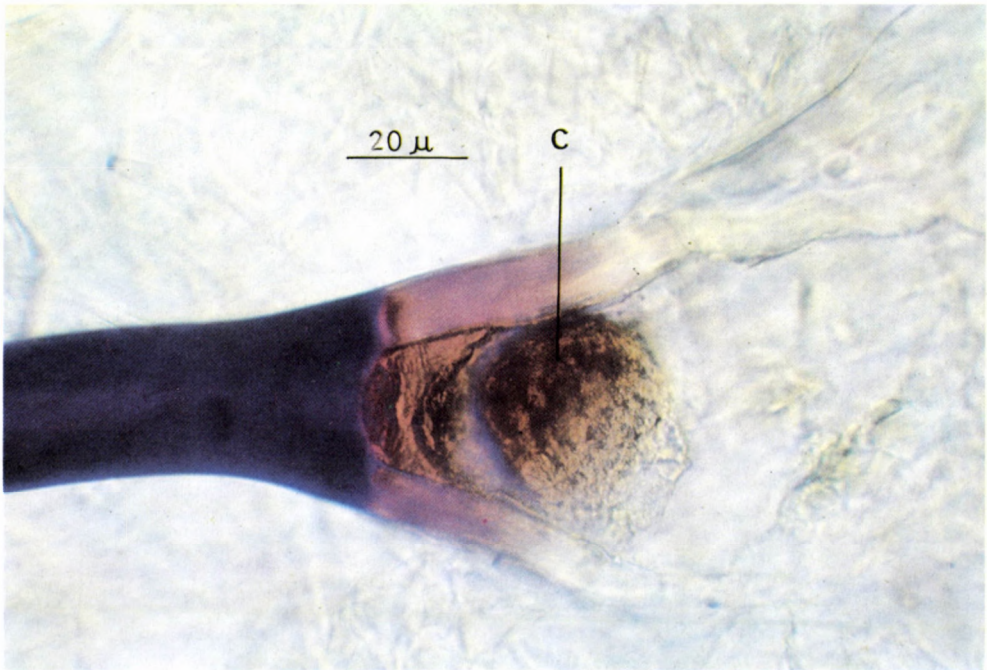

Fig. 4. Non-hashish, covering trichome with crystal

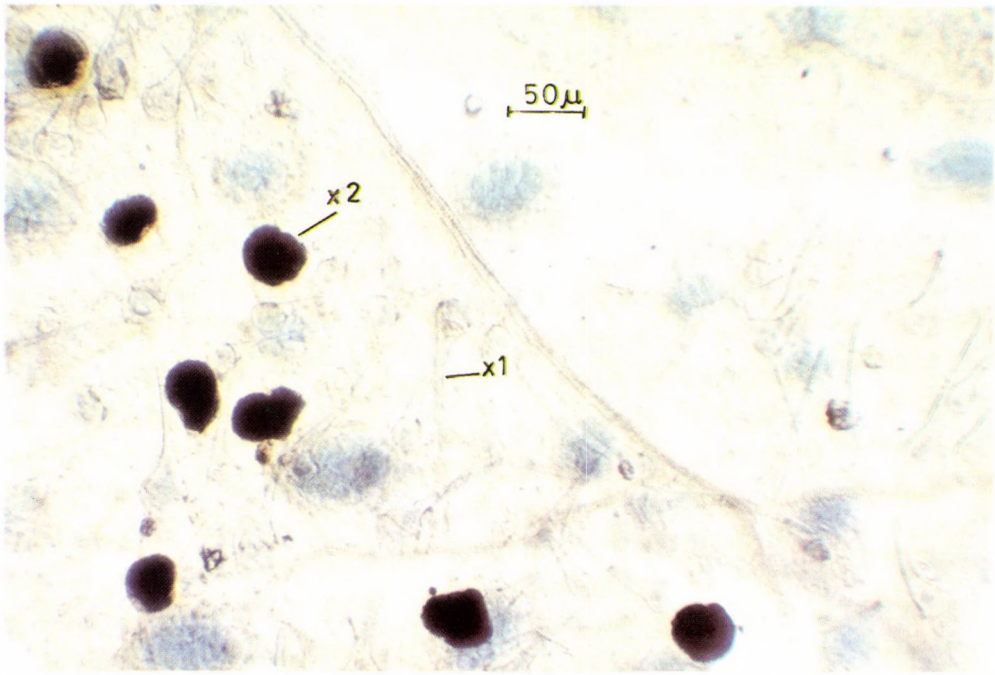

Fig. 5. Chinese hemp sort with higher THC content. Leaf epidermis stained with Toluidine blue. 1) Covering trichome; 2) Glandular hair

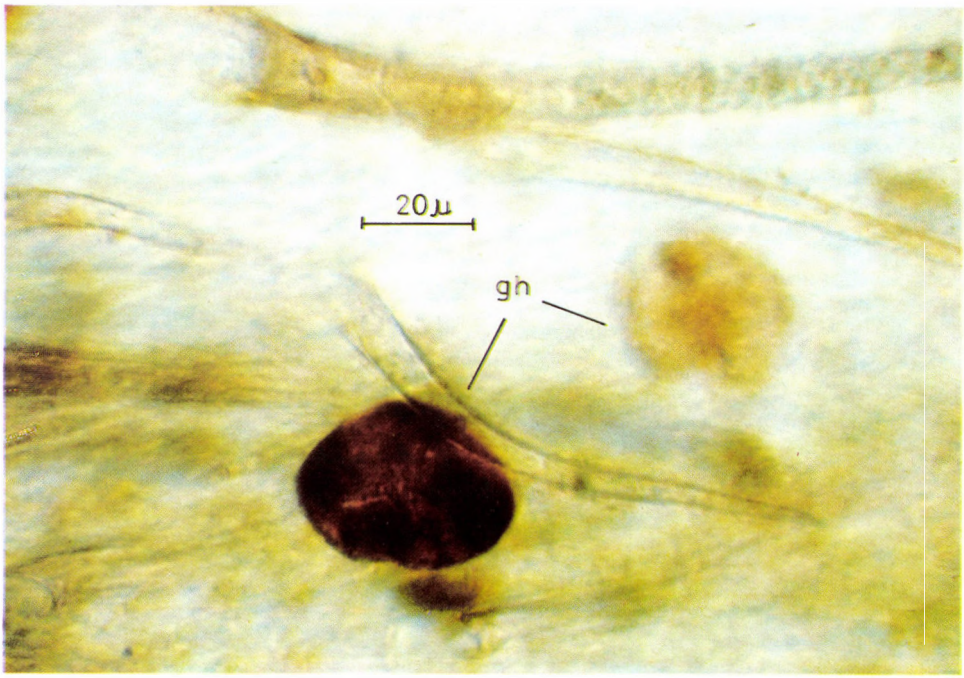

Fig. 6. Chinese hemp sort. Leaf stalk epidermis, treated with FBB reagent. The cannabinoid containing glandular hairs will be red

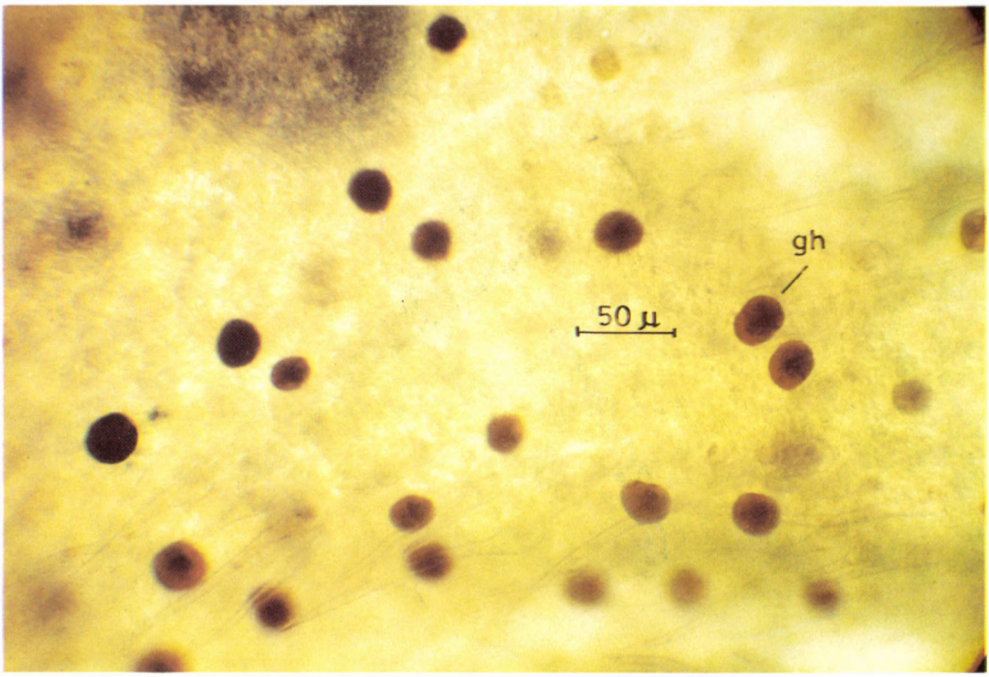

Fig. 7. Thailand hemp sort with many hashish containing glandular hairs (gh) (FBB reaction)

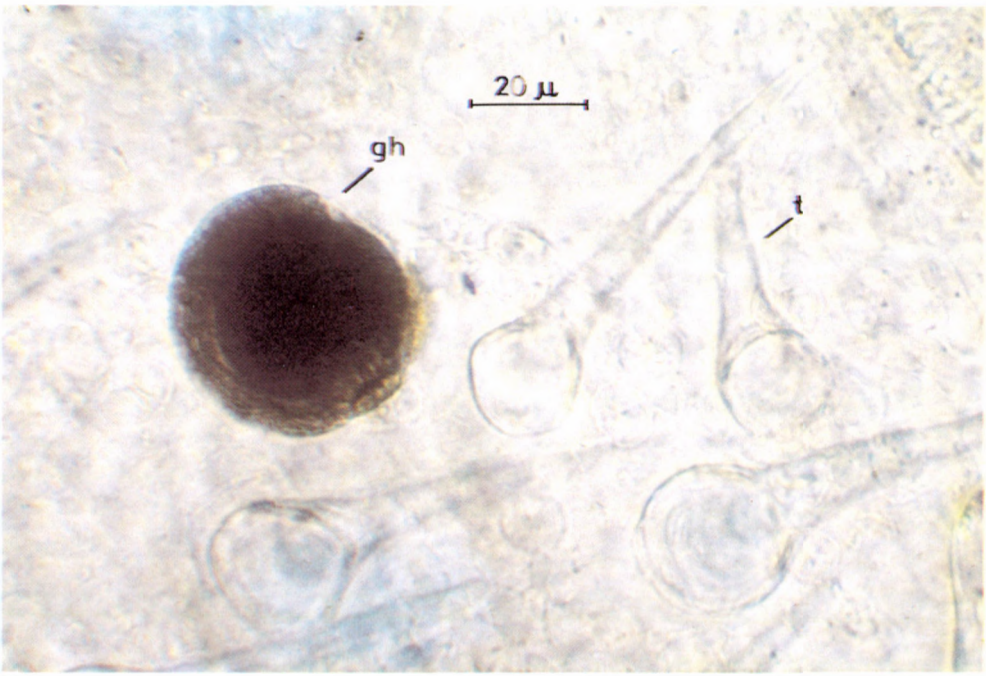

Fig. 8. Mexico hemp sort stained with Toluidine blue

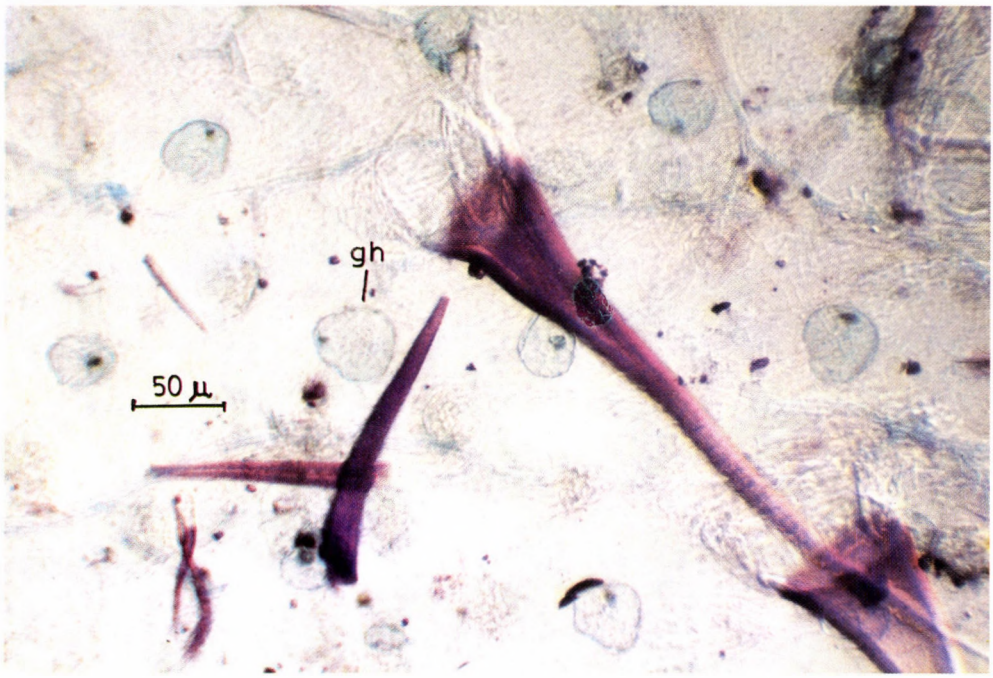

Fig. 9. The same sort stained with Toluidine blue after extraction of cannabinoids

The cannabinoid content of the samples is in a good correlation with the presence of hashish trichomes (Table 1) (Figs 16, 17).

The described histochemical reaction demonstrates the application of the quick method in getting preliminary information as to the characterization of the samples and for the improvement, but these informative examinations do not eliminate the necessity of a quantitative phytochemical characterization.

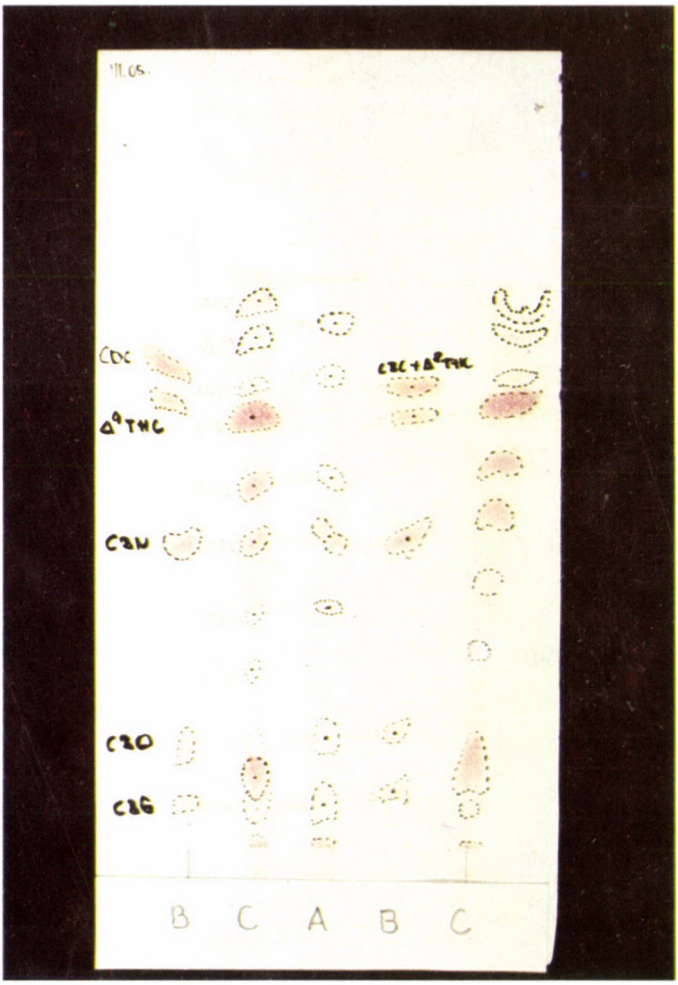

Fig. 10. OPLC chromatogram CBC = Cannabichromene, THC = tetra hydrocannabinoid

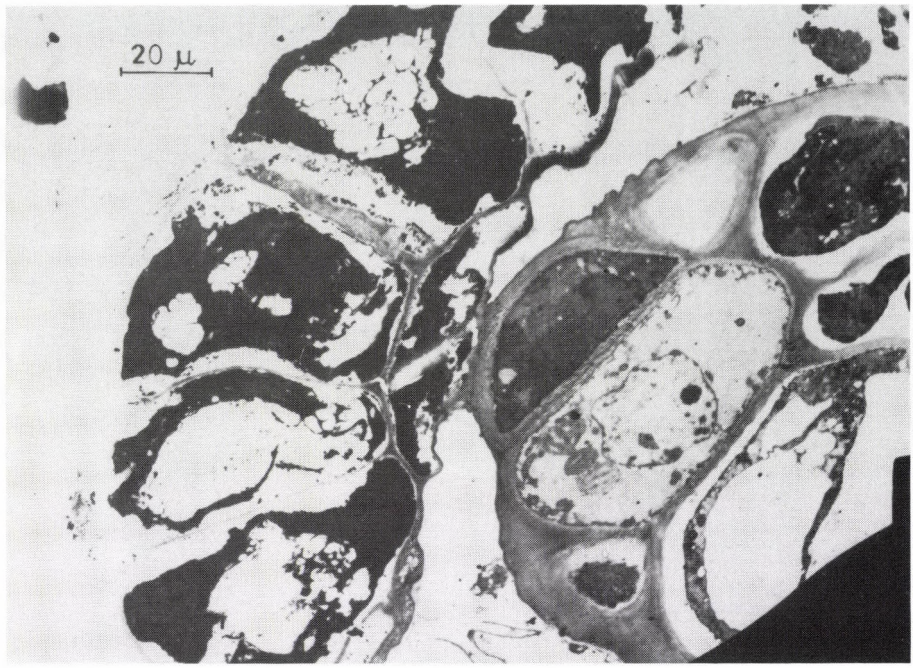

Fig. 11. Electron microscopic picture from the hashish glandular hair

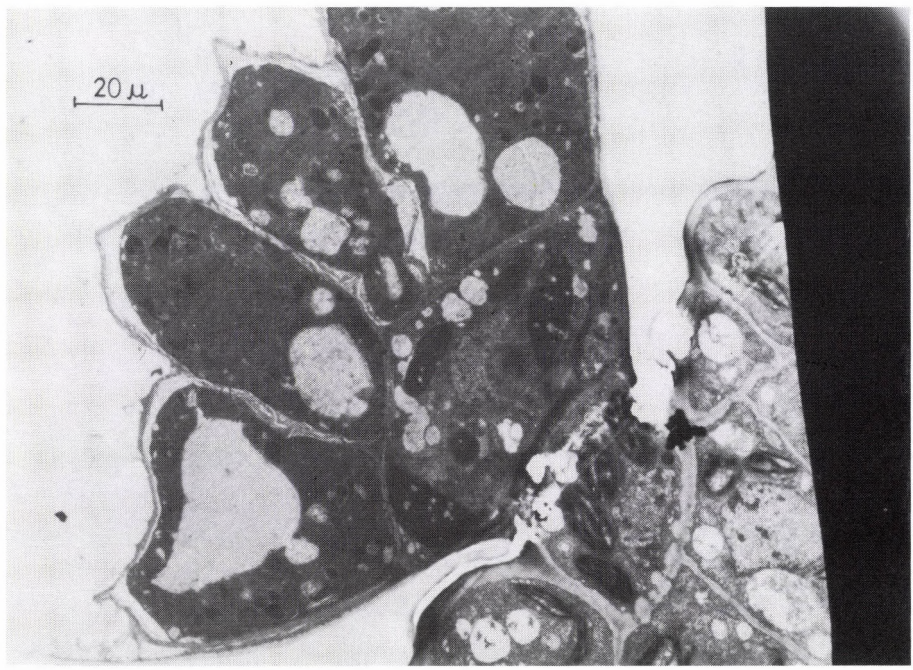

Fig. 12. Electron microscopic picture from upper part of a young glandular hair of Thailand hemp. x 4800

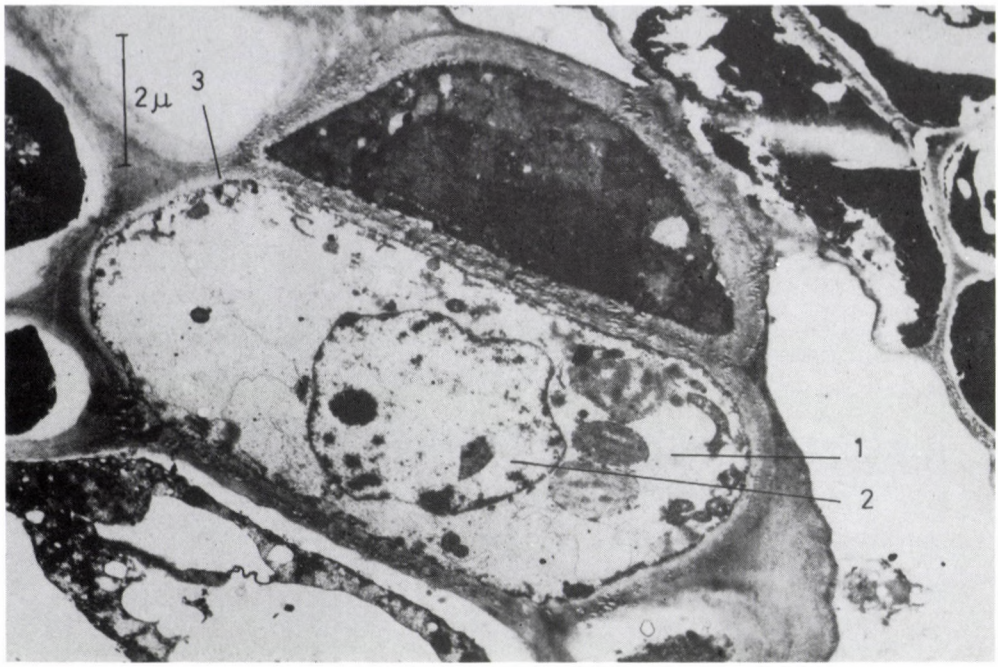

Fig. 13. Basal part from a little hashish hair of Thailand hemp. Chloroplast and other cell organelles are present. x 4200, 1 = chloroplast, 2 = cell, 3 = lysosome

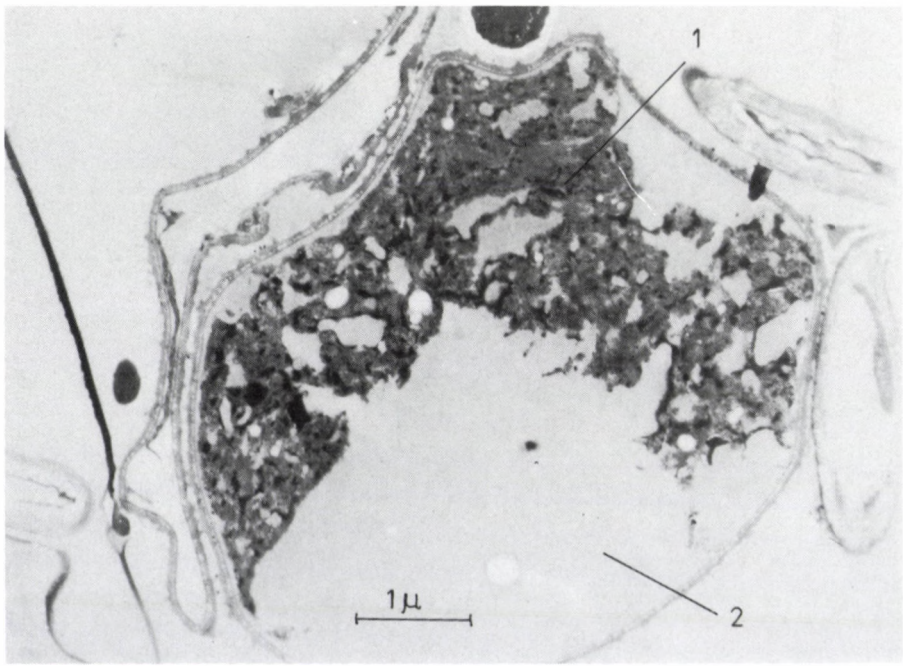

Fig. 14. Small glandular hair with FBB positive reaction from petal of Thailand hashish hemp. x 4800, 1 = chloroplast, 2 = vacuole

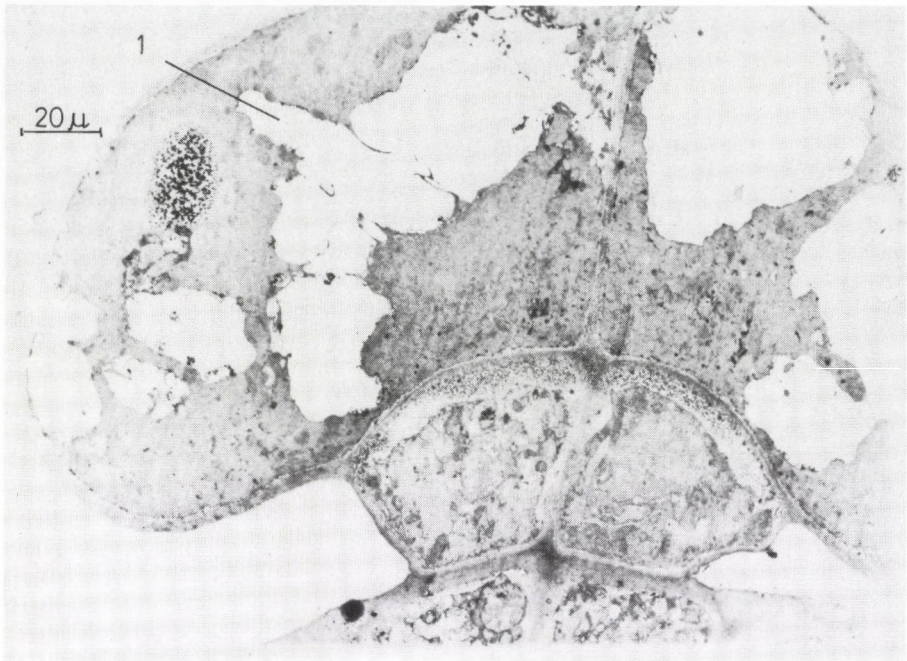

Fig. 15. Electron microscopic picture of hashish glandular hair from Chinese hemp petal. x 4200, 1 = vacuole

Table 1

Relations between trichome types and THC content in different hemp sorts

Hemp sort	Trichomes without glandular reaction	Cystolyth containing trichomes (per 1 mm²)	Small glandular hairs (per 1 mm²)	Labiatae-like (so called hashish type)	THC content %	Phenotype according to Small and Beckstad /8/
"Kompolti" (f)	160	10 – 14	1	-	0.32	II
Spanish (f)	110	10 – 20	1 – 5	-	0.26	II
Mexican (h)	62 – 64	10 – 14	-	7 – 8	1.15	I
Chinese (h)	60 – 70	0 – 1	3 – 4	8	0.94	I

$S_{D5\%}=1.0$

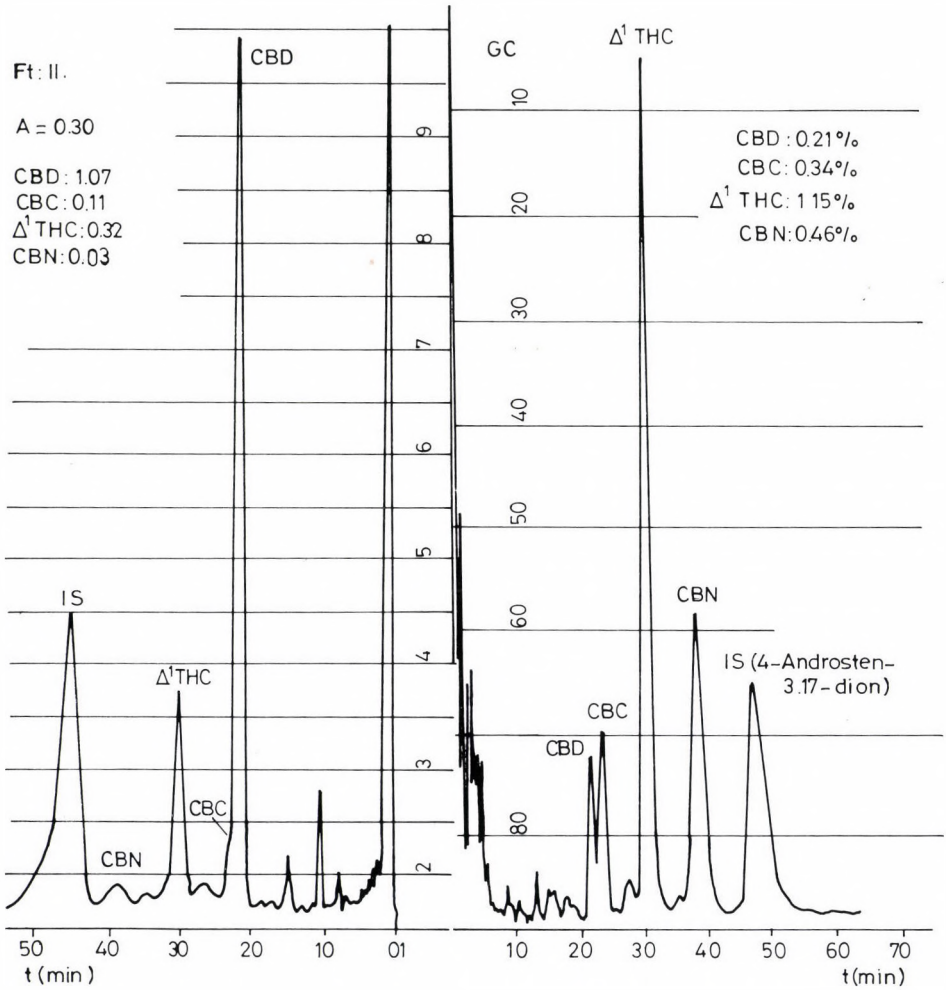

Fig. 17. GC from a fibre hemp sort Fig. 16. GC from the Mexican hemp

DISCUSSION

The cannabinoid spectrum including the THC content is characteristic for the hemp-sorts. The leaves have different segments according to hashish type or fibre type /10/.

In our work with Salvia sclarea (Labiatae) /9/ we have seen a large variety in trichomes in correlation with terpene biosynthesis. Rodriguez et

al. /5/ give many examples from volatile oil – and alkaloid plants, where a correlation exists between production of secondary products and functional intensity of secretory system. This gave the idea to study the production of cannabinoids in connection with the trichome formation. The FBB reagent was a good material for use in histochemistry. So we could detect real cannabinoid hairs. The quantity of these trichomes were in a good correlation with cannabinoid content measured by GC and OPTLC. We think that this comparison can be carried out as a short preliminary method in the identification of hemp types.

REFERENCES

1. André, C., Fercruysse, A. (1976) Histochemical study of the stalked glandular hairs of the female Cannabis plants, using Fast Blue Salt. Plant. Med. 29, 361–366.
2. Mechoulam, R., Lander, N. (1980) Cannabis: a possible source of new drugs. Pharmacy International, 1, 19–21.
3. Mahlberg, P.G., Hammond, C.T., Turner, J.C., Hemphill, J.K. (1984) Structure development and composition of glandular trichomes of Cannabis sativa L. In: Rodriguez, E., Healey, P.L., Mehta, I. (eds) Biology and Chemistry of Plant Trichomes. Plenum Press, New York–London, pp. 23–51.
4. Oroszlán, P., Verzár–Petri, G., Mincsovics, E., Székely, T. (1987) Separation, quantitation and isolation of cannabinoids from Cannabis sativa L. by overpressured layer chromatography, J. Chromatography (In press)
5. Rodriguez, E., Healey, P.L., Mehta, I. (1984) Biology and Chemistry of Plant Trichomes. Plenum Press, New York–London.
6. Sárkány, S., Rákosi–Szentpétery, G., Verzár–Petri, G., Kovács, A. (1969) Licht- und elektronenmikroskopische Untersuchung und chemische Zusammensetzung des aetherischen Öles von Valeriana Collina Wallr. Herba Hung., Budapest.
7. Turner, J.C., Hemphill, J.K., Mahlberg, P.G. (1977) Gland distribution and cannabinoid content in clones of Cannabis sativa L. Amer. J. Bot. 64, 687–693.
8. Turner, J.C., Hemphill, J.K., Mahlberg, P.G. (1978) Quantitative determination of cannabinoids in individual glandular trichomes of Cannabis sativa L. (Cannabaceae) Amer. J. Bot. 65, 1103–1106.
9. Verzár–Petri, G., Then, M. (1975) The study of the localisation of volatile oil in the different parts of Salvia sclarea L. and Salvia officinalis L. by applying ^{14}C-sodium acetate. Acta Pharm. Hung. 45, 167–174.
10. Verzár–Petri, G., Veszki, P., Mészáros, S. (1981) Morphological comparison of local hemp varieties. Acta Agronomica Acad. Sci. Hung. 30, 350–372.

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EFFECT OF HEAVY METALS ON THE GROWTH OF TISSUE CULTURES (II)

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The effect of toxic concentrations of three heavy metal compounds on the growth of the secondary callus tissue of Nicotiana tabacum L. and Ruta graveolens L. was studied. The metal compounds examined were $ZnSO_4$, $NiSO_4$, $CuSO_4$. The metal compounds used were placed in Murashige, Skoog (1962) and White (1943) culture medium at 10^{-6} and 10^{-4} M oncentration, respectively, before autoclaving. The culture media containing macro- and microelements and vitamins were completed with carbon source and regulators (IAA, GA, kinetin for Nicotiana and IAA, 2,4–D for Ruta). The cultures were kept for 4 weeks at 25 (±2) °C under 16/8 h light/dark conditions. The value of pH was 5.6 before the autoclave treatment. The increase in fresh weight of the secondary callus tissue was inhibited by the metal compounds applied with both plant species (to 75–87 % by zinc, 7–97 % by nickel, 5–98 % by copper with tobacco; to 47–69 % by zinc, 5–88 % by nickel, 57–90 % by copper with rue). The cell number and dry weight per g of callus tissue partly increased, partly decreased compared to the control in response to the heavy metal treatment. The growth values obtained with various concentrations of the heavy metals were different in the two plant species due to differences in metabolism and organization potential between them.

Keywords: Heavy metals – cell culture – Nicotiana tabacum – Ruta graveolens

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INTRODUCTION

Two important biological problems of these days: how to increase the organic matter production and how to prevent the environment pollution cannot be tackled without biological basic research /1, 6, 7/. To increase the volume of agricultural and horticultural production use must be made of nutrient sources which have so far been left unexploited although their nutritive value is high and at the same time they decrease the danger of environment pollution /5/. According to experiences gained abroad such material is the sewage-sludge, which, however, needs thorough biological examination not to cause damages. One of the sources of such a danger is e.g. the presence and quantity of toxic heavy metals (Pb, Hg, Cd, Cr, Zn, Ni, Cu, etc.) in it, since the data available on their physiological effects on plants, on their accumulation conditions, and last but not least on the tolerance of the human organism to them are still very few /13, 15, 16/. Investigations into these aspects of heavy metals on the level of basic research have started in many countries. One method is to determine their effect on the plant cell which is easiest carried out by the concentration dependent screening of the growth- and division parameters of cells /4, 8, 10/.

Since so far only intact plants or plant organs have generally been examined for the distribution and toxic effect of heavy metals taken up, it seemed to be reasonable to study the growth damages of tissues and cells forming the organs of the different species /2, 3, 4, 12/. One of the quickest methods to be applied as the first step is to set up tissue cultures of isolated plant parts, whereby damaging effects appearing later in the intact plant can be prognose /8, 10, 14/.

MATERIALS AND METHODS

In the experiment aimed at determining the limit of tolerance shown by the plant cells to heavy metals such heavy metals and concentrations were used which usually occurred in the environment of plants and could be taken up by them /8/. Present paper gives account of the application of zinc nickel and copper in the form of $ZnSO_4$, $NiSO_4$ and $CuSO_4$. The concentrations used ranged from 10^{-6} to 10^{-4} M. The culture media contained Murashige–Skoog's (1962) /11/ and White's (1943) /17/ mineral elements completed with 2 mg

indole acetic acid (IAA), 0.2 mg kinetin (Kin), 1.0 mg gibberellic acid (GA) and 4 mg IAA, 6 mg dichloro-phenoxy acetic acid (2.4–D) per litre, respectively. For testing standard growth secondary callus tissue cultures of Nicotiana tabacum L. and Ruta graveolens L. established in the laboratory of the Station were used /8, 9, 10/. The cultures were grown at 25 ± 2 ^{o}C in a 16/8 h photoperiod for 4 weeks. To check up the effect of the metal concentrations 10 parallels were compared for fresh weight, dry matter content and cell number per unit weight. The results are shown by measured and converted data, and represented as percentages of the control as well.

RESULTS AND DISCUSSION

Effect of zinc ($ZnSO_4$) (Table 1 and Fig. 1). The increase in fresh weight of callus tissue was inhibited by all applied concentrations of zinc in both species. With tobacco the rate of inhibition was very high — 75 per cent even at the lowest concentration. In the case of rue the rate of growth inhibition was substantially lower, only 69–47 per cent compared to the control. The percentage proportion of dry weight, on the other hand, considerably increased compared to the control in the case of tobacco, while it was but slightly below the control value with rue. The number of cells per unit weight (g) was nearly equal to or exceeded by far that of the control in the case of tobacco, while for rue it just reached the corresponding value of the control. Thus, the two species considerably differ in growth response to zinc. Yet, even the highest concentration did not cause lethality in either species.

Effect of nickel ($NiSO_4$) (Table 2 and Fig. 2). The fresh weight increase of the callus tissue showed tendencies similar to those observed in the case of zinc. The only difference was that the lowest concentration of nickel sulphate caused hardly any growth inhibition. On the other hand, the highest concentration used resulted in strong inhibition and tissue necrosis, mainly in the case of tobacco. The percentage proportion of dry weight showed again a tendency opposite to the increase in fresh weight. The values generally are higher than those for the control. The number of cells with almost all concentrations reaches or even exceeds the cell number of the control, particularly in the tobacco callus.

Effect of copper ($CuSO_4$) (Table 3 and Fig. 3). In the callus tissue of tobacco the higher concentrations of copper (10^{-4}, 5.10^{-5}M) caused an

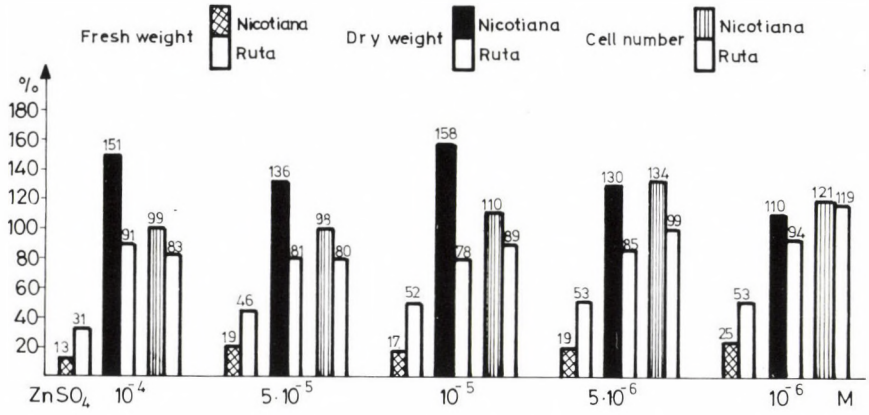

Fig. 1. Effect of various concentrations of zinc sulphate on the callus tissues of tobacco and rue, as percentage of the control

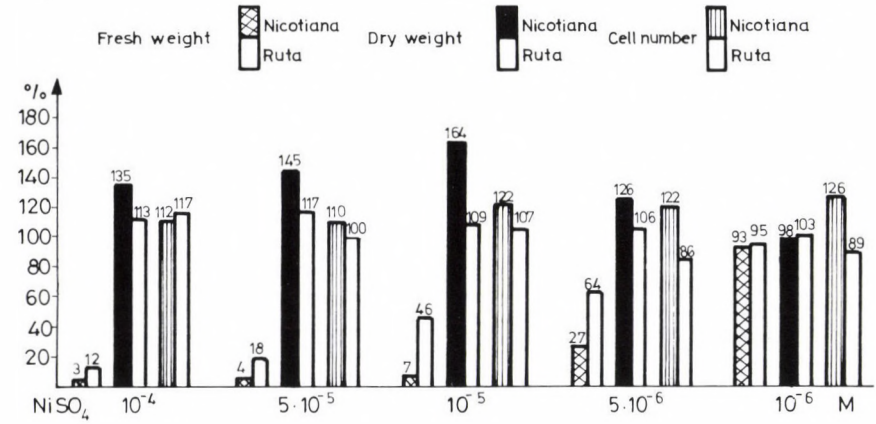

Fig. 2. Effect of various concentrations of nickel sulphate on the callus tissues of tobacco and rue, as percentage of the control

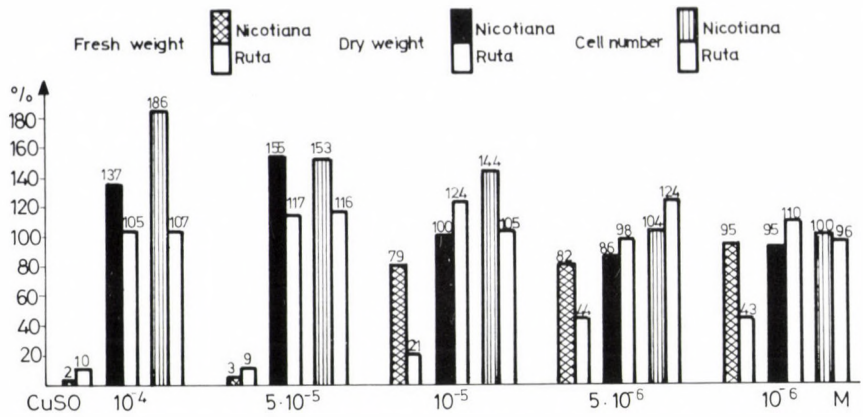

Fig. 3. Effect of various concentrations of copper sulphate on the callus tissues of tobacco and rue, as percentage of the control

almost complete inhibition of weight increase, while lower than 10^{-5}M concentrations resulted in hardly any inhibition. In the case or rue, on the other hand, even the lowest concentration applied (10^{-6}M) caused an about 50 per cent inhibition. With the two highest concentrations a considerable extent of necrosis was observed both in the tobacco- and rue tissue. The tendency of change in the percentage ratio of dry weight was in this case too opposite to that in the fresh weight, and a larger quantity of copper increased the ratio. The same general tendency was observed with the number or cells per unit weight of tissue.

According to the results of several years of experimenting with these cultures under the above conditions the growth parameters of control cultures of the species used in the experiments show realistic values /8, 9, 10/. The growth of the callus of both species was optimum on the culture media used for the control, so they may serve as a suitable basis of comparison in determining the effect of the metal compounds applied. The concentrations of the compounds examined were chosen partly on the basis of literary data, partly according to their possible occurrence in the environment /4, 13, 15/. It is also clear that besides the interactions between environment and intact plant the effects produced by the ever more frequently occurring toxic metals on the structure and metabolism of the cells and tissues of various plant species must be studied as well /14, 16/. Namely, such data may foretell harmful effects appearing but later in the intact

Table 1

Effect of zinc on the growth of Nicotiana tabacum and Ruta graveolens callus tissues

Hormones mg/l	$ZnSO_4$ M		Fresh weight (g/flask)		Dry weight %		Cell number (10^3/g)		Daily increase (mg)		Relative increase	
			Nicotiana	Ruta	Nicotiana	Ruta	Nicotiana	Ruta	Nicotiana	Ruta	Nicotiana	Ruta
	10^{-4}	$\bar{x}$	0.616	0.480	1.00	1.31	2210	4268	14.85	10.00	2.08	1.40
		$\pm s$	0.200	0.171	0.08	0.08	21	27				
Nicotiana tabacum	5.10^{-5}	$\bar{x}$	0.888	0.719	0.90	1.16	2193	4095	24.28	18.53	3.40	2.60
IAA 2.0		$\pm s$	0.121	0.258	0.01	0.10	12	34				
Kin 0.2 GA 1.0	10^{-5}	$\bar{x}$	0.782	0.806	1.04	1.12	2483	4554	20.78	21.64	2.91	3.03
		$\pm s$	0.289	0.146	0.05	0.10	6	39				
	5.10^{-6}	$\bar{x}$	0.897	0.824	0.86	1.22	3004	5086	24.89	22.28	3.48	3.12
		$\pm s$	0.269	0.154	0.12	0.11	88	32				
Ruta graveolens	10^{-6}	$\bar{x}$	1.163	0.829	0.73	1.36	2710	6105	34.39	22.46	4.81	3.14
IAA 4.0		$\pm s$	0.274	0.175	0.28	0.17	13	64				
	Control	$\bar{x}$	4.618	1.551	0.66	1.44	2238	5125	157.78	48.25	22.09	6.75
2,4-D 6.0		$\pm s$	1.176	0.310	0.10	0.10	284	96				

Table 2

Effect of nickel on the growth of Nicotiana tabacum and Ruta graveolens callus tissues

Hormones mg/l	$NiSO_4$ M		Fresh weight (g/flask)		Dry weight %		Cell number (10^{-3}/g)		Daily increase (mg)		Relavite increase	
			Nicotiana	Ruta	Nicotiana	Ruta	Nicotiana	Ruta	Nicotiana	Ruta	Nicotiana	Ruta
	10^{-4}	$\bar{x}$	0.153	0.185	0.89	1.63	2508	5995	-	-	-	-
		$\pm s$	0.008	0.020	0.03	0.16	2	60				
Nicotiana tabacum	5.10^{-5}	$\bar{x}$	0.195	0.306	0.96	1.68	2451	5150	-	3.87	-	0.53
IAA 2.0		$\pm s$	0.031	0.030	0.30	0.10	2	28				
Kin 0.2												
GA 1.0	10^{-5}	$\bar{x}$	0.346	0.706	1.08	1.57	2726	5466	5.21	18.07	0.73	2.53
		$\pm s$	0.109	0.140	0.05	0.15	11	94				
	5.10^{-6}	$\bar{x}$	1.244	0.994	0.83	1.53	2720	4393	37.28	28.35	5.22	3.97
		$\pm s$	0.549	0.100	0.02	0.10	2	19				
Ruta graveolens	10^{-6}	$\bar{x}$	4.297	1.478	0.65	1.48	2824	4568	146.32	45.64	20.48	6.39
IAA 4.0		$\pm s$	1.168	0.510	0.24	0.20	2	35				
2,4-D 6.0	Control	$\bar{x}$	4.618	1.551	0.66	1.44	2238	5125	157.78	48.25	22.09	6.75
		$\pm s$	1.176	0.310	0.10	0.10	284	96				

Table 3

Effect of copper on the growth of Nicotiana tabacum and Ruta graveolens callus tissues

Hormones mg/l	$CuSO_4$ M		Fresh weight (g/flask)		Dry weight %		Cell number (10^3/g)		Daily increase (mg)		Relative increase	
			Nicotiana	Ruta	Nicotiana	Ruta	Nicotiana	Ruta	Nicotiana	Ruta	Nicotiana	Ruta
	10^{-4}	$\bar{x}$	0.114	0.161	0.91	1.51	4130	5502	-	-	-	-
		$\pm s$	0.013	0.057	0.08	0.13	41	21				
Nicotiana tabacum	5.10^{-5}	$\bar{x}$	0.150	0.145	1.02	1.69	3419	5969	-	-	-	-
IAA 2.0		$\pm s$	0.022	0.024	0.07	0.51	33	24				
Kin 0.2	10^{-5}	$\bar{x}$	3.667	0.339	0.66	1.78	3215	5387	123.82	4.96	17.33	0.69
GA 1.0		$\pm s$	0.630	0.074	0.01	0.14	25	33				
	5.10^{-6}	$\bar{x}$	3.796	0.686	0.57	1.41	2319	6377	128.42	17.35	17.98	2.43
		$\pm s$	0.632	0.205	0.04	0.41	15	64				
Ruta graveolens	10^{-6}	$\bar{x}$	4.390	0.674	0.63	1.59	2239	4927	149.64	16.92	20.95	2.37
IAA 4.0		$\pm s$	0.942	0.155	0.06	0.12	29	32				
2,4-D 6.0	Control	$\bar{x}$	4.618	1.551	0.66	1.44	2238	5125	157.78	48.25	22.09	6.75
		$\pm s$	1.176	0.310	0.10	0.10	284	96				

plant, and may at the same time point out the limit of tolerance in the species concerned /6, 7, 14/.

By inhibiting various processes of cell metabolism (enzyme activity, chlorophyll formation, deoxy-ribonucleic acid synthesis) the heavy metals — e.g. Zn. Ni, Cu, etc. — ultimately check the growth and material production of the plant, and when accumulated may even cause damage to animal- and human organisms /3, 12/. The need for preventing the effects of materials contaminating the environment — including the toxic heavy metals —, and producing as much organic matter as possible raises the demand for acquiring a better knowledge of the relations between soil and plant. This purpose is served by the data supplied by our tissue culture experiments. The three metal compounds examined and their divalent cations while usual components of the sterile culture media of plants are — as microelements — very small in quantity. Zinc and copper are considered to be indispensable to the life processes of the plant /2, 3/. However, larger quantities of them may be toxic and may even cause lethality, as was the case with the higher concentrations of Ni and Cu (10^{-4}M) applied in our experiments (Figs 1–3) /3/.

As seen from the results of the experiments all the three metal compounds act on the metabolism parameters examined (fresh- and dry weight, cell number) as a function of concentration. At the same time, there is an essential difference between the two plant species as regards to the metal tolerance of the cells: the callus cells of tobacco are much more sensitive, they show growth inhibition and necrosis in response to the same concentration earlier than the ruta cells. It is a common effect with all the three metal ions that with an increase in the growth inhibition of the tissue the percentage dry weight of tissues increases; and in the case of copper the number of cells per unit weight of tissue also increases. The latter facts partly can be related with the dehydration of the cells and the relative overweight of cell-wall components, partly it is supposed that the checked increase in weight can be traced back to the inhibition of cell expansion rather than to that of cell division (mitosis inhibition). The data of the experiments while indicating the limit of tolerance shown by the plant cells to the metals examined do not inform about the toxic action mechanisms. Further detailed examinations are required to answer this question. Nevertheless, that much is clearly seen from the above experiments that the plant species have different limits of tolerance and give different physiological responses to the heavy metal compounds applied. Furthermore, it is also clear that the so-called toxic metals while at low concentrations

indispensable to the plant metabolism may in larger quantities cause considerable growth inhibition.

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REFERENCES

1. Dässler, H.G.(1979) A légszennyezés hatása a növényzetre (Effect of air pollution on vegetation). Mezőgazdasági Kiadó, Budapest. (In Hungarian)
2. Dijkshoorn, W., von Broekhoven, L.W., Lampe, J.M. (1979) Phytotoxicity of zinc, nickel, cadmium, lead, copper and chromium in three pasture plant species supplied with graduated amounts the soil. Neth. J. Agric. Sci. 27, 241–253.
3. Kovács, J. (1981) Környezetszennyező toxikus nehézfém ionok, illetve vegyületek egyedfejlődésre és a sejtek fehérje szintetizáló apparátusára gyakorolt hatásának vizsgálata (Effect of toxic heavy metal ions and compounds contaminating the environment on ontogeny and on the protein synthetizing apparatus of cells). In: 1979–80. Évi Állami Megbízási Kutatások, pp. 348–350. (In Hungarian).
4. Filippis, de L.F., Hamp, R., Ziegler, H. (1981) The effects of sublethal concentrations of zinc, cadmium, and mercury on Euglena. Growth and pigments. Z. Pflanzenphysiology 101, 37–47.
5. Horváth, L. (1983) Veszélyes hulladékok (Contaminating scraps). Természet Világa 114, 448–451. (In Hungarian).
6. Kovács, M. (ed.) (1985) Pollution Control and Conservation. Akadémiai Kiadó, Budapest.
7. Kovács, M., Podani, J., Tula, Z., Turcsányi, G. (1986) A környezetszennyezést jelző és mérő élőlények (Living organisms environment pollution detecting and monitoring). Mezőgazdasági Kiadó, Budapest (In Hungarian).
8. Maróti, M., Bognár, J. (1985) Growth response of plant callus tissue to toxic heavy metal compounds and ions contaminating the environment. Acta Biol. Hung. 36, 3–13.
9. Maróti, M., Dékány, E. (1978) A ruta kallusz szövetének növekedése és organizációja (The growth and organization of the callus tissue of Ruta). Bot. Közl. 64, 239–247. (In Hungarian).
10. Maróti, M., Pais, I., Bognár, J. (1984) The role of titanium in plant life V. The effect of titanium on the growth of tobacco callus. Acta Agronom. Hung. 33, 315–322.
11. Murashige, T., Skoog, F. (1962) A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant. 15, 473–497.

12. Nag, P., Paul, A.K., Mukherjii, S. (1981) Heavy metal effects in plant tissues involving chlorophyll, chlorophyllase. Hill reaction activity and gel electrophoretic patterns of soluble proteins. Indian J. Exp. Biol. 19, 702–706.

13. Pais, I. (1980) A mikrotápanyagok szerepe a mezőgazdaságban (Role of micronutrients in agriculture). Mezőgazd. Kiadó, Budapest. (In Hungarian).

14. Turcsányi, G. (1986) A környezetszennyeződés jelzése növényi sejtekkel és szövetekkel (Detecting of environment pollution by plant cells and tissues). In: Kovács, M., Podani, J., Tuba, Z., Turcsányi, G. (eds): A környezetszennyezést jelző élőlények (Living organisms environment pollution detecting and monitoring). Mezőgazd. Kiadó, Budapest. pp. 112–143. (In Hungarian).

15. Wallace, A. (1977) Effect of concentration on uptake of some trace metals by plants. Commun. Soil. Sci. Plant Anal. 8, 689–691.

16. Wallace, A., Romney, E.M., Kinnear, J. (1977c) Frequency distribution of several trace metals in 72 corn plants growth together in contamined soil in glasshouse. Commun. Soil. Sci. Plant Anal. 8, 693–698.

17. White, P.R. (1943) A Handbook of Plant Tissue Culture. Jacques Cattel Press, Lancester.

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ACCUMULATION OF CADMIUM BY PLANTS OF ZAWAR MINES, RAJASTHAN, INDIA

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Several plants of the Zawar Zinc Deposits of Rajasthan (India) accumulate very high levels of cadmium. The maximum concentration namely 420 $\mu g\ g^{-1}$ was found in the stems of Crotalaria linifolia. Some other cadmium accumulators with the concentration of the metal in $\mu g\ g^{-1}$ are Impatiens balsamina (380), Dyerophytum indicum (282) and Melhania futteyporensis (245). The sequence of cadmium accumulation in different organs of the same plant species was roots > stems > leaves. Cadmium levels in the leaves of Celosia argentea, Crotalaria linifolia, Impatiens balsamina and Triumfetta pentandra showed a significant (linear) plant-soil relationship. The respective biological absorption coefficients (BAC – concentration of the element in the plant divided by concentration of the same element in the substrate) for these plants were 2.74, 4.13, 5.49 and 4.65.

Keywords: Cadmium – plants – mine territories – India

INTRODUCTION

Though biogeochemical studies have established a number of accumulators for copper /8, 10, 15, 27, 37/, nickel /7, 9, 44/, cobalt /14, 15/, zinc /16/, uranium /43/ and selenium /11/, cadmium accumulators are rather few. There is not much information on cadmium accumulation by plants growing over natural cadmium deposits (cf. Shacklette /35/, Ward et al. /42/, Aery /1/.

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The present paper deals with cadmium accumulation by plants of a mineralized lead-zinc deposit at Zawar Mines, Udaipur, India and the inter-relationships among cadmium levels in plants and soils and with other elements.

STUDY AREA

The study area included the entire expanse of the Zawar Zinc Mines situated between latitudes $24^{o}18'48"$ and $24^{o}22'48"$ N and longitudes $73^{o}40'$ and $73^{o}45'24"$ E. The general elevation is about 337.7 m above MSL. The rocks are part of the Aravalli system of Middle Pre-Cambrian age, consisting of a thick series of Phyllites and slates with subordinate intercalated dolomites, quartzites and conglomerates or their future metamorphosed equivalents[20]. The metallic minerals identified in order of abundance are: Sphalerite, Galena, Pyrite, Arsenopyrite, Chalcopytire and Pyrohotite.

MATERIALS AND METHODS

Leaves and stems of 15 "Characteristic Species" of the community, selected after a preliminary survey (Aery, 1978) and their underlying soils in the mineralized zones of the study area were sampled for their metallic contents. Plants were dried at 110 ^{o}C for 48 h. The powdered plant and soil samples were wet-ashed with ternary acid mixture /6, 21/. The digested samples were atomized in an Atomic Absorption Spectrophotometer (CZ AAS-1) for the analyses of lead, zinc and cadmium.

RESULTS AND DISCUSSION

Cadmium accumulation and its distribution in different plant parts

Though cadmium is not an essential element, it is found as "ballast element" in smaller concentrations in almost all plants, its actual concentration depending upon the genetic variability of the plant species and its contents in the growth medium /22, 24, 30, 32, 38/. This "Luxury Consumption" is common among plants /33/.

Analytical data for the cadmium content in certain species and their underlying soils are given in Table 1 which show that plants of the study

area accumulate quite high levels of cadmium. It may be noted from Table 1 that the presence of cadmium could not be detected in four of the species. In earlier investigations /2, 36/, the present writers had reported the results of elemental analyses for the metal zinc, lead, copper, iron in soils and plants of zinc mines at Zawar. The maximum cadmium concentration of 420 $\mu g\ g^{-1}$ on ash weight basis was found in the stems of Crotalaria linifolia. Other species with abnormally high cadmium content in their tissues are Impatiens balsamina (380 $\mu g\ g^{-1}$), Dyerophytum indicum (282 $\mu g\ g^{-1}$) and Melhania futteyporensis (245 $\mu g\ g^{-1}$).

Further, the concentration of cadmium varies in different parts of the same plant species. Roots have been found to accumulate more cadmium than shoots and leaves the least /13, 17, 22, 40, 41/. However, Patel et al. /31/ and Van Hook et al. /39/ found lower cadmium concentrations in stems than in the leaves. In the present study the sequence of accumulation of cadmium in different organs of the same plant species was roots stems leaves. Flowers and fruits were analyzed for only two species, viz. Impatiens balsamina and Melhania futteyporensis. While in the latter, flowers were richer in cadmium than leaves the reverse was true for the former species. No definite explanation could be thought of for this differential accumulation of cadmium in different parts of the same plant species. According to Brisson et al. /4/ it is due to the blockage of vascular tissues probably by phenolic compounds along the major veins in the leaves at the stem nodes.

The difference in the amounts of cadmium in stems and leaves become manifold when we consider the results on ash-weight basis (Table 1). This is due to the difference present in their ash contents. Leaves when burned, yield about twice as much ash as do stems. This difference in yield is caused by the difference in element content of primary and secondary growth products. Leaves show only primary growth while stems show secondary growth also. Secondary growth produces an increase in 'dry weight' as a result of the synthesis of organic compounds without a corresponding increase in amounts of elements that constitute ash. Therefore, if equal weights of dried stems and leaves are ashed, the organic compounds are oxidized and a greater weight of ash is obtained from the leaves.

On account of this feature, the concentration of metals in equal amounts of dry matter comes to be lower in stems than leaves when expressed on dry weight basis. On an ash weight basis there will not be such difference. Keeping this in view all results have been expressed with the metal contents in ash.

Table 1

Cadmium concentrations in plants and soils from the Zawar Zinc Deposits, Rajasthan, India

S. No.	Name of the plant	Organ	Ash %	n	Mean cadmium concentration ($\mu g\ g^{-1}$) Dry wt.	Ash. wt.	Soil	BAC
1.	Acanthospermum hispidum	Leaf	11.07	10	3.9 ± 1.7	33 ± 14	29.5 ± 13.2	1.11
		Stem	7.52	10	7.9 ± 3.6	105 ± 48		3.55
2.	Aegle marmelos	Leaf	12.89	8	-	-	-	-
		Stem	8.45	8	-	-	-	-
3.	Celosia argentea	Leaf+flower	13.31	11	8.7 ± 4.2	76 ± 46	27.9 ± 16.07	2.72
		Stem	9.25	11	11.0 ± 5.0	119 ± 54		4.26
4.	Crotalaria linifolia	Leaf	9.35	9	23.6 ± 5.4	252 ± 58	61.99± 12.87	4.06
		Stem	5.80	9	24.4 ± 5.3	420 ± 92		6.77
5.	Dyerophytum indicum	Leaf	9.58	9	10.3 ± 1.9	109 ± 18	30.97± 5.36	3.51
		Stem	5.32	9	15.0 ± 2.9	282 ± 55		9.1
6.	Grewia flavescens	Leaf	12.81	8	2.8 ± 0.7	22 ± 6	11.26± 3.12	1.95
		Stem	7.31	8	3.1 ± 0.7	43 ± 10		3.81
7.	Hemigraphis latebrosa	Leaf	19.74	9	1.5 ± 0.5	7 ± 3	10.62± 5.09	0.65
		Stem	6.25	9	2.9 ± 1.2	47 ± 20		4.42
		Root	8.39	9	5.1 ± 1.9	57 ± 20		5.36
8.	Impatiens balsamina	Leaf	13.59	12	21.7 ± 6.7	159 ± 50	28.99± 9.55	5.48
		Stem	8.93	12	32.6 ±10.8	364 ±121		12.55
		Root	11.55	12	43.8 ±14	380 ±121		13.10
		Flower+fruit	6.21	12	12 ± 2	162 ± 45		5.58
9.	Lannea coromandelica	Leaf	7.30	10	-	-	-	-
		Stem	5.61	10	-	-	-	-
10.	Lepidagathis trinervis	Leaf	13.92	9	-	-	-	-
		Stem	3.37	9	-	-	-	-

Table 1 (cont.)

S. No.	Name of the plant	Organ	Ash %	n	Mean cadmium concentration ($\mu g\ g^{-1}$) Dry wt.	Ash. wt.	Soil	BAC
11.	Lindenbergia muraria	Leaf	6.89	9	2.6 ± 1.6	38 ± 24	13.62± 7.46	2.79
		Stem	4.06	9	7 ± 4.2	172 ±103		12.62
12.	Melhania futteyporensis	Leaf	8.27	10	6.7 ± 2	108 ± 69	22 ±13.56	4.90
		Stem	4.55	10	11 ± 6.3	245 ±138		11.13
		Flower+fruit	10.20	10	13.4 ± 7.8	131 ± 76		
13.	Nyctanthes arbortristis	Leaf	8.53	10	-	-	-	-
			6.82	10	-	-	-	-
14.	Triumfetta pentandra	Leaf	5.51	10	9 ± 2.9	163 ± 54	35 ±10.73	4.65
15.	Wrightia tinctoria	Leaf	10.17	10	6.1 ± 5.4	60 ± 53	9.46± 8.22	6.34
		Stem	5.12	10	8.3 ± 7	107 ± 66		11.31

BAC = Biological absorption coefficient

- = Not detectable

That the cadmium is absorbed from the soil and is not due to atmospheric fallout is clear from the following:

(a) Roots and stems were far richer in cadmium than leaves and flowers (Table 1).

(b) High cadmium values were invariably accompanied by high zinc and lead values indicating a synergistic relationship among these metals (Table 2, Fig. 3).

(c) Cadmium levels of leaves and stems show highly positive correlation (Table 2).

(d) Soils were always poor in cadmium levels than plant tissues (Table 1).

Table 2

Showing significance of leaf-soil, leaf-stem and inter-elemental correlations

S. No.	Name of the plant	Leaf×soil	Leaf×stem	Inter-elemental	
				Cd x Pb	Cd x Zn
1.	Acanthospermum hispidum	S	S^{**}	S^{**}	S^{**}
2.	Celosia argentea	S^{**}	S^{**}	S^{**}	S^{**}
3.	Crotalaria linifolia	S	S^{**}	S^{**}	S^{**}
4.	Dyerophytum indicum	NS	S^{*}	NS	S^{**}
5.	Grewia flavescens	NS	S^{**}	S^{**}	S^{**}
6.	Hemigraphis latebrosa	NS	S^{**}	S^{*}	S
7.	Impatiens balsamina	S	S^{**}	S^{**}	S^{**}
8.	Lindenbergia muraria	PS	S^{**}	S^{**}	S^{**}
9.	Melhania futteyporensis	NS	NS	S^{*}	NS
10.	Triumfetta pentandra	S^{*}	S^{**}	NS	NS
11.	Wrightia tinctoria	NS	S^{**}	NS	NS

S^{**} = Very highly significant ($P < 0.001$)

S^{*} = Highly significant ($0.001 < P < 0.01$)

S = Significant ($0.01 < P < 0.05$)

PS = Possibly significant ($0.05 < P < 0.10$)

NS = Not significant ($P > 0.10$)

Plant-soil relationship and relative accumulation

Several workers /12, 23, 25, 33/ have demonstrated that in all such cases where the substrate contains subtoxic levels of cadmium, its concen-

tration in the plant is positively correlated with its concentration in the soil. But almost all these studies have been carried out with nutrient solutions or sand. In the present study, carried on naturally enriched cadmium soils, a significant dependence of plant cadmium on soil cadmium was observed (Table 2, Figs 1,2) in Celosia argentea, Crotalaria linifolia, Impatiens balsamina and Triumfetta pentandra. These plants also showed high relative accumulation. Biological absorption coefficient values (BAC — concentration of the element in the plant divided by concentration of the same element in the substrate) respectively were 2.74, 4.13, 5.49 and 4.65 for these species (Table 1). Though Wrightia tinctoria showed a high relative accumulation with a BAC value of 6.39 (Table 1), it showed a non-significant soil-plant relationship (Table 2).

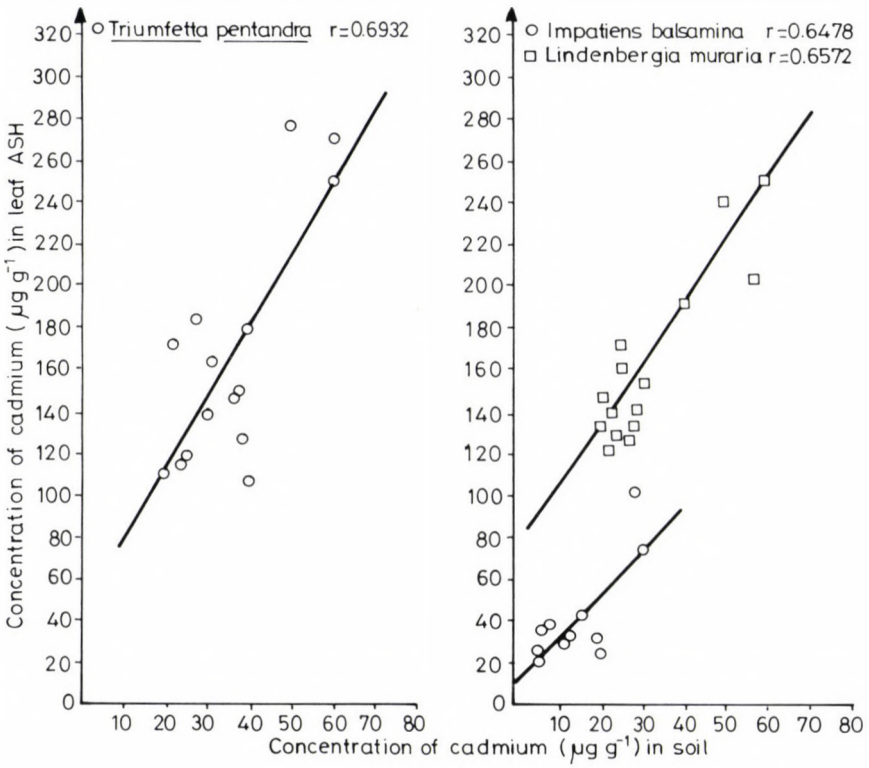

Fig. 1. Concentration of cadmium in leaf ash expressed as a function of the concentration in the soil

No evidence of the presence of any exclusion mechanism due to which the metal contents in the shoot is maintained at constantly low levels, over

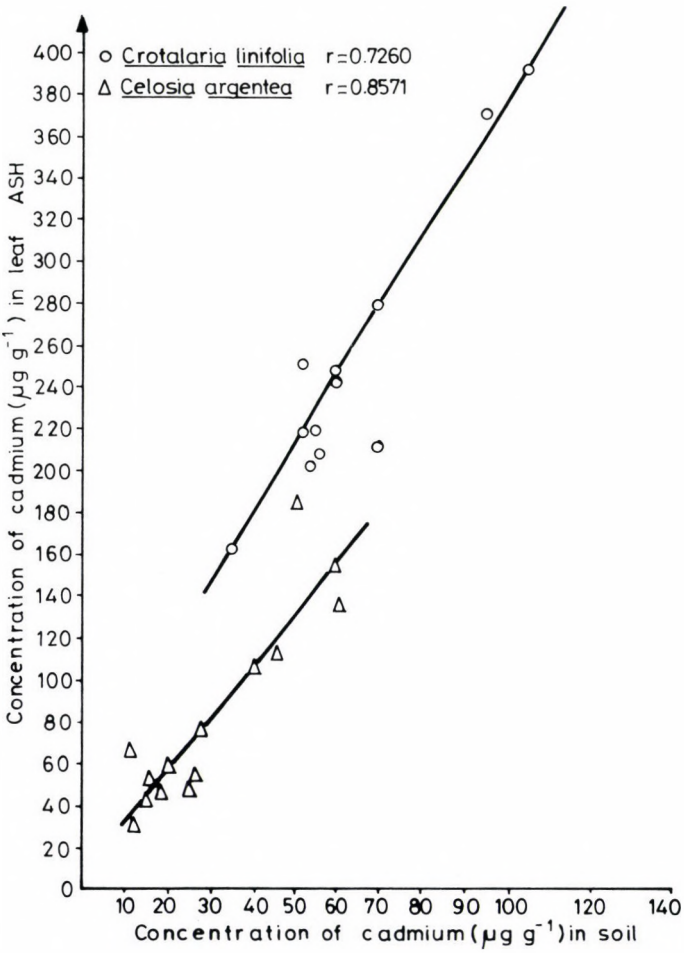

Fig. 2. Concentration of cadmium in leaf ash expressed as a function of the concentration in the soil

a wide range of soil concentrations, up to a critical threshold above which the mechanism breaks down, resulting in unrestricted transport, as reported by Nicolls et al. /29/ and Barry and Clark /3/ could be observed. Neither any absorption barrier as reported by Tiagi and Aery /37/ for copper wherein after attaining a limiting value the concentration of metal in the plant decreases gradually or abruptly with further increase in soil concentration could be observed. Either the soil cadmium concentrations are too low or these mechanisms do not exist at all in the plants of the study area.

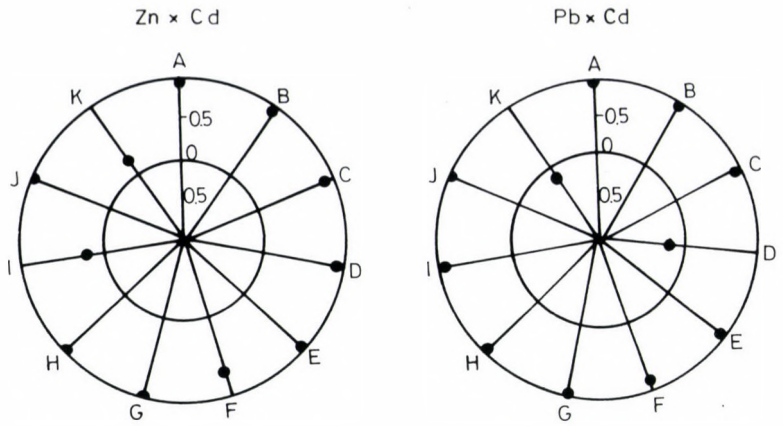

Fig. 3. Graphical summaries of the correlation coefficients for Zn x Cd and Pb x Cd inter-relationships in the leaves of certain plant species of Zawar Mines

A — Acanthospermum hispidum
B — Celosia argentea
C — Crotalaria linifolia
D — Dyerophytum indicum
E — Grewia flavescens
F — Hemigraphis latebrosa
G — Impatiens balsamina
H — Lindenbergia muraria
I — Melhania futteyporensis
J — Triumfetta pentandra
K — Wrightia tinctoria

Inter-elemental relationship

The interaction of cadmium with other cations has been studied in soil and solution culture experiments. While Jarvis /22/ reported antagonism of cadmium with zinc, Maclean /26/ found neither any antagonistic nor a synergistic relationship. Haghiri /18/ reported a synergistic relationship at low zinc levels (5–50 $\mu g\ g^{-1}$) and an antagonistic one at high zinc levels (100–400 $\mu g\ g^{-1}$). In the present study the soil concentration of cadmium ranged from 0.55 to 95.20 $\mu g\ g^{-1}$ and that of zinc from 464 to 3885 $\mu g\ g^{-1}$. Significant positive correlations exist between zinc and cadmium contents

in all the plants studied except Melhania futteyporensis and Wrightia tinctoria indicating a synergistic relationship (Table 2, Fig. 3).

Likewise the concentration of cadmium in plants also correlated well with the concentration of lead in almost all the plant species studied except Dyerophytum indicum and Wrightia tinctoria (Table 2, Fig. 3). A synergistic relationship of the lead and cadmium has also been reported by Hasset et al. /19/, Miller et al. /28/ and Carlson and Bazzaz /12/.

REFERENCES

1. Aery, N.C. (1978) Geobotanical studies of the Regions of Zinc Ore Deposits in the Udaipur Region. Ph.D. Thesis. University of Udaipur, Udaipur, India.

2. Aery, N.C., Tiagi, Y.D. (1987) Biogeochemistry of lead at Zawar Mines, Udaipur (India). Sci. Dev. and Environ, pp. 117–129.

3. Barry, S.A.S., Clark, S.C. (1978) Problems of interpreting the relationship between the amounts of lead and zinc in plants and soil on metalliferous wastes. New Phytol. 81, 773–783.

4. Brisson, J.D., Peterson, R.L., Robb, J., Rauser, W.E., Ellis, B.E. (1977) Correlated Phenolic histochemistry using light, transmission, scanning electron microscopy, with examples taken from phytopathological problems. Scanning Electron Microsc. 2, 667–676.

5. Brooks, R.R. (1977) Copper and cobalt uptake by Haumaniastrum species. Plant and Soil 48, 541–544.

6. Brooks, R.R. (1983) Biological methods of prospecting for minerals. John Wiley and Sons, Inc., Chichester – New York.

7. Brooks, R.R., Wither, E.D. (1977) Nickel accumulation by Rinorea bengalensis (Wall.) O.K.J.Geochem. Explor. 7, 295–300.

8. Brooks, R.R., McCleave, J.A., Malaisse, F. (1977) Copper and cobalt in African species of Crotalaria L. Proc. R. Soc. Lond. B 197, 231–236.

9. Brooks, R.R., Lee, J., Reeves, R.D., Jaffre, T. (1977) Detection of nickeliferous rocks by analysis of herbarium specimens of indicator plants. J. Geochem. Explor. 7, 49.

10. Cannon, H.L. (1960a) The development of botanical methods of prospecting for uranium on the Colorado Plateau. U.S. Geol. Surv. Bull. 1085–A, 1–50.

11. Cannon, H.L. (1960b) Geochemistry of sandstone and related vegetation in the Yellow Cat area of the Thompson district, Grand County, Utah. U.S. Geol. Survey Prof. Paper. 400 B, 96–97.

12. Carlson, R.W., Bazzaz, F.A., Rolfe, G.L. (1975) The effect of heavy metals on plants. 2: Net photosynthesis and transpiration of whole corn and sunflower plants treated with lead, cadmium, nickel and thallium, Environ. Res. 10, 113–120.

13. Cunningham, L.M., Collins, F.W. and Hutchinson, T.C. (1975) Physiological and biochemical aspects of cadmium toxicity in soybean. I. Toxicity symptoms and autoradiographic distribution of Cd in roots, stems and leaves. In: Hutchinson, C.T.C. (ed.) Intern. Conf. Heavy Metals in the Environ. Toronto, pp. 97–120.

14. Duvigneaud, P. (1959) "Cobaltophyte" plants in Haut-Katanga (in French). Soc. Royale Botanique Belgique Bull. 91, 111–134.

15. Duvigneaud, P., Denaeyer de Smet, S. (1963) Cuivre et vegetation au Katanga, Bull. Soc. r. Bot. Belg. 96, 93–231.

16. Ernst, W. (1975) Mehanism der Schwermetallresistenz. In: Muller, P. (ed.) Verhandlungen der Gesellschaft für Ökologie, Erlangen, pp. 187–195.

17. Haghiri, F. (1973) Cadmium uptake by plants. J. Environ. Qual. 2, 93–96.

18. Haghiri, F. (1974) Plant uptake of cadmium as influenced by cation exchange capacity, organic matter, zinc and soil temperature. J. Environ. Qual. 3, 180–813.

19. Hasset, J.J., Miller, J.E., Koeppe, D.E. (1976). Interaction of lead and cadmium on maize root growth and uptake of lead and cadmium by roots. Environ. Pollut. 11, 297–302.

20. Heron, A.M. (1953) The Geology of Central Rajputana. Mem. Geol. Surv. Ind. Vol. 79.

21. Jackson, M.L. (1967) Soil Chemical Analysis. Prentice-Hall of India, New Delhi, p. 498.

22. Jarvis, S.C., Jones, L.F.H., Hopper, M.J. (1976) Cadmium uptake from solution by plants and its transport from roots to shoots. Plant Soil. 44, 179–191.

23. John, M.K. (1972) Uptake of soil applied cadmium and its distribution in radishes. Can. J. Plant Sci. 52, 715–719.

24. John, M.K. (1973) Cadmium uptake by eight food crops as influenced by various soil levels of cadmium. Environ. Pollut. 4, 7–15.

25. John, M.K., Van Laerhoven, C.J. (1976). Differential effects of cadmium on lettuce varieties. Environ. Pollut. 10, 163–173.

26. Maclean, F.I., Lucis, O.J., Shaikh, Z.A., Jansey, E.R. (1972) The uptake and subcellular distribution of cadmium and zinc in microorganisms. Federation Proc. 31, 699.

27. Malaisse, F., Gregoire, J., Brooks, R.R., Morrison, R.S., Reeves, R.D. (1978) Aeolanthus biformifolius De Wild: A Hyperaccumulator of copper from Zaire. Science 199, 887–888.

28. Miller, J.E., Hassett, J.J., Koeppe, D.E. (1977) Interactions of lead and cadmium on metal uptake and growth of corn plants. J. Environ. Qual. 6, 18–20.

29. Nicolls, O.W., Provan, D.M.J., Cole, M.M. and Tooms, J.S. (1965) Geobotany and geochemistry in mineral exploration in the Dugald River Area, Clon curry district, Australia. Trans. Inst. Min. Metal. 74, 695–699.

30. Page, A.L., Bingham, F.T. and Nilson, C. (1972) Cadmium absorption and growth of various plant species as influenced by solution cadmium concentration. J. Environ. Qual. 1, 288–291.

31. Patel, P.M., Wallace, A., Mueller, R.T. (1976) Some effects of copper, cobalt, cadmium, zinc, nickel and chromium on growth and mineral element concentration in Chrysanthemum. J. Am. Soc. Hortic. Sci. 101, 553–556.

32. Pettersson, O. (1976) Heavy metal ion uptake by plants from nutrient solutions with metal ion, plant species and growth period variations. Plant Soil. 45, 445–459.

33. Root, R.A., Miller, R.J., Koeppe, D.E. (1975) Uptake of cadmium its toxicity and effect on the iron ratio in hydroponically grown corn. J. Environ. Qual. 4, 473–476.

34. Shacklette, H.T. (1972) Cadmium in plants. Geol. Surv. Bull. 1314–G.

35. Shacklette, H.T., Sauer, H.I., Miesch, A.T. (1970) Geochemical environments and cardiovascular mortality rates in Georgia. U.S. Geol. Survey Prof. Papers 574–C, 1–39.

36. Tiagi, Y.D., Aery, N.C. (1981) Biogeochemical studies in zinc deposit areas of Zawar Mines, Rajasthan, India. Proc. Indian Nat. Sci. Acad. B 47, 867–887.

37. Tiagi, Y.D., Aery, N.C. (1986) Biogeochemical studies on Khetri copper deposits of Rajasthan, India. J. Geochem. Explor. 26, 267–274.

38. Turner, M.A. (1973) Effect of cadmium treatment on cadmium and zinc uptake by selected vegetable species. J. Environ. Qual. 2, 118–119.

39. Van Hook, R.I., Harris, W.F., Henderson, G.S. (1977) Cadmium, lead and zinc distributions and cycling in a mixed deciduous forest. Ambio 6, 281–186.

40. Wallace, A., Romney, E.M. (1977) Roots of higher plants as a barrier to translocation of some metals to shoots of plant. In: Drucker, H., Wildung, R.E. (eds) "Biological Implications of metals in the Environment". Proceedings of the 15th Annual Hanford Life Sciences Symposium, Richland Wash. 1975, Technical Information Center, Energy Research and Development Administration, CONF– 750929, 370–379.

41. Wallace, A., Romney, E.M., Alexander, G.V., Soufi, S.M., Patel, P.M. (1977) Some interactions in plants among cadmium, other heavy metals and chelating agents. Agron. J. 69, 18–20.

42. Ward, N.L., Brooks, R.R., Reeves, R.D. (1976) Copper, cadmium, lead and zinc in soils, streams, sediments, water and natural vegetation around the Tui Mine, Te Aroha, New Zealand. New Zealand J. Sci. 19, 81–89.

43. Whitehead, N.E., Brooks, R.R. (1969) Aquatic bryophytes as indicators of uranium mineralisation. The Bryologist 72, 501–507.

44. Wither, E.D., Brooks, R.R. (1977) Hyperaccumulation of nickel by some plants of South-east Asia. J. Geochem. Expl. 8, 579–583.

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JOINT ACTION OF NUCLEAR POLYHEDROSIS VIRUS AND CHEMICAL INSECTICIDES AGAINST THE BLACK CUTWORM, AGROTIS IPSILON (HUFN.)

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A polyhedrosis virus disease was isolated from the larvae of Agrotis ipsilon. Isolation of causative agent from the infected larvae again showed to be positive. Microscopic examination of the infected larvae (by means of a light microscope) gave an evidence of the development of the polyhedral inclusion bodies (PIB) in all typical tissues where the virus is known to develop. Examination by an electron microscope showed various irregular shaped polyhedra; their diameter being about 1.5 to 3.5 μ. Most of these polyhedra were hexagonal and they measured about 2.2 μ. Experimental studies were conducted to investigate its pathogenicity alone and when combined with chemical insecticides. The joint action of the nuclear polyhedrosis virus of A. ipsilon (AINPV) combined with four chemical insecticides namely Fenvalerate, Decamethrin, Cypermethrin and Methomyl was assessed against the second instar larvae of A. ipsilon. Results showed that Decamethrin was the most toxic compound followed by Cypermethrin and Fenvalerate while Methomyl was the least toxic, the median lethal concentrations (LC_{50}) were 0.25, 0.77, 14 and 450 ppm, respectively. The LC_{50} value of AINPV was 12×10^6 PIB/ml. Mixtures containing sublethal concentrations (LC_{25} and less) of both AINPV and any of the tested insecticides showed synergistic effects. On the other hand, an additive effect was observed with combinations containing doses of chemical insecticides over the range between LC_{25} and LC_{50} values.

Keywords: Virus disease – insecticides – black cutworm – Agrotis ipsilon

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INTRODUCTION

Many lepidopterous larvae, especially those reared in laboratories, are often attacked by many diseases, but the most detrimental effect was played by viruses Abul-Nasr /14/; Bergold and Flascentrager /8/; Hafez /13/; Vago /30/; Weiser /34/.

Synthetic pyrethroids possess many desirable properties, including high toxicity to insects, low toxicity to mammals and high biodegradation. Hence they are expected to supplement and possibly replace some of the conventional organochlorines, organophosphorous and carbamates against many economically important pests.

The present study deals with the potency of a virus that was found to infect A. ipsilon in the laboratory. Isolation, identification and symptomatology of the virus are considered. The effects of the virus alone and in combination with chemical insecticides on the larval mortality of A. ipsilon are also investigated.

MATERIALS AND METHODS

Larvae and rearing technique

Larvae of A. ipsilon were taken from a standard laboratory colony successfully maintained at 27 ± 2 ^{o}C and reared on an artificial diet developed by Salama (1970). In order to avoid the cannibalism which started from the fourth instar larvae, they were individually distributed in small cylindrical plastic cups (8 cm x 6 cm diameter) with perforated covers. Because of the antiviral activity of formalin (Van der Geest /33/; Vail et al. /32/; David et al. /9/; Moawed /20/ it was not added to the diet ingredients.

Isolation and identification of polyhedra

The principal method of Bergold /7/ for the isolation and purification of arthropod viruses was adopted. The larvae that showed the symptoms of virus disease were suspended in water, blended, filtered and purified by repeated centrifugation. The collected polyhedra were washed with distilled water and kept in a refrigerator at 4 ^{o}C as an aqueous suspension ready for use. Further confirmation was achieved by reinfecting the second instar larvae and reisolating the polyhedra from cadavers. The presence of polyhedra

in different tissues was observed. More identification was made to highly purified polyhedra /27/ using an electron microscope.

Chemical insecticides

The chemical insecticides used represented pyrethroid and carbamate groups. The pyrethroid group was represented by Fenvalerate 20% EC, Decamethrin 5% EC and Cypermethrin 20% EC while the carbamate group was represented by Methomyl 95.9%.

Bioasssay

The toxicological effect of each of the tested insecticides and the pathogenicity of the virus to the second instar larvae were assessed at six different concentrations. The standardization of viral concentrations was based on counting the polyhedra in a haemocytometer (Burker chamber). Castor oil plant leaves (Ricinus communis) were submerged for one minute in the different concentrations, left to dry in open air and then administered to the larvae. The control sample received leaves dipped in distilled water. Twenty second instar larvae were used for each concentration. Mortality counts was recorded daily till the end of the larval period. Mortality rates were corrected according to Abbott's formula /1/. Dose-mortality regression lines were established according to Finney /11/, from which LC_{50} values were figured.

The combined action of each insecticide with the virus was assessed using different mixtures containing sublethal dosages of both, and which independently gave 50% mortality or less. The second instar larvae were allowed to feed on Castor oil plant leaves treated by these mixtures as described previously. For each mixture, the data were obtained from three replicates, 20 larvae per each. The combined action of each mixture was expressed as the cotoxicity factor (C.F.), that was estimated according to the equation given by Mansour et al. /18/:

$$\text{Cotoxicity factor} = \frac{(\%\ \text{Observed mortality} - \%\ \text{Expected mortality})}{\%\ \text{Expected mortality}} \times 100$$

$$\text{C.F.} = \frac{(\%\ \text{O.m} - \%\ \text{E.m})}{\%\ \text{E.m.}} \times 100$$

Percent expected mortality was calculated as the sum of mortalities produced from each of the components of the mixture when applied alone. This

factor was used to differentiate the results into three categories. (1) A positive factor of 20 or more is considered potentiation, (2) a negative factor of 20 or more is considered antagonism, and (3) any intermediate values (i.e. between − 20 and + 20) indicate only additive effect.

RESULTS AND DISCUSSION

Symptomatology of the disease

Polyhedrosis virus preparations administered to the second instar larvae of A. ipsilon led to the development of typical virus symptoms that clearly appeared on the late larval instars. The observed symptoms can be described as follows: The normal green-black colour changed to yellowish 3–4 days after infection. The tint green colour of the abdominal surface changed to pinkish. Late symptoms showed less larval food consumption than the normal the larvae became swollen and lethargic, then the body became flabby, followed by death. The skin of dead larvae was easily ruptured and a milky fluid was liberated. Samples of dead larvae examined with a light microscope, showed large numbers of polyhedra suspended in the body fluids. Also numerous polyhedra were present in the nuclei of infected cells of fat bodies and tracheal matrix. These symptoms of A. ipsilon diseased larvae agree to some extent with those caused by other nuclear polyhedrosis in other lepidopterous insects /4, 20, 21, 28, 29, 35/.

Polyhedra

Electron microscopic examination of highly purified polyhedra revealed that they are of irregular shape and they vary in their diameter from about 1.5 to 3.5 μ. The majority of the polyhedra are hexagonal and they measure about 2.2 μ. These measurements are mostly similar to those mentioned by Bergold and Flascentrager /8/ (1.2 − 3.2 μ) for the PIB of Prodenia litura, but they were smaller than those of Spodoptera littoralis (Abul-Nasr /4/ (5–6 μ); Moawed /20/ (1.3 − 6.3 μ). On the other hand, the size of A. ipsilon PIB were more smaller than the PIB of Heliothis sp. where their diameter ranges between 6 and 10 u (Gregory et al. /12/; Abou-Baker et al. /3/).

Individual bioassay

Results obtained in Table 1 and represented by Fig. 1 showed that the susceptibility of the second instar larvae of A. ipsilon to the NPV and to each chemical toxicant increased considerably by the increase of its concentration. With respect to the LC_{50} values they were found to be 0.25, 0.77, 14 and 450 ppm for Decamethrin, Cypermethrin, Fenvalerate and Methomyl respectively. The LC_{50} value of AINPV was 12×10^6 PIB/ml. These results agree with previous findings on the effect of NPC on other lepidopterous insects, e.g. Lambdina fiscellaria /23/; Autographa californica /32/; Spodoptera littoralis /5, 16, 17, 22/; and Heliothis sp. /3, 6, 26/. The present results of all insecticides application against A. ipsilon are in full agreement with the observation of Harris and Svec /14/, Salama et al. /25/, Abdallah et al. /2/ and Fahmy et al. /10/.

Table 1

Sensitivity of second instar larvae of A. ipsilon to its NPV and chemical insecticides

Insecticide	Total larvae	LC_{50} (95% CL)[a]	Slope ± S.E.
AINPV	120	12(11.06 – 13.76)	1.351 ± 0.147
Fenvalerate	120	14(12.22 – 16.98)	2.381 ± 0.217
Decamethrin	120	0.25(0.19 – 0.36)	3.156 ± 0.635
Cypermethrin	120	0.77(0.62 – 0.95)	0.818 ± 0.016
Methomyl	120	450(370 – 465)	0.778 ± 0.283

[a] LC_{50} values expressed as 1×10^6 PIB/ml for AINPV, and ppm for chemical insecticides, 95% confidence limits in parenthses.

Simultaneous effect

As shown from the values of the cotoxicity factors (Table 2), combinations containing sublethal doses that produced 25% mortality and less (LC_{25} = <), of both AINPV and insecticides, gave high levels of synergism.

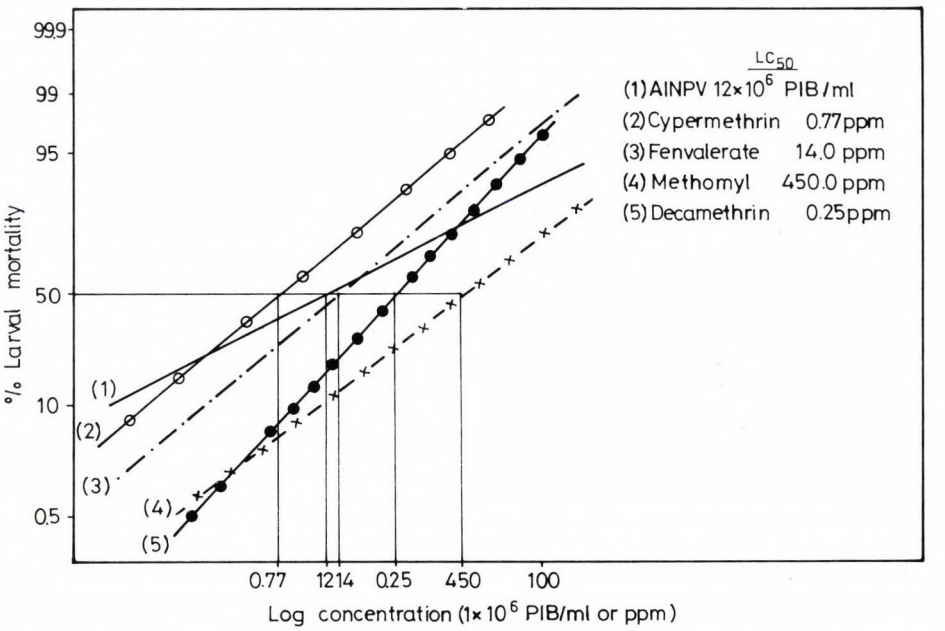

Fig. 1. LD-p lines of AINPV and chemical insecticides applied to the second instar larvae of Agrotis ipsilon

On the other hand, an additive effect was observed with combination which contained concentrations that produced 50% mortality and less ($LC_{25} <= LC_{50}$).

The results of the present investigation confirm the findings of Hassan and Moawed /15/ who demonstrated that Azodrin and Cyolane did not show any decrease in the pathogenicity of the NPC of S. littoralis. The action of combination of virus and chemical insecticides gave higher mortalities than if either component was applied singly. Also, Salama et al. /25/, found that pyrethroids potentiated Bacillus thuringiensis varieties applied against S. littoralis, while carbamates always had an additive effect.

Accordingly, it appears that, for many safety considerations, mixtures containing sublethal doses of AINPV and synthetic pyrethroids may be more potent against A. ipsilon.

Table 2

Effect of combinations of AINPV and chemical insecticides on the second instar larvae of A. ipsilon

Tested mixture	Concentrations $1x10^6$PIB/ml+ppm	Observed[a] mortality %	Cotoxicity factor	Effect
AINPV+Fenvalerate	10 + 12.5	94.12	+ 3.43	Additive
	5 + 6	82.35	+64.7	Synergism
	2.5 + 3	41.77	+81.61	Synergism
AINPV+Decamethrin	10 + 0.2	94.12	+13.40	Additive
	5 + 0.1	70.59	+76.48	Synergism
	2.5 + 0.05	35.29	+68.15	Synergism
AINPV+Cypermethrin	10 + 0.6	88.24	+ 3.81	Additive
	5 + 0.3	64.71	+ 9.68	Additive
	2.5 + 0.15	47.06	+74.30	Synergism
AINPV+Methomyl	10 +225	76.47	+17.65	Additive
	5 +150	64.71	+47.07	Synergism
	2.5 + 75	29.41	+33.68	Synergism

[a]Data obtained from three replicates, 20 larvae per each

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REFERENCES

1. Abbott, W.S. (1925) A method for computing the effectiveness of an insecticide. J. Econ. Entomol. 18, 256–267.

2. Abdallah, S., Omar, A., El-Tantawy, M., Khidr, A., El-Nabarawy, I. (1985) The efficiency of certain insecticides against Spodoptera littoralis (Boisd.) and Heliothis armigera (Hubn.). Alexandria Sci. Exch. 6, 160–174.

3. Abou-Bakr, H.E., El-Husseini, M.M., Tawfik, M.F.S. (1985) On the nuclear polyhedrosis virus of Heliothis armigera in Egypt. Bull. Ent. Soc. Egypt. 14, 29–36.

4. Abul-Nasr, S. (1956) Polyhedrosis virus disease on cotton leafworm, Prodenia litura F. Bull. Ent. Soc. Egypt. 11, 321–332.

5. Abul-Nasr, S., Ammar, E.D., Abul-Ela, S.M. (1979) Effects of nuclear polyhedrosis virus on various development stages of the cotton leafworm, Spodoptera littoralis (Boisd.). Z. ang. Ent. 88, 181–187.

6. Allen, G.E., Ignoffo, C.M. (1969) The nucleopolyhedrosis virus of Heliothis. Quantitative in vivo estimates of virulence. J. Invertebr. Pathol. 13, 378–381.

7. Bergold, G.H. (1947) Die Isolierung des Polyedervirus und die Natur der Polyeder. Z. Naturforsch. 20, 122–143.

8. Bergold, G.H., Flascentrager, B. (1957) The polyhedral virus of Prodenia litura (Fab.) Lepidoptera: Noctuidae. Nature (Lond.) 180, 1046–1047.

9. David, W.A.L., Fllaby, S., Taylo, G. (1969) Formaldehyde as an antiviral agent on granulosis virus of Pieris brassicae. J. Investebr. Pathol. 14, 96–101.

10. Fahmy, H.S.M., Kandil, M.A., Rabei, M.M. (1985) Toxicological studies on synthetic pyrethroids on the cotton leafworm; Spodoptera littoralis (Boisd.). Bull. Ent. Soc. Egypt, 14, 79–86.

11. Finney, D.J. (1962) Probit Analysis. Cambridge University Press, Cambridge and New York.

12. Gregory, B.G., Ignoffo, C.M., Chapiro, M. (1969) Nucleopolyhedrosis of Heliothis: morphological description of inclusion bodies and virions. J. Invertebr. Pathol. 14, 186–193.

13. Hafez, M. (1958) Studies on the polyhedrosis virus disease of the cotton leafworm, Prodenia litura F., in Egypt. Bull. Ent. Soc. Egypt. 42, 357–370.

14. Harris, C.R., Svec, H.J. (1968) Toxicological studies on cutworms. 1-Laboratory studies in the toxicity of insecticides to the dark-sided cutworm. J. Econ. Entomol. 61, 788–793.

15. Hassan, S.M., Moawed, S.M. (1973) Combination of (virus + insecticides) for control of cotton leafworm, Spodoptera littoralis (Boisd.). 1st Pest Control Conf. Assiut. pp. 129–132.

16. Hassan, S.M., Moawed, S.M. (1974) Toxicological studies on the effect of nuclear polyhedrosis for control of the cotton leafworm, Spodoptera littoralis (Boisd.). Symposium on the use of isotopes in pesticides and pest control, Beirut, Lebanon. pp. 175–180.

17. Klein, M., Podoler, H. (1978) Studies on the application of a nuclear polyhedrosis virus to control population of the Egyptian cotton worm, Spodoptera littoralis. J. Invertebr., Pathol. 32, 244–245.

18. Mansour, N.A., El-Defrawi, M.E., Tappozada, A., Zeid, M. (1966) Toxicological studies on the Egyptian cotton leafworm, Prodenia litura. 6: Potentiation and antagonism of organophosphorus and carbamate insecticides. J. Econ. Entomol. 59, 307–311.

19. Merdan, A.I. (1978) Comparative immunological and ultrastructure study of the nuclear polyhedrosis viruses of two Spodoptera spp. 4th Conf. Pest Control. NRC. Cairo, pp. 740–748.

20. Moawed, S.M. (1972) The effect of different insecticides on diseased larvae of the Egyptian cotton leafworm, Spodoptera littoralis (Boisd.). M.Sc. Thesis, Cairo Univ., Fac. Agric., Egypt.

21. Moawed, S.M. (1980) The nuclear polyhedrosis virus of Spodoptera littoralis as biological insecticide. Ph.D. Thesis, Czechoslovak Academy of Sciences, Czechoslovakia.

22. Moawed, S.M., El-Nabarawy, I.M. (1987) Effectiveness of nuclear polyhedrosis virus and insecticides against the cotton leafworm, Spodoptera littoralis (Boisd.). Insect Sci. Applic. (In press).

23. Morris, O.N. (1962) Quantitative infectivity studies on the nuclear polyhedrosis of the western oak looper, Lambdina fiscellaria sominaria (Hulst.). J. Insect Pathol. 4, 207–215.

24. Salama, H.S. (1970) Rearing the corn borer Ostrinia nubilalis Hubn. on a semi-artificial diet. Z. ang. Ent. 65, 216–218.

25. Salama, H.S., Foda, M.S., Zaki, F.N., Moawed, S.M. (1984) Potency of combination of Bacillus thuringiensis and chemical insecticides on Spodoptera littoralis (Lepidoptera: Noctuidae). J. Econ. Entomol. 77, 885–890.

26. Salama, H.S., Moawed, S.M., Megahed, M.I. (1986) Effect of nuclear polyhedrosis virus on the cotton bollworm, Heliothis armigera (Hubner). Z. ang. Ent. 102, 123–130.

27. Scott, H.A., Young III, S.Y., McMasters, J.A. (1971) Isolation and some properties of components of nuclear polyhedra from the cabbage looper, Trichoplusia ni. J. Invertebr. Pathol. 18, 177–182.

28. Steinhaus, E.A. (1949) Principles of Insect Pathology. McGraw – Hill Co., New York. P. 757.

29. Steinhaus, E.A. (1960) Notes on polyhedrosis in Peridroma prodenia colias and other Lepidoptera. J. Insect Pathol. 2. 327–333.

30. Vago, C. (1972) Problemes pathologiques concernant les élevages en masse invertebres. Entomophaga 17, 111–120.

31. Vail, P.V., Henneberry, T.J., Kishaba, A.N., Arakawa, K.Y. (1968) Sodium hypochlorite and formalin as antiviral agents against nuclear polyhedrosis virus in larvae of the cabbage looper. J. Invertebr. Pathol. 10, 84–93.

32. Vail, P.V., Jay, D.L., Hunter, D.K. (1973) Infectivity of a nuclear polyhedrosis virus from the alfalfa looper, Autographa californica after passage through alternate hosts. J. Invertebr. Pathol. 21, 16–20.

33. Van der Geest, L.P.S. (1968) A method for the purification of polyhedra. J. Invertebr. Pathol. 11, 502.

34. Weiser, J. (1977) An Atlas of Insect Diseases. Acad. Sci. Prague, Czechoslovakia.

35. Whitlock, V.H. (1974) Symptomatology of two viruses infecting Heliothis armigera. J. Invertebr. Pathol. 23, 70–75.

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EFFECTS OF VARIOUS OXYGEN CONCENTRATIONS ON ANTIOXIDANT ENZYMES AND THE QUANTITY OF TISSUE PHOSPHOLIPID FATTY ACIDS IN THE CARP

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Analyses were made of the phsopholipid fatty acids and the antioxidant enzymes in the carp (Cyprinus carpio morpha) at three different oxygen concentrations, corresponding to hyperoxia, hypoxia and anoxia. Variations of the oxygen concentration were found to influence the quantities of phsopholipid fatty acids, as well as the antioxidant enzyme activities.

In hyperoxia and hypoxia the amount of polyunsaturated fatty acids in carp liver was higher than in anoxia, but in other tissues there was no significant differences. As to the antioxidant enzyme system, the glutathione peroxidase activity and the lipid peroxidation value increased significantly with decrease of the oxygen concentration, while the total superoxide dismutase activity decreased on lowering of the oxygen level.

Keywords: Oxygen concentrations — phospholipid fatty acids — antioxidant enzymes — carp tissues

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Abbreviations: C-ase=catalase, Cu,Zn-SOD=Cu,Zn-superoxide dismutase, t-SOD=total superoxide dismutase, Mn-SOD=manganese superoxide dismutase, FA or FAs=fatty acid or fatty acids, GP-ase=glutathione peroxidase, GSH=reduced glutathione, LP=lipid peroxidation, MDA=malondialdehyde, PL or PLs=phospholipid or phsopholipids, TBA=thiobarbituric acid, w.t.w.=wet tissue weight

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INTRODUCTION

One of the problems relevant to environmental acclimatization concerns the effect of the water oxygen concentration on fish. It has been observed that the oxygen toxicity is well correlated with the aerobic metabolism in eels (Anguilla anguilla L.) and trout (Salmo gairdneri R.) adapted to different oxygen concentrations /2/. It was reported by Pärt et al. /14/ that variations of the oxygen concentration led to changes in the structure and blood circulation of rainbow trout gill. The blood pH increased in hypoxia, from 7.6 to 8 at a water oxygen tension of 10 mm Hg. Variation of the environmental temperature and oxygen concentration are associated with compositional changes in the phospholipids (PLs) and their acyl groups in the membranes of goldfish (Carassius auratus L.) brain /5, 6/. The aim of the present study was to investigate the influence of variations in the water oxygen concentration from hyperoxia to anoxia on the tissue PL fatty acid (FA) composition and antioxidant enzyme activities in the common carp.

MATERIALS AND METHODS

The experiments were performed with common carp (Cyprinus carpio) weighing 350–500 g, maintained in outdoor pools supplied with running river-water at 21 ^{o}C for at least one week before the experiments. The animals were divided into three groups, then habituated for 1 h in large open plastic containers for aquatic respiration experiments, with ventilation for 5 min with 100% oxygen. The first group of fish were exposed to 99% O_2. The oxygen saturation of the water was 15.51 mg O_2/L, the second group were exposed only to running River Körös water, at an oxygen concentration of 2.01 mg/L, and the third group were exposed to non-aerated non-running water at an oxygen concentration of 0.78 mg/L. The oxygen concentration of the water was measured using a Yellow Springs Instrument Co. 53–2390 oxygen monitor and an OH-814/1 No. 292 potentiometric recorder. After a 17 h exposure, the animals were killed, and their liver, gill and muscle were removed and prepared for the determination of enzyme activities, lipid peroxidation (LP) and PL FAs.

Extraction and separation of lipids

Lipids were extracted by homogenizing the tissue samples with a chloroform-methanol (2:1 v/v) mixture in a BIOMIX homogenizer (MTA KUTESZ, Budapest, Hungary). The crude extract was filtered and washed according to the method of Folch-P. et al. /9/. The sample volume was reduced to about 2 ml under CO_2, to prevent the oxidation of FAs. Aliquots of the lipid extracts were separated by one-dimensional thin-layer chromatography on 20x20 cm plates coated with Kieselgel G. Plates were developed after spotting by the method of Malins et al. /11/, in an n-heptane:diethyl ether:acetic acid mixture (85:15:1 v/v). After development, the plates were dried in a stream of CO_2. PLs (start-spot) from the dried plates were taken in ampoules containing 5% HCl in dry methanol, and transesterified at 80 ^{o}C, within 2–3 h, under CO_2 or N_2. The methyl esters of the FAs were extracted with n-hexene (30–40 ^{o}C) from the esterification mixture after diluting it with an equal volume of distilled water.

Gas chromatography

The methyl esters were separated using a GCV chromatograph (PYE UNICAM, England) fitted with flame ionization detectors. Runs were performed in 2.7 m long glass analytical columns filled with 10% SP-2330 on Chromosorb WAW of 100–200 mesh (Supelco, Bellefonte, PA, USA) at 230 ^{o}C. High-purity nitrogen was applied at a flow rate of 40 ml/min. The dual column system was programmed from 150 ^{o}C to give partial separation of 18:3 and 20:3 (n-9) at a heating rate of 2.5 ^{o}C/min. The detector response obtained at a 250 ^{o}C detector head temperature was recorded at a chart speed of 1 cm/min. For identification of the peaks, calibration standards (e.g. GLC 10, 40 and 90) (Supelco, Bellefonte, PA, USA) were also run at constant temperature (200 ^{o}C). Peaks were identified by plotting log retention time versus the number of carbon atoms /1/ or by plotting log elution temperature (in ^{o}C) versus the number of carbon atoms in the FA chain /16/. The chromatograms were quantitated by the "triangulation technique". The data obtained are given in area percentages, which, according to the observation of Driscoll et al. /8/, are identical to the weight percentage composition within the limits of error.

Enzymatic activities in tissues

The tissues were quickly taken out, about 1 g was weighed and homogenized in saline at 0 ^{o}C, and the enzyme activities and other parameters were determined in the supernatants after centrifugation at 20.000 g.

a. Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined on the basis of inhibition of the pinephrine adrenochrome autocatalytic transformation under basic conditions /12, 13/. Mitochondrial (manganese) SOD (Mn-SOD) activity was determined in the presence of $5x10^{-3}$ M KCN /3/.

b. Glutathione peroxidase (GP-ase, EC 1.11.1.9) activity was determined by the method of Chiu et al. /7/ with cumene hydroperoxide as substrate. The reduced glutathione (GSH) residue after the action of the enzyme was measured by the method of Sedlak et al. /17/ using the Ellman reagent.

c. Catalase (C-ase, 1.11.1.6) activity was determined by the spectrophotometric method of Beers et al. /4/.

LP and protein determination

The amlondialdehyde (MDA) formed in the course of the LP was determined with thiobarbituric acid (TBA) by the method of Placer et al. /15/. The total amount of TBA-positive substance is given in nmol MDA/g wet tissue weight (w.t.w.) or mg protein. The calibration curve for the quantitative assay of LP was prepared with MDA diethyl acetal (Merck, FRG), as described by Placer et al. /15/.

The quantity of protein was determined by the method of Lowry et al. /10/. The Folin reagent was used for measurements, and bovine serum albumin was used in the preparation of the calibration curve.

The results were subjected to statistical evaluation with Student's t-test. All numerical data are given as means±SD. In the enzyme activity and LP measurements, the differences between 4—5 parallel measurements were never in excess of 5%.

RESULTS

The adaptation to the different oxygen concentrations brought about marked differences in the characteristic PL FA pattern and antioxidant enzyme system.

FA content of total PLs

The results in Tables 1/A–C illustrate the quantitative changes in the PL FA pattern in the treated carp tissues.

1. Decrease of the oxygen concentration decreased the palmitic acid (16:0) level in the liver, but increased that in the gill and white muscle at oxygen concentrations I and II. The quantity of palmitoleic acid (16:1 (n-7)) was lower in all organs as compared with the controls.

2. There were slight changes in the stearic acid level in all organs.

3. The amounts of oleic acid (18:1(n-9)) in the liver and gill decreased with the decrease of oxygen concentration, while in the white muscle it increased appreciably at the highest oxygen concentration, but decreased at the low oxygen concentrations.

The arachidonic acid (20:4(n-6)) level increased more in the liver and white muscle than in the gill tissue with decrease of the oxygen concentration.

The polyunsaturated acids, especially docosapentaenoic and docosahexaenoic acids, generally increased in all organs with decrease of the oxygen concentration.

The data in Table 2 indicate the effects of the three different oxygen concentrations on the protein values, LP levels and acyl chain indices.

1. The LP increased significantly and correlated negatively with the oxygen concentration in all investigated tissues, but the protein concentrations decreased in parallel with decrease of the oxygen concentration.

2. The acyl chain indices in the same Table clearly show that decrease of the oxygen concentration generally elevated the ratio $\Sigma(n-3)/\Sigma(n-6)$ in all investigated tissues, and also increased the ratio $\Sigma\%$ satd./$\Sigma\%$ unsatd. FAs in all tissues, except in anoxia.

The biochemical parameters in the carp tissues were changed signifi cantly after oxygen treatment. The results in Table 3 show that the most marked changes were observed in the gill and liver, followed by the white muscle.

1. GP-ase. The activity of this enzyme decreased significantly in hyperoxia in the liver and gill, but increased with the decrease of oxygen concentration in the white muscle homogenate. A positive correlation was observed with decreasing oxygen concentration, i.e. $r=0.97$, $r=0.79$ and $r=0.68$, for the gill, liver and white muscle, respectively.

Table 1/A

Effects of variations of O_2 concentration on phospholipid fatty acid composition in liver tissue

Fatty acids	Oxygen concentration			
	C	I.	II.	III.
	composition (area percentage)			
14:0	0.07±0.03	0.27±0.01	0.44±0.03	0.44±0.00
15:0	0.35±0.08	0.34±0.11	0.50±0.10	0.57±0.21
16:0	26.10±2.30	22.56±1.71	23.82±0.43	23.75±1.11
16:1 (n-7)	8.16±1.00	5.73±2.61	3.02±0.06	5.24±0.90
16:2 (n-7)	0.39±0.04	1.37±0.51	0.30±0.03	0.40±0.04
18:0	5.92±0.13	9.68±0.53	10.83±0.30	10.85±1.31
18:1 (n-9)	13.88±2.14	9.58±0.40	9.76±0.53	11.03±1.40
18:2 (n-6)	2.36±0.26	1.22±0.28	1.93±0.18	1.46±0.11
18:3 (n-6)	-	-	-	-
18:3 (n-3)	0.40±0.06	0.70±0.15	0.35±0.02	0.48±0.00
20:1 (n-9)	2.88±0.48	1.17±0.43	1.30±0.21	0.79±0.01
20:2 (n-6)	0.38±0.08	0.83±0.18	0.37±0.14	0.78±0.00
20:3 (n-9)	-	-	-	-
20:3 (n-6)	1.87±0.23	1.20±0.78	1.72±0.28	0.98±0.21
20:4 (n-6)	10.15±0.16	14.74±0.82	13.53±0.24	12.03±2.51
20:4 (n-3)	0.15±0.06	1.28±0.13	-	0.43±0.10
20:5 (n-3)	-	-	-	-
22:4 (n-6)	2.72±0.48	2.15±0.41	2.58±0.21	2.38±0.21
22:5 (n-6)	1.82±0.10	2.06±0.06	2.10±0.13	1.10±0.10
22:5 (n-3)	2.63±0.43	2.73±0.16	3.67±0.55	2.70±0.21
22:6 (n-3)	14.03±0.80	18.68±0.26	18.00±0.68	18.91±0.71

I., II. and III. 15.51, 2.01 and 0.78 mg/O_2/L

Table 1/B

Effects of variations of O_2 concentration on phospholipid fatty acid composition in gill tissue

Fatty acids	Oxygen concentration			
	C	I.	II.	III
	composition (area percentage)			
14:0	0.35±0.10	0.39±0.03	0.43±0.13	0.61±0.04
15:0	0.50±0.26	0.78±0.07	0.40±0.07	0.91±0.06
16:0	25.13±1.10	27.05±1.09	26.19±5.31	22.90±0.48
16:1 (n-7)	7.34±0.83	3.61±0.41	4.71±0.52	6.03±0.04
16:2 (n-7)	0.88±0.18	0.79±0.20	1.00±0.52	0.53±0.08
18:0	6.93±0.28	10.69±0.21	11.02±1.92	11.04±0.60
18:1 (n-9)	18.37±1.72	15.23±4.05	15.31±1.20	17.39±0.86
18:2 (n-6)	2.36±0.14	1.43±0.22	1.74±0.24	1.33±0.39
18:3 (n-6)	0.24±0.15	-	-	-
18:3 (n-3)	0.29±0.06	0.24±0.02	0.49±0.08	0.28±0.00
20:1 (n-9)	2.23±0.20	1.14±0.17	1.05±0.34	0.68±0.10
20:2 (n-6)	0.43±0.05	0.24±0.02	0.35±0.20	0.13±0.10
20:3 (n-9)	-	-	-	-
20:3 (n-6)	1.31±0.50	0.71±0.07	0.52±0.23	0.53±0.00
20:4 (n-6)	12.26±1.78	13.39±0.60	14.78±0.82	16.17±0.80
20:4 (n-2)	-	-	-	-
20:5 (n-7)	-	-	-	-
22:4 (n-6)	2.66±0.09	3.46±0.10	2.31±0.43	2.82+0.51
22:5 (n-6)	1.75+0.42	1.50±0.09	1.95±0.12	1.11±0.21
22:5 (n-3)	1.75±0.17	2.04±0.16	2.38±0.28	2.90±0.20
22:6 (n-3)	9.39±2.50	10.04±0.66	13.09±2.57	13.40±1.81

I., II. and III: 15.51, 2.01 and 0.78 mg O_2/L

Table 1/C

Effects of variations of O_2 concentration on phospholipid fatty acid composition in white muscle tissue

Fatty acids	Oxygen concentration			
	C	I.	II.	III
	composition (area percentage)			
14:0	0.43±0.04	0.43±0.03	-	0.33±0.18
15:0	0.48±0.12	0.52±0.15	-	0.74±0.03
16:0	21.45±1.48	22.33±1.37	26.13±1.78	19.29±0.56
16:1 (n-7)	7.90±1.46	4.13±0.63	4.54±0.32	5.48±0.74
16:2 (n-7)	0.31±0.09	0.47±0.32	0.07±0.04	0.50±0.24
18:0	4.55±0.08	7.47±1.01	7.26±0.11	6.26±0.19
18:1 (n-9)	16.68±2.54	19.67±0.87	15.61±0.69	13.53±1.22
18:2 (n-6)	6.46±0.34	4.71±1.27	6.16±1.28	3.81±0.31
18:3 (n-6)	-	-	-	-
18:3 (n-3)	0.49±0.16	0.45±0.30	0.17±0.18	0.45±0.07
20:1 (n-9)	1.61±0.21	2.13±0.67	1.13±0.30	1.40±0.34
20:2 (n-6)	0.36±0.08	0.33±0.09	-	0.60±0.13
20:3 (n-9)	-	-	-	-
20:3 (n-6)	1.33±0.11	1.64±0.53	2.10±0.43	0.59±0.33
20:4 (n-6)	10.36±0.36	13.61±0.45	11.42±1.23	14.10±0.81
20:4 (n-3)	-	-	-	-
20:5 (n-3)	-	-	-	-
22:4 (n-6)	1.33±0.23	2.61±0.63	1.36±0.08	1.95±0.12
22:5 (n-6)	1.71±0.24	1.37±0.37	2.40±0.73	1.88±0.55
22:5 (n-3)	2.45±0.18	2.87±0.30	2.85±0.30	3.37±0.09
22:6 (n-3)	7.31±1.36	7.00±0.43	10.32±1.18	8.46±1.04

I., II. and III: 15.51, 2.01 and 0.78 mg O_2/L

Table 2

Variations of protein quantity, lipid peroxidation level and acyl parameters in the organs at different oxygen concentrations

Organs	Prot. mg/g w.t.w.				LP nmoles MDA/g w.t.w.				$\frac{\Sigma\% \text{ n-3}}{\Sigma\% \text{ n-6}}$				$\Sigma\%$ PUFA				$\frac{\Sigma\% \text{ Satd. FA}}{\Sigma\% \text{ Unsatd. FA}}$			
	C	I	II	III	C	I	II	III	C	I	II	III	C	I	II	III	C	I	II	III
Liver	66.69	48.32	29.87	28.62	34.57	121.16	92.85	63.67	0.9	1.06	0.97	1.23	44.30	51.32	50.09	46.21	0.50	0.50	0.58	0.60
	±2.05	±4.03	±2.45	±2.19	±1.40	±15.21	±4.73	±1.61	±0.0	±0.04	±0.01	±0.09	±1.30	±0.80	±1.06	±3.07	±0.01	±0.02	±0.03	±0.03
		$p \ll 0.01$	$p \ll 0.01$	$p \ll 0.01$		$p < 0.3$	$p \ll 0.01$	$p \ll 0.01$		$p \ll 0.01$	$p \gg 0.3$	$p < 0.3$		$p < 0.1$	$p > 0.3$	$p < 0.1$		$p \ll 0.01$	$p > 0.3$	$p < 0.2$
Gill	75.95	27.87	19.88	26.34	101.96	76.25	42.50	0.50	0.59	0.82	0.74	39.50	37.42	40.84	44.84	0.50	0.66	0.67	0.45	0.45
	±5.97	±1.88	±2.49	±1.24	±1.72	±24.41	±12.66	±3.56	±0.01	±0.03	±0.13	±0.05	±3.10	±0.39	±1.94	±0.72	±0.00	±0.03	±0.09	±0.01
		$p \ll 0.01$	$p \gg 0.3$	$p < 0.01$		$p \ll 0.01$	$p \ll 0.01$	$p \ll 0.01$		$p \ll 0.01$	$p \ll 0.01$	$p \ll 0.01$		$p < 0.01$	$p < 0.01$	$p < 0.01$		$p \ll 0.01$	$p \ll 0.01$	$p \ll 0.01$
White	46.56	12.72	12.08	7.21	23.72	66.31	42.30	30.44	0.40	0.39	0.54	0.48	42.10	44.67	40.84	41.97	0.40	0.45	0.54	0.42
muscle	±6.19	±1.05	±1.05	±0.95	±1.49	±3.44	±5.07	±3.39	±0.00	±0.03	±0.03	±0.05	±2.60	±0.68	±1.94	±2.99	±0.00	±0.01	±0.02	±0.01
		$p \ll 0.01$	$p \ll 0.01$	$p \ll 0.01$		$p \ll 0.01$	$p \gg 0.3$	$p > 0.2$		$p < 0.05$	$p < 0.02$	$p > 0.3$		$p < 0.02$	$p \ll 0.01$	$p \ll 0.01$		$p < 0.01$	$p \ll 0.01$	$p \ll 0.01$

I, II and III; 15.51, 2.01 and 0.78 mg O_2/L

The given values are the means ($\pm$SD) of the results measured in 4–5 pieces of tissue of carp

Table 3

Variations of antioxidant enzymes in the organs at different oxygen concentrations

Organs	GP-ase U/g w.t.w.				C-ase BU/g w.t.w. ($\times 10^{-2}$)				total-SOD U/g w.t.w.				Mn-SOD U/g w.t.w.				Cu,Zn-SOD U/g w.t.w.			
	C	I	II	III	C	I	II	III	C	I	II	III	C	I	II	III	C	I	II	III
	35.2	7.7	12.4	24.2	47	52	22	14	1283.9	2183.7	1166.5	1472.8	96.1	120.7	94.3	72.9	1183.4	2062.9	1072.2	1399.9
Liver	±2.1	±0.7	±1.6	±2.1	±5	±3	±4	±0.8	±280.9	±95.9	±75.8	±73.6	±16.1	±14.0	±13.1	±4.7	±213.7	±107.4	±84.8	±78.3
		$p \ll 0.01$	$p \ll 0.01$	$p \ll 0.01$		$p < 0.3$	$p \ll 0.01$	$p \ll 0.01$		$p \ll 0.01$	$p \gg 0.3$	$p < 0.3$		$p < 0.1$	$p > 0.3$	$p < 0.1$		$p \ll 0.01$	$p > 0.3$	$p < 0.2$
	24.9	4.2	27.6	32.4	2	3	7	6	208.2	53.1	67.0	35.9	8.6	5.7	11.6	11.1	199.6	46.4	››.4	24.8
Gill	±2.7	±0.7	±4.0	±1.6	±0.2	±0.1	±1.0	±0.6	±15.1	±3.5	±6.68	±4.1	±1.0	±0.7	±0.7	±0.4	±15.3	±3.1	±7.3	±3.8
		$p \ll 0.01$	$p \gg 0.3$	$p < 0.01$		$p \ll 0.01$	$p \ll 0.01$	$p \ll 0.01$		$p \ll 0.01$	$p \ll 0.01$	$p \ll 0.01$		$p < 0.01$	$p < 0.01$	$p < 0.01$		$p \ll 0.01$	$p \ll 0.01$	$p \ll 0.01$
White	0.3	0.6	1.5	1.2	0.5	0.3	0.4	0.4	32.2	26.3	24.8	22.9	4.5	7.4	15.2	10.2	27.2	18.9	9.5	12.4
muscle	±0.0	±0.1	±0.2	±0.2	±0.0	±0.0	±0.0	±0.0	±3.4	±1.2	±2.5	±1.3	±0.4	±1.3	±0.6	±0.3	±3.5	±1.9	±3.0	±1.8
		$p \ll 0.01$	$p \ll 0.01$	$p \ll 0.01$		$p \ll 0.01$	$p \gg 0.3$	$p \gg 0.3$		$p < 0.05$	$p < 0.02$	$p > 0.3$		$p < 0.02$	$p \ll 0.01$	$p \ll 0.01$		$p < 0.01$	$p \ll 0.01$	$p \ll 0.01$

I, II and III; 15.51, 2.01 and 0.78 mg O_2/L

The given values are the means (±SD) of the results measured in the tissues of 4–5 fish

2. C-ase. The activity of C-ase significantly decreased and correlated negatively (r=-0.91) with the decrease in oxygen concentration for the liver. However, it significantly increased and correlated positively, i.e. r=0.51, for the gill.

3. SOD. The activity of the t-SOD generally decreased significantly with the decrease of oxygen concentration, while the Mn-SOD activity increased significantly and correlated positively, i.e. r=0.81 and r=0.51, for the gill and white muscle, respectively, but in the liver homogenates changes were not linear.

DISCUSSION

The oxygen toxicity is correlates well with the aerobic metabolic rate in poikilothermic animals which are adapted at the same temperature and different oxygen concentrations. Our data showed that variation of the oxygen concentration causes marked changes in the PL FAs, especially the unsaturated and polyunsaturated FAs. Consequently, the membranes become more fluid, which promotes more intensive oxygen exchange, in spite of the lower oxygen concentration. However, the oxygen radicals formed in the metabolism react more readily with the polyunsaturated FAs, as demonstrated by the increased LP on decrease of the oxygen concentration. Naturally, it is also possible that the oxygen radicals influence the PL synthesis directly too, by reacting with some key-enzyme of lipid or PL synthesis.

In the gill and white muscle tissues, the GP-ase increased significantly with the decrease of oxygen concentration. This is due to forced oxygen transfer, which induces changes in the gill structure, flattening of the lamella and damage to the capillary endothelium (14), and indirectly proves the presence of greater amounts of superoxide radicals and H_2O_2.

The C-ase activity in the liver decreased with the decrease of oxygen concentration. This is due to a decrease in the transformation of O_2^- into H_2O_2. However, in the gill and muscle, its activity increased as a result of severe damage to the tissues.

Other changes were also observed in the cells. The protein content decreased at high and at low oxygen concentrations, due to the reaction of enhanced LP products (oxo compounds) with the free $-NH_2$ groups of the amino acid constituents of the protein, forming Schiff bases. The LP increased at increased oxygen concentration, indicating a high generation of O_2^- and $OH^{\cdot}$

radicals, which react with unsaturated FAs and oxidize them to lipid peroxides and oxo compounds /5, 6/.

The Σ%(n-3)/Σ%(n-6)) ratio for PL FAs increased as the oxygen concentration decreased. This may be due to the fact that lipid alterations are associated with changes in oxygen concentration.

We consider it noteworthy that the larger quantity of oxygen radicals produced as a consequence of hyperoxia, hypoxia and anoxia lead to the enhanced aging and decomposition of the cell membranes.

REFERENCES

1. Achmans, G.R., Castell, J.D. (1967) A generalized study of effect of structure and other factors in open tubular gas chromatography of fatty acids on a polyester liquid phase with particular reference to monoethylenic fatty acids. J. Gas Chromatogr. 5, 489–496.
2. Barthelemy, L., Belaud, A., Chastal, C. (1981) A comparative study of O_2 toxicity in vertebrates. Resps. Physiol. 44, 261–268.
3. Beauchamp, C., Fridovich, I. (1971) Superoxide dismutase improved assay and an assay applicable to acrylamide gels. Anal. Biochem. 44, 276–287.
4. Beers, R.F., Sizer, I.W. (1952) Spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195, 133–140.
5. Chang, M.C.J., Roots, B.J. (1985a) The effects of temperature- and oxygen-acclimation on phospholipids of goldfish Carassius auratus L. brain microsomes. Neurochem. Res. 10, 355–375.
6. Chang, M.C. J., Roots, B.J. (1985b) The effects of temperature- and oxygen-acclimation on phospholipids of goldfish (Carassius auratus L.) brain mitochondria. Neurochem. Res. 10, 1231–1246.
7. Chiu, D.T.Y., Stults, F.H., Tappal, A.L. (1976) Purification and properties of rat lung soluble glutathione peroxidase. Biochim. Biophys. Acta 445, 558–566.
8. Driscoll, J.N., Ford., J., Jarmaillo, L.F., Gruber, E.T. (1978) Gas chromatography of hydrocarbons. J. Chromatogr. 158, 171–180.
9. Folch-P, J., Lees, M., Stanley, G.H.S. (1957) A simple method for isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497–509.
10. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 165–175.
11. Malins, D.C., Magold, H.K. (1960) Analysis of complex lipid mixtures by thin-layer chromatography and complementary methods. J. Am. Oil Chem. Soc. 37, 576–578.
12. Matkovics, B., Novák, R., Hoang Duc Hahn, Szabó, L., Varga, I.Sz., Zalesna, G. (1977) A comparative study of some important experimental animal peroxide metabolism enzymes. Comp. Biochem. Physiol. 56B, 31–34.

13. Misra, H.P., Fridovich, I. (1972) The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J. biol. Chem., 247, 3170–3175.

14. Pärt, P., Tuurala, H., Soivio, A. (1982) Structural and physiological changes in perfused trout (Salmo gairdneri) gills. Comp. Biochem. Physiol. 71C, 8–13.

15. Placer, Z.A., Cushman, L.L., Johnson, B.C. (1966) Estimation of product of lipid peroxidation malonyle dialdehyde in biochemical systems. Anal. Biochem. 16, 359–364.

16. Schmit, J.A., Wenner, R.B. (1966) Relative elution temperature. A simple method for measuring peak retention in temperature programmed gas chromatography. J. Gas. Chromatogr. 4, 325–328.

17. Sedlak, I., Lindsay, R.H. (1968) Estimation of total, protein-bound and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal. Biochem. 25, 192–205.

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BOOK REVIEWS

BIOLOGY OF SPERMATOGENESIS AND SPERMATOZOA IN MAMMALS

S.S. Guraya

Springer Verlag, Berlin, Heidelberg, New York, London, Pairs, Tokyo (1987), pp.430, 85 figs

Guraya's monograph containing more than 2600 references is a successful account of the morphological and functional interrelationship between developing germ cells and Sertoli cells and of the differentiation, stucture, chemistry and function of various components of mammalian spermatozoa. Hormonal influences and effects of various exogeneous factors on spermatogenesis are also surveyed. The methodology described in the book including electron microscopy of surface replicas, freeze-cleaving, freeze-etching, autoradiography, histochemistry and biochemistry, biophysics, lectin-binding, immunology, endocrinology, cell culture, etc. represents a wide range. It gives an up-to-date overlook on current knowledge from normal histology to molecular biology of spermatogenesis. The book comprises two parts: Spermatogenesis and Spermatozoa. Spermatogenesis is discussed in the following chapters: the seminiferous epithelium, spermatogonia, spermatocytes, spermatids and spermiogenesis, antigens during spermatogenesis. Part 2 starts with a general description of spermatozoa, and separate chapters are devoted to the head, neck, cytoplasmic droplet, tail, plasma membrane and its surface components of spermatozoa and sperm motility. The excellent schematic drawings and electron micrographs of high quality are of great help in comprehending the text, but the quality of some light micrographs (e.g., Figure 12) is not up to the standard of the book. The names of glycosaminoglycans in Figure 85 and in the related text are unusual. Typography and the quality of printwork are excellent.

The comprehensive review and lucid discussion of recent developments in this field make this book an important contribution to the understanding of mammalian spermatozoa. A special value of the book is its exceedingly rich list of references. It is highly recommended to everybody interested in embryology, reproductive biology, fertility regulation and problems of artificial insemination.

Cs. Hadházy (Debrecen)

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POPULATIONSGENETISCHE GRUNDLAGEN DER GERICHTETEN SELEKTION

(Populationgenetic Basis of Directed Selection)

G. Herrendörfer and L. Schüler

VEB Gustav Fischer Verlag, Jena, 1987, pp. 263, 48 figures and 95 tables, ISBN 0435-284 X

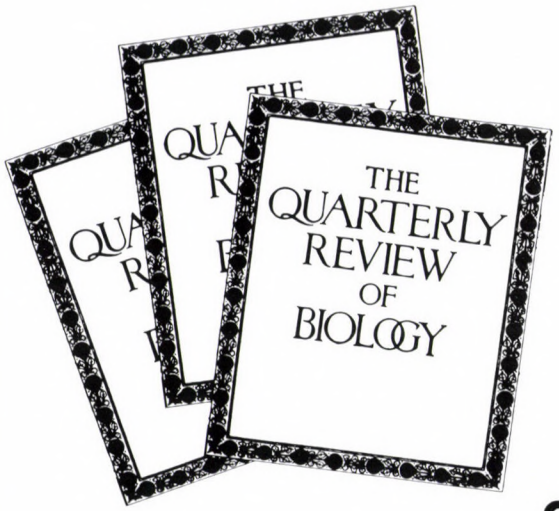
The Populationsgenetische Grundlagen der gerichteeten Selektion is the 14th volume of the well-known and popular series Genetik, Grundlagen, Ergebnisse und Probleme in Einzeldarstellungen edited by Professor Hans Stubbe from Gatersleben. It reflects the vast experience of the authors — Dr. Günter Herrendörfer and Dr. Lutz Schüler — in the field of the theoretical and applied animal breeding as well as in model construction in animal genetics, gained in the research centre of animal production (Forschungszentrum für Tierproduktion) in Dummerstorf — Rostock (GDR). Spurred by the developments in applied animal genetics observed during the last two decades, the authors concentrate on the most rapidly expanding territory, directional selection, and provide a comprehensive overview of its population genetic aspects.

The contents of this volume are arranged as follows: The first two chapters give an introduction to the concept and genetic background of directional selection. This is followed by a brief run through the methods of estimation of genetic parameters. Individual chapters deal with the selection index, the breeding value indices and the estimation of breeding values. The reader is then lead through a description of selection methods, the influence of the maternal effect on the selection gain and the relationship of the genotype x environment interaction and selection. The last part of the volume is devoted to simulated selection and experimental selection, both illustrated with numerous examples from animal breeding.

Full of useful formulae and tables, this book is aimed at readers armed with a thorough knowledge of statistical methods. The authors seem to expect this knowledge from a wide range of potential readers, because they recommend their volume not only to professional breeders but also to "hobby-breeders". Therefore, they omitted the most complicated models and the description of the multi-stage selection methods. Despite these efforts, however, their book is still a hard task to read for those who are not trained in mathematical statistics.

The title is somewhat misleading: it promises a general treatise, but the authors practically restrict the topic on three species such as swine, cattle and laboratory mice. An honest title for this book would be "Populationsgenetische Grundlagen der gerichteten Selektion in Tierzucht" Furthermore, the mode of treatment of the topic and the interpretation of experimental observation and models may also embarrass the reader: this book is clearly more than a text-book but less than a complete and up-to-date review of the field. In spite of these and a few other minor shortcomings, I find it an excellent and exciting text which can be recommended mainly to students of post-graduate courses and to professional breeders about to begin applying the methodology of model construction to their improvement programmes.

M. Sipiczki (Debrecen)



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Acta Biologica Hungarica 39(2–3), pp. 123–125 (1988)

On the occasion of the Second World Congress of the IBRO, which has been held from 17 to 21 August, 1987, in Budapest, we in the Anatomy Department of the Medical School University, Debrecen, organized a precongress satellite symposium with the title Comparative Aspects of Spinal and Brain Stem Neural Mechanisms: Parallelisms and Divergencies in Lower and Higher Vertebrates. The spinal cord and the brain stem contain a number of so-called "ancient" neural structures which are present, though in different forms, in fish as well as in mammals. In many cases, these "ancient" structures are readily accessible for experiments in lower vertebrates; they control a number of activities which, although play an instrumental role, may be concealed in the colourful motor repertoire of higher vertebrates. We are often inclined to take an opportunistic standpoint and ascribe the apparent differences to the account of "species differences" whereas we miss the important point of realizing that the same structure can be better studied in one particular species than in another. A good example for that is the experiments of P. Weiss (1936) in which he showed that the spinal cord itself was capable of generating rhythmic output for the control of co-ordinated limb movements. As the experiments were performed on amphibian species, which behaved so much differently from mammals, this suggestion, quite heretic at that time, remained unnoticed in the dominating realm of the reflex-chain theory interpreting coordinated movements. Today we know that a pattern generating structure is present in the mammalian spinal cord as well, but P. Weiss' experiments are forgotten.

Thus the primary aim of the symposium was to show our experimental

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objects to each other: what are they capable of producing both in organizational and functional aspects: which are the similarities and the divergencies among different animal species. With such objective a conference is bound attract a very heterogeneous audience and may easily disintegrate. Numerous excellent scholars have accepted the invitation, their active participation saved the day and each presentation was followed by a lively discussion.

The program started with presentations on the spinal cord, which have revealed a number of similarities in the synaptic organization of the cat and frog motoneuron. A number of interesting papers were presented on the morphology and physiology of primary afferents and on the sensory-motor coupling at the segmental level. The afternoon was devoted to "Spinal Automatism" meaning the complex movement patterns, scratch reflex and locomotion, which are controlled at the spinal level. The excellent presentations giving account of experiments performed on a wide range of animal species, from crustacea to cats, guaranteed the comparative aspects of this section.

On the following day, the mesencephalic centers of sensory organs (eye, vestibulo-acoustic system, lateral line organ) were the objects of discussion. Michael Gaze, Edinburgh, as a co-organiser brought together an excellent group to discuss the development and regeneration in the visual system. As the reader may have already guessed, the primary problem of the papers was the pathway and target selection by growing optic fibers in fishes and amphibia with a fascinating new touch of an activity dependent mechanism in the selective stabilization of retinotopic synapses. Their papers were followed by presentations on the mesencephalic visual centers; and the audience was presented with interesting accounts on the anatomy and physiology of the optic tectum in fish, frog and cat. On the same day a second group organized by Thomas Szabo, Gif-sur-Yvette, entertained the participants with the vestibulo-acoustic and the lateral line systems. The papers of both the vision group and the vestibular group were followed with great interest and it was interesting to witness how participants of different fields joined in the lively discussion.

Papers on other brain stem structures were on program on the third day. The evolution of the reticular formation and the presentation of evolutionary aspects in the organization of cranial nerve nuclei especially fitted into the scope of the discussion. The symposium was concluded with a general discussion on to what extent the synthesis of data obtained from comparative neurobiology can contribute to the understanding of the brain.

According to many polite comments, the symposium, despite its heterogeneous composition as far as the different fields of interest of the participants are concerned, was a success. The discussion of presentations from the many different points of view proved to be very fruitful in clearing up ambiguous details, or in pointing out the complexity of neural events presented in a simplified manner. About one half of the speakers have sent the manuscript of their presentations to this journal for publication. If the authors felt that their materials were not fully completed, or they wanted to make an *in extenso* publication of them in another journal, they have sent an extended summary of their presentations; however a number of full papers have also been received. Although many interesting papers are missing from these proceedings the reader may still get an impression of the atmosphere of the symposium.

REFERENCE

Weiss, P. (1941) Self-differentiation of the basic patterns of coordination. Comp. Psychol. Monogr. *17*, 1–96.

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SYNAPTIC CONNECTIONS BETWEEN PRIMARY AFFERENTS AND MOTONEURONS IN THE SPINAL CORD OF ANURAN LARVAE

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(Received 1988–01–19)

The relationship between dorsal root afferents and lumbar motoneurons has been studied in the isolated spinal cord of Rana ridibunda tadpoles. It was found that primary afferents do not form direct contacts with "primary" motoneurons innervating the axial musculature used by the larvae in swimming. Monosynaptic connections were revealed only between afferent fibres and lateral motor column motoneurons which innervate the developing hindlimb. The transmission in these synapses was dual: electrical and chemical. During the metamorphic stages when the locomotion is gradually taken over by the developing hindlimbs, an increase of the percentage of mononeurons receiving direct synaptic input from the primary afferents was observed.

Keywords: Anuran larvae, spinal cord, sensory-motor synapses, electrical and chemical transmission

INTRODUCTION

The analysis of unitary potential properties in central synapses of the lower vertebrates, showed the existence of mixed synapses with dual mode of transmission (electrical and chemical). Mainly heterotypic neurons are connected by these mixed synapses. In homologous synapses of higher vertebrates the transmission is purely chemical. These data allowed Shapovalov to put forward a hypothesis that in the course of evolution some synapses of

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the heterotypic neurons develop as dual-action synapses and are likely to be precursors of chemically transmitting synapses /7/.

A good example of this developmental process is a sequence of changes in phylogenesis of transmitter-release mechanisms in the direct sensorymotor synapses of the spinal cord of vertebrates. In lower vertebrates (lamprey, fish and amphibia) transmission in the monosynaptic reflex arc occurs with the help of a joint electrical and chemical machanism. On the other hand, in reptiles and mammals the transmission is purely chemical /7, 8/.

In this paper data are presented about the formation of mixed synapses between dorsal ganglion cells and motoneurons in the ontogenesis. The aim of our investigation was: 1. to study the development of monosynaptic sensory-motor connections in the lumbar spinal cord in the amphibian larvae metamorphosis, 2. to determine the mode of synaptic transmission in different stages of formation of the interneuronal contact.

MATERIALS AND METHODS

Experiments were performed on the isolated spinal cord of tadpoles (Rana ridibunda) ranging in age from stage XI to stage XXV /11/.

The preparatory procedures were described previously /10/. The preparation was kept in an oxigenated Ringer solution at 16–18 °C. The test solution without Ca^{2+} containing 2 mM of Mn^{2+} was applied to eliminate chemically mediated transmission.

The cut ends of the 9th dorsal and the 9th and 10th ventral roots were placed on the stimulating electrodes. Intracellular recordings were made from lumbar motoneurons, using bevelled glass micropipettes with 3 M KCl or 2 M K citrate. Motoneurons were identified by antidromic activation. In some cases, intracellular recordings were obtained with HRP-filled microelectrodes. After this, the HRP was iontophorized into the motoneuron. The microelectrode signals were conventionally amplified, monitored on an oscilloscope and simultaneously digitized by a computer.

Poly- or monosynaptic type of interaction between primary afferents and motoneurons was identified from the time interval between a positive peak of the field potential wave in the motor zone and the onset of EPSP. The time interval was conditionally designated as segmentary delay (SD).

At the same time the projections of primary afferent fibres and motoneuron organization were studied by placing HRP (for 12–24 h) on the cut ends of dorsal and or ventral roots of the spinal cord, using a specially developed technique /9/. The 60 μm sections were treated by the procedure of Graham and Karnovsky /4/.

RESULTS

In metamorphosis of anuran larvae two types of motoneurons, "primary" and "secondary" are found in the lumbar spinal cord. The first is located in the margin of ventromedial gray matter, and innervates the tail and trunk muscles, the second group forms the lateral motor column (LMC) and innervates the developing hindlimb /2/.

Our experiments showed that "primary" motoneurons do not receive a direct synaptic input from the dorsal ganglion cells. Contacts between them were not revealed after parallel HRP-labellings of the afferent fibres and motoneurons. Figure 1 illustrates the result of such an experiment performed on stage XIV. The motoneuron dendrites are located mainly in the lateral and ventral white matter, and are not revealed within the field of primary afferent terminals.

Only polysynaptic EPSPs were evoked in the "primary" motoneurons by dorsal root stimulation. The SD of EPSPs was, as a rule, more than 3 ms, which suggests the existence of more than two synaptic sites in the segment.

On the early stages of metamorphosis (XI—XIII), EPSPs induced in "secondary" motoneurons were also polysynaptic. The first monosynaptic reactions were obtained in LMC motoneurons on stage XIV. It should be mentioned at this point, that on stages XIII—XIV Liuzzi and co-workers /6/ in their histological investigation found first contacts between primary afferents and LMC motoneurons. The averaged monosynaptic EPSP recorded in a motoneuron on stage XIV is shown in Fig. 2A. The EPSP consists of two components, the first having a segmentary delay 0.5 ms, the second — 1.2 ms. After replacement of the normal solution to the Ca^{2+}-free, Mn^{2+}-containing solution, the second component was gradually depressed and finally completely blocked. Unlike the second component, the first remained unchanged. According to these findings the first component of EPSP is electrical in nature, while the second is chemically mediated. The existence of electrical and chemical mechanisms of transmission in monosynaptic EPSPs from the first stages, when synapses start to function indicates that both mechanisms are formed at about the same time.

The monosynaptic EPSPs with electrical and chemical components were recorded after stage XIV (see Fig. 2B). The quantitative estimates of SD in EPSPs recorded in different motor cells are shown in Fig. 3. It is shown that with larvae development, the percentage of motoneurons receiving the direct synaptic input from the primary afferents increases. When the re-

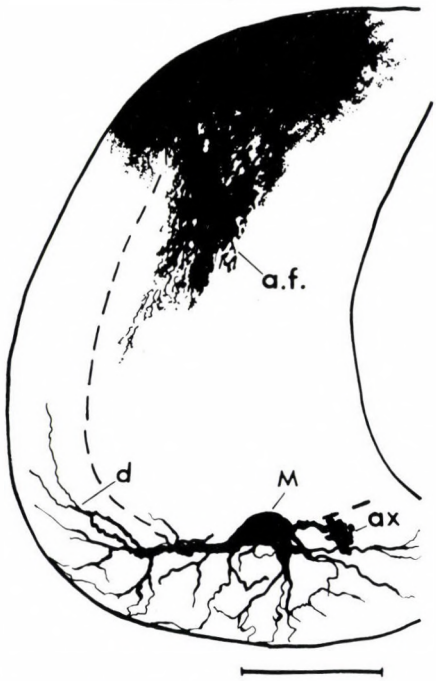

Fig. 1. Camera lucida drawing of a transverse section through the lumbar spinal cord of the stage XIV tadpole, illustrating the dorsal root afferent — "primary" motoneuron relationship. — The 9th dorsal root and axons of "primary" motoneurons (ax) were labelled with HRP simultaneously. Afferent fibres (a.f.) do not reach the dendrites (d) of the "primary" motoneuron (M). Bar: 100 μm

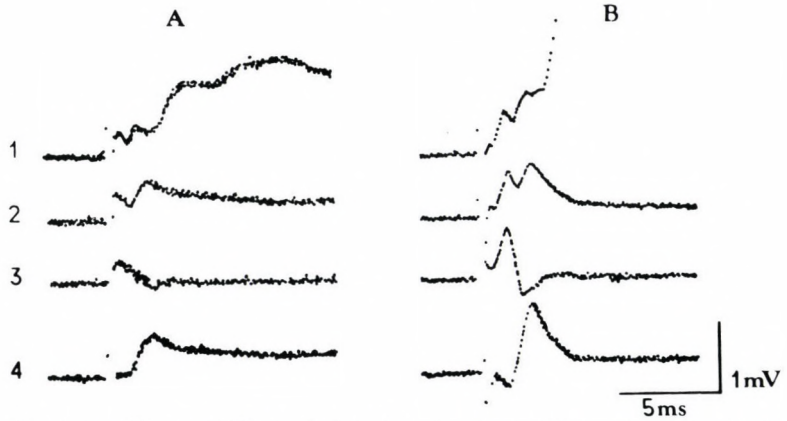

Fig. 2. Monosynaptic reactions induced in lumbar motoneurons by stimulating the 9th dorsal root by single stimuli on stages XIV (A) and XXV (B) of metamorphosis. — 1 - EPSP in normal Ringer solution; 2 - response in motoneuron after 20 min perfusion of the spinal cord with the test solution; 3 - field potential; 4 - result of subtraction of extracellular tracing (3) from intracellular tracing (2)

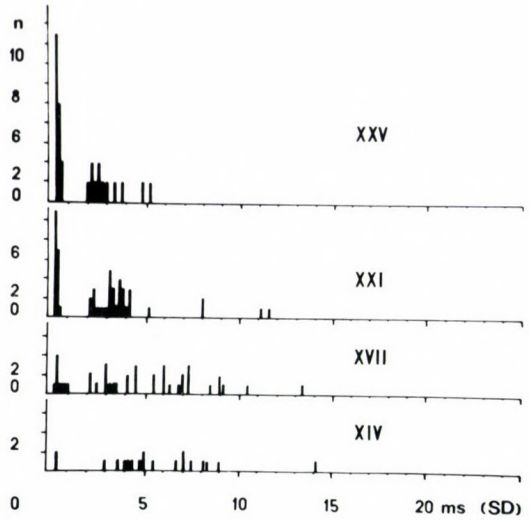

Fig. 3. Histograms of segmentary delay distribution of EPSPs on various stages of development. SD — the segmentary delay in ms; n — number of neurons. Roman numbers — stages of metamorphosis. SD of monosynaptic EPSPs form 0.4 to 1.1 ms

cordings were done on prometamorphic stages (XIV—XVII), the monosynaptic EPSPs were registrated only in some cases. On the final climax stage (XXV) monosynaptic responses were obtained in more than 50% motoneurons studied. This indicates that the formation of sensory-motor synapses continues throughout the late stages of metamorphosis. All monosynaptic dorsal root EPSPs are found to have electrical as well as chemical components. This is another evidence that both mechanisms of transmission begin to function at the same time.

The increase of the number of motoneurons which receive direct synaptic input from the dorsal ganglion cells in the climax stages is supported by our morphological investigations. Labeling contacts between LMC motoneurons and primary afferents on these stages revealed them to be located mainly in the intermediate gray matter /5, 6/. In our experiments, an increase in the number of collaterals and terminals of primary afferents in this region was observed. Figure 4A, B shows the projections of the 9th dorsal root fibres on stages XVII and XXV. It is shown that the ventral field of primary afferent terminals (indicated by arrow) increase during development.

There was an increase of branching of motoneuron dendrites in the

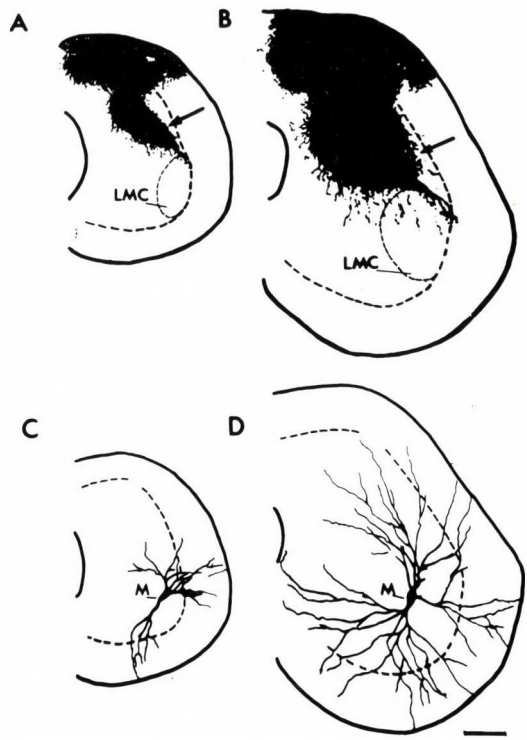

Fig. 4. A and B. The ventral projections of the 9th dorsal root fibres filled with HRP in the lumbar spinal cord of the tadpoles on stages XVII (A) and XXV (B). Arrows indicate the ventral terminal field of primary afferent axons. LMC – lateral motor column. Camera lucida drawings from 60 μm thick sections. C and D. Dendritic arborizations of HRP-labeled LMC motoneurons (M), where monosynaptic dorsal root EPSPs were recorded (stages XVII (C) and XXV (D)). Reconstructions from sections containing dendrites directed to the zone of primary afferent terminals

intermediate gray matter. Figure 4C, D illustrates partial reconsturctions of two motoneurons on stages XVII and XXV where monosynaptic EPSPs were recorded. The reorganization of dendritic trees directed in sensory afferent neuropil are clearly shown in the figure. This is in a good agreement with the results of Jackson and Frank /5/ who obtained very similar histological data on the brachial spinal cord of anuran larvae.

DISCUSSION

Our experiments show that the monosynaptic reflex arc in the lumbar spinal cord of anuran larvae is formed only between dorsal ganglion cells and motoneurons of the lateral motor column. The formation of the first functional synapses begins on the prometamorphic stages, when the development of the hindlimb being completed and it can be involved in locomotion. In the first stages on which monosynaptic contacts were revealed, transmission in the synapses was realized by the electrical and chemical mechanism.

This observation is supported by the results of our ultrastructural study /1/ that in some functionally developed synapses on stage XIV both active zones and gap junctions are present. Our ontogenetic investigations are in a good agreement with the data of Frank and Westerfield /3/. They succeeded in recording electrical and chemical transmission in sensory-motor synapses in the developing brachial spinal cord of Rana catesbieana tadpoles during climax stages. Our results confirm the hypothesis of Shapovalov /7/ about the evolution of transmission mechanisms in this type of interneuronal synapse.

There is no doubt that the functional significance of the structural co-existence of the two mechanisms of transmission in monosynaptic connections between primary afferents and motoneurons is of great importance. At present, there is no explanation for the functional role of this co-existence requiring further studies to be elucidated.

REFERENCES

1. Adanina, V.O., Shupliakov, O.V. (1987) Ultrastructure of terminals of the primary afferents in segment IX of the lumbar spinal cord of the tadpole Rana ridibunda. Zh. Evol. Fisiol. Bioch. 23, 399–401 (In Russian).

2. Forehand, C.J., Farel, P.B. (1982) Spinal cord development in anuran larvae: I. Primary and secondary neurons. J. Comp. Neurol. 209, 386–394.

3. Frank, E., Westerfield, M. (1983) Development of sensory-motor synapses in the spinal cord of the frog. J. Physiol. 343, 593–610.

4. Graham, R.C., Karnovsky, M.J. (1966) The early stages of absorption of injection horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. J. Histochem. Cytochem. 14, 291–302.

5. Jackson, P.C., Frank, E. (1987) Development of synaptic connections between muscle sensory and motor neurons: anatomical evidence that postsynaptic dendrites grow into a preformed sensory neuropil. J. Comp. Neurol. 255, 538–547.

6. Liuzzi, F.J., Beattie, M.S., Bresnahan, J.C. (1985) The development of relationship between dorsal root afferents and motoneurons in the larval bullfrog spinal cord. Brain Res. Bull. 14, 377–392.

7. Shapovalov, A.I. (1980) Interneuronal synapses with electrical, dual and chemical mode of transmission in vertebrates. Neuroscience 5, 1113–1124.

8. Shapovalov, A.I. (1983) Types of synaptic connections between nervous cells and their evolution. Zh. Evol. Fisiol. Bioch. 19, 341–350 (In Russian).

9. Shupliakov, O.V. (1985) Suction electrode for morpho-physiological studies in thin and short nerve trunks in vitro. Sechenov. J. Physiol. USSR. 71, 804–807 (In Russian).

10. Shupliakov, O.V. (1986) Transmission in sensory-motor synapses in the lumbar spinal cord of the tadpoles Rana ridibunda. Zh. Evol. Fisiol. Bioch. 22, 174–180 (In Russian).

11. Taylor, A.C., Kollros, J.J. (1946) Stages in the normal development of Rana pipiens larvae. Anat. Rec. 94, 7–23.

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THE USE OF FETAL MOUSE SPINAL CORD-DORSAL ROOT GANGLION EXPLANTS TO STUDY THE FACTORS UNDERLYING SELECTIVE CONNECTIONS IN VITRO

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(Received 1988–01–19)

Dorsal root ganglion (DRG) afferents selectively project to dorsal spinal cord (SC) explants when grown in a medium containing serum or a serum-free medium (CDM) containing galactose compounds. SC-DRG explants grown in CDM retain their gross morphological characteristics over several months in vitro, greatly facilitating the mapping of the sensory afferents within given regions of the cord explant. Explants grown in CDM without the addition of galactose show no such selective preference for dorsal cord, and terminate equally throughout the cord. This lack of selectivity also occurs in galactose-grown SC-DRG provided tetrodotoxin (TTX) is added to the medium sufficient to block all recordable spontaneous bioelectric activity (SBA), viz., action potential discharges and slow waves. Gangliosides are galactose-containing compounds found in neural membranes which may be involved in cell-cell recognition/adhesion. The addition of a mixture of gangliosides to the CDM results in the preferential restriction of the sensory afferents within the dorsal cord regions even in the presence of TTX. It is concluded that gnagliosides may be able to compensate for the absence of SBA during periods of development associated with the formation of selective interneuronal connections.

Keywords: Spinal cord-DRG, organotypic explants, selectivity, gangliosides, bioelectric activity

One in vitro model currently being used ot examine factors underlying the formation of selective connections within the central nervous system (CNS) is an organotypic explant of fetal mouse spinal cord-dorsal

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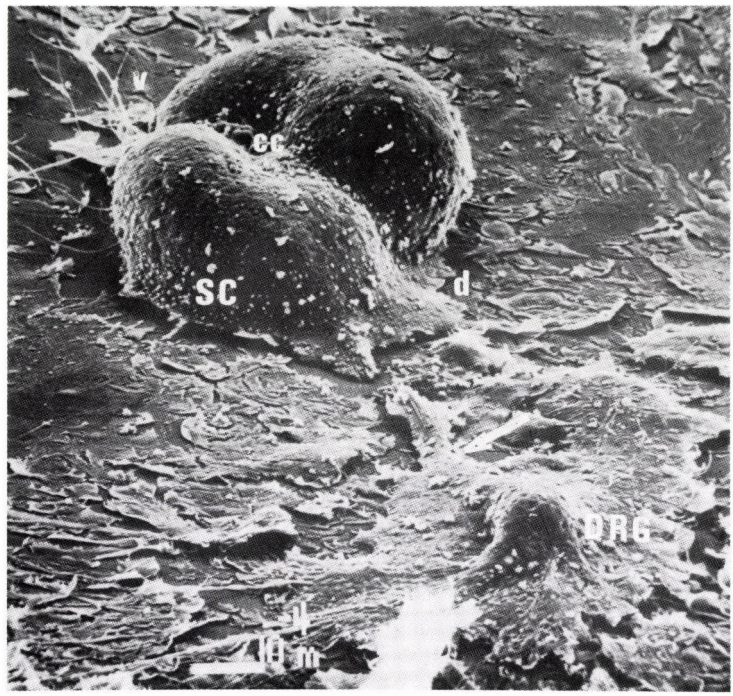

Fig. 1. Scanning electron micrograph of a SC-DRG organotypic explant 27 days in vitro, grown in a chemically defined medium containing 10 µg/ml galactose-1-phosphate. The typical cross-sectional morphology of the spinal cord (SC) is readily apparent (c = central canal). The dorsal root ganglion (DRG) has migrated some distance away from the SC but is connected to the cord by a bundle of sensory afferent fibres (arrow). d = dorsal side; v = ventral side

root ganglia (SC-DRG, Fig. 1). The dimensions of the fetal spinal cord are such that an entire cross-section can be optimally maintained in vitro for periods of several months. Since an entire organotypic cross-section is used, spinal areas not associated with processing incoming afferent information are open to innervation by the DRG sensory afferents, thus providing alternative (i.e., incorrect) domains for synapse formation. Lastly, the sensory afferents preferentially terminate within the substantia gelatinosa and dorsal horn of the cord in situ /15, 16, 28, 29/. Such afferents have also been shown to exhibit similar dorsal cord preferences within organotypic cord explants when cultured under the appropriate conditions in vitro /3, 11, 26/.

The successful use of serum-free media for long-term culturing of neural tissues has an obvious advantage over media supplemented with sera or

embryo extracts: every constituent within the medium is known and can be regulated to suit the requirements of the tissues being cultured. As such, the effects of compounds added to a chemically defined medium (CDM) can be studied more reliably without fear of contaminating influences from the serum. This can be particularly crucial when the compounds under scrutiny are themselves normal constitutents of sera and embryo extracts. The basic CDM used in our laboratory is a modification of a medium developed by Bottenstein and Sato and Baker et al. /9 and 3/.

A number of experiments using SC-DRG organotypic explants taken from 13-day-old mouse fetuses have now been reported. One of the most striking features of these explants is the superb retention of a typical cross-sectional appearance by the spinal cord tissues even after several months in vitro (Fig. 1). The in vitro localization of sensory afferents has been determined anatomically (HRP-filled fibers) and electrophysiologically (fixed latency discharges, representing afferent terminations), revealing projection patterns which reflect the environment under which the explants were grown. Sensory afferents display a significant preference for the dorsal regions of the spinal cord explant when chronically exposed to serum /3/. Afferents grown in a simple CDM, on the other hand, show no selective preference for any region of the cord explant, but terminate equally in dorsal and ventral cord. This has led us to conclude that a serum-borne factor(s) is responsible for the expression of afferent selectivity for dorsal cord /3/.

What is this factor and how does it operate? It is widely held that selective cell-cell adhesion and/or recognition is accomplished via macro-molecules which include glycoproteins and glycolipids. Both are produced in the soma of the neuron and transported via axoplasmic flow to and from the terminals where they are inserted into the membranes /see 12, 13, 20/.

Both glycoproteins and glycolipids possess oligosaccharide chains which extend from the plasma membrane into the extracellular spaces. The great diversity in oligosaccharide composition and configuration at the cell surface make these molecules ideally suited to function as receptors or recognition factors. Thus,it is the oligosaccharides that represent the "business end" of intercellular recognition and/or adhesion /17, 24, 25/.

The discovery of non-selectivity in CDM-grown SC-DRG explants has provided us with a useful model on which to test various compounds suspected of being involved in the development of selective connectivity in this model system. Several experiments have now been carried out to this end and are

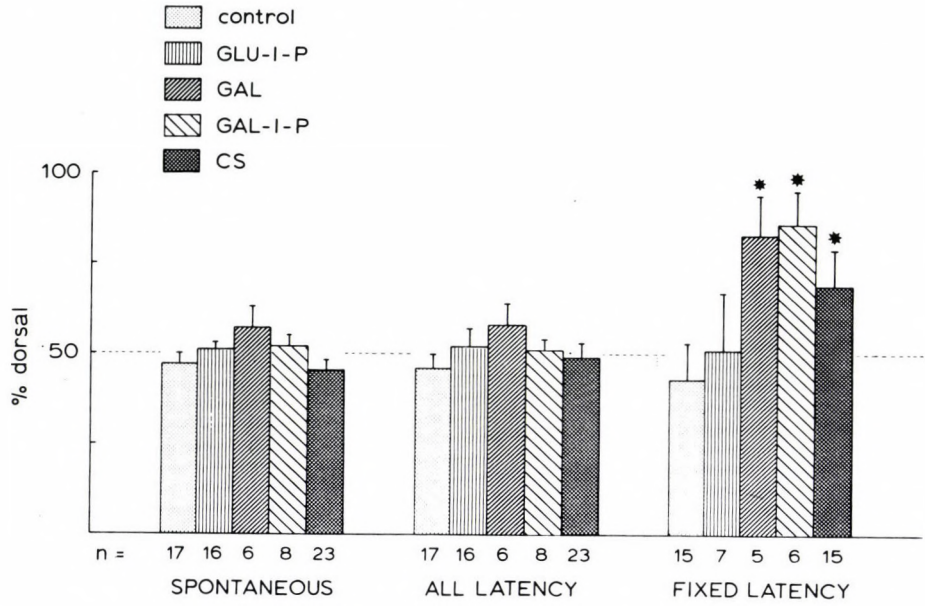

Fig. 2. Bar graph representing the distribution of sensory DRG afferents within organotypic explants of fetal mouse spinal cord. Days in vitro: control, 27–33; glucose-1-phosphate (glu-1-P), 28–33; galactose-1-phosphate (gal-1-P) 25–26; condroitin sulfate (CS), 25–35. Spontaneous refers to spontaneous bioelectric activity measured from 16 sites evenly distributed across the surface of the cord explant. All latency refers to evoked responses recorded in the cord following DRG stimulation, whose latencies varied between 1 and 20 ms. Fixed latency responses which had a latency "jitter" of less than 0.5 ms. * differs significantly from 50% and control values; o differs significantly from gal-1-P ($P < .05$); n represents the number of cultures in each group

beginning to give us an insight into what sorts of molecules are involved in the expression of DRG afferent preferences for dorsal cord in vitro.

Galactose is an important hexose sugar in glycoprotein and glycolipid oligosaccharide chains. None of the media used in our initial experimented CDM displayed preferences for dorsal cord regions that were similar to those observed in serum-grown cultures /4/; (Fig. 2). The substitution of other hexose sugars for galactose in the membrane macromolecules might change the adhesion and/or recognition characteristics for these molecules (see /32/). We concluded, therefore, that galactose was required for the development of afferent selectivity for SC-DRG explants grown in CDM, though these studies did not enlighten us regarding which compounds underlie selectivity in this model nor say anything about the pathways through which

galactose travels in order to affect the biochemical changes responsible for the restoration of dorsal cord preferences.

A growing body of literature has recently highlighted the dramatic effects gangliosides exert on nervous tissues, both in vivo and in vitro. Gangliosides are glycospingolipids, which are produced in the soma and transported to terminals where they appear to accumulate (see /19/). All major gangliosides have in common an oligosaccharide chain containing several molecules of galactose. The fatty acid-sphingosine base of the ganglioside molecule is embedded in the outer leaflet of the neural plasma membrane and the oligosaccharide chain extends into the extracellular space where its negative charges contribute strongly to ionic interactions associated with various cations, such as those involved in neurotransmission (see /30/).

The qualitative and quantitative patterns of gangliosides change considerably during mammalian and avian development, stabilizing at adult levels once the period of synaptogenesis has been completed (see /20/). Exogenously added mixtures of gangliosides as well as individual gangliosides promote extensive neuritogenesis and neuritic elongation in most types of primary nerve cell cultures and neuroblastoma cell lines (see /31/). They also promote a rapid return of function in many lesioned areas of the CNS, presumably by the same phenomena (see /31/). In addition, gangliosides have been implicated in selective adhesion between retinal-geniculate neurons /8/, retinal-tectal neurons /21/, and neuromuscular junctions /23/.

In view of the heavy concentration of galactose in the ganglioside molecule and the implications of gangliosides in synaptogenesis and selective adhesion, it was a logical next step in our own SC-DRG studies to examine the effects of ganglioside addition to our CDM. Moreover, since gangliosides are present in sera (see /14, 18, 26, 33/). they could have accounted for the sensory afferent selectivity observed in SR-DRG cultures grown in the presence of these fluids /3/.

SC-DRG explants chronically exposed to an unknown mixture of gangliosides dissolved in CDM displayed sensory afferent projection patterns which were similar to those reported in the above serum-supplemented and CDM-galactose studies /1/. The percentage of HRP-stained or electrophysiologically identified sensory afferent terminals within dorsal cord reached levels reported for serum-grown and galactose-grown explants (Fig. 3). These levels were attained even if the spontaneous bioelectric discharges in the cultures were chronically silenced by tetrodotoxin throughout the culturing

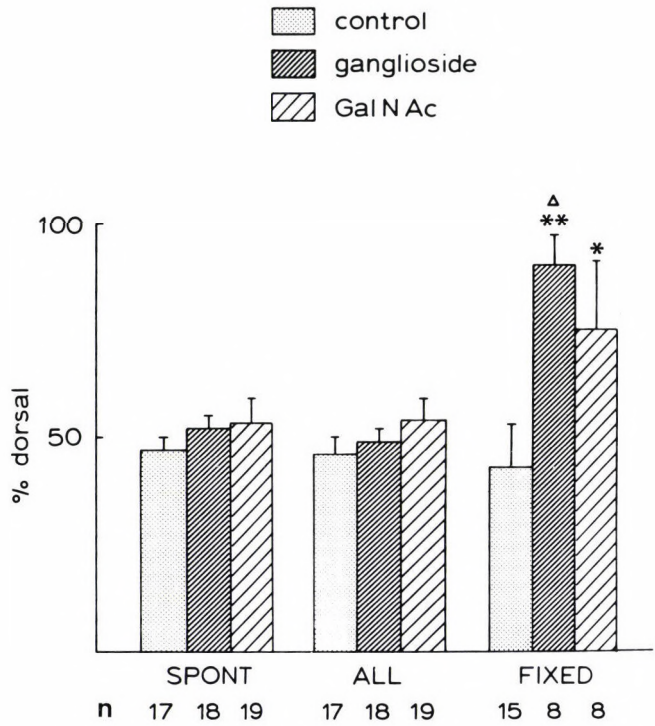

Fig. 3. Bar graph representing the dorsal percentages of spontaneous (SPONT), all-latency (ALL) and fixed-latency (FIXED) evoked responses (± S.E.M.) in control and experimental cultures (see Fig. 2 for description of each category). n – represents the number of cultures used in each group; * – less than $p < .05$ (with respect to control cultures); ** – less than $P < .005$ (with respect to controls); $P < .0005$ (with respect to the 50% level)

period /6/. Since the loss of spontaneous activity abolished dorsal cord preferences by sensory afferents grown in the presence of galactose /2/, we must conclude that mixtures of gangliosides can circumvent the bioelectrically dependent synthesis or transport processes, so as to support selective neuronal matching even in "virgin" networks.

One major question raised by the above results is whether an individual ganglioside is capable of eliciting the selective connections observed between the DRG afferents and dorsal cord, or whether a mixture of gangliosides is required. We have recently determined that the answer to the first part of this question is that not all gangliosides are capable of eliciting the selective response in our model (Baker and Gaasbeek-Janzen, in preparation). The chronic exposure of Sc-DRG explants to purified GM1 fails

to eovke the same dorsal cord selectivity that was observed when the unknown mixture of gangliosides was used. Instead, the spread of the sensory afferents from their point of entry into the cord occurred with equal frequency dorsally and ventrally as was seen in control CDM cultures.

The results of the experiments described above and of others dealing with the role of function in selective connectivity (see /5, 6, 10/). Demonstrate the usefulness of the SC-DRG model in the determination of the underlying bases of specific cell-cell connections in this portion of the CNS. Our studies have implicated gangliosides in this phenomenon.The SC-DRG explant, therefore, should continue to serve as a model for studies designed to determine which of several functions performed by neuronal gangliosides is most involved in the expression of selectivity.

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REFERENCES

1. Baker, R.E. (1983) Effects of gangliosides on the development of selective afferent connections with fetal mouse spinal cord explants. Neurosci. Letts. 41, 81–84.

2. Baker, R.E. (1985) Horseradish peroxidase tracing of dorsal root ganglion afferents within fetal mouse spinal cord explants chronically exposed to tetrodotoxin. Brain Res. 334, 357–360.

3. Baker, R.E., Habets, A.M.M.C., Brenner, E., Corner, M.A. (1982) Influence of growth medium, age in vitro and spontaneous bioelectric activity on the distribution of sensory ganglion-evoked activity in spinal cord explants. Dev. Brain Res. 5, 329–341.

4. Baker, R.E., Corner, M.A., Kleiss, M. (1983) Effects of chemical additives on functional innervation patterns in mouse spinal-cord-ganglion explants in serum-free medium. Neurosci. Letts. 41, 321–324.

5. Baker, R.E., Corner, M.A., Habets, A.M.M.C. (1984) Effects of chronic suppression of bioelectric activity on the development of sensory ganglion evoked responses in spinal cord explants. J. Neurosci. 4, 1187–1192.

6. Baker, R.E., Van der Togt, C. (1986) Gangliosides restore the specificity of afferent projection patterns in spinal cord explants chronically exposed to tetrodotoxin. Neurosci. Letts. 67, 285–288.

7. Barondes, S.H. (1970) Brain glycomacromolecules and interneuronal recognition. In: Quarton, G.C., Melchenuk, T., Schmidt, F.O. (eds) The Neurosciences A Second Study Program. Rockefeller University Press, New York.

8. Blackburn, C.C., Swank-Hill, P., Schnaar, R.L. (1986) Gangliosides support neural retina cell adhesion. J. Biol. Chem. 261, 2873–2881.

9. Bottenstein, J.E., Sato, G.H. (1979) Growth of a rat neuroblastoma cell line in serum-free supplemented medium. Proc. Natl. Acad. Sci. USA 76, 514–517.

10. Corner, M.A., Habets, A.M.M.C., Baker, R.E. (1987) Bioelectric activity is required for regional specificity of sensory ganglion projections to spinal cord explants cultured in vitro. Roux's Arch. Dev. Biol. 196, 133–136.

11. Crain, S.M., Bornstein, M.B., Peterson, E.R. (1968) Maturation of cultured embryonic CNS tissues during chronic exposure to agents which prevent bioelectric activity. Brain Res. 8, 363–372.

12. Edwards, D.L., Grafstein, B. (1983) Intraocular tetrodotoxin in goldfish hinders optic nerve regeneration. Brain Res. 269, 1–14.

13. Elam, J.S. (1982) Composition and subcellular distribution of glycoproteins and glycosaminoglycans undergoing axonal transport in garfish olfactory nerves. J. Neurochem. 39, 1220–1229.

14. Fishman, P.H., Bradley, R.M., Moss, J., Manganiello, V.C. (1978) Effects of serum on ganglioside uptake and choleragen responsiveness of transformed mouse fibroblasts. J. Lipid Res. 19, 77–81.

15. Fitzgerald, M. (1985) The post-natal development of cutaneous afferent fibre input and receptive field organization in the rat dorsal horn. J. Physiol. 364, 1–18.

16. Fitzgerald, M., Swett, J. (1983) The termination pattern of sciatic nerve afferents in the substantia gelatinosa of neonatal rats. Neurosci. Letts. 43, 149–154.

17. Gahmberg, C.G. (1981) Membrane glycoproteins and glycolipids: structure, localization and function of the carbohydrate. In: Finean-Michell (ed.) Membrane Structure. Elsevier.

18. Ladisch, S., Gilard, B., Wong, C., Ulsh, L. (1983) Shedding and immunoregulatory activity of YAC-1 lymphoma cell gangliosides. Cancer Res. 43, 3808–3813.

19. Ledeen, R. (1985) Gangliosides of the neuron. Trends in Neurosci. 8, 169–174.

20. Ledeen, R.W., Aquino, D.A., Sbaschnig-Agler, M., Gammon, C.M., Vaswani, K.K. (1987) Fundamentals of neuronal transport of gangliosides. Functional implications. In: Rahman, H. (ed.) Gangliosides and Modulation of Neuronal Functions. Springer-Verlag, Heidelberg.

21. Marchase, R.B. (1977) Biochemical investigations of retinotectal adhesive specificity. J. Cell Biol. 75, 237–257.

22. McDonough, J.P., Springer, W.R., Barondes, S.H. (1980) Species-specific cell cohesion in cellular slime molds. Exp. Cell Res. 125, 1–14.

23. Obata, K., Oide, M., Handa, S. (1977) Effects of glycolipids on in vitro development of neuromuscular junction. Nature 266, 369–371.

24. Overton, J. (1982) Inhibition of desmosome formation with tunicamycin and with lectin in corneal cell aggregates. Dev. Biol. 92, 66–72.

25. Pfenninger, K.H., Maylié-Pfenninger, M.-F., Friedman, L.B., Simkowitz, P. (1984) Lectin labeling of sprouting neurons. III. Type-specific glycoconjugates on growth cones of different origin. Dev. Biol. 106, 97–108.

26. Skipski, V.P., Katapodis, N., Prendergast, J.S., Stock, C.C. (1975) Gangliosides in blood serum from normal rats and morris hepatome 5132tc bearing rats. Biochem. Biophys. Res. Comm. 67, 1122–1127.

27. Smalheiser, N., Peterson, E.R., Crain, S.M. (1981) Specific neuritic pathways and arborizations formed by fetal mouse dorsal root ganglion cells within organized spinal cord explants in cultures: a peroxidase-labeling study. Dev. Brain Res. 2, 383–396.

28. Smith, C.L. (1983) The development and postnatal organization of primary afferent projections to the rat thoracic spinal cord. J. Comp. Neurol. 220, 29–43.

29. Stelzner, D.J. (1971) The normal postnatal development of synaptic end feet in the lumbosacral spinal cord and of responses in the hind limbs of the albino rat. Exp. Neurol. 31, 337–357.

30. Tettamanti, G., Sonnino, S., Ghidoni, R., Masserini, M., Venerando, B. (1985) Chemical and functional properties of gangliosides. Their possible implication in the mmebrane-mediated transfer of information. In: Degiorgio, V., Corti, M. (eds) Physics of Amphiphiles: Micelles, Vesicles and Microemulsions. Soc. Italiana di Fisica, Bologna.

31. Tettamanti, G., Ledeen, R.W., Sandhoff, K., Nagai, Y., Toffano, G. (1986) "Gangliosides and Neuronal Plasticity". Fidia Research Series, Vol. 6, Liviana Press, Padova, Italy.

32. Tolvanen, M., Gahmberg, C.G. (1986) In vitro attachment of mono- and oligosaccharides to surface glycoconjugates of intact cells. J. Biol. Chem. 261, 9546–9551.

33. Yu, R.K., Ledeen, R.W. (1972) Gangliosides of human, bovine and rabbit plasma. J. Lipid Res. 13, 680–686.

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THE NEURONAL BASES OF LOCOMOTION IN LAMPREY – IN VITRO STUDIES OF THE BRAINSTEM-SPINAL CORD

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Many basic vertebrate motor patterns have evolved gradually during phylogeny and basic features of their underlying neural organization appears to have been retained through evolution /19, 20, 23/. Although much knowledge can be gained from studies of higher vertebrates concerning the general neural organization controlling different patterns of behaviour, it has so far been impossible to deduce the cellular bases of mammalian behaviour. Lower vertebrate nervous systems contain fewer neurones and are more accessible to a cellular approach, particularly when they can be studied in vitro. The brainstem-spinal cord of the lamprey can be maintained in vitro for several days /33/ and the isolated nervous system can be made to generate the neural activity underlying different patterns of behaviour such as locomotion (cf/24/) and respiration /34/. The lamprey CNS has a basic vertebrate organization, even though the lamprey during phylogeny has been separated from other vertebrates for approximately 450 million years. With an aim to understand the neuronal organization underlying locomotion we have explored the lamprey brainstem-spinal cord preparation. We will briefly mention the different areas which have been investigated and give reference to direct the reader to the detailed studies.

The descending control from the brainstem is executed mainly by reticulospinal neurones originating in the mesencephalic and the anterior,

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middle and posterior rhombencephalic reticular nuclei. Large (Muller cells) and medium-sized reticulospinal neurones utilize excitatory amino acid transmission /7, 9/, whereas other medium-sized and small cells may contain 5-HT /5/, CCK- or PYY-like peptides /6, 7/. Stimulation near the middle and posterior reticular nuclei elicits locomotor activity in the spinal cord /30, 31/. Many reticulospinal neurones are rhythmically active during locomotion in phase with the ventral root bursting on the ipsilateral side /18, 28/.

The reticulospinal neurones may initiate locomotion by increasing the excitability in three types of spinal premotor interneurones: the excitatory premotor interneurone /11/, the CC interneurone (crossed axon) which inhibits contralateral interneurones, and the inhibitory lateral interneurone /8, 32/. The interaction between these interneurones can generate rhythmic burst activity, provided that all interneurones have a sufficiently high excitability level, making the individual neurones discharge when not actively inhibited. When the interneurones on one side are active the CC-interneurones will inhibit all neurones on the contralateral side. The activity on one side will be terminated by activity in the ipsilateral lateral interneurones, which inhibits the ipsilateral CC interneurones /11, 21, 24/. This leads to a disinhibition of the contralateral neurones and a subsequent discharge. When the network is modelled (18 neurones) (Grillner, Buchanan and Lansner, unpubl.) and the different types of neurones are given realistic properties (cf /29/) and experimentally established connections, the network model provides the appropriate pattern of activity in all types of neurones within the upper part of the normal frequency range (5–10 Hz). In the lower part bistable membrane properties induced by an activation of NMDA receptors most likely are important /2, 3, 4, 22, 35, 36, 37/.

The excitatory synaptic transmission is due to an activation of excitatory amino acid transmission, both with regard to reticulospinal projections /9/, spinal excitatory interneurones /11, 15/ and sensory dorsal cells /1/. The inhibitory premotor interneurones are glycinergic /8/ but there are also GABA-ergic neurones in the spinal cord (Brodin, et al., unpubl.). The 5-HT innervation of the spinal cord is derived from both descending, afferent and intrinsic spinal neurones. 5-HT neurones depresses the Ca dependent potassium conductance responsible for the after-hyperpolarization following the action potential in motoneurones and interneurones. A functional consequence of an activation of 5-HT neurones is that they may control the gain on the output side of the spinal cord /16, 17/.

Although the spinal cord can produce rhythmic alternating locomotor activity in isolation /14, 26, 38/, the spinal networks are normally affected by movement-related sensory signals which powerfully entrain the activity of the central pattern generating network /27/. Intraspinal stretch receptor neurones take part in this feedback control /25/. Activation of sensory touch and pressure neurones can also affect the swimming motor activity /10, 12, 13, 30/.

In conclusion. The available information of the lamprey CNS is sufficient to explain several aspects of the cellular bases of locomotion and how alternating burst activity is generated in a vertebrate nervous system.

REFERENCES

1. Brodin, L., Christenson, J. and Grillner, S. (1987) Single sensory neurons activate excitatory amino acid receptors in the lamprey spinal cord. Neurosci. Lett. 75, 75–79.

2. Brodin, L., Grillner, S. (1985) The role of putative excitatory amino acid neurotransmitters in the initiation of locomotion in the lamprey spinal cord. I. The effects of excitatory amino acid antagonists. Brain Research 360, 139–148.

3. Brodin, L., Grillner, S. (1985) The role of putative excitatory amino acid neurotransmitters in the initiation of locomotion in the lamprey spinal cord. II. The effects of amino acid uptake inhibitors. Brain Research 360, 149–158.

4. Brodin, L., Grillner, S. (1986) Effects of magnesium on fictive locomotion induced by activation of N-methyl-D-aspartate (NMDA) receptors in the lamprey spinal cord in vitro. Brain Research 380, 244–252.

5. Brodin, L., Buchanan, J.T., Hökfelt, T., Grillner, S., Verhofstad, A.A.J. (1986) A spinal projection of 5-hydroxytryptamine neurons in the lamprey brainstem; evidence from combined retrograde tracing and and immunohistochemistry. Neurosci. Lett. 67, 53–57.

6. Brodin, L., Buchanan, J.T., Hökfelt, T., Grillner, S., Rehfeld, J.F., Frey, P., Verhofstad, A.A.J., Dockray, G.J., Walsh, J.H. (1987) Immunohistochemical studies of cholecystokinin (CCK)-like peptides and their relation to 5-HT, CGRP and Bombesin immunoreactivities in the brainstem and spinal cord of lampreys. J. Comp. Neurol. (In press).

7. Brodin, L., Grillner, S., Dubuc, R., Ohta, Y., Kasicki, S., Hökfelt, T. (1987) Reticulospinal neurons in lamprey. Transmitters, synaptic interactions and their role during locomotion. Arch. Ital. Biol. (In press).

8. Buchanan, J.T. (1982) Identification of interneurons with contralateral caudal axons in the lamprey spinal cord: Synaptic interactions and morphology. J. Neurophysiol. 47, 961–975.

9. Buchanan, J.T., Brodin, L., Dale, N., Grillner, S. (1987) Reticulospinal neurones activate excitatory amino acid receptors. Brain Research 408, 321–325.

10. Buchanan, J.T., Cohen, A.H. (1982) Activities of identified interneurons, motoneurons, and muscle fibers during fictive swimming in the lamprey and effects of reticulospinal and dorsal cell stimulation. J. Neurophysiol. 47, 948–960.

11. Buchanan, J.T., Grillner, S. (1987) A new class of spinal premotor interneurones activates excitatory amino acid receptors during fictive locomotion in lamprey. Science (In press).

12. Christenson, J., Boman, A., Lagerbäck, P-Å., Grillner, S. (1987) The dorsal cell, one class of primary sensory neuron in the lamprey spinal cord. I. Touch, pressure but no nociception — a physiological study. Brain Research (In press).

13. Christenson, J., Lagerbäck, P-A., Grillner, S. (1987) The dorsal cell, one class of primary sensory neuron in the lamprey spinal cord. II. A light- and electron microscopical study. Brain Research (In press).

14. Cohen, A.H., Wallén, P. (1980) The neuronal correlate of locomotion in fish. "Fictive swimming" induced in an in vitro preparation of the lamprey spinal cord. Exp. Brain Res. 41, 11–18.

15. Dale, N., Grillner, S. (1986) Dual component synaptic potentials in the lamprey mediated by excitatory amino acid receptors. J. Neurosci. 6, 2653–2661.

16. van Dongen, P.A.M., Grillner, S., Hökfelt, T. (1986) 5-Hydroxytryptamine (serotonin) causes a reduction in the afterhyperpolarization following the action potential in lamprey motoneurons and premotor interneurons. Brain Research, 366, 320–325.

17. van Dongen, P.A.M., Hökfelt, T., Grillner, S., Verhofstad, A.A.J., Steinbusch, H.W.M. (1985) Possible target neurons of 5-HT fibres in the lamprey spinal cord: Immunohistochemistry combined with intracellular staining with Lucifer yellow. J. Comp. Neurol. 234, 523–535.

18. Dubuc, R., Grillner, S. (1987) The activity of reticulospinal neurones during fictive locomotion in the lamprey: The contribution of spinal locomotor networks. Acta Physiol. Scand. (In press).

19. Grillner, S. (1981) Control of locomotion in bipeds, tetrapods and fish. In: Brooks, E.V. (ed.). Handbook of Physiology, Sect. 1. The Nervous System II. Motor Control. Waverly Press, Maryland, pp. 1179–1236.

20. Grillner, S. (1985) Neurobiological bases of rhythmic motor acts in vertebrates. Science 228, 143–149.

21. Grillner, S., Buchanan, J.T., Wallén, P., Brodin, L. (1987) Neural control of locomotion in lower vertebrates. From behavior to ionic mechanisms. In. Cohen, A.H., Rossignol, S., Grillner, S. (eds) Neural Control of Rhythmic Movements. John Wiley and Sons, New York.

22. Grillner, S., Wallén, P. (1985) The ionic mechanisms underlying NMDA receptor induced, TTX resistant membrane potential oscillations in lamprey neurons active during locomotion. Neurosci. Lett. 60, 289–294.

23. Grillner, S., Wallén, P. (1985) Central pattern generators for locomotion, with special reference to vertebrates. Ann. Rev. Neurosci. 8, 233–261.

24. Grillner, S., Wallén, P., Dale, N., Brodin, L., Buchanan, J., Hill, R. (1987) Transmitters, membrane properties and network circuitry in the control of locomotion in lamprey. Trends in Neurosci. 10, 34–41.

25. Grillner, S., Williams, T., Lagerbäck, P.-A. (1984) The edge cell, a possible intraspinal mechanoreceptor. Science 223, 500–503.

26. Grillner, S., McClellan, A., Sigvardt, K., Wallén, P., Wilén, M. (1981) Activation of NMDA-receptors elicits "fictive locomotion" in lamprey spinal cord in vitro. Acta Physiol. Scand. 113, 549–551.

27. Grillner, S., McClellan, A., Perret, C. (1981) Entrainment of the spinal pattern generators for swimming by mechano-sensitive elements in the lamprey spinal cord in vitro. Brain Research 217, 380–386.

28. Kasicki, S., Grillner, S. (1986) Muller cells and other reticulospinal neurones are physically active during fictive locomotion in the isolated nervous system of the lamprey. Neurosci. Lett. 69, 239–243.

29. Lansner, A. (1986) Investigations into the pattern processing capabilities of associative nets. Tech. Rep. TRITA-NA-8601, Dept. of Numerical Analysis and Computing Science, The Royal Institute of Technology, Stockholm, Sweden. (Thesis).

30. McClellan, A.D., Grillner, S. (1984) Activation of "fictive" swimming by electrical microstimulation of "locomotor command regions" in the brainstem of the lamprey. Brain Research 300, 352–362.

31. McClellan, A.D. (1986) Command systems for initiating locomotion in fish and amphibians: Parallels to initiation systems in mammals. In: Grillner, S., Stein, P.S.G., Stuart, D., Forssberg, H., Herman, R. (eds). Neurobiology of Vertebrate Locomotion. MacMillan Press, London.

32. Rovainen, C.M. (1974) Synaptic interactions of identified nerve cells in the spinal cord of the sea lamprey. J. Comp. Neurol. 154, 189–206.

33. Rovainen, C.M. (1979) Neurobiology of lampreys. Physiol. Rev. 59, 1007–1077.

34. Rovainen, C.M. (1985) Respiratory bursts at the midline of the rostral medulla of the lamprey. J. Comp. Physiol. 157, 303–309.

35. Sigvardt, K.A., Grillner, S., Wallén, P., van Dongen, P.A.M. (1985) Activation of NMDA receptors elicits fictive locomotion and bistable membrane properties in the lamprey spinal cord. Brain Research 336, 390–395.

36. Wallén, P., Grillner, S. (1985) The effect of current passage on N-methyl-D-aspartate-induced, tetrodotoxin-resistant membrane potential oscillations in lamprey neurons active during locomotion. Neurosci. Lett. 56, 87–93.

37. Wallén, P., Grillner, S. (1987) N-methyl-D-aspartate receptor-induced, inherent oscillatory activity in neurons active during fictive locomotion in the lamprey. J. Neurosci. 7, 2745–2755.

38. Wallén, P., Williams, T.L. (1984) Fictive locomotion in the lamprey spinal cord in vitro compared with swimming in the intact and spinal animal. J. Physiol. 347, 225–239.

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SPINAL LOCOMOTION: A COMPARISON OF THE KINEMATICS AND THE ELECTROMYOGRAPHIC ACTIVITY IN THE SAME ANIMAL BEFORE AND AFTER SPINALIZATION

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To assess the recuperation of locomotor functions of the hindlimbs after spinal section at T13, EMGs and kinematics were analyzed in a chronically implanted adult cat before and up to 14 days after spinalization. At this time, the cat was capable of coordinated walking, plantar foot contact and weight support of the hindquarters for several step cycles. There was however a foot drag early in swing and the steps tended to be shorter. Most muscles showed similar timing characteristics although there were some changes in amplitude. It is concluded that the spinal cord can generate a detailed locomotor pattern in the hindlimbs even in the adult animal.

Keywords: Spinal cats, locomotion, electromyography, kinematics

INTRODUCTION

After spinalization at T 13, kittens can walk with the hindlimbs on a treadmill /4, 5/. Contrary to reports by others /3, 7/, it has been shown more recently that cats spinalized as adults can also walk if they are regularly and adequately trained /1, 6, 8, 9/. About three weeks post-spinalization, these animals can walk up to 1 m/s, with plantar foot placement and weight support of the hindquarters. Kinematic and electromyographic (EMG) analyses have shown that the walking pattern is within normal limits

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/1/ although the method of acute implantation of EMG wires did not permit a stringent comparison between the pre- and post-spinalization EMG pattern. We have made such a before versus after comparison in one chronically implanted cat and we report here the data obtained 14 days after spinalization when the cat just started to support its weight during walking.

MATERIALS AND METHODS

To record EMGs, up to 28 pairs of Teflon-insulated stainless steel wires were sewn into the bellies of selected extensor and flexor muscles of both hindlimbs. The wires were led subcutaneously to multi-pin connectors cemented to the skull of the animal. The gain of each recording channel was kept the same in each recording session before and after spinalization. EMGs were recorded on a 14 channel tape recorder and digitized at 1KHz on a PDP 11/34 computer. Using interactive software, the same muscles and the same number of walking cycles were selected for comparison before and after spinalization. In each recording session, the animal was also video-taped using a rotating shutter camera. The limb trajectories and the angular movements were reconstructed from the X and Y coordinates of light-reflecting spots placed on the hindlimb. The movements and the EMG data were synchronized by means of a frame-oriented time code (see /1, 2/ and /8/).

After several weeks of recording in intact conditions, the cat was spinalized under barbiturate anaesthesia by removing a segment of the spinal cord at T 13. Training on the treadmill was started rapidly a few days only after surgery. The present report deals with data obtained 14 days after spinalization when the animal just started to support its weight and is intended to show the locomotor capabilities of these animals at a very early stage of the recuperation process.

RESULTS AND DISCUSSION

Walking movements of the hindlimbs at 14 days were well-coordinated in this cat; there was a strict alternation between the two sides and the animal walked with a plantar foot contact and could largely support its weight. At a given speed of the treadmill, the overall step length was shorter and consequently the cadence was higher than in the prespinal condition. This was associated with a smaller total excursion of the hip and the knee. On the other hand, the amplitude of the ankle movement was clearly augmented and the yield during the second extensor phase was generally reduced. In the first part of swing, the foot tended to drag on the belt before being clearly lifted.

The observed changes in the EMG patterns can account for most of the kinematical defects. Although the timing of the onset of most extensor

muscles was similar before and after spinalization, there was however an important reduction of the amplitude and duration of hip and knee extensors which may account for the reduced step length. On the other hand, the ankle and digit extensors had a greater amplitude which may be related to the greater ankle excursion after spinalization. There was an increase in the overall amplitude of flexor muscles, especially at the hip joint. Hip flexors tended to start earlier and knee flexors later. This may account for the fact that the leg started to move forwards before knee flexion had actually lifted the foot above the treadmill belt (see foot dragging above).

These results strongly support the view that the locomotor pattern results from activity generated within the spinal cord even in adult animals. However, the loss of descending control leads to a number of deficits which are reflected in some aspects of the pattern. Most of these changes can be attributed to the well known imbalance between flexors and extensors whereby a predominance of flexor activity and a reduction of extensor activity is seen even after acute spinalization. The consequence of the latter is a reduction in weight support and a shorter step length. The result of the former, is a phase advancement of hip flexion over more distal joints so that the hip flexes before the foot is lifted above ground thus leading to a foot drag.

Some, if not all, of these defects may disappear after prolonged interactive training /1/. The animal can bear the weight of its hindquarters for several minutes of walking and the step length may increase to normal values for a given speed. The foot drag can be minimal at optimal speeds (0.4 – 0.6 m/s). Some noradrenergic /2, 8/ and serotonergic /8/ drugs may also be used to improve some aspects of the pattern such as weight support or foot drag.

REFERENCES

1. Barbeau, H., Rossignol, S. (1987) Recovery of locomotion after chronic spinalization in the adult cat, Brain Research, 412, 84–95.

2. Barbeau, H., Julien, C., Rossignol, S. (1987) The effects of clonidine and yohimbine on locomotion and cutaneous reflexes in the adult chronic spinal cat. Brain Research 437, 83–96.

3. Eidelberg, E., Story, J.L., Meyer, B.L., Nystel, J. (1980) Stepping by chronic spinal cats. Exp. Brain Res., 40, 614–630.

4. Forssberg,H., Grillner, S., Halbertsma, S. (1980) The locomotion of the low spinal cat. I. Coordination within a hindlimb, Acta Physiol. Scand., 108, 269–281.

5. Forssberg, H., Grillner, S., Halbertsma, S., Rossignol, S. (1980) The locomotion of the spinal cat. II. Interlimb coordination, Acta Physiol. Scand., 108, 283–295.

6. Lovely, R.G., Gregor, R.J., Roy, R.R., Edgerton, V.R. (1986) Effects of training on the recovery of full weight-bearing stepping in the adult spinal cat. Exp. Neurol., 92, 421–435.

7. Robinson, G.A., Goldberger, M.E. (1986) The development and recovery of motor function in spinal cats. I. The infant lesion. Exp. Brain. Res., 62, 373–386.

8. Rossignol, S., Barbeau, H., Julien, C. (1986) Locomotion of the adult chronic spinal cat and its modification by monoaminergic agonists and antagonists. In: Goldberger, M.E., Gorio A. and Murray M (eds), Development and Plasticity of the Mammalian Spinal Cord, Liviana Press, Padova, pp. 323–346.

9. Smith, J.L., Smith, A., Zernicke, R.F., Hog, M. (1982) Locomotion in exercised and non-exercised cats cordotomized at two or twelve weeks of age. Exp. Neurol., 16, 393–413.

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MOTOR MECHANISMS IN THE TURTLE SPINAL CORD

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We reveal the intrinsic motor capacity of the spinal cord by examining motor behaviours produced by spinal segments caudal to a complete transection of the spinal cord. The turtle spinal cord generates three forms of the scratch reflex in the absence of neural inputs from supraspinal structures. Each form exhibits a characteristic motor neuron discharge pattern. We test the ability of the spinal cord to generate organized motor patterns in the absence of movement-related sensory feedback by examining motor neuron discharge patterns in spinal preparations that are immobilized with a neuromuscular blocking agent. The motor neuron discharge pattern associated with each form is observed in the spinal immobilized preparation. Each of these motor patterns is therefore generated centrally within the spinal cord.

Keywords: Spinal cord, turtle, central pattern generators, scratch reflex, motor pattern

Motor mechanisms intrinsic to the vertebrate spinal cord can be revealed by examining the motor capacity of spinal segments caudal to a complete spinal cord transection. The motor patterns responsible for swimming, stepping, scratching, and the flexion reflex are generated by spinal segments in the absence of supraspinal input /7, 27–29/. A wide variety of vertebrates including lamprey, stingray, tadpole embryo, turtle, chick, and cat have been studied. We have utilized the turtle, Pseudemys scripta elegans, as our experimental animal. We have examined the swim /14,

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26, 33/, the scratch /4, 5, 13, 16–24, 27–35/, and the flexion reflex /4, 5, 21, 24, 36/.

The shell of the turtle /36/ is a bony structure that offers important experimental advantages. There are ten dorsal (= postcervical and presacral) vertebrae fused to the dorsal carapace. Removal of the shell along the dorsal midline of the carapace permits experimental access to these spinal segments. The shell is innervated with tactile receptors whose activation elicits a scratch reflex directed toward the receptive field of the sensory neurons. The dermal markings of the turtle shell in one individual are similar to that of other individuals of the same species; the experimenter therefore can compare results of stimulation of a given site among different preparations. In addition, the metabolic characteristics of the turtle offer advantages for physiological studies /12/. The turtle is both a diver and a hibernator; its nervous system can function under anaerobic conditions. This feature is particularly useful for in vitro studies of the nervous system /3, 9–11, 13, 15/.

The spinal turtle with a complete transection of the spinal cord just posterior to the forelimb enlargement will respond to gentle tactile stimulation of specific sites on the shell or skin caudal to the level of the complete transection /17, 22, 34/ with a scratch reflex in which the ipsilateral hindlimb reaches toward and rubs against the stimulated site. We discriminate three different types or "forms" of the scratch reflex. For each form of the scratch, the turtle utilizes a distinct portion of the limb to rub against the site that has received the tactile stimulus. For the rostral scratch, the dorsum of the foot and toes are used; for the pocket scratch, the side of the thigh, knee, and/or calf are used; for the caudal scratch, the heel or the side of the foot is used. The receptive field for the rostral scratch lies mainly on the shell bridge, the piece of shell at the midbody level that joins the dorsal carapace with the ventral plastron. The receptive field for the pocket scratch lies in the pocket region just anterior to the hip and posterior to the shell bridge. The receptive field for the caudal scratch lies in the region posterior to the hip.

Each form of the scratch exhibits a characteristic movement strategy /17/. Common to all three forms is the rhythmic alternation between protraction of the hip and retraction of the hip. Distinct for each form is the timing of knee extension in the movement cycle of the hip. For the rostral scratch, knee extension occurs during the latter portion of hip protraction; for the pocket scratch, knee extension occurs during hip re-

traction; for the caudal scratch, knee extension occurs after hip retraction is completed. The pattern of electromyographic (EMG) activity recorded from hip protractor, hip retractor, and knee extensor muscles is distinct for each form of the scratch /22/. The timing of these muscles corresponds to the timing of the corresponding joint movements revealed during the movement analyses.

The motor pattern for each of the forms of the scratch is generated centrally within the spinal cord and is not dependent upon phasic sensory feedback from the hindlimb /22/. This is demonstrated in the spinal turtle by the technique of blocking the neuromuscular junctions with gallamine; this immobilizes the turtle's musculature and prevents movement-related sensory feedback. The electroneurographic (ENG) motor pattern is recorded from the specific nerves innervating the muscles previously utilized for the EMG studies. The ENG motor pattern recorded in the immobilized preparation in response to stimulation of a specific site on the body surface is an excellent replica of the EMG motor pattern recorded in the preparation with limb movement. This proves that the motor pattern for each of the three forms of the scratch is generated within the spinal cord. The spinal cord in the immobilized preparation selects the proper scratch form in response to stimulation of a site in a particular receptive field. Selection of an appropriate motor pattern is therefore a characteristic of the spinal cord.

Intracellular recordings from motor neurons innervating muscles of the hindlimb reveal that during each scratch cycle a motor neuron receives both synaptic excitation and synaptic inhibition. The synaptic excitation depolarizes the membrane and triggers action potentials; the synaptic inhibition maintains the membrane voltage at a subthreshold level and prevents the occurrence of action potentials /21, 23, 24, 35/. In each motor neuron during each scratch form, there is rhythmic alternation between synaptic excitation and synaptic inhibition. For certain motor neurons during some forms of the scratch, e.g., hip protractor motor neurons (VP-HP) and monoarticular knee extensor motor neurons (FT-KE) during the rostral scratch, there is combined synaptic excitation and inhibition that maintains the membrane voltage at a subthreshold level for a portion of the scratch cycle /21, 23–25/.

There are five segments of the spinal cord that contain motor neurons innervating hindlimb musculature: the three pre-sacral segments, D8, D9, and D10 and the two sacral segments S1 and S2 /25/. The dorsal roots of the D8 segment contain primary cutaneous afferents sensitive to mechanical

stimulation of the posterior ventral portion of the pocket scratch receptive field /16, 18, 19/. Three segments of the spinal cord, D8–D10, can produce a pocket scratch motor pattern in response to natural tactile stimulation of primary afferents entering the cord via the D8 dorsal roots /16, 18, 20/. Key elements of the central pattern generator for the scratch reflex are located in the anterior parts of the hindlimb enlargement. Similar observations have been made for stepping /8/ and scratching /1, 2, 6/ in the cat.

The immobilized turtle with a complete transection of the spinal cord will produce a flexion reflex motor pattern in response to a tactile stimulus applied to the dorsum of the foot /4, 5, 21, 24, 35/. The flexion reflex motor pattern is characterized by a robust activation of the hip protractors (flexors) and a potent inhibition of the knee extensors. In addition, tactile stimulation of the contralateral dorsum of the foot that elicits a flexion reflex in the contralateral hip protractors also suppresses ongoing activity in ipsilateral hip protractors /4, 5/. If stimulation is applied to either dorsum of the foot during an ongoing scratch motor behaviour, then the flexion reflex response will interrupt and may reset the scratch motor rhythm /4, 5/. Since the reset of the scratch rhythm occurs in the immobilized turtle, this implies that the flexion reflex neural circuitry interacts with the scratch motor pattern circuitry. One possible mechanism for this interaction may be shared neuronal elements in the flexion reflex pathways and scratch pathways.

The mechanisms underlying the neuronal generation of the scratch reflex in the spinal turtle are therefore partially understood. Further work will be required to understand completely the mechanisms responsible for the spinal generation of coordinated movements in the turtle.

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REFERENCES

1. Arshavsky, Y.I., Gelfand, I.M., Orlovsky, G.N. (1986) Cerebellum and Rhythmical Movements, Berlin, Springer-Verlag.

2. Arshavsky, Y.I., Gelfand, I.M., Orlovsky, G.N., Pavlova, G.A., Popova, L.B. (1984) Origin of signals conveyed by the ventral spino-cerebellar tract and spino-reticulo-cerebellar pathway, Exp. Brain Res. 54, 426–431.

3. Connors, B.W., Kriegstein, A.R. (1986) Cellular physiology of the turtle visual cortex: distinctive properties of pyramidal and stellate neurons, J. Neurosci. 6, 164–177.

4. Currie, S., Stein, P.S.G. (1987) Interaction of flexion and scratch reflexes in the turtle. Neuroscience 22, S653.

5. Currie, S., Stein, P.S.G. (1987) Interactions between cutaneous flexion reflex pathways and scratch central pattern generators in the turtle. Soc. Neurosci. Abstr. 13, 823.

6. Deliagina, T.G., Orlovsky, G.N., Pavlova, G.A. (1983) The capacity for generation of rhythmic oscillations is distributed in the lumbo-sacral spinal cord of the cat. Exp. Brain Res. 53, 81–90.

7. Grillner, S. (1981) Control of locomotion in bipeds, tetrapods, and fish, In: Brooks, V.B. (ed.) Handbook of Physiology, Sect. 1, The Nervous System, Vol. 2, Motor Control. Bethesda, Maryland, American Physiological Society, pp. 1179–1236.

8. Grillner, S., Zangger, P. (1979) On the central generation of locomotion in the low spinal cat. Exp. Brain Res. 34, 241–261.

9. Hounsgaard, J., Kiehn, O. (1985) Ca^{++} dependent bistability induced by serotonin in spinal motoneurons. Exp. Brain Res. 57, 422–425.

10. Hounsgaard, J., Kiehn, O., Mintz, I. (1988) Response properties of motoneurones in a slice preparation of the turtle spinal cord. J. Physiol. (Lond.) 398, 575–589.

11. Hounsgaard, J., Mintz, I. (1988) Calcium conductance and firing properties of spinal motoneurones in the turtle. J. Physiol. (Lond.) 398, 591–601.

12. Jackson, D.C. (1979) Respiration. In: Harless, M. Morlock, H. (eds) Turtles, Perspectives and Research, New York, John Wiley and Sons, pp. 165–191.

13. Keifer, J., Stein, P.S.G. (1983) In vitro motor program for the rostral scratch reflex generated by the turtle spinal cord. Brain Res. 266, 148–151.

14. Lennard, P.R., Stein, P.S.G. (1977) Swimming movements elicited by electrical stimulation of turtle spinal cord. I. Low-spinal and intact preparations. J. Neurophysiol. 40, 768–778.

15. Mori, K., Nowycky, M.C., Shepherd, G.M. (1981) Electrophysiological analysis of mitral cells in isolated turtle olfactory bulb. J. Physiol. (Lond.) 314, 281–294.

16. Mortin, L.I. (1987) Mechanisms of Sensorimotor Integration in the Spinal Cord: Three Forms of Scratch Reflex in the Turtle, Washington University in St. Louis, Doctoral Thesis.

17. Mortin, L.I., Keifer, J., Stein, P.S.G. (1985) Three forms of the scratch reflex in the spinal turtle: movement analyses. J. Neurophysiol. 53, 1501–1516.

18. Mortin, L.I., Stein, P.S.G. (1985) Segmental properties of three forms of scratch reflex in the spinal turtle: localization of input dermatomes and central pattern generating elements. Soc. Neurosci. Abstr. 11, 1021.

19. Mortin, L.I., Stein, P.S.G. (1989) Segmental cutaneous dermatomes for initiating three forms of the scratch reflex in the spinal turtle. (Submitted for publication)

20. Mortin, L.I., Stein, P.S.G. (1989) Spinal segments containing key elements of the central pattern generators for three forms of the turtle scratch reflex. J. Neurosci. (In press).

21. Robertson, G.A. (1986) Synaptic control of motor neurons in the production of three forms of the fictive scratch in the turtle. Washington University in St. Louis, Doctoral Thesis.

22. Robertson, G.A., Mortin, L.I., Keifer, J. Stain, P.S.G. (1985) Three forms of the scratch reflex in the spinal turtle: central generation of motor patterns. J. Neurophysiol. 53, 1517–1534.

23. Robertson, G.A., Stein, P.S.G. (1985) Integration of excitatory and inhibitory synaptic drive in motor neurons during production of the fictive scratch in the turtle. Soc. Neurosci. (Abstr.) 11, 477.

24. Robertson, G.A., Stein, P.S.G. (1988) Synaptic control of hindlimb motoneurones during three forms of the fictive scratch reflex in the turtle. J. Physiol. (Lond.) 404, 101–128.

25. Ruigrok, T.J.H., Crowe, A. (1984) The organization of motoneurons in the turtle lumbar spinal cord. J. Comp. Neurol. 228, 24–37.

26. Stein, P.S.G. (1978) Swimming movements elicited by electrical stimulation of the turtle spinal cord: the high spinal preparation. J. Comp. Physiol. 124, 203–210.

27. Stein, P.S.G. (1983) The vertebrate scratch reflex. Symp. Soc. Exp. Biol. 37, 383–403.

28. Stein, P.S.G. (1984) Central pattern generators in the spinal cord. In: Davidoff, R.A. (ed.) Handbook of the Spinal Cord, Vols 2 and 3. Anatomy and Physiology, New York, Marcel Dekker, pp. 647–672.

29. Stein, P.S.G. (1985) Neural control of the vertebrate limb: multipartite pattern generators in the spinal cord. In: Strumwasser, F. and Cohen, M. (eds), Comparative Neurobiology: Modes of Communication in the Nervous System. New York, John Wiley and Sons, pp. 245–253.

30. Stein, P.S.G. (1987) Scratch reflex, In: Adelman, G. (ed.), Encyclopedia of Neuroscience. Boston, Birkhauser, p. 1077–1078.

31. Stein, P.S.G., Camp, A.W., Robertson, G.A., Mortin, L.I. (1986) Blends of rostral and caudal scratch reflex motor patterns elicited by simultaneous stimulation of two sites in the spinal turtle. J. Neurosci. 6, 2259–2266.

32. Stein, P.S.G., Grossman, M.L. (1980) Central program for scratch reflex in turtle. J. Comp. Physiol. 140, 287–294.

33. Stein, P.S.g., Johnstone, K.M. (1986) Motor patterns during rostral scratching and forward swimming in the turtle. Soc. Neurosci. (Abstr.) 12, 790.

34. Stein, P.S.G., Mortin, L.I., Robertson, G.A. (1986) The forms of a task and their blends, In: Grillner, S., Stein, P.S.G., Stuart, D.G., Forssberg, H., Herman, R.M. (eds), Neurobiology of Vertebrate Locomotion. Hampshire, England, Macmillan Press, pp. 201–216.

35. Stein, P.S.G., Robertson, G.A., Keifer, J., Grossman, M.L., Berenbeim, J.A., Lennard, P.R. (1982) Motor neuron synaptic potentials during fictive scratch reflex in turtle, J. Comp. Physiol. 146, 401–409.

36. Zangerl, R. (1969) The turtle shell, In: Gans, C. (ed.), Biology of the Reptilia. Vol. 1. New York, Academic Press, pp. 311–339.

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RETICULOSPINAL NEURONS, LOCOMOTOR CONTROL AND THE DEVELOPMENT OF TAILSWIMMING IN XENOPUS

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The development of early swimming in Xenopus occurs early during the embryonic period and within a few hours. Between stages 25 and 33 the central nervous system reaches a state of 'critical mass' at which the for swimming necessary body structures have partly developed, thus enabling the embryo to move through the water. The pattern of undulatory body movements is formed within the pattern generators in the central nervous system (CNS) involving different types of neurons in the spinal cord and brainstem. Tailswimming in Xenopus embryos can be evoked by tactile stimuli, light or vibrations. Here the development of brainstem-spinal connections and their possible role in swimming caused by external stimuli will be discussed. It is now clear that reticulospinal neurons are among the first neurons that differentiate within the CNS, their axons enter the spinal cord when the first swimming movements occur, that they are active in a motoneuron-like fashion, during — and involved in the control of early tailswimming. Among the reticulospinal neurons only the Mauthner cell seems to serve a command function.

Keywords: Amphibian development, tailswimming, locomotor control, reticulospinal neuron, Mauthner cell

INTRODUCTION

An important premise in the study of animal locomotion is that rhythmic muscle activity during locomotion originates from the central

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nervous system (CNS). This rhythmic muscle activity is supposed to reflect the central neural activity which must therefore also be rhythmic (for reviews see /9, 20/). The vertebrate spinal cord and brainstem contain neuronal circuitry capable of generating neural activity necessary for coordinated tail or body movements during swimming. Rhythmic motor activity is generated in so-called central pattern- or rhythm generators (for review see /29/).

Within the neural network that controls locomotor behaviour two compartments can be distinguished: 1) the central rhythm generators which produce the rhythmic motor activity, and 2) the 'command' system which initiates locomotion by activation of the rhythm generators /14/. The central locomotor rhythm generators, assumingly segmentally repeating small neuronal networks of still largely unknown configuration, can be activated or modulated by so-called command neurons. The coordination among the rhythm generators can be produced by centrally derived signals, sensory derived signals, or both /27, 28/. Command neurons have been defined by Wiersma and Ikeda /40/ as a class of neurons which when stimulated will produce a complex stereotyped behaviour like locomotion (for review see /6/). In higher vertebrates no cases are known in which stimulation of a single neuron does result in locomotor behaviour. However, in lower vertebrates like fish and amphibia examples of command neurons have been found, e.g. a single Mauthner or Müller cell will, when stimulated, produce a coordinated motor output /5, 22/. So in the lamprey some of the large reticulospinal neurons like the Müller cells which were recently described by Kasicki and Grillner /12/ might 'fit' the description of locomotor command neurons. Single interneurons can elicit locomotor behaviour but it has been suggested that rather than a single neuron a small collection of command neurons is activated by sensory input during natural locomotor behaviour (for review see /28/).

Although it has been shown in extenso that the neural circuitry for the basic swimming rhythm generation is present within the spinal cord, less is known about the role the brainstem plays during swimming. Clearly the embryonic central nervous system (CNS) in Xenopus provides a good model to study the concurrent development of brainstem-spinal connections, the spinal rhythm generators and tailswimming.

Here we will review what we know now on the development of the myotomal musculature and tailswimming combined with a few observations on the concurring development of descending projections from the brainstem to

the spinal cord of Xenopus. Furthermore we will discuss evidence for a possible role of reticulospinal neurons in the control of early swimming.

TAILSWIMMING, MYOTOMES AND PRIMARY MOTONEURONS

Undulatory body movements are assumed to be a primitive or simple way of locomoting in vertebrates, occurring most frequently in lower vertebrates. In Xenopus embryos and larvae swimming is indeed accomplished by lateral undulating alternate movements of the axial body musculature comprising two components: 1) a side to side alternation of body flexions, and 2) a head to tail progression of waves of bending /10, 23, 32/.

Swimming behaviour can be evoked by several types of external stimuli. Identation of the skin can cause swimming episodes /10, 11, 17/. Roberts and co-workers /3/ have shown that mechanical stimulation of the skin activates Rohon-Beard cells by way of their free nerve endings in the skin, and that single Rohon-Beard cells upon electrical stimulation will initiate swimming. Light, or rather the sudden absence of light is known to be detected by the pineal eye /8/. This diencephalic photoreceptive structure is able to trigger a swimming episode when a sudden drop in the light intensity occurs. When Xenopus larvae are exposed to unexpected vibrations of appropriate frequency /35/ they will show a remarkable startle response and swim away from the stimulus with a powerful tail-flip.

In Xenopus the development of tailswimming starts early during the embryonic period (Fig. 1) around stage 22 (6-8 myotomes present) with the occurrence of brief random contractions in the rostral myotomes. Between stages 24 and 33, a period of some 18 h, the number of myotomes increases from 15 to 30, the first muscle contractions rapidly develop into more rhythmic movements. Simultaneous extra- and intracellular recordings from ventral roots between the myotomes and primary motoneurons within the spinal cord have shown that the rhythmicity of the muscle contractions is also present in the activity of primary motoneurons /10, 25, 32, 33/. Intracellular labeling then enables morphological identification of the recorded neuron type (Fig. 4G). Thus extracellular ventral root recordings provide an accurate means of monitoring the activity of the central nervous system during swimming.

The first (extracellularly recorded) spontaneous ventral root activity occurs around stage 25 between the rostralmost myotomes. Short bursts can be voked by violent stimulation of the skin in the neck region.

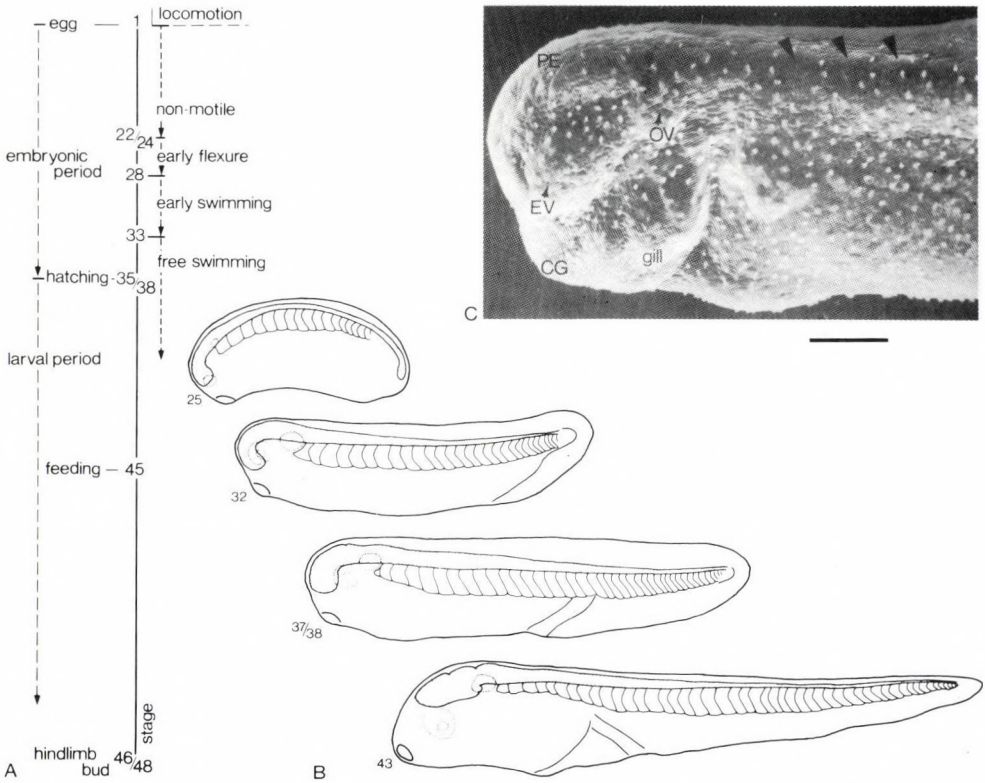


Fig. 1. The embryonic and early larval development of Xenopus. A) Tail-swimming starts early in the embryonic period, around stage 22/24. In the early flexure stage embryos bend their heads upon skin stimulation, during the early swimming stage the first short swimming episodes occur, while in the free swimming stage the embryos move freely through the water. B) The development of the myotomal muscles starts around stage 17 /15, 30/ caudally of the otic vesicle; myotomes are segregated in rostrocaudal direction along both sides of the neural tube. Between stages 25 and 43 the number of myotomes increases from approximately 15 to 45. Part of the myotomal musculature has been removed to expose the underlying developing central nervous system (shaded). C) Scanning electron micrograph showing the rostral half of a stage 32 embryo. The otic- and the eye vesicle can be seen under the overlying skin. Arrowheads indicate the position of a few myotomes. CG, cement gland; EV, eye vesicle; OV, otic vesicle; PE, pineal eye. Scale bar= 100 μm for B and 300 μm for C

However, the first rhythmic activity does not appear before stage 28 and only after vigorous stimulation of the dorsal skin /32, 33/. The first spontaneous rhythmic swimming activity appears at stage 29/30. Between stages 29/30 and 33 the embryos swim frequently and become free swimming. During this period, starting at stage 30/31 the embryos become sensitive to light stimuli by way of the pineal eye and start to swim away in response to light dimming /8/. Although having an important direct excitatory effect on behaviour it is not considered to be very powerful since only 40–50% of the embryos respond in their most sensitive period stage (33 to 36).

After hatching, between stages 40 and 45, larvae become sensitive to vibrational stimuli and show a type of avoidance behaviour known as the startle reflex. Vibrational stimuli applied to a tank containing Xenopus larvae cause these larvae to make a fast start of the C-type /35/ during which the body curves away from the stimulus thereby taking the form of a 'C' before muscle contractions on the stimulus side of the body occur (see /7/). From stage 46 on almost 100% of the larvae responds to vibrational stimuli with a fast C-start. Tailswimming remains the only mode of locomoting through water until during metamorphosis the developing hindlimbs take over.

Embryonic tailswimming development is preceded by the formation of myotomes on both sides of the neural tube and spinal cord /30/. During this period many Rohon–Beard cells, primary motoneurons and interneurons differentiate within the spinal cord /13, 30/. Approximately 75% of the primary motoneurons that differentiate between stages 10 and 50 do so before stage 24, before the early flexure stage /32/. Innervation of the myotomal musculature starts before stage 22 as was shown by Blackshaw and Warner /1/ who observed the first endplate activity in the rostral myotomes of stage 22/23 embryos. Application of α-Bungarotoxin labeled with fluorescein shows that innervation of the myotomes progresses in caudal direction. The innervation is followed by the appearance of ventral root activity in the intermyotomal clefts (Table 1; /32/). Primary motoneurons mature in the lateral motor columns in rostrocaudal direction by extending an axon into the myotomes and gradually more dendrites into the spinal marginal zone (Fig. 4G, H, I; /38/). The primary motoneurons have long axons (Fig. 4F) and are usually situated 1–1.5 myotome rostral to the myotomes they innervate. Occasionally they innervate more than 4 myotomes /32/.

Table 1

Development of the myotomal musculature, its innervation and activation in Xenopus embryos and larvae. The values indicate the rounded average number of myotomes present at five different stages, how many of these were innervated, and how many showed ventral root activity (from /32/)

Stage	Formed myotomes	Innervated myotomes	Ventral root activity
17	-	-	-
25	16	5	3
32	27	19	16
37/38	40	31	25
43	45	39	36

GENERATION OF SWIMMING MOTOR PATTERNS

The generation of swimming motor patterns within the central nervous system involves different types of neurons (for review see /20, 21/). These different types of neurons are organized in a network which because of its capability of generating rhythmic motor activity has been called the central motor pattern generator. In 1911 Brown proposed a neural model for the control of alternating contraction of antagonistic muscles. The main feature of this model was the necessity of reciprocal inhibition between the two 'half-centres' during each contraction cycle (Fig. 2B). If one introduces that each half-centre feeds excitation back onto itself (from /21/) then Brown's model for the spinal motor pattern generator would be self-sustaining once triggered, needing no continuous background excitation. However, it cannot account for how the length of a swimming episode is determined or how swimming stops.

The embryonic spinal cord of Xenopus is capable of generating swimming motor patterns, even when separated from the rest of the CNS and in the absence of movement related sensory input (see /10, 24/). As soon as Xenopus embryos (stage 29/30 or older) start swimming transection of the spinal cord just behind the brain stem causes behavioral disorders (like the temporary disappearance of all movement; van Mier, unpublished). Roberts and Alford /18/ have recently shown that transection of the 1st to the 5th postotic CNS segment leads to progressive reduction in the swimming episode duration and the initial swimming fequency. They concluded from

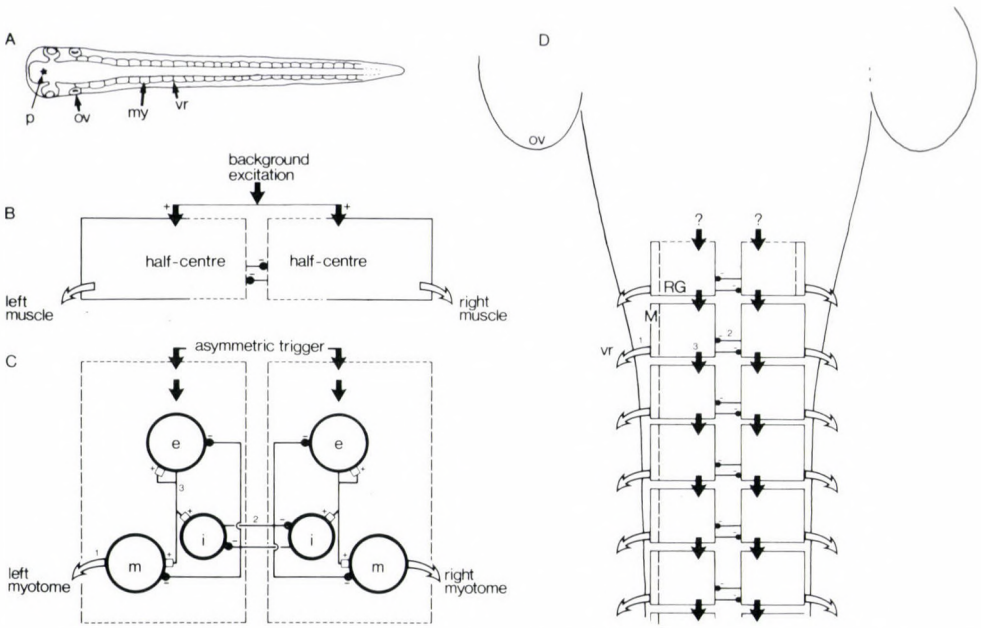


Fig. 2. Schematized possible reciprocal and longitudinal organization of the brainstem and spinal cord which are involved in the generation of swimming patterns. A) Dorsal view of a Xenopus embryo showing the longitudinal arrangement of myotomes along the spinal cord caudal to the otic veiscle (ov) and the position of the ventral roots (vr) between the myotomes (my). B) Schematized model of Brown's half-centre' reciprocal inhibition hypothesis (redrawn form /23/), and C) a modification of it which could account for the self-sustained rhythm generation in the Xenopus embryonic spinal cord (see /21/). e, excitatory descending interneuron; i, inhibitory commissural interneuron; m, motoneuron. D) Longitudinal arrangement of a number of pattern or rhythm generators (RG) showing the primary motoneurons (M) which innervate the myotomes (1), inhibitory connections which cross the spinal cord (2) and descending pathways (3) which can propagate the rhythmic motor activity along the cord after initiation by unknown (sensory?) input

their findings that tonic excitation during swimming must come from a population of descending interneurons which are phasically active during swimming. Midsagittal lesion of the brainstem and spinal cord also affects the central motor pattern. Kahn and Roberts /11/ could show in lesion experiments that both halves of the spinal cord, when separated, are still capable of generating their own swimming motor pattern but that these two programs are no longer 'linked' to each other.

These findings imply that longitudinal swimming patterning is still

possible through longitudinal connections (descending interneurons) while reciprocal connections in the spinal cord are responsible for the presize alternation of the motor patterns of the left and right half of the spinal cord.

DEVELOPMENT AND ROLE OF DESCENDING PROJECTIONS

The development of reticulospinal neurons starts around stage 10 during the early gastrulation period /13, 33/. /^{3}H/-thymidine autoradiography experiments demonstrated that the first large basal plate cells are 'born' between stages 10 and 13. More particularly, of the cells that differentiate between stages 10 and 50 in the brainstem reticular formation as much as 55% is born before stage 24, before the early felxure stage. In the interstitial nucleus of the medial longitudinal fascicle differentiation is even higher, approximately 70% of the cells differentiates before stage 24. The Mauthner cells differentiate between stages 10 and 12 /13, 33, 39/. Using the combination of /^{3}H/-thymidine application at early embryonic stages with the application of HRP at older stages to the rostral spinal cord revealed the presence of many double labeled cells in the brainstem (Fig. 4E), meaning that many of the early-born reticular neurons indeed do project to the spinal cord at later stages. The same series of experiments made it clear that later on many of these cells die during metamorphosis (van Mier et al., 1988).

In Xenopus embryos the first reticulospinal axons appear in the rostral spinal cord around stage 27/28 (Fig. 3; see /36/). HRP staining of axons that pass the rostral spinal cord show that the first ingrowing reticulospinal axons arise from cells, organized in two longitudinal columns, in the caudal and medial part of the brainstem /16, 36, 37/. So during the flexure stage (22/24–28) only cells from the caudal reticular formation project to the spinal cord. In the early swimming stage (28–33) axons in the rostral spinal cord also arise from more rostrally situated brainstem neurons like the Mauthner cells and mesencephalic reticulospinal neurons in the interstitial nucleus of the flm (Fig. 3). This does not change until stage 35/36 which means that at stage 33, when the embryos are quite capable of rhythmic swimming for periods up to 100 s /32/, only reticulospinal neurons in the caudal brainstem, the Mauthner cells and a few reticulospinal cells in the mesencephalon together with the developing spinal cord take part in the generation of tailswimming. After some time, at

stage 43, just before the onset of hindlimb bud formation, more brainstem neurons in the superior- and isthmic reticular nuclei and some cells in the vestibular nuclear complex start to project to the spinal cord (Fig. 3).

Application of HRP to the caudal brainstem of stages 28 to 43 embryos and larvae shows that axons which arise from cells in the brainstem grow quickly into the spinal cord during the development of early tail-swimming /37/. Similar observations concerning the growth rate of reticulospinal axons have been obtained previously using antibodies against serotonin which stained serotonergic raphespinal neurons in the brainstem and their descending axons in the spinal cord (Figs 3, 4A, B; /34/). At the end of the early flexure stage (stage 38) the first reticulospinal axons reach into the rostral spinal cord, down to the level of the 3rd myotome. At stages 30, 32, 37/38 and 43 the majority of ingrowing axons reach down to the level of respectively the 5th, 12th, 17th and the 24th myotome (average values). This ingrowth of reticulospinal axons keeps pace with the development of myotomes, their innervation and activation by primary motoneurons /37/.

Intracellular recordings in the caudal brainstem of curarized late Xenopus embryos revealed in the caudal brainstem a class of neurons which are phasically active during swimming /31, 37/, in a motoneuron-like fashion, firing one spike each swimming cycle (Fig. 5). Intracellular staining with Lucifer Yellow revealed that these cells (all with an ipsilateral descending axon) closely resemble reticulospinal neurons described by van Mier and ten Donkelaar /36; see also 16, 19, 34/. They show the same morphological features as descending interneurons in the spinal cord (Fig. 4D, mixed population of neurons with ipsilaterally descending spinal axons), which makes it difficult to determine whether or not these reticulospinal neurons and spinal descending interneurons /19/ form one homogeneous population /18, 30, 37/. However, their functional likeness with descending interneurons seems to favour the unification of both types of neurons into one functional population of descending interneurons.

Double labeling experiments in which HRP was applied to the caudal brainstem and the rostral myotomal musculature showed that synaptic contacts could be present between reticulospinal axons and primary motoneurons (Fig. 4I), which was confirmed by electron microscopical re examination of these contact sites /37/. However, at the current stage of our investigations nothing can be said about contacts between reticulospinal neurons and other spinal neurons. Findings of Dale and Roberts /4/ from paired intracellular

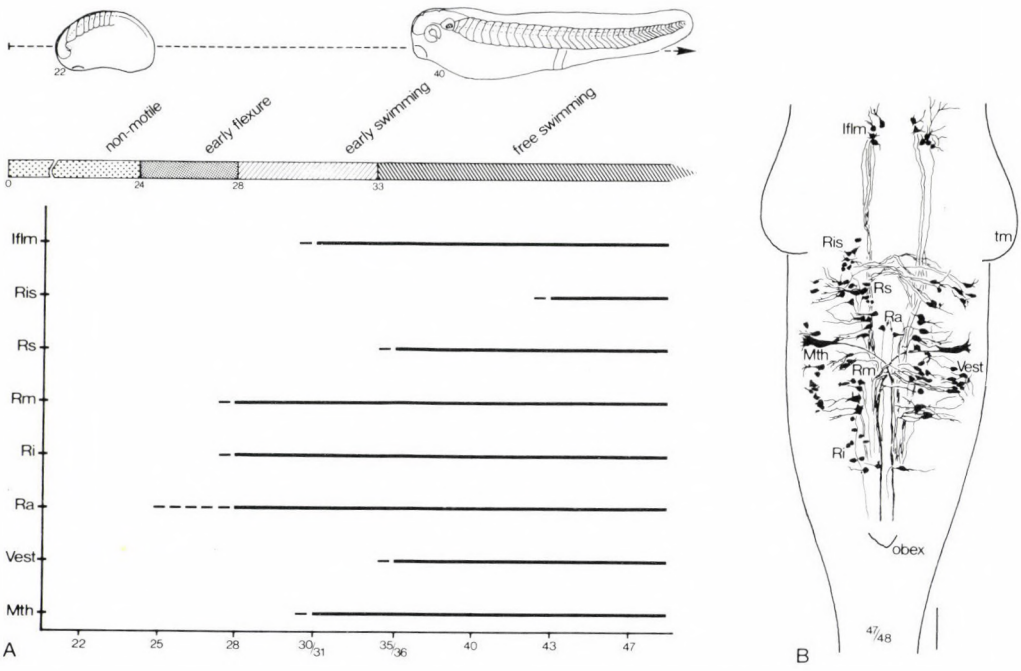
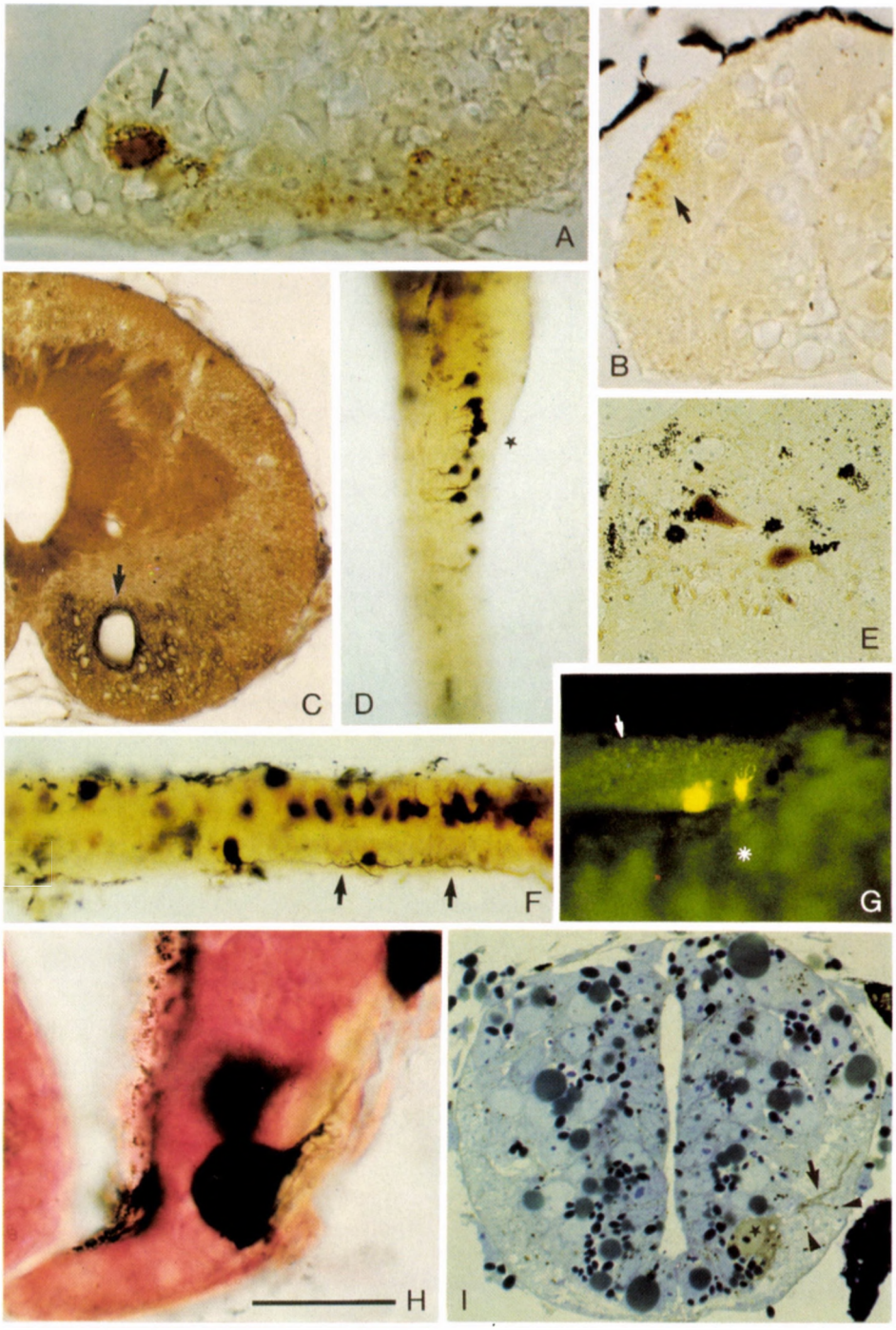


Fig. 3. A) Diagram illustrating the concurring development of descending supraspinal pathways and tailswimming. Lines indicate the different cell groups in the brainstem which where consistently labeled after HRP application to the rostral spinal cord. The presence of raphespinal projection was detected using antibodies against serotonin (see also Fig. 4A,B; from /34/). B) Distribution of HRP labeled neurons in the brainstem of a stage 47/48 larva (from /36/). Iflm, interstitial nucleus of the flm; Mth, Mauthner cell; Ra, nucleus raphes; Ri, Rm, Rs, Ris, nucleus reticularis inferior, -medius, -superior and -isthmi; tm, tectum mesencephali; Vest, vestibular nuclear complex. Bar = 100 µm

Fig. 4. A) The use of antibodies against serotonin enabled the location of raphe neurons before their axons reached the spinal cord (see also Fig. 3A; /34/). Raphespinal neuron close to the midline in a stage 39 embryo brainstem. B) Distribution of descending raphespinal axons in the spinal cord at stage 40. C) Transverse section of the larval spinal cord at stage 46 (courtesy of J. van der Linden), showing the ventral position of many large reticulospinal axons among which the Mauthner axon (arrow). D) HRP labeled descending interneurons and reticulospinal neurons on the border between spinal cord and brain stem, forming one continuous population? Asterisk indicates the obex. E) /^{3}H/-thymidine (grains) and HRP (brown precipitate) double-labeled reticulospinal neuron in the caudal brainstem of a stage 56/57 larva which received thymidine at stage 28 and HRP at stage 56/57. F) Two primary motoneurons with axons (arrows) which travel over considerable length along the spinal cord before leaving it. G) Intracellularly Lucifer Yellow labeled motoneurons photographed in vivo in a stage 40 embryo spinal cord (arrow). One with dorsal dendrites is partly visible above the musculature (asterisk). H) HRP labeled primary motoneuron in the ventral stage 33 spinal cord, extending its dendrites into the lateral marginal zone (white matter). I) Contacting primary motoneuron dendrites (arrow) and descending reticulospinal axons (arrowheads) in a stage 33 spinal cord visualized by applying HRP to the caudal brainstem and several myotomes. Bar=15 µm for I, 25 µm for H, 30 µm for A,B,E, 75 µm for C, 150 µm for F, and 200 µm for D and G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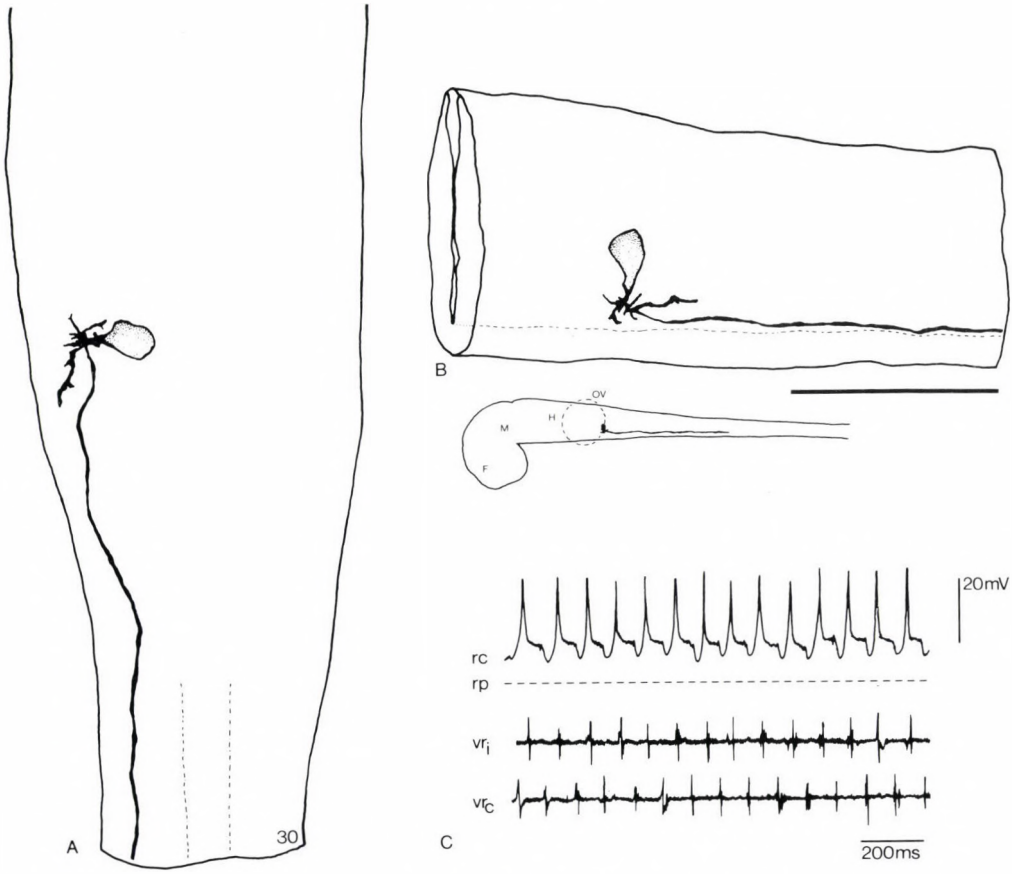

Fig. 5. Anatomy and physiology of an embryonic reticulospinal neuron (see also /37/). A) Reticulospinal neuron in the caudal brainstem of a stage 30 Xenopus embryo (ventral view), visualized by intracellular labeling with Lucifer Yellow. B) The neuron was situated in the hindbrain reticular formation close to the otic vesicle (OV), giving rise to a 590 µm long ipsilaterally descending axon. F, forebrain; H, hindbrain; M, midbrain. C) In response to electrical skin stimulation this neuron (resting potential = -68 mV) fired at an average frequency of 11 Hz, one spike each swimming cycle, in phase with the motor discharge of the ipsilateral 5th ventral root (bilateral ventral root recordings were made with suction electrodes). rc, reticulospinal cell; rp, resting potential; vr_i and vr_c, ipsilateral and contralateral ventral root

recordings from primary motoneurons and descending interneurons revealed the existence of excitatory amino-acid-dependent synapses onto motoneurons thereby confirming our findings.

Around stage 30 the contralaterally descending axons of the Mauthner cells have reached the rostral spinal cord /34/. At stage 37 these axons have passed the level of the 17th myotome /16/. However, it is not before stage 43 that the large Mauthner cells become active and a startle reflex appears /35/. Extracellular recordings in the brainstem showed that the larval Mauthner cell fires one spike after vibrational stimulation, which when conducted along its descending axon excites the contralateral primary motoneurons and thus initiates a fast C-shaped contraction of the contralateral body musculature. This even happens while larvae are already swimming.

RETICULOSPINAL NEURONS AND THE INITIATION OF SWIMMING

We know now that reticulospinal neurons and spinal descending interneurons are active during tailswimming in a motoneuron-like fashion firing one spike each swimming cycle /4, 37/. This and the fact that progressively more caudal lesions in brainstem and spinal cord lead to a decrease in the swimming episode duration /18/ suggest that reticulospinal neurons and descending interneurons play an excitatory role in sustaining swimming over time. We have some ideas about how reticulospinal neurons, after receiving for instance sensory information from the pineal eye or Rohon-Beard cells, could influence the spinal swimming pattern generators. A few of our ideas are illustrated diagrammatically in Fig. 6.

1) Touching of the skin causes the rostral myotomes in young stage 25 embryos to contract /17/. In older embryos (stage 28 on) skin stimulation evokes swimming, involving the Rohon-Beard cells /3/. The ascending axons of the Rohon-Beard cells and of dorsolateral interneurons (excited by Rohon-Beard cells; see /21/), which both project to the caudal brainstem may excite reticulospinal neurons in the brainstem. These will then excite primary motoneurons and other spinal neurons /14, 26, 37/, thus influencing the spinal swimming pattern generators.

2) Lowering the light causes a rise in the firing rate of the photoreceptors in the pineal eye /8/. From stage 32 on this results in brief swimming episodes. Axons emerging from the pineal eye have been shown to descend to the ventral caudal part of the mesencephalon where cells of the

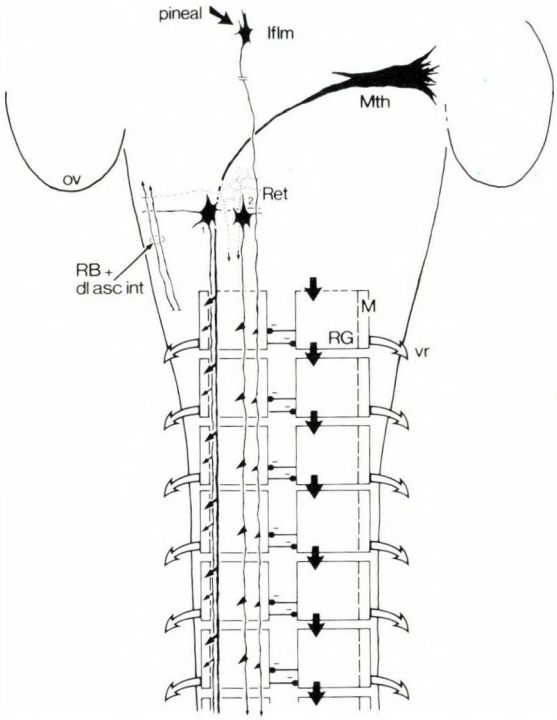

Fig. 6. Diagram showing possible neuronal pathways through which the different changing environment might evoke initiation of tailswimming in Xenopus embryos and larvae (for explanation see text). Arrows indicate synaptic contacts of descending axons throughout the spinal cord. Iflm, interstitial nucleus of the flm; dl asc.int., axons of dorsolateral ascending interneurons; M, primary motoneurons; Mth, Mauthner cell; ov, otic vesicle; RB ascending Rohon-Beard axons; Ret, reticulospinal neuron; RG, rhythm generator; vr, ventral root

interstitial nucleus of the flm are situated. Iflm neurons are, at that stage of development, the only mesencephalic neurons which project to the spinal cord /36/ and therefore the only pathway from the pineal eye to the spinal swimming pattern generators.

3) Unexpected vibrations in the surrounding water causes larvae (from stage 44 on) to startle (fast C-start). The Mauthner cells, which are active during the fast C-start /37/, make contact with the ventral side of primary motoneurons very close to the axon /38/. Thus they can exert a powerful excitatory influence on the contralateral motoneuron population and a gating function on any other excitatory input of the primary motoneurons (startle reflexes can be evoked during swimming; see /35/).

CONCLUSION

In this brief review we have focussed on the development of descending pathways in Xenopus embryos. The present evidence suggests that reticulospinal neurons in the caudal brainstem and mesencephalon are involved in tailswimming conveying sensory input which these cells receive from the Rohon–Beard cells or the pineal eye. During a swimming episode unexpected vibrational stimuli cause the Mauthner cells to overrule all rhythmic swimming and trigger a startle response. Among the reticulospinal neurons only the Mauthner cell can when active by itself generate a unique type of behaviour, and is therefore the only reticulospinal neuron that seems to fit the command neuron concept.

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REFERENCES

1. Blackshaw, S., Warner, A. (1976) Onset of acetylcholine sensitivity and endplate activity in developing myotome muscles of Xenopus. Nature 262, 217–218.
2. Brown, T.G. (1911) The intrinsic factor in the act of progression of the mammal. Proc. R. Soc. Lond. B 84, 308–319.
3. Clarke, J.D.W., Hayes, B.P., Hunt, S.P., Roberts, A. (1984) Sensory physiology, anatomy and immunohistochemistry of Rohon–Beard neurons in embryos of Xenopus laevis. J. Physiol. 348, 511–525.
4. Dale, N., Roberts, A. (1985) Dual-component amino-acid-mediated synaptic potentials: excitatory drive for swimming in Xenopus embryos. J. Physiol. (Lond.) 363, 35–59.
5. Diamond, J. (1971) The Mauthner cell. In: Hoar, W.S., Randall, D.J. (eds) Fish Physiology. Academic Press, New York, pp. 265–346.
6. Eaton, R.C., DiDomenico, R. (1985) Command and the neural causation of behavior: a theoretical analysis of the necessity and sufficiency paradigm. Brain Behav. Evol. 27, 132–164.
7. Eaton, R.C., Hackett, J.T. (1984) The role of the Mauthner cell in fast-starts involving escape in teleost fishes. In. Eaton, R.C., (ed.) Neural Mechanisms of Startle Behavior. Plenum Press, New York, pp. 213–266.

8. Foster, R.G., Roberts, A. (1982) The pineal eye in Xenopus laevis embryos and larvae: a photoreceptor with a direct excitatory effect on behavior. J. Comp. Physiol 145, 413–419.

9. Grillner, S. (1981) Control of locomotion in bipeds, tetrapods and fish. In. Brooks, V. (ed.) Handbook of Physiology – The Nervous System Am.Physiol. Soc., Bethesda, Sect. 1, Vol. 2, pp. 1179–1236.

10. Kahn, J.A., Roberts, A. (1982a) The central nervous origin of the swimming motor pattern in embryos of Xenopus laevis. J. Exp. Biol. 99, 185–196.

11. Kahn, J.A., Roberts, A. (1982b) Experiments on the central pattern generator in amphibian embryos. Phil. Trans. R. Soc.(Lond.) 296, 229–243.

12. Kasicki, S., Grillner, S. (1986) Müller cells and other reticulospinal neurons are phasically active during fictive locomotion in the isolated nervous system of the lamprey. Neurosci. Lett. 69, 239–243.

13. Lamborghini, J.E. (1980) Rohon–Beard cells and other large neurons in Xenopus embryos originate during gastrulation. J. Comp. Neurol. 189, 323–333.

14. McClellan, A.D. (1986) Command systems for initiating locomotion in fish and amphibians: parallels to initiation systems in mammals. In: Grillner, S., Herman, R.M., Stein, P.S.G., Stuart, D., Forssberg, H. (eds) Neurobiology of Vertebrate Locomotion. McMillan, London.

15. Nieuwkoop, P.D., Faber, J. (1976) Normal table of Xenopus laevis (Daudin). North Holland, Amsterdam.

16. Nordlander, R., Bader, S.T., Ryba, T. (1985) Development of early brainstem projections to the tail spinal cord of Xenopus. J. Comp. Neurol. 231, 519–529.

17. Roberts, A. (1971) The role of propagated skin impulses in the sensory system of young tadpoles. Z. Vergl. Physiol. 75, 388–401.

18. Roberts, A., Alford, S.T. (1986) Descending projections and excitation during fictive swimming in Xenopus embryos: neuroanatomy and lesion experiments. J. Comp. Neurol. 250, 253–261.

19. Roberts, A., Clarke, J.D.W. (1982) The neuroanatomy of an amphibian embryo spinal cord. Phil. Trans. R. Soc.(Lond.) B 296, 195–212.

20. Roberts, A., Soffe, S.R., Clarke, J.D.W., Dale, N. (1983) Initiation and control of swimming in amphibian embryos. In: Roberts, A., Roberts, B. (eds) Neural Origin of Rhythmic Movements. Cambridge University Press, Cambridge, pp. 261–284.

21. Roberts, A., Soffe, S.R., Dale, N. (1986)Spinal interneurons and swimming in frog embryos. In: Grillner, S., Stein, P.S.G., Stuart, D.G., Forssberg, H., Herman, R.M. (eds) Neurobiology of Vertebrate Locomotion. McMillan, London.

22. Rovainen, C.M. (1967) Physiological and anatomical studies on large neurons of the central nervous system of the sea lamprey (Petromyzon marinus). I. Müller and Mauthner cells. J. Neurophysiol. 30, 1000–1023.

23. Soffe, S.R. (1985) Central coordination of swimming in lower vertebrates. In: Bush, B.M.H., Clarac, F. (eds) Coordination of Motorbehavior. Cambridge University Press, Cambridge, pp. 141–162.

24. Soffe, S.R., Clarke, J.D.W., Roberts, A. (1983) Swimming and other centrally generated motor patterns in newt embryos. J. Comp. Physiol. 152, 535–544.

25. Soffe, S.R., Roberts, A. (1982a) Activity of myotomal motoneurons during fictive swimming in frogs. J. Neurophysiol. 48, 1274–1278.

26. Soffe, S.R., Roberts, A. (1982b) Tonic and phasic synaptic input to spinal cord motoneurons during fictive locomotion in frog embryos. J. Neurophysiol. 48, 1279–1288.

27. Stein, P.S.G. (1977) A comparative approach to the neural control of locomotion. In: Hoyle, G. (ed.) Identified Neurons and Behavior in Arthropods. Plenum Press, New York, pp. 227–239.

28. Stein, P.S.G. (1978) Motor systems, with special reference to the control of locomotion. Ann. Rev. Neurosci. 1, 61–81.

29. Stein, P.S.G. (1984) Central pattern generators in the spinal cord. In: Davidoff, R.A. (ed.) Handbook of the Spinal Cord, Vols 2 and 3: Anatomy and Physiology. Marcel Dekker, New York, pp. 647–672.

30. van Mier, P. (1986) The development of the motor system in the clawed toad, Xenopus laevis. PhD thesis, University of Nijmegen, Nijmegen.

31. van Mier, P. (1987) The development of early swimming in Xenopus embryos. In: Elsner, N., Creutzfeld, O. (eds) New Frontiers in Brain Research. Georg Thieme, Stuttgart, pp. 39.

32. van Mier, P., Armstrong, J., Roberts, A. (1988a) The development of early swimming in Xenopus laevis embryos: Myotomal musculature, its innervation and activation. (In preparation).

33. van Mier, P., Dederen, P.J.W.C., van Rheden, R., ten Donkelaar, H.J. (1988b) Neurogenesis in the brain stem and spinal cord of Xenopus laevis: the time of neuron origin revealed by /^{3}H/-thymidine. (In preparation).

34. van Mier, P., Joosten, H.W.J., van Rheden, R., ten Donkelaar, H.J. (1986) The development of serotonergic raphespinal projections in Xenopus laevis. Int. J. Dev. Neurosci. 4, 465–475.

35. van Mier, P., Stoop, R. (1988) The development of Startle behaviour in Xenopus laevis larvae. (In preparation).

36. van Mier, P., ten Donkelaar, H.J. (1984) Early development of descending pathways from the brain stem to the spinal cord in Xenopus laevis. Anat. Embryol. 170, 295–306.

37. van Mier, P., ten Donkelaar, H.J. (1988) Structural and functional properties of reticulospinal neurons in the early-swimming stage Xenopus embryo. J. Neurosci. (In press)

38. van Mier, P., van Rheden, R., ten Donkelaar, H.J. (1985) The development of the dendritic organization of primary and secondary motoneurons in the spinal cord of Xenopus laevis. Anat. Embryol. 172, 311–324.

39. Vargas-Lizardi, P., Lyser, K.M. (1974) Time of origin of Mauthner's neuron in Xenopus laevis embryos. Dev. Biol. 38, 220–228.

40. Wiersma, C.A.G., Ikeda, K. (1964) Interneurons commanding swimmeret movements in the crayfish, Procambarus clarkii (Girard). Comp. Biochem. Physiol. 12, 509–525.

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ROLE OF CELL DISPLACEMENT, CELL DIVISION, AND FRAGMENT SIZE IN PATTERN FORMATION DURING EMBRYONIC RETINAL REGENERATION IN XENOPUS

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Studies on the regenerating vertebrate retina provide, for the first time, a detailed view of how cells respond to removal of 2/3 of the eyebud, how their displacements, new juxtapositions, and extra mitotic activity correlate with stable changes in the retino-tectal projection. They provide insight into how retinal ganglion cells encode "position", and how they use this information to form specific neural connectivity patterns.

Keywords: Retina, regeneration, cell responses, Xenopus

How specific patterns of neural connections are generated is one of the central questions of neurobiology and certainly the focus of work done on the retino-tectal system /4/. One approach has been to examine changes in neural connectivity patterns resulting from perturbations of the embryonic Xenopus eyebud. In past Xenopus retinotectal studies, unpredicted neural projections to the midbrain optic tectum were obtained after manipulations such as removal of the nasal or temporal 2/3 of the eyebud. After healing and subsequent regeneration, the eye fragment innervated the midbrain optic tectum with 2 mirror-image, overlapping projections (pattern duplication) instead of one partial projection. In the following paper, I summarize work which shows how healing and related intercalary cell division are intimately linked to the pattern formation which underlies pattern duplication of neural projections.

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The presence of displaced cells during early healing correlates with the formation of pattern duplicated retino-tectal projections

After removal of 2/3 of the eye primordium at stage 32 /20/, the remaining fragment will heal and, within several days, restore an appropriate yet miniature retinal pattern /11/. From this point, many operated eyes undergo accelerated growth and attain the size of control eyes within several weeks post-surgery. Regeneration in the embryonic retina, then, consists of an early restoration of the anatomical retinal pattern followed by growth to restore the normal size of the eye.

In addition, during the growth phase and through metamorphosis, approximately an 8-week period, retinal ganglion cell axons grow into and innervate the midbrain optic tectum. In regenerating retinas, the pattern of visuotectal connections to the optic tectum correlates with the specific healing mode which occurred during the first 2 days after surgery /11/. One healing mode, termed "tongue formation" is characterized by partial rounding-up of the remaining eyebud fragment concurrent with cell emigration into the region of the ablation to form a supernumerary retina; the supernumerary retina appears similar to a "tongue" protruding from the "lips" of the closing fragment. The fragment and the "tongue" fuse to form a single eyeball over the first several days of healing. However, later, in the metamorphosed frog, the anterior and the posterior halves of the retina each map over the whole optic tectum as if each were a whole eye. The two projections appear as mirror images to one another, and are thus, termed pattern duplicated projections /5, 9, 10/. Another healing mode, in which the fragment simply "rounds-up" to close the wound, with no associated cell movements into the region of the ablation, in most cases, gives rise to unduplicated (often normal) visuotectal projections /11/.

To better understand the healing process at the cellular level, and to further characterize its relationship to visuotectal pattern formation, we next performed a gross anatomical and histological description and subcategorization of observed healing modes /13/. We showed that several "tongue" subcategories and several "rounded-up" subcategories form during the first 24 h of healing. We also characterized several subcategories which represent healing modes "intermediate" between the "rounded-up" healing mode and the "tongue" healing mode.

In the "rounded-up" healing mode, the cut edges of the fragment contract to close the wound; retinal cell type layers (pigmented retinal epithelium (pre), photoreceptors, interneurons, ganglion cells) and a lens

are present by 24 h post surgery. No extraneous or disorganized cells are present either internal or external to the fragment. Correlated electrophysiology experiments showed that rounded-up fragments formed pattern duplicated projections only 17% of the time /13/. In the "intermediate" healing mode, wound closure is not complete by 24 h post surgery and groups of disorganized cells are present in the fragment and amassed between the healing cut edges. "Intermediate" healing mode fragments formed pattern duplicated projections 72% of the time. In the "tongue" healing mode, an ectopic mass of cells, contiguous with the main body of the fragment, forms a supernumerary retina in the region of the ablation. At 24 h post surgery, the cells of the main body fragment form retinal layers the cells of the "tongue", excluding the presence of differentiated pre cells, remain undifferentiated, resembling ciliary margin. "Tongue" healing mode fragments (in nasal and temporal 1/3 sized fragments) formed pattern duplicated projections 100% of the time.

Thus, healing modes which establish the regenerating retinal structure, occur along a continuum, with apparent epithelial contraction more pronounced ("rounded-up" mode) at one extreme, and massive cell movements more prevalent at the other extreme ("tongue" mode). "Intermediate" mode fragments, which show both epithelial contraction and some cell movements fall between the two extremes. Duplicative pattern formation follows the presence of displaced cells in nasal and temporal fragments.

In addition, nasal fragment derived duplicated points appeared most often in the posterior region of the tectum, the normal site of innervation of the nasal retina. This differed significantly from temporal fragment derived duplicated points which appeared most often in the front of the tectum, the normal site of innervation by temporal retina /13/. The fact that nasal and temporal duplicated points were found most often in tectal areas appropriate for normal nasal and temporal innervation indicated that pattern duplication may involve local interactions among cells according to specific positional values remaining in the fragment during early healing and regeneration.

To determine if simple maturational factors (such as time of outgrowth of fibers) might account for duplicated projections, we crushed the optic nerves of frogs known to have duplicated projections, recorded no activity in the tectum immediately after nerve crush, and then recorded duplicated projections 7 months later /14/. After nerve crush, optic fibers re-innervated the tectum, all growing in at the same time, free of develop-

mental timing constraints. These data are compatible with the idea that retinal ganglion cells carry positional markers, which play a role in forming tectal connections. Taken together, the described studies confirmed and extended the correlation between healing mode and pattern formation /11/ and provided an anatomical paradigm for dissecting specific cell behaviour underlying pattern formation during regeneration.

Cell movements during early healing correlate with local cell division in retinal regions undergoing visuotectal pattern duplication

That both "intermediate" and "tongue" healing modes gave qualitatively similar duplicated patterns suggested that the duplication process might involve more than, e.g., a minimal explanation of cell movements and simple sorting-out of cells according to position /11/. To explore this possibility, we performed another study /12/, in which we first deleted large "tongues" which had formed during the first 24 h post surgery. We found that these fragments duplicated less often than fragments where the "tongue" was left intact, and that if an identifiable "tongue" reformed, duplication was restored. These studies confirmed that "tongue" formation during early wound healing is linked to pattern duplication.

We also injected healing embryos with tritiated thymidine and performed autoradiography at 60 h post surgery. We showed qualitatively that, in nasal 1/3 sized fragments, the naso-ventral retinal region (the site of displaced cells) contained more cells undergoing cell division /12/. To measure exactly where in this region cell division significantly exceeded control values, we used image analysis to measure tritiated thymidine labeling quantitatively /15/. The labeling indices for the front (ciliary margin) and mid-retinal regions were equivalent in operated and control eyes; however, operated eyes showed a statistically significant increase in thymidine labeling in the rear 1/3 of the eye, the retinal area showing the most cellular disorganization and clear presence of displaced cells.

We also measured thymidine labeling in "rounded-up" dorsal 1/3 sized fragments (dorsal 1/3 sized fragments do not form pattern duplicated projections /15/). These fragment types showed no significant increase in labeling over control eyes in any retinal region. A rare dorsal 1/3 "tongue" fragment, however, showed increased labeling in the ventral (tongue) region of the eye, even though these fragment types do not form duplicated projections (explained below).

We measured the mean volume ratio of operated eyes to control eyes for nasal 1/3 sized fragments vs. dorsal 1/3 sized fragments /15/. We found,

after 3 days of healing, that nasal fragments were considerably larger (compared to control eyes) than dorsal fragments. We believe this increase in volume was due to cell division and migration of cells.

Thus, nasal fragments showed increased thymidine labeling in healing regions, and pattern duplication of the projection. "Rounded-up" dorsal fragments showed no increase over control values in thymidine labeling, no pattern duplication of the projection, and were smaller than nasal fragments after 3 days of healing. These data suggested that displaced cells and correlated "extra" cell division in nasal fragments were tightly linked to pattern formation which brings about duplicated neural projections.

In a different approach, we recently showed that nasal 1/3 sized fragments made from fully differentiated retina (as late as stage 48) also regenerated to form pattern duplicated projections /22, 23/. Healing in these differentiated fragments also involved cell displacements, prominent in the naso-ventral retinal arc, similar to displacements in fragments made at stage 32. Again, the source of these displaced cells, and the dynamics of their growth are central to understanding healing and related pattern formation.

Finally, to determine the source of cells which give rise to the "tongue" which forms during early healing, whole eyes were reciprocally grafted between albino and pigmented embryos (Fig. 1A). Grafts were done at stage 28, before the optic stalk migrates to form the ventral retina. The grafts were allowed to heal until stage 34–35, at which time nasal 1/3 sized fragments were created (Fig. 1B). A day later (stage 42), external examination revealed a "tongue" of cells forming between the cut edges of the fragment (Fig. 1C, arrow). Compared to an albino control eye, stage 47 (Fig. 1D), the chimeric eye contained pigmented pre over about 1/2 its extent, and a large "tongue", made of unpigmented tissues and contiguous with the optic stalk. This result indicated that the "tongue" may be primarily composed of cells of ventral origin, probably derived from the optic stalk.

To this point, the best explanation regarding pattern duplication in the regenerating retina lies in a "cell movement: intercalary cell division hypothesis" /15/ which is a variation of the Polar Coordinate Model of French et al. /6/ and Bryant et al. /1/

In Fig. 2A, B, an N1/3 fragment is represented by a partial clockface containing values postulated to remain after surgery. Since grafting experiments and histology studies have shown that displaced cells in N1/3 fragments are, for the most part, derived from ventral retinal

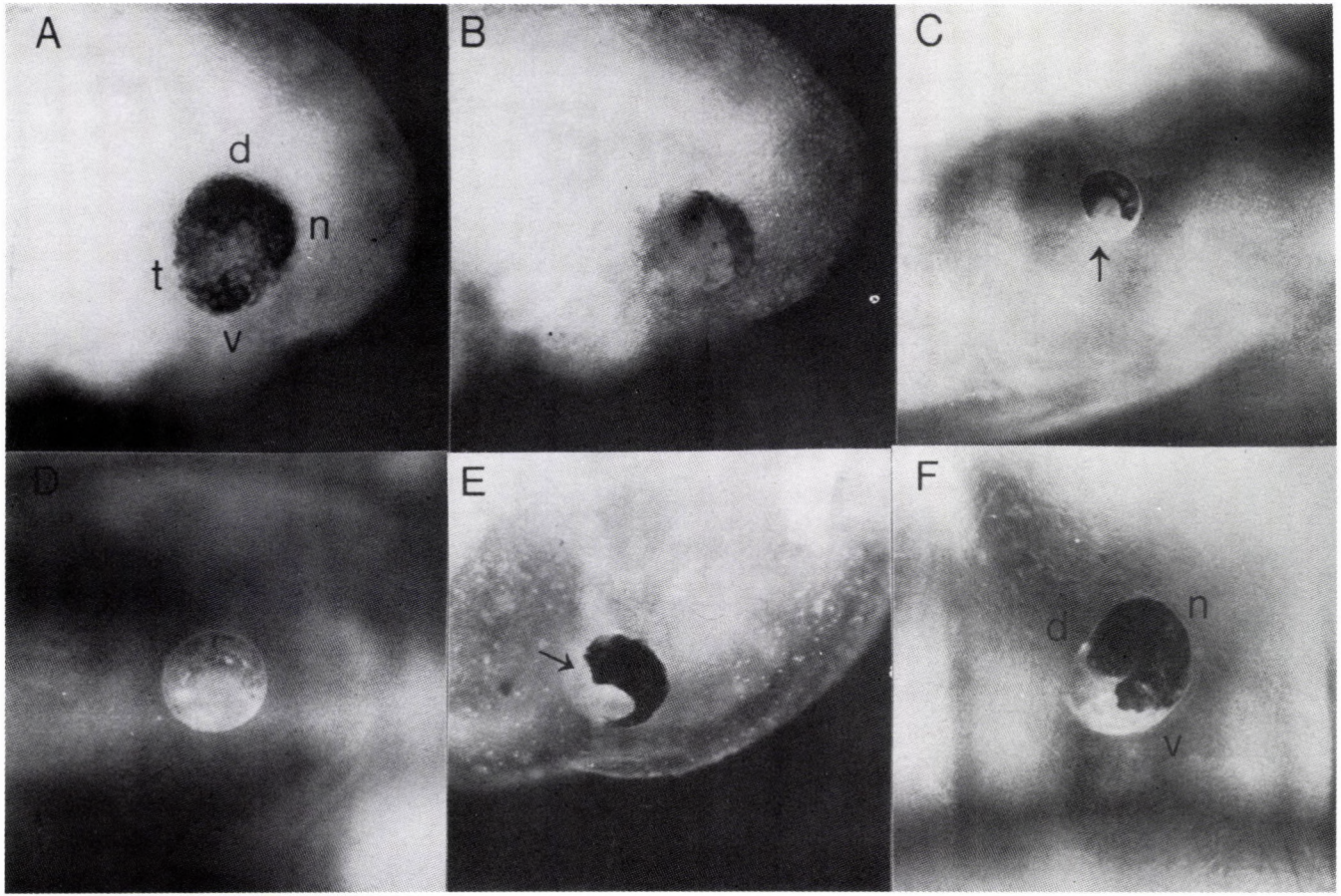
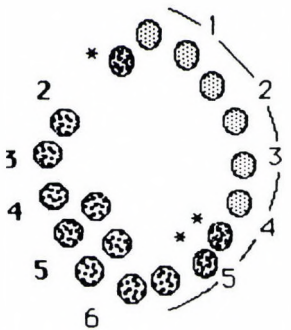


Fig. 1. A. Pigmented eye on albino host, stage 34. B. Nasal 1/3 sized fragment created at stage (34–35). C. A "tongue" (arrow) of displaced cells forms during early healing, stage 42. D. Albino control eye at stage 47. E. Dorsal view; arrow shows the "tongue"-optic stalk connection, stage 47. F. ventral view shows that the "tongue" forms the regenerating temporal half eye (d = dorsal, n = nasal, v = ventral, t = temporal)

tissues and the underlying optic stalk (derived from the ventrum; Holt, /8/), we assume that displaced cells appearing ventrally in the fragment have a clockface value of "6". In the simplest case, displaced cells with value "6" might interact with cells of value "1" to produce a fully mirror duplicated pattern of ganglion cell positional values. Intermediate values "2, 3, 4, 5" would be created via intercalary cell division, and the resulting retinal pattern would be "1, 2, 3, 4, 5, 6, 5, 4, 3, 2". If cells with value "6" interacted with cells of value "3", only values "4, 5", would be duplicated via intercalary growth. This would produce a partial duplicate pattern similar to those which have been reported /9, 11, 16/. In all cases intercalary growth would fill in values intermediate to values of interacting cells; the resulting ciliary margin of the new retina would contain both normal and duplicated values. These values, during subsequent normal retinal growth, would undergo "distal transformation". As each new retinal ring was added to the growing ciliary margin, each angular value on the clockface would be associated with a more distal radial value /17/.

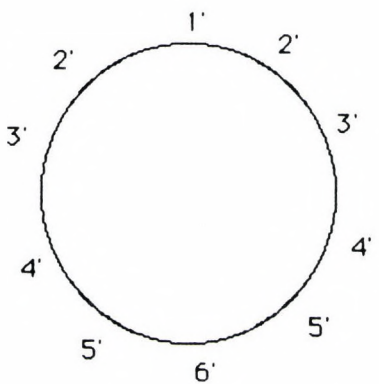
Dorsal fragments, on the other hand, in most cases do not show the presence of displaced cells during healing. We might assume that in the "rounded-up" healing mode, no cells interact to produce intercalary growth; hence no extra or local thymidine labeling is evident (Fig. 2C). Cells remaining in the fragment may undergo distal transformation, as they do during normal growth (Fig. 2D). The assumption that distal transformation can proceed without the presence of a full circle is an exception to the polar coordinate model as defined by French et al /6/ and Bryant et al /1/. In rounded-up fragments, only a partial set of distally transformed values are postulated to exist in the regenerated eye. This, however, would be difficult to detect because axons from a partial set of retinal ganglion cells sometimes expand over and innervate more than their normal tectal territories /18, 21/.

According to the polar coordinate model, as discussed above, the rare D1/3 fragment which shares the orbit with a "tongue" (assumed value of "6") would not form a duplicated projection. In N1/3 fragments, "6" juxtaposes with the only values present, those on the right hand side of the clock face and duplication via the shortest circle rule is predicted. In D1/3 fragments "6" would have opportunity to juxtapose with values on both the right and left hand sides of the clockface. Thus, a circle with all values would be regenerated. A full normal projection would be formed during

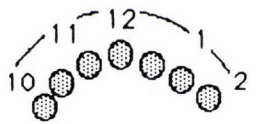


A.

N1/3 SHORTEST CIRCLE
DUPLICATION

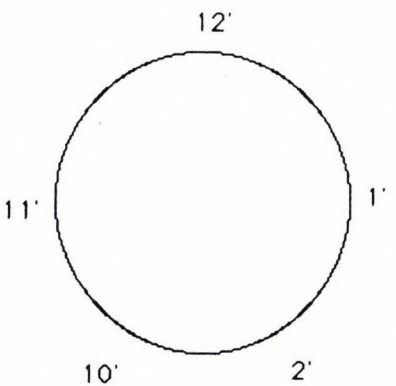


B.

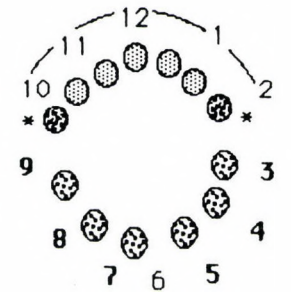


C.

D1/3 "ROUNDED-UP"
DISTAL TRANSFORMATION
ONLY

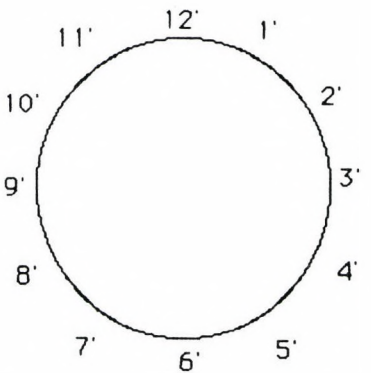


D.



E.

D1/3 "TONGUE"
FULL CIRCLE



F.

regeneration, and this projection would be distally transformed during subsequent growth (Fig. 2E, F).

The fact that D 1/3 fragments are, on the average smaller in volume than N 1/3 fragments, yet do not form duplicated projections, seems paradoxical in terms of the polar coordinate model which predicts that smaller fragments should duplicate more often than larger fragments. A simple explanation is that nasal fragments increase in size during healing, due to cel displacement from the ventrum (possibly from the optic stalk) and due to related increased cell division. "Rounded-up" dorsal fragments neither increase in volume due to cell displacement from the ventrum, nor do they show increased cell division. Thus, cell displacement during healing may be as important as fragment size in determining, in the regenerating retina at least, whether pattern duplication occurs.

In support of this hypothesis, specific healing interactions and local cell division have been shown to correlate with anatomical pattern duplication in a number of other systems (Drosophila imaginal discs; /2, 3/; amphibian limbs /7,19/, where the polar coordinate model remains the best explanation for duplication phenomena.

ACKNOWLEDGEMENTS

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Fig. 2. Possible interpretation using the polar coordinate model of how cell displacement: intercalary growth data might explain visuotectal pattern formation. A. In N1/3 fragments, displaced cells derived from the ventrum, and thus, of positional value "6", undergo intercalary cell division after interacting with cells of value "1" (dark stippling represents thymidine labeling). Most cells undergoing division to create positional values between "6" and "1" are displaced cells, but several cells near the cut edge of the fragment might also participate (dark stippled cells marked by *; duplicated values appear in boldface). B. After intercalary growth fills in a complete circle of angular positional values, the next generation of cells in the retinal ring are distally transformed by one radial value (1', 2', etc.). C. In rounded-up D1/3 fragments, healing does not juxtapose cells of differing positional value; no intercalary cell division occurs. D. The remaining cells in the D1/3 rounded-up fragment undergo distal transformation, i.e., the next ring of cells contains only a partial set of values. E. In D1/3 "tongue" fragments, displaced cells from the ventrum (of value "6") interact with cells in the fragment (of value "10" and value "2") to produce intercalary intermediates (dark stippling = thymidine labeling; cells marked by * = cells in the fragment also undergoing intercalalry cell division; intercalated values appear in boldface). F. After complete circle is filled by intercalary growth, distal transformation produces a full set of values in the next retinal ring

REFERENCES

1. Bryant, S.V., French, V., Bryant, P.J. (1981) Distal regeneration and symmetry. Science 212, 993–1002.

2. Dale, L., Bownes, M. (1980) Is regeneration in Drosophila the result of epimorphic regulation, Wilhelm Roux's Archiv. 189, 91–96.

3. Dale, L., Bownes, M. (1985) Pattern regulation in fragments of Drosophila wing discs which show variable wound healing. J. Embryol. 85, 95–109.

4. Easter, Jr., S.S., Purves, D., Rakic, P., Spitzer, N.C. (1985) The changing view of neural specificity. Science 230, 507–511.

5. Feldman, J.D., Gaze, R.M. (1975) The development of half-eyes in Xenopus tadpoles. J. Comp. Neur. 162, 13–22.

6. French, V., Bryant, P.J., Bryant, S.V. (1976) Pattern regulation in epimorphic fields. Science 193, 969–981.

7. Gardiner, D.M., Muneoka, K., Bryant, S.V. (1986) The migration of dermal cells during blastema formation in axolotls. Developmental Biology 118, 488–493.

8. Holt, C. (1980) Cell movements in Xenopus eye development. Nature 287, 850–852.

9. Ide, C.F., Kosofsky, B.E., Hunt, R.K. (1979) Control of pattern duplication in the retinotectal system of Xenopus: Suppression of duplication by eye-fragment interactions. Develop. Biol. 69, 337–360.

10. Ide, C.F., Fraser, S., Meyer, R. (1983) Dominance columns from an isogenic frog eye. Science 221, 293–295.

11. Ide, C.F., Reynolds, P., Tompkins, R. (1984) Two healing patterns correlate with different adult neural connectivity patterns in regenerating embryonic Xenopus retina. J. Exp. Zool. 230, 71–80.

12. Ide, C.F., Blankeneau, A., Morrow, J., Tompkins R. (1986) Cell movements correlate with novel cell division patterns during pattern formation in regenerating embryonic retina. In: Harold C. Slavkin (ed.), Progress in Clinical and Biological Research, Vol. 217B, Alan R. Liss, Inc.

13. Ide, C.F., Wunsh, L., Lecat, P., Kahn, D., Noelke, L. (1987a) Healing modes correlate with pattern formation in regenerating embryonic Xenopus retina. Develop. Biol., 124, 316–330.

14. Ide, C.F., Noelke, L., Nelson, P. (1987b) Regional asymmetries in the production of pattern duplicated projections during regeneration of Xenopus embryonic eye fragments. (Submitted).

15. Ide, C.F., Wunsh, L., VanDecar-Ide, A. (1987c) Local cell division during early healing in Xenopus eyebud fragments correlates with regeneration of a pattern duplicated visuotectal projection. (Submitted).

16. Ling, R.T.K., Ide, C.F., Hunt, R.K. (1979) Control of pattern duplication in the retinotectal system of Xenopus: Induction of duplication in eye fragments by secondary cuts. Dev. Biol. 69, 361–374.

17. MacDonald, N. (1975) In: Feldman, J.D. and Gaze, R.M. The development of half-eyes in Xenopus laevis tadpoles. J. Comp. Neurol. 162, 13–22.

18. Meyer, R.L. (1978) Deflection of selected optic fibers into a denervated tectum in goldfish. Brain Res. 155, 213–227.

19. Muneoka, K., Holler–Dinsmore, G.V., Bryant, S.V. (1986) Pattern discontinuity, polarity, and directional intercalation in axolotl limbs. J. Embryol. Exp. Morph. 93, 51–72.

20. Nieukoop, P.D., Faber, J. (1956) Normal table of Xenopus laevis (Daudin). North Holland Publishing Company, Amsterdam.

21. Schmidt, J.T., Easter, S.S. (1978) Independent biaxial reorganization of the retinotectal projections: A reassessment. Exp. Brain Res. 31, 155–162.

22. Wunsh, L., Ide, C.F. (1987a) Differentiated Xenopus retina undergoes regeneration and pattern duplication of the visuotectal projection. Soc. Neuroscience Abstracts.

23. Wunsh, L., Ide, C.F. (1987b) Fully differentiated Xenopus eye fragments regenerate to form pattern duplicated visuo-tectal projections. (Submitted).

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SPECIFICITY AND RETINOTECTAL PROJECTIONS OF QUARTER-EYE FRAGMENTS IN XENOPUS LAEVIS

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Three quarters of the eye anlage in Xenopus embryos of stage 33/34 were eliminated in three different sets of experiments. The remaining quadrant originated from the nasoventral part of the retina, from its ventral portion, or from the temporo-ventral area of the retina. All the fragments developed into small eyes of normal shape. The retinotectal connections did not deviate from those found in the control groups, even though mirror-image duplication was fairly frequent. For all fragments the tectal projection fields were rather limited. There was some indication of fragments retaining their original specificity. Irrespective, however, of their different origins, the optic projections always occupied the rostro-lateral area of the tectum.

Keywords: Xenopus, retinotectal projection, eye fragment, state of retinal specification

INTRODUCTION

In lower vertebrates, eye and tectum opticum initially develop independently. According to Sperry /6/, the retinal ganglion cells and tectum cells form complementary chemical markers which establish positional information and later guarantee precise retinotopic connections.

Based on the chemospecificity hypothesis it would have to be expected that the projections of fragmentary eyes will be restricted to the

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corresponding tectum areas only. However, investigations on compound and half eyes revealed that their projections covered the whole surface of the tectum /2, 4/. The explanation that the fragments respecified into normal eyes is fairly unlikely, because Gaze and Straznicky /5/ were able to demonstrate, at least for compound eyes, that these eyes keep their original specificity. As half eyes and compound eyes often develop into eyes of normal size, it cannot be excluded that the projection expands on account of the large quantity of ingrowing fibres.

In order to examine the latter explanatory possibility, we reduced the fragment size to such an extent that eyes of normal size could no longer be obtained.

At stage 33/34 we eliminated three quarters of the eye anlage. The remaining quarter originated from the naso-ventral (NV), ventral (V), or temporo-ventral (TV) part of the retina. Within one day the fragments rounded up and developed into normal-looking eyes. Their size, however, was only 25–75% the size of the normal eye (Degen and Brändle /1/).

SIZE AND LOCATION OF THE PROJECTION FIELD

We studied the optic projections of quarter-eyes at stage 60, shortly before metamorphosis, by means of electrophysiological mapping and cobalt filling.

The size of the projection field was considerably smaller than that of our control group. In case the quarter-eye fragments had respecified, the existence of tectal markers would have led us to expect an exapnsion of the projections over the whole tectal surface. If the fragments, however, would have kept their original speicificity, we could have predicted that the projections occupy different areas of the tectum, in accordance with the different origin of the optic fibres.

None of the above expectations was fulfilled. The projection always occupied the rostrolateral portion of the tectum, irrespective of the type of fragment (Fig. 1).

MAPPING OF THE PROJECTION FIELDS

Electrophysiological mapping of the projection fields resulted in consistently orderly patterns, but the maps were rarely normal (Fig. 2a). In many cases we obtained mirror-image duplicated projections (Fig. 2b), the

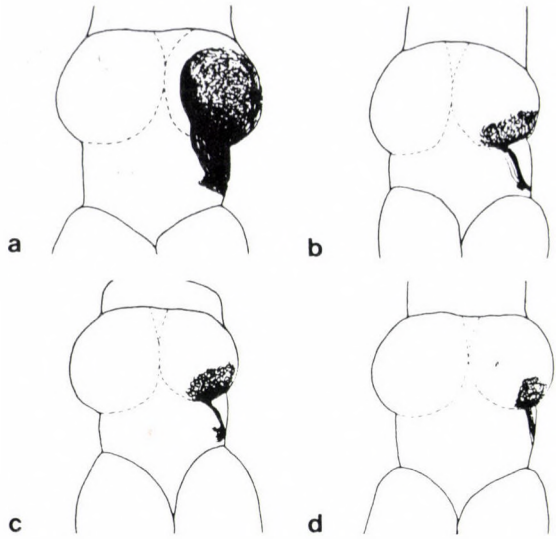

Fig. 1. Drawings of the optic projections marked by cobalt fillings. Top view of the tectal surface at stage 61, innervated by a) normal eye, b) temporo-ventral fragment, c) naso-ventral fragment, d) ventral fragment

duplicated portion was often restricted to a small cone of the visual field (Fig. 2c).

We are inclined to assume that duplicated projections develop in accordance with the shortest intercalar rule, a model suggested by French, Bryant and Bryant /3/.

THE PROBLEM OF SPECIFICITY OF QUARTER EYES

For interpretation of these findings, the state of specification of the fragments should be known. We found some indication that quarter eyes retain their original specificity:

Shortly before metamorphosis, quarter eyes of all fragment types vary considerably in size. This size was estimated by measuring the eye radius r and calculating the eye area $r^2 \times \pi$. Fibre countings in cobalt preparations indicated that the value of the eye area corresponded to the number of fibres in the eye stalk. When the eye area was considered in relation to the projection field, the result has been a linear correlation (Fig. 3a). A comparison of the slopes of the straight lines for different fragment types demonstrated that the increase of the projection area with

increasing eye size was significantly smaller for temporo-ventral eyes than for naso-ventral and ventral eyes ($p < 0.01$). In other words, for temporo-ventral eyes, the projection remains more concentrated in the rostro-lateral area of the tectum.

With respect to the various fragment types, we also studied the directions into which the projection fields expanded with incrasing size. For numerical description of the expansion direction of each projection field, we proceeded as follows:

First, the shape of the tectum was standardized by calculation. Then we determined a vector relative to the rostro-lateral corner of the tectum, for each individual recording location that yielded a positive response. By summation of the vectors, we obtained a mean vector, the angle of which was proportional to the shape of the field (Fig. 3b). A homogeneous expansion of the projection field in medial and caudal directions would not change the angle of the mean vector. However, any expansion in mainly caudal direction would result in a decrease of the angle towards 90 degrees.

A classification of the mean angles on the basis of fragment types revealed for naso-ventral and temporo-ventral eyes that again there were linear relationships between the size of the projection fields and the values of the angles (Fig. 3c). With increasing field size, the expansion in caudal direction was more pronounced than that in medial direction. For ventral eyes, however, the expansion in the caudal and medial directions was the same, and the angle did not change (Fig. 3d).

All these findings are in good agreement under the following assumptions: Quarter eyes retain their original speicificity. The optic fibres have directional preferences while trying to reach their normal areas of projection. In other words, the temporal fibres concentrate in a rostral area, the nasal fibres tend to grow into a more caudal area, and the ventral fibres into a more medial area of the tectum according to their specificity.

CONCLUSIONS

Our results concerning the projection of quarter-eyes can be interpreted as follows:

1) Quarter eyes seem to retain their original specificity. Their projections, however, never covered the appropriate area of the tectum. Therefore it is unlikely that tectal markers are involved in determining the

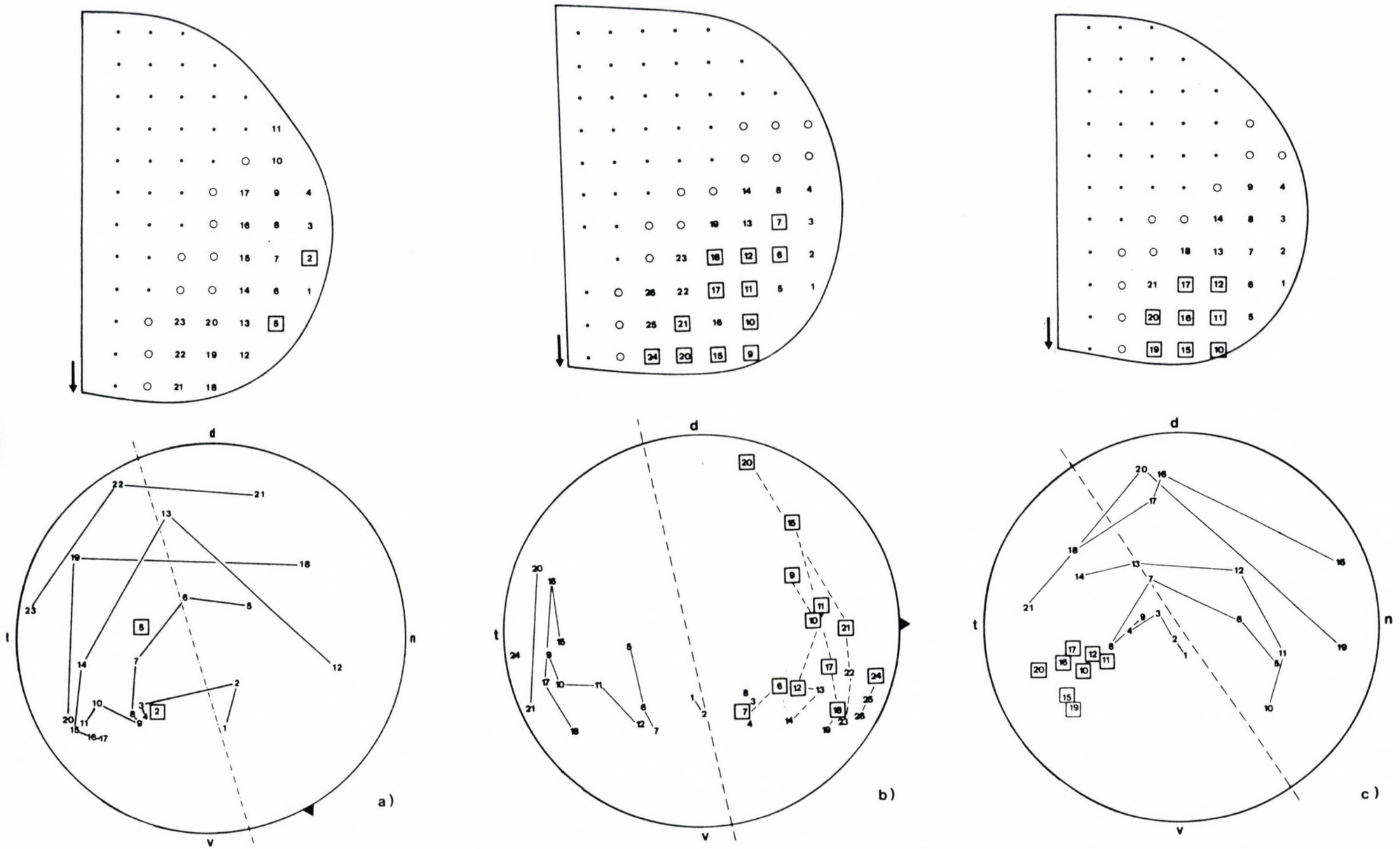

Fig. 2. Patterns of the retinotectal projections of quarter eyes, mapped electrophysiologically at stage 60. Top: left tectal surface with recording sites; the arrow points to the rostral pole. Bottom: right visual field, ▼ represents the position of the ventral fissure. • : no response. o : response only to stimulation with intermittently changing light. Numbers: single projections from the visual field. Numbers marked by squares: duplicated projections. Dotted line: principal plane of the duplicated projections. a) temporo-ventral fragment. Normal projection with two duplicated recording points. b) naso-ventral fragment. Mirror-image duplicated projection is restricted to a small cone of the visual field

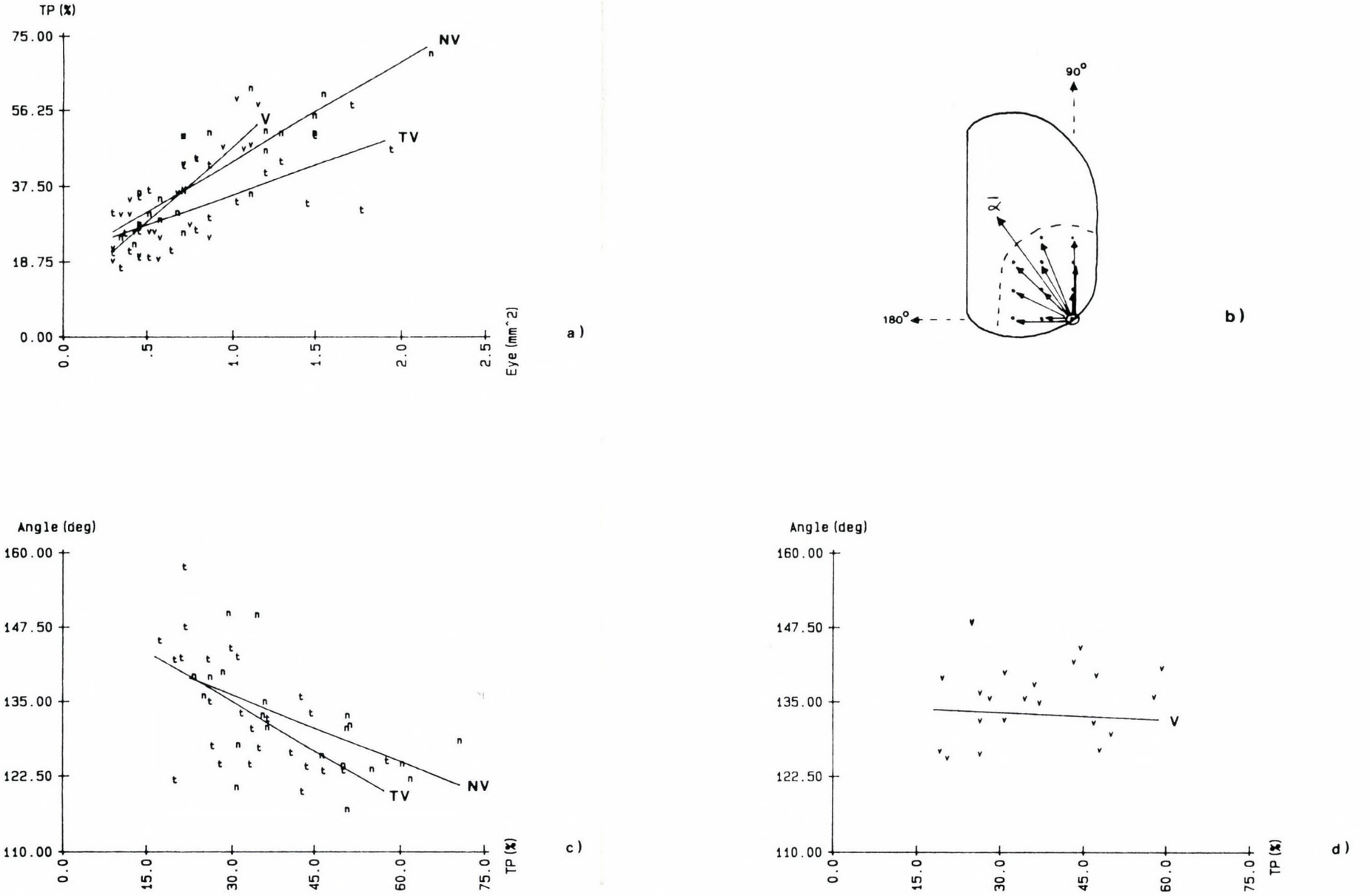

Fig. 3. a) Linear correlation between eye area and tectal projection area (TP). b) Determination of the mean vector angle (see text). c) Linear correlation between tectal projection area (TP) and mean vector angle for naso-ventral and temporo-ventral fragments. d) Linear correlation between tectal projection area and mean vector angle for ventral fragments.
NV,n = naso-ventral fragments, TV,t = temporo-ventral fragments, V,v = ventral fragments

location of the projection field during the first ingrowth of the optic fibres. This does not exclude, however, that the tectum provides general compass-like information , such as 'caudal' or 'medial' for orientation of the optic fibres.

2) Despite of the unusual location of the projection field, orderly maps were consistently formed. This indicates that the fibres separate in accordance with their specificity, which results in retinotopic patterns of projection. It is still unsettled whether this separation is brought about by independent fibre growth in their preferred directions, or by interaction of the fibres among themselves, or both.

If the process of sorting out is the result of interactions between the fibres, one would expect that shifting apparently ends when the fibres have reached a certain maximum distance from each other. This seems to occur earlier in the case of a minor fibre invasion, which would help to explain why the projection field of quarter eyes is restricted to the rostro-lateral part of the tectum. This would also provide indication that the fibres communicate with each other by means of signals that cover only short distances.

REFERENCES

1. Degen, N., Brändle, K. (1986) The retinotectal projection of quarter eyes in Xenopus laevis. Develop. Brain Res. 29, 141–143.

2. Feldman, J.D., Gaze, R.M. (1975) The development of half-eyes in Xenopus tadpoles. J. Comp. Neurol. 162, 13–22.

3. French, V., Bryant, P.J., Bryant, S.V. (1976) Pattern regulation in epimorphic fields. Science 193, 969–981.

4. Gaze, R.M., Jacobson, M., Székely, G. (1965) On the formation of connections by compound eyes in Xenopus. J. Physiol. 176, 409–417.

5. Gaze, R.M., Straznicky, C. (1980) Regeneration of optic nerve fibres from a compound eye to both tecta in Xenopus: evidence relating to the state of specification of the eye and the tectum. J. Embryol. Exp. Morph. 60, 125–140.

6. Sperry, R.W. (1963) Chemo-affinity in the orderly growth of nerve fibre patterns and connections. Proc. Natl. Acad. Sci. USA 50, 703–710.

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SYNAPTIC DEVELOPMENT OF REGENERATING RETINOTECTAL PROJECTION OF GOLDFISH

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The purpose of the present study is to examine the post-synaptic phenomena underlying the target finding process of re-generating retinal fibers. To perform this, retinotectal mapping was made using field potentials generated by small spots of light fixed in the visual field. The results show that regenerating retinal fibers first make functional but temporary, unspecific and diffuse synapses on the rostral tectum, disregarding the positions of their final target area, before making the sharpened and retinotopic pro-jections.

Keywords: Retinotectal projections, regeneration, post-synaptic potentials, goldfish

INTRODUCTION

The retinotectal visual system has been used to study how the growing nerve fiber finds its specific target. Many investigators have examined the retinotectal projection during optic nerve regeneration in normal or operated (half-retina, half-tectum etc.) animals as well as during development. The mapping technique used in these experiments was to record action potentials of optic fiber terminals and determine the recep-tive field. Since this technique is not able to detect any postsynaptic

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phenomenon, we developed a new mapping technique using field potentials. With this method, we estimated the position, size and topography of the area of functional synapses formed by regenerating retinal fibers coming from a small portion of the retina.

MATERIALS AND METHODS

Common goldfish (Carassius auratus) were used (10 cm in body length and 30–40 g in body weight). The left optic nerve was crushed under MS222 anesthesia. An experimental animal was anesthetized with MS222 and immobilized with gallamine triethiodide. The fish was kept in the air and the gills were perfused with water (20–23 ^{o}C). The optic tectum was exposed and the brain was covered with liquid paraffin.

Three LED lamps (1^{o} in visual angle) were positioned in the anterior, central and posterior visual fields, and they were sequentially illuminated every 5 s. The optic tectum was divided into a coordinate system having a unit interval of 200 μm. The field potential for each of the lamps was recorded on these grid points at a depth of 200 μm below the tectal surface, where the negative peak amplitude was maximum. Glass micropippets with the resistance of 2–5 M Ω were used for recording. As shown in Fig. 1, contour lines were drawn for each projection using the magnitude of the negative peak amplitude.

RESULTS AND DISCUSSION

To ascertain that the visually induced slow potential recorded from the optic tectum is postsynaptic in origin, Ringer solution containing 100 mM $MgCl_2$ was applied to the surface of the optic tectum. The potential disappeared after application of $MgCl_2$, but it recovered after washing.

Rostral, central and caudal projections of a normal fish (control) are shown in Fig. 1A. Experiments on regenerating projections were performed in the 2nd (n=3), 3rd (n=2), 4th (n=5), 6th (n=5), 10th (n=5) and 20th (n=2) weeks after nerve crush. No synaptic potential could be detected from any of the 2nd week group. In the 3rd week, synapses were formed only on the rostral part of the optic tectum independent of the final destination of the optic fibers (Fig. 1B). Fig. 1C shows an example of the 4th week group. The maps are still diffuse, but during the 4th week the projections began to

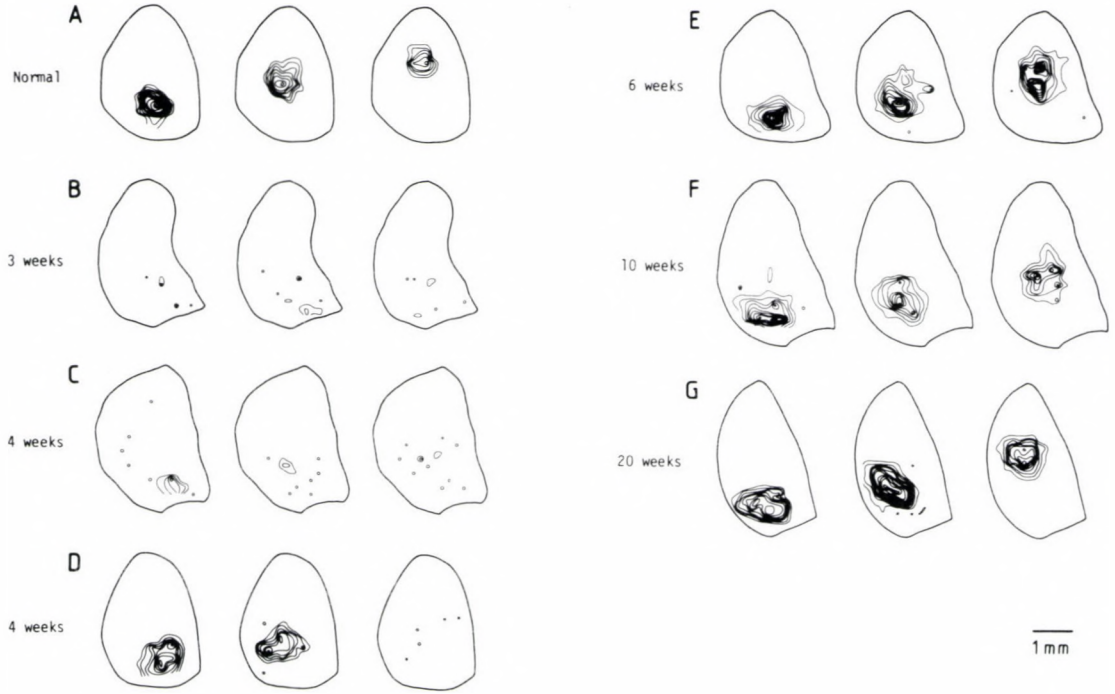

Fig. 1. Retinotectal projections mapped with field potentials. A: projections in a normal fish. B: projections in the 3rd week of regeneration. C and D: projections in the 4th week of regeneration, E: projections in 6th week of regeneration. F: projections in the 10th week of regeneration. G: projections in the 20th week of regeneration. Interval of contour lines: 100 μV

become localized (Fig. 1D). In the 6th week (Fig. 1E), separation and localization of the projection was observed in all fish examined, although the response areas were still larger than those in normal fish. It seems that the response areas get smaller in the later stages (Fig. 1F and G), but they are still larger than those in control animals (even in the 20th week, the response area is about 1.3–1.4 larger than the control area).

Initial disorder of regenerating retinotectal connections in the frog has been reported by Gaze and Jacobson /3/. Humphrey and Beazley /4/ later examined the receptive field size as well as the retinotopicity in Hyla. Those experiments indicated that regenerated fibers first distributed diffusely on the tectum, and that they returned to their original positions later.

Early projection patterns in the present study have clearly shown that regenerating optic fibers first make temporary, unspecific and diffuse synapses in the rostral tectum, and later retinotopic synapses develop first in the rostral tectum without waiting for the formation of synapses in the more caudal part of the tectum. Anatomical and physiological studies have shown the shifting of retinal fiber terminals during development /1, 5/. Fujisawa et al. /2/ have shown that the regenerating terminals of the optic fibers frequently branch before reaching the target area. Our present results suggest that such branches make functional but temporary synapses with tectal neurons.

REFERENCES

1. Chung, S.H., Keating, M.J., Bliss, T.V.P. (1974) Functional synaptic relation during the development of the retinotectal projection in amphibians. Proc. Roy. Soc.(Lond.) B 187, 449–459.
2. Fujisawa, H., Tani, N., Watanabe, K., Ibata, Y. (1982) Branching of regenerating retinal axons and preferential selection of appropriate branches for specific neuronal connection in the newt. Developmental Biol. 90, 43–57.
3. Gaze, R.M., Jacobson, M. (1963) A study of the retinotectal projection during regeneration of the optic nerve in the frog. Brain Res. 21, 183–195.
4. Humphrey, M.F., Beazley, L.D. (1982) An electrophysiological study of early retinotectal projection patterns during optic nerve regeneration in Hyla moorei. Brain Res. 239, 595–602.
5. Reh, T.A., Constantine-Paton, M. (1984) Retinal ganglion cell terminals change their projection sites during larval development of Rana pipiens. J. Neurosci. 4, 1045–1051.

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A GLIAL CELL LINE PROMOTES THE OUTGROWTH OF NEURITES FROM EMBRYONIC XENOPUS RETINA

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A glial cell line (XR1 cell line) derived from Xenopus retinal neuroepithelium was examined for neurite outgrowth promoting activity. A monolayer of the XR1 cells serves as an excellent substrate upon which embryonic retinal explants attach and freely elaborate neurites. The XR1 neurite outgrowth promoting activity is not secreted into the medium, but is laid down directly on the substrate where it remains active after lysing the cells by hypoosmotic shock. A polyclonal antiserum raised against membranes of the XR1 cells was effective in blocking neurite outgrowth on XR1 conditioned collagen. It is proposed that the neurite outgrowth promoting factors produced by the XR1 cells are associated with the extracellular matrix and possibly glial specific.

Keywords: XR1 cells, glial cell line, neurite outgrowth, retina

INTRODUCTION

A major goal in neural development is the elucidation of mechanisms which are involved in the outgrowth and guidance of axons and growth ones. In developing embryos growth cones are often found in intimate association with glial cells and it has been hypothesized that such interactions may play important roles in supporting neurite growth /20, 25, 28, 30/. For example, in vivo studies in vertebrates have indicated glial cells playing a part in guiding axons or migrating neurons in the developing and regener-

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ating visual system, and also in the corpus callosum and spinal cord /3, 20, 25, 27/. In addition, in vitro studies have also demonstrated preferential outgrowth of neurites on glial cells /8, 9, 13, 18/.

In the embryonic vertebrate visual system serial reconstructions or electron micrographs have recently revealed associations between retinal growth cones and glial endfeet within the retina (D. Meissner and U. Schwarz, personal communication) and optic pathway /12, 26/, and C. Holt, personal communication. The examination of these associations in culture may lead to a better understanding of the cellular basis of these interactions.

In the present communication the neurite outgrowth promoting activity of a retinally derived glial cell line (XR1 cell line) is described. Embryonic Xenopus retinal tissue can effectively use the XR1 cells or substrates conditioned by the XR1 cells as a substrate on which to extend neurites. A polyclonal antiserum generated against membrane components of the XR1 cells is effective in blocking this activity. Combining components of the developing retinotectal pathway in vitro and studying the cellular basis of their interactions may provide a better understanding of neural pathfinding in vivo.

MATERIALS AND METHODS

Xenopus laevis embryos were produced in the laboratory by gonadotropin induced spawnings and staged according to the normal Xenopus table /17/. The isolation and immunohistological characterization of the XR1 cell line has been described /22, 23/.

Culture preparation

A number of cell lines were examined for their ability to support the outgrowth of retinal neurites. Xenopus cell lines (XR1, XR8, XR9, A6 and XTC) were plated onto collagen coated plastic culture dishes and allowed to grow to a confluent monolayer. In most experiments the monolayer of cells were lysed by hypoosmotic shock (48 h incubation with sterile water). Substrates prepared in this fashion are referred to as cell line conditioned collagen substrates. Retinal tissue from stage (24 to 27) Xenopus laevis embryos were dissected and carefully transferred by pipette and explanted to culture dishes. In some experiments substrates were incubated with antibodies (pre- and postimmune IgG NOB1 and Fab NOB1; Sakaguchi et al., submitted) for 5 h. Substrates were then rinsed, culture media /12/ added and embryonic eye tissue explanted.

RESULTS

Isolation and characterization of XR1 cells, a glial cell line

Neurites from primary explants of embryonic retinas plated on collagen surfaces extend to the border of a growing layer of flat, non-neuronal cells. The retinal neurites and growth cones strongly prefer the surface of the flat cells to that of the collagen substrate (Fig. 1A). To examine neurite outgrowth promoting factors associated with the non-neuronal cells they were harvested and maintained for many generations. Extensive serial dilutions were carried out to isolate several relatively homogeneous morphological cell lines, three of which were used in the present communication (XR1, XR8, and XR9). One of these cell types, the XR1 cells has been cloned and appears to be glial in nature /11, 22/. The morphology of these cells in culture, that of large flattened, polygons with radial extensions is similar to descriptions of cultured astrocytes from other experimental preparations /1, 8, 9/. The XR1 cells also stain with a number of antibodies specific for glial cells (/22, 23/ such as αGFAP /2/, αVimentin /2, 15, 24/, αS-100 /10/ and R5 /6/. In addition, the XR1 cells possess another feathre of glial cells in that they promote neurite outgrowth /11, 22/.

Neurite outgrowth and the XR1 cells

Embryonic retinal tissue explanted onto a monolayer of XR1 cells freely elaborate neurites onto the carpet of glial cells (Fig. 1B). The extent of neurite outgrowth was assessed quantitatively by calculating the percentage of retinal explants with neurites growing on the experimental substrate after eight days in culture (Fig. 2). To determine whether the XR1 cells were excreting a neurite promoting factor into the medium the effect of XR1 cell conditioned medium (CM) and XR1 conditioned collagen were investigated (Fig. 2A). Culture medium conditioned by the XR1 cells /23/ possessed no demonstrable neurite outgrowth promoting activity (Fig. 2A). The conditioned collagen substrate from which a monolayer of XR1 cells had been lysed by a 48 h incubation in distilled water, however, showed significant outgrowth promoting activity (Figs 1C and 2A). Two additional cell lines derived from the embryonic Xenopus retina, XR8 and XR9, were also tested and showed some activity, but considerably less than the XR1 cells (Fig. 2A). In contrast, collagen conditioned by a monolayer of A6 cells, a Xenopus kidney epithelial cell line or XTC cells /19/, another established

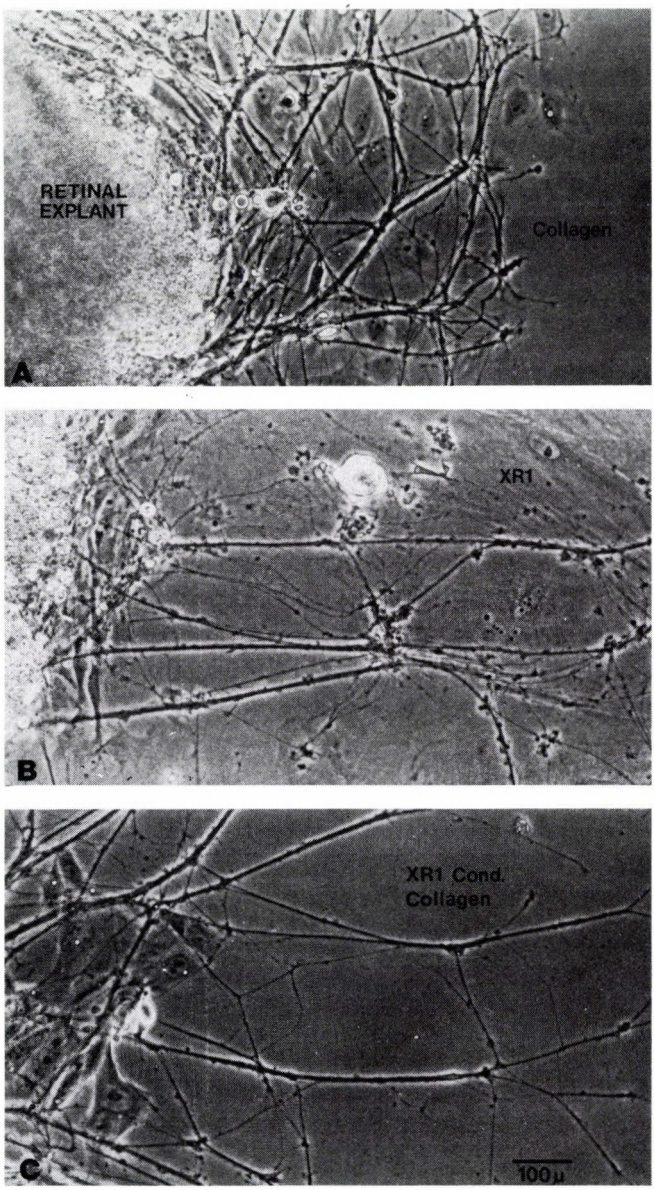

Fig.1. Neurite outgrowth from embryonic retinal explants cultured on collagen (A), a confluent monolayer of XR1 cells (B), and on collagen conditioned by XR1 cells (C) at eight days in culture. (A), Neurites growing from the retinal explant confine themselves to the non-neuronal, flat cells which grow out from the explanted tissue and seldom venture onto the surrounding collagen substrate. (B),Neurites from explants cultured on a confluent layer of XR1 cells or (C), on XR1 conditioned collagen show extensive outgrowth away from the explant. Calibration bar: 100 μm.

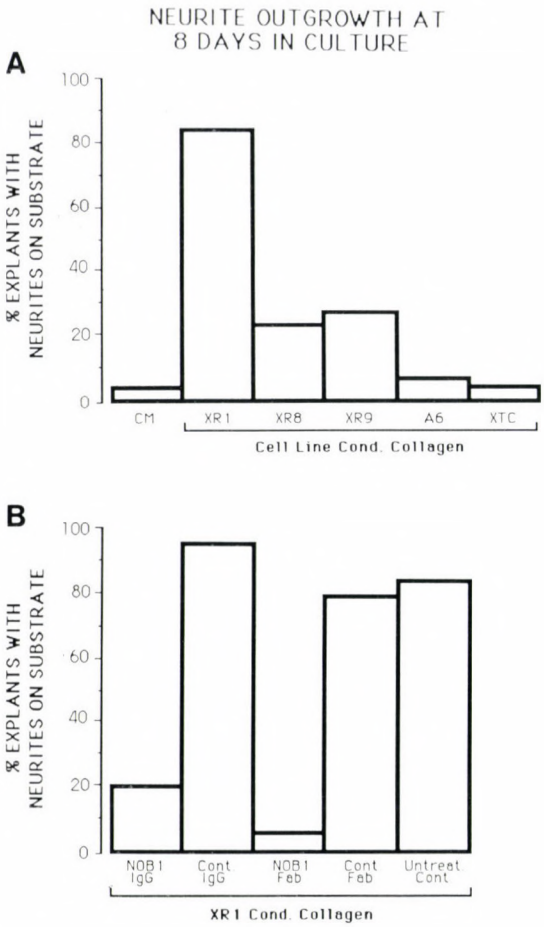

Fig. 2. Neurite outgrowth from retinal explants growing on various substrates. A scoring system was devised to quantify the ability of various substrates to support neurite outgrowth. If neurites grew out from an explant onto the test culture surface the explant was scored positively; if the neurites remained confined to the edges of the explant, it was scored as a negative. This system was used to calculate the percentage of explants with neurites growing on particular substrates. (A) and (B), the vertical axes shows the percentage of retinal explants with neurites extending onto the experimental substrates after 8 days in culture. On the horizontal axes are the different substrates and conditions. (A), Neurite outgrowth on various cell line derived substrates. CM = collagen with XR1 conditioned medium (n=56 explants); XR1 = collagen conditioned by XR1 cells (n=125); XR8 = collagen conditioned by XR8 cells (n=24); XR9 = collagen conditioned by XR9 cells (n=99); A6 = collagen conditioned by A6 cells (n=79) XTC = collagen conditioned by XTC cells (n=48). (B), Neurite outgrowth on XR1 conditioned substrates treated with antibodies. IgG NOB1 = IgG fraction of NOB1 antiserum from a rabbit which was immunized with XR1 membranes (n=32); Cont IgG = IgG fraction of preimmune serum (n=32); Fab NOB1 = Fab fragments from NOB1 IgGs (n=68); Cont Fab = Fabs from preimmune IgGs (n=47)

Xenopus cell line showed no neurite outgrowth promoting activity (Fig. 2A). Thus, the XR1 cells or a substrate conditioned by XR1 cells showed substantial outgrowth promoting activity when compared with a variety of other Xenopus cell line associated substrates.

To begin searching for molecules which may mediate growth cone-glial cell interactions polyclonal antibodies were generated in rabbits against components of the XR1 cells /23/. Affinity purified IgGs as well as monovalent fragments of antiserum NOB1 were effective in blocking neurite outgrowth on XR1 conditioned substrates (Fig. 2). In contrast, neurites grew freely on substrates treated with the control, preimmune IgG and Fabs at the same concentrations (Fig. 2B).

DISCUSSION

The XR1 cells appear, by a number of criteria, to be glial in nature. Morphologically the cells are similar to descriptions of cultured astrocytes from other in vitro preparations /1, 8, 9/. The XR1 cells are also immunoreactive to a variety of glial cell specific antibodies /23/. In addition, recent studies have shown that XR1 cells transcribe large amounts of the presumed glial form of N-CAM mRNA (C. Kintner, personal communication). And lastly, the XR1 cell line promotes and supports the outgrowth of neurites from embryonic retinal tissue.

Although numerous studies have focused on the role of the extracellular matrix molecule laminin in neurite extension /7, 16, 21/, and laminin substrates are useful for neurite outgrowth in this system /23/, the outgrowth promoting activity associated with the XR1 cells may be different from laminin. In a number of systems laminin is secreted from non-neuronal cells and can be recovered from the media /5, 14/, however, the neurite outgrowth promoting activity of the XR1 cells is associated with the cell membranes or remains on the culture surface where the cells attached to the substrate and was not recovered from medium conditioned by the XR1 cells (Fig. 2A). In addition, Fabs effective in blocking XR1 promoted outgrowth do not block outgrowth on laminin, nor do anti-laminin antibodies block outgrowth on XR1 conditioned substrates (data not shown). Other recent studies have also shown that neurites can extend on non-neuronal cells /4, 29/ via laminin independent mechanisms.

The neurite outgrowth promoting activity associated with the XR1

cells, studied in vitro, may be an integral part of axon elongation during pathway development in the embryonic CNS. Serial reconstruction of electron micrographs have revealed early growth cones of retinal ganglion cells intimately associated with glial endfeet in the chick retina (D. Meissner and U. Schwarz, personal communication) and optic tract of the chick and frog (/11, 26/ and C. Holt, personal communication. In addition, the preferential adherence of neurons on glial cells has been demonstrated in a number of in vitro systems as well /8, 9, 12, 18, 29/. The endfeet of glial cells may form a substrate for the initial pathway by providing cell surface associated molecules which promote adherence and axonal extension from embryonic neurons. By providing a preferred adhesive substrate for neurite growth, glial cell components could play an important role in helping to organize the complex development of the nervous system.

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REFERENCES

1. Adler, R., Manthorpe, M., Varon, S. (1979) Separation of neuronal and non-neuronal cells in monolayer cultures from chick embryo optic lobe. Dev. Biol. 69, 424–435.

2. Bignami, A., Eng, L.F., Dahl, D., Uyeda, C.T. (1972) Localization of the glial acidic protein in astrocytes by immunofluorescence. Brain Res. 43, 429–435.

3. Bohn, R.C., Reier, P.J., Sourbeer, E.G. (1982) Axonal interactions with connective tissue and glial substrata during optic nerve regeneration in Xenopus larvae and adults. Amer. J. Anat. 165, 397–419.

4. Cohen, J., Burne, J.F., Winter, J., Bartlett, P. (1986) Retinal ganglion cells lose response to laminin with maturation. Nature 322, 465–467.

5. Davis, G.E., Manthorpe, M., Engvall, E., Varon, S. (1985) Isolation and characterization of rat schwannoma neurite-promoting factor: Evidence that the factor contains laminin. J. Neurosci. 5, 2662–2671.

6. Dräger, U.C., Edwards, D.L., Barnstable, C.J. (1984) Antibodies against filamentous components in discrete cell types of the mouse retina. J. Neurosci. 4, 2025–2042.

7. Faivre-Bauman, A., Puymirat, J., Loudes, C., Barret, A., Tixier-Vidal, A. (1984) Laminin promotes attachment and neurite elongation of fetal hypothalamic neurons grown in serum-free medium. Neurosci. Lett. 44, 83-89.

8. Fallon, J. (1985a) Neurite guidance by non-neuronal cells in culture: Preferential outgrowth of peripheral neurites on glial as compared to non-glial cell surfaces. J. Neurosci. 5, 3169-3177.

9. Fallon, J. (1985b) Preferential outgrowth of central nervous system neurites on astrocytes and Schwann cells as compared with non-glial cells in vitro. J. Cell Biol. 100 198-207.

10. Haan, E.A., Boss, B.D., Cowan, W.M. (1982) Production and characterization of monoclonal antibodies against the "brain-specific" proteins 14-3-2 and S-100. Proc. Natl. Acad. Sci. (USA) 79, 7585-7589.

11. Harris, W.A. (1985) Outgrowth of embryonic retinal cell axons in Xenopus is promoted by a factor, probably not laminin, on the surface of a glial cell line. Soc. Neurosci. Abstr. 11, 762.

12. Harris, W.A., Holt, C.E., Smith, T.A., Gallenson, N. (1985) Growth cones of developing retinal cell in vivo, on culture surfaces, and in collagen matrices. J. Neurosci. Res. 13, 101-122.

13. Hatten, M.E., Leim, R.K.H., Mason, C.A. (1984) Two forms of cerebellar glial cells interact differently with neurons in vitro. J. Cell Biol. 98, 193-204.

14. Lander, A.D., Fujii, D.K., Reichardt, L.F. (1985) Laminin is associated with the "neurite outgrowth-promoting factors" found in conditioned media. Proc. Natl. Acad. Sci. (USA). 82, 2183-2189.

15. Lemmon, V., Rieser, G. (1983) The developmental distribution of vimentin in the chick retina. Devel. Brain Res. 11, 191-197.

16. Manthorpe, M., Engvall, E., Ruoslahti, E., Longo, F.M., Davis, G.E., Varon, S. (1983) Laminin promotes neuritic regeneration from cultured peripheral and central neurons. J. Cell Biol. 97, 1882-1890.

17. Nieuwkoop, P.D., Faber, J. (1956) Normal tables of Xenopus lavis. (Daudin), North-Holland, Amsterdam.

18. Noble, M., Fok-Seang, J., Cohen, J. (1984) Glia are a unique substrate for the in vitro growth of central nervous system neurons. J. Neurosci. 4, 1892-1903.

19. Pudney, M., Varma, M.G.R., Leake, C.J. (1973) Establishment of a cell line (XTC-2) from the South African clawed toad, Xenopus laevis. Experientia. 29, 466-467.

20. Rakic, P. (1971) Neuron-glia relationship during granule cell migration in developing cerebral cortex: A Golgi and electron microscopic study in Macacus rhesus. J. Comp. Neurol. 141, 283-312.

21. Rogers, S.L., Letourneau, P.C., Palm, S.L., McCarthy, J., Furcht, L.T. (1983) Neurite extension by peripheral and central nervous system in response to substratum-bound fibronectin and laminin. Dev. Biol. 98, 212-220.

22. Sakaguchi, D.S., Moeller, J., Harris, W.A., Gallenson, N. (1987) Growth cone interactions with glial cells in cultures from embryonic Xenopus retina. Neuroscience. 22, S283.

23. Sakaguchi, D.S., Moeller, J.F., Coffman, C.R., Gallenson, N., Harris, W.A. Growth cone interactions with a glial cell line from embryonic Xenopus retina. (Submitted).

24. Schnitzer, J., Franke, W.W., Schachner, W. (1981) Immunocytochemical demonstration of vimentin in astrocytes and ependymal cells of developing and adult mouse nervous system. J. Cell. Biol. 90, 435–447.

25. Silver, J. (1984) Studies on the factors that govern directionality of axonal growth in the embryonic optic nerve and at the chiasm of mice. J. Comp. Neurol. 223, 238–251.

26. Silver, J., Rutishauser, U. (1984) Guidance of optic axons in vivo by a preformed adhesive pathway on neuroepithelial endfeet. Dev. Biol. 106, 485–499.

27. Singer, M., Nordlander, R.H., Egar, M. (1979) Axonal guidance during embryogenesis and regeneration in the spinal cord of the newt: The blueprint hypothesis of neuronal pathway patterning. J. Comp. Neurol. 185, 1–22.

28. Smith, G.M., Miller, R.H., Silver, J. (1986) Changing role of forebrain astrocytes during development, regenerative failure, and induced regeneration upon transplantation. J. Comp. Neurol. 251, 23–43.

29. Tomaselli, K.J., Reichardt, L.G., Bixby, J.L. (1986) Distinct molecular interactions mediate neuronal process outgrowth on non-neuronal cell surfaces and extracellular matrices. J. Cell Biol. 103, 2659–2672.

30. Varon, S.S., Somjen, G.G. (1979) Neuron-glia interactions. Neurosci. Res. Prog. Bull. 17, 1–239.

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COLUMNAR ORGANIZATION OF THE OPTIC TECTUM IN THE FROG

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Cobaltous lysine complex was used to label tectal cells. Cobalt soaked into a piece of filter paper and placed onto the surface of the tectum labelled neurons in the whole thickness of the tectum below the filter paper. The labelled area was sharply demarcated from the unlabelled tectal tissue. Focal cobalt injections into different tectal layers labelled small groups of cells and the cobalt-filled structures were perpendicularly oriented to the surface of the tectum. Efferent axons could be followed into layer 7, but other lateral connections were very sparse. These results support the hypothesis that the tectum has columnar organization similar to that of the mammalian neocortex.

Keywords: Cobalt labelling, tectal columns, frog

In simple histological preparations, like Nissl stained sections, the neurons in the optic tectum appear as rows of perikarya arranged in strata oriented parallel with the surface of the brain. Optic fibres and terminals show a similar stratification (Fig. 1). Laminar organization in layer 9 is even more striking, when the location of a number of peptides is taken into consideration /2/. Efferent fibres are also gathered in laminae in layer 7, and to a lesser extent, in the deep part of layer 9.

Sharply contrasting this pattern, most of the tectal cells project their dendrites and axons perpendicularly to the surface, crossing layers

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of perikarya and the laminae of retinal terminals (Fig. 2). Only a few cells with horizontally oriented dendrites can be found in layers 9 and 7. Most of the dendrites of the ganglionic cells, the largest in the tectum, are oriented obliquely to the tectal surface.

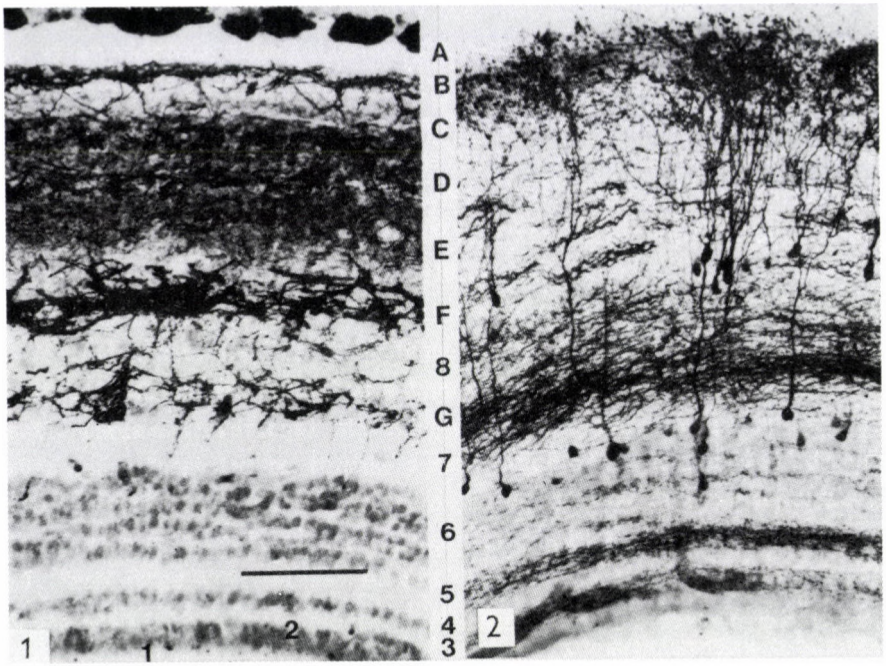
The dominant vertical orientation of dendrites suggested that tectal cells are arranged in columns, similarly to cortical neurons of mammals /1, 5/, and such columns may serve as functional units in the tectum /3, 6/.

To study columnar organization in the tectum, I used the cobalt-filling techniques /4/. In one group of frogs (Rana esculenta), a piece of filter paper, soaked with cobaltous lysine complex was placed on the surface of the tectum. In such a preparation, a large number of tectal cells below the filter paper became labelled with cobalt (Fig. 3). Most of the dendrites were oriented toward the surface and the borders of the labelled tectal spot sharply contrasted with unlabelled tectal tissue. A few cells, which send obliquely oriented dendrites or axons into the heavily labelled cell group, were also filled.

In the second group of animals, cobalt was iontophoretically injected into various layers of the tectum. This type of focal cobalt injection into layer 9 resulted in heavy labelling of cells in a column in the deeper tectal layers (Fig. 4). In layer 9, around the cobalt focus, mainly horizontally oriented structures were labelled. Most of them are probably fibres of retinal origin.

Cobalt, injected into layers 7 and 6, labelled a dendritic column peripheral to the injection site, and perikarya in the deeper layers. Labelled fibres could be followed for long distances in layer 7 (Fig. 5), but in layer 9 only a few cobalt-containing axons and axon terminals could be identified. The length of such horizontally oriented axons was only 300–350 μm measuring from the periphery of the labelled dendritic column (Fig. 5). When the focus was confined to layers 5 to 1, heavy labelling of probably basal dendrites and non-optic afferents was observed in layers 5 and 3. The topographic organization of efferent tectal axons also suggests a columnar organization of projection neurons. When two cobalt foci were placed in the tectum, (Fig. 6), two cobalt-labelled spots in layer 7 indicated that the axons originated from two, clearly separated groups of cells (Fig. 7).

Among the dendrites of tectal cells large numbers of fine, beaded, chiefly vertically oriented axons form loose networks suggesting that most of the synaptic contacts of an axon are restricted to a narrow column of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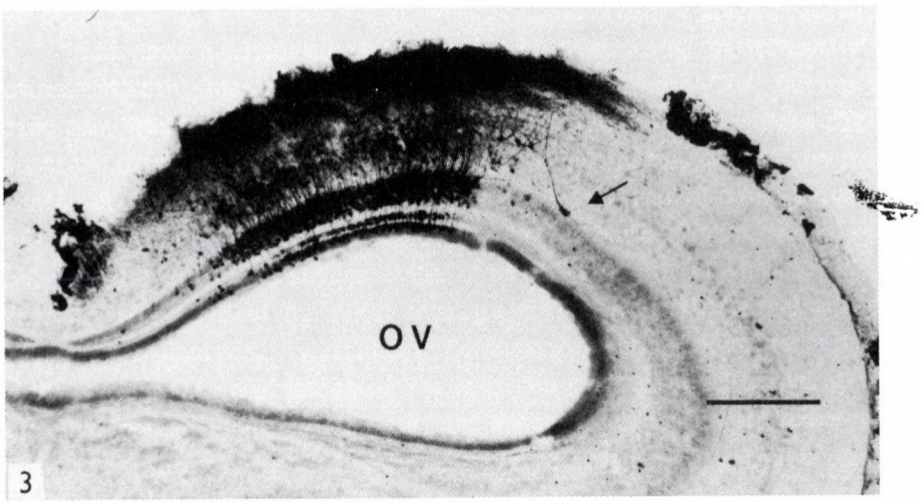
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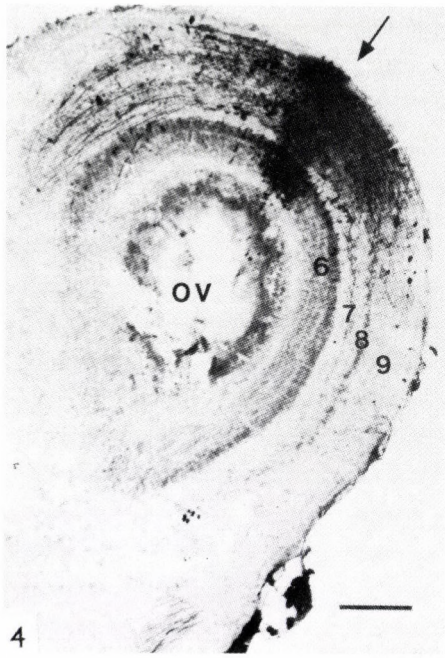

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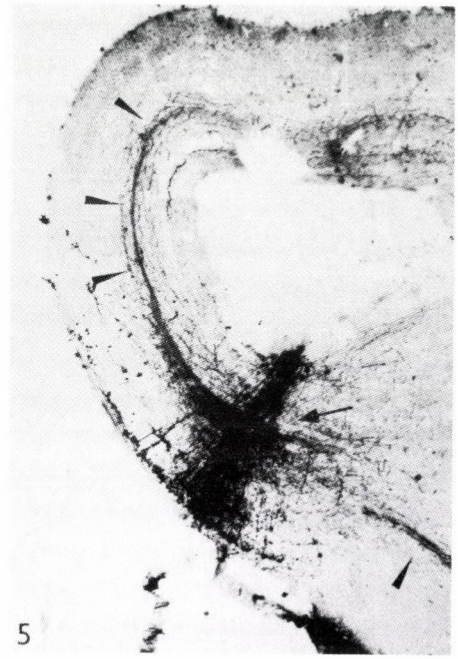

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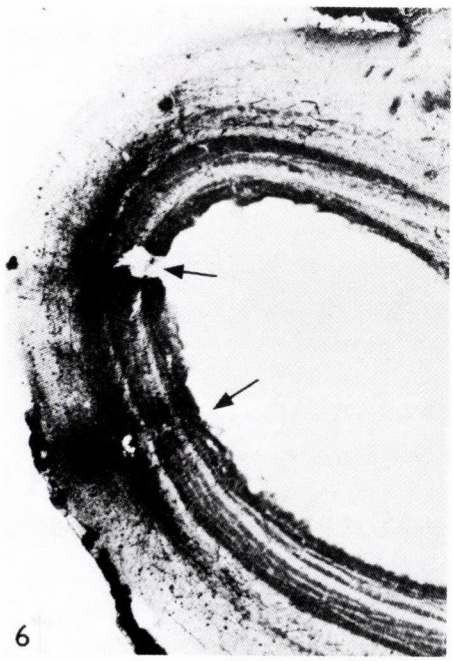

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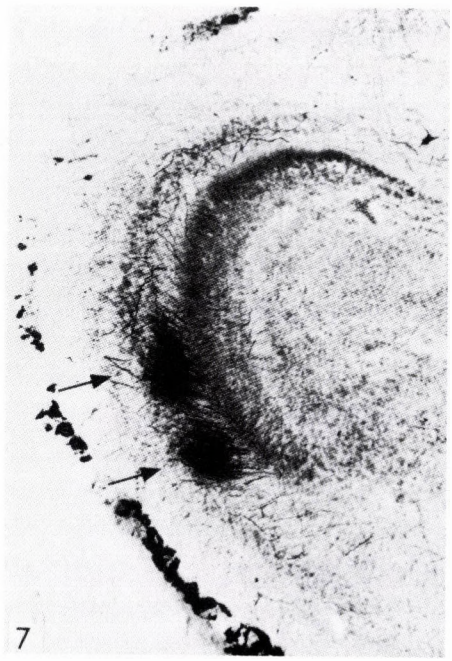

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cells and lateral connections are limited. These results give further evidence in favour of columnar organization of the optic tectum. The output element of a column is probably a projection neuron. Depending on the type of this neuron, columns of different sizes may exist. Large ganglionic neurons may be the output cells of columns with a diameter of 350–1000 μm. Large piriform cells and pyramidal cells may belong to smaller columns (150–350 μm in diameter), and layer 8 projection neurons may express the activity of the smallest columns (50–150 μm in dimater).

It is not identified yet, what kind of neurons establish synaptic contacts within a column, and what is the relationship between local circuit neurons and projection neurons. For further steps in the analysis of tectal circuitry, synaptological studies are urgent.

Fig. 1. Laminar organization of the optic tectum. Cobalt-filled myelinated retinal fibres terminate in four laminae oriented parallel with the surface (B, D, F, G). Unmyelinated fibres occupy three laminae (A, C, E). Fibres in lamina A were not filled in this preparation. Toluidine blue counterstaining shows the location of neurons which form layers (8, 6, 4, 2). The innermost layer (1) is the ependymal lining of the optic ventricle. The other layers contain non-optic afferents (3, 5) and the efferent axons leave the tectum through layer 7. Capitals and numerals on the right refer also to Fig. 2. The scale bar is 100 um and applies also to Fig. 2.

Fig. 2. The orientation of dendrites of most of the tectal cells is perpendicular to the surface. Cobaltic-lysine was injected into rostrally projecting tectal axons in the caudal one third of the diencephalon and thalamic fibres terminating in the tectum

Fig. 3. Tectal cells labelled with cobaltous-lysine complex soaked in a piece of filter paper and put on the surface of the tectum. Note the sharp boundary between labelled and non-labelled tectal tissue. The large ganglionic cell (arrow) was filled through a dendrite reaching the cobalt focus. Transverse section. OV - optic ventricle. The scale bar is 250 μm

Fig. 4. Focal cobalt injection in layer 9 in a transverse section of the optic tectum (arrow). A column-like group of neurons and large number of fibres (chiefly optic terminals) were filled in layer 9. OV — optic ventricle, 6, 7, 8, 9 — tectal layers. The scale bar is 250 μm and applies to all figures in this plate

Fig. 5. Focal cobalt injection in layers 6 and 7 of the optic tectum (arrow). Note the narrow labelled cell-column and efferent axons in layer 7 and the tegmentum (arrow heads). Transverse section

Fig. 6. Two cobalt injection sites in the optic tectum (arrows). Horizontal section. Rostral is upward

Fig. 7. Two bundles of cobalt-labelled tectal efferent axons in layer 7 (arrows) originating from cobalt-filled cells which are located in the injection sites shown in Fig. 5. Horizontal section 480 μm ventral to the plane of cobalt foci

REFERENCES

1. Hubel, D.H., Wiesel, T.N. (1962) Receptive fields, binocular interaction and functional architecture in the cat's visual cortex. J. Physiol. (Lond.) 160, 106–154.

2. Kuljis, O.R., Karten, H.J. (1982) Laminar organization of peptide-like immunoreactivity in the anuran optic tectum. J. Comp. Neurol. 212, 188–201.

3. Lázár, Gy., Székely, Gy. (1967) Golgi studies on the optic center of the frog. J. Hirnforsch. 9, 329–344.

4. Lázár, Gy., Tóth, P., Csank, Gy., Kicliter, E. (1983) Morphology and location of tectal projection neurons in frogs: a study with HRP and cobalt-filling. J. Comp. Neurol. 215, 108–120.

5. Mountcastle, V.B. (1957) Modality and topographic properties of single neurons of cat's somatic sensorycortex. J. Neurophysiol. 20, 408–434.

6. Székely, Gy., Lázár, Gy. (1976) Cellular and synaptic architecture of the optic tectum. In: Llinas, R. and Precht, W. (eds) Frog Neurobiology. Springer-Verlag Berlin, Heidelberg, pp. 407–434.

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SYNAPTIC ORGANIZATION OF RETINOTECTAL CONNECTIONS OF THE FROG
APPLICATION OF PULSE TRIGGERED AVERAGING

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Pulse-triggered averaging technique was applied to retinotectal connections of the frog. An extracellular single unit was first isolated from the terminals of retinal fibers, and then intracellular responses were recorded from a tectal neuron in the vicinity of the extracellular recording electrode. Intracellular potentials in response to a moving stimulus were averaged by triggering with the isolated presynaptic impulses. The results show that "on–off" retinal fibers monosynaptically excite E–E type (EPSP at "on" and "off" of light) and EI–EI type (EPSP–IPSP at "on" and "off" of light). One of the E–E type neurons was identified as a large ganglionic neuron in layer 8.

Keywords: Retinotectal connections, extracellular and intracellular recording, frog

INTRODUCTION

Retinal ganglion cells are divided into 4 classes according to their response properties to visual stimuli. Tectal neurons which receive retinal inputs are also divided into several classes according to extracellular recording experiments. Recent intracellular studies combined with cellular labelling technique /1, 4/ made it possible to correlate physiological properties with cellular morphology. Until now, however, it has remained un-

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known which class of retinal fibers make synaptic contacts with a particular type of tectal neurons. To elucidate this point, simultaneous recording from a retinal fiber ending (extracellular) and a tectal neuron (intracellular) was carried out. Postsynaptic potentials were then analyzed by applying pulse triggered averaging method.

MATERIALS AND METHODS

Experiments were carried out on adult frogs (Rana catesbeiana). During methanesulfonate anaesthesia, the left optic tectum was exposed and immobilized with succinylcholine. Action potentials of optic fiber terminals were recorded with tungsten electrodes, and intracellular responses of tectal neurons, with glass micropipettes filled with 2M K-citrate. To identify morphologically the tectal neurons, ethidium bromide was injected.

A tungsten electrode was first inserted into the tectum and a single or multiple "on–off" retinal unit was isolated using the diffuse light as stimulus. A glass microelectrode was then inserted in the vicinity of the tungsten electrode, and intracellular responses were recorded from a tectal neuron simultaneously with the presynaptic impulses. Potentials were stored on tape.

Postsynaptic potentials were averaged by triggering with a single presynaptic unit. Averaging was performed using a moving square as the stimulus, since "on–off" of diffuse light more or less synchronously fires "on–off" ganglion cells in the whole visual field.

RESULTS AND CONCLUSIONS

The criteria for recording from ganglion cell axon terminals were i) small receptive field ($< 8^{o}$); ii) very little or no adaptation to repeated visual stimulation; iii) short and fixed latency ($<$ 1.4 ms) of the spike in response to electrical stimulation of the optic tract and iv) agreement of the recording position on the tectum with established retinotectal maps.

If the presynaptic impulses show autocorrelation or cross-correlation, we cannot be sure about the latency of the postsynaptic potentials or the shape of the potential might be deformed. Autocorrelograms and cross-correlograms were calculated using presynaptic impulses, but neither significant autocorrelation nor cross-correlation was detected.

Responses of tectal neurons to "on–off" of diffuse light are divided into some classes. In the present study, only E–E type (EPSP at "on" and "off") and EI–EI type (EPSP-IPSP at "on" and "off") were examined. Figure 1A shows an "on–off" response of presynaptic terminals and simultaneously

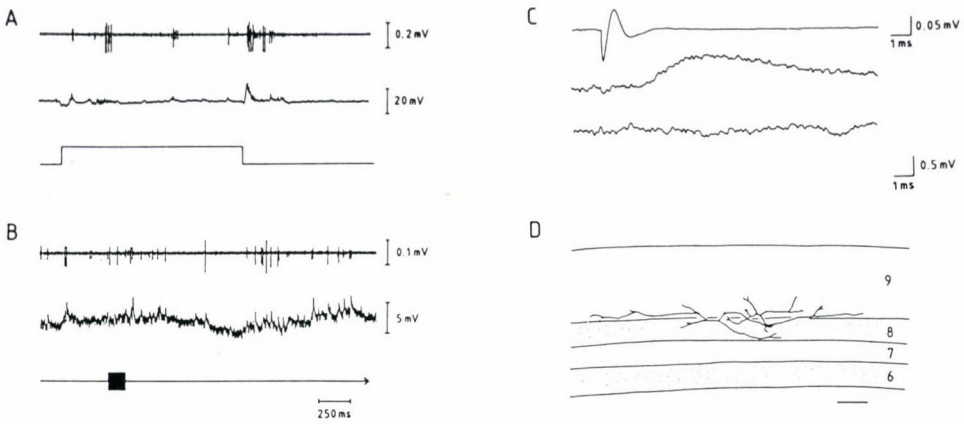

Fig. 1. A: Simultaneous representation of impulses of an "on–off" retinal fiber (top trace) and a postsynaptic potential of an E–E type tectal neuron (middle trace). The duration of illumination is indicated by upward deflection in the bottom trace. B: Presynaptic and postsynaptic potentials to a moving $2^{O}x2^{O}$ square. C: Averaged presynaptic (top trace) and postsynaptic (middle trace) potentials. The bottom trace is an averaged field potential recorded just outside of the neuron. D: A large ganglionic neuron in layer 8 which showed an E–E type response to diffuse light. Calibration: 100 μm

recorded intra-cellular response (E–E type) of a tectal neuron. Figure 1B shows the responses of the same pair to a moving stimulus. The top and the second traces in Fig. 1C are an averaged presynaptic impulse and the related post-synaptic potential, respectively. The third trace shows the field potential just outside of the neuron.

EPSPs were detected in 11 cells. In each of them, one or two fibers were effective, and the number of pre- and postsynaptic pairs with positive correlation was 15. Four cells were E–E type and seven cells were EI–EI type. The largest distance between the two recording sites was about 500 μm. The latency of the EPSPs was measured. Most frequently observed value was about 1.4 ms. This is comparable to the synaptic delay time in the central nervous system of the frog /2, 3/ and the EPSP in Fig. 1C was concluded to be monosynaptically generated by "on–off" retinal fibers. One of the E–E type neurons which gave rise to a monosynaptic EPSP was successfully stained and identified as a large ganglionic neuron in layer 8.

REFERENCES

1. Antal, M., Matsumoto, N., Székely, G. (1986) Tectal neurons of the frog: Intracellular recording and labeling with cobalt electrodes. J. Comp. Neurol. 246, 238–253.

2. Brookhart, J.M., Fadiga, E. (1960) Potential fields initiated during monosynaptic activation of frog motoneurons. J. Physiol. 150, 638–655.

3. Matsumoto, N., Bando, T. (1980) Excitatory synaptic potentials and morphological classification of tectal neurons of the frog. Brain Res. 192, 39–48.

4. Matsumoto, N., Schwippert, W.W., Ewsert, J.-P. (1986) Intracellular activity of morphologically identified neurons of the grass frog's optic tectum in response to visual stimuli. J. Comp. Physiol. 159, 721–739.

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DENDRITIC ANATOMY AND ELECTROTONIC TRANSFER PROPERTIES OF CAT SUPERIOR COLLICULUS NEURONS

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Detailed morphometrical and corresponding electrotonic characteristics on three classes of cat superior colliculus (SC) neurons have been derived. The sample of cells selected for analysis comprised ascending projection neurons (APNs), inter-layer neurons (ILNs) and tecto-reticulo-spinal neurons (TRSNs) recorded intracellularly and stained with HRP. Superficial SC neurons (APNs, ILNs) could be attached to the allo- and idiodendritic type while deep layer neurons (TRSNs) belong to the isodendritic type. For each neuron, the branching pattern, lengths and diameters of the dendritic trees were determined. These data served as input to the computer program "DENDRIT" from which electrotonic membrane and transfer properties were calculated. Both the morphometrical data and the electrotonic properties underline the contrasting features of superficial vs deep layer neurons in the SC. Our results support the hypothesis that on the neuron level a close relationship between dendritic pattern and neuron function might exist.

Keywords: Superior colliculus neurons, dendritic anatomy, segmental cable modeling, electrotonic properties, structure – function relationship

INTRODUCTION

The present work was undertaken in the course of a detailed study of neurons located in the upper as well as in the deep superior colliculus (SC) layers of the cat. It is pertinent to three problems: First, quantita-

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tive data on dendritic dimensions and branching patterns are presented essential to the characterization of neuron classes in the SC; Second, criteria for classification of nerve cells according to their dendritic morphology are tested; Third, the dendro-somatic architecture is quantified in order to explore the structure-dependent functions of neurons from different SC layers by mathematical modeling.

MATERIALS AND METHODS

All data were obtained from neurons recorded intracellularly and stained with HRP as described earlier /1, 2/. The sample of cells selected for analysis comprised 3 ascending projection neurons (APNs), 2 inter-layer neurons (ILNs) and 3 tecto-reticulo-spinal neurons (TRSNs).

For each dendritic tree, length (l) and diameter (d) of its segments were measured up to processes of 1 μm diameter; l having been allowed for section thickness. Measurements of d were performed under oil immersion (2000-fold), and a mean segment diameter was determined.

Branching structure, lengths and diameters of segments served as input to the FORTRAN computer program "DENDRIT" /7/. Based on measurements of the input resistance ($R_{N,exp}$), estimates of membrane resistance (R_m), electrotonic length (L), dendro-somatic conductance ratio (ϱ) and efficacy of synaptic current (T_s) and voltage transfer were calculated. In addition, total neuron surface area (A_N), dendro-somatic surface area ratio (A_D/A_S) and branch power (n) were computed.

RESULTS

Superficial SC neurons (APNs, ILNs) can be sorted to the allo- or idiodendritic type, while deep layer neurons (TRSNs) meet the criteria for isodendritic neurons as shown in detail elsewhere /8/.

Statistical evaluation of branch power revealed that only in the case of TRSNs the sample mean estimates the population expectation n = 1.5, a prerequisite for the application of Rall's "equivalent cylinder" model /4/. Further, the dependence of number, length and diameter of segments on branching order was determined for TRSNs. Terminal segments were shown to be longer in the average than intermediate ones. A significant loss of segments with increasing branching order could be stated.

Exemplified by a representative of each neuron class under study, the morphometrical data in Table 1 illustrate the different features of superficial vs deep layer SC neurons.

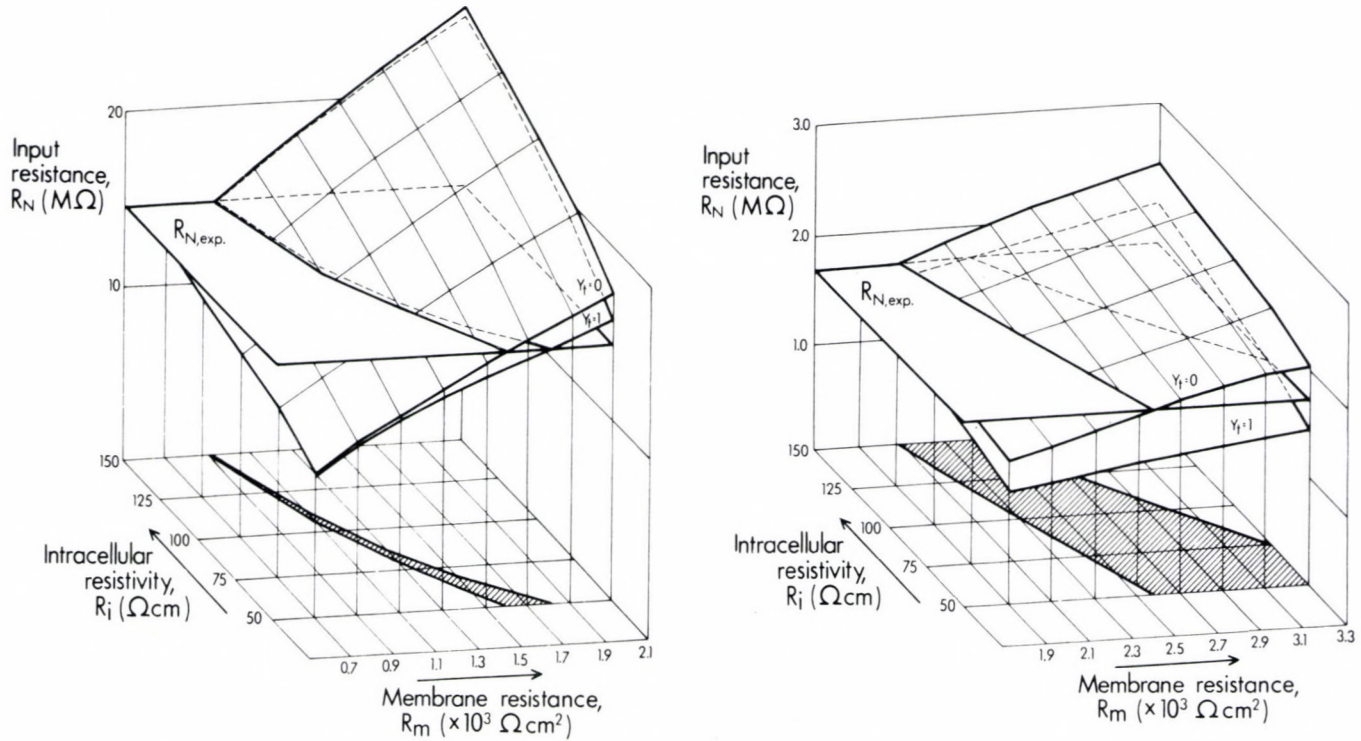

Fig. 1. 3-dimensional plots of calculated R_N vs assumed R_m and R_i for "sealed" (Y_t= 0) and "open" (Y_t=1) dendritic terminations. The intersections of the two corresponding R_N-surfaces with the plane $R_N = R_{N,exp.}$ denote the parameter region which is consistent with the experimental R_N-measurements. Left: APN, right: TRSN

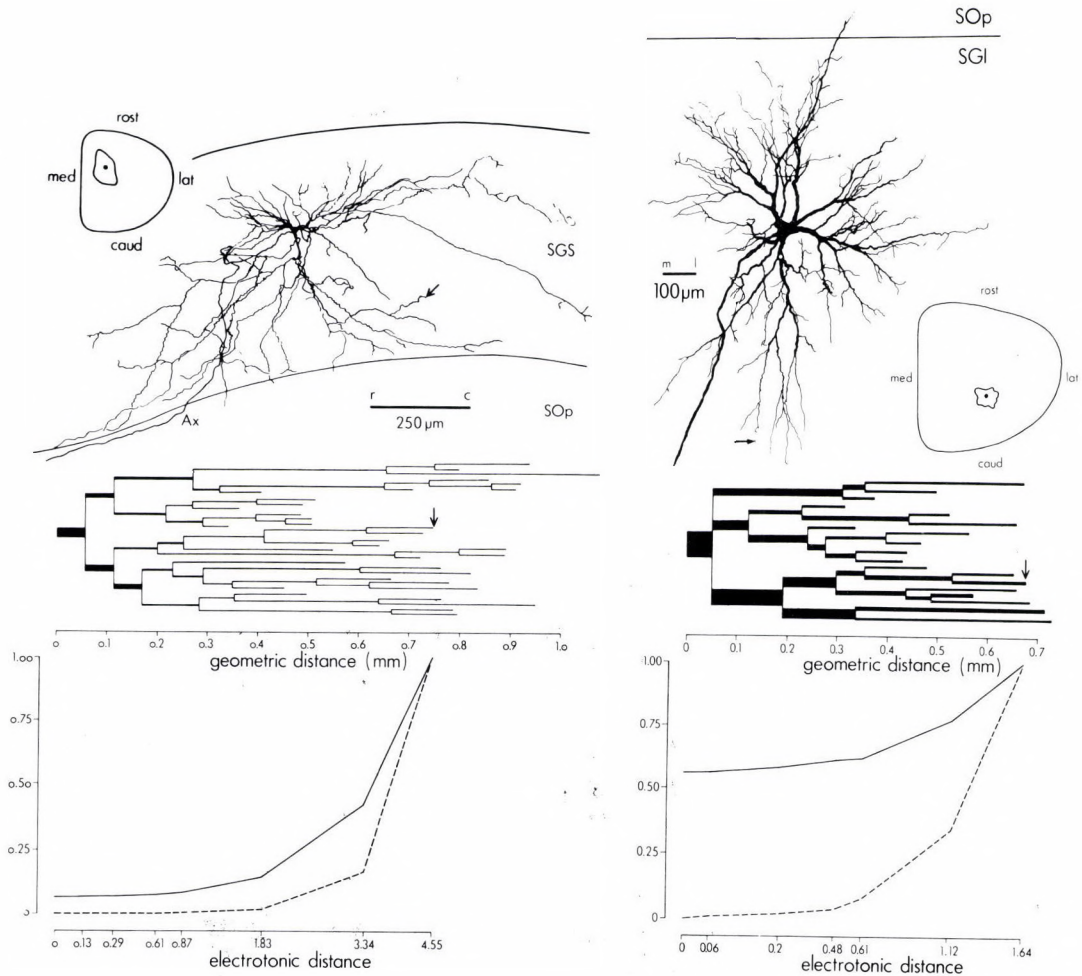

Fig. 2. Dendritic ramification patterns and transfer properties of the cells used in the calculations of Fig. 1. For each neuron the plane reconstruction, the Sholl diagram of one dendrite and the transfer ratios for current (T_c, continuous line) and voltage (dashed line) for a tonic synapse (arrow) on that dendrite are displayed

Table 1

Morphometrical characteristics*

Cell No.	Neuron type	No. of main dendrites	Extension of dendrites (μm)	$A_N(\mu m^2)$	A_D/A_S	n
6/2	ILN	3	220–850	43400	13.7	1.75
7/4	APN	4	250–1000	23500	20.6	1.60
28/2	TRSN	7	400–700	179000	21.4	1.44

The subsequent computation of electrotonic cell parameters by segmental cable modeling represents mathematically an inverse problem, i.e., comparison of experimental with theoretical input resistance value yields a parameter configuration (R_m, R_i, Y_t) which is consistent with the experimental data (Fig. 1). On the whole, the differences between superficial and deep layer neurons are even more distinct with regard to electrotonic properties (Table 2).

Table 2

Electrotonic properties*

Cell No.	$R_{N,exp.}$ (MΩ)	R_M (Ω cm^2)	L	ρ	T_S (%) at X=L/2	T_S (%) at soma
6/2	9.5	1850	1.96	5.7	47	24
7/4	14.6	1150	2.31	7.5	22	7
28/2	1.7	2300	0.95	15.7	68	56

DISCUSSION

One of the attempts at classifying nerve cells on the basis of their dendritic patterns is due to Ramón–Moliner /5/. As a criterion permitting the distinction of generalized (isodendritic) neurons from allo- and idiodendritic neurons, he noted that in the former daughter branches are, as a

*For explanations of symbols, see text

rule, longer than the parent branches. Our findings, however, revealed a pattern of almost constant branch length which is in agreement with the "terminal growth hypothesis" /10/. This pattern has been also established in an analysis of intrinsic and extrinsic determinants of neuronal form /3, 9/.

The branching exponent (n) has been turned out as an additional criterion with discriminative power in this context. For TRSNs n = 1.5 could be confirmed as expected for isodendritic neurons. In superficial SC neurons (APNs, ILNs), however, always succeeded this value, in conformity with findings on other neurons with specialized dendrites.

Allo- and idiodendritic neurons have been denoted as the appropriate "logical instrument for discriminative functions" /6/. By contrast, isodendritic cells are assumed to represent a suitable substrate for integrative functions. Our results support these suggestions. In particular, superficial SC neurons revealed an electrotonic anatomy which suggests that either dendritic spike generation serves to increase the amount of charge reaching the soma, or only specific spatio-temporal input patterns are effective in influencing the somatic spike trigger zone. Most probably, both mechanisms are acting in concert.

TRSNs show an electrotonically compact structure combined with high dendritic dominance, thus enabling an effective integration of their multimodal afferences (Fig. 2).

ACKNOWLEDGEMENT

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REFERENCES

1. Grantyn, R., Grantyn, A., Schierwagen, A. (1983) Passive membrane properties, afterpotentials and repetitive firing of superior colliculus neurons studied in the anesthetized cat. Exp. Brain Res. 50, 377–391.

2. Grantyn, R., Ludwig, R., Eberhardt, W. (1984) Neurons of the superficial tectal gray. An intracellular HRP-study on the kitten superior colliculus in vitro. Exp. Brain Res. 55, 172–176.

3. Hillman, E.E. (1979) Neuronal shape parameters and structures as a basis of neuronal form. In: Schmitt, F.O. and Worden, F.G. (eds) The Neurosciences. Fourth Study Program. MIT Press Cambridge, Mass., pp. 477–498.

4. Rall, W. (1977) Core conductor theory and cable properties of neurons. In: Kandel, E.R. (ed.) Handbook of Physiology. The Nervous System, Vol. 1, Part 1. Amer. Physiol. Soc., Bethesda, MD, pp. 39–97.

5. Ramón–Moliner, E. (1966) An attempt at classifying nerve cells on the basis of their dendritic patterns. J. Comp. Neurol. 119, 211–227.

6. Ramón–Moliner, E., Nauta, W.J.R. (1966) The isodendritic core of the brain stem. J. Comp. Neurol. 126, 311–336.

7. Schierwagen, A. (1986) Segmental cable analysis of electrotonic transfer properties of deep superior colliculus neurons in the cat. J. Hirnforsch. 27, 679–690.

8. Schierwagen, A., Grantyn, R. (1986) Quantitative morphological analysis of deep superior colliculus neurons stained intracellularly with HRP in the cat. J. Hirnforsch. 27, 611–623.

9. Székely, G., Antal, M. (1981) Significance of dendritic pattern in the function of the neurons. In. Székely, G. (ed.) Neural Communication and Control. Akadémiai Kiadó, Budapest, pp. 171–182.

10. Van Pelt, J., Verwer, R.W.H. (1985) Growth models (including terminal and segmental branching for topological binary trees. Bull. Math. Biol. 47, 323–336.

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COMPARATIVE VIEW OF THE CENTRAL ORGANIZATION OF AFFERENT AND EFFERENT CIRCUITRY FOR THE INNER EAR

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In all vertebrates, eighth nerve fibres from the inner ear distribute to target nuclei situated in the dorsolateral wall of the rhombencephalon. In amniotes, primary auditory and vestibular nuclei are readily delineated in that acoustic nuclei lie dorsal and sometimes rostral to vestibular nuclei. Fishes and aquatic amphibians have, in addition to labyrinthine organs, hair cell receptors in the lateral line system. Eighth nerve and lateral line fibres from these sense organs project to the octavolateralis region of the rhombencephalon. In this region, the primary nuclei cannot be easily divided into functionally distinct units. However, modality-specific zones seem to be present for auditory as well as lateral line projections lie dorsal and sometimes rostral to those from vestibular organs.

Projections from the primary auditory and vestibular nuclei to higher order centres follow pathways which are conservative in their architecture among vertebrates. Ascending auditory fibres project either directly or via relay nuclei to a large midbrain center, the torus semicircularis (inferior colliculus) and hence to the forebrain. In fishes and aquatic amphibians, the lateral line system also sends a projection to the midbrain and information from this system may be integrated with auditory input at that level. The organization of vestibulospinal and vestibulo-ocular pathways shows little variation throughout vertebrate phylogeny.

The sense organs of the inner ear of all vertebrates and of the lateral line system of anamniotes receive an efferent innervation. In anamniotes and some reptiles, the efferent supply origi-

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nates from a single nucleus (Octavolateralis Efferent Nucleus) while that of "higher" vertebrates arises from separate auditory and vestibular efferent nuclei. The biological significance of this innervation for all vertebrates is not yet understood. However, an important feature common to all is the association of the efferent system with the motor centres of the hindbrain.

Keywords: Auditory, vestibular, labyrinth, olivocochlear bundle, torus semicircularis

INTRODUCTION

The hair cells in the auditory and vestibular structures of the inner ear receive both an afferent and an efferent innervation. The sensory fibres project into the central nervous system to target nuclei that are located within the lateral rhombencephalic wall while the centrifugal nerve supply originates from neurons that lie deep in the hindbrain tegmentum. Our knowledge of the primary connections and higher order pathways of the inner ear of mammals have advanced considerably since Lorente de Nó /26/ first traced auditory and vestibular fibres to their destinations using the Golgi method. In the past decade or so there has been a surge of interest in how these pathways are organized in non-mammalian vertebrates. Nevertheless, the literature on auditory and vestibular connections in "lower" vertebrates, namely fishes and amphibians, remains extremely sparse and few studies have examined the organization from a comparative point of view.

In fishes, it is difficult to delineate specific auditory and vestibular centres in the hindbrain because in contrast to other vertebrates, there is no distinctive auditory organ in the ear; indeed, the otolithic organs are thought to be multimodal /42/. Furthermore, the presence of another, closely related sense in fishes, the lateral line, underlines the

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A: anterior nucleus, AC: auditory cortex, ANG: angular nucleus, Cb: cerebellum, D: descending nucleus, DCN: dorsal cochlear nucleus, DL: dorsolateral nucleus, I: intermediate nucleus, IC: inferior colliculus, L: lateral nucleus, LL: lateral lemniscus, LSO: lateral superior olive, M: medial nucleus, Mg: magnocellular nucleus, MG: medial geniculate body, MSO: medial superior olive, NLL: nucleus of the lateral lemniscus, OL: octavolateralis region, PT: posterior portion of thalamus, Rf: reticular formation, S: superior nucleus, SC: superior colliculus, SO: superior olive, T: tangential nucleus, tb: trapezoid body, Te: mesencephalic tectum, Tel: telencephalon, TS: torus semicircularis, VCN: ventral cochlear nucleus, VL: ventrolateral nucleus, VM: ventromedial nucleus, Y: nucleus Y

difficulty of deciding which organs are auditory. Presumably, sound and other disturbances in the water are detected by both the ear and the lateral line in fishes /5, 45/. Recent studies have suggested that the lateral line may function together with the otolithic organs in detecting different components of acoustic stimuli /5/. This would indicate that information from labyrinthine and lateral line detectors is integrated in the central nervous system and any study of their central connections should carefully consider how these centres are organized with respect to each other.

The aim of this review is to compare the central organization of the auditory and vestibular systems of fishes to those of other vertebrates. The preparation of this topic is based on several important questions: 1) Are separate auditory and vestibular centres to be found in fishes as in other vertebrates? 2) Do auditory and vestibular pathways of fishes resemble those of higher vertebrates? and 3) Is the efferent nerve supply organized in a similar manner throughout vertebrates?

CENTRAL ORGANIZATION OF PRIMARY AUDITORY AND VESTIBULAR CONNECTIONS

On entering the medulla, eighth nerve (VIIIn) fibres form bundles which ascend or descend to nuclei, which, in amniotes, can be readily divided into auditory and vestibular nuclei. The fascicles give off collateral fibres into the central neuropil of the nuclei. In mammals, the two primary auditory nuclei (Fig. 1D) are laminated and inputs are tonotopic, representing a highly ordered map of specific locations along the basilar membrane /22/. In birds, the nuclei angularis and magnocellularis (Fig. 1B) are probably homologous to the dorsal and ventral cochlear nuclei, respectively, of mammals /6/. Inputs are spatially ordered from the basilar papilla of birds and the nuclei are tonotopically organized /6/. In reptiles, there is some confusion over the number and location of the primary auditory centres /6, 13, 25, 51). Recently, Barbas-Henry and Lohman (personal communication) have shown that, at least in lizards, there are three primary auditory nuclei, which lie dorsal, but not rostral to vestibular centres. We know virtually nothing of frequency representation in the auditory zone of reptiles, except in crocodiles, where a tonotopic organization of the auditory nuclei has been reported /28/. The story in amphibians is complicated by the presence of more than one auditory structure at the inner ear. Most auditory fibres project to the dorsal medullary

nucleus /6, 56/ but there are also projections to other primary nuclei such as nucleus saccularis and nucleus caudalis and, in aquatic amphibians, to the intermediate nucleus, a region of lateral line termination /55, 56/. The dorsal medullary nucleus has been shown to be tonotopically organized in frogs /17/.

The organization of the primary auditory centres for most vertebrates, therefore, involves two (or more) nuclei which receive tonotopically organized inputs. A comparison to the arrangement in fishes is complicated, not only by the multiple roles of peripheral sense organs in these animals, but also by the way in which the primary sensory region is organized. Indeed, it seems unlikely that modality-specific nuclei exist at all in fishes.

The target zone for auditory, vestibular and lateral line fibres in fishes is termed the octavolateralis (OL) region and is composed of three cell columns: dorsal, intermediate and ventral /39/. The dorsalmost column, which is associated with electroreception, is absent in the vast majority of fishes and will, therefore, be excluded here. The intermediate or lateralis column lies dorsalmost in the OL region of non-electroreceptive fishes and is composed of nucleus medialis (Fig. 1A) and, in some fishes, nucleus caudalis /39/. Nucleus medialis is termed nucleus intermedius in cartilaginous fishes and aquatic amphibians /47,55/. The lateralis zone primarily receives input from mechanoreceptive lateral line receptors. The most ventral column is composed of three to five octaval nuclei (Fig. 1A) -anterior, magnocellular, tangential, descending, and posterior /39/. The octavus zone serves as the end station for most VIIIn fibres. As in other vertebrates, entering bundles of fibres, from VIIIn and lateral line nerves, bifurcate and ascend or descend and each fibre gives off abundant, irregularly spaced collateral branches (Fig. 2B) throughout the rostrocaudal extent of the region /33/. The neurons in the OL region are not grouped or arranged in laminae (Fig. 2A), except for the layer of large "Purkinje-like" or principal neurons that lie directly under the cerebellar crest and all neurons have extensive dendrites which often extend into the neighbouring cell column (Fig. 2A). Inputs from labyrinthine or lateralis organs or both are therefore likely to converge on the same cells (Fig. 2C) even though the incoming fibre bundles from VIIIn or the lateral line nerves appear to project to separate zones as illustrated in Fig. 3A.

Although many studies have now been conducted which examined primary input from VIIIn and lateral line nerves for several fishes /27/, few /2, 3, 32, 33, 37/ have looked at projections from individual labyrinthine organs.

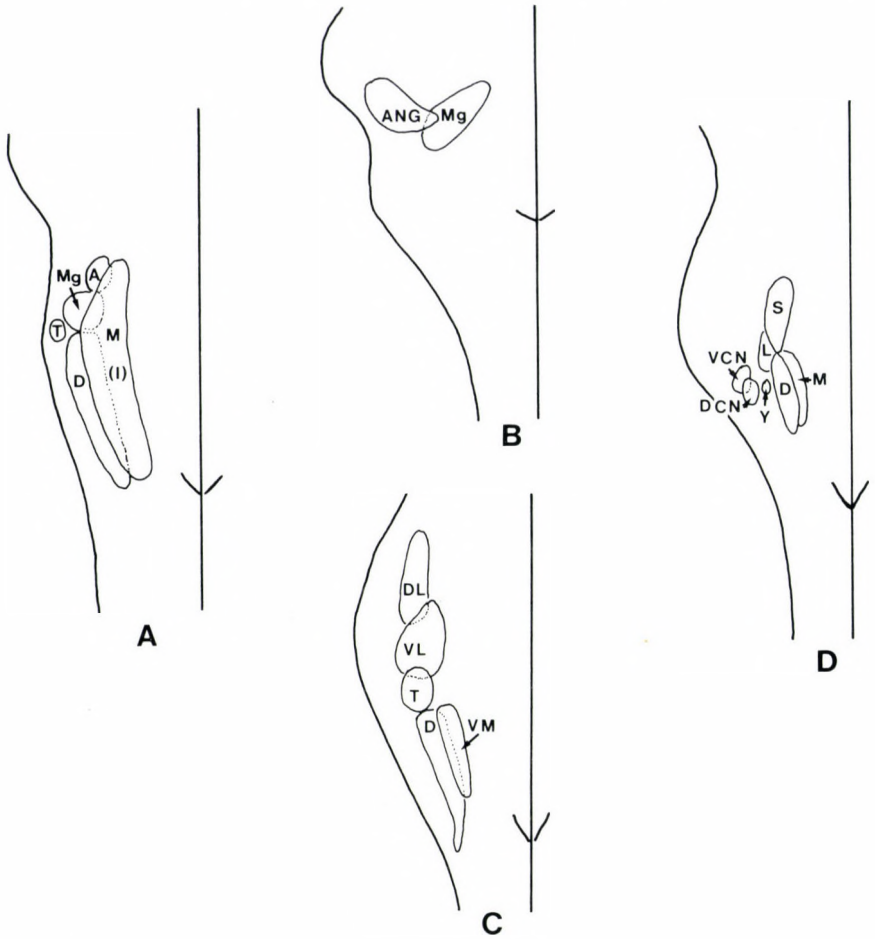


Fig. 1. Schematic diagrams of the locations of acoustic and vestibular nuclei (unilateral drawings) in different vertebrates as viewed from the dorsal surface of the medulla. Rostral is top in all drawings. (A) Octavolateralis region (fish—teleost). The lateralis zone is dorsal to the octavus zone. (B) Acoustic nuclei (bird). (C) Vestibular nuclei (reptile—lizard; drawing courtesy of H. Barbas-Henry). (D) Acoustic and vestibular nuclei (mammal)

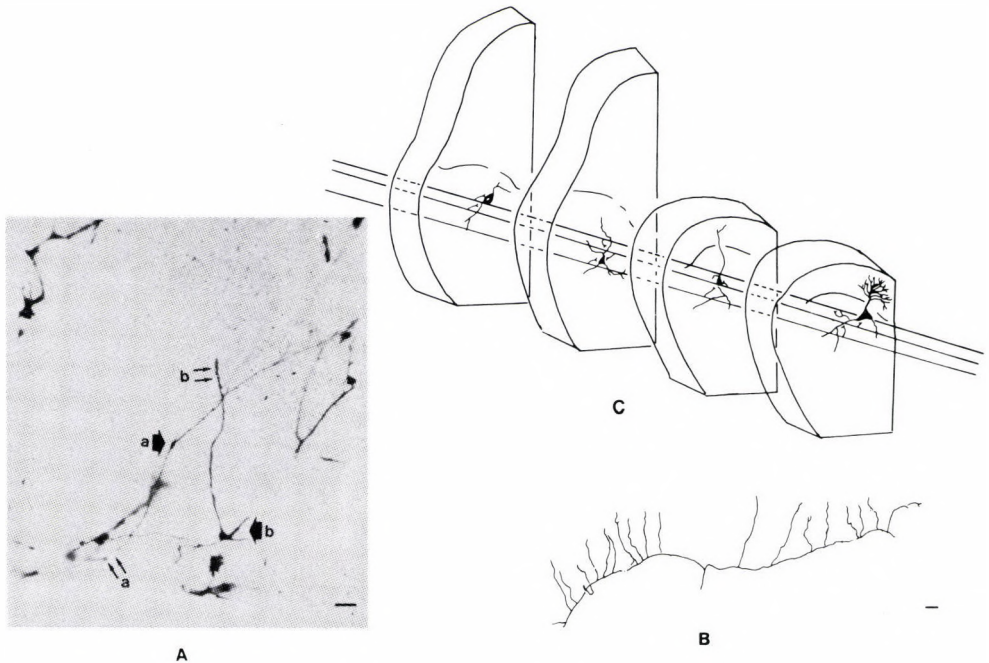


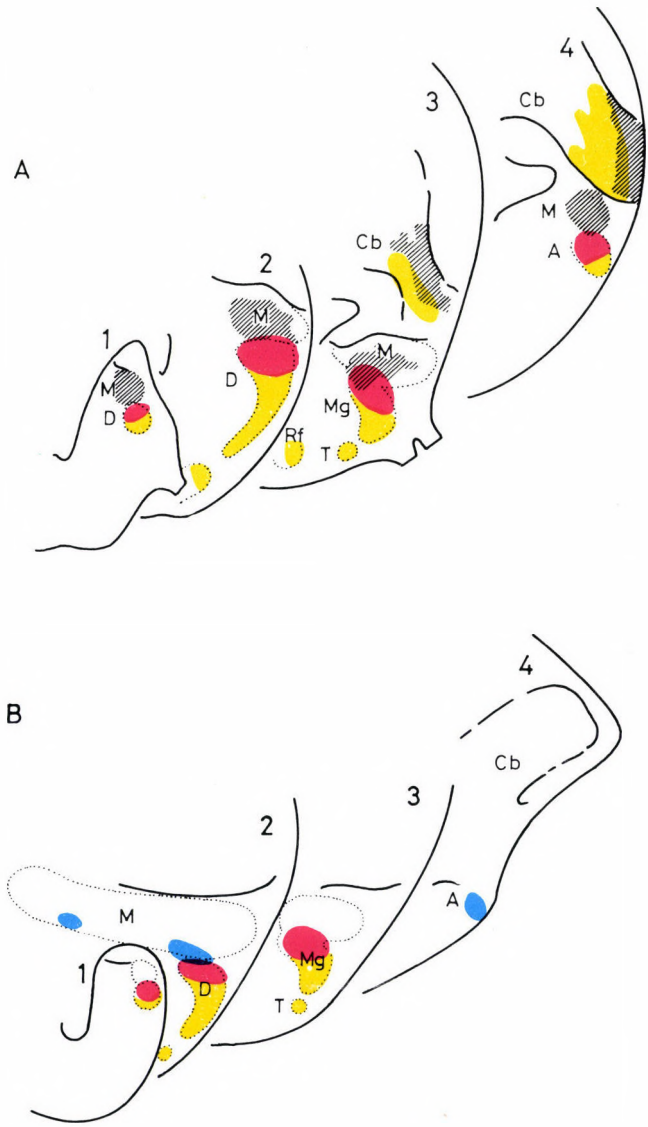
Fig. 2. (A) Photomicrograph of Golgi-impregnated neurons in the OL region of the fish, Astronotus ocellatus. The cell body of neuron "a" (thick arrow) lies in nucleus medialis and sends a ventral dendrite (small arrows) into the octavus column. The cell body of neuron "b" (thick arrow) lies in nucleus magnocellularis and sends its dorsal dendrite (small arrows) into the lateralis column. Bar scale is 100 μm. (B) camera lucida drawings made from serial horizontal sections to illustrate the trajectory, in the hindbrain, of two single nerve fibres from the anterior lateral line nerve of A. ocellatus. Note the extensive, medially-directed collaterals of each fibre. (C) Schematic diagram of transverse sections through the hindbrain (rostral is left) of A. ocellatus which illustrate how VIIIn and lateral line fibres can contact the same neurons within the OL region. Lower fibre is from VIIIn and upper 2 fibres from lateral line nerves

Therefore, our knowledge of how auditory and vestibular projections are arranged, is limited. Nevertheless, certain consistent features of organization emerge from these studies. For example, all nuclei of the octavus cell column, except the tangential nucleus, receive projections from all labyrinthine organs and the presumed auditory zone lies dorsal to the vestibular region (Fig. 3A). Some VIIIn fibres project into the lateralis column, where they overlap with fibres from the mechanoreceptive lateral line receptors. Lateral line fibres also intermingle with VIIIn projections in the nucleus magnocellularis of the octavus column (Fig. 3A).

In some teleosts, peripheral specializations can enhance acoustic sensitivity. For example, an air-filled cavity, functioning as a pressure transducer and sound amplifier, may be coupled to an otolithic organ /42/ as in the case of mormyrid fish or clupeids. An examination of the primary projections from the identified "acoustic" organs, therefore, could shed light on which OL nuclei or subdivisions are important for acoustic processing. Auditory projections in these fishes /3, 32/ do, indeed, terminate in dorsal portions of the octavus cell column (Fig. 3B) but some fibres also terminate rostrally in the octavus column, rostral to the vestibular projection zone (Fig. 3B–4) and others project bilaterally into nucleus medialis /3, 32/ (Fig. 3B–2). This projection pattern implies that the anterior nucleus, or its rostral portion, together with a portion of nucleus medialis are acoustic centres (Fig. 3B). This pattern may be unique to these fishes, however, for, in cartilaginous fishes, where the sacculus and macula neglecta have been identified as the acoustic organs /9/, the auditory projections /2/ more closely resemble those of bony fishes /33, 37/ that lack obvious auditory specializations (Fig. 3A). Moreover, the anterior nucleus does not receive an exclusive auditory projection nor is there a bilateral projection to the lateralis column. The picture is further complicated in that fibres from the macula neglecta intermingle extensively with vestibular projections from the semicircular canal cristae /2/.

If we turn our attention to the output side of the OL region we find that cells in the anterior nucleus and a portion of the descending nucleus project to the torus semicircularis (TS) /2, 3, 10, 11, 14/ — presumed homologue of the inferior colliculus /29/ — in the midbrain and therefore may be involved in the ascending auditory pathway. In addition, the TS also receives a projection from the lateral line center, nucleus medialis. The relationship of this pathway to the ascending auditory system, however, is not fully understood.

The primary auditory region in fishes, therefore, seems to lie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dorsal or rostral to the vestibular zone and is composed of 2 or 3 cell groups. However, no single nucleus, with the exception of the anterior nucleus of fishes with auditory specializations, can strictly be termed an auditory nucleus and data are still too limited to establish the presence of a defined auditory region which is the same for all fishes.

In contrast to the variability of auditory organization, vestibular centres are constructed more conservatively in vertebrates. The vestibular nuclei lie ventral or medial to acoustic centres in the alar plate and there are six vestibular nuclei in mammals /18/ — four major and two minor ones (Fig. 1D); six in birds /29/; five to six in reptiles /51/ (Fig. 1C); four in amphibians /56/; and in fishes, all octavus nuclei are involved in vestibular processing /2, 3, 33, 37/ (Fig. 1A). In all vertebrates, vestibular fibre bundles enter the hindbrain, bifurcate and distribute to the primary nuclei. In mammals, fibres from the semicircular canals have the most extensive distribution although they overlap consistently with fibres from the otolithic organs. Some fibres from all vestibular organs, except the saccule, project to the cerebellum and reticular formation /18/. Additionally, nucleus "Y" receives input exclusively from saccular fibres /18/. Basically, the organization of vestibular projections in other amniotes is similar /29/. There seems, however, to be no exclusively "saccular" nucleus /18/ except for nucleus sacculari s of amphibians /31, 55/ which, in contrast to nucleus "Y", seems to be a mixed nucleus involved in processing auditory and vibratory information (Will, personal communication).

In fishes, as in other vertebrates, vestibular terminations lie ventral to auditory areas (Fig. 3A) and some vestibular fibres course into the vestibulolateral lobe of the cerebellum and into the reticular formation. In contrast to other amniotes, octavus nuclei in fishes are multimodal in function and only the tangential nucleus serves exclusively as a vestibular nucleus /33/. Unlike mammals, the terminal fields from semicircular

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Fig. 3. (A) Drawings of transverse sections (rostralmost is section 4) through the octavolateralis region of bony fish, A. ocellatus. Stippled area represents the termination zone of lateral line fibres; red illustrates the presumed "acoustic" zone; and yellow denotes the vestibular field. Note the overlap of lateral line and VIIIn fibres in nucleus magnocellularis in section 3. (B) Drawings of transverse sections (rostralmost is section 4) through the octavolateralis region of a bony fish, Clupea harengus. Vestibular zone is indicated by yellow, auditory zone by red and the blue color indicates the areas which receive inputs exclusively from the acoustic sense organ

canal fibres in fishes are equal in rostrocaudal extent to those from the otolithic organs and the overlap between canal and saccular fibres is somewhat limited for saccular fibres are distributed only to dorsal portions of octavus nuclei. However, like mammals, fibres from the sacculus do not enter the cerebellum or reticular formation /2, 3, 33, 37/.

ORGANIZATION OF HIGHER ORDER AUDITORY AND VESTIBULAR CONNECTIONS

In most vertebrates, two hindbrain relay centres, the superior olivary nucleus and the nucleus of the lateral lemniscus, are interposed in the pathway to the midbrain /6, 22, 29/ (Fig. 4). These centres may also be present in the OL pathway of fishes /2, 4, 10, 11, 14, 35/ (Fig. 4). The first of these centres, the superior olive, varies in its degree of differentiation for different vertebrate species. The organization in mammals is complex; the region is composed of the trapezoid body and lateral and medial subdivisions of the superior olive /22/. This complexity is probably related to the role of the superior olive in resolving binaural time and intensity differences for different vertebrates /30/.

In non-mammalian groups, the superior olive is a single nucleus lying deep in the brainstem tegmentum; there is no trapezoid body but, in many species, another nucleus, situated some distance away, is thought to be homologous to the medial subdivision of the superior olive (MSO) of mammals. In birds and some, but not all, reptiles /6, 13, 25/, it is the nucleus laminaris, which lies in the primary auditory zone, that is the probable homologue to the MSO /6, 25, 40/. However, in contrast to the mammalian MSO, nucleus laminaris receives some primary auditory input /6, 40/. Indeed, nucleus laminaris in the lizard, Varanus, seems to receive a major primary input (Barbas-Henry and Lohman, personal communication). In amphibians, there is a nucleus saccularis which lies close to the primary auditory zone /31, 56/; in cartilaginous fishes, there is a single lamina of neurons (designated C1 or C2 /47/) in a similar location; and in a catfish, the medial auditory nucleus lies dorsally in the OL region /14/. The location and connections of these nuclei of amphibians and fishes, suggests that they are homologous to nucleus laminaris but since these nuclei in anamniotes and some reptiles receive a large primary input, they may not be homologous to the MSO of mammals. Further investigations are needed before we can fully appreciate how the "superior olive" of non-mammalian species is organized.

The higher order centres of the OL pathway of fishes have been

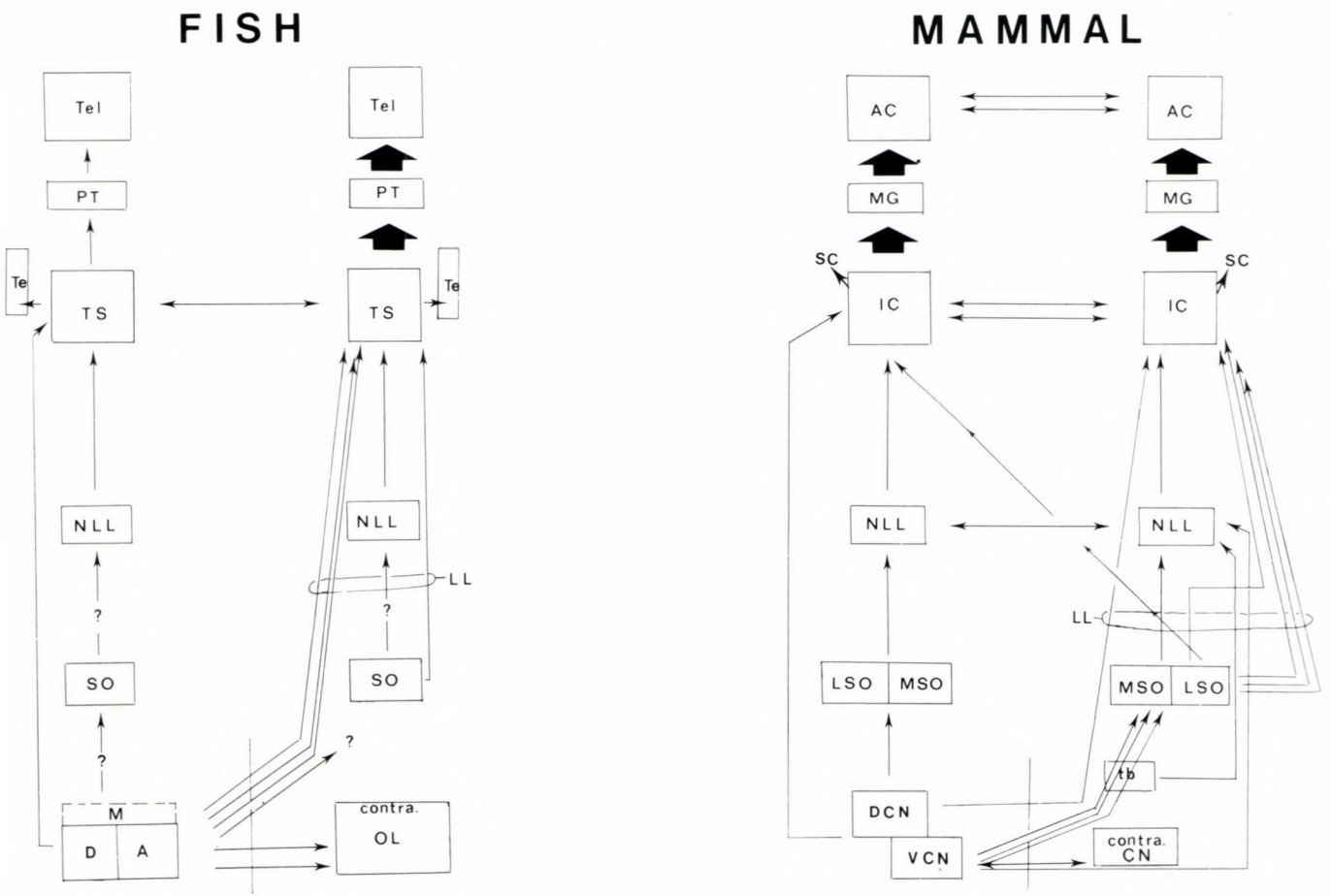

Fig. 4. Schematic diagrams of the auditory pathway in fishes (left) and mammals (right). These pathways are bilaterally organized. However, here they are illustrated as unilateral originating in rhombencephalic nuclei on the lower left of each diagram

little investigated. Nevertheless, we know that the TS is the mesencephalic target of the ascending auditory and lateral line pathways and that it receives direct input from the medial, anterior and descending nuclei of the OL region, the superior olivary nucleus and the nucleus of the lateral lemniscus /2, 4, 10, 11, 14/. Some investigators have suggested that acoustic information remains fully separate from lateral line input at the TS, i.e., auditory information processed medial to lateral line input /11, 14, 39/. However, recently, evidence has emerged which shows that there is considerable convergence of lateralis and auditory information at this level /10, 38, 55, 57/. This is not unreasonable if we presume that the lateral line and ear function together detect acoustic signals /5, 45/ and that the information from these receptors converge centrally. The TS, which is an important multisensory centre, could be the place of such convergence /38, 57/.

In most vertebrates, neurons in the midbrain acoustic centre (inferior colliculus of mammals and torus semicircularis of other vertebrates) project to the mesencephalic tectum (superior colliculus of mammals) and to the posterior portion of the thalamus (Fig. 4). The thalamic region goes by a variety of names /6/ e.g., medial geniculate body (mammals), nucleus ovoidalis (birds) and nucleus reunions (reptiles). In fishes, the TS also connects with the mesencephalic tectum and the posterior thalamus, termed the lateral posterior nucleus of elasmobranchs /47/ and central posterior nucleus of teleosts /11, 39/. Furthermore, the OL pathway in fishes, just as in other vertebrates, continues forward from the thalamus to the telencephalon /24, 39, 47/ (Fig. 4). Whether or not these projections in fishes are homologous to those in mammals or other vertebrates has yet to be investigated.

If we turn our attention to higher order vestibular pathways, we find that comparisons are even more difficult due to the sparsity of data from anamniotes. The ascending connections of the vestibulo-ocular circuit have been examined in the goldfish and an elasmobranch /20, 21/. These projections resemble those described for other vertebrates /29/. The vestibulospinal pathways are also similar in all vertebrates. In bony fishes, as in other vertebrates, vestibulospinal fibres arise from the octavus nuclei situated close to the entry of VIIIn (magnocellular, tangential and the rostral pole of the descending nucleus in fishes (Fig. 1A)) and course to the spinal cord in a medial pathway within the descending MLF. In cartilaginous fishes /2, 47/, as in mammals /18/, there are two pathways to

the spinal cord, one medial and the other lateral via Steida's fasciculus /47/. Other projections of the vestibular nuclear complex, which are well known in mammals and some other vertebrates — amniotes and anamniotes alike — such as the commissural connections with the contralateral vestibular nuclei and the vestibulo-cerebellar and vestibulo-olivary connections, have yet to be investigated in fishes.

CENTRIFUGAL INNERVATION OF THE INNER EAR

A centrifugal or efferent innervation of the OL sense organs, arising from neurons grouped in the rhombencephalic tegmentum, has been reported for all classes of vertebrates. The efferent neurons form separate auditory and vestibular groups in mammals /53, 54/, birds /46, 50/ and certain reptiles /48/ but in fishes /3, 8, 23, 34, 36/, amphibians /8, 15, 16, 41/ and other reptiles /49/, the efferent neurons form a single nucleus called the octavolateralis nucleus (OEN) /34/. The precise location of these neurons varies considerably between species (Fig. 5). Nevertheless, the cells are always close to or are contained within the lateral motor column of the brainstem and show a special relationship to facial motoneurons /34, 36, 41/ (Fig. 5). The primary neurotransmitter in the efferent system is thought to be acetylcholine but recent work in mammals has shown that other transmitter molecules such as GABA and certain peptides, e.g., enkephalins, may be involved /1, 12/.

The efferent supply originates bilaterally in all vertebrates, except certain amphibians /16. 41/ and the contralateral component is approximately 5—10% of the total projection in bony fishes /3, 23, 36/, up to 40% in a cartilaginous fish /34/, and as much as 55% in mammals /52, 53, 54/. The axons of efferent neurons may branch to supply more than one end organ /8, 44, 46/ and the same neuron may even innervate receptors of different modalities /3, 8, 34, 36/.

Functional (auditory and vestibular) subdivisions which can be recognized in the efferent system of terrestrial vertebrates, are harder to make in fishes. As we have seen, this problem relates to the fact that sharp separation of roles cannot be made for the OL end organs. The OEN has been tentatively subdivided in the toadfish where the efferent innervation of each vestibular organ may originate from different sets of efferent neurons /23/ and in the European eel, where the rostral pole of OEN may constitute

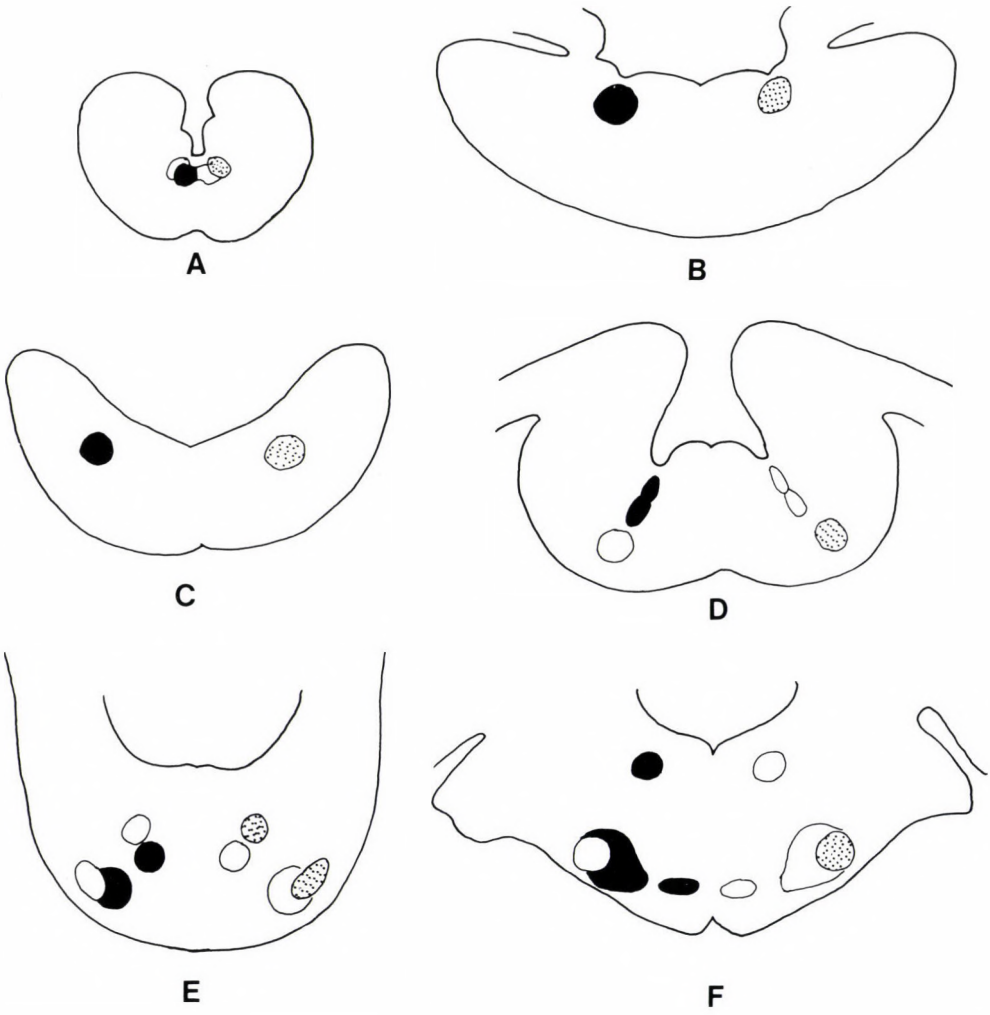

Fig. 5. Diagrams, from different vertebrates, of a transverse section through the medullary efferent zone. These illustrate the relationship between the facial motor nucleus — shown as a stippled area on the right side of each drawing — and the efferent nuclei — shown in black on the left side of each drawing. Sources: (A), teleost fish /36/; (B), elasmobranch /34/; (C), anuran amphibian /41/; (D), reptile /48, 49/; (E), bird /50/; (F), mammal /53/

an auditory subdivision /35, 36/. Data from anamniotes, however, are still too limited to establish how ΠEN reflects the functional modalities of the OL system.

The numbers of efferent neurons in most vertebrates are small when compared to those found in mammals (see Table 1). It is estimated that, in the mammalian labyrinth, one efferent neuron supplies approximately 40 hair cells /19/. In contrast, in the dogfish lateral line, one efferent neuron innvervates up to 400 hair cells /34/ and in the utriculus of the eel, one efferent neuron supplies approximately 850 hair cells (unpublished observations). The high numbers of olivocochlear neurons in mammals may relate to the fact that the efferent projection is tonotopically organized /43/ but we have no clear idea of why numbers of efferent neurons are different for other sense organs. Certainly, more data are needed before we can begin to explain the differences in efferent supply among different species.

We now have a small body of knowledge about the location and conformation of the centrifugal innervation of the inner ear for several different species and we find that the efferent system is clearly a basic feature of brainstem organization. There are, however, many questions which remain unanswered. For example, we know nothing of the central connections of the efferent neurons; we have no coherent interpretation of the role of the efferent system in sensory function nor do we understand how the system is activated.

CONCLUDING REMARKS

Studies of peripheral and central auditory and vestibular pathways in vertebrates, have for the most part, proceeded independently. Since audition and postural mechanisms are different for an aquatic animal, we might predict that the central wiring from the inner ear would reflect differences at the periphery. However, as this review has shown, much of the organization of the brainstem is conservative and the centres of the auditory and vestibular pathways are longstanding, basic features of the brains of vertebrates. The story, however, is far from complete. Although we know what constitutes the primary OL region of fishes, more information is certainly needed to establish which otolithic organs are responsive to sound and moreover, which primary projections are the acoustic ones. Further experimental studies are needed in non-mammalian vertebrates to determine how higher order

Table 1

Numbers of efferent neurons

(Numbers of neurons which supply a single labyrinth)

Animal	Total efferents to single labyrinth	auditory efferent neurons	vestibular efferent neurons	saccular efferent neurons	all semi-circular canals
FISH:					
Scyliorhinus canicula	24				
Anguilla anguilla				40	
Opsanus tau				131	
AMPHIBIA:					
Bufo bufo	25				
Salamandra salamandra	22				
Ichthyophis khotaoensis	8				
Boulengerula boulengeruli	13				
REPTILIA:					
Terapene ornata		78			63
Caiman crocodilus		263			196
AVES:					
Gallus domesticus	294		116		
Columba livia					200
MAMMALIA:					
Felis domesticus		1746	420		
Saumiri sciureus*		1750	502		
guinea pig		1234			
albino rat		578	228		

*Numbers of cochlear efferents from Thompson and Thompson /52/; vestibular efferents from Goldberg and Fernandez /19/.

Sources: Scyliorhinus /34/; Anguilla /36/; Opsanus /23/; Bufo /41/; Salamandra /15/; Ichthyophis /16/; Boulengerula /16/; Terapene /49/; Caiman /48/; Gallus /50/; Columba /46/; Felis /53/; Saumiri /19, 52/; guinea pig /44/; albino rat /54/.

OL pathways are organized. We have, as yet, little information about acoustic representation in the telencephalon and diencephalon of fishes and our data on vestibular connections are limited to the vestibulo-ocular and vestibulospinal pathways. The largest gap in our knowledge of the OL system, however, must lie in the organization of the efferent innervation. We know little of the basic significance of this system, its central connections, or how it might be related to the sensory pathways. Herein lies a major challenge for the next decade!

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REFERENCES

1. Altshuler, R.A., Parakkal, M.H., Rubio, J.A., Hoffman, D.W., Fex, J. (1984) Enkephalin-like immunoreactivity in the guinea pig organ of Corti: ultrastructural and lesion studies. Hearing Res. 16, 17–31.

2. Barry, M.A. (1987) Afferent and efferent connections of the primary octaval nuclei in the clearnose skate, Raja eglanteria. J. Comp. Neurol. 246, 457–477.

3. Bell, C. (1981) Central distribution of octavolateral afferents and efferents in a teleost (Mormyridae). J. Comp. Neurol. 195, 391–414.

4. Bell, C. (1981) Some central connections of medullary octavolateral centers in a mormyrid fish. In: Tavolga, W.N., Popper, A.N., Fay, R.R. (eds) Hearing and Sound Communication in Fishes. Springer-Verlag, New York.

5. Blaxter, J.H.S., Denton, E.J., Gray, J.A.B. (1981) Acousticolateralis system in clupeid fishes. In: Tavolga, W.N., Popper, A.N., Fay, R.R. (eds) Hearing and Sound Communication in Fishes. Springer-Verlag, New York.

6. Campbell, C.B.G., Boord, R.L. (1974) Central auditory pathways of non-mammalian vertebrates. In: Kreidel, W.D., Neff, W.D. (eds) Handbook of Sensory Physiology, Vol. V, Part 1. Springer-Verlag, New York.

7. Cant, N.B., Gaston, K.C. (1982) Pathways connecting the right and left cochlear nuclei. J. Comp. Neurol. 212, 313–326.

8. Claas, B., Fritzsch, B., Münz, H. (1981) Common efferents to lateral line and labyrinthine hair cells in aquatic vertebrates. Neurosci. Lett. 27, 231–235.

9. Corwin, J.T. (1981) Peripheral auditory physiology in the lemon shark: evidence of parallel otolithic and nonotolithic sound detection. J. Comp. Physiol. A 142, 379–390.

10. De Wolf, F.A., Schellart, N.A.M., Hoogland, P.V. (1983) Ocatvolateral projections to the torus semicircularis of the trout, Salmo gairdneri. Neurosci. Lett. 38, 209–213.

11. Echteler, S.M. (1984) Connections of the auditory midbrain in a teleost fish, Cyprinus carpio. J. Comp. Neurol. 230, 536–551.

12. Fex, J., Altschuler, R.A., Kachar, B., Wenthold, R.W., Zempel, J.M. (1986) GABA visualized by immunocytochemistry in the guinea pig cochlea in axons and endings of efferent neurons. Brain Res. 366, 106–117.

13. Foster, R.E., Hall, W.C. (1978) The organization of central auditory pathways in a reptile, Iguana iguana. J. Comp. Neurol. 178, 783–832.

14. Finger, T.E., Tong, S.-L. (1984) Central organization of eighth nerve and mechanosensory lateral line systems in the brainstem of ictalurid catfish. J. Comp. Neurol. 229, 129–151.

15. Fritzsch, B. (1981) Efferent neurons to the labyrinth of Salamandra salamandra as revealed by retrograde transport of horseradish peroxidase. Neurosci. Lett. 26, 191–196.

16. Fritzsch, B., Crapon De Caprona, D. (1984) The origin of centrifugal inner ear fibers of gymnophions (amphibia). A horseradish peroxidase study. Neurosci. Lett. 46, 131–136.

17. Fuzessery, Z.M., Feng, A.S. (1981) Frequency representation in the dorsal medullary nucleus of the leopard frog, Rana p. pipiens. J. Comp. Physiol. 143, 339–349.

18. Gacek, R.R. (1969) The course and central termination of first order neurons supplying vestibular endorgans in the cat. Acta Oto-Laryngol. (Stockh.) (Suppl.) 254, 1–66.

19. Goldberg, J.M., Fernandez, C. (1980) Efferent vestibular system in the squirrel monkey: anatomical location and influence on afferent activity. J. Neurophysiol. 43, 986–1025.

20. Graf, W., Brunken, W.J. (1984) Elasmobranch oculomotor organization: Anatomical and theoretical aspects of the phylogenetic development of vestibulo-oculomotor connectivity. J. Comp. Neurol. 227, 569–581.

21. Graf, W., McGurk, J.F. (1985) Peripheral and central oculomotor organization in the goldfish, Carassius auratus. J. Comp. Neurol. 239, 391–401.

22. Harrison, J.M. (1978) Functional properties of the auditory system of the brain stem. In: Masterton, R.B. (ed.) Handbook of Behavioral Neurobiology, Vol. I, Sensory Integration. Plenum Press, New York.

23. Highstein, S.M., Baker, R. (1986) Organization of the efferent vestibular nuclei and nerves of the toadfish, Opsanus tau. J. Comp. Neurol. 243, 309–325.

24. Ito, H., Murakami, T., Fukuoka, T., Kishida, R. (1986) Thalamic fiber connections in a teleost (Sebastiscus marmoratus): Visual, somatosensory, octavolateral, and cerebellar relay region to the telencephalon. J. Comp. Neurol. 250, 215–227.

25. Künzle, H. (1986) Projections from the cochlear nuclear complex to rhombencephalic auditory centers and torus semicircularis in the turtle. Brain Res. 379, 307–319.

26. Lorente de Nó, R. (1933) Anatomy of the eighth nerve. I. The central projection of the nerve endings of the internal ear. Laryngoscope 43, 1–38.

27. McCormick, C.A. (1983) Organization and evolution of the octavolateralis area of fishes. In: Northcutt, R.G., Davis, R.E., (eds) Fish Neurobiology. Vol. I: Brain Stem and Sense Organs. Univ. Mich. Press, Ann Arbor.

28. Manley, G.A. (1970) Comparative studies of auditory physiology in reptiles. Z. vergl. Physiol. 67, 363–381.

29. Masterton, R.B., Glendenning, K.K. (1978) Phylogeny of the vertebrate sensory systems. In: Masterton, R.B. (ed.) Handbook of Behavioral Neurobiology. Vol. I. Sensory Integration. Plenum Press, New York.

30. Masterton, B., Heffner, H., Ravizza, R. (1969) The evolution of human hearing. J. Acoust. Soc. Amer. 45, 966–985.

31. Matesz, C. (1979) Central projection of the VIIIth cranial nerve in the frog. Neuroscience 4, 2061–2071.

32. Meredith, G.E. (1985) The distinctive central utricular projections in the herring. Neurosci. Lett. 55, 191–196.

33. Meredith, G.E., Butler, A.B. (1983) Organization of eighth nerve afferent projections from individual endorgans of the inner ear in the teleost, Astronotus ocellatus. J. Comp. Neurol. 220, 44–62.

34. Meredith, G.E., Roberts, B.L. (1986) Central organization of the efferent supply to the labyrinthine and lateral line receptors of the dogfish. Neuroscience 17, 225–233.

35. Meredith, G.E., Roberts, B.L. (1986) The relationship of saccular efferent neurons to the superior olive in the eel, Anguilla anguilla. Neurosci. Lett. 68, 69–72.

36. Meredith, G.E., Roberts, B.L. (1987) The distribution and morphological characteristics of efferent neurons innervating end organs in the ear and lateral line of the European eel. J. Comp. Neurol. 265, 494–506.

37. Meredith, G.E., Roberts, B.L., Maslam, S. (1987) The distribution of afferent fibers in the brainstem from end organs in the ear and lateral line in the European eel. J. Comp. Neurol. 265, 507–520.

38. Niderstigt, L.J.A., Schellart, N.A.M. (1986) Acousticolateral processing in the torus semicircularis of the trout Salmo gairdneri. Pflügers Archiv. 406, 151–157.

39. Northcutt, R.G. (1981) Audition in the central nervous system of fishes. In: Tavolga, W.N., Popper, A.N., Fay, R.R. (eds) Hearing and Sound Communication in Fishes. Springer-Verlag, New York.

40. Parks, T.N., Rubel, E.W. (1978) Organization and development of the brain stem auditory nuclei of the chicken: primary afferent projections. J. Comp. Neurol. 180, 439–448.

41. Pelligrini, M., Ceccotti, F., Magherini, P. (1985) The efferent vestibular neurons in the toad (Bufo bufo L.): their location and morphology. A horseradish peroxidase study. Brain Res. 344, 1–8.

42. Popper, A.N., Coombs, S. (1980) Auditory mechanisms in teleost fishes. Am. Sci. 68, 429–440.

43. Robertson, D., Gummer, M. (1985) Physiological and morphological characterization of efferent neurones in the guinea pig cochlea. Hearing Res. 20, 63–77.

44. Robertson, D., Cole, K.S., Harvey, A.R. (1987) Brainstem organization of efferent projections to the guinea pig cochlea studied using the fluorescent tracers fast blue and diamidino yellow. Exp. Brain Res. 66, 449–457.

45. Sand, O. (1981) The lateral line and sound reception. In: Tavolga, W.N., Popper, A.N., Fay, R.R. (eds) Hearing and Sound Communication in Fishes. Springer-Verlag, New York.

46. Schwarz, I.E.,Schwarz, D.W.F., Frederickson, J.M., Landolt, J.P. (1981) Efferent vestibular neurons: a study employing retrograde tracer methods in the pigeon (Columba livia). J. Comp. Neurol. 196, 1–12.

47. Smeets, W.J.A.J., Nieuwenhuys, R., Roberts, B.L. (1983) The Central Nervous System of Cartilaginous Fishes. Springer-Verlag, New York.

48. Strutz, J. (1981) The origin of centrifugal fibers to the inner ear in Caiman crocodilus. A horseradish peroxidase study. Neurosci. Lett. 27, 95–100.

49. Strutz, J. (1982) The origin of efferent fibers to the inner ear in a turtle (Terapene ornata). A horseradish peroxidase study. Brain Res. 244, 165–168.

50. Strutz, J., Schmidt, C.L. (1982) Acoustic and vestibular efferent neurons in the chicken (Gallus domesticus). A horseradish peroxidase study. Acta Otolaryngol. 94, 45–51.

51. Ten Donkelaar, H.J., Bangma, G.C., Barbas-Henry, H.A., De Boer-Van Huizen, R., Wolters, J.G. (1987) The brain stem in a lizard, Varanus exanthematicus. Adv. Anat. Embryol. Cell Biol. 107, 1–168.

52. Thompson, G.C., Thompson, A.M. (1986) Olivocochlear neurons in the squirrel monkey brainstem. J. Comp. Neurol. 254, 246–258.

53. Warr, B.W. (1975) Olivocochlear and vestibular efferent neurons of the feline brain stem; their location, morphology and number determined by retrograde axonal transport and acetylcholinesterase histochemistry. J. Comp. Neurol. 161, 159–182.

54. White, J.S., Warr, W.B. (1983) The dual origins of the olivocochlear bundle in the albino rat. J. Comp. Neurol. 219, 203–214.

55. Will, U. (1987) Organization and projections of the area octavolateralis in amphibians. In: Fritzsch, B. et al. (eds) Evolution of the Amphibian Auditory System. Wiley and Sons, New York.

56. Will, U., Luhede, G., Görner, P. (1985) The area octavo-lateralis in Xenopus laevis I. The primary afferent projections. Cell Tiss. Res. 239, 147–161.

57. Zitlau, K.E., Claas, B., Münz, H., Görner, P. (1985) Multisensory interaction in the torus semicircularis of the clawed toad Xenopus laevis. Neurosci. Lett. 60, 77–81.

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PROCESSING OF WAVE PATTERNS IN THE LATERAL LINE SYSTEM PARALLELS TO AUDITORY PROCESSING

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Processing of wave patterns in the lateral line system is reviewed with particular reference to similarities with auditory processing. Four levels are considered: the receptor cell, stimulus parameter encoding in the afferent nerve, the neural organization of stimulus localization, and analysis of complex waves. The high degree of parallelism at all these levels to auditory processing is considered as a strong evidence for a common evolutionary origin of these two systems.

Keywords: Lateral line system, auditory system, afferent encoding, stimulus localization, complex stimuli

The vertebrate auditory and lateral line systems have long been considered as subsystems of one common system, the acousticolateral or octavolateralis system; recently, however, their common evolutionary origin has been questioned /5, 30/. Until now, the relationship between these two systems has been discussed only on anatomical and developmental grounds, because knowledge about stimulus processing in the mechanoreceptive lateral line system was lacking. Only the electroreceptive lateral line system has been investigated in detail /7/. That system, however, is highly specialized and thus seems to be less suitable for the evaluation of common principles of stimulus processing in the acousticolateral system than the mechanoreceptive lateral line system. The goal of this paper, therefore is to present

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the results of recent investigations of stimulus analysis in the mechanoreceptive lateral line system that demonstrate considerable parallels to analysis in the auditory system of vertebrates. Hence, the term 'lateral line system' will be used only with regard to the mechanoreceptive lateral line system.

The morphological similarities, even at the electron microscopic level, of the sensory hair cells in the auditory, vestibular, and lateral line systems originally led to the suggestion of a uniform mechanism of mechano-electrical transduction in these cells /17/. This has recently been confirmed by intracellular recordings /21, 22, 37/. These recordings, though not yet providing a conclusive proof, provide strong evidence that the mechano-electrical transduction that occurs from the deflection of the sensory hairs generating the hair cell's receptor potential, results from the same mechanisms and follows common rules, whether in the auditory, vestibular, or lateral line system. The deflection of the cilia in one direction entails depolarization of the receptor potential, deflection in the opposite direction entails hyperpolarization, and deflections in other directions produce potential changes that correspond in size to the cosine between the deflection and the axis of maximal sensitivity. The change of the potential follows the deflection immediately, with time constants being fractions of a millisecond. The depolarization voltage increases with the magnitude of hair deflection over a wide range, whereas hyperpolarization saturates soon. These common properties of the primary processes of stimulus transduction have been treated in detail by recent reviews /21, 22, 37/ and will therefore not be discussed here further.

Mechano-electrical transduction at the receptor level is, however, but one link in the chain from the external stimulus to encoding of its parameters in the afferent discharge, which is the basis for central nervous system processing. At the beginning, there are mechanical links or accessory structures through which the stimulus waves are transformed into deflection of the sensory hairs, and these links can differ strongly both between the sensory systems and their submodalities and within a system between species. At the end, there is the transformation from the receptor potential of the hair cell to the discharge of the afferent fiber. One might ask therefore, whether there exist common principles of stimulus encoding in auditory and lateral line afferent fibers.

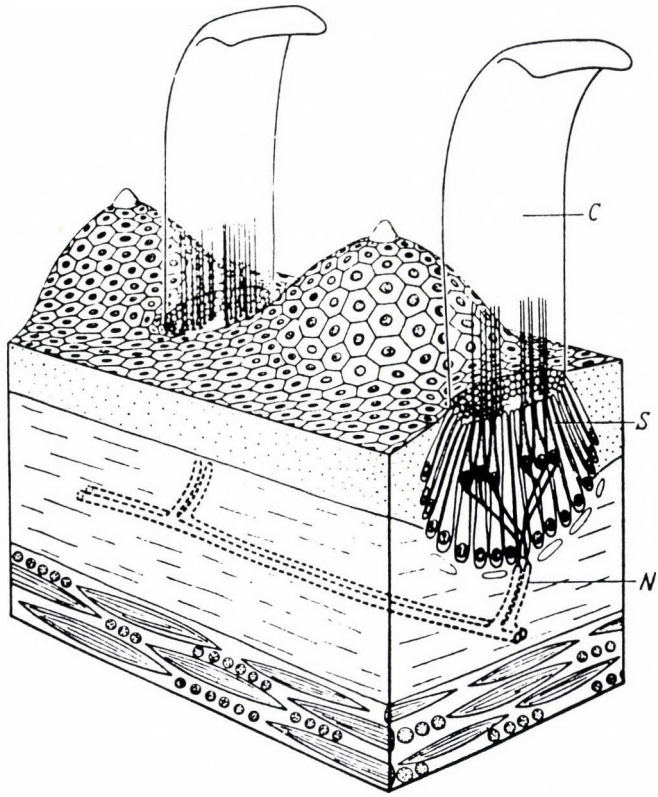
We have recorded, in the aquatic clawed frog, Xenopus laevis, the responses of lateral line afferent fibers to water waves under natural

conditions /14/. The immobilized frog was suspended on a holder in an approximately natural posture in the center of a water basin. Surface waves were produced by a vibrator at 12 cm distance from the frog, and the responses of single afferent fibers were recorded.

The lateral line organs of Xenopus are epidermal neuromast organs, and their cupulae protrude for approximately 100 µm into the surrounding water (Fig. 1). Thus, impinging waves can directly deflect the cupula and with it the enclosed sensory hairs of the receptor cells. The 100–500 hair cells of each neuromast organ consist of two populations: the cells of the one population are maximally depolarized by deflection of the cupula in one direction, the cells of the other population by deflection in the opposite direction. The cells of each population contact one of the organ's two afferent neurons, respectively. These neurons then project into the medulla /19, 36/. Thus, the anatomical organization of this organ is just the opposite to that of the mammalian ear: it is mechanically simple without intermediate auxiliary structures, but many hair cells are contacted by a single afferent fiber. The ear, however, possesses a complicated conducting apparatus in the middle and inner ear, but the afferent neurons from inner hair cells contact only one hair cell, respectively. This anatomical difference notwithstanding, we found in the lateral line system the same form of afferent stimulus encoding as is known in the auditory system.

Without stimulation, the afferent lateral line fibers of Xenopus fire spontaneously 5–25 imp/s at irregular intervals. With increasing stimulus intensity the discharges become more and more phase coupled to the wave cycle. The degree of phase coupling increases linearly with the logarithm of stimulus intensity. The slope of the increase is independent of stimulus frequency. At 25–30 dB above response threshold, phase coupling saturates at vector strengths of 0.75–0.90 (Fig. 2A). The mean firing rate changes little at low stimulus intensities because increased firing at one phase of the wave cycle is compensated by reduced firing at the opposite phase. When, however, phase coupling has reached its maximum – which implies that firing suppression in the opposite phase intervals has reached its maximum as well – firing rate during the excitatory phase of the response cycle increases further with increasing stimulus intensity, and then, consequently, the mean firing rate increases as well. Like for phase coupling, the firing rate increases linearly with the logarithm of stimulus intensity and the slope of the increase is independent of stimulus frequency.

A



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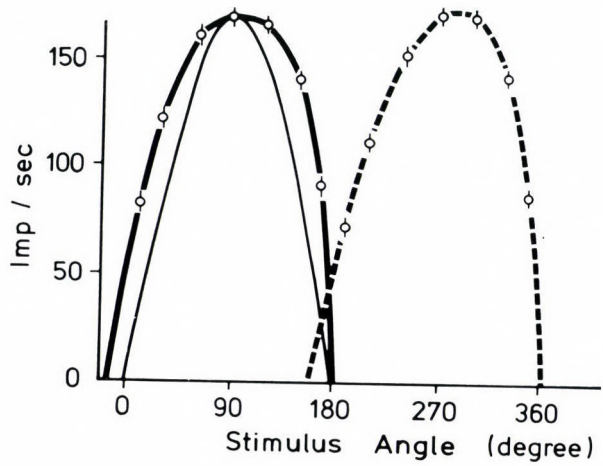

This dual kind of intensity encoding, low intensities by phase coupling and high intensities by mean firing rate, with both encoding mechanisms following a logarithmic function of stimulus intensity at a slope that is independent of stimulus frequency, coincides exactly with intensity encoding in mammalian low-frequency auditory afferent fibers below their characteristic frequency /18, 24, 32, 38/.

For the analysis of water waves and their low frequencies, the mean firing rate of the response measured over the whole cycle of the stimulus wave is inappropriate for encoding stimulus intensity because at high intensities all spikes concentrate at one phase so that firing is completely suppressed for up to 70% of the cycle, which e.g. for a 5 Hz wave would mean 140 ms. Therefore we measured the firing rate in the 30% of the cycle during which firing was maximal and called this 'excitatory firing rate'. The advantage of this parameter is that it can be used appropriately at high and low stimulus intensities, so that one can replace the two codes of stimulus intensity, phase coupling and mean firing rate, by one code. It turned out that this code increases,over the whole tested 80 dB,linearly with the logarithm of stimulus intensity (Fig. 2B). It would be interesting to see whether the same applies also for auditory responses to low frequencies. An interpretation of excitatory firing rate is that the time of the response peaks and the firing rate indicate the time of occurrence and amplitude of individual wave peaks, respectively. This has similarly been suggested for the responses of auditory afferent fibers /6, 33, 34/.

Additional details corroborate the existence of common principles of stimulus encoding. In both the auditory and lateral line systems the response peaks are slightly skewed at higher stimulus intensity so that their leading edge rises faster than their trailing edge falls /24, 32/. Further, in lateral line afferent fibers the response phase does not change with stimulus intensity (Fig. 2C). In auditory afferent neurons, phase shifts with stimulus intensity have been reported (e.g. /2, 3/), but these shifts can apparently be attributed to the motion of the basilar membrane so that the response phase relative to the basilar membrane oscillation

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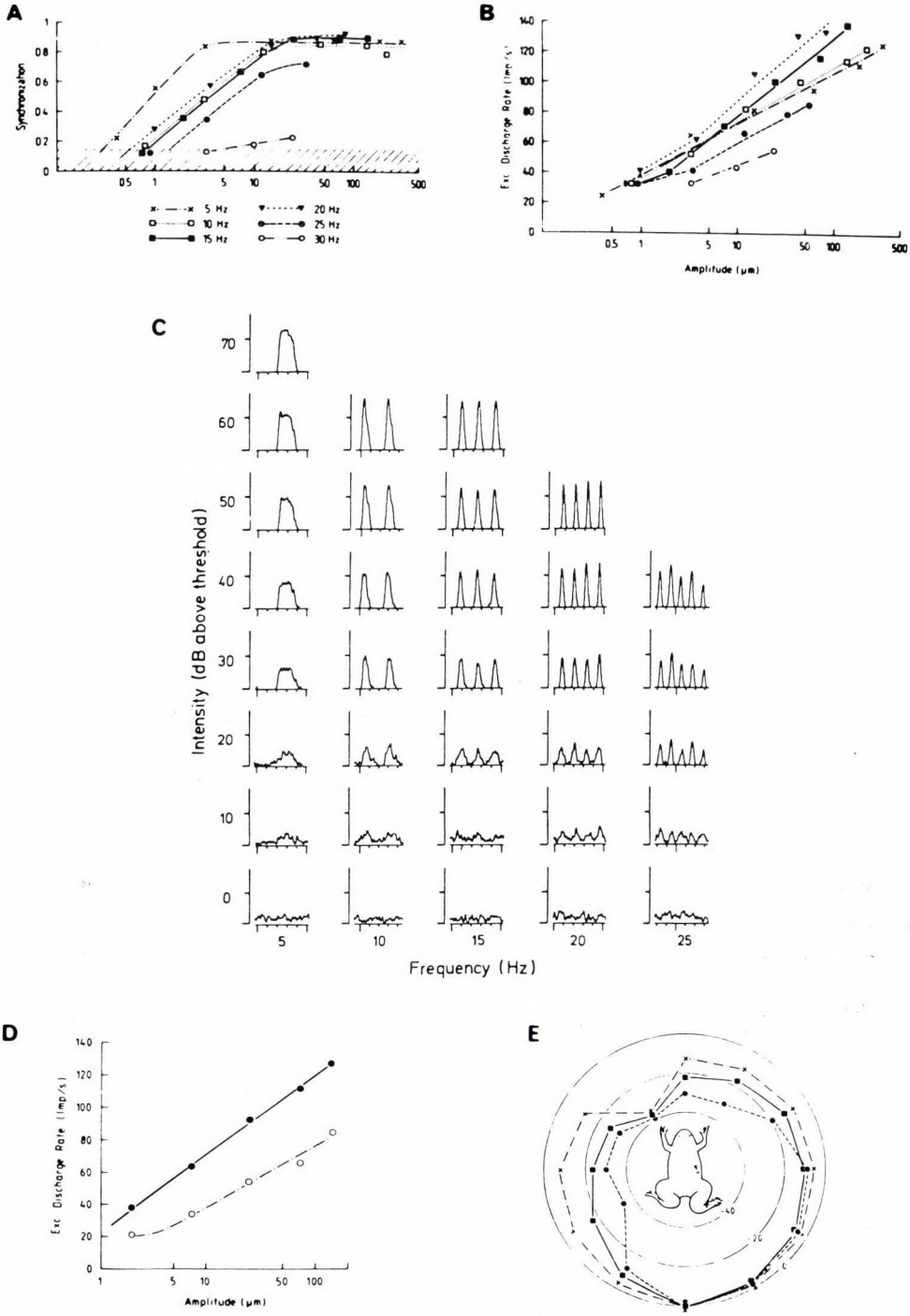
Fig. 1A. Schematic view of the lateral line organ of Xenopus. C — cupula, S — sensory hair cells, N — afferent fibers

B: Sinusoidal directional response characteristic of the organ's two afferent fibers to water jets from various directions. The thinner line indicates, for comparison, a sinus curve (after 19)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Frequency (Hz)
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Exc. Discharge Rate (Imp/s)
Amplitude (µm)
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does not shift with stimulus intensity over a wide range /35/, as in lateral line afferent fibers. Finally, we find the sensitivity of the response and the slope of the rate-intensity function to be positively correlated with the afferent neuron's spontaneous discharge rate (Fig. 2D). This has also been reported for auditory afferent fibers, /29, 31, 34, 38/. Thus, stimulus intensity is encoded in the same way in single afferent fibers of the lateral line and auditory systems.

The next question concerns the encoding of stimulus frequency. In the cochlea, the afferent fibers are aligned tonotopically so that their characteristic frequency decreases with the distance from the base of the cochlea. Below approximately 5 kHz, however, the afferent discharges are also phase coupled to the stimulus, and it is a matter of controversy whether frequencies below 5 kHz are encoded according to the 'place principle' (i.e. which fiber is excited most by a tone) or to the 'time principle' (i.e. the timing of phase coupled discharges). In Xenopus all lateral line organs have identical tuning curves with maximal sensitivity at about 20 Hz /27/, so that wave frequency discrimination cannot be based on differential frequency sensitivity. Behavioural analysis in Xenopus, however, has demonstrated wave frequency discrimination with an acuity that is equivalent to vertebrate auditory discrimination /15/. Thus, in the lateral line system we have proof for frequency discrimination of auditory quality

Fig. 2. Response characteristics of lateral line afferent fibers of Xenopus to water waves. Data in subfigures from one unit, respectively.

A: Phase coupling increases linearly with the logarithm of wave amplitude until it saturates.

B: The discharge rate during the wave cycle's 30%-sector with maximal firing rate increases for all intensities linearly with the logarithm of stimulus intensity.

C: PST-histograms of a neuron's responses at various stimulus intensities and frequencies. Histograms were started at identical phase of the stimulus wave. Irrespective of stimulus frequency, response phase does not change with intensity.

D: Change of sensitivity in correlation with altered spontaneous firing rate. Broken line — 15 imp/s, continuous line — 23 imp/s. The measurements differed by about three hours.

E: Directional sensitivity, indicated relative to maximal sensitivity. The numbers on the circles indicate dB. The small mark in the frog indicates the recorded organ, the arrowhead indicates the direction of local water movement at the cupula that produced maximal excitation of the recorded afferent fiber. Frequencies are indicated as in A (after 14)

based on the time principle. Frequency discrimination with lateral lines is not limited to the low frequencies below 30 Hz to which Xenopus has adapted: the surface feeding fish Aplocheilus lineatus can discriminate wave frequencies up to 150 Hz /4/.

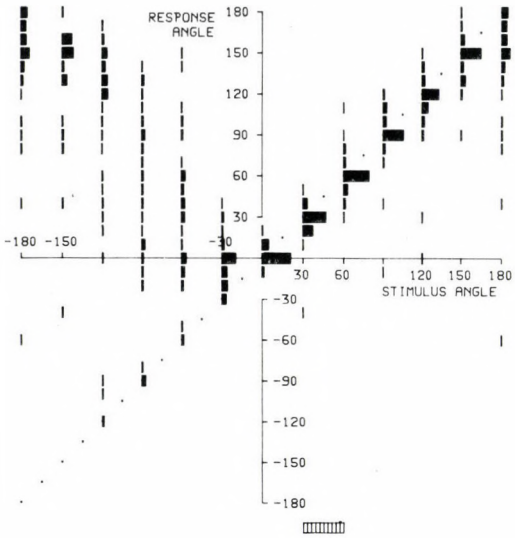
The third parameter besides intensity and frequency that affects the afferent response is stimulus direction. In textbooks, the directional sensitivity of the lateral line organ is described as sinusoidal, reflecting the directional sensitivity of the hair cells and their connections to the afferent fiber (Fig. 1B). However, this relates to directional sensitivity with regard to local water movements at the organ. The directional sensitivity of the organ in situ and with regard to stimuli of distant origin is completely different. It is not sinusoidal, but is determined by the animal's wave shadow, so that the sensitivity is minimal to waves impinging from an opposite direction with regard to the organ's position on the animal (Fig. 2E). This kind of directional sensitivity is similar to that known in hearing. An additional parallel is the fact that the wave shadow is larger for higher stimulus frequency.

Thus, despite the completely different organization of their respective organs, lateral line and low-frequency auditory afferent fibers apencode stimulus parameters in the same way: intensity and frequency encoding is identical, and the dependence of the response on stimulus direction is the same. It should be mentioned, however, that differing types of auditory encoding exist in fish and frogs (e.g. /16, 28/). The reasons for these differences remain to be elucidated.

Very little is known about central nervous mechanisms of wave analysis so far. Only the organization of wave localization in Xenopus has been investigated to some detail. By means of its lateral line system, Xenopus can detect the direction of impinging water waves with an accuracy of $\pm 5^{\circ}$. It responds to them by turning toward the wave's origin /8, 26/. This response has been used to analyze the neural organization of wave localization. As in hearing, but unlike other systems, stimulus localization with the lateral lines depends on a comparison of the inputs from several organs because each organ can be stimulated from all directions and thus provides by itself no directional cues. Sound localization is accomplished by comparison of inputs from two ears so that destruction of one ear abolishes comparison and, consequently, localization ability (behavioural strategies may be neglected). For wave localization, however, Xenopus possesses approximately 180 lateral line organs distributed over its body, and

it has been shown that a variety of organ subgroups – even unilateral ones – are sufficient for accurate localization of all wave directions /8, 9, 20/. This complex peripheral organization notwithstanding, we find a topological organization in the midbrain with regard to wave direction /10, 12, 13/. Unilateral ablation of the sensory midbrain (i.e. tectum and torus) abolishes localization of contralateral waves, and smaller lesions result in localization failure within sectors of the contralateral hemifield (Fig. 3). The exact location of this topology in the midbrain nuclei is as yet unclear. Neurons that respond selectively to waves impinging from certain directions were recorded in the tectum of Xenopus, and these neurons are in register with the tectal visual map /39/. However, Xenopus can orient to waves from all directions after tectal removal /12, 13/. These results correspond closely to data on sound localization. Unilateral ablation of the sensory midbrain in the cat abolishes localization of contralateral sound /23/. In the barn owl and several mammals, some neurons in the tectum and superior colliculus, respectively, respond specifically to sound from particular directions, and these neurons show a map-like organization with regard to stimulus direction (reviewed in /25/). On the other hand, sound localization is possible after ablation of the superior colliculus /1/. Thus, despite the different arrangement of the sensory organs, both systems possess the same midbrain organization for stimulus localization: a topological and chiasmatic organization with regard to stimulus direction rather than to receptor topology, and in addition a map-like projection to the tectum or superior colliculus that is apparently not essential for localization.

A fourth parallel between the lateral line and auditory systems concerns the analysis of complex signals. When Xenopus is stimulated with two waves simultaneously, it can determine the direction of the component waves if they differ by at least 30° (Fig. 4). Even at interstimulus angles of only 10° or 20°, the animal responds often by turning exactly toward one of the wave sources /11/. If the frog is trained to respond to a certain wave frequency, it will detect the rewarded frequency in the interference pattern and will turn toward it. What are the mechanisms that enable the frog to do this? Differential frequency tuning can be ruled out for two reasons: First, the organs have identical frequency tuning /27/, and second, localization of the wave sources is also possible when both waves have the same frequency /11/. Further, at interstimulus angles of 30° or less, the animal's wave shadow is insufficient to let a considerable number of organs be stimulated by one but not by the other wave. Thus, the only information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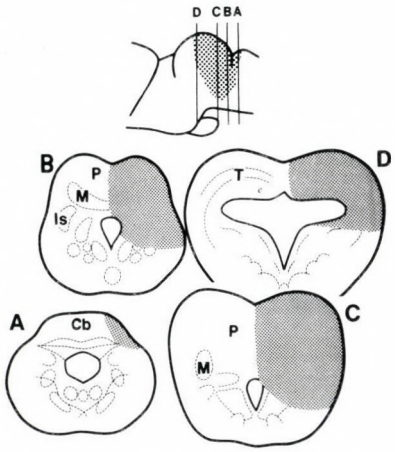
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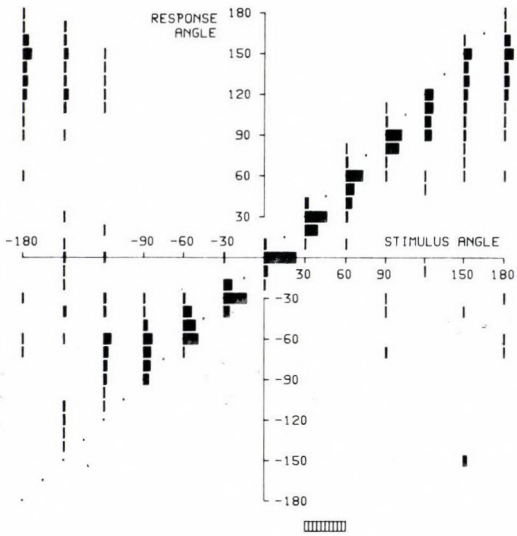
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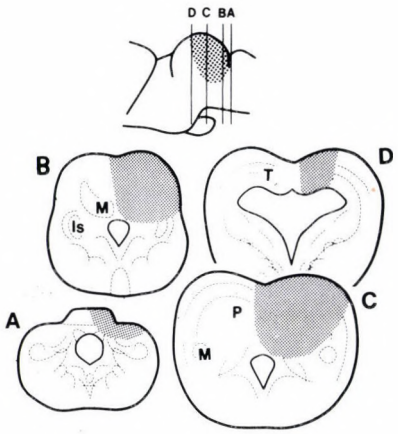
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that the frog can obtain from its lateral line organs is information about the wave patterns that result from the superposition of the two stimulus waves at the locations of the respective organs. Identification and localization of a component wave, on the basis of such inputs, requires differential analysis of the temporal structure of the superposition at several organs, taking also into account their locations on the body.

This strategy for complex-wave analysis in Xenopus may be compared with the computations that are required in analyzing complex tones with the ear. If a multi-frequency tone reaches our ear, the various frequency components are distributed by the inner ear's travelling wave to the afferent fibers with the corresponding best frequencies. However, frequency resolution in the cochlea is not so precise that every afferent fiber is stimulated only by one frequency. Instead the hair cells are stimulated, according to their tuning curve, by other frequencies as well, and the discharge patterns of the afferent fibers correspond to the wave forms that result from the superposition of the frequency components at the respective locations on the basilar membrane /6, 33, 34/. Thus, the problem to be solved in the central nervous system for analysis of complex tones is basically the same as in the mentioned two-wave experiment with Xenopus: Computation of the frequency components and their directions from superpositioned waves along the basilar membrane. Thus, in both the lateral line and the auditory systems we find the ability to detect and localize frequency components in complex stimuli, and the method for it is a central nervous comparison of the superposition patterns at several locations of the body or basilar membrane, respectively. In view of this coincidence, and of the ad-

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Fig. 3. Representative samples of the effect of midbrain lesions on wave localization in the clawed frog, Xenopus.

A: After unilateral removal of tectum and torus semicircularis all ipsilateral but no contralateral waves are localized.

B: After small midbrain lesions wave localization is abolished within sectors of the contralateral hemifield.

Explanation of figures: The stippled brain areas indicate which parts have been eliminated. In the response histograms, 0^{0} indicates frontal of the frog, and positive and negative angles indicate left and right side, respectively. The dots along the major diagonal indicate where accurate response turns would be. The animal was stimulated until it had responded 50 times to every stimulus angle. The length of the hatched bar below the histogram indicates 100% of the responses to a stimulus angle. Cb — Cerebellum, IS — nucleus isthmi, M — magnocellular nucleus of the torus semicircularis, P — principal nucleus of the torus semicircularis, T — tectum (from 12, 13)

Fig. 4. When Xenopus is stimulated with two waves simultaneously, it turns toward the wave for which it had been trained (in the sample to 20 Hz rather than to 12 Hz). This implies that it can localize and identify the component waves in the interference pattern (from 11)

ditional close correspondence in afferent stimulus encoding and in stimulus localization as mentioned above, it seems not too speculative to suggest that the central nervous comparison of the input patterns from various afferent neurons might be done by basically the same mechanisms in both systems.

In summary, our knowledge of wave processing with lateral lines is still incomplete. Aspects, however, that have been investigated are strikingly similar to mechanisms of auditory analysis. It appears most reasonable to explain this similarity at several levels of stimulus processing by homology, that is by a common evolutionary origin.

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REFERENCES

1. Allard, T.T., Jenkins, W.M. (1986) Sound localization after lesions of the cat superior colliculus. Soc. Neurosci. Abstr. 12, 1323.

2. Allen, J.B. (1983) Magnitude and phase-frequency response to single tones in the auditory nerve. J. Acoust. Soc. Am. 73, 2071–2092.

3. Anderson, D.J., Rose, J.E., Hind, J.E., Brugge, J.F. (1971) Temporal position of discharges in single auditory nerve fibers within the cycle of a sine-wave stimulus: frequency and intensity effects. J. Acoust. Soc. Am. 49, 1131–1139.

4. Bleckmann, H., Waldner, I., Schwartz, E. (1981) Frequency discrimination of the surface-feeding fish Aplocheilus lineatus – a prerequisite for prey localization? J. Comp. Physiol. 143, 485–490.

5. Boord, R.L., McCormick, C.A. (1984) Central lateral line and auditory pathways: a phylogenetic perspective. Amer. Zool. 24, 765–774.

6. Brugge, J.F., Anderson, D.J., Hind, J.E., Rose, J.E. (1969) Time structure of discharges in single auditory nerve fibers of the squirrel monkey in response to complex periodic sounds. J. Neurophysiol. 32, 386–401.

7. Bullock, T.H., Heiligenberg, W. (eds) (1986) Electroreception. Wiley, New York, Chichester.

8. Elepfandt, A. (1982) Accuracy of taxis response to water waves in the clawed toad (Xenopus laevis Daudin) with intact or with lesioned lateral line system. J. Comp. Physiol. 148, 535–545.

9. Elepfandt, A. (1984a) The role of ventral lateral line organs for water wave localization in the clawed toad (Xenopus laevis). J. Comp. Physiol. A 154, 773–780.

10. Elepfandt, A. (1984b) Topological representation of water wave directions in the lateral line midbrain projection of the clawed toad, Xenopus laevis Daudin. Neurosci. Letters (Suppl.), 18, S241.

11. Elepfandt, A. (1986) Detection of individual waves in an interference pattern by the clawed frog, Xenopus laevis Daudin. Neurosci. Letters, Suppl., 26, S380.

12. Elepfandt, A. (1988a) Central organization of wave localization in the clawed frog, Xenopus. I. Involvement and bilateral organization of the midbrain. Brain Behav. Evol. 31, 349–357.

13. Elepfandt, A. (1988b) Central organization of wave localization in the clawed frog, Xenopus. II. Midbrain topology for wave localization. Brain Behav. Evol. 31, 358–368.

14. Elepfandt, A., Wiedemer, L. (1987) Lateral-line responses to water surface waves in the clawed frog, Xenopus laevis. J. Comp. Physiol. A 160, 667–682.

15. Elepfandt, A., Seiler, B., Aicher, B. (1985) Water wave frequency discrimination in the clawed frog, Xenopus laevis. J. Comp. Physiol. A 157, 255–261.

16. Fay, R.R. (1981) Coding of acoustic information in the eighth nerve. In: Tavolga, W.N., Popper, A.N., Fay, R.R. (eds) Hearing and Sound Communication in Fishes. Springer Verl. New York–Berlin–Heidelberg, pp. 189–221.

17. Flock, A. (1971) Sensory transduction in hair cells. In: Loewenstein, W.R. (ed.) Principles of Receptor Physiology, Vol. I Handbook of Sensory Physiology, Springer Verl. Berlin–Heidelberg–New York, pp. 396–441.

18. Geisler, C.D., Rhode, W.S., Kennedy, D.T. (1974) Responses to tonal stimuli of single auditory nerve fibers and their relationship to basilar membrane motion in the squirrel monkey. J. Neurophysiol. 37, 1156–1172.

19. Görner, P. (1963) Untersuchungen zur Morphologie und Elektrophysiologie des Seitenlinienorgans vom Krallenfrosch (Xenopus laevis Daudin). Z. Vergl. Physiol. 47, 316–338.

20. Görner, P., Moller, P., Weber, W. (1984) Lateral-line input and stimulus localization in the African clawed toad Xenopus sp. J. Exp. Biol. 108, 315–328.

21. Hudspeth, A.J. (1983) Mechanoelectrical transduction by hair cells in the acousticolateralis sensory system. Annu. Rev. Neurosci. 6, 87–215.

22. Hudspeth, A.J. (1985) The cellular basis of hearing: The biophysics of hair cells. Science 230, 745–752.

23. Jenkins, W.M., Masterton, R.B. (1982) Sound localization: effect of unilateral lesions in central auditory system. J. Neurophysiol. 47, 987–1016.

24. Johnson, D.H. (1980) The relationship between spike rate and synchrony in responses of auditory-nerve fibers to single tones. J. Acoust. Soc. Am. 68, 1115–1122.

25. Knudsen, E.I., du Lac, S., Esterly, S.D. (1987) Computational maps in the brain. Annu. Rev. Neurosci. 10, 41–65.

26. Kramer, G. (1933) Untersuchungen über die Sinnesleistungen und das Orientierungsverhalten von Xenopus laevis DAUD. Zool. Jb. Physiol. 52, 629–676.

27. Kroese, A.B.A., Van der Zalm, J.M., Van den Bercken, J. (1978) Frequency response of the lateral-line organ of Xenopus laevis. Pflügers Arch. 375, 167–175.

28. Lewis, E.R. (1986) Adaptation, suppression and tuning in amphibian acoustical fibers. In: Moore, B.C.J., Patterson, R.D. (eds) Auditory Frequency Selectivity. Plenum, New York London, pp. 129–136.

29. Liberman, M.C. (1978) Auditory-nerve response from cats raised in a low-noise chamber. J. Acoust. Soc. Am. 63, 442–455.

30. Northcutt, R.G. (1980) Central auditory pathways in anamniotic vertebrates. In: Popper, A.N., Fay, R.R. (eds) Comparative Studies of Hearing in Vertebrates. Springer Verl. New York–Berlin–Heidelberg, pp. 79–118.

31. Palmer, A.R., Evans, E.F. (1980) Cochlear fibre rate-intensity functions: no evidence for basilar membrane nonlinearities. Hearing Res. 2, 319–326.

32. Palmer, A.R., Russell, I.J. (1986) Phase-locking in the cochlear nerve of the guinea-pig and its relation to the receptor potential of inner hair-cells. Hearing Res. 24, 1–15.

33. Rose, J.E., Brugge, J.F., Anderson, D.J., Hind, J.E. (1969) Some possible neural correlates of combination tones. J. Neurophysiol. 32, 404–423.

34. Rose, J.E., Hind, J.E., Anderson, D.J., Brugge, J.F. (1971) Some effects of stimulus intensity on response of auditory nerve fibers in the squirrel monkey. J. Neurophysiol. 34, 685–699.

35. Ruggero, M.A., Rich, N.C. (1987) Responses of cochlear afferents to low-frequency tones: intensity dependence. Neuroscience (Suppl.) 22, S130.

36. Russell, I.J. (1976) Amphibian lateral line receptors. In: Llinas, R., Precht, W. (eds) Frog Neurobiology. Springer Verl. Berlin–Heidelberg–New York, pp. 513–550.

37. Russell, I.J. (1981) The responses of vertebrate hair cells to mechanical stimulation. In: Roberts, A., Bush, B.M.H. (eds) Neurones without Impulses: Their Significance for Vertebrate and Invertebrate Nervous Systems. Cambridge Univ. Press, Cambridge, pp. 117–145.

38. Sachs, M.B., Abbas,P.J. (1974) Rate versus level functions for auditory-nerve fibers in cats: tone-burst stimuli. J. Acoust. Soc. Am. 65, 1835–1847.

39. Zittlau, K.E., Claas, B., Münz, H.(1986) Directional sensitivity of lateral line units in the clawed toad Xenopus laevis Daudin. J. Comp. Physiol. A 158, 469–477.

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FINE STRUCTURE OF THE PRIMARY AFFERENT VESTIBULOCOCHLEAR TERMINALS IN THE FROG

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The synaptology of the primary afferent vestibulocochlear fibers of the frog's nucleus vestibularis lateralis (NVL), nucleus cochlearis dorsalis (NCD) and nucleus saccularis (NS) was studied with the aid of cobalt labeling technique. On the basis of their sizes and morphological characters, two types of boutons could be distinguished. The type one large boutons making only axosomatic contacts were found in the NVL and NS. The form of contact in these boutons was mainly attachement plaque without vesicle accumulation in the contact zone. The second type of bouton having a smaller diameter and spheric vesicles, were engaged both in axosomatic and axodendritic synapses in all three nuclei studied. There was about the same number of axosomatic and axodendritic boutons in the NCD and NS; while the numerical distribution of type one and type two axosomatic boutons was similar in the NVL and NS. On the basis of our results it is suggested that the NS may be a transitory form between the NVL and NCD.

Keywords: Cobalt labeling, vestibulocochlear nuclei, sacculus, synaptology, frog

INTRODUCTION

In an earlier light microscopical work investigating the central projections of the VIIIth cranial nerve, we /10/ came to the conclusion that the same primary vestibulocochlear nuclei can be found in the frog as in

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higher vertebrates. Of the two nuclei showing the cochlear type of termination pattern, the nucleus cochlearis dorsalis (NCD) receives fibers from the cochlear primordia, that is, the basilar and amphibian papillae and the lagena. The second nucleus, the nucleus saccularis (NS), receives primary afferent terminals solely from the sacculus. On the basis of the similarity of the termination pattern in these two nuclei we have postulated that the nucleus saccularis may be the primordium of the nucleus cochlearis ventralis in mammals. This hypothesis seems to be in agreement with earlier results which suggest the role of the sacculus in hearing /4, 16, 18/. It is also known that the lagena, when it appears in phylogenesis, buds off from the wall of the sacculus /5/. On the other hand, the sacculus gives rise also to vestibular type of termination pattern in vestibular nuclei /10/; it is known that this organ is sensitive to linear acceleration in mammals.

This experiment was designed with the purpose to investigate the fine structure of the vestibulocochlear nuclei, since such data are not available for these structures of the frog. The cobalt labeling technique proved to be an adequate tool in the identification of primary afferent terminals in a series of electron microscopic investigation of the spinal cord /9, 21/ and the medulla /20/. In the present work, we have applied the same technique to the primary afferent fibers of the VIIIth nerve and studied the pattern of termination in an acoustic nucleus, in a vestibular nucleus and in the nucleus saccularis. Through the comparison of the fine structural features of labeled terminals, we expect to obtain data which either support, or contradict, our suggestion about the cochlear nature of the nucleus saccularis.

MATERIALS AND METHODS

Nineteen common water frogs, Rana esculenta, were anaesthetized with MS 222 (tricaine metanesulfonate), and the VIIIth cranial nerve was prepared through a pharyngeal approach. The mucous membrane overlying the roof of the mouth was cut and the parasphenoidal bone was clipped away. In order to prepare the branches of the VIIIth nerve, the bony otic capsule has to be also opened. Either the whole VIIIth nerve, or its posterior branch, or the saccular ramus, were separately filled with cobalt. The appropriate nerve was introduced into a small plastic tube containing 0.11 M $CoCl_2$ solution. The animal was kept in the refrigerator for one day and then sacrified. The brainstem was quickly removed and put for 2 min into a 0.11 M orthophosphat buffer saturated with H_2S. The brainstem was fixed in a mixture of 1% glutaraldehyde, 1% paraformaldehyde in phosphate buffer at pH 7.4 for 2 h, and 1 mm thick slices were cut at the level of the nucleus vestibularis lateralis, nucleus cochlearis dorsalis and nucleus saccularis. The

specimens were osmicated and embedded in araldite and semithin sections were used for orientation. The blocks were trimmed and the cobalt sulfide precipitate was intensified with the Timm's physical developer for 8–10 min /21/. The electron micrographs were made with the TESLA 100BS microscope. For the semiquantitative investigation the cobalt-filled terminals were selected, with the magnification of x10 000 diameter, and the axosomatic and axodendritic synaptic relations were counted on 300 boutons in each nuclei.

RESULTS

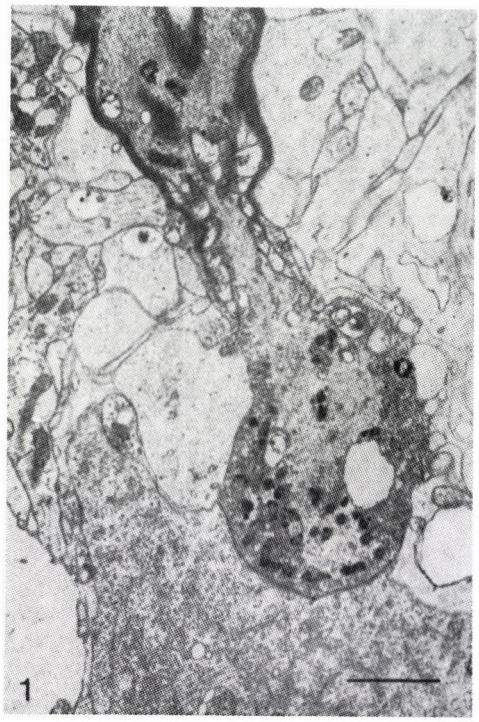
The nucleus vestibularis lateralis (Deiters) /NVL/

On the basis of their diameters and morphological characters, two types of boutons can be recognized in the NVL. The first type is a large bouton (4–6 μm) which has a neurofilamentous core and numerous mitochondria (Figs 1 and 2). The round-shaped vesicles are mainly found at the perimeter of the bouton. They establish multiple specialized junction in which the prae- and postsynaptic dense materials were visible without regular accumulation of vesicles. This type of contact is known as the attachement plaque, and is regarded as a characteristic vestibular type of synaptic contact in mammals /11, 19/. Sometimes a regular gap junction is made by these large boutons. In many cases these terminals directly emerge from their myelin sheaths and immediately make synaptic contact. The first type of bouton can be only found in axosomatic junctions.

The second type of bouton in the NVL is small, usually 1–2 μm in diameters (Figs 3 and 4). The cytoplasm of these boutons is electronlucent because of the small quantity of the neurofilaments. The mitochondria are not as numerous as they are in large boutons. These terminals contain round vesicles which accumulate at the synaptic contacts. These contacts are 0.3–0.6 μm long and the membrane thickening can be detected both at the pre- and postsynaptic sites. These boutons are involved both in axosomatic and axodendritic engagements and usually establish more than one synapses with the same postsynaptic profile (Fig. 4). In axodendritic contacts, these boutons are more frequently found on proximal than on distal dendrites, the size of the postsynaptic dendrite is therefore usually larger than that of the terminal bouton.

The nucleus cochlearis dorsalis (NCD)

The primary afferent terminals of this nucleus are unfirom, they have the same characteristic features as the second type of bouton in the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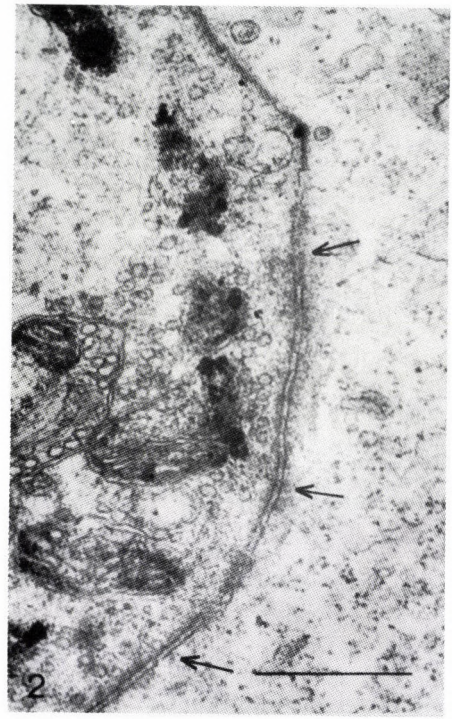
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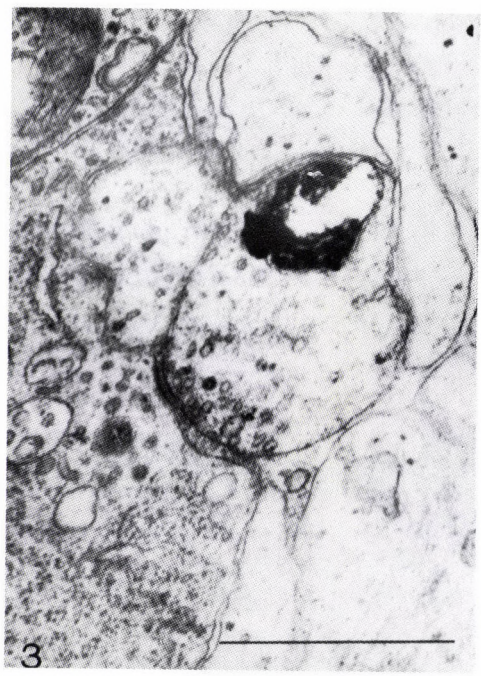
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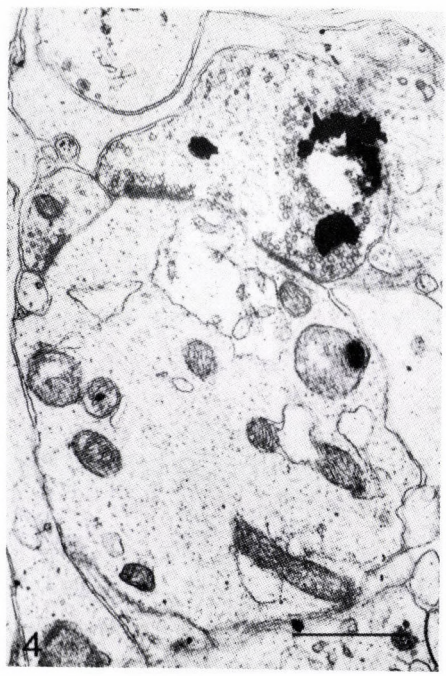
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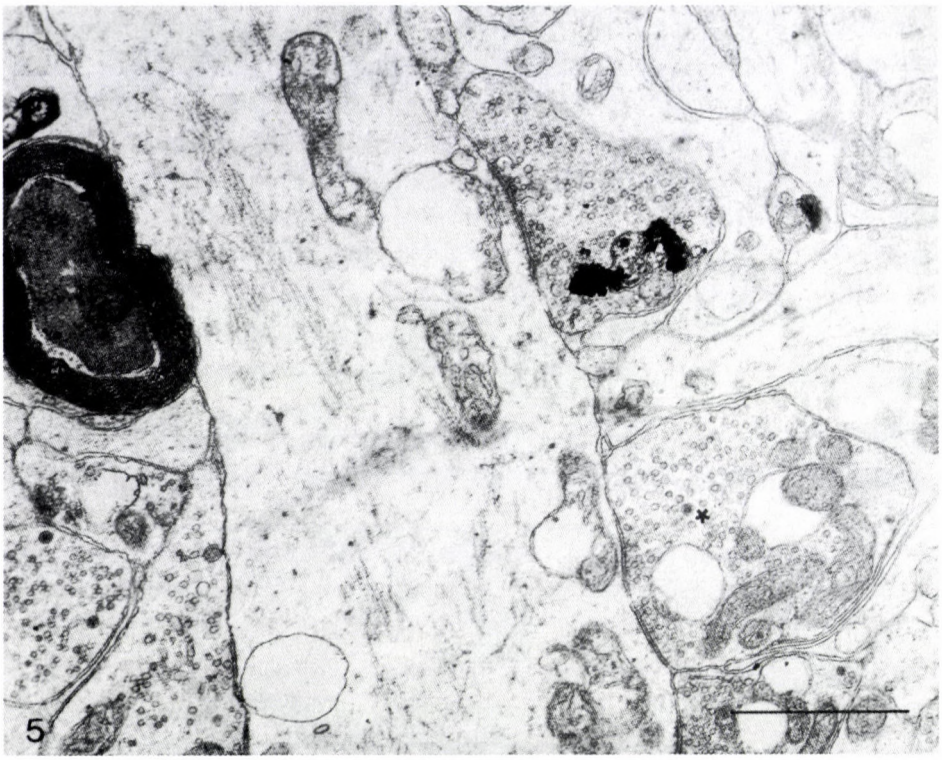
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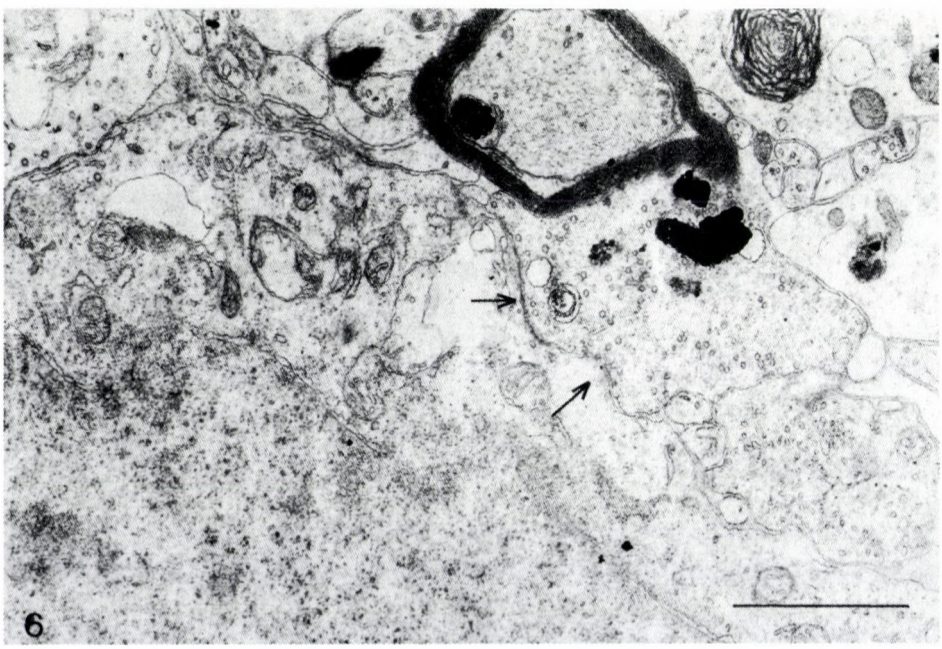
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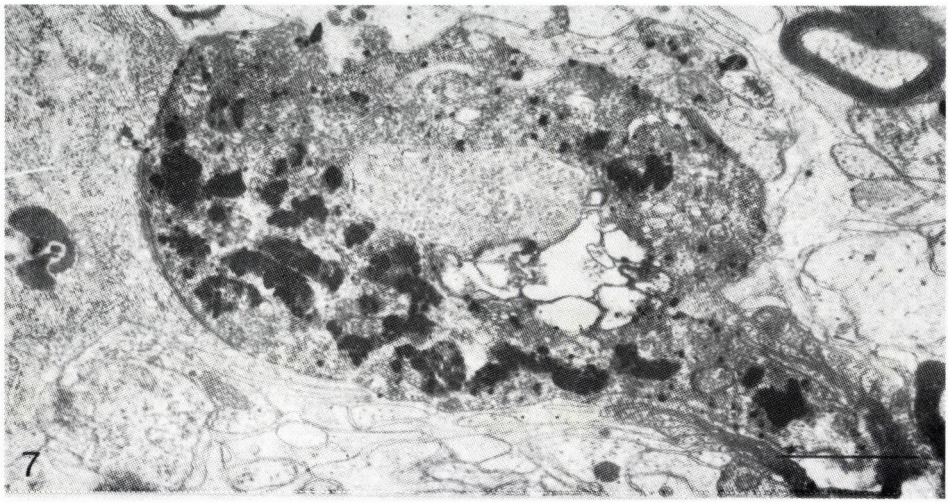
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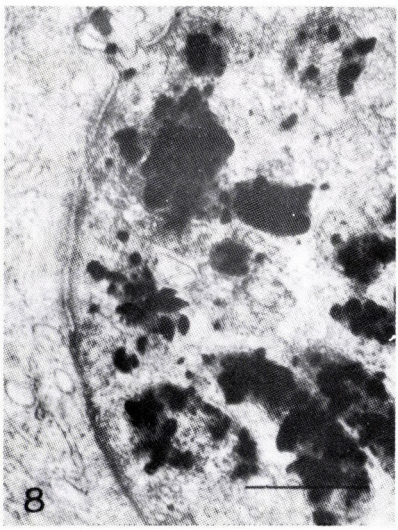
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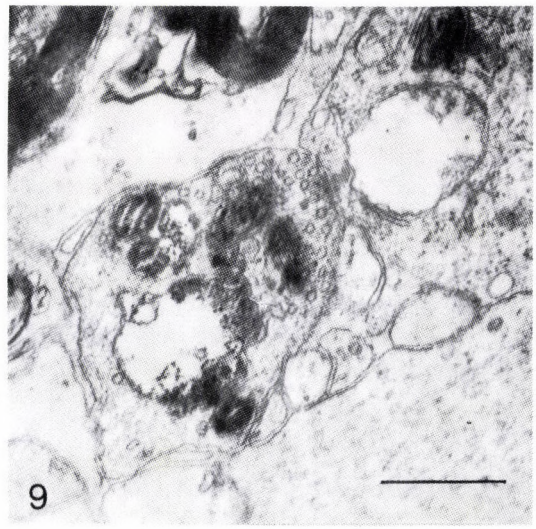
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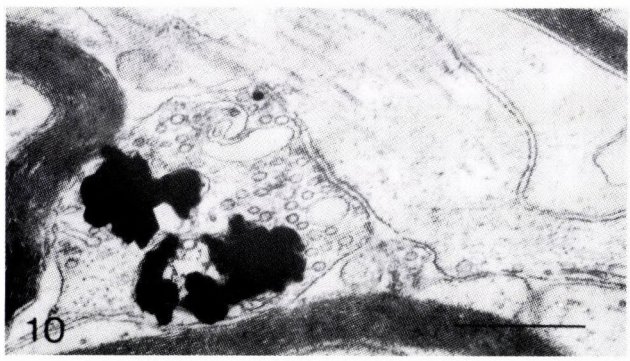
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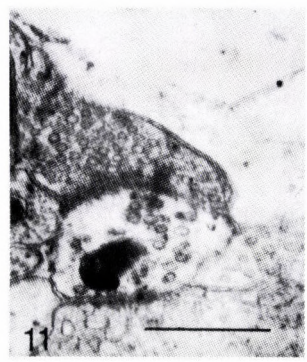
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NVL (Figs 5 and 6). These boutons are 1–2 μm in diameter and establish asymmetric synapses both with somata and dendrites. Like in the NVL, there are often more than one active zones of these boutons in contact with the same postsynaptic profile. In axodendritic engagements, the postsynaptic dendrite usually have a larger diameter than the presynaptic bouton.

The nucleus saccularis (NS)

The primary afferent fibers to this nucleus give rise to the same two types of boutons as described for the NVL. The type 1 large terminals measuring 2–2.5 μm in diameters are somewhat smaller than their counterparts in the NVL. They establish multiple attachement plaques, without a clear sign of vesicle accumulation, exclusively with somata (Figs 7, 8). The second type of bouton is engaged both in axosomatic and axodendritic synapses (Figs 9 and 10). The boutons are 0.5–1.0 μm in diameter, and the length of their synaptic contacts measures between 0.2 and 0.4 μm. The only axoaxonic engagement found in this experiment is encountered in this nucleus: a labeled type 2 bouton can be seen in the postsynaptic position to an axon terminal containing small and round vesicles (Fig. 11).

Figs 1 and 2. Large axosomatic endings from the NVL with lower and higher magnification. The direct emergence of the bouton from the myelin sheath is demonstrated in Fig. 1; arrows show attachement plaques in Fig. 2. Scale indicates 2 μm at Fig. 1 and 0.25 μm at Fig. 2

Fig. 3. Small bouton (type 2) is in axosomatic contact in the NVL

Fig. 4. An axodendritic synapse is shown from the NVL. There are two active zones in the bouton. Scale indicates 1 um at Figs 3-4

Fig. 5. Axodendritic synapses from the NCD. Asterisk indicates an unlabeled terminal

Fig. 6. Axosomatic synapse from the NCD. Arrows point to two synaptic sites. Calibration bar is 1 um at Figs 5 and 6

Figs 7 and 8. Large axosomatic terminals in the NS with lower and higher magnification. The center of bouton in Fig. 7 is indented by an axonal profile. Note the similarity with the Figs 1 and 2. Scale indicates 1 um at Fig. 7 and 0.5 μm at Fig. 8

Fig. 9. Axosomatic synapse (type 2 bouton) from the NS

Fig. 10. Axodendritic synapse (type 2 bouton) from the NS

Fig. 11. Axoaxonic synapse from the NS. The labeled bouton makes a synapse on a dendrite. Scale indicates 0.5 μm at Figs 9-11

Semiquantitative results

Of the 300 labeled boutons counted in each nucleus, 79 establish axosomatic and 221 axodendritic contacts in the Deiters nucleus. In the NCD these numbers are 130 and 170, whereas in the NS 102 and 198, respectively. In the NVL, 60% of the axosomatic contacts belong to the type one, and in the NS this ratio is 61.94% (Table 1).

Table 1

Distribution of the axosomatic and axodendritic boutons in the nucleus vestibularis lateralis, nucleus cochlearis dorsalis and nucleus saccularis, respectively. The number of type one bouton is shown in parentheses

	Nucleus vestibularis lateralis	Nucleus cochlearis dorsalis	Nucleus saccularis
Axosomatic boutons	79 (47)	130 (-)	102 (63)
Axodendritic boutons	221	170	198
Total	300 (47)	300	300 (63)

DISCUSSION

Earlier electron microscopical investigations of the vestibulocochlear nuclei were carried out on normal and degenerated materials which derived from mammalian and avian species /2, 6, 7, 8, 11, 12, 13, 19/. The fine structure of the vestibulocochlear nuclei of lower vertebrates has not been investigated so far either in normal or in experimental materials. Our results have shown that the frog's primary afferent terminals of the NVL and NCD are almost the same as the terminals found in higher vertebrates. This supports our light microscopical findings /10/ that the frog's vestibulocochlear center is also composed of similar nuclei as found in the mammalian brainstem. In the present work, we have used the cobalt labeling technique for the identification of primary vestibulocochlear fibers and terminals in the NVL, NCD and NS. Labeled material renders possible an unequivocal identification of primary boutons and terminals. This technique resulted in also a reliable selectivity in labeling of primary terminals since the

cobalt never could be seen passed through the synaptic sites into the postsynaptic profile.

In the NVL, two types of primary vestibular terminals can be recognized on the basis of their morphological features. One type, the large bouton, seems to be characteristic of the vestibular nuclei as described in the Dieters nucleus of the rat and cat /11, 19/. This type of bouton probably corresponds to the spoon-like endings of the chicken's Deiters nucleus /13, 14, 15/. The second type of bouton which establishes multiple synaptic contacts with the same postsynaptic profile, are found in all species studied /11, 13, 14, 15, 19/. The multiple synaptic formation with the same postsynaptic structure probably has a special function in the vestibular nuclei, because it can be rarely detected in other sensory cranial nerve nuclei and in the spinal cord of the frog /9, 20/. The gap junction generated by primary vestibular boutons is not as frequently found in the frog and the rat /19/, as in the chicken nucleus vestibularis tangentialis where this type of contact is predominant /15/. The probable content of synaptic vesicles and the mechanism of impulse transmission is not known in this type of terminal.

The nucleus cochlearis dorsalis contains only the second type of bouton. Similar morphology of terminals has been described in the chicken /6, 7, 8, 12/ and the cat /3, 17/ nucleus cochlearis. The spheric vesicles of the primary cochlear terminals contain excitatory amino acids as neurotransmitter /22/.

The nucleus saccularis, as a separate termination area of the saccular fibers, was first recognized in the frog with light microscopical studies /10/. The existence of this nucleus was confirmed in Xenopus laevis /23/ and hagfish /1/. In our light microscopic investigations, the terminal formation of primary saccular fibers resembled very much the pericellular basket termination pattern of cochelar fibers, we came therefore to the conclusion that the nucleus saccularis may have been the primordium of the nucleus cochlearis ventralis in mammals. This earlier conclusion has obtained support from the suggestion that the sacculus, which is known as a receptor of equilibrium in mammals, has a dual function in lower vertebrates /4, 5/. The present electron microscopical findings appears to contradict this conclusion since the same types of terminals are found in the NS as in NVL. The ratio of the first and second types of terminals is also similar in the axosomatic engagements in the two nuclei. However, taking into account the ratio of axosomatic and axodendritic terminals, the NS with its rela-

tively large number of axosomatic terminals resembles more the NCD than the NVL. The many axosomatic boutons may have simulated the cochlear type basket-like termination pattern in the NS in our light microscopical investigations. These observations suggest that the NS may represent a transitory form between the vestibular type and cochlear type of nuclei, as far at least as the termination patterns are concerned. From this point of view, it would be interesting to extend these studies to the pattern of termination of saccular fibers in the mammalian brain stem.

REFERENCES

1. Amemiya, F., Kishida, R., Goris, R.C., Onishi, H., Kusunoki, T. (1985) Primary vestibular projections in the hagfish, Eptatretus burgeri. Brain Res. 337, 73–79.
2. Bird, S.J., Gulley, R.L., Wenthold, R.J., Fex, J. (1979) Kainic acid injections result in degeneration of cohlear nucleus cells innervated by the auditory nerve. Science 202, 1087–1089.
3. Cant, N.B., Morest, D.K. (1979) The bushy cells in the anteroventral cochlear nucleus of the cat. A study with the electron microscope. Neurosci. 4, 1925–1945.
4. Capranica, R.R., Moffat, A.J.M. (1975) Selectivity of the peripheral auditory system of spade-foot toads (Scaphiopus couchi) for sounds of biological significance. J. Comp. Physiol. 100, 231–249.
5. Ihle, J.E.W., van Kampen, P.N., Nierstrasz, H.F., Versluys, J. (1971) Vergleichende Anatomie der Wierbeltiere. Springer, Berlin, pp. 489–499.
6. Jhaveri, S., Morest, D.K. (1982) Neuronal architecture in nucleus magnocellularis of the chicken auditory system with observations on nucleus laminaris: a light and electron microscopic study. Neurosci. 7, 809–836.
7. Jhaveri, S., Morest, D.K. (1982) Sequential alterations of neuronal architecture in nucleus magnocellularis of the developing chicken: an electron microscopic study. Neurosci. 7, 855–870.
8. Lenn, N.J., Reese, T.S. (1966) The fine structure of nerve ending in the nucleus of the trapezoid body and the ventral cochlear nucleus. Am. J. Anat. 118, 375–390.
9. Lévai, G., Székely, G., Matesz, C. (1982) Fine structure of dorsal root terminals in the dorsal horn of the frog spinal cord. Acta Biol. Acad. Sci. Hung. 33, 231–246.
10. Matesz, C. (1979) Central projection of the VIIIth cranial nerve in the frog. Neurosci. 4, 2061–2071.
11. Mugnaini, E., Walber, F., Hauglie-Hanssen, E. (1967) Observations on the fine structure of the lateral vestibular nucleus (Deiters' nucleus) in the cat. Exp. Brain Res. 4, 146–186.

12. Parks, Th.N. (1981) Morphology of axosomatic endings in an avian cochlear nucleus: nucleus magnocellularis of the chicken. J. Comp. Neurol. 203, 425–440.

13. Peusner, K.D. (1981) An ultrastructural study of the development of synaptic endings in the nucleus vestibularis tangentialis of the chick embryo. Neurosci. 6, 2335–2350.

14. Peusner, K.D. (1984a) The development of synapses and "spoon" synaptic terminal space in the tangential vestibular nucleus: a quantitative electron microscope study. J. Comp. Neurol. 230, 372–385.

15. Peusner, K.D. (1984b) Development of synaptic organization in the tangential vestibular nucleus: a quantitative electron microscope study. J. Comp. Neurol. 230, 386–392.

16. Popper, A.N., Platt, C., Saidel, W.M. (1982) Acoustic function in the fish ear. TINS 5, 276–280.

17. Ryugo, D.K., Fekete, D.M. (1982) Morphology of primary axosomatic endings in the anteroventral cochlear nucleus of the cat: a study of the endbulbs of Held. J. Comp. Neurol. 210, 239–257.

18. Saidel, W.M., Popper, A.N. (1983) The saccule may be the transducer for directional hearing of nonostariophysine teleosts. Exp. Brain Res. 50, 149–152.

19. Sotelo, C., Palay, S.L. (1970) The fine structure of the lateral vestibular nucleus in the rat. II. Synaptic organization. Brain Res. 18, 93–115.

20. Székely, G., Lévai, G., Matesz, C. (1983) Primary afferent terminals in the nucleus of the solitary tract of the frog: an electron microscopic study. Exp. Brain Res. 53, 109–117.

21. Székely, G., Kosaras, B. (1976) Dendro-dendritic contacts between frog motoneurons shown with the cobalt labeling technique. Brain Res. 108, 194–198.

22. Wenthold, R.J., Zempel, J.M., Parakkal, M.H., Reeks, K.A., Altschuler, R.A. (1986) Immunocytochemical localization of GABA in the cohlear nucleus of the guinea pig. Brain Res. 380, 7–18.

23. Will, U., Luhede, G., Görner, P. (1985) The area octavo-lateralis in Xenopus laevis. I. The primary afferent projections. Cell Tiss. Res. 239, 147–161.

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SPATIAL COORDINATION OF COMPENSATORY EYE MOVEMENTS IN VERTEBRATES: FORM AND FUNCTION

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The semicircular canals of the labyrinth of vertebrates provide one way of motion detection in three-dimensional space. The fully developed form of the vertebrate labyrinth consists of six semicircular canals, three on each side of the head, whose spatial arrangement (vertical canals are placed diagonally in the head, horizontal canals are oriented earth horizontally) follows three interconnected principles: 1) bilateral symmetry, 2) push-pull operational mode, and 3) mutual orthogonality. Other sensory and motor systems related to vestibular reflexes, such as the extraocular muscles or the "optokinetic" coordinate axes encoded in the activity of the visually driven cells of the accessory optic system, share the same geometrical framework. This framework is also reflected in the anatomical networks mediating compensatory eye movements, linking each of the semicircular canals to a particular set of extraocular muscles (so-called principal vestibulo-ocular reflex connections to yoke muscles). These classical vestibulo-oculomotor relationships have been verified at many levels of the vertebrate hierarchy, including lateral- and frontal-eyed animals.

The particular spatial orientation of the semicircular canals requires further comment and phylogenetic evaluation. The spatial arrangement of the vertical canals is already present in fossil ostracoderms, and is also exemplified in lampreys, the modern forms of once abundant agnathan species that populated the Silurian and Devonian oceans. The lampreys and ostracoderms lack horizontal canals, which appear later in all descendant vertebrates. The fully developed vertebrate labyrinth with its six semicircular canals displays distinct differences that are obvious when comparing distant taxa (e.g. elasmobranchs versus other vertebrates). Whereas the common crus of the semicircular canals in teleosts through

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mammals is formed between the anterior and the posterior semicircular canal, it occurs between the anterior and the horizontal canal in elasmobranchs. However, despite this morphological difference, these two vertebrate labyrinth prototypes constitute a functionally identical solution. A similar analysis holds for certain invertebrate species (crab, octopus, squid), which display an even wider variety in the physical expressions of movement detection systems when compared to vertebrates.

Although the physical expressions of motion detection systems differ in the animal kingdom, the functional solutions (providing the best signal-to-noise ratio) with adherence to bilateral symmetry, push-pull operational mode, and mutual orthogonality are identical. Furthermore, this functional principle is reflected in the intrinsic organization of related sensory and motor systems.

Keywords: Semicircular canals, vestibulo-ocular reflex, reference frame, vertebrates, invertebrates

The semicircular canals of the labyrinth of vertebrates provide one mechanism for motion detection in three-dimensional space. This function is a necessary prerequisite if animals are to move effectively in their habitat. Of the many vestibular output systems, the vestibulo-ocular reflex system of vertebrates is a model paradigm to investigate brain function by viewing it in its functional context and by searching for and describing common features which were retained throughout phylogeny. One of these features includes the uniform architecture of the spatial orientation of the semicircular canals in the head among vertebrate species /8, 13, 17, 30/. The most developed form of the vertebrate labyrinth system consists of six semicircular canals, three on each side of the head, whose spatial arrangement (vertical canals placed diagonally in the head, horizontal canals placed horizontally with respect to the earth) follows three interconnected principles: 1) bilateral symmetry, 2) mutual orthogonality, and 3) push-pull operational mode (Fig. 1). The same geometrical framework is found in the spatial arrangement of the extraocular muscles (see /8, 21, 22/), and in the coordinate axes of the accessory optic system /20, 23/ which provides another important sensory input for the performance of the reflex. The same reference frame is also reflected in the anatomical networks mediating compensatory eye movements,in particular the three-neuron reflex arc /26, 27/, which links each one of the semicircular canals to a particular set of extraocular muscles (see below).

The first systematic experiments dealing with spatial aspects of vestibular function were conducted by Flourens in the early 19th century

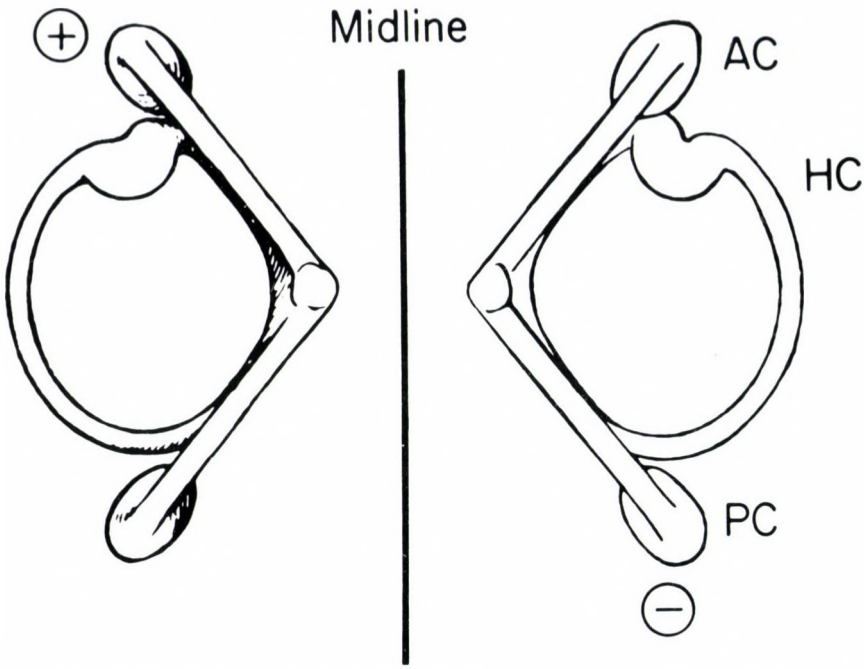


Fig. 1. Top view of an idealized vertebrate semicircular canal system (modified after Werner /30/). Anterior and posterior canals are oriented vertically, horizontal canals are oriented horizontally. Note bilateral symmetry of the system, mutual orthogonality between canals, and the push-pull operational mode illustrated for the right posterior and the left anterior canal. When one canal becomes excited (+), its coplanar counterpart becomes inhibited (-). The combined excitatory and inhibitory responses of all canals during head movements produce a meaningful activity pattern to represent a movement vector in physical space. AC: anterior canal; HC: horizontal canal; PC: posterior canal

/9, 10/. Following single semicircular canal lesions, he described head and/or eye movements in the plane of this particular canal. These experiments originally conducted in pigeons were later verified in a number of different vertebrates. For more than 50 years after Flourens' discovery, his experiments were replicated in order to find an explanation for the existence of his pigeons' "miraculous movements" (as it was described by some authors). The underlying anatomical network that does explain Flourens' results — at least for eye movements — is given in the firm wiring of the three-neuron vestibulo-ocular reflex arc /26, 27, see also 3, 4/, which connects the anterior canal in an excitatory fashion to the ipsilateral superior rectus and the contralateral inferior oblique muscle, the posterior canal to the ipsilateral superior oblique and the contralateral inferior

rectus muscle, and the horizontal canal to the ipsilateral medial rectus and the contralateral lateral rectus muscle. Inhibitory connections link the antagonists to the above muscles to the same semicircular canals. This classical vestibulo-oculomotor relationship,as first described by the Hungarian scientist Szentágothai /26/ (Fig. 2), has now been verified at all levels of the vertebrate hierarchy, including lateral – and frontal – eyed animals (for review see /21, 22/). Furthermore, the above-mentioned muscle pairs are closely aligned with the semicircular canal from which they receive their principal input.

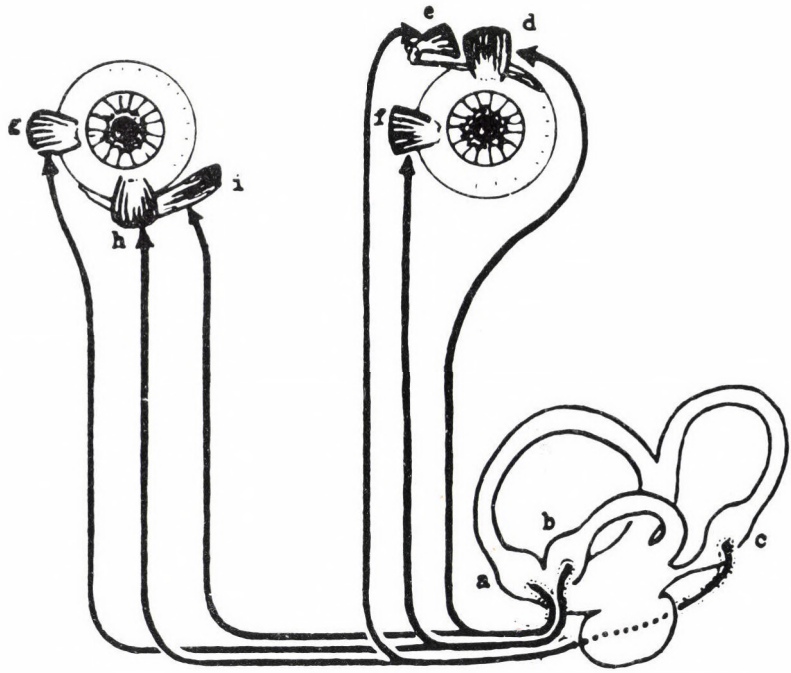
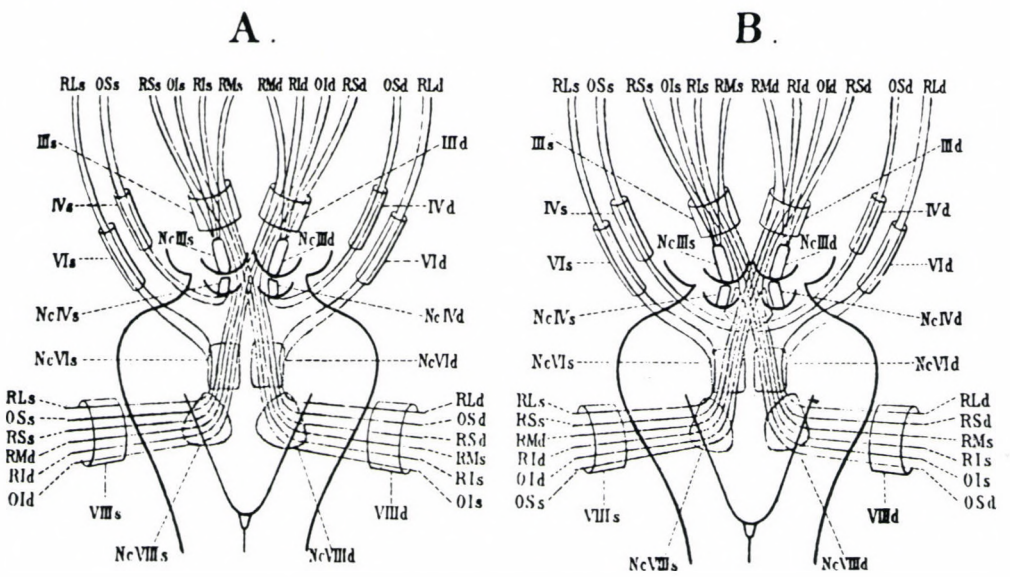


Fig. 2. Classical three-neuron-arc vestibulo-ocular reflex connections (from Szentágothai /27/). Each canal excites one particular extraocular muscle in each eye. a: anterior canal; b: horizontal canal; c: posterior canal; d: superior rectus; e: superior oblique; f: medial rectus; g: lateral rectus; H: inferior rectus; i: inferior oblique



A szemmozgás associáló centrum schémája

M.T.Akad. Ert. a Természettud. köréből, 1884. XIV k. 9 sz.

Fig. 3. Vestibulo-oculomotor relationships as envisioned by Hőgyes /15/. The two subsections depict one scheme with an ipsilateral trochlear nerve projection (A), and one with the – correct – crossed trochlear nerve (B). The superior rectus motoneurons are incorrectly projecting to the ipsilateral eye. Hőgyes also assumed – incorrectly – that ampullofugal endolymph current would excite the horizontal canals. However, given the techniques of the time, his wiring diagram was a remarkable achievement

Szentágothai's discovery established a solid scientific basis for the studies of vestibulo-oculomotor relationships, and thus of spatial coordination of compensatory eye movements. However, about 60 years earlier, another Hungarian investigator, Högyes /15/, had published a wiring diagram of the vestibulo-ocular reflex connectivity which, based on "innovative speculation" /26/, strikingly enough had led him quite close to the actual network of canal-eye muscle relationships (Fig. 3).

The three-neuron-arc reflex network as outlined above constitutes a generalized wiring plan for all vertebrates regardless of how the eyes are positioned in the skull. Identical canal-to-eye muscle connections in animals with varying optic axis orientations function because of a constant geometrical relationship between a given canal and a particular set of extraocular muscles (the yoke muscles named above connected to a particular canal) /22/. Furthermore, second-order vestibular neurons projecting to oculomotor motoneuron pools always contact their two respective principal eye movers in the excitatory as well as the inhibitory vestibulo-oculomotor link, a relationship that is particularly obvious in the vertical systems /11, 12, 28, 29/. This tight coupling defines the operation of yoke muscles for conjugate eye movements, and is in accordance with Hering's law of equal innervation of yoke muscles.

The particular spatial orientation of the semicircular canals requires further comment and phylogenetic evaluation since it provides a physical and operational framework for other subsystems of the vestibulo-ocular reflex system (see also /5, 19, 22/). Unfortunately, there is no fossil record testifying to the origin of the semicircular canal system, and the most primitive living chordate, Amphioxus, lacks a labyrinth. The earliest record for the spatial arrangement of the vertical canals is available from fossil agnatha, the ostracoderms /24/. The same geometrical organization is exemplified in the lampreys, the modern descendants of once abundant Silurian and Devonian species. However, lampreys and ostracoderms lack horizontal canals. Horizontal semicircular canals appeared later, and are present in all extant vertebrates examined. The acquisition of horizontal semicircular canals may have provided an advantage within the Devonian vertebrates, in particular when considering compensatory conjugate eye movements during typical teleost and elasmobranch swimming movements /7, 14/. These swimming movements occur about a vertical axis, predominantly stimulating the — now present — horizontal semicircular canals. However, different physical expressions of horizontal semicircular canal realizations

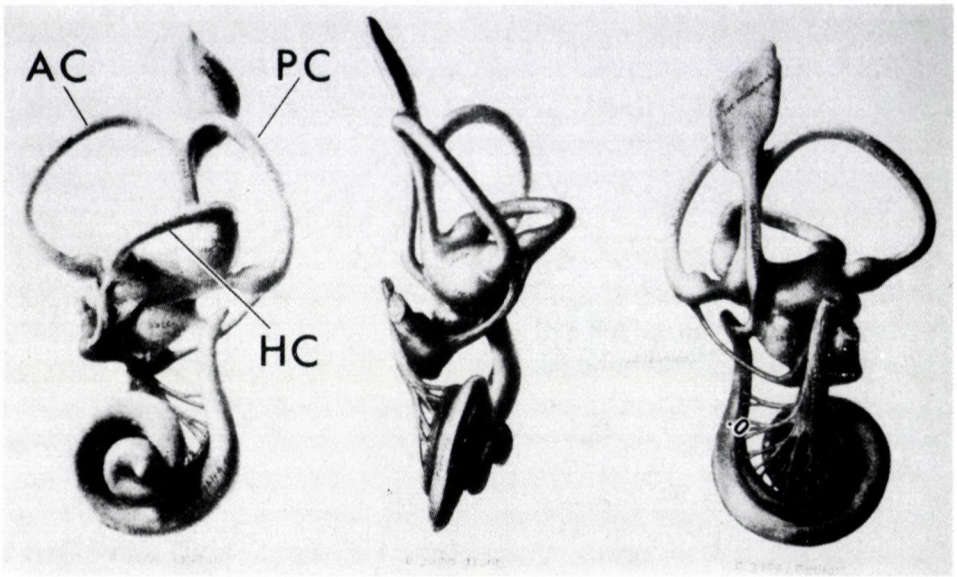


Fig. 4. Labyrinth of a 30 mm human embryo (from Streeter /25/). Note common crus formed between anterior and posterior canals. AC: anterior canal; HC: horizontal canal; PC: posterior canal

are found within the vertebrate subphylum when comparing distant taxa (e.g. elasmobranchs versus other vertebrates). In vertebrates of the "bony fish line" (teleosts through mammals) a so-called common crus is formed between the anterior and the posterior semicircular canal (Fig. 4), i.e. the two canals share a segment of their circular structure. In elasmobranchs, the posterior canal is completely separate and the anterior and the horizontal canal share a common segment (Fig. 5). Despite this morphological difference, these two vertebrate labyrinth prototypes are functionally identical solutions. In regard to these differences the question about a mono- or polyphyletic horizontal canal development remains to be answered (see also /1, 17/).

A similar analysis holds for certain invertebrate species (crab, octopus, squid), which display an even wider variety in the physical expressions of movement detection systems when compared to vertebrates. Nevertheless adherence to the above-mentioned three characteristics of bilateral symmetry, push-pull operational mode and orthogonality are readily detectable (Fig. 6) (for a detailed discussion see /22/). Incidently, the extraocular muscle arrangement in the octopus is almost identical to that in lateral-eyed vertebrates (Fig. 7).

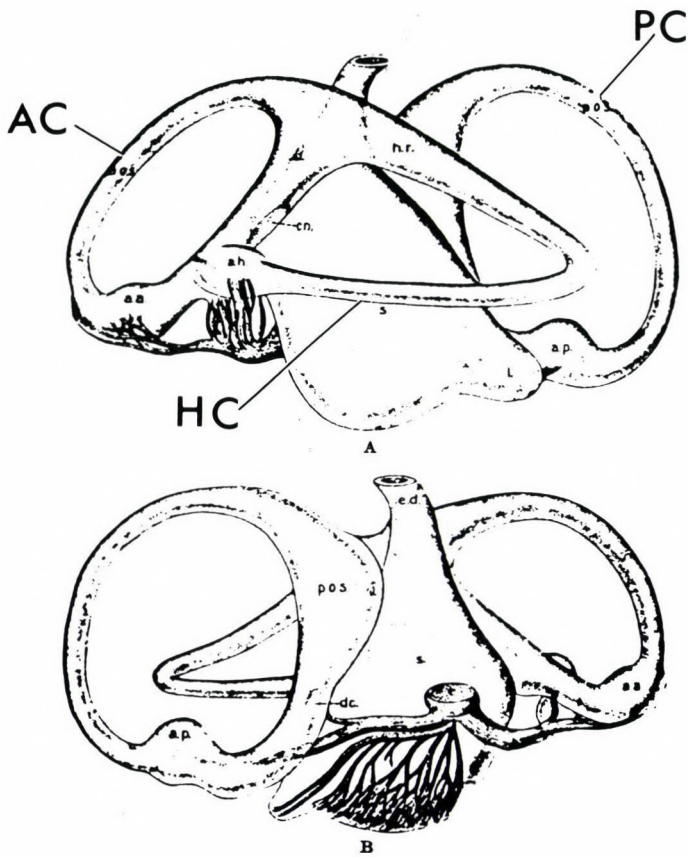


Fig. 5. Elasmobranch labyrinth (from Daniel /6/). Note separate posterior canal and link between anterior and horizontal canals. AC: anterior canal; HC: horizontal canal; PC: posterior canal

The realized semicircular canal systems constitute examples of a very limited number of possible solutions for an "ideal" (i.e. accurate, fast and economical) movement detection in physical space /18/ (Fig. 6), and thus were presumably selected during phylogeny. Species-specific specializations may obscure a common conservative plan /2/ at one level or another, but nevertheless the functional similarity of vestibulo-ocular reflex organization at almost every phylogenetic level in vertebrate and — to some degree — also invertebrate history is obvious. We conclude that possibly several motion detection system prototypes developed independently from each other during vertebrate and invertebrate phylogeny. Presumably

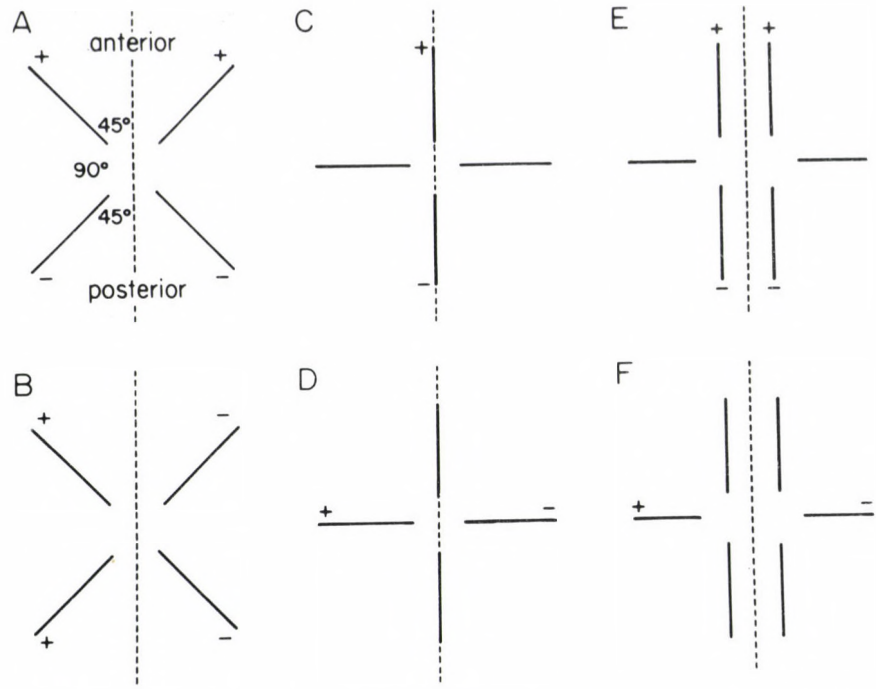


Fig. 6. Schematic of the only two semicircular canal arrangement prototypes (for the vertical system) as projected onto the horizontal plane which provide bilateral symmtery, push-pull (+/-) operational mode, and mutual orthogonality. The midsagittal plane is indicated by broken lines. The typical vertebrate canal arrangement and activity pattern during pitch-down movement (A) and a roll movement to the left (B) are contrasted to an activity pattern that would be observed in a system where the canals are rotated by 45 deg into the main body planes (C, D). In fact, such an arrangement is realized in the squid (E, F). However, for possible developmental and mechanical reasons, semicircular canals never appear as midline structures. Thus, the squid possesses six vertical canal-like structures, of which two are oriented in the transverse and four in parasagittal body planes. Nevertheless, this arrangement preserves bilateral symmetry, orthogonality, and push-pull operational mode for both pitch (E) and roll (F) movements (modified after Simpson and Graf /22/). Incidentally the directional polarities in the squid system are the opposite from that found in vertebrates. Whereas the vertical canals in vertebrates are excited by ampullofugal endolymph current, they are inhibited in the squid, and vice versa. Similarly, ampullopetal endolymph current in the vertebrate horizontal canals is excitatory, whereas in the squid it is inhibitory, and vice versa. Functionally, however, the two system prototypes are identical

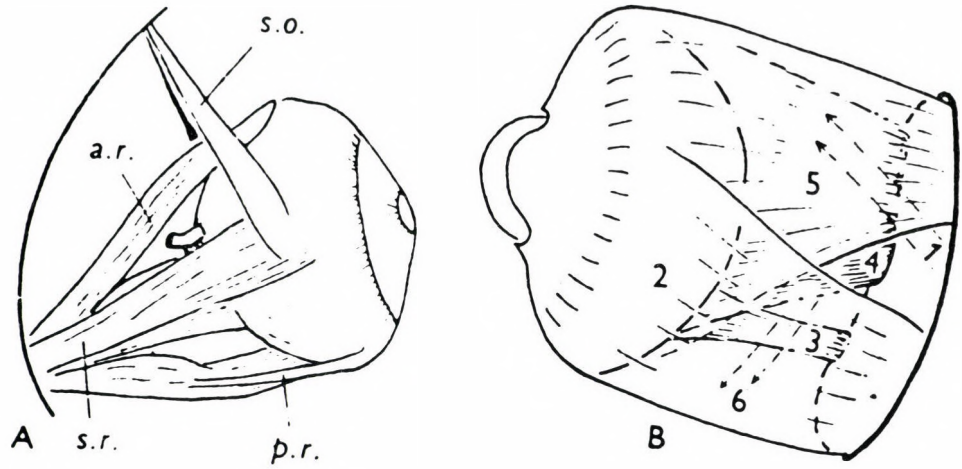


Fig. 7. Extraocular muscle arrangement in an elasmobranch (A) and in the octopus (B). Note similarity in the spatial arrangement of the two muscle systems. In the octopus, the original thirteen muscles are reduced to six (from Packard /16/). s.o.: superior oblique; a.r.: medial rectus; s.r.: superior rectus; p.r.: lateral rectus; 2: posterior muscle; 5: superior muscle

the most successful solutions, i.e. one or the other of the two "ideal" prototypes (Fig. 6) provided one distinct advantage, and thus were retained in all descendant radiations.

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REFERENCES

1. Baird, I.L. (1974) Some aspects of the comparative anatomy and evolution of the inner ear in submammalian vertebrates. Brain, Behav., Evol. 10, 11–36.

2. Baker, R., Delgado-Garcia, J.M., Graf, W., Spencer, R.F. (1987) Vestibular ocular organization in vertebrates is a largely conserved phylogenetic plan. Soc. Neurosci.(Abstr.) 13, 49.10.

3. Cohen, B., Suzuki, J.I. (1963) Eye movements induced by ampullary nerve stimulation. Am. J. Physiol. 204, 347–351.

4. Cohen, B., Suzuki, J.I., Bender, M. (1964) Eye movements from semicircular canal nerve stimulation in the cat. Ann. Oto-Rhino-Laryngol. 73, 153–165.

5. Cohen, B., Goto, K., Shanzer, S., Weiss, A.H. (1965) Eye movements induced by electric stimulation of the cerebellum in the alert cat. Exp. Neurol. 13, 145–165.

6. Daniel, J.F. (1928) The Elasmobranch Fishes. University of California Press, Berkeley.

7. Easter, S.S., Johns, P.R., Heckenlively, D. (1975) Horizontal compensatory eye movements in goldfish Carassius auratus I. The normal animal. J. Comp. Physiol. 92, 23–35.

8. Ezure, K., Graf, W. (1984) A quantitative analysis of the spatial organization of the vestibulo-ocular reflexes in lateral- and frontal-eyed animals. I. Orientation of semicircular canals and extraocular muscles. Neuroscience 12, 85–93.

9. Flourens, P. (1825) Expériences sur le système nerveux. Crevot, Paris.

10. Flourens, P. (1826) Expériences sur les canaux semi-circulaires de l'oreille, dans les mamifères. Mem. Acad. Roy. Sciences de l'Institut de France 9, 467–477.

11. Graf, W., Ezure, K. (1986) Morhplogy of vertical canal related second order vestibular neurons in the cat. Exp. Brain Res. 63, 35–48.

12. Graf, W., McCrea, R.A., Baker, R. (1983) Morphology of posterior canal related secondary vestibular neurons in rabbit and cat. Exp. Brain Res. 52, 125–138.

13. Gray, A.A. (1908) The Labyrinth of Animals, Including Mammals, Birds, Reptiles and Amphibians, Vols I, II. Churchill, London.

14. Harris, A.J. (1965) Eye movements in the dogfish Squalus acanthias L. J. Exp. Biol. 43, 107–130.

15. Hőgyes, E. (1880, 1881, 1884) Az associált szemmozgások idegmechanismusáról. Értekezések a természettudományok köréből. X, 18, 1–62; XI, 1, 1–100; XIV, 9, 1–84. German translation: Hőgyes, A. (1912) Über den Nervenmechanismus der assoziierten Augenbewegungen. Mschr. Ohrenheilk. 46, 685–740; 809–841; 1027–1083; 1353–1413; 1554–1571.

16. Packard, A. (1972) Cephalopods and fish: The limits of convergence. Biol. Rev. 47, 241–307.

17. Retzius, G. (1881) Das Gehörorgan der Wirbeltiere: morphologisch-histologische Studien. I. Das Gehörorgan der Fische und Amphibien. Samson und Wallin, Stockholm.

18. Robinson, D.A. (1982) The use of matrices in analyzing the three-dimensional behavior of the vestibulo-ocular reflex. Biol. Cybern. 46, 53–66.

19. Schaefer, K.-P., Schott, D., Meyer, D.L. (1975) On the organization of neuronal circuits involved in the generation of the orienting response (visual grasp reflex). Fortschr. Zool. 23, 199–212.

20. Simpson, J.I. (1984) The accessory optic system. Ann. Rev. Neurosci. 7, 13–41.

21. Simpson, J.I., Graf, W. (1981) Eye muscle geometry and compensatory eye movements in lateral-eyed and frontal-eyed animals. Ann. N.Y. Acad. Sci. 374, 20–30.

22. Simpson, J.I., Graf, W. (1985) The selection of reference frames by nature and its investigators. In: Berthoz, A., Melvill Jones, G. (eds) Reviews of Oculomotor Research Vol. 1, Elsevier/North-Holland Biomed. Press, Amsterdam – New York – Oxford. pp. 3–16.

23. Simpson, J.I., Graf, W., Leonard, C. (1981) The coordinate system of visual climbing fibers to the flocculus. In: Fuchs, A., Becker, W. (eds) Progress in Oculomotor Research. Developments in Neuroscience Vol. 12, Elsevier/North-Holland Biomedical Press, New York – Amsterdam – Oxford. pp. 475–484.

24. Stensiö, E.A. (1927) The Downtonian and Devonian Vertebrates of Spitsbergen, Part 1. Family Cephalaspidae. Skr. om Swalboard og Nordischavet Nr. 12. Norske Vidensk. Akad., Oslo.

25. Streeter, G.L. (1906/1907) On the development of the membranous labyrinth and the acoustic and facial nerves in the human embryo. Am. J. Anat. 6, 139–166.

26. Szentágothai, J. (1943) Die zentrale Innervation der Augenbewegungen. Arch. Psychiatr. Nervenkr. 116, 721–760.

27. Szentágothai, J. (1950) The elementary vestibulo-ocular reflex arc. J. Neurophysiol. 13, 395–407.

28. Uchino, Y., Hirai, N., Suzuki, S., Watanabe, S. (1980) Axonal branching in the trochlear and oculomotor nuclei of single vestibular neurons activated from the posterior semi-circular nerve in the cat. Neurosci. Lett. 18, 283–288.

29. Uchino, Y., Hirai, N., Suzuki, S. (1982) Branching pattern and properties of vertical- and horizontal-canal related excitatory vestibulo-ocular neurons in the cat. J. Neurophysiol. 48, 891–903.

30. Werner, C.F. (1960) Das Gehörorgan der Wirbeltiere und des Menschen. VEB Georg Thieme Verlag, Leipzig.

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RESPONSES OF AUDITORY BRAINSTEM NEURONS IN THE GRASSFROG TO CLICKS

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Single unit recordings were made in the dorsal medullary nucleus and in the torus semicircularis of the immobilized grassfrog. The natural calls have a periodic pulsatile structure. To investigate the coding of pulse repetition rate periodic click trains with varying pulse repetititon rate and an ensemble of cliks distributed randomly in time were used as stimuli. In the dorsal medullary nucleus strong time-locking to clicks was found. Most units showed an activation followed by suppression response. Some units showed a preference for pulse repetition rates matching their low-frequency sensitivity. In the torus semicircularis part of the units showed responses similar to dorsal medullary nucleus units. Other response types were activation irrespective of pulse repetition rate, and suppression followed by activation. The responses to the two stimulus ensembles were more compatible in the dorsal medullary nucleus than in the torus semicircularis.

Keywords: Anuran, click, dorsal medullary nucleus, temporal processing, torus semicircularis

INTRODUCTION

The natural calls of the male grassfrog (Rana temporaria L.) consist of pulse trains with a periodic structure /3, 9/. The dominant fre-

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Abbreviations: CCH crosscoincidence histogram; DMN dorsal medullary nucleus; FRH first-order recurrence-time histogram; PRR pulse repetition rate; SPL sound pressure level; TS torus semicircularis

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quency in a pulse is about 0.5 kHz. A pulse has a rise time of 2 ms and a fall time of about 10 ms. The pulse repetition rate ranges from 10 to 40 Hz, depending on temperature (Walkowiak and Brzoska, unpublished results). Walkowiak /12/ has used pulse trains to investigate the coding of PRR in the torus semicircularis of the grassfrog. His pulse trains with a duration of 0.5 s consisted of 10 ms pulses with 2.5 ms rise and fall times, and were presented at best frequency. With respect to average rate the responses could be classified as low-pass, band-pass, high-pass, bimodal or non-selective as a function of PRR. As an abstraction of the pulses click stimuli have been used by Epping and Eggermont /4/ to investigate the coding of PRR. Using trains of 10 equidistantly spaced clicks they also reported a diversity of average-rate responses. The synchronization capability to individual clicks or pulses mostly was a low-pass function of PRR. Thus it seems that information about PRR is conveyed mainly in the average rate of TS neurons and is not preserved well in the fine-temporal structure of spike trains. To investigate the mechanisms which underlie the observed temporal selectivities an ensemble of randomly distributed clicks has been used /2, 4/. In a forward-correlation approach, by which the spikes following a click are analyzed on basis of the cross-coincidence histogram, Bibikov /2/ determined the time-course of activation and suppression in the spike-generating region of a neuron. In a reverse-correlation approach Epping and Eggermont /4/ analyzed the configuration of clicks preceding a spike on basis of first- and second-order system kernels. Again various combinations of activation, suppression and facilitation by clicks were observed.

In this report responses to click stimuli from neurons in the dorsal medullary nucleus are presented, using the same stimuli as Epping and Eggermont /4/ used in the torus semicircularis. The results in both brainstem nuclei are compared. An attempt is made to relate the responses of a neuron to the two different stimulus ensembles. In a forthcoming paper (Epping, in preparation) the possibility of predicting the responses to click trains using the responses to the random click ensemble will be evaluated.

MATERIALS AND METHODS

Animal preparation and recording procedure

The methods are described extensively in Epping and Eggermont /4/ and Van Stokkum /10/. A brief description will be given here. Adult grassfrogs (Rana temporaria L.) from Ireland were anaesthetized with a 0.05%

solution of MS-222. A hole was drilled into the parietal and exoccipital bones, exposing the dura above the brainstem. The animal was allowed to recover overnight and the next day it was immobilized with an intralymphatic injection of Buscopan (0.12 mg per gram bodyweight). A local anaesthetic, Xylocaine 4%, was applied to the wound margins. The animal was placed in a sound attenuated room (IAC type 1202A) onto a damped vibration-isolated frame. Temperature was maintained around 15°C and the skin was kept moist to aid cutaneous respiration. The animal's condition was monitored with help of ECG recording and by examination of the blood flow in superficial vessels below the dura. A successful preparation remained intact for two days. The preparation of the dorsal medullary nucleus turned out to be much more difficult than that of the torus semicircularis.

The position of the DMN and of the TS were determined from anatomical studies /5, 7/. Parylene-c coated microelectrodes (Micro Probe Inc.) with a 1 kHz impedance of 1–5 M Ω were used for extracellular recording. Using hydraulic microdrives 1 or 2 microelectrodes were lowered into the brainstem. This again was much more difficult for the DMN than for the TS because of the tissue lag caused by the choroid plexus. Waveform features and spike epochs were stored on a PDP 11/34 with a resolution of 10 μs, and analyzed off-line with a PDP 11/44.

Acoustic stimulus presentation

The acoustic stimuli were generated by a programmable stimulus generator. They were presented to the animal by two electrodynamic microphones (Sennheiser MD211N) coupled to the tympanic membrane using a closed sound system. The sound pressure level was measured in situ with a half inch condenser microphone (Brüel and Kjaer 4143) connected to the coupler. Stimuli were presented ipsilaterally to the DMN and contralaterally to the TS, at a sound pressure level of 100 or 110 dB peak.

The condensation click had a duration of 0.7 ms, and its amplitude spectrum was flat within 5 dB for the range of interest /4/. Two different stimulus ensembles were used:

1. Periodic click trains. Trains of 10 equidistantly spaced clicks were presented every 3 s. A sequence consisted of 16 different PRRs presented pseudorandomly. The PRRs were chosen logarithmically equidistant between 7.8 and 250 Hz, which corresponds with interclick intervals between 128 and 4 ms. The sequence was repeated 10 times. The total duration of the stimulus was 480 s, in which 1600 clicks were presented.

2. Random clicks. Stimulus ensemble of clicks with an average rate of 16/s. The interclick intervals are drawn independently from a negative exponential distribution with a minimum interval of 1 ms. The interval distribution corresponds to a Poisson distribution with a dead-time of 1 ms. The total duration of the stimulus was 750 s, in which 11755 clicks were presented.

The response to the periodic click trains is presented as an event-display reordered according to PRR. Temporal integration of the response results in an average rate histogram as function of PRR. A measure for the degree of time-locking of spikes to the individual clicks in the periodic trains can be determined from the period histogram and is called the synchronization index. A value of 1 means that all spikes coincide in one bin of the period histogram, whereas a value of 0 indicates a flat period histogram.

The response to the random clicks is presented in two ways. The first usually non-stationary 30 s of the response were discarded from the stimulus-response analysis. The remaining 720 s generally were reasonably

stationary. Averaging the forward cross correlation function obtained from the clicks and the spikes over time results in a cross-coincidence histogram. In addition to the CCH a first-order recurrence-time histogram was calculated, representing the distribution of the first spike after a click. To analyze the fine-structure of the CCH its power spectrum was calculated with a resolution of 3.9 Hz. In a few cases the time-dependent cross-correlation function /11/ was determined in order to investigate the stationarity of the response.

RESULTS

This paper is based on the responses of 24 DMN units and 43 TS units recorded in 26 grassfrogs. Only TS units responsive to both stimulus ensembles have been analyzed.

The lowest thresholds found were 60 to 70 dB peak SPL. The stimulus intensities used were usually well above threshold.

Responses from three DMN units are presented in Fig. 1. The reordered event displays of unit 281, 1, 0 show strong time-locking to the clicks. The unit responds with one spike to each click at a latency of 4 ms up to a PRR of 156 Hz. The rate histogram of unit 281, 1, 0 shows a cut-off PRR of 200 Hz. The cut-off PRR is defined as the PRR were the rate response is half the maximum. The synchronization-index histogram shows a low-pass character, with again a cut-off PRR of 200 Hz. Note that the synchronization index is practically 1, up to 125 Hz. The cross-coincidence histogram of unit 281, 1, 0 shows an activation followed by suppression response. The activation latency is 4 ms. The suppression duration is 5 ms. The power spectrum of the CCH is mainly low-pass. Its half maximum is reached at 400 Hz. Unit 306, 0, 2 responds in a similar way. It has a cut-off PRR of 25 Hz, and a suppression duration of 32 ms. A different type of response is shown by unit 298, 2, 0, which was sensitive for low-frequency tonepips. Its rate histogram is tuned to a PRR of 62–200 Hz. The latency of 50 ms to the click trains indicates integration of multiple clicks. The CCH shows a double peak. These peaks consist of first spikes after a click, as indicated by the shaded FRH. Therefore it is plausible that each spike is a reponse to two or more clicks. The power spectrum of the CCH at the lower right shows a peak between 100 and 200 Hz. A response to the random clicks with multiple peaks in the CCH and a peak in its power spectrum is called tuning. In Fig. 3a the time-dependent cross correlation function obtained from the spikes and the clicks is plotted, together with the CCH and the FRH. In Fig. 3 the reverse correlation is shown. Vertically at $\tau = 0$ s an event display of the spikes

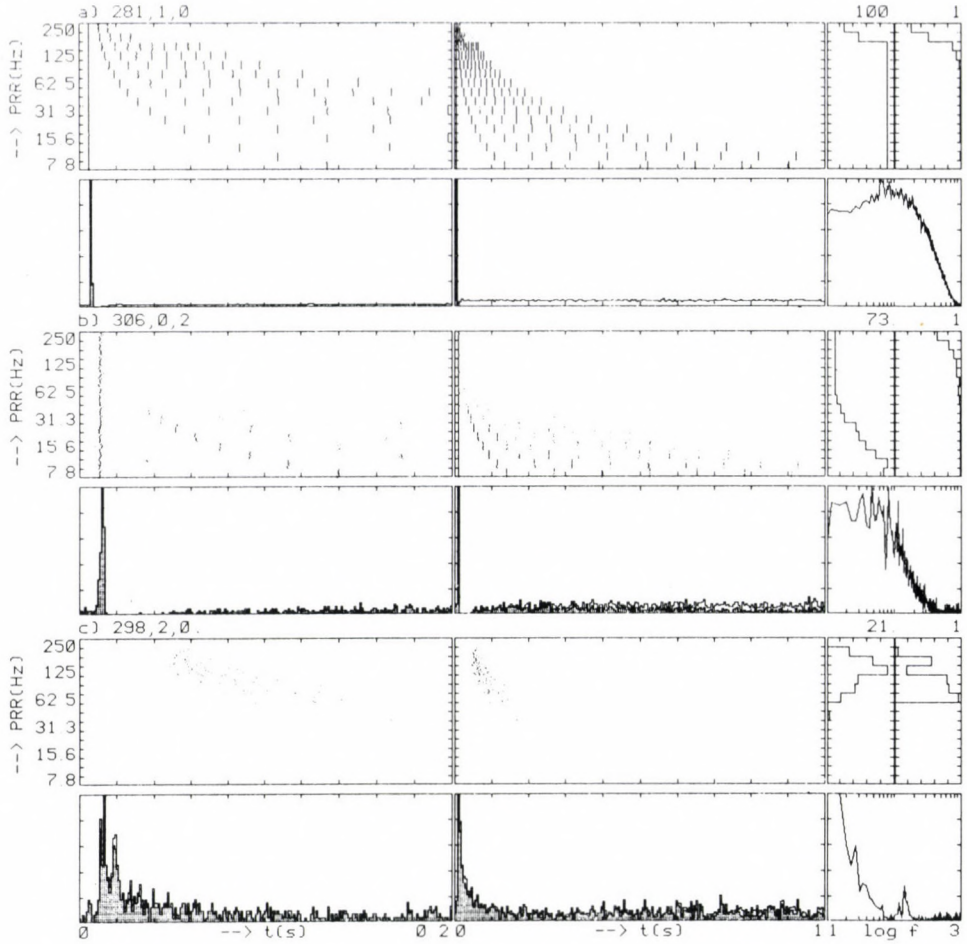

Fig. 1. Responses of three DMN units to click stimuli with an intensity of 110 dB peak SPL. Unit number is indicated at the left. In the upper row from left to right: reordered event display of response to the periodic click trains on a timebase of 0.2 and 1 s, rate histogram and synchronization-index histogram. The number above the rate histogram indicates the maximum number of events elicited with 10 click trains. The synchronization-index histogram is scaled from 0 to 1. In the lower row from left to right: CCH obtained from the randomly distributed clicks and the spikes on a timebase of 0.2 and 1 s. The shaded FRH indicates the distribution of the first spike after a click. Both histograms have been scaled to the maximum of the CCH. The power spectrum of the CCH is shown at the right, with frequency varying logarithmically from 10 to 1000 Hz

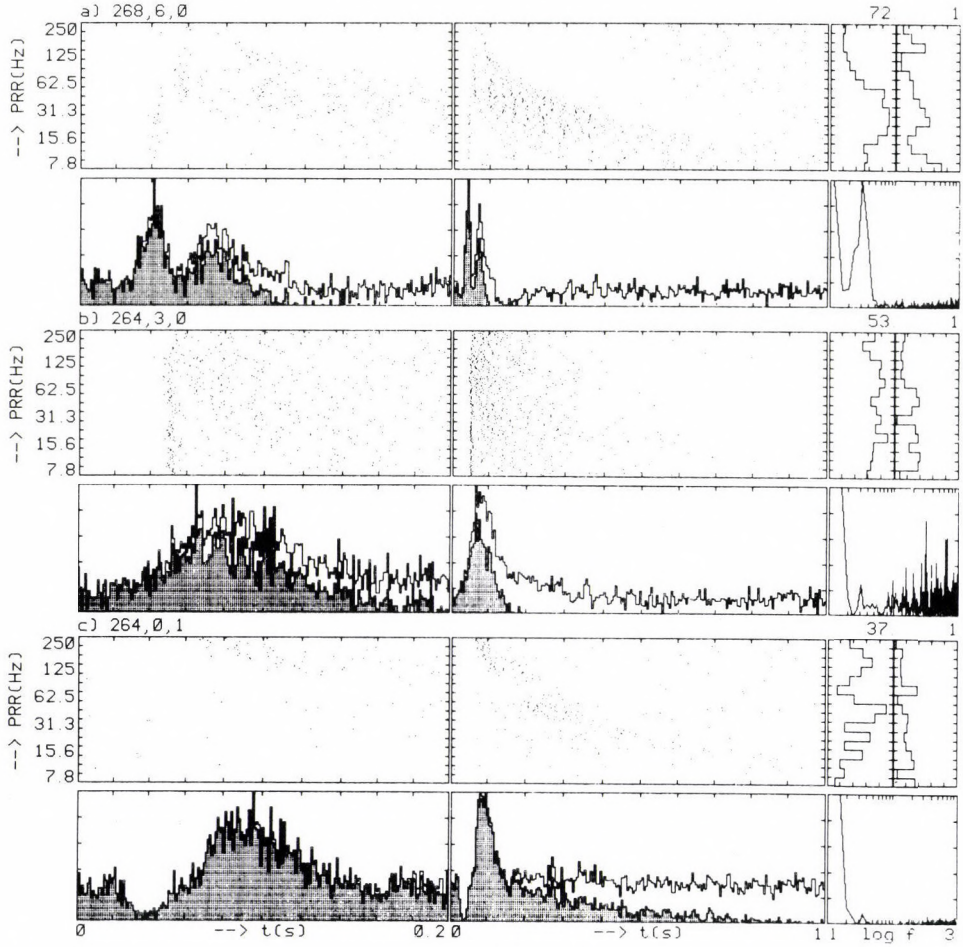

Fig. 2. Responses of three TS units to click stimuli with an intensity of 100 dB peak SPL. See legend Fig. 1

may be envisioned. Each spike functions as trigger for the clicks, and all clicks within 0.2 s before the spike are plotted horizontally. Integration of this function over time results in the CCH shown underneath. This CCH is the mirror image of the CCH in Fig. 1c. The FRH representing the distribution of the first clicks preceding the spikes is indicated by the shaded histogram. This is different from Fig. 1c, because the first spike after a click is not necessarily related to the first click before a spike. The facilitation by multiple clicks is visible in the clusters of clicks before τ/τ_1 = 0 s and in the FRH which extends up to τ/τ_1 = -18 ms. To the left thereof higher-order click recurrence-times contribute to the CCH. After t = 200 s it can be seen that each spike is a reponse to two or more clicks, confirming the suggestion derived from the FRH in Fig. 1c. Looking along the absolute-time axis a gradual decrease of response is seen. This unit thus did not exhibit a steady-state response after 30 s.

Another response type to the random clicks found in the DMN is activation. In this case there was also facilitation by multiple clicks.

In the TS part of the units shows responses similar to DMN units /4/. Three different response types of TS units are presented in Fig. 2. The response of unit 268, 6, 0 to the click trains shows a preference for intermediate PRRs. The rate histogram has a weakly band-pass character. The FRH is double peaked indicating a response to multiple clicks with an appropriate temporal relation. The powerspectrum of the CCH shows a peak at 30 Hz. Unit 264, 3, 0 shows a practically non-selective response to PRRs. An onset spike at a latency of 50 ms is followed by tonic activation. The CCH shows a very broad activation peak. A qualitatively different response is shown by unit 264, 0, 1. In its CCH suppression is followed by activation. The rate response to the click trains shows a multimodal histogram with a minimum at 79 Hz. The peak in the FRH is almost equal to the peak in the CCH. Analogous to Fig. 1c spike elicitation by multiple clicks is therefore plausible. To investigate this further the time-dependent cross-correlation function is shown in Fig. 3b. The distribution of clicks before the spikes shows a high density of clicks between τ = -0.12 s and τ = -0.07 s. The large difference between the CCH and the shaded FRH indicates the contribution of higher-order click recurrence-times to the peak in the CCH.

In Fig 4a, b latency histograms are shown for the DMN and for the TS. The DMN latency histogram shows a peak between 4 and 5 ms, probably reflecting the shortest transmission of activation via the papillae and the auditory nerve. In Fig. 4c the relation is shown between the suppression

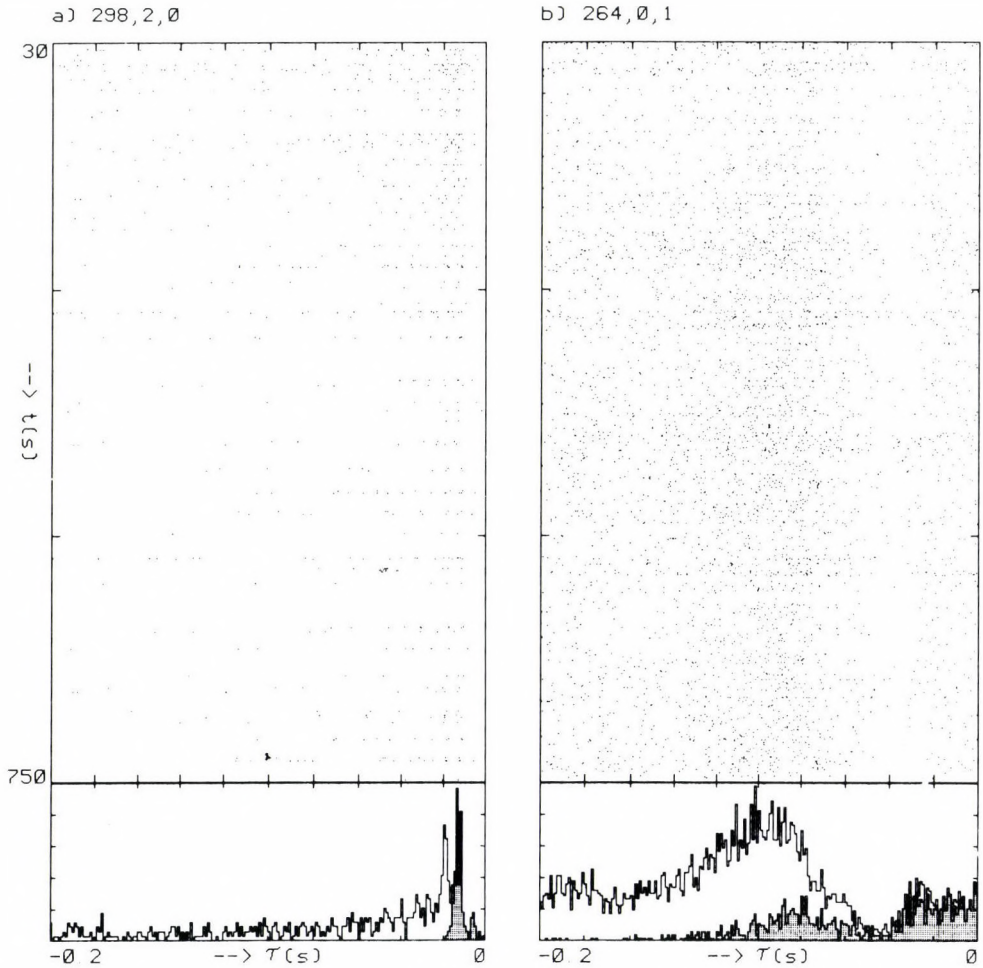

Fig. 3. Diagram of time-dependent cross-correlation function obtained from the spikes and the clicks. Underneath CCH and shaded FRH. Further explanation in text. a) DMN-unit 298,2,0. 115 spikes, 11262 clicks; b) TS-unit 264,0,1. 895 spikes, 11262 clicks

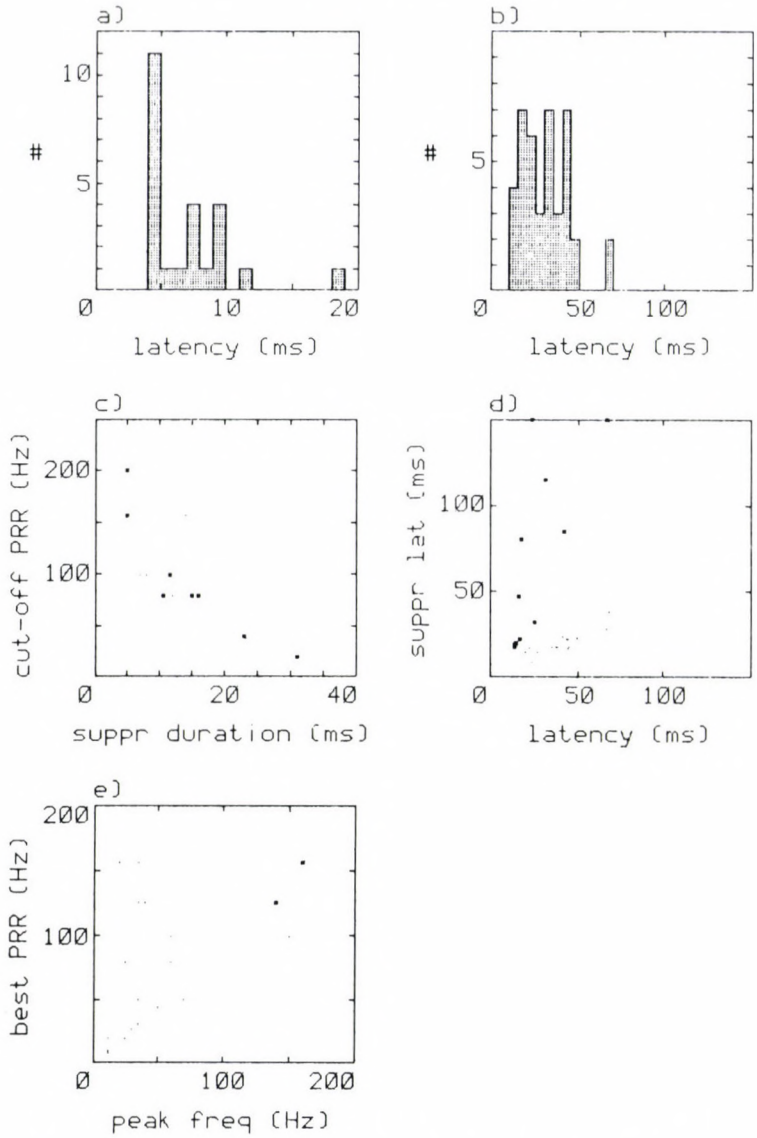

Fig. 4. a) Latency histogram of DMN units to clicks; b) Latency histogram of TS units to clicks; c) Scatterdiagram of duration of suppression as determined from response to random clicks and cut-off PRR as determined from rate response to periodic click trains. Squares indicate DMN units, points indicate TS units; d) Scatterdiagram of latency and suppression latency for TS units. Both latencies are determined from response to random clicks. Squares indicate units showing an activation-suppression response, points indicate a suppression-activation response; e) Scatterdiagram of peak frequency in power spectrum of CCH and best PRR as determined from rate histogram. Squares indicate DMN units, points indicate TS units

duration of units showing an activation followed by suppression response pattern to random clicks, and the cut-off PRR of low-pass rate responses to periodic click trains. The relation is reciprocal, except for one TS unit. Figure 4d shows the range of combined activation and suppression latencies found with TS units. The units with a suppression-activation response, indicated by points, show a weak correlation between these latencies. Finally, in Fig. 4e the relation between the preference for the PRR of click trains and the peak frequency of the CCH power spectrum is shown. For the two DMN units (squares) both measures show a strong correlation. About half of the TS units exhibit good correlation, for the others the correlation is not so clear. An overview of the responses to the random click ensemble is shown in Table 1. Units with a suppression or suppression-activation response are only found in the TS.

Table 1

Responses to the random click ensemble

	DMN	TS
activation-suppression	11	6
tuning	4	11
activation	3	9
suppression-activation		15
suppression		2
	18	43

DISCUSSION

The minimum latency to clicks of 4 ms found for DMN units is compatible with minimum latencies of 2–4 ms found for auditory fibers /6/. DMN units show two basic response types, as revealed by the responses to the random clicks. A click that is above threshold elicits a time-locked response (see Fig.1a, b). After this activation usually suppression can be observed in the CCH, with a range of durations (See Figs 1a, b and 4c). This suppression is most probably responsible for the low-pass rate response to the periodic click trains.

When a click is subthreshold more clicks are needed with an appropriate temporal relation. In that case the power spectrum of the CCH and the rate histogram of the response to periodic click trains may show tuning (see Figs 1c and 4e). These DMN units are also sensitive to low-frequency tonepips. At least three mechanisms can account for the activation followed by suppression response type: refractory mechanisms, adaptation of input supplying units, and inhibition. This is discussed elaborately in Van Stokkum /10/. The activation response type is probably an intermediate between the other two types.

In the DMN Schneider-Lowitz /8/ used the same pulse train stimulus as Walkowiak /12/. He found that 28% of the units showed a non-selective rate response up to PRRs of 100 Hz. The other units responded in a manner comparable with the low-pass rate responses found with the periodic click trains. It is remarkable that he did not observe such responses in the auditory nerve. He concluded that the temporal information processing starts in the DMN.

In the TS part of the units shows responses similar to DMN units. These units usually have latencies under 20 ms. Their time-locking to clicks is also strong, with synchronization indices between 0.8 and 1. A preference for PRRs around 30 Hz as shown by unit 268, 6, 0 (Fig. 2a) may be useful for coding natural calls. As discussed by Epping and Eggermont /4/ the range of best PRRs as found with clicks seems to be wider than the PRR-range of the natural calls. However Walkowiak /12/ found a better match between units showing preference for PRR and natural-call PRR. This may be due to the difference between his 10 ms pulses and our 0.7 ms clicks. Also his free field stimulation versus our monaural closed sound system may contribute to the PRR selectivity.

A large group of TS units shows a suppression followed by activation response to the random clicks (see Figs 2c and 4d), which points at neural inhibition. According to the latencies found (Figs 4b and 4d) this inhibition may be present within the TS. The depth of the trough in the CCH was often down to zero, suggesting that spike generation was preceded by inhibition.

In the DMN good compatibility of responses to periodic click trains and to random clicks was found (Fig. 4c, e). In the TS this relation often is not so clear. Epping and Eggermont /4/ already discussed that the different adaptation levels caused by the two stimulus ensembles may be responsible for this. Another reason for the incompatibility of the

responses may be the absence of response to a single click. In the DMN most units respond to a single click, whereas in the TS more often integration of several clicks is necessary to elicit a spike. The temporal sequence of suppressive and facilitatory influences necessary to elicit a spike (e.g. Fig. 2c) is even harder to investigate.

The rate histogram to the periodic click trains and the power spectrum of the CCH in general only showed qualitative agreement. The peaks in the power spectra of Figs 1c and 2a coincide with the peaks in the rate histograms. Quantitatively however there are differences, as can be seen in Fig. 1a, b. There are two possible explanations for the discrepancies. Firstly the neuron under study is too complex for a first-order analysis. Therefore Epping and Eggermont /4/ have extended the analysis up to second order. Secondly the power spectrum of the CCH has methodological disadvantages. A histogram only has positive contributions. This leads to a large DC-term in the power spectrum, and may also lead to frequency-doubling effects. Negative contributions to the firing probability can only be seen as far as they reduce the background. The interpretation of the suppression in the CCH as a kind of dead-time in Fig. 4c was more successful to explain the low-pass rate histogram than the power spectrum. It can be concluded that the power spectrum of the CCH is useful to investigate qualitatively a multiply peaked histogram.

In this paper a time-averaged analysis of the responses to the random clicks was presented. But some units in the DMN as well as in the TS responded in a non-stationary way. Non-stationarities found were adaptation, partially or totally, and disinhibition. A proper way to analyze these responses is the time-dependent correlation function /11/. In Fig. 3 of this paper and in Fig. 4 of /11/ and Fig. 4c of /10/ examples of this analysis are shown. In most cases it appeared that the combination of the CCH with the FRH preserved the main response characteristics. A time-dependent analysis as shown in Fig. 3 is useful for a detailed analysis of the clicks preceding a spike and of non-stationarities in the response.

The relevance of click stimuli for investigation of the auditory system of the grassfrog seems to be mainly methodological. As Bibikov /2/ already pointed out, the time course of activation and suppression in the spike-generating region of a neuron may be revealed with the random click ensemble. The presence of inhibition is clear when there is initial suppression in the CCH (Fig. 2c). This underlines the importance of analysis of the temporal structure of responses. The difference between the results

of Walkowiak /12/ using 10 ms pulses, and our results with clicks points at another mechanism for PRR selectivity. The pulseform itself and the on-off time ratio may also contribute to selectivity for natural calls: Bibikov /1/ found that the optimal pulse duration at a PRR of 27 Hz was 6–12 ms.

ACKNOWLEDGEMENTS

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REFERENCES

1. Bibikov, N.G. (1980) The reaction of torus semicircularis units of Rana temporaria to the signals simulating the mating call temporal characteristics. Zool. Zh. 59, 577–586.

2. Bibikov, N.G. (1981) Cross-correlation analysis of the activity of the auditory neurones on exposure to sound clicks. Biophysics 26, 346–352.

3. Brzoska, J., Walkowiak, W., Schneider, H. (1977) Acoustic communication in the grassfrog (Rana t. temporaria L.): calls, auditory thresholds and behavioral responses. J. Comp. Physiol. 118, 173–186.

4. Epping, W.J.M., Eggermont, J.J. (1986) Sensitivity of neurons in the auditory midbrain of the grassfrog to temporal characteristics of sound. I. Stimulation with acoustic clicks. Hearing Res. 24, 37–54.

5. Hermes, D.J., Eggermont, J.J., Aertsen, A.M.H.J., Johannesma, P.I.M. (1982) Spectro-temporal characteristics of single units in the auditory midbrain of the lightly anaesthetised grass frog (Rana temporaria L.) investigated with tonal stimuli. Hearing Res. 6, 103–126.

6. Hillery, C.M., Narins, P.M. (1987) Frequency and time domain comparison of low-frequency auditory fiber responses in two anuran amphibians. Hearing Res. 25, 233–248.

7. Opdam, P., Kemali, M., Nieuwenhuys, R. (1976) Topological analysis of the brainstem of the frogs Rana esculanta and Rana catesbeiana. J. Comp. Neurol. 165, 307–332.

8. Schneider-Lowitz, B. (1983) Neuronale Verarbeitung einfacher und komplexer Schallsignale in der Peripherie und den unteren Stationen der Hörbahn des Grasfrosches (Rana t. temporaria L.). Thesis Bonn.

9. Van Gelder, J.J., Evers, P.M.G., Maagnus, G.J.M. (1978) Calling and associated behaviour of the common frog, Rana temporaria, during breeding activity. J. Anim. Ecol. 47, 667–676.

10. van Stokkum, I.H.M. (1987) Sensitivity of neurons in the dorsal medullary nucleus of the grassfrog to spectral and temporal characteristics of sound. Hearing Res. 29, 223–235.

11. van Stokkum, I.H.M., Johannesma, P.I.M., Eggermont, J.J. (1986) Representation of time-dependent correlation and recurrence time functions. A new method to analyse non-stationary point processes. Biol. Cybern. 55, 17–24.

12. Walkowiak, W. (1984) Neuronal correlates of the recognition of pulsed sound signals in the grass frog. J. Comp. Physiol. A 155, 57–66.

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THE AMPHIBIAN OCTAVO-LATERALIS SYSTEM AND ITS REGRESSIVE AND PROGRESSIVE EVOLUTION

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The phylogenetic and ontogenetic changes in the octavolateralis system of sarcopterygian fish and tetrapods, presumed to be important for the formation of an amphibian auditory system, are reviewed. The lateral line system shows rudimentation of lines and loss of ampullary electroreceptors in many amphibians; in some amphibians it never develops. The metamorphic changes of the lateral-line system show different patterns in the different amphibian lineages with metamorphic retention in most urodeles and metamorphic loss in most anurans. The multitude of both ontogenetic and phylogenetic changes of the lateral line system among amphibians do exclude any prediction as to how this system might have changed in ancestral amniotes.

The most important auditory epithelium of the tetrapod inner ear, the basilar papilla, seems to be primitively present in all tetrapods and Latimeria. In two amphibian lineages there is a trend towards rudimentation and loss of the basilar papilla. Only in the third order, the anurans, a tympanic ear develops and the inner ear shows a progressive evolution of the auditory epithelia. Together with the known differences in the periotic labyrinth of amphibians and amniotes, this scenario suggests a parallel evolution of the amniotic and anuran auditory periphery.

All mechanoreceptive hair cells of the lateral line system and the inner ear appear to receive a common and bilateral efferent innervation. Among amphibians this pattern is represented only in some urodeles, whereas anurans show a derived pattern with loss of a bilateral component and presumably also of a common neuromast/inner ear component.

Changes in the rhombencephalic nuclei which receive octavolateralis afferent fibers show a trend towards development of auditory nuclei only in the anuran lineage. The phylogenetic appearance of an auditory nucleus in this lineage coincides with the

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complete absence of formation of ampullary electroreceptors. In contrast, the earlier claim of a correlation between a metamorphic loss of the lateral line system and the formation of an auditory nucleus is not supported by more recent data: an auditory nucleus develops in anurans already prior to metamorphosis and is present in all anurans even when they retain the neuromast system. In anurans with a metamorphic loss of the neuromasts, the second order neurons degenerate as well. This independence of the auditory and the second order lateral line nuclei is further substantiated by their separate projection to other brain areas, like the torus semicircularis of the midbrain, and their functional properties.

A new hypothesis for the phylogenetic origin of the anuran auditory nucleus is proposed which suggests that the loss of ampullary electroreceptors and their afferents has caused an instability in the rhombencephalic alar plate which has led to a rearrangement of cells to form the auditory nuclei in anurans. Based on the unique features of the anuran octavo-lateralis system it is argued that this scenario provides no information about the origin of the amniotic auditory nuclei but reflects a parallel evolution of auditory nuclei of both groups.

Keywords: Octavo-lateral system, evolution, amphibia

INTRODUCTION

In the course of tetrapod evolution the octavo-lateralis system, consisting of the lateral line and inner ear, has been changed in various ways. The lateral line, with its neuromasts and ampullary organs, of primary aquatic vertebrates has disappeared in all amniotic vertebrates. In these organs, the apical part of the secondary sensory cells is exposed to the water the animal lives in and perceive the ambient stimuli directly. It is clear that no sensory cell can sustain prolonged exposure to air and that a protection by a cover of epidermal cells is not easily compatible with an ongoing function of these cells. For these reasons the sensory organs can not adapt to the terrestrial environment and hence have been lost in terrestric amniotes. Interestingly, a change in function has been suggested for the neuromasts of some amphibic fish /51/.

In contrast, the inner ear has long been known to show progressive development in tetrapods leading to the formation of an auditory middle and inner ear, in particular the basilar papilla /32/. In connection with the changes of the inner ear and the evolutionary appearance of the auditory epithelium of tetrapods, the brainstem developed the auditory nuclei and the superior olive (Fig. 1).

The coincidence between the phylogenetic loss of the neuromasts and

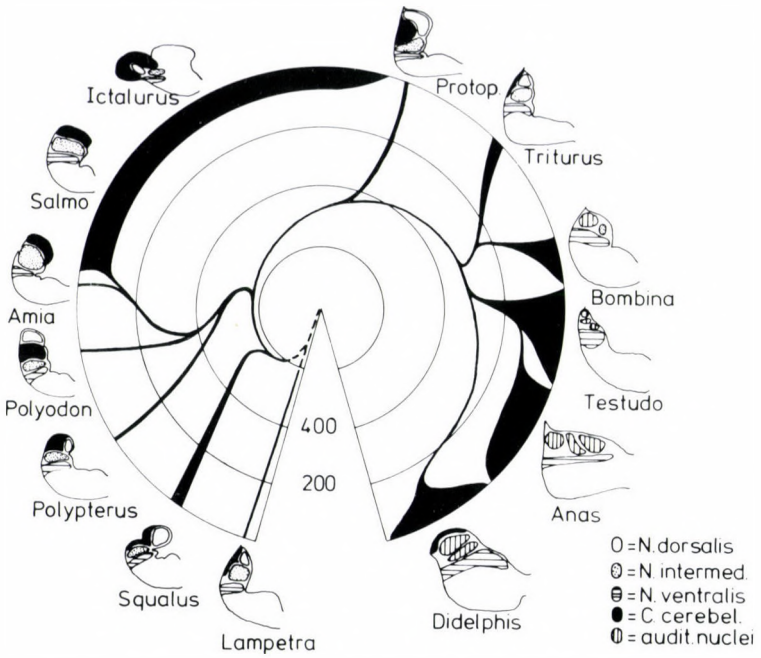


Fig. 1. Schematic representation of the alar plate and the phylogenetic relationship of vertebrates. Numbers of recent members in each vertebrate lineage are indicated by the size of the black area, million years are indicated by circles. Reduction of the dorsal, electroreceptive nucleus occurs independently in three lineages, the teleosts (Amia, Salmo), the anurans (Bombina) and all amniotes (Testudo, Anas, Didelphis). Loss of neuromast organs and the intermediate nucleus occurs during metamorphosis in many anurans. No amniote is known to develop neuromasts and intermediate nuclei. Only anurans and amniotes have delineable auditory nuclei

the first appearance of the auditory epithelia and the auditory nuclei in tetrapods has stimulated speculations about a possible link between these two events. Already Ariens-Kappers /1/ argued that the second order neurons of the neuromast system are used as auditory nuclei once the neuromasts and their afferents are lost. In a detailed study on the metamorphic changes in an anuran species of the genus Hyla, Larsell /28/ concluded that the auditory nuclei of frogs have partly developed from second order lateral line neurons during metamorphosis. Although skeptical at first, Herrick /23/ later even considered this remarkable change in function to be similar to the phylogenetic history of the formation of auditory nuclei in tetrapods. Regarding the increasing evidence in invertebrates about a metamorphic change in function of some neurons /29/, it would be interesting to show

that we simply hear by transformed second order lateral-line neurons and that metamorphosis in frogs recapitulates the phylogeny of the auditory nuclei of tetrapods. If this is true, we can study the formation of a phylogenetically new vertebrate nucleus during anuran metamorphosis.

In order to evaluate the hypothesis outlined above, I shall first describe the phylogenetic changes in the lateral line system, the inner ear and in the efferent system, and test the suggestion that the phylogenetic loss of the lateral line and the development of the basilar papilla coincide. Secondly I shall compare metamorphic changes in all three amphibian orders to find out whether amphibian metamorphosis shows a coincidence between the loss of the lateral line and the development of auditory nuclei. Thirdly, I shall present data on the second order connections of the lateral line and the inner ear, and some functional data, to explain why the hypothesis of Larsell /28/ is hardly compatible with our current knowledge. Finally, I will propose an alternative hypothesis.

PHYLOGENETIC RUDIMENTATION

1. The lateral line

Lungfish, Latimeria and most non-teleost fish possess a lateral line composed of two types of receptors, the mechanoreceptive neuromasts and the electroreceptive ampullary organs /39/. This primitive pattern is retained in larval and most adult urodeles and larval gymnophionans, whereas anurans have lost the electroreceptive ampullary organs and their afferents /17/. This loss correlates in tadpoles with a change from a predatory to a omniphagic food exploration. Both types of organs seem to develop from placodal material /15/ but the loss of ampullary organs in anurans indicates some independence for both "anlagen". This suggestion is backed up by the loss of neuromasts and retention of ampullary organs in some gymnophionans /19/. It is unclear why the posterior placodes do not develop ampullary organs nor is it clear how the two types of organs are differently formed by the anterior placodes.

Compared to lungfish, Latimeria shows a reduced number of lateral line canals both on the head and the trunk (Fig. 2) but this interpretation may be revised if we know more about the epidermal neuromasts /36/. If we assume that the pattern of lungfish is primitive for Sarcopterygians, only Siren among urodeles and pipids among anurans have retained the primitive

pattern with its four trunk lines. Regression in trunk line numbers results in three lines in most amphibians, two lines in some anurans and in a single line in gymnophionans. The adaptive significance of this regression is difficult to evaluate since we do not know the function of individual trunk lines. In contrast, loss of lines on the head seems to be related to the changes in the mouth of tadpoles /16, 26/. Besides a reduction in line numbers, there is shortening of lines /10/ and a reduction in stitch formation. Moreover, there appears to be changes in the direction of stitch formation and in the orientation of neuromasts and stitches /10, 26, 27/. All these changes may be interpreted as rudimentation /40/.

In utero embryos of terrestric urodeles develop at least neuromasts /10/ in utero, and direct developing embryos of some gymnophionans develop ampullary organs /19/ and the embryos in the skin of the aquatic anuran Pipa develop neuromasts /10/. In contrast, no amphibian species which develops in ovo on land has been shown to develop neuromasts. Interestingly, these are the only amphibians known to resemble amniotic vertebrates in that they develop independently of water and never have a lateral line. However, direct developing amphibians parallel amniotic vertebrates but are unlikely to exemplify the primitive amphibian condition because they are considered to be derived in all three lineages /7/.

In conclusion, phylogenetic loss of the lateral line system shows three levels of reduction:

(i) Rudimentation by loss or shortening of lines and loss of stitch formation.

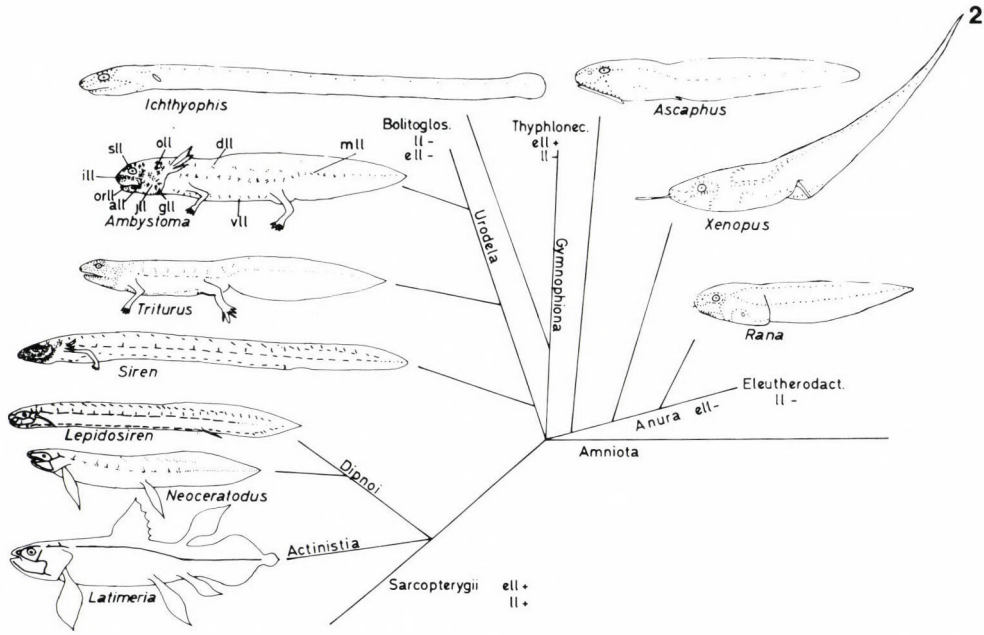
(ii) Complete absence of ampullary organs, of neuromasts or of both.

(iii) Metamorphic loss of the larval system (see below).

Considering this diversity in regressive patterns, we might gain knowledge about general principles of lateral line regression by studying amphibians. However, we will not be able to identify with this approach the pattern of lateral line loss in amniotic ancestors because we know nothing about their embryonic development and the adaptive constraints imposed on the development of their lateral line organs.

2. The inner ear

Hearing in aquatic anamniotic vertebrates may be achieved with different labyrinthine organs /42/ whereas the basilar papilla is the major auditory epithelium in tetrapods /2/. Recent evidence indicates that in


2
Ichthyophis
Ascaphus
Bolitoglos.
II -
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e II +
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oII
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iII
orII
aII
gII
vII
Ambystoma
Urodela
Gymnophiona
Xenopus
Triturus
Rana
Siren
Eleutherodact.
II -
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Neoceratodus
Actinistia
Latimeria
Sarcopterygii e II +
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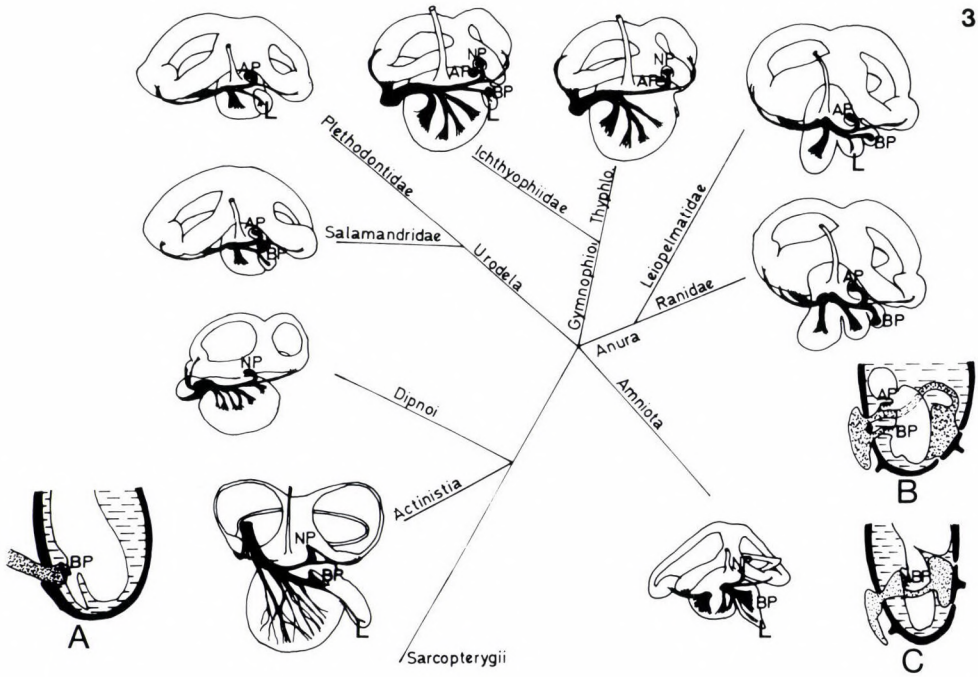
3
AP
NP
BP
L
Plethodontidae
Ichthyophiidae
Thyphlo.
Salamandridae
Urodela
Gymnophiona
Leiopelmatidae
Ranidae
Anura
Amniota
Dipnoi
Actinistia
Sarcopterygii
A
B
C

Latimeria there is an epithelial specialization which in position, innervation pattern and structure resembles the basilar papilla of tetrapods, in particular of amniotes /13/. In addition to the development of the auditory sensory epithelium, the development of the so-called periotic labyrinth is necessary for terrestrial hearing /32/. Latimeria has a perilymphatic space underlying the basilar papilla like in amniotes, whereas the basilar papilla of amphibians is in a blind ending relation to the periotic system /2/. The sound conducting periotic labyrinth appears to be largely absent in Latimeria and differently developed in tetrapods /32/ (Fig. 3). These differences among tetrapods may be related to different patterns of phylogenetic development of a tympanic middle ear. Impedance matching problems may have rendered the basilar papilla of amphibian ancestors comparatively unresponsive to airborn sound before a tympanic ear was evolved. In the course of subsequent rudimentation the basilar papilla might have changed its position with respect to the periotic sac.

Fig. 2. The distribution of lateral line neuromasts (ll+) and ampullary organs (ell+) is shown for Sarcopterygii. There are differences in lateral line numbers on the trunk and the head between Actinistia and Dipnoi. Some Amphibians (Siren, Xenopus) have four trunk lines, but three trunk lines prevail. Rudimentation leads to two lines (Ascaphus) or one line (Ichthyophis). Note the longitudinal stitches in Lepidosiren and Siren and the changed stitch orientation in Xenopus. Direct developing urodeles (Bolitoglossa) do not develop neuromasts (ll-) and ampullary organs (ell-), some gymnophionans (Typhlonectes) develop no neuromasts (ll-) but ampullary organs (ell+) and all anurans lack ampullary organs (ell-). The lateral line organs of anurans may either disappear during metamorphosis or may never develop (Eleutherodactylus, ll-). Continuous lines indicate canals, short lines stitches and dots neuromasts. Abbreviations: all = angular line, dll = dorsal line, gll = gular line, ill = infraorbital line, jll = jugular line, mll = medial line, oll = occipital line, orll = oral line, sll = supraorbital line, vll = ventral line. Drawings are modified according to Escher (1925) /10/ and own data

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Fig. 3. The auditory epithelia, their innervation and their relation to the periotic labyrinth is shown for Sarcopterygii. Latimeria, but no lungfish, has a basilar papilla (BP) similar to tetrapods in location and innervation. Structurally (A) it resembles the BP of amniotes (C) more than the BP of amphibians (B) because it rests on a membrane that separates a perilymphatic (stippled) from an endolymphatic space (white). In contrast, in amphibians both the BP and amphibian papilla (AP) are in an indirect relation to the perilymphatic system (B). The BP is lost in derived urodeles (Plethodontidae) and derived gymnophionans (Typhlonectidae). Innervation pattern of the BP changes in the anuran lineage from the primitive pattern (BP fibers run with fibers to the lagena) to a unique derived pattern (BP fibers run with AP fibers). Amniotes are represented by a turtle ear. Drawings are modified after Baird /2/, Will and Fritzsch /49/, Fritzsch /13/ and own data. L = lagena

Further rudimentation in urodeles and gymnophionans, which may primitively lack a tympanic ear, has led to the loss of the basilar papilla in many derived urodeles /31/ and gymnophionans /46/. Besides mammals, some gymnophionans are the only tetrapods which have also lost the lagena. Because of the loss of the basilar papilla in some amphibians and of the lagena in some gymnophionans and in most mammals, the absence of a lagena and a basilar papilla in lungfish can be interpreted as either secondarily derived by loss or as primitively absent. In contrast, most anurans have a tympanic ear. Primitive anurans have auditory epithelia like urodeles whereas derived anurans show a progressive increase of the auditory epithelia /30/.

In conclusion, the basilar papilla is present already in Latimeria and therefore before the rudimentation of the lateral line system in amphibians and amniotes started (Figs 2, 3). Apart from this lack of coincidence between the phylogenetic development of the basilar papilla and the loss of the lateral line, there is a regression and loss of the basilar papilla in some gymnophionans and urodeles which have incidentally also lost the lateral line. In contrast, anurans increase the auditory epithelia in derived species and evolve a tympanic ear. This interpretation suggests that the evolution of the anuran auditory periphery parallels rather than precedes the evolution of the amniotic auditory periphery.

3. Central pathways and nuclei

The lateral line afferents enter the alar plate of the rombencephalon rostral (head lateral line) or caudal (trunk lateral line) to the octavus nerve /39/. Afferents reach the ventral zone (octavus afferents), the intermediate nucleus (neuromast afferents) and the dorsal nucleus (ampullary organ afferents) in lungfish /39/ larval gymnophionans and larval and most adult urodeles (/14/; Fig. 4). Direct developing urodeles lack the lateral line afferents and show no intermediate and dorsal nuclei. Some direct developing and some lifebearing gymnophionans possess ampullary organs and a projection of afferents to a dorsal nucleus /33/. Other gymnophionans have no identifiable dorsal or intermediate nucleus /49/. As in direct developing urodeles, the alar plate is in these gymnophionans dominated by the inner ear afferents but shows no specialization into an auditory nucleus /49/. Urodeles and gymnophionans which lose their lateral line during metamorphosis show no reduction of the dorsal and intermediate nucleus (see below).

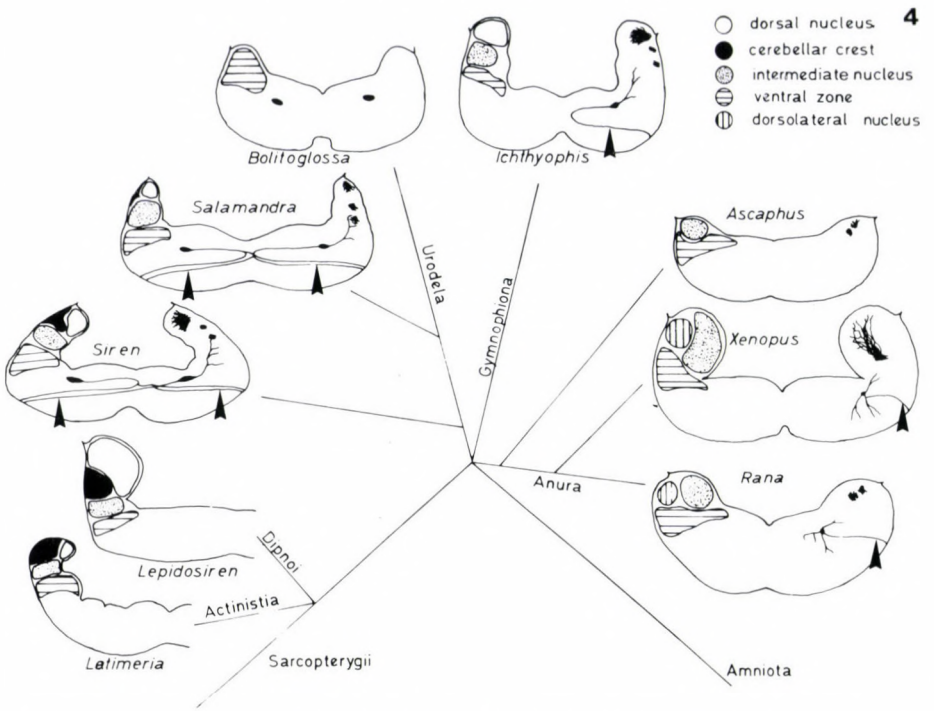

Fig. 4. The nuclei of the alar plate, the pattern of lateral line afferents and the efferent cells are shown. Note the similar alar plate pattern in Dipnoi, Actinistia and Siren, Salamandra has a lateral line only as larvae, whereas Bolitoglossa never develops a lateral line and lacks identifiable dorsal and intermediate nuclei. A projection of ampullary organs to the dorsal nucleus is also present in larval gymnophinans but is absent in all anurans. The octavo-lateralis efferent cells are bilateral with bilateral collaterals only in urodeles but are unilateral in gymnophionans and anurans with characteristic differences in the course of the axon (arrowhead). Ascaphus seems to lack neuromast efferents. Bolitoglossa may have retained the lateral line efferents because of their connection to the inner ear whereas Rana may lose efferents innervating exclusively to the lateral line during metamorphosis. Compiled after Fritzsch /14/ and Will and Fritzsch /49/

In contrast, anuran larvae have only an intermediate nucleus (neuromast afferents) and a ventral zone (labyrinthine afferents) but no recognizable dorsal nucleus /20/. Already in premetamorphic tadpoles a new nucleus, the dorsolateral nucleus /49/, appears in a submeningeal position (Fig. 4). It receives afferents of auditory epithelia /25/. A similar pattern exists in frogs which retain the lateral line system like Xenopus /49/. In adult frogs which never had or have lost the lateral line there is no intermediate nucleus (see below) and only a dorsolateral (auditory) nucleus and a ventral zone may be recognized /49/.

In conclusion, among amphibians primitive urodeles, gymnophionans, and ancestral amniotes resemble the primitive Sarcopterygian pattern of the alar plate (Fig. 4). In both amphibian orders, changes in the alar plate correlate with the reduction in the lateral line but not with changes in the inner ear auditory epithelia. The evolution of the dorsolateral nucleus in anurans coincides with the loss of a recognizable dorsal nucleus. Premetamorphic tadpoles and all adult anurans, irrespective of the neuromast system, have a dorsolateral nucleus. In contrast to Larsell's hypothesis /28/ it is the phylogenetic loss of the ampullary organs, and not of the neuromasts, which coincides with the formation of the dorsolateral nucleus in anurans.

4. The efferent system

The neuromasts and most labyrinthine epithelia receive a centrifugal or efferent innervation /49/. Moreover, this innervation is common for both systems in many anamniotes including urodeles /5, 34/. The neuromast and inner ear efferent perikarya in the rhombencephalon are bilateral and collaterals reach organs on both sides. In amphibians only urodeles display this primitive vertebrate pattern /18/. Anurans and gymnophionans are derived and have perikarya located unilaterally /16, 48/. Further, collaterals are restricted to one side of the body, axons show characteristic differences between gymnophionans and anurans /16/, and decisive evidence for common lateral line and inner ear efferents in anurans is still lacking /20/. There is also some indication that the lateral line of Ascaphus has no efferents (Fritzsch, unpublished data; Fig. 4). The physiological significance of these changes is not clear but unilateralization occurs also in teleosts /5, 16/. The absence of an efferent innervation to the basilar papilla in anurans seems to reflect the loss of an innervation present in urodeles, gymnophionans and most amniotes /18, 45/. Loss of efferent innervation like in anurans occurs also in parts of the cochlea of bats /4/ and some reptiles /35/.

The data on the efferent system clearly shows another case of rudimentation in the octavo-lateralis system and tends to support the conclusions presented for the other parts of the octavo-lateralis system: urodeles display the primitive and anurans the derived amphibian pattern whereas in gymnophionans the relationship is not as clear but may be intermediate with unique specializations.

METAMORPHIC CHANGES

1. The lateral line

During metamorphosis larval structures, such as the lateral line, either regress or transform to be suitable for adults. No loss of lateral line organs occurs in most urodeles. Only the complete terrestrial adults of Salamandra and a few other species of urodeles lose the lateral line organs and most of its nerves /10, 13, 14, 22/. The retention of lateral line organs in most urodeles may be important for foraging or for courtship in later aquatic life phases. In gymnophionans the larval lateral line organs and nerves are lost during metamorphosis /19, 38/. Most anurans lose the neuromasts and the lateral line nerves during metamorphosis /43/, but some aquatic anurans retain parts of the lateral line system either for foraging or for courtship /21/. The loss of organs is rapid and shows different patterns in different amphibians /10/, whereas the degeneration of lateral line nerves is much slower. Metamorphic changes of urodeles and gymnophionans may be closer to those of ancestral amphibians and those of anurans are suggested to be derived /44/. However, metamorphosis may not recapitulate the changes leading to the lateral line loss in ancestral amniotes, because nothing is known about metamorphosis in the amniotic lineage.

2. The inner ear

The inner ear in anurans shows virtually no changes during metamorphosis. There is, however, a postmetamorphic increase in number of hair cells /6/ and changes in the physiological characteristics of the auditory receptors /41/. The tympanic ear of anurans develops at or after metamorphosis /24/. Metamorphic changes in the inner ear of urodeles and gymnophionans have not yet been studied.

3. Central pathways and nuclei

Urodeles and gymnophionans which lose their lateral line organs apparently retain the second order neurons /49/. There is little evidence for an expansion of the inner ear projection to the dorsal nucleus, but expansion may occur to the intermediate nucleus. In contrast, in anurans, neuromasts, their afferents and a substantial part of the second order neurons degenerate /43/, and there is no evidence of second order lateral line neurons transforming into auditory nucleus cells. Only the caudal part

of the intermediate nucleus may survive metamorphosis and has been recognized in adult anurans /49/. Further, the auditory nuclei are already present in tadpoles /25/. Other anuran species retain most neuromasts, their afferents and the intermediate nucleus, but nevertheless develop an auditory nucleus /21, 50/. The metamorphic loss of the intermediate nucleus in many anurans as well as the presence of the intermediate and dorsolateral nucleus in larval and some adult anurans makes the hypothesis of a transformation of intermediate nucleus cells to dorsolateral nucleus cells highly unlikely.

4. The octavo-lateralis efferents

Metamorphic changes in the efferents have not been studied. Given their widespread collaterals to the lateral line and inner ear in urodeles /18/, it is likely that they do not degenerate even in species which lose the lateral line during metamorphosis. In anurans and probably gymnophionans, at least some lateral line efferents have no collaterals to the inner ear /16, 20, 48/. It is conceivable that these cells may degenerate when their target disappears. However, like in insects /29/ they could be re-used in other neuronal connections.

ASCENDING CONNECTIONS

There are additional arguments against the replacement hypothesis of Larsell /28/ if one considers the ascending connections and their function. Transformation of second order lateral line neurons into auditory nuclei neurons would lead to comparable ascending projections of the intermediate and the dorsolateral nucleus. The ascending connections of the dorsolateral nucleus are mainly to the contralateral dorsolateral nucleus, to the superior olive and to the principal toral nucleus /3, 11, 47/. In contrast, the intermediate nucleus projects to the contralateral intermediate nucleus and to the magnocellular toral nucleus but not to the superior olive /50/. The projection to the torus appears to be as distinct as the nuclei and afferent input to the alar plate (Fig. 5). Moreover, it has recently been shown that some lateral line information reaches the tectum, presumably via the magnocellular torus, and provides a directional map that is in register with the visual map /52/. In contrast, auditory information has never been found to reach the tectum in frogs. This segrega-

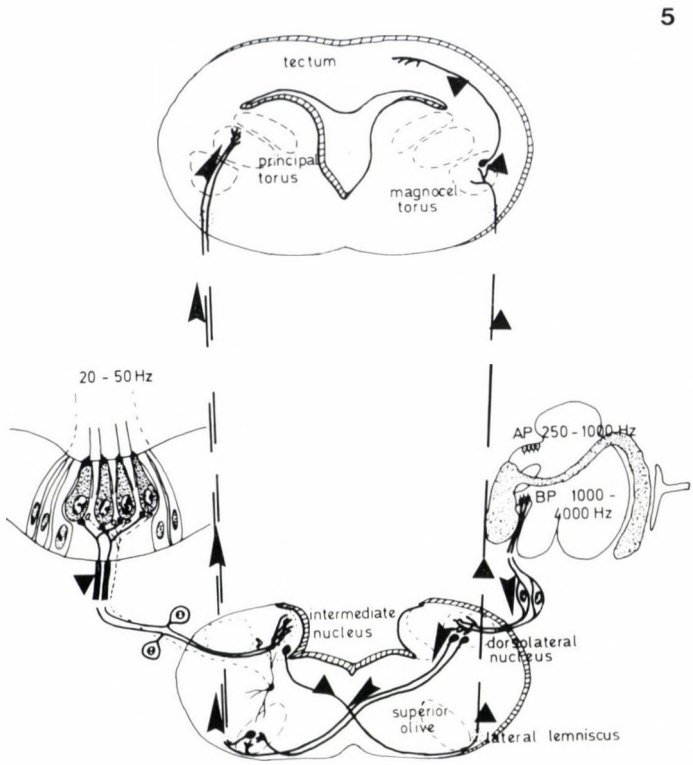

Fig. 5. This scheme shows the ascending connections of the lateral line (triangle) and of the auditory epithelia (arrowheads) in Xenopus. Afferents of neuromasts are in contact with hair cells of two opposite polarities. They are stimulated by low frequency signals. Axons of pseudounipolar afferent cells innervate neurons of the intermediate nucleus. These cells send axons via the lateral lemniscus to the magnocellular toral nucleus. A further projection to the tectum has been proposed. In contrast, the basilar papilla (BP) and amphibian papilla (AP) receive rather high frequency stimuli. The axons of the bipolar afferent cells terminate in the dorsolateral nucleus. Ascending information flows either direct or via superior olive neurons to the principal toral nucleus. Efferents to the neuromasts are indicated by a broken line. No efferents exist to the BP. Compiled according to Münz et al. /37/, Wilczynski and Capranica /47/, Will et al. /50/, Zittlau et al. /52/ and own data

tion of the ascending lateral line and auditory pathways was also reported for teleosts /8/ and may be primitive for Sarcopterygians.

In contrast to the directional information of the auditory system, which is most likely generated by a pressure gradient mechanism /9/, the directional information of the lateral line is generated by an interaction

of the information of numerous receptors /52/ which are unlikely to involve a pressure gradient mechanism. Furthermore, auditory directionality is represented already in superior olive neurons and also in a complex spatiotopic map in the torus but not in the tectum /47/. In contrast, little directional information is reported for medullary and toral lateral line units but a clear directionality is present in tectal lateral line units /52/. In addition, spectral information processing is in the frequency range of about 100 to 4000 Hz in the anuran auditory system with different receptors devoted for different frequency ranges /47/. However, there is only a limited frequency range of about 20–80 Hz to which amphibian neuromasts respond /37/.

In conclusion, the second order connections are anatomically and most likely physiologically distinct for the auditory and the lateral line in anurans. This argues against a replacement hypothesis for the evolution of the auditory nucleus and supports the conclusions derived from the phylogenetic and metamorphic changes outlined above. Considering how difficult it already is to unravel the differences in connections and changes of the octavo-lateralis system in amphibians, it is premature to speculate which auditory nucleus in the rhombencephalon and the torus of mammals and birds is homologous to which nucleus in these areas of amphibians or processed lateral line and inner ear information in ancestral amniotes. More studies on connectivities and their development are necessary, particularly on basic reptiles, before we can tackle this problem.

ORIGIN OF THE DORSOLATERAL AUDITORY NUCLEUS OF ANURANS

Having ruled out the ontogenetic or phylogenetic transformation of the intermediate nucleus into an auditory nucleus, there remains the phylogenetic coincidence between the disappearance of ampullary organs and the formation of the dorsolateral nucleus in anurans. These events have been suggested to be causally linked /20/. The available ontogenetic studies indicate that the dorsolateral nucleus may have two sources, the ventral zone and an undifferentiated dorsal part of the alar plate. The dorsal zone has been speculated to be homologous to the proliferation zone of the dorsal electroreceptive nucleus /20/. However, loss of ampullary organs and their afferents might have resulted in a phylogenetic instability of the alar plate which might have led to the de novo formation of this dorsal proliferation zone.

Formation from ventral zone proliferation areas is another likely source of the dorsolateral nucleus /20/. The efferent connection via a superior olive to the torus /11, 50/ is very similar to the ascending connections of the teleostean ventral zone /12/ and tends to support this assumption. This suggestion implies continuity with the area that may receive the afferents of the basilar papilla in Latimeria, urodeles and gymnophionans and was presumably involved in the processing of vibratory signals.

If both suggestions are true, the dorsolateral nucleus of anurans presents a heterogeneous cell population derived from two phylogenetically old sources and may be new only with respect to its position. This double origin was possible because of the selective phylogenetic loss of the ampullary organs in ancestral anurans whereas ontogenetic changes of the neuromast system do not contribute to the origin of auditory nuclei. Since the possible origin of the auditory nuclei of amniotes from ancestral intermediate or dorsal nuclei has never been demonstrated, it is premature to discuss about homologies with anurans. Moreover, because the anuran auditory system appears to be derived among amphibians in nearly every respect, I suggest that the anuran auditory system developed in parallel to the amniotic auditory system instead of reflecting the ancestral conditions for amniotes.

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REFERENCES

1. Ariens Kappers, C.U. (1920) Vergleichende Anatomie des Nervensystems. E.F. Bohn, Haarlem.
2. Baird, I.L. (1974) Anatomical features of the inner ear in submammalian vertebrates. In: Keidel, W.D., Neff, W.D. (eds) Handbook of Sensory Physiology. Vol. V/1. Springer Verlag, Berlin, pp. 159–212.
3. Boord, R.L. McCormick, C.A. (1984) Central lateral line and auditory pathways: a phylogenetic perspective. Amer. Zool. 24, 765–774.
4. Bruns, V., Schmieszek, E. (1980) Cochlear innervation in the greater horseshoe bat: demonstration of an acoustic fovea. Hearing Res. 3, 27–43.

5. Claas, B., Fritzsch, B., Münz, H. (1981) Common efferents to the lateral line and labyrinthine systems in aquatic vertebrates. Neurosci. Lett. 27, 231–235.

6. Corwin, J.T. (1985) Perpetual production of hair cells and maturational changes in hair cell ultrastructure accompany postembryonic growth in an amphibian ear. Proc. Natl. Acad. Sci. USA. 82, 3911–3915.

7. Duellman, W.E., Trueb, L. (1986) Biology of Amphibians. McGraw-Hill, New York.

8. Echteler, S.U. (1985) Organization of central auditory pathways in a teleost fish. Cyprinus carpio. J. Comp. Physiol. 156, 267–280.

9. Eggermont, J. (1988) Mechanisms of sound localization in Anurans. In: Fritzsch, B., Ryan M., Wilczynski, W., Hetherington, T.E., Walkowiak, W. (eds), The Evolution of the Amphibian Auditory System. Wiley and Sons, Chichester, pp. 307–336.

10. Escher, K. (1925) Das Verhalten der Seitenorgane der Wirbeltiere und ihrer Nerven beim Übergang zum Landleben. Acta Zool. (Stockh.). 6, 307–414.

11. Feng, A.S. (1986) Afferent and efferent innervation patterns of the cochlear nucleus (dorsal medullary nucleus) of the leopard frog. Brain Res. 367, 183–191.

12. Finger, T.E., Tong, S.-L. (1984) Central organization of eighth nerve and mechanosensory lateral line system in the brainstem of ictalurid catfish. J. Comp. Neurol. 229, 129–151.

13. Fritzsch, B. (1987) The inner ear of the coelacanth fish Latimeria has tetrapod affinities. Nature. 327, 153–154.

14. Fritzsch, B. (1988) The inner ear projections of larval and adult urodeles. Brain, Behav. Evol. 31, 325–348.

15. Fritzsch, B., Bolz, D. (1986) On the development of the ampullary organs in the mountain newt, Triturus alpestris. Amphibia – Reptilia 7, 1–9.

16. Fritzsch, B., Crapon de Caprona, M.-D. (1984) The origin of centrifugal inner ear fibers of gymnophions. A horseradish peroxidase study. Neurosci. Lett. 46, 131–136.

17. Fritzsch, B., Münz, H. (1986) Electroreception in Amphibians. In. Bullock, T.H., Heiligenberg, W. (eds) Electroreception. Wiley and Sons, New York, pp. 483–496.

18. Fritzsch, B., Wahnschaffe, U. (1987) Electron microscopical evidence for bilateral and common lateral line and inner ear efferents in urodeles. Neurosci. Lett. 81, 48–52.

19. Fritzsch, B., Wake, M.H. (1986) A note on the distribution of ampullary organs in Gymnophions. J. Herpetol. 20, 90–93.

20. Fritzsch, B., Nikundiwe, A.M., Will, U. (1984) Projection patterns of lateral line afferents in anurans. A comparative study using transganglionic transport of HRP. J. Comp. Neurol. 229, 451–469.

21. Fritzsch, B., Drewes, R.C., Ruibal, R. (1987) On the retention of the lateral line nucleus in adult anurans. Copeia, 1, 127–135.

22. Göppert, E. (1929) Untersuchungen am Lateralissystem der Amphibien. Die Kopfganglien der Urodelen vor und nach der Metamorphose. Gegenbaurs Morph. Jahrb. 62, 507–542.

23. Herrick, C.J. (1948) The Brain of the Tiger Salamander. university of Chicago Press, Chicago.

24. Hetherington, T.E. (1988) Metamorphic changes in the middle ear. In: Fritzsch, B., Ryan, M., Wilczynski, W., Hetherington, T.E., Walkowiak, W. (eds) The Evolution of the Amphibian Auditory System. Wiley and Sons. Chichester, pp. 339–358.

25. Jacoby, J., Rubinson, K. (1983) The acoustic and lateral line nuclei are distinct in the premetamorphic frog, Rana catesbeiana. J. Comp. Neurol. 216, 152–161.

26. Lannoo, M.J. (1987a) Neuromast topography in urodele amphibians. J. Morphol. 191, 247–263.

27. Lannoo, M.J. (1987b) Neuromast topography in anuran amphibians. J. Morphol. 191, 115–129.

28. Larsell, O. (1934) The differentiation of the peripheral and central acoustic apparatus in the frog. J. Comp. Neurol. 60, 473–527.

29. Levine, R.B. (1984) Changes in neuronal circuits during insect metamorphosis. J. Exp. Biol. 112, 27–44.

30. Lewis, E.R., Leverenz, E.L. Bialek, W. (1985) The vertebrate inner ear, CRC Press, Boca Raton. p. 256.

31. Lombard, R.E. (1977) Comparative morphology of the inner ear in salamanders (Caudata: Amphibia). Contrib. Vert. Evol. 2, 1–143.

32. Lombard, R.E., Bolt, J.R. (1979) Evolution of the tetrapod ear: an analysis and reinterpretation. Biol. J. Linn. Soc. 11, 19–76.

33. McCormick, C.A., Braford, M.R. (1987) Central connections of the octavo-lateralis system. In: Atema, J., Fay, R.R., Popper, A.N., Tavolga, W.N. (eds), Sensory systems in aquatic animals. Springer Verl., New York. (In press).

34. Meredith, G.E., Roberts, B.L. (1986) Central organization of the efferent supply to the labyrinth and lateral line of the dogfish. Neuroscience. 17, 225–233.

35. Miller, M.R. (1980) The reptilian cochlear duct. In: Popper, A.N., Fay, R.R. (eds) Comparative Hearing in Vertebrates. Springer Verl., Berlin.

36. Millot, J., Anthony, H. (1965) Anatomie de Latimeria chalumnae. Vol. II. Systeme nerveux et organes des sens. Centre National de la Recherche, Paris.

37. Münz, H., Claas, B., Fritzsch, B. (1984) Electroreceptive and mechanoreceptive units in the lateral-line of the axolotl Ambystoma mexicanum. J. Comp. Physiol. 154, 33–44.

38. Norris, H.W., Hughes, S.P. (1918) The cranial and anterior spinal nerves of the caecilian amphibians. J. Morphol. 31, 489–569.

39. Northcutt, R.G. (1986) Electroreception in nonteleost bony fishes. In: Electroreception. Bullock, T.H., Heiligenberg, W. (eds). Wiley and Sons, Chichester.

40. Sewertzoff, A.N. (1931) Morphologische Gesetzmässigkeiten der Evolution. Fischer Verlag, Jena.

41. Shofner, W. (1987) Postmetamorphic changes in the auditory system. In: Fritzsch, B., Ryan, M., Wilczynski, W., Hetherington, T., Walkowiak, W. (eds) Evolution of the Amphibian Auditory System. Wiley and Sons, Chichester, pp. 377–392.

42. Tavolga, W.N., Popper, A.N., Fay, R.R. (1981) Hearing and sound communication in fishes. Springer Verl., New York.

43. Wahnschaffe, U., Bartsch, U., Fritzsch, B. (1987) Metamorphic changes within the lateral line system of Anura. Anat. Embryol. 175, 431–442.

44. Wassersug, R.J., Hoff, K. (1982) Developmental changes in the orientation of the anuran jaw suspension. Evol. Biol. 15, 223–246.

45. White, J.S. (1986) Morphological and fine structural features of the basilar papilla in ambystomatid salamanders (Amphibia: Caudata). J. Morphol. 187, 181–199.

46. White, J.S., Baird, I.L. (1982) Comparative morphological features of the caecilian inner ear with comments on the evolution of amphibian auditory structures.Scann. Electron Micr. 3, 1301–1312.

47. Wilczynski, W., Capranica, R.R. (1984) The auditory system of anuran amphibians. Progr. Neurobiol. 22, 1–38.

48. Will, U. (1982) Efferent neurons of the lateral line system and the VIIIth cranial nerve in the brainstem of anurans. Cell Tissue Res. 225, 673–685.

49. Will, U., Fritzsch, B. (1988) The eighth nerve of amphibians: Patterns of afferents and efferents. In. Fritzsch, B., Ryan, M., Wilczynski, W., Hetherington, T., Walkowiak, W. (eds) The Evolution of the Amphibian Auditory System. Wiley and Sons, Chichester, pp. 159–184.

50. Will, U., Luhede, G., Görner, P. (1985) the area octavo-lateralis of Xenopus laevis. Part II. Second order projections and cytoarchitecture. Cell. Tissue Res. 239, 147–161.

51. Zander, C.D. (1984) Ökologische Faktoren und Regression. Fortschr. zool. Syst. Evol.-forsch. 3, 101–115.

52. Zittlau, K.E.,Claas, B., Münz, H. (1986) Directional sensitivity of lateral line units in the clawed toad Xenopus laevis Daudin. J. Comp. Physiol. 158, 469–477.

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RARE CLINICAL DISTURBANCES OF THE BRAIN STEM MECHANISM AS CEREBELLAR FITS AND DROP ATTACKS

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Cerebellar Fits (CF) and Drop Attacks (DA) are rare pathophysiological disorders of the Brain Stem (BS) and of the Cerebellar System (CS). Both have various etiologies (traumatic, vascular of tumoural). Both manifestations have an individual character. In this respect I would like to make 5 clinical observations (3 CF and 2 DA). These cases were verified clinically as well as by such methods as NMR (Nuclear-Magnetic-Resonance) CT-scan (Computer-Tomography-scan examination), BAEP (Brainstem-Auditive-Evoked-Potential), CCG (Cranio-Corpo-Graphy-based on a modified Unterberg's Test) and TDS (Transcranial-Doppler-Sound-examination), etc. In this short paper I wish to present some particular clinical apsects of the CF and DA.

Keywords. Brainstem lesions, Cerebellar Fits, Drop Attacks

The aim of this short paper is to offer scientific material for a discussion concerning a difficult problem of neuroscience. Cerebellar Fits (CF) and Drop Attacks (DA) are rare pathophysiological disorders of the Brainstem (BS) and of the Cerebellar System (CS). CF and DA have various etiologies (traumatic, vascular or tumoral), sometimes widespread sites of origin within the Central Nervous System (CNS) and have a multiple clinical individual manifestation. For this reason CF and DA are highly complex disorders. First of all, the CF is a rare clinical complex manifestation. Perhaps it is because of the individual character of the manifestation that

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no monograph has been available summarizing this difficult clinical problem.

The term of CF was introduced by Hughlings Jackson 1906 (see Ref. 4). Since this time many authors have discussed this rare clinical problem (see /1/ and /3/), now commonly called as CF. Clinically CF refers to sudden vertigo accompanied by vegetative symptoms as nausea or head-skin-perspiration, sometimes vomiting, but no convulsions or myoclonies or loss of consciousness. Characteristic by the CF is the sudden loss of the leg strength and fall if not supported. The duration of a CF is a few seconds or minutes (see /2/). Each patient with CF has an individual attack (A personal manifestation). The CF is not accompanied by psychologic anxious states.

CASE No. 1.: V.H. 36-year-old male (police sergeant). In 1983 he has suffered a closed head injury with lose of consciousness for a short time, later with hard of hearing an tinnitus at the right ear, cerebellar ataxia, sleep disturbances and poor concentration. Since summer 1984 intermittent weakness of legs, headaches and sometimes falling if not supported. No convulsions, no myoclonies or loss of consciousness. Duration of a CF was a few minutes followed by general tiredness. Frequency 1984 = 9 times, 1985 = 7 times, 1986 = 6 times and 1987 = 4 times (information data Sept. 1987).

The NMR from 1985 specially performed for the BS examination: no morphological changes. The BAEP (Brainstem-Auditive-Evoked-Potential) from 1984 indicates a wave instability and curve latency at right. The BAEP from 1985 (control examination) demonstrates a curve amelioration, but diminution of the wave amplitude at right. The CCG (Cranio-Corpo-Graphy-based on a modified Unterberg's Test) from 1985 has given indication for a central vestibular instability at right. The TDS (Transcranial-Doppler-Sound-examination) from 1985 without pathological blood-flow-velocity modifications.

CASE No. 2.: G.B. 41-year-old male (insurance agent) since 1981 suffering from cardiac arrhythmia; for this reason a pacemaker was implanted in October 1983, later arrhythmia amelioration. But 1984 intermittent weakness of legs, bilateral tinnitus, falling (sometimes) if not supported, without convulsions, myoclonies or loss of consciousness. At the beginning of CF, the frequency was 1 or 2 times weekly, duration few minutes. 1986 = once in a month and 1987 no CF. By this case no vegetative symptoms. The NMR — was not possible (acemaker). The BAEP from 1985 has

demonstrated a wave amplitude diminution predominant at right side. The CCG from 1985 has indicate light central vestibular disturbances at right.

CASE No. 3.: F.B. 46-year-old male (surveying engineer). 1981 operated for an epidermoid tumour in the clivus region. Since 1983 he developed vertigo accompanied by nausea, sometimes vomiting and intermittent weakness of legs and falling if not supported. No convulsions or loss of consciousness. Duration of CF was a few seconds. Frequency at the beginning: 1 or 2 CF daily; 1986 = one CF in a week. At present no CF. The clinical examination has revealed an auditory weakness at right, cerebellar gait ataxia at left and sleep disturbances. The NMR – examination in different geometrical projections have demonstrated tumour-rest anterior and lateral (at left) of the BS and clivus region. The control NMR – examination (1986) revealed unchanged tumour-rest dimension and localization (see Ref. 2).

In comparison with the CF, the DA are most different. The DA are an extremely rare clinical manifestation. The frequency of the DA shows great individual differences. No convulsions, no myoclonies, no vegetative symptoms or loss of consciousness. No vertigo! Characteristic of the DA is the falling backwards. Duration: few seconds.

CASE No. 1.: U.K. 55-years-old male (pensioner) has been suffering since 1978 from an Arteria-Basilaris-Insufficiency. A By-pass-operation from the left external occipital Artery to the left posterior-inferior-Cerebellar Artery was performed in April 1985. In May of the same year Lumbothecal-drainage-operation followed. Since June 1985 he developed DA with falling backwards once or twice daily. The clinical examination has revealed a gait ataxia at the right, converged strabism at left and Diplopia. After DA he was anxious and gloomy. Duration: few seconds, no convulsions or loss of consciousness. He was able to walk only with the help of a special device. The CT-scan and NMR – examinations showed no morphological alterations, but BAEP has demonstrated a wave instability at the left. The CCG – examination was not possible. Only the TDS has given indication concerning a blood-flow-velocity modification in the posterior temporal Artery at left and in the vertebral Artery at left. In the Arteria Basilaris diminution of the blood-flow-velocity was observed.

CASE No. 3.: M.Cl. a 34-years-old woman (pensioned) was suffering from a very rare intranatal-brain-hypoxia complicated at the age of 4 years by a post-Pertussis-Encephalitis. After this last infection she developed Epilepsy. For this reason she was operated (1958) left temporo-parieto-occipital. A successful epilepsy-focus-resection was performed. After this

procedure the various epileptical seizures (a) Petit-Mal, b) focal seizures at right) remained under anticonvulsivant treatment unchanged for the last 29 years. In the meantime she has developed DA (falling backwards if not supported — without convulsions or myoclonies) frequency once or twice DA in a week, duration few seconds. The CT — scan in different geometrical projections (sagittal, coronal and transversal) give indications of a left parieto-occipital-porencephaly and at the same time, a global cerebral and cerebellar atrophy. In the operated field (s.a.) several metal clips. For this reason NMR was not possible. The BAEP and CCG have demonstrated functional BS-disturbances at left.

CONCLUSIONS

CF and DA are brief involuntary clinical disturbances of the BS and CNS, without convulsions, myoclonies or loss of consciousness. Their duration is a few seconds or minutes. The onset is sudden, however CF is accompanied by vertigo and vegetative symptoms. By the DA characteristic is falling backwards if not supported. The diagnosis is difficult, the BAEP and CCG give us indication of a BS and vestibular-central-disturbances. Due to the rarity of the symptoms it is very difficult to differenciate the pathophysiological aspects of CF and DA. As a hypothesis perhaps it is a disturbance in the intimate mechanism of the reticular formation (descendent pathways-especially) at the level of the BS.

REFERENCES

1. Dow, R.St., Moruzzi, G. (1958) The Physiology and Pathology of Cerebellum. University of Minnesota Press, Minneapolis, pp. 408–410.
2. Kisch, G. (1987) Cerebellar Fits bei Epidermoidresten in Clivusbereich. In: Bock, W.J., Schirmer, M. (eds) Differentialdiagnose in der Neurochirurgie. Urban und Schwarzenberg, München — Wien — Baltimore, pp. 70.
3. Penfield, W., Jasper, H. (1954) Epilepsy and functional Anatomy of the Human Brain. Little, Brown and Company, Boston, pp. 378–381.
4. Robert, M., Geiner, L. (1921) Cerebellar Fits. Arch. of Neurology and Psychiatry 410, 296–304.

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EVOLUTION OF THE RETICULAR FORMATION

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The reticular formation of mammals contains numerous nuclei which can be recognized by their projection patterns, cytoarchitectonics, and neuropeptide/neurotransmitter content. We have identified reticular nuclei in representatives from numerous reptilian groups and ascertained presence or absence of these reticular nuclei in an attempt to use neuronal occurrence as a tool to determine phylogenetic relationships. Recently these studies have been extended to two elasmobranchs, a galeomorph shark and a ray.

In this report, we concentrate on three medullary spinal projecting reticular nuclei, reticularis gigantocellularis, reticularis magnocellularis, and reticularis paragigantocellularis. We found that all three nuclei were present in rats, lizards, and elasmobranchs, but one nucleus was absent in crocodilians, and two nuclei were absent in turtles. Thus brain organization may give us clues to phylogenetic relationships. Moreover, these three reticular nuclei exhibited remarkably similar cellular morphology in mammals, reptiles, and elasmobranchs.

Keywords: Motor nuclei, medulla, cladistics, spinal cord, reptiles, elasmobranchs, mammals

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The reticular formation is considered to be a phylogenetically old part of the brain which influences diverse physiological mechanisms such as sleep /24/, arousal /14/, eye movements /12/, autonomic function /10/, and motor output /20/. While the various reticular nuclei subserve many disparate functions, reticular formation neurons have certain basic cytoarchitectonic features in common (isodendritic morphology and non-specific projections /21/). Thus, scientists have tended to lump together nuclei in the core of brainstem and simply call them "reticular formation". Yet detailed studies of connectivity and cytoarchitectonics in mammals indicate that many distinct nuclei can be identified /1, 2/. Indeed recent studies in rat /16, 17/ have identified twenty-six distinct nuclei. Our studies on various species of reptiles /5, 6, 18, 19/ and, more recently, elasmobranchs /7, 9/, have led us to the conclusion that cell groups or nuclei homologous to those found in rat reticular formation can be identified in reptiles, elasmobranchs, and probably other diverse groups. In this paper, we will focus on three medullary reticular groups and discuss the evidence which has led us to the postulation of homology. The groups are reticularis gigantocellularis, reticularis paragigantocellularis lateralis and reticularis magnocellularis (called respectively reticularis inferioris dorsalis or RID, reticularis ventralis lateralis or RVL, and reticularis inferioris ventralis or RIV, in reptiles, see Table 1). Our initial goal was to characterize descending reticular projections in various reptilian species by injecting a retrograde tracer, horseradish peroxidase, into different spinal cord funiculi and levels /19/. One means of distinguishing the above mentioned three groups is by projection patterns: RID projects bilaterally to the spinal cord while RIV and RVL project unilaterally. We were able to identify all three groups, RID, RIV, and RVL in the Tegu lizard, Tupinambus, which is considered to be a fairly advanced lizard /3, 6/. In crocodilians (Caiman crocodilius) however, RVL is missing, while in turtles (Chrysemys, Pseudemys, and Chelydra), both RVL and RIV were absent /6/. In the course of these investigations, we became increasingly convinced that these three groups were so distinct cytoarchitectonically, that the cell groups could be distinguished, at least in reptiles, in good quality Nissl stained material. These three reticular nuclei are located in the medulla and each has a characteristic neuronal morphology. For example, reticularis gigantocellularis (corresponding to RID) contains very large cells which have multipolar dendritic arborizations and are found to either side of the midline. Their thick axons course dorsomedially. Reticularis magnocell-

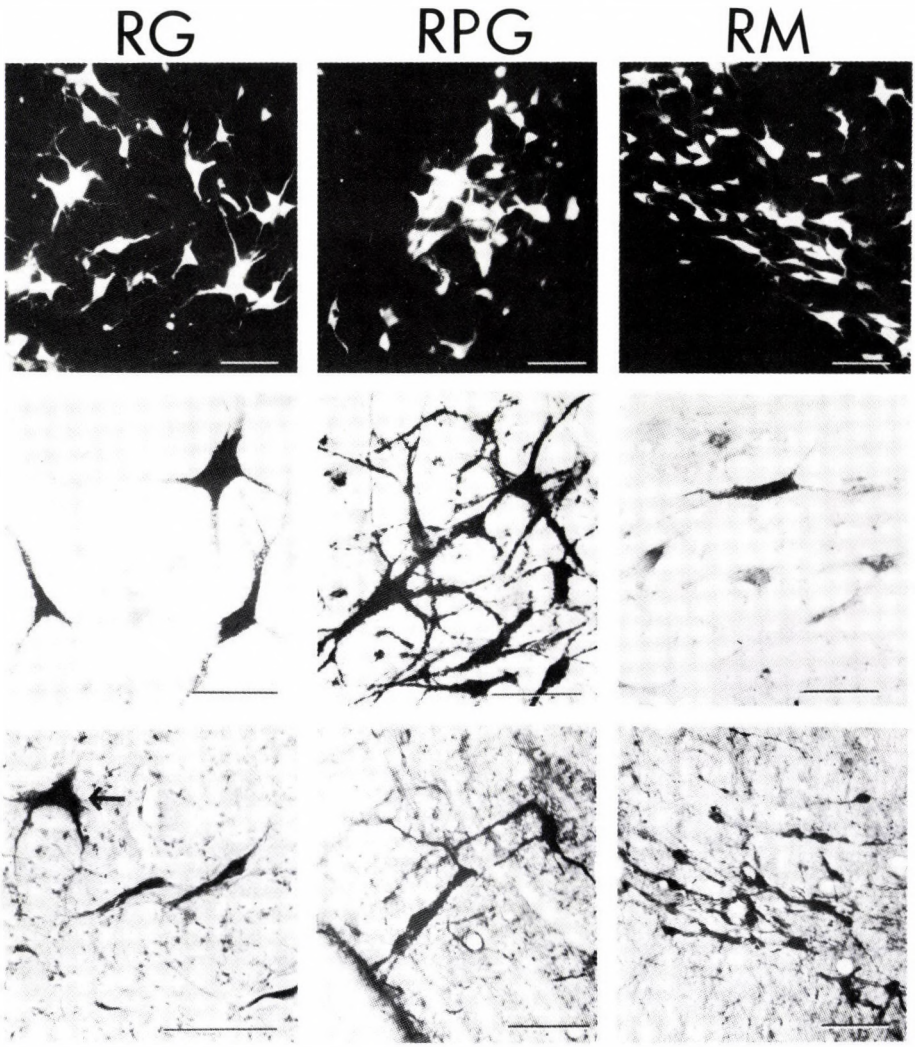
ularis (corresponding to RIV) consists of extremely flattened cells with horizontal processes. These cells tend to be oriented from dorsomedial to ventrolateral and send their axons laterally. The third cell group, reticularis paragigantocellularis lateralis (corresponding to RVL) consists of widely separated cells some of which have a very characteristic shape: the cell bodies are multipolar and the processes twist around the soma to form a pinwheel shape. Other paragigantocellularis lateralis cells have a more fusiform shape, and these are more difficult to distinguish, however, this cell group tends to be segregated from other reticular groups (Fig. 1).

Table 1

Reticular Nomenclature in Mammals, Reptiles, and Elasmobranchs

Name in Mammals and Elasmobranchs	Name in Reptiles
reticularis gigantocellularis (RG)	reticularis inferioris dorsalis (RID)
reticularis paragigantocellularis lateralis (RPG)	reticularis ventralis lateralis (RVL)
reticularis magnocellularis (RM)	reticularis inferioris ventralis (RIV)

We proceeded to survey various vertebrate taxa for the presence or absence of these three reticular nuclei /6/. Some of our conclusions were based on analysis of brainstems after HRP injections into the cord, some on Golgi material, and some of our conclusions were based solely on Nissl material (including published cases of other investigators and museum material). By surveying a large number of taxa, we postulated phylogenetic relationships based on the presence or absence of these reticular nuclei. Superimposing our reticular data on cladograms of presumed vertebrate and reptilian relationships /3, 15/, we found that RVL was present in birds and mammals but absent in all reptiles except a group of closely-related lizards /6/. Since modern birds, reptiles, and mammals evolved from older reptilia /15/, one of two hypotheses must hold true (1), either RVL arose separately and independently several times (e.g. in mammals, birds, and a few lizard families) or (2) RVL (and RIV in turtles) was lost in many reptilian groups during evolution. In the first case, nuclei identified as RVL would not be homologous, but in the second case, they would be. The


RG
RPG
RM

question of homology is important because it allows us to make certain assumptions about possible cell function by extrapolating from studies in one group of animals to another.

We have recently extended our study on the reticular formation to the elasmobranchs, a phylogenetically old group of fishes which has been in existence for at least 300 million years /4, 22/ but which has many contemporary representatives with well developed brains. To date we have studied two species: a galeomorph shark, Heterodontus francisci and a ray, the thornback guitarfish, Platyrhinoidis triseriata. In addition to cytoarchitectonics and spinal projections, we have added another experimental technique to our armamentarium, immunohistochemistry. If a given nucleus in two separate groups of animals not only has a characteristic morphological appearance, location in the brainstem, and spinal projections, but also contains similar chemicals, one is even more convinced that the nuclei are homologous. To date, we have identified all three medullary reticular nuclei, RID, RIV, and RVL in both Heterodontus and Platyrhinoidis. The identification in Heterodontus /8/ is based on cytoarchitectonics and injections of HRP (in collaboration with Dr. Glenn Northcutt) and a newer retrograde fluorescent tracer, fluorogold /23/, into the spinal cord. The identification in Platyrhinoidis /7, 9/ (also in collaboration with Dr. Northcutt) is based on cytoarchitectonics, HRP injections, and immunohistochemistry using antibodies made in rabbit to serotonin or to tyrosine hydroxylase, a catecholamine-synthesizing enzyme. A proportion of cells in reticularis paragigantocellularis lateralis and reticularis magnocellularis contain tyrosine hydroxylase or serotonin, however, cells in reticularis gigantocellularis contain neither. Amazingly, the cells in these three reticular groups in elasmobranchs exhibit a similar morphology to those in

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Fig. 1. Identification of three medullary reticular nuclei in three genuses of animals. Rat has been chosen as a mammalian representative, Tegu lizard as a reptilian, and Platyrhinoidis as an elasmobranch representative. The representative reticular group in each genus is shown using only one technique for identification, although in all cases, more than one technique was used. The cells illustrated were treated or identified as follows: Top row: Rattus – fluorogold injection into the lower cervical spinal cord /23/; Middle row: Tupinambus – horseradish peroxidase injection into the lower cervical spinal cord and tissue processed with tetramethyl benzidine /13/; Lower row: Platyrhinoidis – RG (arrow), horseradish peroxidase injection into spinal cord and tissue processed with Hanker–Yates reagent /11/, RPG and RM, sections incubated with rabbit anti-serotonin primary antibody and tissue processed using the avidin-biotin reaction (Vectastain Kit).
All bar scales represent 100 μm. See Table 1 for abbreviations

rats and in reptiles (Fig. 1). This would indicate that not only has the clustering of these cells into specific nuclei been conserved hundreds of millions of years, during which time numerous species have evolved, but also the morphology of their processes, their projection patterns, and presumably their putative transmitters have also been conserved. One wonders if the functions of these three reticular cell groups have remained similarly unchanged. Our goal is to survey the reticular formation of many more elasmobranchs and to use this information to construct hypotheses about possible phylogenetic relationships. For although most of the twenty-six identified mammalian reticular nuclei seem to be present in Heterodontus and Platyrhinoidis, some nuclei are not. Perhaps as we correlate the presence or absence of reticular nuclei with the presence or absence of other morphological or functional characteristics, we will obtain further clues to the roles of these nuclei. At the very least, we will characterize nuclei, some of which have not been previously recognized, and this information will provide a morphological basis to interpret other experimental studies.

REFERENCES

1. Brodal, A. (1981) Neurological Anatomy in Relation to Clinical Medicine. Oxford, London , (3rd Ed.), pp. 394–447.
2. Brodal, A. (1958) The Reticular Formation of the Brain Stem. Anatomical Aspects and Functional Correlations. Oliver and Boyd, London.
3. Camp, C.L. (1923) Classification of the lizards. Bull. Amer. Mus. Nat. Hist., 48, 289–481.
4. Compagno, L.J.V. (1977) Phyletic relationships of living sharks and rays. Amer. Zool. 17, 303–322.
5. Cruce, W.L.R., Newman, D.B. (1981) Brain stem origins of spinal projections in the lizard Typinambis nigropunctatus. J. Comp. Neurol. 198, 185–207.
6. Cruce, W.L.R., Newman, D.B. (1984) Evolution of motor systems: the reticulospinal pathways. Amer. Zool. 24, 733–753.
7. Cruce, W.L.R., Northcutt, R.G. (1985) Spinal projecting nuclei in the brainstem of an elasmobranch, the thornback guitarfish, Platyrhinoidis triseriata. Neurosci. Abs. 11, 1313.
8. Cruce, W.L.R., Northcutt, R.G. (1986) Spinal projecting nuclei in the brainstem of a galeomorph shark, Heterodontus francisci. Neurosci. Abs. 12, 1546.
9. Cruce, W.L.R., Stuesse, S.L., Northcutt, R.G. (1987) Tyrosine hydroxylase and serotonin immunoreactivity in the brain of an elasmobranch, Platyrhinoidis triseriata. Neurosci. Abs. 13, 131A.

10. Dampney, R.A.L., Goodchild, A.K., Robertson, L.G., Montgomery, W. (1982) Role of ventrolateral medulla in vasomotor regulation: A correlative anatomical and physiological study. Brain Res. 249, 223–236.

11. Hanker, J.S., Yates, P.E., Metz, C.B., Rustioni, A. (1977) A new specific, sensitive and non-carcinogenic reagent for the demonstration of horseradish peroxidase. Histochem. J., 9, 789–792.

12. Langer, T.P., Kaneko, C.R.S. (1983) Efferent projections of the cat oculomotor reticular omnipause neuron region: An autoradiographic study. J. Comp. Neurol. 217, 288–306.

13. Mesulam, M. (1978) Tetramethyl benzidine for horseradish peroxidase neurochemistry: A non-carcinogenic blue reaction product with superior sensitivity for visualizing neural afferents and efferents. J. Histochem. Cytochem. 26, 106–117.

14. Moruzzi, G., Magoun, H.W. (1949) Brainstem reticular formation and activation of the EEG. E.E.G. Clin. Neurophysiol. 1, 455–473.

15. Nelson, G.J. (1969)Gill arches and the phylogeny of fishes, with notes on the classification of vertebrates. Bull. Amer. Mus. Nat. Hist., 141, 475–552.

16. Newman, D.B. (1985) Distinguishing rat brainstem reticulospinal nuclei by their neuronal morphology. I. Medullary nuclei. J. Hirnforsch, 26, 187–226.

17. Newman, D.B. (1985) Distinguishing rat brainstem reticulospinal nuclei by their neuronal morphology. II. Pontine and mesencephalic nuclei. J. Hirnforsch. 26, 385–418.

18. Newman, D.B., Cruce, W.L.R. (1982) The organization of the reptilian brainstem reticular formation: a comparative study using Nissl and Golgi techniques. J. Morphol. 173, 325–349.

19. Newman, D.B., Cruce, W.L.R., Bruce, L.L. (1983) The sources of supraspinal afferents to the spinal cord in a variety of limbed reptiles. I. Reticulospinal systems. J. Comp. Neurol. 215, 17–32.

20. Peterson, B.W. (1980) Participation of pontomedullary reticular neurons in specific motor activity. Chairman's overview of part III. In: Hobson, J.A. and Brazier, M.A.B. (eds) The Reticular Formation Revisited, Vol. 6, Raven Press, New York, pp. 171–192

21. Ramon-Moliner, E., Nauta, W.J.H. (1968) The isodendritic core of the brainstem. J. Comp. Neurol. 126, 311–336.

22. Schaeffer, B., Williams, M. (1977) Relationship of fossil and living elasmobranchs. Amer. Zool., 17, 293–302.

23. Schmued, L.C., Fallon, J.H. (1986) Fluorogold: a new fluorescent retrograde axonal tracer with numerous unique properties. Brain Res. 377, 147–154.

24. Vertes, R.P. (1984) Brainstem control of the events of REM sleep. Prog. Neurobiology, 22, 241–288.

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3. Umbreit, W. E., Burris, R. H., Stauffer, I. F. (1957) *Manometric Techniques.* Burgess Publishing Co., Minneapolis.

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LYSOSOMAL ENZYME RELEASE FROM MACROPHAGES: A MODEL OF FOOD YEAST TOXICITY EVALUATION

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The role of lysosomal enzyme released by macrophages was examined in relation to the toxic effect caused by food yeast. Mouse peritoneal macrophages exposed to yeast in culture showed marked release of N-acetyl glucosaminidase, beta-galactosaminidase and beta-glucuronidase below the median lethal dose (LD50).

LD50 was measured from the dose response curves of the cytoplasmic lactate dehydrogenase enzyme. Saccharomyces cerevisiae showed the highest LD50 followed by Kluyveromyces fragilis and Candida utilis yeast. LD50 values obtained as well as the in vitro lysosomal release by mouse peritoneal macrophages may be relevant to assess the toxic capacity of food yeast intended for human consumption.

Keywords: Macrophage — lysosomal enzymes — food yeast — toxicity

INTRODUCTION

The use of yeast as a source of protein for human nutrition has become an object of intensive research mainly in the field of toxicology. At present, food yeasts generally in use for this purpose are obtained from Saccharomyces and Candida genera /11/. Earlier reports have pointed out that the yeast cell wall and nucleic acid content are responsible for the effect of yeast /8, 13/.

Many biological tests are used to assess the safety of yeast proposed and employed in many parts of the world /11/. However, closely controlled "in vitro" tests are needed for precise and rapid answers.

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In a previous study we found that rat nephrocalcinosis produced by yeast diets was associated with an increase of urine lysosomal enzymes (unpublished data). Whether lysosomal enzyme release has any relationship with toxic effects evoked by yeast is still a question to be studied.

Our aim was to analyse, in a suitable system, the relation between the toxic effect of yeast and the hydrolase secretion from macrophages. The convenience of this model is presented and discussed.

MATERIALS AND METHODS

Industrial Candida utilis, Saccharomyces cerevisiae and Kluyveromyces fragilis dried yeast were individually mixed in 0.9% (w/v) NaCl and sonicated 30 min in a MSE apparatus with 1 min intervals of cooling. With this treatment the total disruption of cells was achieved.

Peritoneal macrophages from Balb/c mice were collected in 5 ml of M199 containing 100 U/ml of penicillin and streptomycin and 10 IU/ml of heparin. One and a half-millilitre aliquots of the peritoneal exsudate cell suspension containing 0.5–1.0 x 10^6 cells/ml were distributed into 24 well Nunc plates and incubated in 5% (v/v) CO_2 in air at 37°C. After washing to remove non-adherent cells, the macrophages were cultured in 1.5 ml of M199 containing 10% (v/v) fetal calf serum and 0.1 ml of the corresponding sterile yeast solution or 0.9% (w/v) NaCl. At the end of each incubation period the medium was removed and the adherent cells were then released by 0.1% (w/v) Triton X-100 in saline solution and scraped with a rubber policeman. The activities of various enzymes were assayed in both the medium and cell-containing fractions. Enzyme release was expressed as the percentage of total cell enzyme activity, obtained by dividing the activity in the medium by total cell (medium plus lysed cells) activity. Basal secretion was substracted from the corresponding values.

The activities of N-acetylglucosaminidase(NAG), beta-glactosaminidase (bCal) and betaglucuronidase (bGlu) were assayed as described by Den Tandt et al. /6/ using 4-methylumbellyferil (4MU), 2-acetamide 2-deoxy-beta-D glucopyranoside, 4MU 2-acetamide 2-deoxy-beta-D-galactopyranoside and 4MU-beta-D-glucuronide, respectively. Lactate dehydrogenase (LDH) activity was measured by Boehringer Mannheim sets.

LD50 was measured as a function of LDH enzyme release, a marker of cellular death, at 24 and 48 h of macrophage incubation with C. utilis yeast. Fifty per cent value of released LDH was taken as the LD50 value.

Cytochalasin B was obtained commercially (SIGMA) and was dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO in culture medium was 0.1% (v/v).

Biochemical results are epxressed as geometrical mean and standard error of the geometrical mean of four or five cultures. These data were analyzed by the variance /22/ and statistical significance as established by Duncan's test /7/.

RESULTS

LD50 values of C. utilis in macrophage cultures

The dose-response of the concentration of C. utilis required for achieving the LD50 is shown in Fig. 1. We obtained typical dose response curves which indicate an LD50 with 100 and 75 micrograms of yeast at 24 and 48 h of incubation, respectively.

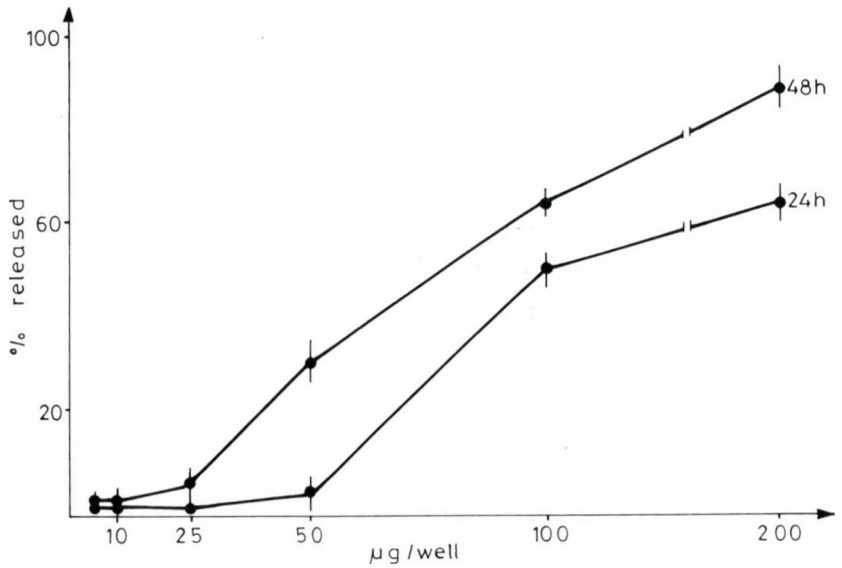

Fig. 1. LDH values by means of LDH release from macrophages cultured for 24 and 48 h with Candida utilis yeast. The fity per cent of released LDH was taken as LD50 value

Effect of C. utilis on macrophage cultures

The effect of C. utilis on the macrophage cultures at non-lethal dose concentration was studied by measuring the effect of the yeast on the release of the NAG and bGal. The half-LD50 was used for 24 h of incubation (Fig. 1). This was the highest dose of C. utilis added to the cultures.

Significantly increased amounts of both lysosomal enzymes were recovered from medium when cells were incubated with yeast from 5 to 50 µg/1.5 ml (Fig. 2). In the 24 h incubation cultures, negligible LDH was measured in the controls. However, the cultures incubated for 48 h showed conspicuous release of LDH at 50 ug/1.5ml. For this reason, further experiments were

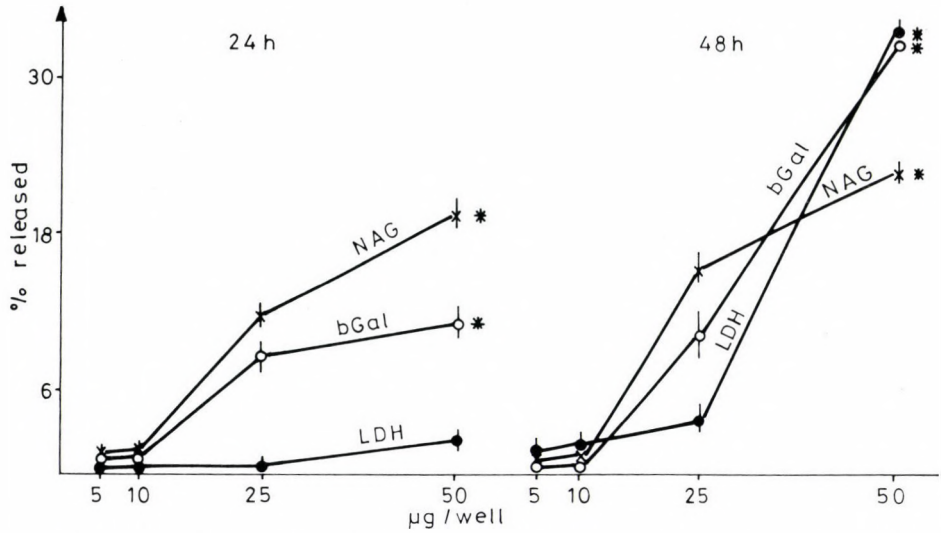

Fig. 2. Lysosomal enzyme release from macrophages cultured for 24 and 48 h with Candida utilis yeast. *p 0.001

then performed using an incubation of period of 24 h, in which case the non-lethal toxic level is higher.

Influence of medium calcium level on the enzyme release

To test if a higher content of calcium in the extracellular fluid might provoke itself the observed enzymatic release, cells were incubated for 24 h with different amounts of $CaCl_2$ in the culture medium. As seen in Table 1, significant release of NAG, bGal and bGlu was not found.

Combined effect of calcium and yeast on enzyme release

In order to find out whether the induction of enzyme release was only due to yeast or was associated with both calcium and yeast, the macrophages were incubated with different amounts of yeast and only one concentrations of calcium. As shown in Fig. 3, when calcium concentration was fixed (3A), the dose-response curves were similar to those shown in Fig. 2. On the other hand, dose-response curves were not obtained with fixed yeast amount (3B) in the presence of different calcium concentrations. Although statistical differences were not manifested, the lysosomal activities tended to be lower with the increase in the calcium concentration.

Table 1

Effect of calcium content on lysosomal enzyme release

nmol Ca/1.5 ml	NAG	bGal	bGlu	LDH
0.000	6.1±1.3	5.3±0.9	10.0±1.1	4.3±1.1
0.753	5.4±1.1	6.4±1.1	11.8±0.9	4.9±1.1
1.882	7.2±1.0	7.6±1.3	11.6±1.1	4.4±1.2
3.764	6.4±1.2	7.2±1.0	10.2±0.8	5.4±1.3
7.527	6.6±1.4	4.7±0.9	10.1±1.1	5.3±1.5
18.818	6.8±1.2	3.2±1.0	11.8±1.3	3.2±1.1

Macrophages were incubated for 24 h with different medium calcium concentrations. Geometrical mean and standard error of the mean are determined from the percent value of the total activity in the supernatant

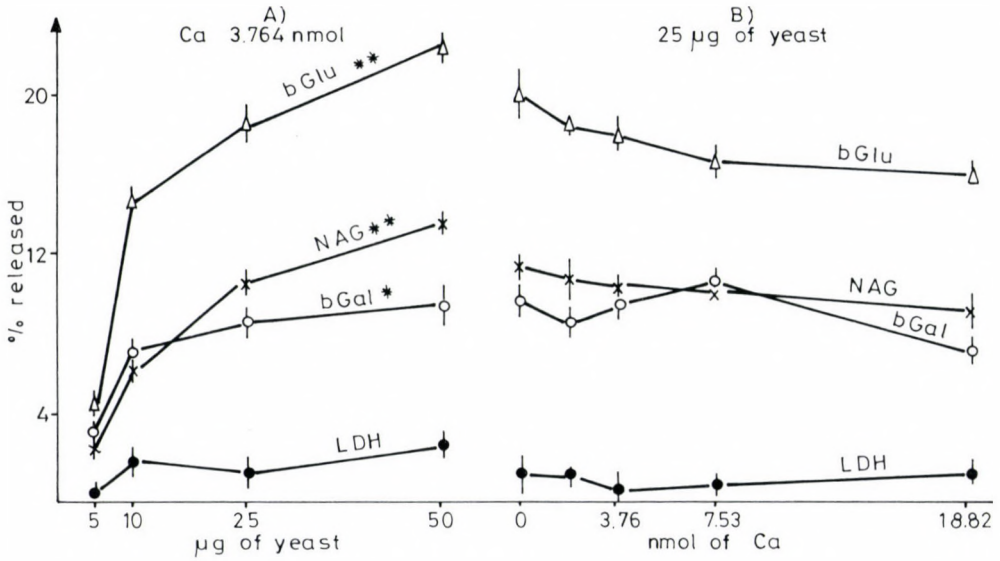

Fig. 3. Effect of calcium and Candida utilis yeast on macrophage exocytic activity. (A) with fixing medium calcium concentration to 3.764 nmol and (B) with fixing medium yeast concentration to 25 µm. $^{*}p < 0.01$

Enzyme release in the presence of cytochalasin B

To elucidate whether enzyme release from the peritoneal macrophage cultures exposed to yeast is associated with phagocytic function or yeast induction, the cells were preincubated with cytochalasin B at a concentration inhibiting 100% phagocytosis /1/. After 15 min, yeast was added and incubated 24 h. The results shown in Fig. 4 demonstrate that the release of lysosomal enzyme is directly proportional to the dose of the material used. Significantly higher amounts of all the three enzymes were recovered from the medium. Under these conditions hydrolase secretion was not accompanied by a loss of cell viability. After a 24 h exposure to cytochalasin B as well as C. utilis yeast no significant increase of the cytoplasmic enzyme lactate dehydrogenase was detectable in the culture medium.

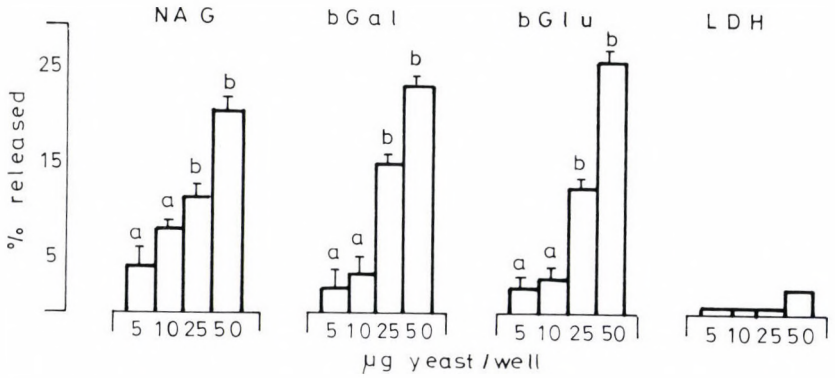

Fig. 4. Cytochalasin B (CB) effect on enzyme release from macrophages cultured for 24 h with Candida utilis yeast. Macrophage cells were previously treated with a CB concentration of 1.26×10^{-5}/1.5 ml. Bars not sharing a common superscript letter are significantly different ($p < 0.01$)

Toxic effect of other yeast strains

This series of experiments is suggestive of the fact that lysosomal enzyme release at non-lethal dose is a cell response to toxicity caused by the yeast. The ability of other yeast strains, with more or less toxic capacity than the C. utilis strain /2, 12, 23/, to produce lysosomal enzyme exocytosis was measured using different yeast amounts in the incubation medium. Figure 5 demonstrates the results obtained under the above mentioned conditions for 24 h of incubation. Saccharomyces cerevisiae, considered the

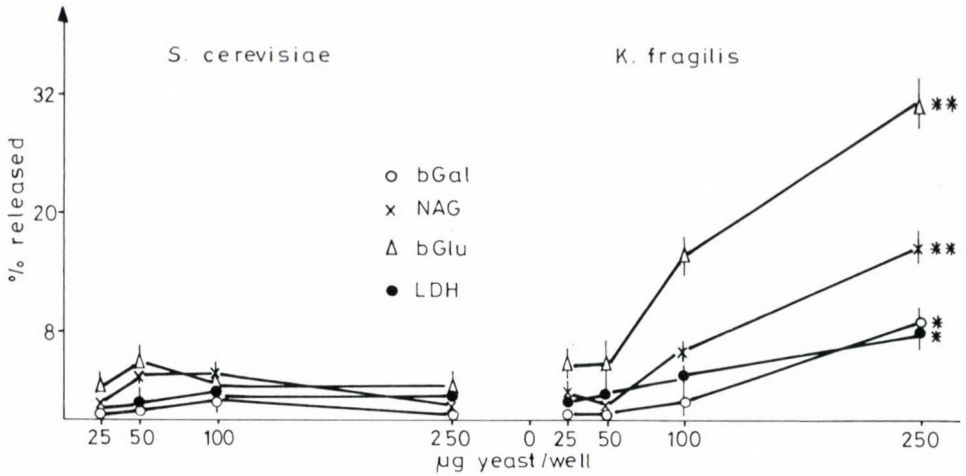

Fig. 5. Enzyme release from macrophages cultured for 24 h with Saccharomyces cerevisiae and Kluyveromyces fragilis yeasts. * $p < 0.05$; ** $p < 0.01$

least toxic yeast, showed no significant release of either three lysosomal enzymes tested, not even at the higher dose of 250 µg/1.5 ml. LDH activities were very low in all doses, indicating that the lethal dose for this yeast is higher for the C. utilis. Furthermore, Kluyveromyces fragilis, a yeast which is less toxic than C. utilis but more toxic than S. cerevisiae, exhibited a similar behaviour to that observed for the C. utilis, though this was found when applying higher doses of yeast. In this case, the LDH activities suggest an LD50 value higher than that of the C. utilis and lower than of the S. cerevisiae.

DISCUSSION

Dose-response relationships regarding yeast influence on lysosomal enzyme release in macrophage cultures have been examined to determine whether there is any correlation between hydrolase exocytosis and toxic effect evoked by yeast. It has been demonstrated that release of lysosomal enzymes from polymorphonuclear leukocytes /3, 10/ and peritoneal macrophages /19/ occurs when the cells are stimulated in any of several ways, and this was associated with a decrease in the amount of cell-associated enzymes /24/. In the same way, several agents which induce chronic inflammation, including streptococcal cell wall /4/, immune complex /15/ and carrageenan

/5/, induce secretion of hydrolytic enzymes from macrophages. We have found that certain yeasts activate macrophages in vitro to release their lysosomal enzyme content. This was a selective release, since no changes were observed in the LDH.

It is clearly established that calcium movement across the cell membrane plays an essential role in triggering the release of transmitters or of proteins packaged in membrane-bound vesicles /16/. Some investigators found that an early response to certain agents in connection with the rabbit granulocyte /9/, is the release of membrane-associated calcium from intracellular stores; others pointed out that an influx of calcium favours enzyme release /14/. However, the exact role of calcium and the molecular events associated with membrane fusion and exocytosis are not certain /17/. Calcium alone is unable to trigger hydrolase secretion as shown in Table 1 and Fig. 3.

During the process of phagocytosis, rabbit polymorphonuclear leukocytes /3/ and mouse peritoneal macrophages /18/ release lysosomal enzymes into the extracellular medium. Inhibition of phagocytosis by cytochalasin B did not affect neither hydrolase release nor the extent of the activity enzymes. These results are in agreement with other authors /20, 21/, who found that enzyme release is independent of phagocytosis and therefore, cytochalasin B does not affect their release.

The dose-response curves of the cytoplasmic lactate dehydrogenase may be taken as a measure of lethal toxic effect of yeast on macrophage cells. This observation was drawn by means of comparing the approximate LD50 values of the three types of yeast tested.

From these experiments, it is not possible to elucidate which mechanisms are involved in toxic cell death. However, we may suppose yeast toxicity is induced, perhaps, by the activation of the alternative pathway /18/ which may cause a series of additional events including calcium movement and exocytosis, and also a loss of macrophage adhesive capacity. In addition, an overproduction of lipid peroxides may lead to cell death /16/. The toxic agent is not known as yet; it might be a yeast cell wall component with a structure similar to glucan, mannan or zymosan which are potent inducers of macrophage hydrolase release in vitro /19/.

Finally, the present study contributes to the observation that dose-response curves of yeast strains at half-LD50, will be a useful tool for toxic screening tests of food yeasts intended for human consumption.

REFERENCES

1. Axline, S.G., Reaven, E.P. (1974) Inhibition of phagocytosis and plasma membrane mobility of the cultivated macrophage by cytochalasin B. J. Cell Biol. 62, 647–659.

2. Contreras, C.R., Valdés, I. (1984) Yeast selection for human consumption. Biologia (Bratislava) 39, 707–715.

3. Davies, P., Fox, R.I., Polyzinis, Z.M., Allison, A.C., Haswell, A.D. (1973) The inhibition of phagocytosis and facilitation of exocytosis in rabbit polymorphonuclear leukocytes by cytochalasin B. Lab. Invest. 28, 16–22.

4. Davies, P., Page, R.C., Allison, A.C. (1974) Changes in cellular enzyme levels and extracellular release of lysosomal acid hydrolases in macrophages exposed to group A streptococcal cell wall substances. J. Exp. Med. 139, 1262–12828.

5. Davies, P., Allison, A.C. (1976) Secretion of macrophage enzymes in relation to the pathogeneis of chronic inflammation. In Nelson, D.S. (ed.). Immunbiology of the Macrophage. Acad. Press Incorporated, New York.

6. Den Tandt, W.R., Lassilla, E., Phillipart, M. (1974) Leroy's I-cell disease: Markedly increased activity of plasma acid hydrolases. J. Lab. Clin. Med. 83, 403–408.

7. Duncan, D.B. (1955) Multiple-range and multiple F test. Biometrics 11, 1–42.

8. Engel, C. (1972) Analyse des essais toxicologiques effectues sur les levures cultivées sur alcanes. In Gounelle de Pontanel, H. (ed.). Les levures cultivées sur alcanes. Nouvelles sources de proteines alimentaires. Symp d'Aix en Provence, Paris.

9. Gallin, J.I., Rosenthal, A.S. (1974) The regulatory role of divalent cations in human granulocyte chemotaxis. J. Cell Biol. 62, 594–609.

10. Henson, P.M., Oades, Z.G. (1973) Enhancement of immunologically induced granule exocytosis from neutrophils by cytochalasin B. J. Immunol. 110, 290–293.

11. Hernández Rosales, F. (1984) Yeast as a source for human nutrition. Acta Microbiol. Hungarica. 31, 159–172.

12. Holzachu, D.L., Chancler, F.W., Ajello, L., Ahearn, D.G. (1979) Evaluation of industrial yeasts for pathogenicity. Sabourandia, 17, 71–78.

13. Kihlberg, R. (1972) The microbe as a source of food. Ann. Rev. Microbiol. 26, 427–466.

14. Kramer, C.M., Franson, R.C., Rubin, R.P. (1984) Regulation of phosphatidylinositol turnover, calcium metabolism and enzyme secretion by phorbol dibutyrate in neutrophils. Lipids. 19, 315–323.

15. Moises, T., Finlay, C. (1983) Actividad enzimática de macrófagos cultivados sobre, miliporos tratodos con inmunocomplejos a los cuales se les anadió clofazimina. Revista CENIC, Ciencias Biológicas 14, 49–57.

16. Morrinson, A.R., Pascoe, N., Rauk, N., Kennerly, D. (1984) Biochemical alterations of membrane lipids associated with renal injury. Fed. Proc. 43, 2811–2814.

17. Publicover, S.J., Duncan, C.J. (1981) Membrane protein mobility and exocytosis. Comp. Biochem. Physiol. 70B, 261–263.

18. Schorlemmer,H.U., Edwars, J.H., Davies, P., Allison, A.C. (1977) Macrophage response to mouldy hay dust, Mycropolyspora faeni and zymosa, activators of complement by the alternative pathway. Clin. Exp. Immunol. 27, 198–207.

19. Seljelid, R., Bogwald, J., Lundwall, A. (1981) Glycan stimulation of macrophages in vitro. Exp. Cell. Res. 131, 121–129.

20. Skosey, J.L., Damgaard, E., Chow, D., Sorensen, L.B. (1974) Modification of enzymes from human polymorphonuclear leukocytes by cytochalasin B. J. Cell. Biol. 62, 625–634.

21. Skosey, J.L., Chow, D., Damgaard, E., Sorensen, L.B. (1973) Effect of cytochalasin B on response of human polymorphonuclear leukocytes to zymosan. J. Cell. Biol. 57, 237–240.

22. Snedecor, G.W., Cochram, W.G. (1980) Statistical Methods. The Iowa State University Press, Ames, Iowa.

23. Stanley, V.C., Hurley, R. (1967) Growth of Candida species in cultures of mouse epithelial cells. J. Path. Bact. 94, 301–315.

24. Wright, D.C., Malawista, S.E. (1972) The mobilization and extracellular release of granular enzymes from human and extracellular release of granular enzymes from human leukocytes during phagocytosis. J. Cell. Biol. 53, 788–797.

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LEVELS OF ZINC, CADMIUM AND LEAD IN SOME MARINE ALGAE FROM AQABA-RED SEA

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(Received 1988–04–15)

Jordan has witnessed a rapid industrial development in the last twenty years. This has lead to the release of waste materials or pollutants into the marine environment, particularly nearby Aqaba Port. The present study investigates the levels of zinc, cadmium and lead in four brown algae, three red algae and four green algal species collected from Aqaba. Three different levels of lead and zinc concentrations were found: the highest level of both metals is exhibited among brown algae; intermediate level is exhibited among red algae and the lowest level is seen among the green algae. Very low concentrations of cadmium were found in all examined algal species. The results indicate that the brown algal species Cystosira myrica, Sargassum asperifolium, Sargassum neglectum, and Sargassum subrepandum always contain the highest concentrations of lead and zinc, but these algae are less contaminated than brown algae from industrial European seas.

Keywords: Heavy metals — zinc — cadmium — lead — marine algae — Red Sea

INTRODUCTION

In contrast to the constant ratio between salts in seawater, the heavy metals show marked fluctuations. This is due to the increased pollution in certain regions /11/.

Jordan has witnessed a rapid industrial development in the last twenty years. This has lead to a multiple increase in the number of transportation vehicles in the country and to the utilization of heavy machinary for various industrial purposes all of which cause the release of waste materials or pollutants into the environment. Among these pollutants are the heavy metals such as lead, cadmium and zinc. Aqaba Port is a potential source of metal contamination where increased shipping, domestic sewage,

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fertilizer industry and desert dust blown by prevailing northerly winds has increased the health hazard /18/. As happened in various Western industrial countries, the industrial use of water from rivers, canals, and springs could cause pollution of water with various metals /2, 4, 17/.

Seaweeds, in particular the brown algae, have been used as indicators of heavy metal pollution in estuaries and coastal waters due to their ability to bind heavy metals strongly /3, 10/. As shown by Bryan /2/ the metal uptake in Laminaria digitata is a gradual accumulation process and not accompanied by exchange between plant and the seawater. Results of Gutknecht /8/, Black and Michell /1/, Fuge and James /7/ suggested that the heavy metal concentrations in seaweeds are directly related to those in the seawater. The previous studies show great variation in tissue content of metals in different species and environments /13, 14/.

The present study investigates the levels of zinc, cadmium and lead in the red algal species; Hypenea musciformis — (Wulfen) Lamouroux, Galaxaura rugasa (Solander) Lamouroux and Galaxaura fastigata (Solander) Lamouroux, the brown algal species Cystosira myrica (Gmel) C. Ag, Sargassum asperifolium Hering et Mart ex. J. Ag., Sargassum neglectum C. Ag. and Sargassum subrepandum J. Ag., and the green algal species Codium tomentosum Stackh, Caulerpa serrulata (Forssk.) J. Ag., Valonia macrophysa Kütz and Hydroclathrus clathratus (C. Ag.) Howe, which were collected from the coast of Aqaba, Jordan. Also this study aims to asses differences in metal levels between the different algal species mentioned above.

MATERIALS AND METHODS

All of the above mentioned species of algae were collected from the Red Sea coast opposite the Marine Science Station in Aqaba City. This station lies nearby a cement floating berth and only 2 km south of the sewage plant and the phosphate loading berth. The algae were lyophylized separately using a christ lyophylizer (Model Beta I). 30 mg of the lyophylized algae samples and the whole lyophylized algae samples were digested in perchloric acid for 10 h at gradual increments in temperature from 70 to 180°C. After digestion the material was diluted with glass distilled water (4 X-distillation). Then the lead, zinc and cadmium contents were determined by the standard addition method using a pye — Unicam atomic absorption spectrophotometer fitted with its hollow cathode lead, Zinc or cadmium lamp. Results are expressed in ppm on dry weight basis.

RESULTS AND DISCUSSION

The concentrations of lead, zinc and cadmium in the algal species used in this study are given in Table 1. Three different levels of lead concentrations were found: a) highest level is exhibited among brown algae; b) intermediate level is exhibited among red algae, and c) the lowest level is seen among the green algae. The concentrations of zinc also reflect three different levels similar to that for lead except in the red alga Hypenea musciformis which contains higher zinc concentration than brown algal species. However, Cadmium concentrations were found to be always very low in all algal species due to the scarcity of Cadmium ions in the seawater of the Gulf of Aqaba which is about 0.024 ± 0.004 $\mu g ml^{-1}$ /18/.

These results indicate that the brown algal species always contain the highest concentration of lead and zinc. They provide examples of organisms which are unable to regulate their heavy metal contents, due to the presence of large amount of alginic acids in their cell walls /12/. Differences in accumulation abilities between the brown, red and green algal species are probably related to differences in rates of metal uptake and/or in efficiencies of binding the metals on their cell wall; and the later possibility seems to be well accepted /14/. Trullope and Evans /16/ observed in Chlorella fusca a rapid bonding through adsorption, as well as energy dependent accumulation and a passive entrance of cadmium in these cells by means of diffusion. Findennegg et al. /5/, supposed that cadmium uses the active sites for zinc accumulation. Where as reported studies of Mesmar /9/ indicate selectivity of the brown alga Fucus vesiculosus in absorbing more lead than cadmium; since alginic acid constitute about 80% of cell wall so it is postulated that brown algae have a greater affinity for lead binding. Thorell /15/ also reported a higher affinity of Fucus alginate to lead than to cadmium.

A comparison of metal concentration in this study with those reported earlier for various algal species from North Sea coast of UK indicates that the algal species of Aqaba are less contaminated than algal species from UK. So it is suggested that the water of Aqaba Sea is not highly polluted /6/. Since there is a reasonably direct relationship between metal concentrations in the algae and seawater /3, 10/ metal levels in the algae examined may be used as bio-indicators of the seawater quality in general and the Aqaba Sea in specific.

Table 1

Concentrations of zinc, cadmium and lead as ppm dry weight of tissue in algae collected from Aqaba. (Values are mean $\pm$S.D. of three samples)

Organism	Zinc (ppm)	Cadmium (ppm)	Lead (ppm)
Pheophycophyta (brown-algae)			
1. Cystosira myrica	244 $\pm$ 9.22	7.66 $\pm$ 0.21	8.97 $\pm$ 1.4
2. Sargassum asperifolium	193 $\pm$ 7.4	6.81 $\pm$ 0.11	9.43 $\pm$ 1.7
3. Sargassum neglectum	211 $\pm$10.3	7.2 $\pm$ 0.64	7.88 $\pm$ 0.64
4. Sargassum subrepandum	203 $\pm$ 9.76	6.9 $\pm$ 0.14	9.1 $\pm$ 0.72
Rhodophycophyta (red-algae)			
1. Hypenea musciformis	249 $\pm$ 6.8	1.96 $\pm$ 0.21	2.54 $\pm$ 0.79
2. Galaxaura rugasa	42 $\pm$ 0.92	4.60 $\pm$ 0.83	4.32 $\pm$ 1.06
3. Galaxaura fastigata	43 $\pm$ 1.2	3.11 $\pm$ 0.35	2.26 $\pm$ 0.64
Chlorophycophyta (green-algae)			
1. Codium tomentosum	10.7 $\pm$ 0.110	1.17 $\pm$ 0.164	2.36 $\pm$ 0.45
2. Caulerpa serrulata	8.104 $\pm$ 0.036	1.08 $\pm$ 0.150	2.17 $\pm$ 0.13
3. Valonia macrophysa	13.61 $\pm$ 0.14	1.28 $\pm$ 0.379	1.27 $\pm$ 0.63
4. Hydroclathrus clathratus	37.1 $\pm$ 4.08	3.68 $\pm$ 0.872	3.86 $\pm$ 1.45

The present studies has provided us an insight into the presence of heavy metals in algal species which can easily provide a big health hazard to the general coastal population since they rely on fish food.

REFERENCES

1. Black, W.A.P., Michell, R.L.(1952) Trace elements in the common brown algae and seawater, J. Mar. Biol. Ass. UK. 30, 575–584.

2. Bryan, G.W. (1976), In: Lockwood, A.P.M. (ed.) Effects of Pollutants on Aquatic Organisms. Cambridge Univ. Press. Cambridge.

3. Bryan, G.W., Hummerstone, L.G. (1973) Brown seaweeds as indicators of heavy metals in estuaries in South-West England. J. Mar. Biol. Ass. UK 53, 705–720.

4. Durphy, B. (1967) Acute occupational cadmium poisoning: A critical review of the literature J. OCC. Med. 9, 16–46.

5. Findennegg, G.R., Paschinger, H., Broda, E. (1971) Untersuchung der Lichtabhängigkeit der Aufnahme von Rubidium, Zn, Co, Pb and Cd durch Chlorella nach einer Flussmethode, Planta (Berlin) 99, 163–173.

6. Förstner, A., Witton, G. (1981) Metal Pollution in the Aquatic Environment, Springer-Verlag, Berlin, p. 289.

7. Fuge, R., James, K.H. (1974) Trace metal concentration in Fucus from Bristol Channel, Marine Poll. Bull. 5, 9–12.

8. Gutknecht, J. (1965) Uptake and retention of cesium 137 and zinc 65 by seaweeds. Limnol. Oceanogr. 10, 58–66.

9. Mesmar, M.N. (1987) Distribution of lead and cadmium in trophic levels of some marine organisms. Acta Biol. Hungarica 38, 155–159.

10. Morris, A.W., Bale, A.J. (1975) The accumulation of Cd, Cu, Mn and Zinc by Fucus vesiculosus in the Bristol channel. Estuarine and Coastal Marine Science, 3, 153–163.

11. Munda, I. (1979) Temperature dependence of zinc uptake in Fucus virsoides (Don) J. Ag. and Enteromorpha prilifera (O.F. Mull) J. Ag. from the Adriatic Sea. Bot. Mar. XXII, 149–152.

12. Prescott, G.W. (1968) The Algae: A review. Houghton Mifflin Company. Boston, pp. 207–208.

13. Saenko, G.N., Koryakova, M.D., Makienko, V.F., Dobrosmyslova, I.G. (1976) Concentration of polyvalent metals by seaweed in Vostok Bay, Sea of Japan, Mar. Biol. 34, 169–170.

14. Seeliger, U., Edwards, P. (1977) Correlation coefficients and concentration factors of copper and lead in seawater and benthic algae. Mar. Pollut. Bull. 8, 16–19.

15. Thorell, L. (1977) Pollutants from Swedish municipal and industrial outlets into the Baltic. In: Akerbolom, A. (ed.) 3rd Soviet-Swedish Symposium on the Pollution of the Baltic. Ambio Spec. Rep. 5, 213–218.

16. Trullope, D., Evans, B. (1976) Concentration of Cu, Fe, Pb, Ni and Zn in fresh water algae blooms. Environm. Pollution. 11, 109–116.

17. Tsuchia, K. (1981) Cadmium in the Environment. Part II (Health Effects). Nriagu, J.O. (ed.). John Wiley and Sons, New York.

18. Wahbeh, M.I., Mahasneh, D.M., Mahasneh, I. (1985) Concentrations of zinc, manganese, copper, cadmium, manganese and iron in ten species of algae and seawater from Aqaba, Jordan. Marine Environm. Res. 16, 95–102.

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EFFECTS OF GRAMOXONE®-INDUCED REACTIVE OXYGEN RADICALS ON EICOSANOID SYNTHESIS OF MOUSE LUNG

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1. Studies were made to prove that a shift in the eicosanoid metabolism plays a role in the lung-damaging action of paraquat, the active ingredient of Gramoxone®, in mice.

2. An attempt was made to inhibit the eicosanoid metabolism with acetylsalicylic acid (Aspisol). Among others, it was found that the metabolic effect impaired by paraquat is influenced in a favourable direction by Aspisol treatment, including the % of survival, the lipid peroxidation values and the superoxide dismutase activity in the lung.

3. At the same time, as a prostacyclin synthesis activator, paraquat participates in the efforts of the organism to eliminate the toxic material, but it later weaknes the defence mechanisms by enhancing thromboxane A_2 synthesis, and it initiates pulmonary fibrosis, which cannot then be averted with Aspisol.

Keywords: Gramoxone® – Oxygen radicals – Lung antioxidant enzymes – Eicosanoids

INTRODUCTION

At the International Symposium and Colloquium-School organized by the Bulgarian Academy of Sciences in 1986 ("Activated oxygen species in biological systems"), we reported on work showing how paraquat (PQ) influences the prostanoid metabolism in mouse lung /3/.

A pathological chain-reaction is induced by the harmful effects of PQ on the pulmonary alveolar epithelial cells, and the most characteristic of the acute clinical signs are pulmonary edema and haemorrhages; these can

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be explained well in terms of a change in the prostanoid matabolism equilibrium. The redox cycle of PQ leads to the formation of oxygen radicals /6/. These radicals may not only be responsible for these acute symptoms, but also interfere with prostaglandin synthesis.

A certain shift in the redox-cycling has been observed in response to PQ in the perfused lung of guinea-pig /10/. Recently, Shibamoto et al. /15/ carried out prostanoid metabolism measurements following acute PQ infusion in awake sheep. They found that the contents of thromboxane A_2 (TXA_2; determined in the form TXB_2) in the plasma and lymph gradually increase after ca. 30 minutes in the course of the PQ infusion. The same was true for the prostacyclin metabolism, which was measured as 6-ketoprostaglandin F_1 (6-keto-PGF_1).

The aim of the present investigation was to follow the changes in the lipid peroxidation (LP), the total superoxide dismutase (SOD; EC 1.15.1.5) activity, the quantity of TXA_2 and the prostacyclin (PG I) metabolism in mice in response to the oral LD_{50} dose of PQ (150 mg/kg). The weight of the lung and the % of survival rate were determined in all cases.

A study was also made of how a single dose of acetyl salicylic acid (Aspisol (ASP)) as an eicosanoid metabolism inhibitor, influences the metabolism.

MATERIALS AND METHODS

Mice of the CFLP strains in both sexes were used. These were of the same age and nearly the same weight 25—30 g and reared under identical conditions /8/.

Experimental groups contained 10 animals and all experiments were repeated three times. Reported results are avareged values calculated from the three examinations.

Gramoxone (ICI, U.K.) preparation contained 25% paraquat (PQ) and applied in a 2.5% dilution orally in a dose of 120 mg/kg.

After 24 hours and the marked other hours the experimental animals were killed and enzyme activity and the LP were determined in the lung tissue (in the target organ).

The total SOD activity was determined by a modification of the method involving SOD-induced inhibition of the epinephrine —— adrenochrome transformation /8, 10/.

The LP was measured as the total amount of thiobarbituric acid (TBA)-active substances. The quantitative measurements used were closest to that described by Placer et al. /12/, which we simplified somewhat. The precipitation mixture and the reagent (10% perchloric acid solution is saturated with TBA) were added together to an aliquot of the total homogenate, and the mixture was subsequently heated for 20 minutes in a boiling water-bath.

The mixture was next cooled to room temperature, and the amount of TBA-active substance was determined photometrically at 532 nm.

In the lung homogenates, the eicosanoid metabolites were determined with a radioactive kit. The tissue homogenate was extracted with absolute ethanol, the ethanol was evaporated under N_2 and the residue was dissolved in the kit buffer. In the use of the kit, the prescription provided by the Isotope Institute of the Hungarian Academy of Sciences (Izinta, Budapest, Hungary), was followed. The two RIA kits used were denoted ^{125}J-TXB_2RK and ^{125}J-6-ketoprostaglandin F_1 RK. The eicosanoid metabolites were in all cases determined 24 hours after the administration of PQ and ASP. Each experimental value is the mean of 10 parallel determinations. The difference between the measured results never exceeded 15%. The 6-keto-PGF_1 and TXB_2 values are expressed in units of pg/g wet lung tissue weight.

The quantity of protein was measured by the method of Lowry et al. /7/.

Aspisol® (ASP; acetyl salicylic acid) is an injection product of Bayer (Leverkusen, FRG) and its effective dose was found to be 5 mg/kg administered i.p. parallel with PQ intoxication (SIN treatment /9/).

The chemicals used were generally of p.a. quality, bought from the most suitable sources.

The results were subjected to statistical evaluation with the Student-t test. All numerical data are given as means $\pm$ S.E.M. In the enzyme activity and LP measurements, the differences between 4—5 parallel measurements were never in excess of 5%.

RESULTS

Figure 1 shoes how the aucte =72 hours) survival rate following Gramoxone LD_{50} treatment is influenced by ASP administration in mice (see Fig. 1). As concerns the data, the ASP treatment increase the survival % of PQ LD_{50} to about its half.

Figure 2 depicts the change in lung wet weight in the controls, in response to the LD_{50} of PQ, and to the combined administration of PQ and ASP.

In this Figure (Figure 2) notable is, that ASP together with PQ LD_{50} significantly decrease the lung wet weight, which is a typical effect of PQ poisoning. This influence is most characteristic between 72—96 hours.

Of the antioxidant enzyme activities, only that of SOD in the lung was determined when the mice were treated with the LD_{50} of PQ, with ASP, and with together PQ LD_{50} + ASP (see Fig. 3).

Figure 3 presents the total-SOD activity changes during treatments. Between these circumstances and time the lung total-SOD activity increased after the PQ administration, however, the ASP alone decreased the lung t-SOD activity. The same tendency is observable during the ASP + PQ treatment.

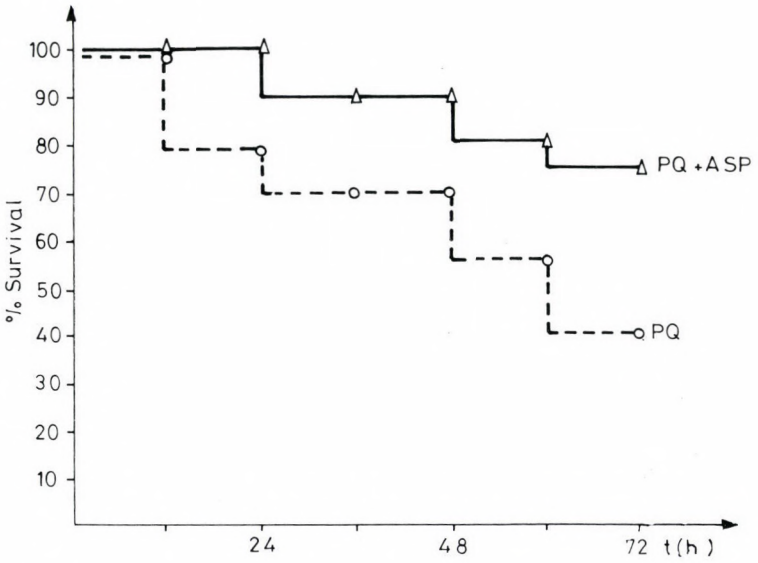

Fig. 1. The effect of 72 hours survival % of Gramoxone LD_{50} (o— — —o) and and Gramoxone LD_{50} + Aspisol (Δ——Δ) treated mice

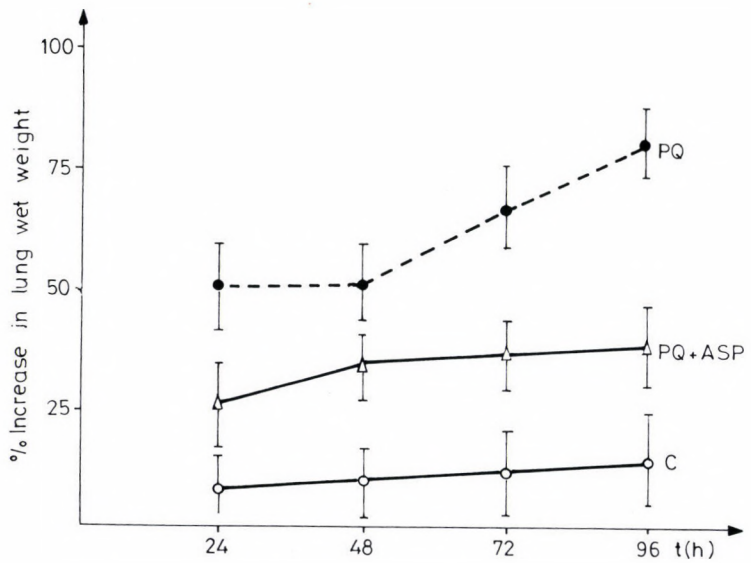

Fig. 2. The % increase of lung wet weight after PQ LD_{50}(●— — —●), PQ LD_{50} + ASP (Δ——Δ) treatment and in control mice (o——o)

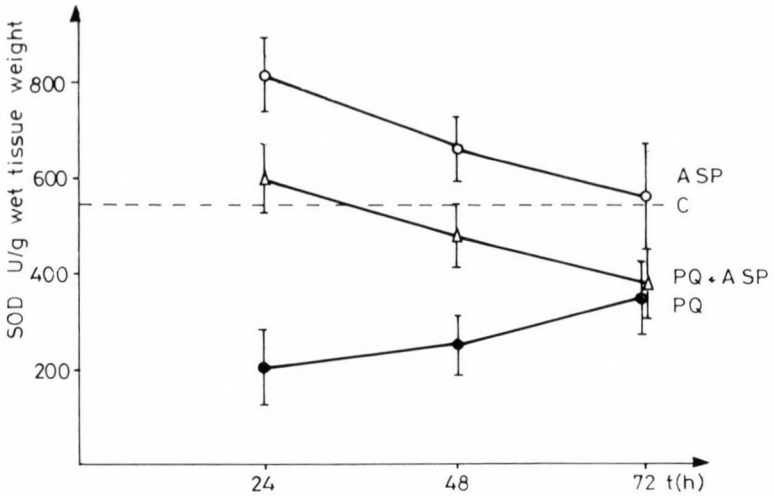

Fig. 3. Total SOD activities on mouse lung homogenates after Gramoxone (PQ ●——●) and Aspisol (ASP o——o) administration and the two substances together (Δ——Δ). (C = normal control activities)

Figure 4 compares the LP values under the conditions mentioned in Figure 3.

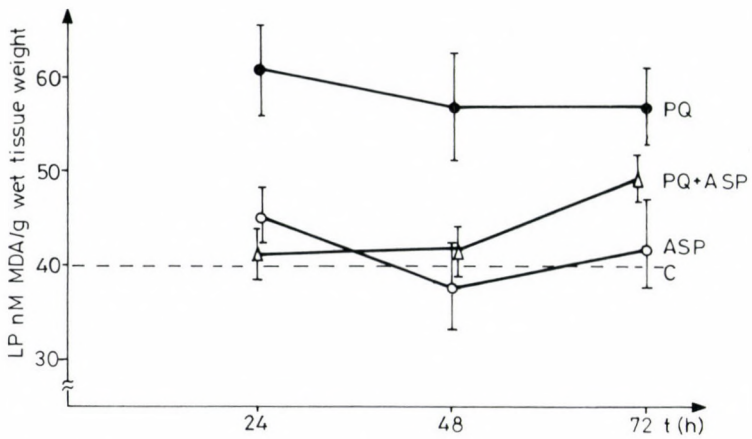

Fig. 4. LP values of mouse lung homogenates after adminstration of Gramoxone (PQ ●——●) and Aspisol (ASP o——o) and Aspisol treatment of Gramoxone poisoning (PQ+ASP Δ——Δ). (C = normal control values)

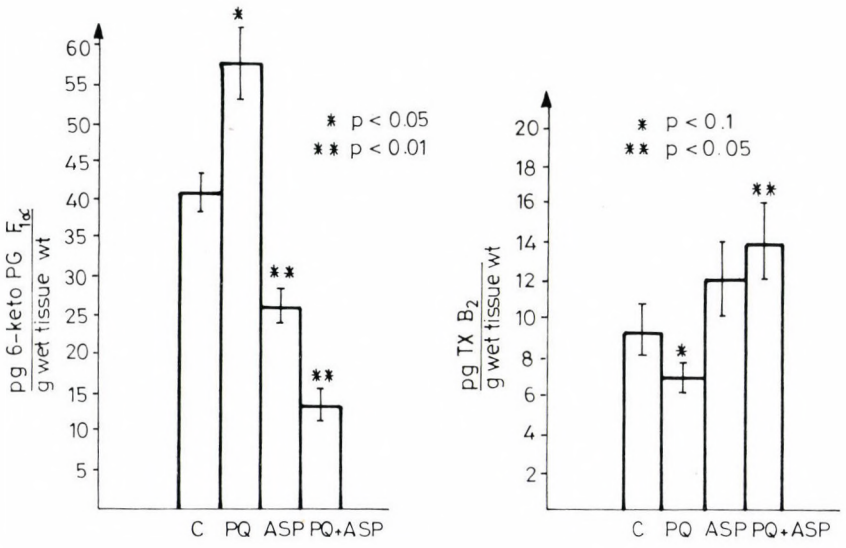

Fig. 5. Influence of the PQ LD_{50}, ASPISOL and PQ+ASP treatments on the amount of PG F_1 and TX B_2 in mice lung

PQ LD_{50} alone enlarges significantly the amount of malonyldialdehyde (MDA) in the lung homogenates. But in the presence of ASP, only after 48 h is PQ capable to increase the MDA values.

Figure 5 presents the eicosanoid metabolism values for mouse lung, i.e. the amounts of 6-keto-PGF_1 and TXB_2 in values of pg/g wet lung tissue weight 24 h after treatment with the LD_{50} of PQ, with ASP, and with LD_{50} of PQ + ASP together. Significant differences compared to the controls are indicated by* or ** asterisk. After 24 h PQ LD_{50} enhance the PG F_1 and decrease the TXB_2 amount in the lung homogenates. However ASP alone decreases the PG F_1 and increases TXB_2 syntheses (change contrary to that of the 6-keto-PGF_1/TXB_2 ratio). In the case of ASP+PQ the mentioned changes are more pronounced.

All the differences in parameters depicted on the Figures are significant.

All the differences in parameters depicted on the Figures are significant.

DISCUSSION

Sedor /13/ states that, the oxygen radicals and H_2O_2 reacting with polyunsaturated fatty acids play important roles in the eicosanoid metabolism by the utilization of the products fromed in the LP. The papers of Warso et al. /18/ and Bunting et al. /4/ indicated that the eicosanoid metabolism plays a significant role in the regulation of hyperalgesia, inflammation and thrombosis, through its effects on the TXA_2 and prostacyclin pathways. They proved that higher lipid hyperoxide concentrations inactivate the prostacyclin synthese and enhance the TXA_2 synthese function. An increase in the quantity of TXA_2 enhances the blood platelet aggregation and increases the presence of activated white blood cells providing the oxygen radicals. Glutathione peroxidase participates in the regulation of hydroperoxide concentration.

Another correlation that should be pointed out is that the cyclic GMP / cyclic AMP ratio in the cells depends on the TXB_2 / 6-keto-PG F_1 ratio /1, 5, 19/. This latter ratio rises in acute respiratory failure /16/.

Mention should be made of the results of Seeger et al. /14/, who observed that the oxidant release due to the leukocytes in rabbit is responsible for the pulmonary vasoconstruction and edema. However, they also state that the lung responds to H_2O_2 with increases in the Ca-calmodulin system activity and in the TX synthesis, and these changes give rise to the vasoconstruction. The organism attempts to compensate these phenomena through the pulmonary GSH-redox cycle.

Shibamoto et al. /15/ examined the effect of PQ in sheep lung from the aspects of the pulmonary fluid equilibrium and the eicosanoid metabolism. They found that PQ causes very early pulmonary endothelial damage (after ca 3.5 h): it increases the production of TXA_2 and prostacyclin; they considered that the prostacyclin production enhancement is the cause of the vascular and pulmonary damage.

On the basis of the above findings, we evaluate our results as follows. Figures 1 and 2 convincingly demonstrate that ASP treatment reduces the death rate and lung weight increases due to PQ LD_{50} besides the effect on the eicosanoid metabolism, the compensation brought about by ASP features in the decrease of the PQ-induced inhibition of the antioxidant enzyme (SOD) and of the LP enhancing effect (see Figs 3 and 4), i.e. it contributes to the improvement of the effect of the eicosanoid metabolism.

In our present experiments, a correlation was found between the

toxic effect of the LD_{50} of PQ and the simultaneous administration of ASP; initially, a significant prostacyclin synthesis increase occurred in response to the PQ poisoning, and it was still significant 24 hours later, this increase being accompanied by a simultaneous significant fall in the TXA_2 synthesis. Here, therefore, there is obviously a prostacyclin predominance. ASP itself enhances the TXA_2 synthesis, while there is a significant decrease in the prostacyclin effect (i.e. the above ratio decreases). PQ and ASP behave synergistically in inhibiting the prostacyclin synthesis and in enhancing the TXA_2 synthesis. Hence, the ratio increases and the TX effects take over, with the resulting enhanced tendency to thrombosis and the shortening of the bleeding and clotting times, as proved in our experiments /2/. Thus, one of the general effects of PQ, the active ingredient of Gramoxone, is the enhancement of the tendency to thrombosis, which is mainly responsible for the endothelial damage to the pulmonary vascular wall in the high oxygen concentration, and for the haemorrhages.

The findings of Smith et al. /17/ suggest that the situation here may involve the loss of prostacyclin protection, for it has been demonstrated that prostacyclin protects the lung against the damage caused by hyerpoxia.

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REFERENCES

1. Balla, Gy., Karmazsin, L. (1982) A prosztanoidok és néhány gyermekgyógyászati vonatkozásúak. (Some paediatric relations of prostanoids.) Az Orvostud. aktuális problémái. 44, 57–83. (In HUngarian).

2. Barabás, K., Matkovics, B., Berencsi, G. (1983) New consideration on the time-dependence of toxic changes caused by paraquat poisoning. Gen. Pharmac. 14, 381–383.

3. Barabás, K., Németh, J., Matkovics B. (1986) Effect of Gramoxone®-induced oxygen radicals on prostaglandin synthesis in mouse lung. Abstracts, p. 22. Internat. Symp. and colloquium – School, 29th Sept.–5th October, Varna, Bulgaria.

4. Bunting, S., Moncada, S., Vane, J.R. (1983) The Prostacyclin – thromboxane A_2 balance. Pathophysiological and therapeutic implications. Br. Med. Bulf. 39, 271–276.

5. Chi-Ying Wu-Wang, Craig-Schmidt, M.C., Faircloth, S.A. (1987) Conversion of arachidonic acid to prostanoids by lung microsomes from rats fed varying amounts of vitamin E. Prostaglandinds Leukoterienes Med. 26, 291–298.

6. Kappus, H., Sies, H. (1981) Toxic drug effects associated with oxygen metabolism: Redox cycling and lipid peroxidation. Experientia. 37, 1233–1241.

7. Lowry, O.H., Rosebrough, N.L., Farr, A.L., Randall, R.I. (1951) Protein measurement with the Folin phenol reagent. J. biol. Chem. 193, 265–275.

8. Matkovics, B., Novák, R., Hoang Duc Hahn, Szabó, L., Varga, Sz.I., Zalesna, G. (1977) A Comparative study of some more important experimental animal peroxide metabolism enzymes. Comp. Biochem. Physiol. 56B, 31–34.

9. Matkovics, B., Barabás, K., Szabó, L., Berencsi, G. (1980) In vivo study of the mechanism of protective effects of ascorbic acid and reduced glutathione in paraquat poisoning. Gen. Pharmac. 11, 455–461.

10. Matkovics, B., Barabás, K., Kálmán, E., Puppi, A. (1985) In vivo redox changes in some organs in response to Gramoxone®. Acta Biol. Hung. 36, 301–303.

11. Misra, H.P., Fridovich, I. (1972) The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J. biol. Chem. 247, 3170–3175.

12. Placer, Z.A., Cushman, L., Jonson, B.C. (1966) Estimation of product of lipid peroxidation malonyl dialdehyde in biochemical systems. Analyt. Biochem. 16, 359–364.

13. Sedor, J.R. (1986) Free radicals and prostanoid synthesis. J. Lab. Clin. Med. 108, 521–532.

14. Seeger, W., Suttop, N., Schmidt, F., Neuhof, H. (1986) The glutathione redox cycle as a defense system against hydrogen-peroxide-induced prostanoid formation and vasoconstruction in rabbit lungs. Am. Rev. Respir. Dis. 133, 1029–1036.

15. Shibamoto, T., Kobayashi, T. (1986) Acute effect of paraquat on lung fluid balance and prostanoid production in awake ship. Am. Rev. Respir. Dis. 134, 1252–1257.

16. Slotman, G.J., Burchard, K.W., Gann, D.S. (1985) Thrombosane and prostacyclin in clinical acute respiratory failure. J. Surg. Res. 39, 1–7.

17. Smith, L.I., Sommers, E., Hunt, C.E., Pachman, L. (1986) Hyperoxid lung usury in mice; A possible protective role for prostacyclin. J. Lab. Clin. Med. 108, 479–488.

18. Warso, M.A., Lands, W.E.M. (1983) Lipid peroxidation in relation to prostacyclin and thromboxane physiology and pathophysiology. Br. Med. Bull. 39, 277–280.

19. Wolim, M.S., Burke, T.M. (1987) Hydrogen peroxide elicits activation of bovine pulmonary arterial soluble guanylate cyclase by a mechanism associated with its metabolism by catalase. Biochem. Biophys. Res. Comm. 143, 20–25.

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FOOD DEPRIVATION AFFECTS REPRODUCTION IN ADULT FEMALE MICE (MUS MUSCULUS) AND THE AGE OF PUBERTY FOR THEIR FEMALE PROGENY

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Three experiments were designed to test the effects of food deprivation during various phases of the reproductive cycle on fertility and fecundity of the dams and on the age of sexual maturation and body growth of their female progeny. Food deprivation consisted of removal of all food every other day. Animals were deprived of food either during the period prior to pairing, during the period between pairing and conception or during gestation. Both fertility and fecundity were affected by food deprivation in some, but not all manipulations. The female progeny of food-deprived females reached puberty significantly later than the progeny of non-deprived dams when the food deprivation occurred during the week prior to pairing and up until successful insemination after pairing with a fertile male, but not when food deprivation occurred at other times during the reproductive cycle. Body growth did not differ in the daughters of food-deprived dams across the treatments for any of the experiments.

Keywords: Mus musculus – puberty – food deprivation – fecundity – fertility, pregnancy – gestation

INTRODUCTION

Any social, environmental or other factors that influence fertility, fecundity and/or puberty will have important consequences for the growth or decline of a population. This is particularly true for rodents that have

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opportunistic life history strategies and can reproduce rapidly under appropriate conditions. For individual animals, fertility is a measure of whether they successfully reproduce or not. Fertility is also used as a measure of the proportion of females in a population that are successfully reproducing at a particular time. Fecundity is a measure of the number of progeny produced by those reproductively active females. Generation time is that interval from the birth of an animal until it produces offspring. Puberty is a measure of the age at which young animals in the population are capable of reproducing. For many rodents first reproduction occurs at or near the time of puberty and thus puberty can be used as a measure of the generation time.

A variety of factors can affect fertility and fecundity in house mice, including light /3, 29/, temperature /1, 16, 24/, seasons /23, 28, 31/ social variables /26/, and genetic and maternal influences /5, 15/. Also, a variety of factors can affect the timing of puberty in female mice, including body growth /21/, social factors and chemosignals /14, 33/, diet /34/, genetics /12, 13/, litter size /6, 11, 17/, and day length /9, 10/. Previously, both McClure /19/ and Bronson and Marsteller /4/ have demonstrated that food deprivation affects reproduction in mice. Further, severe food restriction will result in complete blockage of the reproductive development of female house mice /18/.

In the present paper we report on three experiments designed to test the effects of food deprivation on the fertility and fecundity of female house mice and on the puberty of the female progeny of the deprived dams. Further, these experiments tested whether food deprivation at different periods of the reproductive cycle would differentially influence fertility, fecundity or the age of puberty in female progeny. Food deprivation during lactation, particularly during the first 10 days after parturition, results in dams consuming their pups /4/. Thus, we have not tested food deprivation during lactation in the present sequence of experiments.

METHODS AND MATERIALS

All of the mice used in these experiments were from a randomly bred, closed colony of ICR/Alb house mice (Mus musculus). All colony and test mice were maintained in shoe-box cages of polypropylene measuring 15x28x15 cm deep with opaque sides and fitted wire lids. A bedding of ground wood shavings was changed once each week. Pregnant females and young female test mice were provided with cotton for making nests. Purina Mouse Chow and water

were supplied ad libitum throughout all procedures and experiments, except as specified in experimental protocols involving food deprivation. All of the breeding and experimental testing were conducted at 21–25°C and 30–60% relative humidity on a 12L:12D daily regime with overhead fluorescent lights on from 06:00 to 18:00 h.

Food deprivation manipulations were performed by removing all food from the hopper located in the wire cage lid for 24 h and then replacing it for 24 h before being removed again. When the food was removed, the cage floor was also checked for any remaining food particles. This method of food deprivation was selected as it may better simulate fluctuations in food availability or abundance for free-living mice than procedures that provide the animals with the same limited quantity of food each day, or artificially maintain their body weight at some fixed percentage of an initial body weight. In nature mice may often be confronted with an opportunity to eat one day and a lack or near lack of food the next. For purposes of investigating physiological mechanisms and related phenomena, it may be desriable to use a deprivation scheme that controls the animal's food intake at a specified level, or maintains the animal's body weight at a prescribed percentage of some initial body weight. However, other schemes for deprivation, such as the every-other-day deprivation regime used here should also be investigated; such schemes may be much like what happens in the natural world of the mouse on some occasions. We have previously shown /20/ by weighing dams, that when food deprivation occurs every other day, the dams do not loose body weight, but compensate for weight loss on a day of deprivation by consuming additional food on days when the food is present and thus regain the small amount of lost weight. As a control procedure for our deprivation manipulation, the food for cages with control females was removed and immediately replaced each time the food was removed from experimental mice.

Each test female was paired with an adult, fertile male and checked daily for the appearance of a seminal plug. Upon finding a seminal plug, the male was removed and the female remained individually caged. All cages were checked daily and births were recorded. Each litter was counted and all pups were sexed. In an attempt to insure that the observed pup counts were not influenced by food-deprived dams consuming pups prior to the counting procedure, cages with pregnant females were checked 3–4 times daily during the daylight portion of the cycle, when most births occur. Also, we never noted the remains of any partially consumed pups and no obviously pregnant females were later found to have delivered and consumed all of the litter. Using the foregoing procedure, data were obtained on both fertility and fecundity.

To determine the age of puberty of female progeny of the dams from various treatment conditions, one female pup was removed at random from each litter upon weaning at 21 days of age. These young females were placed into individual cages and each mouse was examined daily from day 21 until the occurrence of vaginal introitus. Starting on the day of vaginal introitus a vaginal lavage was made each day until the occurrence of first vaginal estrus. The wet-mount vaginal smears were examined immediately with a light microscope and the cellular contents were judged to determine the stage of the oestrous cycle /27, 32/. To ascertain whether the various treatments produced any differences in body growth, each of the test females was weighed to the nearest 0.1 g upon weaning at 21 days of age, at the age of first vaginal estrus and at 42 days of age.

In each of the three experiments, the data on fertility were analyzed using Chi-square tests. For all other dependent variables in each experiment, one-way analyses-of-variance were used, followed by Duncan's New Multiple Range Test, with $\alpha = 0.02$.

EXPERIMENT I

Purpose. The purpose of this first experiment was to test the effects of food deprivation of adult female dams during the three weeks prior to pairing on their fertility and fecundity and on the puberty of their female progeny.

Methods. Seventy-four adult, virgin females (aged 90–120 days at the start of the experiment) were assigned at random to 1 of 4 treatments: (1) control females (n = 18) on ad libitum food and where the food in the hopper was removed and immediately replaced every other day for 3 weeks, (2) 1-week deprived females (n = 20) where the mice were on ad libitum food for 2 weeks and then had the food taken away every other day for 1 week (4 food removals), (3) 2-week deprived females (n = 20) where the mice were on ad libitum food for 1 week and then had the food taken away every other day for 2 weeks (7 food removals), or (4) 3-week deprived females (n = 16) where the mice had the food taken away every other day for all 3 weeks (10 food removals). At the end of the 3-week period all females were paired with adult, fertile males for up to 1 week. The males were removed upon finding a seminal plug and the females were monitored for litters as described in General Methods. For those females that gave birth to litters, a single female offspring was selected at random on day 21 and tested for first vaginal oestrus and body weight.

Results. There were no significant differences in the fertility of the mice deprived for 1 (50% produced litters), 2 (80%) or 3 (75%) weeks prior to pairing relative to control females (83%), though the percentage of females that became pregnant was lower for the mice deprived for 1 week and the overall Chi-square test approached statistical significance (Table 1). There were no significant differences in the average litter size produced by the female mice across the four treatment conditions (Table 1). Female progeny of dams that were food deprived for 1 week prior to pairing attained first vaginal oestrus significantly later than the female progeny of control dams or dams that were food deprived for 3 weeks prior to pairing (Table 1). The daughters of dams that were food deprived for 2 weeks before pairing were intermediate in age of first oestrus. There were no significant differences in the mean body weights at 21 or 42 days of age for the daughters of the food-deprived females across all four treatments (Table 1). Daughters of females that were deprived for 1 week prior to pairing weighed significantly more at the age of first oestrus than daughters of control females or

Table 1

Fertility and fecundity for control and food-deprived dams; ages of puberty and body weights at three different ages for their daughters. Dams were food-deprived for 1, 2 or 3 weeks prior to pairing

Treatment	N	No. of litters	Mean litter Size (± 1 SEM)	Daughter's mean age of puberty (± 1 SEM)	Daughter's mean body weight (± 1 SEM) 21 days	42 days	1st oestrus
Control	18	15	11.5(0.9)	34.9^{a}(1.1)	9.8(0.3)	22.2(0.5)	18.2^{a}(0.3)
Deprived 1 Week	20	10	10.9(1.0)	40.9^{b}(1.5)	10.1(0.4)	22.2(0.7)	21.8^{c}(0.6)
Deprived 2 Weeks	20	16	11.6(1.8)	$37.7^{a,b}$(1.0)	9.8(0.3)	22.2(0.6)	$20.5^{b,c}$(0.5)
Deprived 3 Weeks	16	12	10.2(0.9)	36.4^{a}(0.9)	9.4(0.3)	22.0(0.9)	$19.3^{a,b}$(0.4)
F =			0.591	4.641	1.442	0.118	13.423
d.f. =			3.49	3.49	3.49	3.49	3.49
prob.			n.s.	1.12	n.s.	n.s.	0.001

No. litters (fertility): X^2 (d.f. = 3) = 6.52; $0.05 < p < 0.10$

In each vertical column those means not marked with the same superscript letter are significantly different at the 0.02 level; Duncan's New Multiple Range Test

daughters of females that were deprived for 2 or 3 weeks prior to pairing (Table 1). Body weight at first oestrus also was not different for daughters from dams deprived for 1 or 2 weeks prior to pairing.

EXPERIMENT II

Purpose. The second experiment was designed to test whether food deprivation just prior to pairing, during pairing or in the variable interval between pairing and insemination would affect the fertility or fecundity of the dams or the age of puberty of the female progeny of the deprived dams.

Methods. Seventy five adult, virgin females (aged 90–120 days of age at the start of the experiment) were assigned at random to one of four treatment conditions: (1) control mice (n = 19) where the food supply was removed and immediately replaced every other day during the week prior to pairing, (2) females (n = 20) where the food was removed every other day for one week prior to pairing (4 food removals), (3) females (n = 18) where the food supply was present for the entire week prior to pairing, but was taken away every other day beginning on the day of pairing, until the appearance of a seminal plug (variable number, 1 to 4, of food removals), or (4) females (n = 18) where the food supply was removed every other day for the week prior to pairing and after pairing until the appearance of a vaginal plug (variable number, 5 to 8 removals). All females in all treatments were checked daily for the presence of a vaginal plug and the males were removed when the plug was found. The females were monitored for litter production as described in General Methods and a single female progeny from each litter was selected at random and tested for first vaginal oestrus and body weight.

Results. There were no significant differences in the numbers of females conceiving and producing litters in the four treatments (Table 2). Females that were food deprived from the time of pairing until the appearance of a seminal plug and those that were food deprived from 1 week prior to pairing through the appearance of a seminal plug after pairing had significantly smaller litters than control females and females that were deprived only for the 1 week prior to pairing (Table 2). Daughters of females that were deprived for 1 week prior to pairing and of females that were deprived for that week and then up until the appearance of a seminal plug after pairing attained first oestrus significantly later than daughters of control females and daughters of females that were deprived from the time

Table 2

Fertility and fecundity for control and food-deprived dams, and ages of puberty and body weights at three different ages for their daughters. Dams were food-deprived for various designated intervals during the period from 1 week prior to pairing until the detection of a seminal plug after pairing

Treatment	N	No. of litters	Mean litter Size (± 1 SEM)	Daughter's mean age of puberty (± 1 SEM)	Daughter's mean body weight (± 1 SEM) 21 days	42 days	1st oestrus
Control	19	19	12.2^{b}(0.4)	34.6^{a}(0.8)	9.7(0.2)	22.4(0.4)	19.2^{a}(0.4)
Deprived 1 Week prior to pairing	20	17	12.2^{b}(0.5)	40.4^{b}(0.9)	10.0(0.3)	22.6(0.7)	21.6^{b}(0.5)
Deprived from pairing to plug	18	15	10.9^{a}(0.5)	36.1^{a}(1.3)	9.7(0.3)	22.9(0.3)	$19.7^{a,b}$(0.5)
Deprived for 1 Week prior to pairing & to plug	18	11	10.3^{a}(0.6)	40.0^{b}(0.9)	9.5(0.3)	22.0(0.9)	21.4^{b}(0.4)
F =			3.547	9.473	0.526	0.385	6.878
d.f. =			3.56	3.56	3.56	3.56	3.56
prob.			0.05	0.001	n.s.	n.s.	0.001

No. litters (fertility): X^2 (d.f. = 3) = 3.65; $p > 0.20$

In each vertical column those means not marked with the same superscript letter are significantly different at the 0.02 level; Duncan's New Mutliple Range Test

of pairing until the appearance of a seminal plug (Table 2). There were no significant differences in the mean body weights at 21 or 42 days of age for the daughters of food-deprived females across all four treatments (Table 2). Daughters of control females and daughters born to females that were deprived during the period from pairing until the appearance of a seminal plug had significantly lighter mean body weights at the time of first oestrus than daughters born to females that were deprived for one week prior to pairing or for during the period from one week prior to pairing until the appearance of a seminal plug after pairing (Table 2).

EXPERIMENT III

Purpose. The purpose of the final experiment was to determine whether food deprivation during gestation would influence fertility or fecundity or the age of puberty of female progeny.

Methods. Seventy-six adult virgin females (aged 90–120 days of age at the start of the experiment) were assigned at random to 1 of 4 treatments. In each treatment all of the females were paired with fertile, adult males and monitored daily for the presence of a seminal plug. The treatment procedures were initiated beginning on the day the seminal plug was present. These procedures consisted of (1) control females (n = 19) where the food supply was removed every other day and immediately replaced from the day of insemination until birth, (2) females (n = 18) where the food supply was removed on the first, third and fifth days after insemination and then ad libitum food was given until birth (3 removals), (3) females (n = 19) where the food supply was ad libitum for the first 5 days after insemination and was then removed every other day from day 6 after insemination until birth (8 or 9 removals), or (4) females (n = 20) where the food supply was removed every other day from the day of insemination until birth (10 removals). In all cases the females were on ad libitum food throughout lactation. Each female was monitored for the birth of a litter as described in General Methods and a single test female was removed from each litter at 21 days of age for determination of the age of first vaginal oestrus and body weight.

Results. All three food deprivation treatments resulted in significant decrements in litter production relative to control females (Table 3). The treatment involving food deprivation throughout gestation resulted in only 1 litter (5% fertility), deprivation from insemination to day 5 of

Table 3

Fertility and fecundity for control and food-deprived dams, and ages of puberty and body weights at three different ages for their daughters. Dams were food deprived for varying periods during gestation

Treatment	N	No. of litters	Mean litter Size (± 1 SEM)	Daughter's mean age of puberty (± 1 SEM)	Daughter's mean body weight (± 1 SEM) 21 days	42 days	1st oestrus
Control	19	16	13.1(0.5)	34.7(1.2)	9.2(0.2)	22.7(0.4)	19.8(0.4)
Deprived from insemination to day 5 of gestation	18	10	14.9(0.9)	35.7(0.7)	9.4(0.3)	22.3(0.6)	20.2(0.5)
Deprived from day 5 of gestation to parturition	19	8	12.5(1.0)	36.4(1.6)	9.5(0.5)	22.3(0.5)	20.8(0.8)
Deprived from insemination to parturition	20	1	(12)	---	---	---	---
F =			2.261	0.480	0.412	0.204	0.595
d.f. =			2.31	2.31	2.31	2.31	2.31
prob.			n.s.	n.s.	n.s.	n.s.	n.s.

No. litters (fertility): X^2 (d.f. = 3) = 24.98; $p < 0.001$

gestation (56%) and deprivation from day 5 of gestation to parturition (42%) also resulted in fewer litters than for non-deprived control females (84%). There were no significant differences in litter size across the three treatments where this parameter could be tested (Table 3). Also, there were no significant differences in the ages of puberty for daughter's of dams in the three experiments treatments where puberty was measured. There were no significant differences in mean body weights at 21 or 42 days of age, or at the age of first oestrus for daughters of females in the three treatment conditions with sufficient sample size to be tested (Table 3).

DISCUSSION

These experiments provide data for three major conclusions: (1) Fertility in mice is not affected by food deprivation that occurs during the period prior to pairing with a male or during the time when mating may occur, but fewer females bear litters when they are food deprived during gestation. (2) For those females that do produce litters fecundity is not affected when food deprivation occurs either prior to pairing or during gestation, but food deprivation during the period when the female may be conceiving lowers the average litter size. (3) The age of sexual maturation in daughters of food deprived females is delayed when the dams are food deprived in the week prior to pairing or during that week and during the time when conception may occur, but puberty in the daughters is not delayed when the food deprivation occurs for moderately long periods prior to pairing or during gestation.

Food deprivation may represent a relatively stressful environmental condition. We would thus expect that reproductive physiology would be affected in any of several ways in food-deprived animals. Stress has been shown to affect reporductive biology in various rodents, e.g., rats (Rattus norvegicus; 2), prairie deermice (Peromyscus maniculatus; 30), mice (Mus musculus; 24, 25), and gerbils (Meriones unguiculatus; 22). In most instances it is likely that evolution by natural selection has influenced the reproductive physiology and related behaviours of the rodents such that there is a tendency to maximize lifetime reproduction. Mice may therefore avoid expending energy on reproduction in times of food shortage in order to sustain their own health for possible future reproductive efforts. This

hypothesis is consistent with some, but not all of the findings in the present sequence of experiments.

The most pronounced effects of food deprivation on fertility were recorded during gestation. The results of the different treatments in Experiment III suggest that at least two mechanisms are involved. First, some fertilized embryos fail to implant, and second, there are additional embryo resorptions occurring after implantation. These mechanisms appear to be 'all-or-none' in that the female either carries the litter to term and produces a litter with the same number of pups as untreated control females or the pregnancy results in no litter at all. This finding is similar to that reported for rats /2/; rats on restricted food intake either produced full-sized litters or all implants resorbed between days 8 and 11 of gestation. If food conditions are relatively poor during gestation the female might have a long-term advantage in fitness if that pregnancy was terminated and the female's physiology dictated a delay in reproductive efforts until better conditions prevailed. This delay appears to be the case, whether the mechanism is failure of implantation or embryo resorption or both.

It is curious that with respect to fertility, no diminution was observed in the rates of successful reproduction for females that were food deprived prior to or at the time of pairing. In the latter case it is possible that the mechanism for inhibiting pregnancy under conditions of food scarcity does not have sufficient time to activate during the relatively brief period that the females are subjected to the every other day deprivation regime. However, 48 h of food deprivation prior to pairing can result in lowered rates of mating behaviourand fertility /19/. Further, the effects of 48 h of food deprivation on mouse ovulation are related to whether the female was in diestrus or estrus at the time the deprivation began /4/. We do not have a full explanation as to why females that are food deprived for 1-to-3 weeks prior to pairing should conceive and bear litters in spite of the food deprivation conditions. This is particularly interesting in light of the findings from Experiment II that suggest that fecundity may be affected even though fertility is not for some treatments. Food deprivation during the week prior to pairing results in more female biased litters than for control mice that are not deprived or for females that are deprived for 2 weeks prior to pairing /20/. In 2-week deprived mice there are apparently compensatory mechanisms that facilitate adjustment to the food deprivation regime; the sex ratio for litters conceived by deprived females returns to the pattern observed in untreated mice with more male biased litters. In the

work reported by Meikle and Drickamer /20/ and in the present Experiments I and II the food deprivation regimes did not result in any significant loss of fertility when the deprivation occurred prior to pairing.

Experiment II revealed that the critical time period for influencing litter size (fecundity) through food deprivation is during the time that the female is paired with a male. Two possible mechanisms for the observed decline in litter size for females that are food-deprived during pairing are; /1/ fewer ova may be ovulated, and /2/ fewer ova are successfully fertilized. Further investigations will be needed to decide whether one or both of these mechanisms, or perhaps some other mechanism is involved in the diminution in litter size. Again, as with fertility it should be noted that food deprivation prior to pairing or during gestation does not affect the litter size. No clear explanation exists as to why this should be the case, though the results are in general agreement with the earlier findings of Berg /2/ for rats.

For both fecundity and fertility there are apparently internal processes that, given certain environmental conditions, trigger physiological mechanisms that reduce the numbers of pups being born. A female may be increasing her overall fitness by delaying all or part of her output from one litter to conserve resources for future reproductive efforts. However, it is curious that the mechanisms effecting reductions in fertility and fecundity appear to act at different times during the reproduction sequence. If natural selection has operated in such a manner as to maximize the reproductive success of the females then we might expect to discover more boardly based mechanisms acting to reduce the numbers of litters and numbers of pups per litter when environmental stressors, such as food scarcity, are present.

Our final, and perhaps most significant, conclusion concerns the finding that food deprivation, affecting a dam even before she conceives, and also during the early stages of pregnancy, can alter the timing of reproductive development of her female progeny. Female progeny of food-deprived dams in certain treatments were delayed in reaching first vaginal oestrus relative to daughters from non-deprived, control females. These findings are reminiscent of the earlier work by Denenberg and colleagues using rats /7, 8/, in which experiences, in the form of early handling provided to dams, influenced the open-field activity of their progeny. Two aspects of our present findings deserve comment. First, the age of sexual maturation was not altered for daughters when the dams were food-deprived

for 2 or more weeks prior to pairing. As noted previously, females that are food-deprived for 2 weeks prior to pairing appear to exhibit some adjustment to this form of environmental stress /20/. The adjustment is apparently transmitted to their daughters such that the reproductive development of the young females is not altered by the food deprivation conditions. One week of deprivation is not sufficiently long enough for the dams' physiology to make such an adjustment. Second, daughters of dams deprived during gestation mature at the same age as daughters of non-deprived, control dams. Whatever the mechanism by which the dams are influencing the reproductive physiology of their daughters, conceived or about to be conceived, that mechanism is no longer operative after conception has occurred and gestation commences. The data for body weights at days 21 and 42 and at the age of first oestrus are consistent with the hypothesis that the observed effects on sexual maturation are independent of any effects of body growth. In each experiment, there were no significant differences in body weights at 21 or 42 days of age, indicating that the mice were all growing at the same rate. That there should be differences in body weights at first oestrus follows directly from the fact that the mice are attaining first oestrus at significantly different ages. Thus, the effects of food deprivation on the dams that are being transmitted to their daughters, are not influencing the program for overall morphological development, though there is a significant influence on the program for sexual development.

If external conditions are less than adequate for successful reproduction it may be that the evolution of physiological mechanisms regulating reproduction has involved selection for a process by which a female effects a delay in the sexual development of her progeny such that they do not reproduce until conditions have improved. But, if that is the case, why doesn't this or some similar mechanism produce the same delay for all young females of food-deprived dams, particularly those that are in gestation at the time of the food stress?

Mice, it would appear, have evolved a variety of mechanisms that are triggered by food deprivation and which can influence various aspects of reproduction, including fertility, fecundity and the age of sexual maturation of their progeny. The foregoing experiments are an initial series of steps in sorting out the nature and extent of these mechanisms. In addition to exploring the reasons for such wide variation with regard to which time periods for food stress influence which parameters of reproduction, it may

also become important to explore the role(s) of other environmental stressors in affecting these same parameters of reproduction.

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REFERENCES

1. Barnett, S.A. (1962) Total breeding capacity of mice at two temperatures. J. Reprod. Fert. 4, 327–335.
2. Berg, B.N. (1965) Dietary restriction and reproduction in the rat. J. Nutrition 87, 344–348.
3. Bronson, F.H. (1979) Light intensity and reproduction in wild and domestic house mice. Biol. Repro. 21, 235–239.
4. Bronson, F.H., Marsteller, F.A. (1985) Effect of short-term food deprivation on reproduction in female mice. Biol. Repro. 33, 660–667.
5. Butler, L. (1958) The inheritance of litter size, body weight, and variability, in a cross between two inbred strains of mice. Can. J. Zool. 36, 969–983.
6. Crozier, W.J., Enzmann, E.V. (1935) On the relation between litter size, birth weight, and rate of growth, in mice. J. Gen. Physiol. 19, 249–263.
7. Denenberg, V.H., Whimbey, A.E. (1963) Behavior of adult rats is modified by the experiences their mothers had as infants. Science 142, 1192–1193.
8. Denenberg, V.H., Rosenberg, K.M. (1967) Nongenetic transmission of information. Nature 216, 549–550.
9. Drickamer, L.C. (1975a) Daylength and sexual maturation in female house mice. Develop. Psychobiol. 8, 561–570.
10. Drickamer, L.C. (1975b) Female mouse maturation: relative importance of social factors and daylength. J. Reprod. Fert. 44, 147–150.
11. Drickamer, L.C. (1976) Effect of size and sex ratio of litter on the sexual maturation of female mice. J. Reprod. Fert. 46, 369–374.
12. Drickamer, L.C. (1981) Selection for age of sexual maturation in mice and the consequences for population regulation. Behav. Neural Biol. 32, 82–89.
13. Dirckamer, L.C. (1983) Mice selected for age of puberty: reverse selection and reproductive characteristics. Biol. Reprod. 28, 843–848.
14. Drickamer, L.C. (1986) Urinary chemosignals that influence puberty in mice: ecological and evolutionary considerations. In Duvall, D., Muller-Schwarze, D. and Silverstein, R.M. (eds), Chemical Signals in Vertebrates, Vol. 4. New York, Plenum Press.
15. Eisen, E.J., Durant, B.S. (1980) Genetic and maternal environmental factors influencing litter size and reproductive efficiency in mice. J. Anim. Sci. 50, 428–441.

16. Garrard, G., Harrison, G.A., Weiner, J.S. (1974) Reproduction and survival of mice at 23° and 32°C. J. Repro. Fert. 37, 287–298.

17. Gates, W.H. (1925) Litter size, birth weight, and early growth of mice (Mus musculus). Anat. Rec. 29, 183–193.

18. Hamilton, G.D., Bronson, F.H. (1985) Food restriction and reproductive development in wild house mice. Biol. Reprod. 32, 773–778.

19. McClure, T.J. (1966) Infertility in mice caused by fasting at about the time of mating. Reprod. Fert. 12, 243–248.

20. Meikle, D.B., Drickamer, L.C. (1986) Secondary sex ratio variation and food availability in wild and laboratory house mice (Mus musculus). J. Reprod. Fert. 7, 587–591.

21. Montiero, L.S., Falconer, D.S. (1966) Compensatory growth and sexual maturity in mice. Anim. Prod. 8, 179–192.

22. Payman, B.C., Swanson, H.H. (1980) Social influence on sexual maturation and breeding in the female Mongolian gerbil (Meriones unguiculatus). Anim. Behav. 28, 528–535.

23. Pennycuik, P.R. (1972) Seasonal changes in reproductive productivity growth rate, and food intake in mice exposed to different regimes of day length and environmental temprature. Aust. J. Biol. Sci. 25, 527–635.

24. Pennycuik, P.R. (1973) Behaviour of mice house in groups at 4, 21, and 33°. Aust. J. Biol. Sci. 26, 917–926.

25. Pryor, S., Bronson, F.H. (1981) Relative and combined effects of low temperature, poor diet, and short daylength on the productivity of wild house mice. Biol. Reprod. 25, 734–743.

26. Rowe, F.P., Taylor, E.J., Chudley, A.J.H. (1964) The effect of crowdin on the reproduction of the house mouse (Mus musculus) living in corn ricks. J. Anim. Ecol. 33, 477–483.

27. Rugh, R. (1968) The Mouse, Its Reproduction and Development. Minneapolis, Burgess Publ. Co.

28. Russell, E.S. (1954) Search for new cases of parental and seasonal influences upon variations within inbred strains. Annals. N.Y. Acad. Sci. 57, 597–605.

29. Stoddart, R.C. (1970) Breeding and growth of laboratory mice in darkness. Lab. Anim. 4, 13–16.

30. Terman, C.R. (1965) A study of population growth and control exhibited in the laboratory by prairie deermice. Ecology 46, 890–895.

31. Thibault, C., Courot, M., Martinet, L., Mauleon, P., du Mesnil du Buisson, F., Ortavant, R., Pelletier, J., Signoret, J.P. (1966) Regulation of breeding season and estrous cycles by light and external stimuli in some mammals. J. Anim. Sci. (Suppl.) 25, 119–142.

32. Vandenbergh, J.G. (1969) Male odor accelerates female sexual maturation in mice. Endocrinology 84, 658–660.

33. Vandenbergh, J.G. (1983) Pheromonal regulation of puberty. In Vandenbergh, J.G. (ed.), Pheromones and Reproduction in Mammals. New York, Academic Press.

34. Vandenbergh, J.G., Drickamer, L.C., Colby, D.R. (1972) Social and dietary factors in the sexual maturation of female mice. J. Reprod. Fert. 28, 397–405.

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LIGHT MICROSCOPIC, ENZYME BIOCHEMICAL AND STEROID ANALYTICAL INVESTIGATIONS OF FOLLICULAR ATRESIA IN THE OVARY OF DOMESTIC GOOSE

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Follicular atresia in the ovary of the domestic goose was investigated by light microscopic, steroid RIA and lysosomal enzyme activity measuring methods during the spring reproduction cycle. Degenerative processes are associated with the transformation and proliferation of granulosa, internal and external thecal cells in the follicle. Seven types of atretic follicles were identified on the basis of the presence, absence or dominance of cells containing lipids and synthesizing steroids.

Conclusive evidence for the relation between cell type and hormone content was found only in one type: in type 6, stromal glandular cells show an extremely intensive PROG synthesizing activity. In the other types it was shown that glandular type of cells which become proliferative during atresia possess a relatively uniform steroid synthesizing ability. This uniformity is also seen in the high activity of lysosomal enzymes regardless of the size and type of atretic follicles.

Keywords: domestic goose – follicular atresia – light microscopy – steroid synthesis – lysosomal enzymes

INTRODUCTION

At the beginning of each reproductive cycle the number of developing follicles in the avian ovary exceeds the number reaching the stage of ovulation. The number of growing follicles is genetically limited and species specific and a significant number of the follicles present in the ovary will

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subsequently undergo atresia. In laying hens with continuous egg production this number may be as high as 20% /12/.

During the advance of the breeding season the incidence of follicular atresia increases, reaching a peak during the period of incubation /9, 10, 16, 18/. The role of atresia which brings about far-reaching changes in ovarian structure has been studied by several workers /17, 25, 27/. Summarizing their results, Erpino /10/ suggests that also degenerating follicles produce sexual steroids.

The results Gupta and Maiti /14/ are not in agreement with these data. They have found that very few atretic follicles showed 3-β-HSDH activity in the pied myna's (Sturnus contra contra) ovary. The steroid secretory cells appear only during the early stages of atresia and later they undergo abortive luteinization and do not secrete sexual steroids.

Gilbert et al. /12/ studying the ovulatory cycle in laying hens found that follicular atresia played a role in establishing the follicular hierarchy in the ovary.

In this study the structure and steroid content of atretic follicles as well as changes in the activity of lysosomal enzymes involved in degenerative processes were investigated. It was intended to use the data obtained in an attempt to interpret the morphogenesis and functional significance of characteristic types of follicular atresia.

MATERIALS AND METHODS

Two-year-old Landes laying geese with an average body weight of 5 kg were used. The birds were housed in sheds with runs under a natural system of management. They were fed ad libitum a maintenance ratio containing 11% protein.

Fifteen birds were killed at different dates during the spring breeding cycle, from 11 February to 28 April. The follicles were removed and placed on filter paper moistened with physiological saline for macroscopic investigation.

Atretic follicles were separated from developing ones according to Gilbert et al. /12, 13/ on the basis of their deformed shape, irregular and convoluted surface, flaccid touch or the presence of haemorrhages. Their size, because of their irregular shape, could be measured only with an error of 1 to 2 mm. The atretic follicles (AF) were subsequently cut into halves.

One half of each AF was fixed in Bouin's fluid for light microscopic investigation by Péterffy's double embedding method. After embedding, 5 μm sections were made with a Reichert microtome and the sections were stained with haematoxilin-eosin. Microphotos were taken using a Reichert "Zetopan" photomicroscope.

The other half of the follicle was used for determinations of sexual steroids by radioimmuno-assay (RIA). The follicular halves were homogenized

after addition of physiological saline in a teflon glass homogenizer at 0–4 °C.

From the suspension obtained, progesterone (PROG) was first extracted with petroleum ether and subsequently testosterone (TEST) and 17-β-oestradiol (E_2) were extracted with diethyl ether. The triticum-labelled samples were measured by means of a LKB-Wallac scintillation spectrometer. The results obtained in pg refer to 100 mg wet weight. Student's "t" test was used for statistical evaluation.

Among lysosomal marker enzymes the activity of acid phosphatase, β-galactosidase, β-glucuronidase and catepsin D was examined.

Acid phosphatase was assayed as described by Barrett /3/. Reaction mixtures, containing 800 ul of 0.2 M Na-citrate buffer (pH 4.8), 100 µl 5×10^{-3} mM p-nitrophenylphosphate and 100 µl of sample, were incubated for 60 min at 39 °C. The reaction was stopped by the addition of 2 ml of TRIS-SDS solution. After centrifugation the absorbance of supernatans was measured at 410 nm. Dilutions of p-nitrophenol were used as standards.

β-galactosidase was determined according to Barrett /3/. Reaction mixtures contained 800 µl of 0.2 M Na-citrate buffer (pH 4.3), 100 µl 5×10^{-3} mM p-nitrophenyl-β-D-galactopyranoside and 100 µl of sample, were incubated for 60 min at 39 °C. The reaction was terminated by the addition of 2 ml of TRIS-SDS solution. Dilutions of p-nitrophenol were used as standards.

β-glucuronidase was assayed as described by Barrett /3/. Reaction mixtures contained 800 µl of 0.2 M Na-citrate buffer (pH 5.0), 100 µl 3×10^{-3} mM p-nitrophenyl-β-D-glucuronide and 100 µl of the sample and were incubated for 60 min at 39 °C. The reaction was terminated by the addition of 2 ml of TRIS-SDS solution. After centrifugation the absorbance of the supernatans was measured at 405 nm.

Catepsin D was measured by a modification of the assay of Barrett /3/. Reaction mixtures, containing 500 µl of 0.2 M Na-citrate buffer (pH 4.0), 400 µl of 8% haemoglobin and 100 µl of sample, were incubated for 90 min at 39 °C. The reaction was stopped by the addition of 1 ml of 10% TCA. After 30 min at 4 °C, the samples were centrifuged, and 1 ml of the supernatans were used to determine soluble peptides using a protein-dye assay /4/. A unit of the enzyme represented 1 µg of TCA-soluble peptides released from haemoglobin per min.

Protein determinations from the crude homogenates were based on the method of Bradford /4/ with bovine serum albumin fraction V as the standard.

RESULTS

Histology, hormone production and enzyme activity were investigated in 91 AFs from 15 geese.

Morphology

In evaluating the results of light microscopic histological study the observations made by Erpino /10/ were heavily drawn upon.

Seven types of AFs were identified according to the presence, absence or dominance of certain cells and the degree of degeneration in the yolk.

Type 1 comprises the AFs that were classed as early-stage AFs by Erpino. No change is observed in the thecal layer. The granulosa loses its monolayer character, proliferates and produces invaginations several cell layers deep in the yolk (Fig. 1). This type also comprises AFs where the process is further advanced, and ex-granulosa cells (including macrophages) invade the yolk. The ex-granulosa cells are of various shapes, slightly swollen, their nuclei are picnotic. Their structure indicates synthesis and storage of lipid sbustances.

Type 1 atresia frequently occurs in follicles that at first sight give the impression of viable white follicles, although they are less transparent, have a dull colour and are soft to the touch.

Type 2 AFs are characterized by the transformation of the connective-tissue cells of the theca interna into glandular elements and the hyperplasia of such elements. Glandular cells lie close together in 3 or 4 layers between the connective tissue cell layer around the basal lamina that has remained of the theca interna and the theca externa. The cells have light vacuolized cytoplasm and their nuclei contain a prominent nucleolus (Fig. 2). The uniform cell layer may be broken by fibrous trabeculae.

Transformation of the theca interna is always associated with proliferation of the granulosa. Sporadically, "nest-like" cells, which characterize Type 3 AFs, may occur in the theca externa.

The macroscopic appearance of Type 2 atresia is rather heterogeneous and this type cannot be identified on visual observation.

Type 3 AFs are similar in their basic structure to Type 2 AFs. The difference lies in the great number of "nest-like" cells present in the theca externa. These cells accompany the development of viable follicles from a size of about 500 μm to a size of 9–10 mm, in the latter stage they can only be found in the theca externa on rare occasions. Thecal glandular cells are situated close together in nests consisting of connective tissue, their nuclei are round or oval and usually contain a prominent nucleolus. Their cytoplasm is light and often exhibits a foamy texture (presumably containing lipids) (Fig. 3).

Thus, Type 3 comprises three cell types of different origin, characterized by proliferation and distinct cytoplasmic vacuolization as shown by light microscopy. The dissolved lipid droplets, at least partially, suggest the accumulation of steroid precursor cholesterol esthers.

Further development of this type may be represented by forms in which a thick, compact fibrous connective tissue ring emerges in the theca

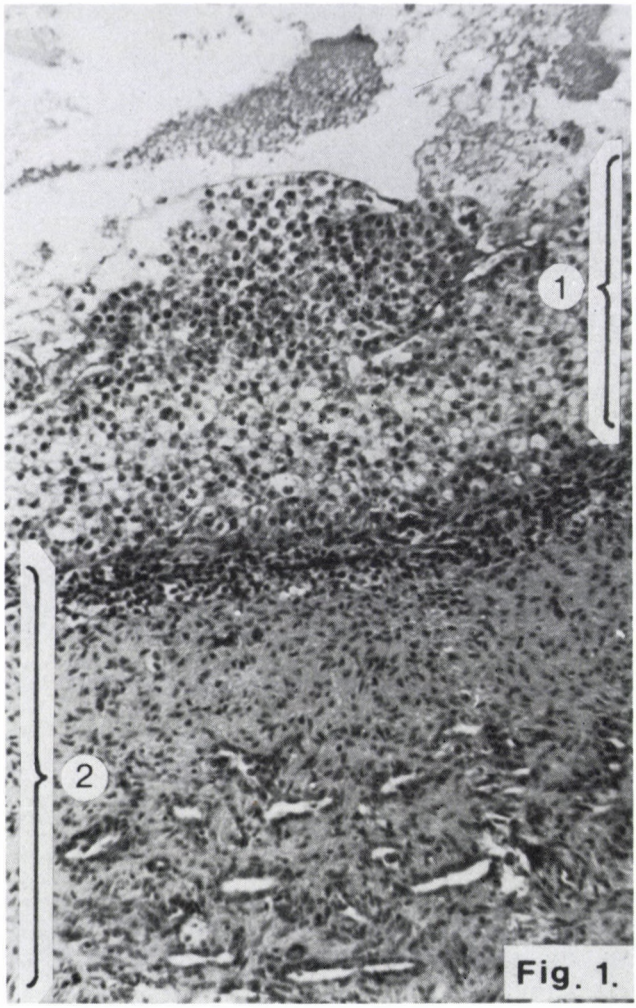

Fig. 1. First type of atretic follicles. The granulosa (1) loses its monolayer character and have started invading the ooplasm. In the theca (2) thecal glandular cells are absent. X 100

interna, which suppresses the glandular cells of the theca interna. The end product of this process is the proliferated granulosa mass filling the yolk and the presence of swollen theca externa nests pressed closely together and separated from each other by a thick connective tissue ring.

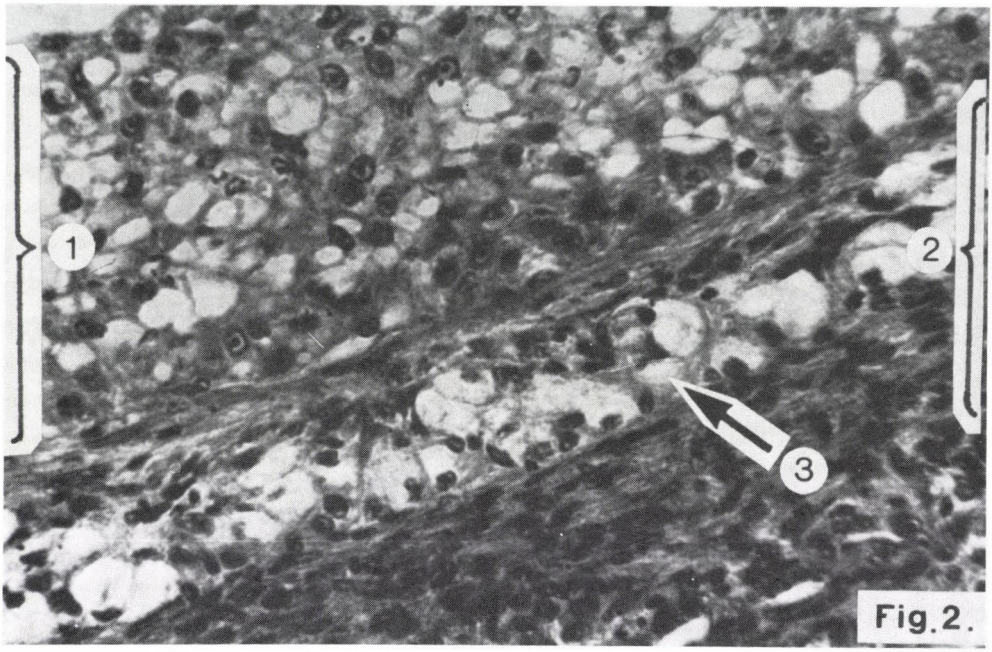

Fig. 2. Second type of atretic follicles. The multi-layered granulosa (1) shows numerous vacuolized degenerating granulosa cells. Note the hypertrophied thecal gland cells (3) in the theca interna (2). X 250

Fig. 3. Third type of atretic follicles. Enlarged and hyperplastic follicular epithelium with picnotic nuclei are seen in the ooplasm (1). The internal gland cells (2) markedly differentiated and in the theca externa large amount of nest-like gland cells are appeared (3). X 250

Fig. 4. Fourth type of atretic follicles. Note the presence the cords of theca external origin gland cells (2). The yolk is completely filled with hyperplastic granulosa cells (1). Fibrous trabeculae (3). X 70

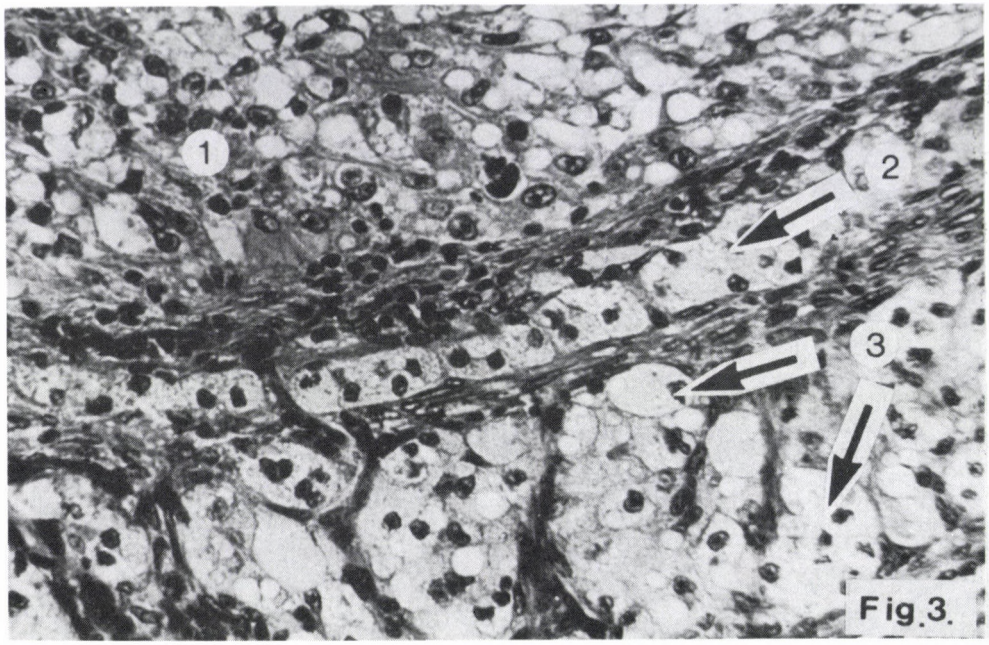

Fig.3.

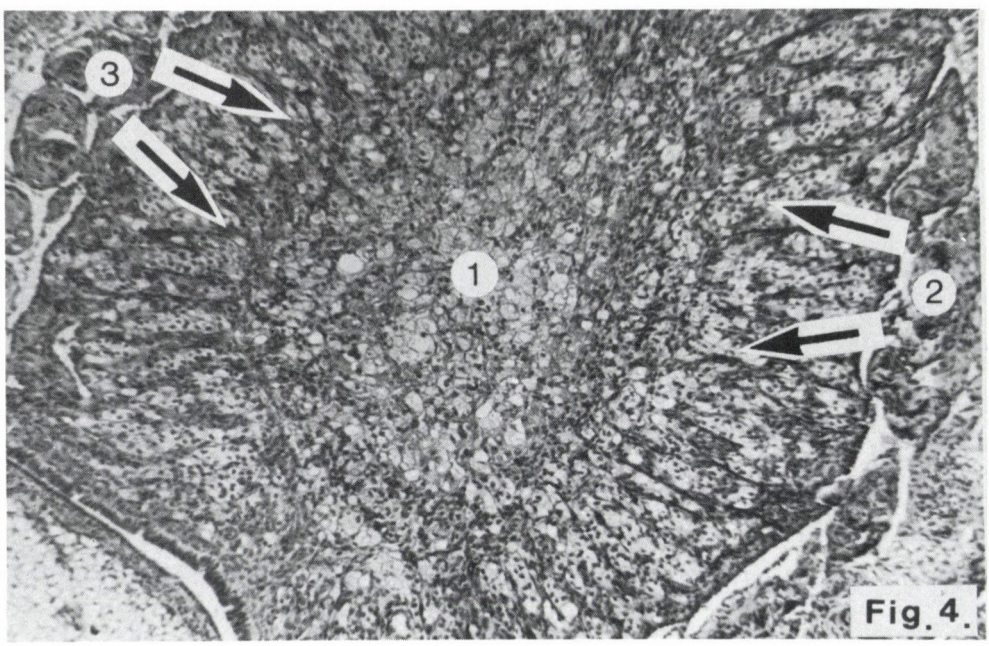

Fig.4.

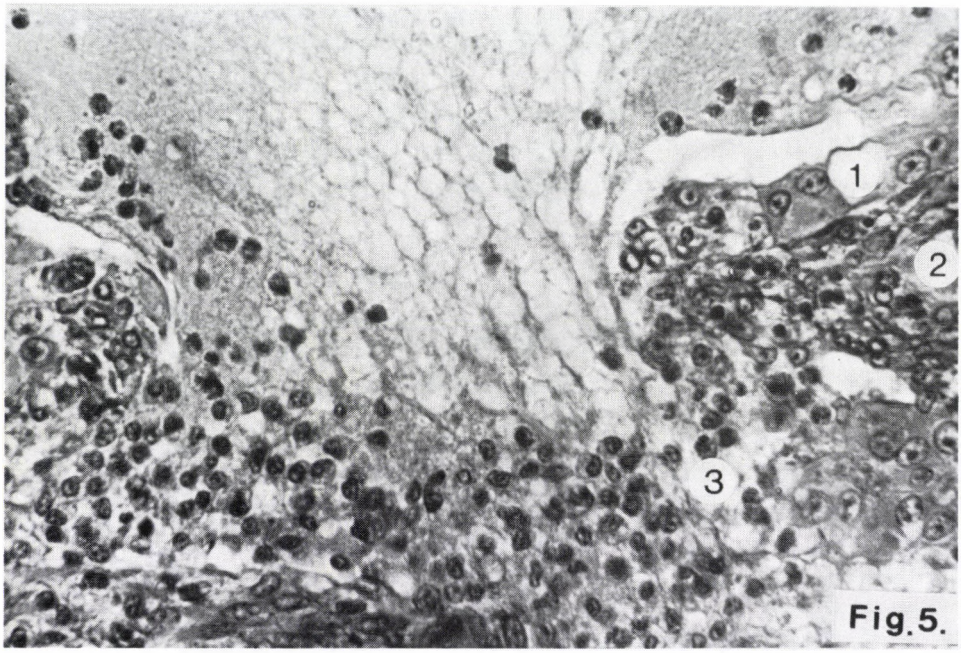

Fig. 5. Fifth type of atretic follicles (bursting atresia). Note the rupture of thecal layers (2) and extrusion of yolk and granulosa cells (3). Granulosa layer (1). X 250

Type 4 AFs were often observed in follicles above size 0.51–1.00 mm, rising out of the ovarian surface this type of atresia does not occur, while the collection of AFs smaller than this was technically impracticable. This type is characterized by intensive proliferation of glandular-type cells arranged in nests in the theca externa. Cell multiplication proceeds radially along connective tissue fibres towards the centre of the degenerating follicle, growing over the frequently hyperplastic theca interna and granulosa cells found there (Fig. 4).

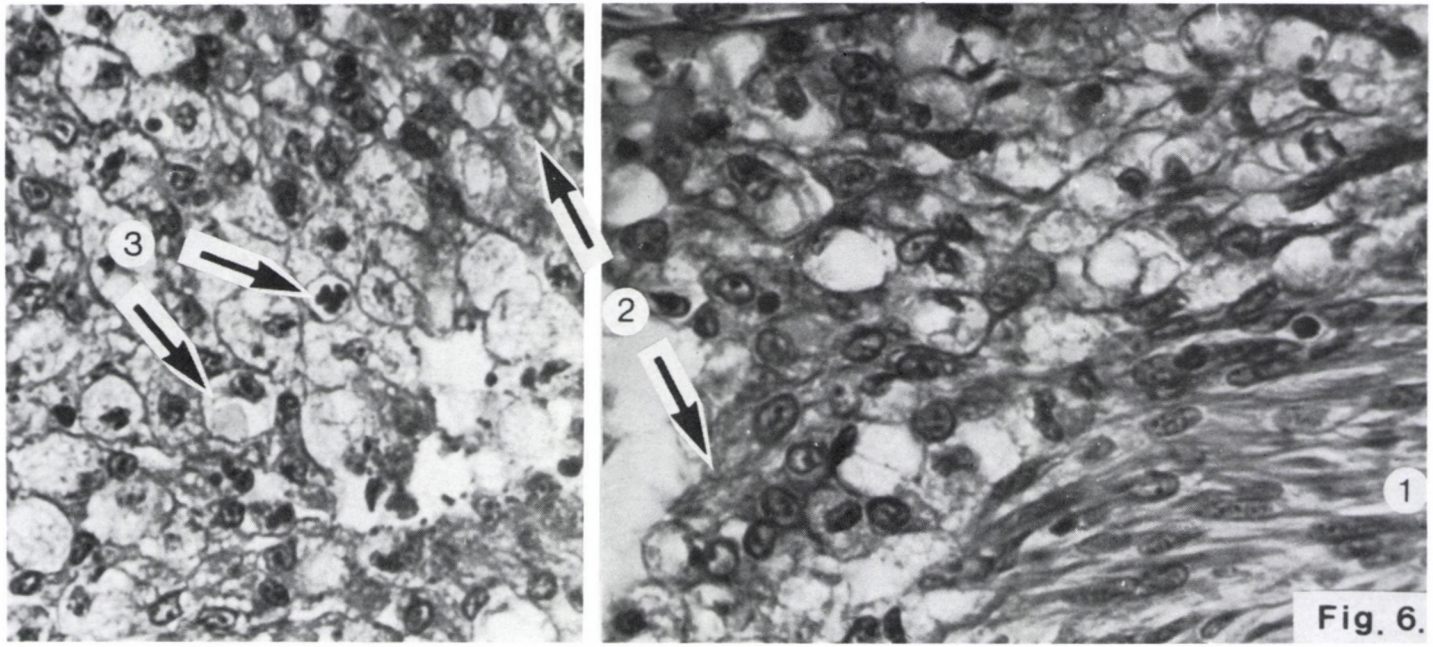

Fig. 6. Fifth type of atretic follicles (bursting atresia). Montage: Note the transformation of connective tissue elements (1) into stromal gland cells (2). Yolk droplets (3). X 400

Rings of compact fibrous connective tissue cannot be observed. Thus it appears that the development of spoke-like connective hedges precludes the organization of compact-fibrous connective tissue arranged in rings.

Type 5 AFs are known as "bursting" atresias and are characterized by the rupture of the egg and the surrounding layers brought about by the degenerative processes, which is followed by the appearance of yolk, erythrocytes and plasma among the connective-tissue fibres of the follicle. The yolk extruding from the egg was invariably surrounded by stromal cells. The appearance of yolk seemed to induce their emergence (Fig. 5).

Stromal cells are polymorphic and quite distinct from the surrounding connective tissue cells. They have large, round or oval nuclei with a prominent nucleolus. Their cytoplasm is lightly stained and often vacuolized. In the cytoplasm of some cells, phagocytized yolk droplets can be seen. Lipoidal degeneration of stromal cells is not yet pronounced in this type (Fig. 6). In the other cell types found in the follicle, degenerative processes are dominant. The granulosa is monolayered, broken and only occasionally stratified. Due to the inactivity of proteases responsible for follicular rupture and the spilling out of yolk the close contact between the granulosa and the lamina basalis ceases and yolk appears both between them and among the granulosa cells. This process can also be seen in the cells of the theca interna. The yolk appearing among the dissociated cells and fibres laterally swamps these areas, causing trophic disruptions and accelerating the degenerative processes.

Thus, in this type it is only the stromal cells appearing outside the thick, fibrous connective tissue (theca externa) that carry on lipid synthesizing activity.

The processes taking place in the follicle are also indicated by the macroscopic appearance of degradation. The signs of yolk spilled out from the egg and of consequent haemorrhages are obvious. AFs of this type have a distinctive "undulating" surface, and are soft to the touch, while their central part can be compared to a hard ball. According to the nature of the liquid spilled out their surface is usually yellow, scarlet or bloodstained. The stigma is conspicuously thick, white and has sharp contours.

The rupture of the egg cell and granulosa layer and the extrusion of yolk into the loose fibrous connective tissue underlying the germinal epithelium was observed in AFs up to 19 mm in diameter. The wall of larger AFs ruptured in their whole thickness and the yolk extruded into the abdominal cavity.

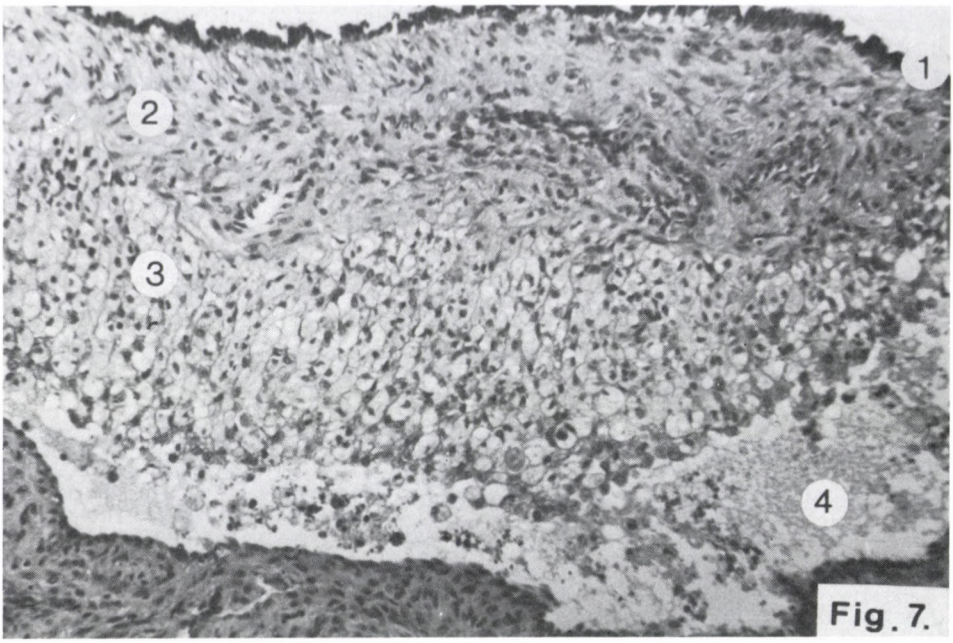

Fig. 7. Sixth type of atretic follicles. Note the intrusion of yolk (4) into the stromal tissue. Germinal epithelium (1). Stromal connective tissue (2). Stromal gland cells (3). X 100

Type 6 atresia is characterized by an invasion of active lipid-synthetizing stromal cells. The original structure of the follicle is indicated only by a few cells or clusters of cells. The connective tissue fibres of the theca are torn and found only in traces (Figs 7, 8). Most of the AFs is filled by stromal cells and yolk containing a great number of heterophylic granulocytes, lymphocytes and erythrocytes. In type 6 atresias lipoidal degeneration of stromal cells affecting an area of variable size is frequently encountered. This type can be relatively reliably identified even macroscopically. Up to a size of 6 to 7 mm in diameter their colour is a dull

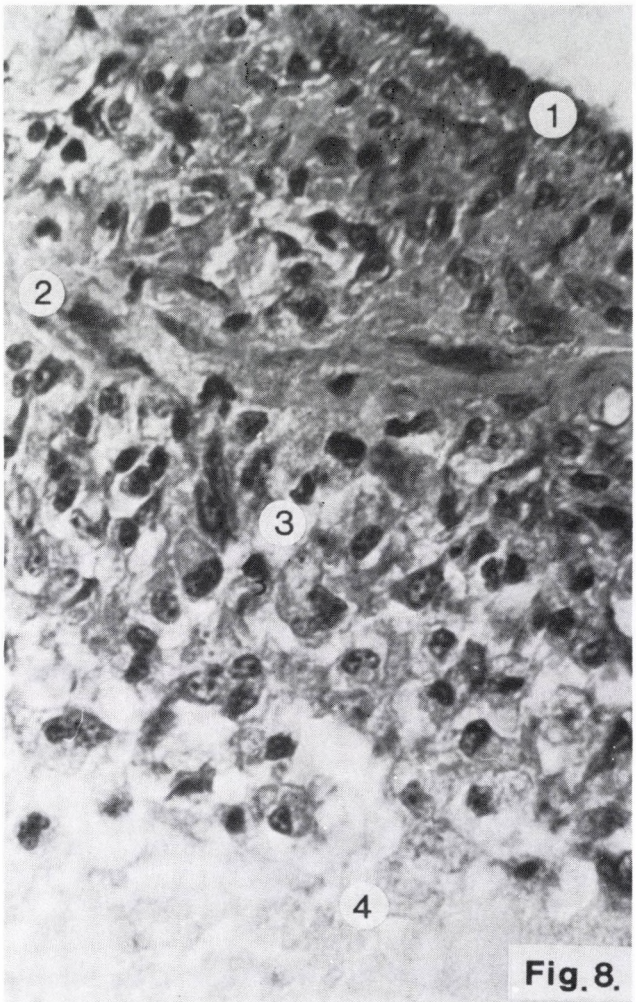

Fig. 8. Sixth type of atretic follicles. A detail of active stromal glandular tissue. Germinal epithelium (1). Fibroblasts (2) and stromal gland cells (3). Yolk (4). X 400

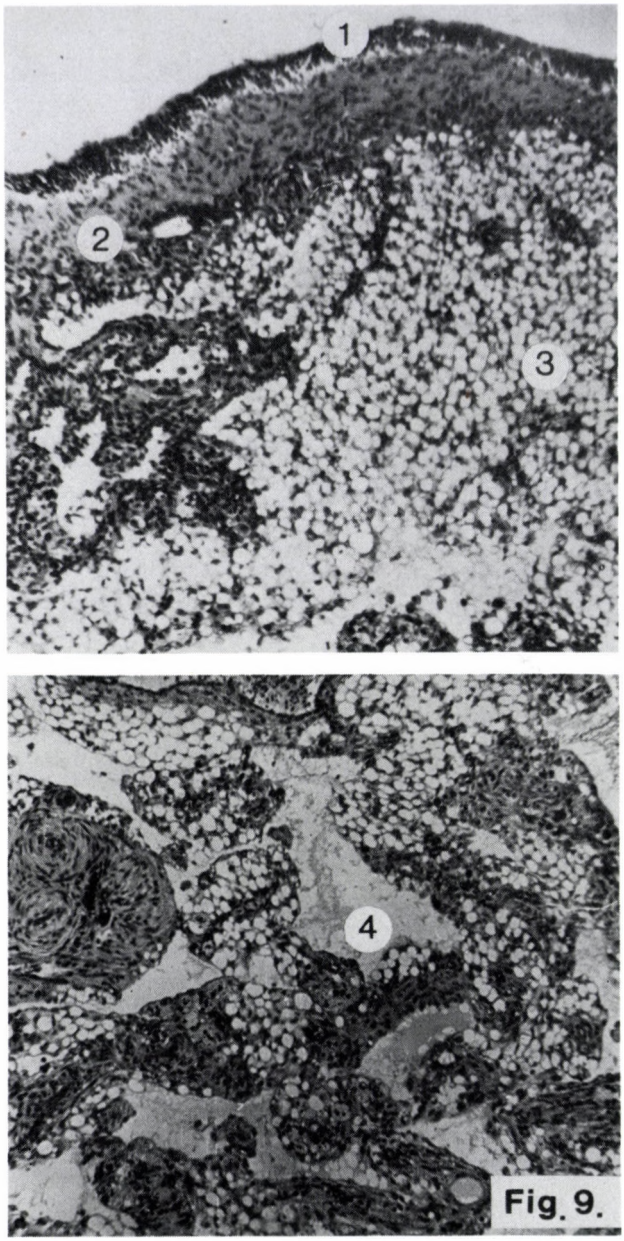

Fig. 9. Seventh type of atretic follicles. The most of the stromal glandular cells undergo lipoidal degeneration. Germinal epithelium (1), connective tissue of an ovarian lobule (2), univacuolar type of degenerating stromal gland cells (3) and extruded yolk (4). X 80

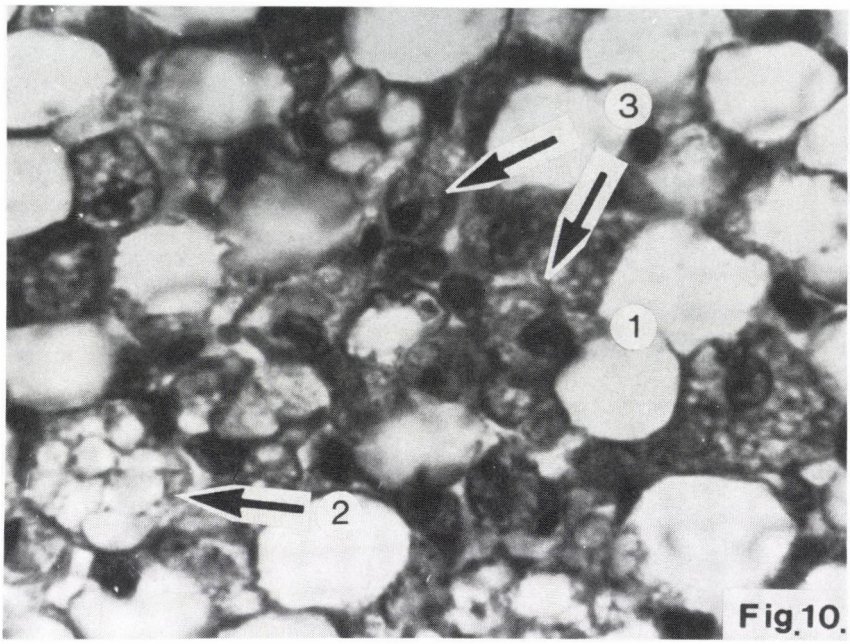

Fig. 10. Seventh type of atretic follicles. Types of lipoidal degeneration of stromal cells. Univacuolar lipoidal degeneration (1), plurivacuolar lipoidal degeneration (2) and active stromal cells (3). X 600

dark yellow with a conspicuously thick and sharp white stigma line. These AFs are only very rarely spherical in shape: as a rule, they are obovate or dilated. Their surface is sometimes convoluted, which, together with the other symptoms described, indicates shrinkage.

Type 6 AFs larger than 7 mm in diameter cannot be safely identified because their appearance is rather heterogeneous.

Type 7 atresia is the last stage of degeneration. In this type, active stromal cells can only be found sporadically. The overwhelming majority of cells are transformed into univacuolar or plurivacuolar lipid tissue with lipoidal degeneration. In many cases this process is not confined to the AF but extends to whole ovarian lobules (Figs 9, 10).

There is no typical, macroscopically visible indication of Type 7 atresia. Its appearance is heterogeneous.

It should be noted that in addition ot the atresia types listed above it has been observed that sometimes the follicular wall ruptures in its whole thickness and the yolk spills into the abdominal cavity. This occurs only with very large follicles (40 to 50 mm) and even then very rarely.

The above classification does not include the final stage of atresia, marked by the development of scarry connective tissue. It is this "atypical" structure that develops at the end of all degenerative processes. Most of it consists of concentrically arranged compact fibrous connective tissue. Its size decreases gradually, and then, simultaneously with its secondary vascularization its structure opens up and merges with the medullar stroma.

Steroid determinations

Hormone contents were measured in different types of atretic follicles during the spring reproductive cycle.

Among sexual steroids it is PROG that exhibits the most pronounced changes across the seven types of follicular degradation. In most types its value (200–400 pg/100 mg) does not show appreciable differences, except for Type 6, where this value is exceptionally high, over 2000 pg/100 mg.

The level of 17-β-oestradiol is at its highest in Type 6, too (279 pg/100 mg). The second highest concentration (203 pg/100 mg) can be found in Type 1, while E_2 contents are low in the other types and there are no appreciable differences among them.

Testosterone content is the highest in Type 3 (220 pg/100 mg) and the lowest in Type 2 and 7 (26 pg/100 mg and 25 mg/100 mg respectively) (Fig. 11).

Determinations of sexual steroids were made not only for different types of follicular degeneration, but also as a function of time. The experimental period between February 11 and April 28 was divided into three stages (February 11 to February 20 — peak activity, March 4 to March 18 — onset of decline in sexual activity, and April 6 to April 28 — the close of the spring cycle), and average hormone contents of atretic follicles within these three stages were determined. As the breeding season advances, progesterone content in degenerating follicles increases considerably. TEST content decreases, while E_2 content, after a temporary decline, shows an upward trend (Fig. 12).

With the hormone contents of developing and atretic follicles known, comparisons were made between steroid contents in developing and degenerating follicles. Since there was no sufficient number of large-sized

atretic follicles available, these comparisons involved follicles up to 18 mm in diameter only.

A comparison of hormone contents in follicles 1 to 2 mm in diameter showed that PROG content was almost six times higher in atretic follicles than in developing ones. In follicles 3 to 5 mm in diameter PROG content was 4 times, in follicles 6 to 8 mm it was 5 times higher in atretic follicles than in normal ones. Up to size 18 mm the ratio shows a downward trend, but even so PROG content in atretic follicles exceeds that in developing ones.

In some cases E_2 content is lower in atretic follicles than in normal ones (1–2 mm, 9–10 mm, 11–18 mm), and in two cases (3–5 mm and 6–8 mm) it exceeds the values for developing follicles.

The value of the TEST ratio is highest with size 1–2 mm follicles: in this class the atretic contain twice as much test as normal ones. Upwards of this size the value of the TEST ratio decreases up to size class 6–8 mm, while from here to size 18 mm it again increases (Fig. 13).

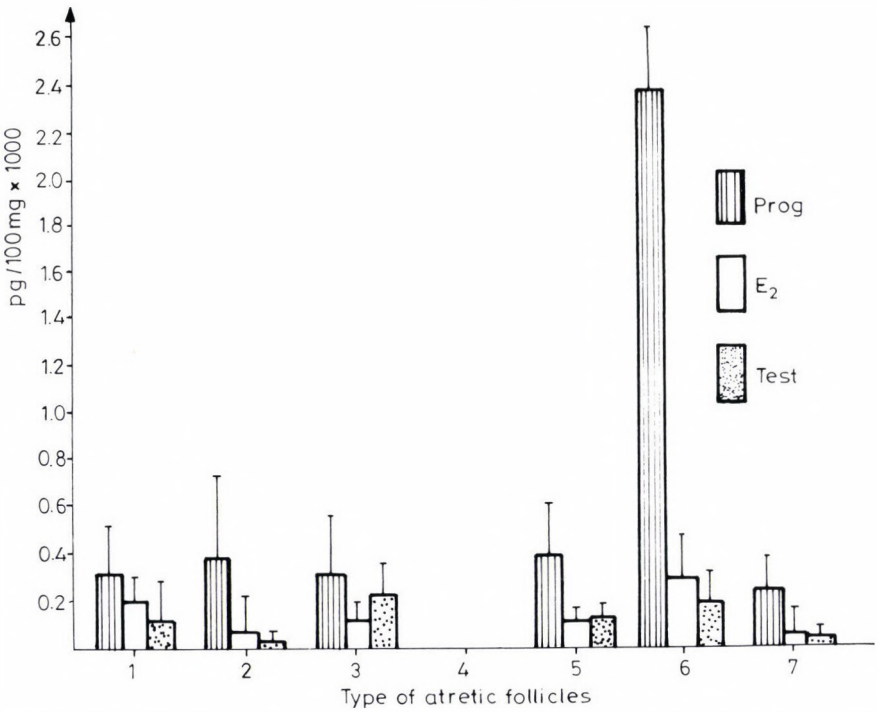

Fig. 11. Steroid content of different type of atretic follicles

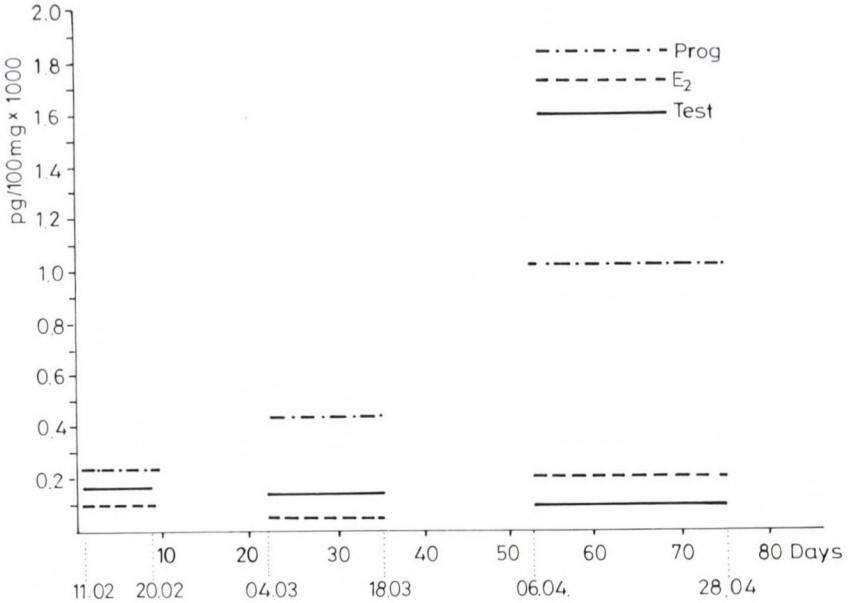

Fig. 12. Steroid content of the atretic follicles in the breeding season

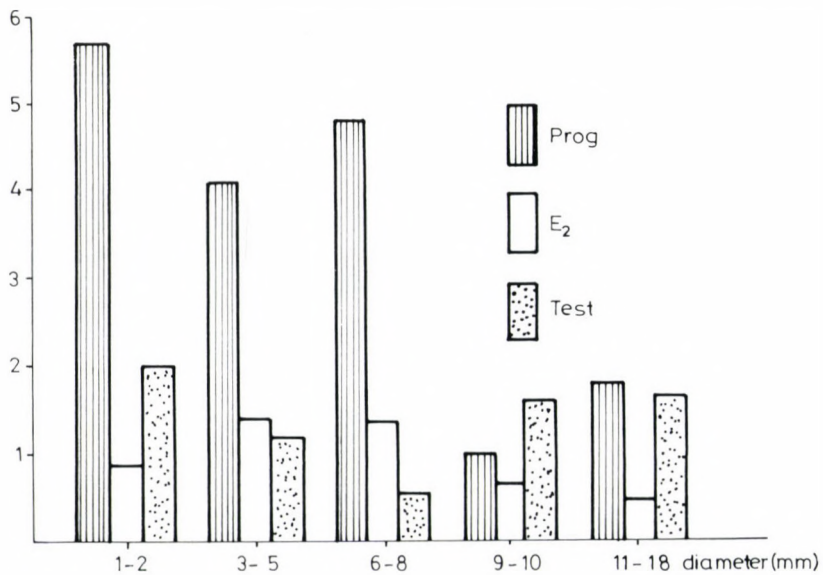

Fig. 13. Relationship between steroid content in the same diameter's atretic and developing follicles

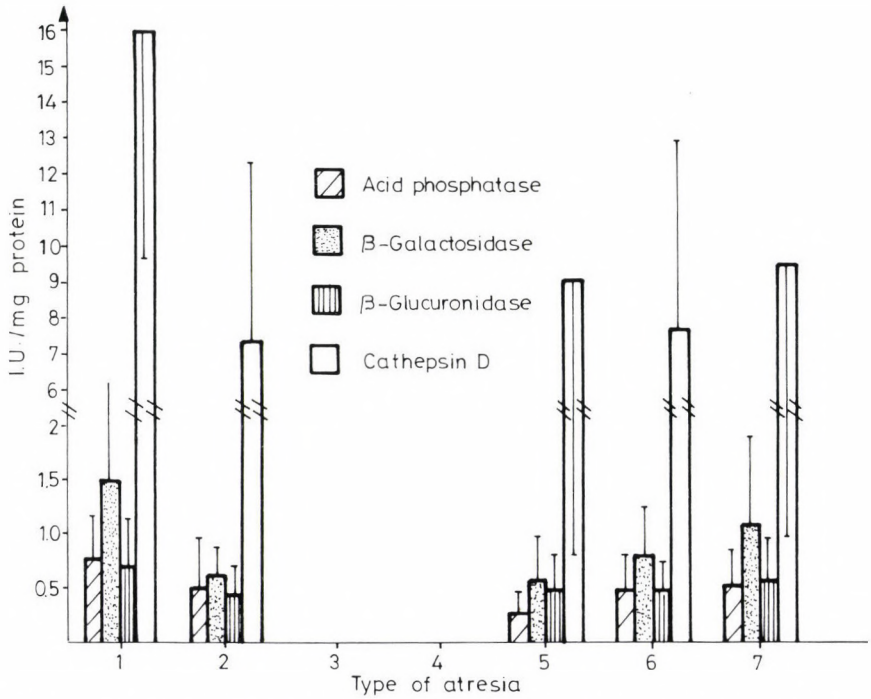

Fig. 14. Lysosomal enzyme activity in different types of atretic follicles

Results of enzyme activity measurements

As a first step, lysosomal enzyme activity in developing follicles from F_9 to F_1 was determined. The activity of all the four enzymes (acid phosphatase, β-galactosidase, β-glucuronidase and catepsin D) gave a curve with the same slope that could be divided into discrete sections. From F_9 to F_7 enzyme activity was uniformly low, from F_7 to F_4 it was on the increase, with a definite peak at F_4.

In the largest follicles, from F_3 to F_1, activity was low again, and the enzyme activity values obtained here were virtually identical with those for small follicles.

Lysosomal enzyme activity in degenerating follicles was studied in two approaches. In the first one, investigation was based on light microscopic histological appearance in each type of degeneration. The results showed that there was no significant difference in enzyme activity between the histological types (Fig. 14).

In the second approach AFs were grouped according to the size categories of developing follicles, and because sample size was small, in this

Table 1

Relationship between lysosomal enzyme activity in the same diameter's atretic and developing follicles

Diameter	Acid phosphatase I.U./mg/protein			β-galactosidase I.U./mg protein			β-glucuronidase I.U./mg protein			Cathepsin D I.U./mg protein		
	AF	F	AF/F	AF	F	AF/F	AF	F	AF/F	AF	F	AF/F
	n=9	n=4		n=9	n=4		n=9	n=3		n=9	n=4	
(F_9)	0.5	0.08	6.2	0.78	0.13	5.2	0.42	0.106	3.9	7.2	2.48	2.9
3–5 mm	±0.4	±0.05		±0.71	±0.08		±0.3	±0.04		±5.6	±0.88	
	n=4	n=3		n=4	n=3		n=4	n=4		n=4	n=3	
(F_8)	0.4	0.13	3.1	0.63	0.3	2.1	0.34	0.14	2.4	5.1	2.63	1.9
6–8 mm	±0.2	±0.02		±0.52	±0.1		±0.14	±0.04		±1.96	±0.45	
	n=1	n=7		n=1	n=7		n=1	n=7		n=1	n=7	
(F_7)	0.74	0.14		1.6	0.26		0.88	0.145		18.2	2.39	
9–10 mm		±0.05			±0.1			±0.07			±0.4	
	n=6	n=2		n=6	n=2		n=6	n=2		n=6	n=2	
(F_6)	0.67	0.33	2.0	1.31	0.75	1.7	0.61	0.43	1.4	10.6	5.42	1.9
11–18 mm	±0.3	±0.23		±0.7	±0.63		±0.4	±0.38		±6.6	±4.27	
	n=1	n=2		n=1	n=2		n=6	n=2		n=1	n=2	
(F_5)	0.45	0.4		0.78	0.78		0.61	0.45	1.4	7.6	5.83	
19–24 mm		±0.02			±0.11		±0.4	±0.11			±2.58	

case again enzyme activity was only determined for atretic follicles with a diameter size of 3 to 18 mm (the size range corresponding to the categories F_9, F_8, F_7 and F_6).

In AFs of different sizes no significant differences were found in enzyme activity values, however, compared to the values for normal follicles those for atretic follicles were considerably higher, especially in the 3–5 mm category (Table 1).

It can be concluded that, on one hand, neither the size nor the structure of AFs influences lysosomal enzyme activity and there are no significant differences between individual enzyme activites, on the other hand, lysosomal enzyme concentrations are considerably higher for AFs than for developing follicles.

DISCUSSION

The processes of follicular maturation and egg development in birds are always accompanied by follicular atresia. In the continuously laying domestic hen the process of atresia can be observed all the year round, while in seasonally breeding birds only in the reproductive cycle.

Extensive investigations of follicular atresia provide ample proof that atresia is not just a device that serves to eliminate egg overproduction (which ensures maintenance of the species), but has a much more complex role.

This is indicated by the high degree of diversity and versatility of the cells constituting the follicles, which enables them to shift their function in response to hormonal influences during the breeding season.

Such functional changes occur also physiologically. Thus, at a given stage of follicular maturation the connective tissue cells of the theca interna are transformed into the glandular type and, presumably, carry on steroid synthesis (Dahl /6/). This transformation can be induced artificially in the theca interna cells as done by Dahl /7/ by treating the domestic fowl with clomiphene citrate or gonadotropin. Our investigations of follicular atresia showed that transformations of this type are common.

Shifts towards the glandular cell type explain the changes in follicular hormone contents. The glandular character and the rich vascular system that develops in atretic follicles suggested that these elements secrete steroids, but there are conflicting views about the kinds of steroids produced and the exact site of their origin even in normal developing follicles.

According to Chieffi and Botte /5/, glandular theca interna cells in the domestic hen contain a considerable degree of 3-β-HSDH activity, which provides conclusive evidence for the presence of PROG synthesis. Mori et al. /21/ drew attention to the metabolizing ability rather than the synthesizing capacity of the theca interna cells, whereby PROG is transformed through several stages into androstenedione and testosterone. This is supported by Asem's /2/ measurements, with the difference that he was able to show the presence of oestrogen as well as androstenedione during the process of metabolisation. As regards the main site of PROG synthesis taking place in the granulosa cells the literature is unanimous /1, 20, 22, 26/. Androgens are produced in the interstitial cells which are in the ovarian stroma and have an epithelial or stromal origin /8, 15/.

In atretic follicles cholesterol-rich lipids were found in different type of cells by Woods and Domm /27/, Sayler et al. /23/ and Marshall and Coombs /19/, which indicates the presence of steroidogenetic enzymes in granulosa and in thecal elements.

The hormonal values measured in our investigations for the various types of atretic follicles were insufficient to ascertain the hormone synthesizing activity in the various cell types, except for Type 6 and Type 3 atretic follicles. The glandular cells that develop during atresia (theca interna cells and stromal glandular cells) probably possess a high degree of flexibility and are capable of synthetizing nearly all the steroids. It is important to note here that the name "thecal glandular cell" does not seem to be precise, because lipoglandular cells can be found in both thecal layers at a certain stage of follicular development as indicated by Kern /18/, too.

The exceptionally high PROG level of AFs in Type 6 shows conclusively that proliferating stromal glandular cells are carrying on this hormone production. Their function is as yet unclear, but in contrast to our results, Taber /24/ and Woods and Domm /27/ claim that they are responsible for androgen production.

Our observations show that the incidence of stromal glandular cells in degenerating follicles is more dominant in the final part of the reproductive cycle signifying their role in the evolution of seasonal cycles. The increase of their quantity might be the reason of elevation in PROG content in AFs towards the end of the reproductive cycle.

In discussing their origin, we can refer to the work of Erpino /10/. He stated that stromal glandular cells were derived from the glandular cells

of the theca interna and connective tissue elements from the ovarian stroma.

In atretic follicles, type 3, the hyperplasia of glandular cells in the theca externa can be interferred with the increased concentrations of testosterone. This type of atresia was only found in small white follicles 5 mm in diameter, consistent with our previous data /11/ which indicated that the glandular cells of the theca externa can only be found up to 6 to 8 mm in diameter even in developing follicles.

Comparing the hormone values for AFs and normal follicles of equivalent sizes it was found that hormone production in degenerating small follicles was frequently many times over that in white and yolky follicles.

This is also reflected in the histological structure of the two kinds of follicles following different evolutionary pathways. Small white follicles are at the initial stage of development and are relatively inactive. In degenerating small white follicles, due to mechanisms as yet unknown, strong proliferation of lipoglandular and hormonally active cells begins, whose steroid production is definitely in excess of that found in developing follicles. During follicular atresia, even though proliferative processes are in progress, it is still the dgenerative processes that are dominant, which is accompanied by strong lysosomal enzyme activity.

Investigations of enzyme activity in normal follicles showed that this was at an equally low level both in the white and the largest yolky follicles.

This can be explained by the fact that enzyme activity values are relatively constant since most of the growth comes from yolk deposition, which is indifferent from the point of view of enzyme activity, while growth of the follicular wall is not appreciable. The values for enzyme activity show a considerable increase from F_6 to F_4, with a definite peak at F_4. This increase in enzyme activity may be related to two histological changes. In F_5 follicles the hyperplasia of the glandular theca interna cells-which was observed around the F_6 stage-begins to fall off, and at the F_4 size the cells show a significant regression, acquiring again a connective tissue character. On the other hand, rich capillarization in the theca interna supporting a more intensive functioning of the granulosa becomes striking. Both processes involve cell degeneration, which may account for an increase in lysosomal activity.

In AFs, regardless of size and degeneration type, enzyme activity values are at an approximately constant level without any major differences.

This level is considerably higher than that measured for developing

follicles which strongly suggests that degenerative processes, decomposition and eventual recycling of substances previously incorporated in the egg cell are taking place.

Summing up our results, the most important conclusion that emerges is that on the basis of the light microscopic histological appearance two opposite processes are taking place in the atretic follicles.

One of the processes is, indeed, of a degenerative character, bringing about the elimination of the egg, the other process, however, taking place in the follicular wall is of a proliferative character. The number of cells in the follicular wall increases and rearrangement may take place with new cell types appearing. AFs are hormonally very active in this state, however, the process ends in scarry tissue or lipoidal degeneration.

In the hormonally active phase of follicular degeneration, especially in the last third of the reproductive cycle, high PROG concentration in the follicular tissues may escape into the blood, contributing to an increase in the level of progestagen produced by the adrenal, which has a key role in triggering of postnuptial moult. It could also be hypothesized that the increased PROG content of AFs acts as a lysosomal enzyme activator.

REFERENCES

1. Asem, E., Marrone, B.L., Hertelendy, F. (1985) Steroidogenesis in ovarian cells of the Japanese quail (Coturnix coturnix japonica). Gen. Comp. Endocrinol. 60, 353–360.
2. Asem, E.K., Hertelendy, F. (1986) Role of calcium in luteinizing hormone-induced progesterone and cyclic AMP production in granulosa cells of the hen (Gallus domesticus). Gen. Comp. Endocrinol. 62, 120–128.
3. Barrett, A.J. (1972) Lysosomal enzymes. In Dingle, J.T. (ed.) Lysosomes. A Laboratory Handbook. Elsevier, New York, pp. 46–135.
4. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt. Biochem. 72, 248–254.
5. Chieffi, G., Botte, V. (1965) The distribution of some enzymes involved in the steroidogenesis of hen's ovary. Experientia 21, 16–17.
6. Dahl, E. (1970) Studies of the fine structure of ovarian interstitial tissue. 2. The ultrastructure of the thecal gland of the domestic fowl, Z. Zellforsch. 109, 195–211.
7. Dahl, E. (1970) Studies of the fine structure of ovarian interstitial tissue 6. Effects of Clomiphene on the thecal gland of the domestic fowl. Z. Zellforsch. 109, 227–244.

8. Dahl, E. (1972) Studies of the fine structure of ovarian interstitial tissue. VII. The postnatal evolution of the gland in the domestic fowl. Z. Zellforsch. 135, 553–561.

9. Erpino, M.J. (1969) Seasonal cycle of reproductive physiology in the black-billed magpie. Condor 71 , 267–279.

10. Erpino, M.J. (1973) Histogenesis of atretic ovarian follicles in a seasonally breeding bird. J. Morphol. 139, 239–250.

11. Forgó, V., Afanasiev, G., Péczely, P. (1988) Structural and hormonal changes during follicular maturation in the ovary of the domestic goose. Acta Physiol. Hung. 39, (In press)

12. Gilbert, A.B., Perry, M.M., Waddington, D., Hardie, M.A. (1983) Role of atresia in establishing the follicular hierarchy in the ovary of the domestic hen (Gallus domesticus). J. Reprod. Fert. 69, 221–227.

13. Gilbert, A.B., Perry, M.M., Walker, M.A., Waddington, D. (1985) Identification of atresia in small ovarian follicles of the domestic hen (Gallus domesticus). IRCS Med. Sci. 13, 732–733.

14. Gupta, S.K., Maiti, B.R. (1986) Study of atresia in the ovary during the annual reproductive cycle and nesting cycle of the pied myna. J. Morphol. 190, 285–296.

15. Guraya, S.S. (1976) Morphological and histochemical observations on follicular atresia and interstitial gland tissue in the columbid ovary. Gen. Comp. Endocrinol. 30, 534–538.

16. Győrváry, I., Péczely, P. (1983) Vizsgálatok tojó és kotló pulyka petefészkének szerkezetéről és működéséről. (Investigations on the structure and function of the ovary of egg-laying and breeding turkey). (In Hungarian). MÁL 38, 414–419.

17. Jones, R.E. (1971) The incubation patch of birds. Biol. Rev. 46, 315–339.

18. Kern, M.D. (1972) Seasonal changes in the reproductive system of the female white-crowned sparrow, Zonotrichia leucophrys gambelii, in captivity and in the field. Z. Zellforsch. 126, 297–319.

19. Marshall, A.J., Coombs, C.J.F. (1957) The interaction of environmental, internal and behavioural factors in the rook, Corvus f. frugilegus Linnaeus Proc. Zool. Soc. Lond. 128, 545–589.

20. Mori, M., Kohmoto, K., Shoda, Y. (1984) Role of granulosa and theca cells on in vitro progesterone production in preovulatory follicles of the Japanese quail. Japan. Poult. Sci. 21, 206–214.

21. Mori, M., Aoki, F., Kohmoto, K., Shoda, Y. (1985) Metabolism of steroid hormones in vitro by follicular tissues of Japanese quail. Biol. Reprod. 33, 11–20.

22. Phillips, A., Scanes, C.G., Hahn, D.W. (1985) Effect of androgens and gonadotropins on progesterone secretion of chicken granulosa cells. Comp. Biochem. Physiol. 81 A, 847–852.

23. Sayler, A., Dowd, A.J., Wolfson, A. (1970) Influence of photoperiod on the localization of 5-3-hydroxysteroid dehydrogenase in the ovaries of maturing Japanese quail. Gen. Comp. Endocrinol. 15, 20–30.

24. Taber, E. (1951) Androgen secretion in the fowl. Endocrinology 48, 6–16.

25. van Tienhoven, A. (1961) Reproductive endocrinology in birds. In: Young, W.C. (ed.) Sex and Internal Secretions. Vol. 2. Williams and Wilkins, Baltimore, pp. 1088–1169.

26. Wells, J.W., Walker, M.A., Culbert, I., Gilbert, A.B. (1985) Comparison of the response in vitro to luteinizing hormone and follicle stimulating hormone of the granulosa of six follicles from the ovarian hierarchy in the chicken (Gallus domesticus). Gen. Comp. Endocrinol. 59, 369–374.

27. Woods, J.E., Domm, L.V. (1966) A histochemical identifications of the androgen-producing cells in the gonads of the domestic fowl and albino rat. Gen. Comp. Endocrinol. 7, 559–570.

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STRUCTURAL AND HORMONAL CHANGES DURING FOLLICULAR MATURATION IN THE OVARY OF THE DOMESTIC GOOSE

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Follicle maturation in the ovary of sexually mature domestic geese in the spring reproductive cycle was investigated by histological methods and steroid-RIA. The single-layer granulosa of primary follicles temporarily transformed in the growing white follicles into several layers or a simple membrana granulosa with nuclei at several different levels in the cell. In the yolky follicles the granulosa represents a cuboidal epithelium (F_4–F_3) and subsequently a high cylindrical epithelium (F_1). The originally connective tissue-like cells of the theca interna show a glandular proliferation in the largest white (F_7) and the small yolky follicles (F_6–F_5). Glandular cell nests in the theca externa are typical in the generation of small white follicles and are absent in the wall of yolky follicles.

Progesterone-content in the follicular wall (granulosa+theca) is the highest in the F_1–F_2 and F_6–F_5 types and is low in small white follicles (F_8, F_9 and F_{10}). E_2 concentration shows only slight variations between F_1–F_{10}. TEST content shows a slight increase between F_1 and F_3 and is high in medium-sized white follicles (F_8–F_9). The results suggest that in addition to the granulosa, the theca interna is also capable of an intensive progesterone synthesis.

Keywords: domestic goose – ovarian follicles – morphogenesis – light microscopy – sexual steroids

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INTRODUCTION

Birds, with the exception of a few species, are unique in that their ovary develops only on the left side.

In the ovary of sexually mature birds a hierarchy of large yolky and small white follicles can be found. The largest yolky follicle (F_1) is nearing ovulation, while the next one will usually rupture in 24 h time.

The number of follicles which mature is species specific: in Gallinaceae which have high rate of lay, it is usually 6 to 12, while in pigeons and birds of prey which lay only few eggs it is 2 to 4.

Most studies of the structure of the avian ovary and follicles have been made in domestic hen (see /4, 5, 11, 12/ for reviews). Relatively few studies have been made in domestic turkey /6/ and, apparently, no detailed studies have been made to date of the anatomical and histological structure of the ovary in water fowls (ducks and geese).

Investigations of the light and electron microscopic structure of the avian ovary have yielded a static description of endocrine cell types and the follicular wall. There are few data on the morphogenetic changes taking place in the course of follicular development. Besides morphological investigations, a number of workers have reported on in vitro hormone production in the follicular wall and the isolated granulosa and thecal cells, respectively /7, 9, 13, 17/, as well as on the hormone production of individual follicles, studied on the basis of the hormone-content of effluent venous blood /3/.

There is relatively little known about the actual hormone content of the follicular wall because the data obtained to date reflect, almost exclusively, the values for the first 4 to 5 (yolky) follicles /19/. Hormone production in the walls of white follicles remains to be explored, and no detailed studies of the relations between histological structure and steroid content have been made.

The present study was undertaken to examine the light microscopic structure of the ovary in domestic goose during the ovulation cycle and to compare the morphogenesis of the ovary with changes in progesterone, 17-β-oestradiol and testosterone concentration as measured in the follicular wall by radioimmunoassay (RIA).

MATERIALS AND METHODS

Two-year-old laying geese of the Landes breed with an average body weight of 5 kg were used in this study. The birds were housed in sheds with runs and maintained under a conventional system of management. They were fed ad libitum a maintenance ration with a protein content of 11%.

In the spring reproductive cycle, from February 11 to April 28, 15 birds were killed. The follicles in the ovary were removed and each follicle was cut into halves. Based on their diameter, the follicles were divided into 10 groups, from F_1 to F_{10}. The largest preovulatory follicles were classified as F_1 with the smallest white follicles designated as F_{10}.

One half of each follicle was fixed in Bouin's fluid and embedded according to Péterffy's double method. Five µm thick sections were made with a Reichert microtome and were subsequently stained with haematoxilin-eosine. The microphotos were made with a Reichert "Zetopan" photomicroscope.

The other half of each follicle was used for determination of sexual steroids by RIA. After removal of the yolk and addition of physiological saline the follicular walls were homogenized at 0 to 4 °C in a teflon-glass homogenizer. From the suspension progesterone (PROG) was first extracted with petroleum ether, while testosterone (TEST) and 17-β-oestradiol (E_2) were subsequently extracted with diethyl ether. The tritium-labelled samples were measured using a LKB-Wallac scintillation spectrometer. The results obtained in pg refer to 100 mg wet weight.

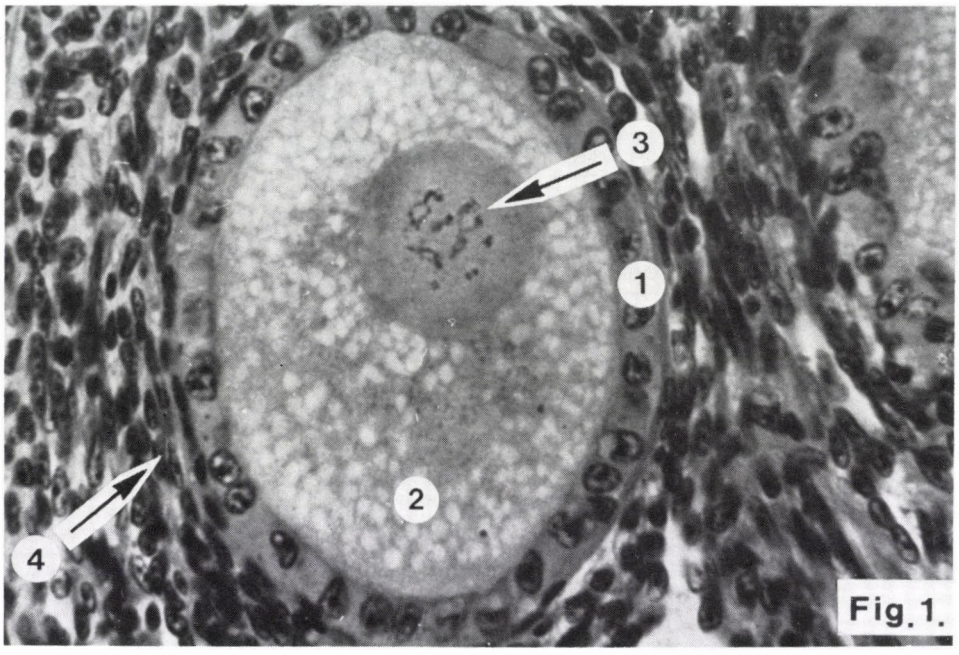
Student's t-test was used for statistical evaluation.

RESULTS

As the development of follicles progresses from microscopic primary follicles to large preovulatory ones, their structure changes.

The structure of primary follicles is very simple. The nucleus of the egg is embedded in a small amount of yolk and is surrounded by a single layer of granulosa cells in the stroma of cortical tissue. These follicles do not contain a theca layer. The primordial theca is represented by a single layer of fibroblasts surrounding the basal lamina. The primary follicles are 50–100 µm in diameter (Fig. 1).

The smallest white follicles (500–1000 µm) are characterized by a thin layer of theca interna surrounding the granulosa cells and by the presence in the theca externa of so-called nest-like or glandular cells, arranged in the form of a nest. Their most important feature is that the cells within a nest are closely connected and their nuclei are round or oval in shape and contain, as a rule, a prominent nucleolus. Their cytoplasm is light in colour, often exhibiting a foam-like structure and containing a number of empty vacuoles from which the lipid had apparently been extracted during the preparation procedure. In this way, these cells acquire a



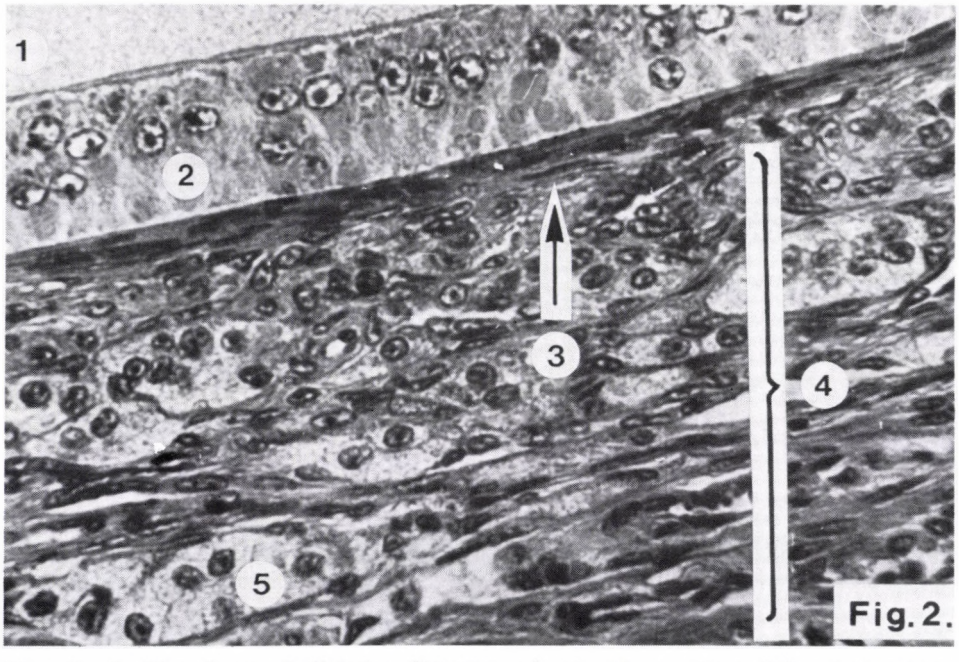


Fig. 1. Small primary follicles (50–100 μm). In the nucleus (3) embedded in a small amount of yolk (2) chromosomes in the configuration of lamp brush are clearly seen. The granulosa is single-layered. The stroma (4) contains fibroblast-like cells. X 630

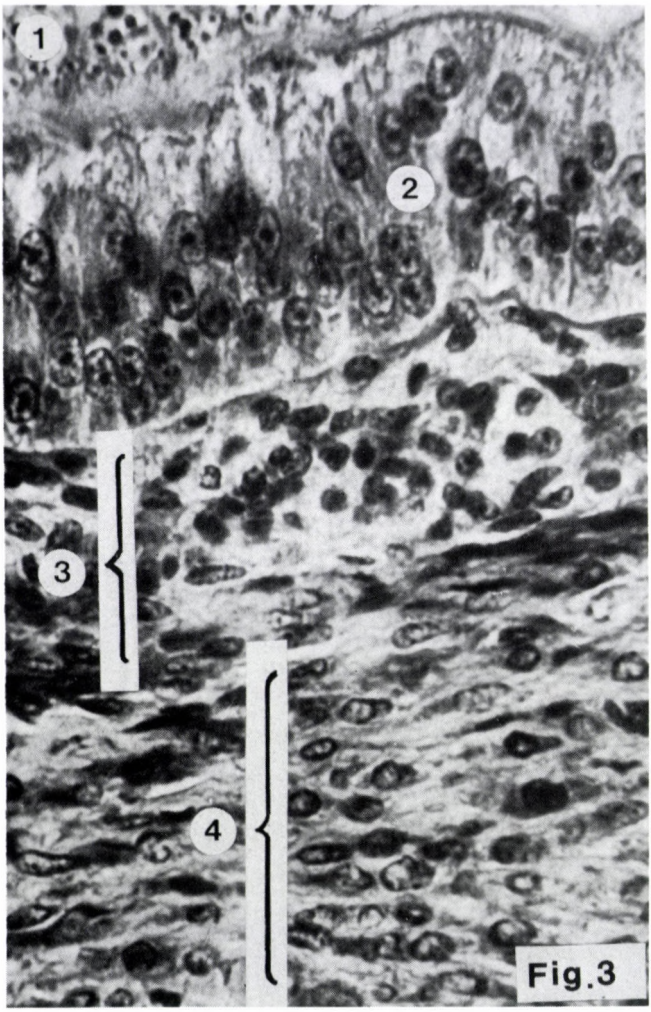


Fig. 3. Large white follicle (8mm, F_8). The granulosa (2) confining the yolk (1) is multi-layered with the nuclei at different levels; the theca interna (3) is of the glandular type and hyperplastic; the glandular cells of the theca externa (4) are not visible. X 400

←

Fig. 2. Small white follicle (500–1000 µm). In the granulosa (2) surrounding the yolk (1) are the nuclei at different levels, the theca interna (3) has connective-tissue character; the theca externa (4) has grown thicker and contains many nest-like glandular cells (5). X 400

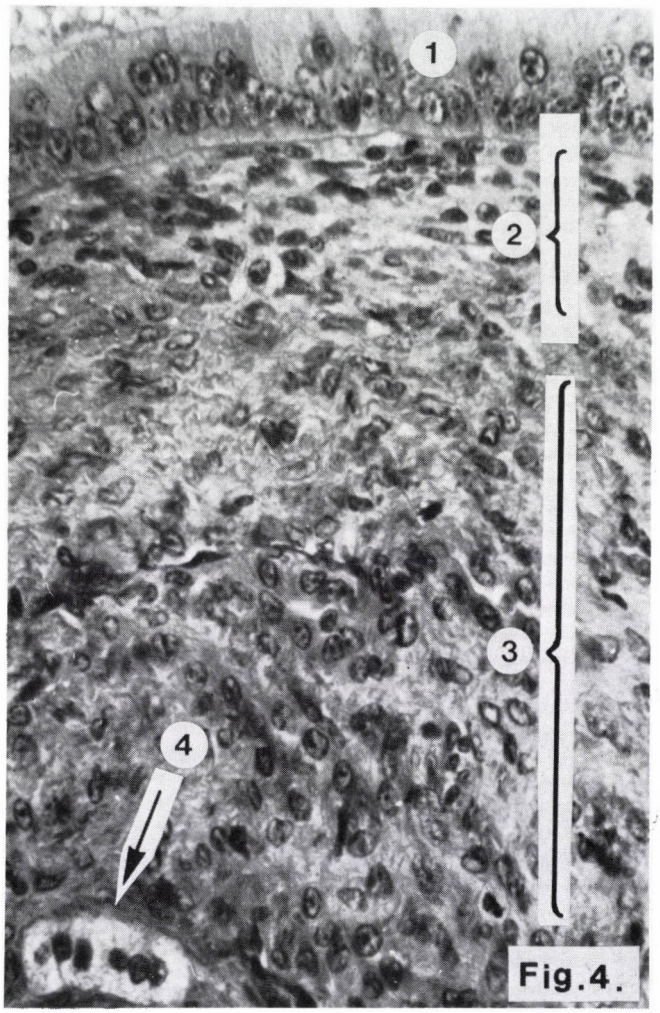


Fig. 4. Large white follicle (10 mm, F_7). The nuclei in the granulosa (1) are at different levels; the glandular character of the theca interna (2) is on the decline; there are only a few scattered glandular nest-like cells (4) in the theca externa (3). X 400

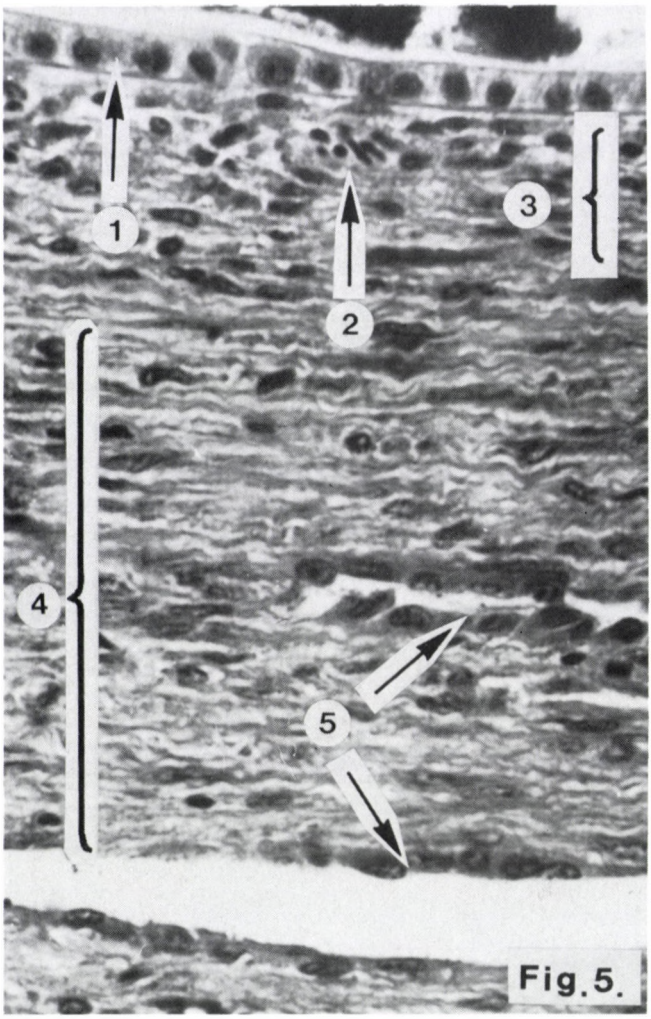


Fig. 5. Developing yolky follicle (23 mm, F_5). The granulosa (1) has the character of cuboidal epithelium, the theca interna (3) is acquiring connective-tissue character with capillaries (2) developing in it. The theca externa (4) is thick, containing dilated veins (5). X 400

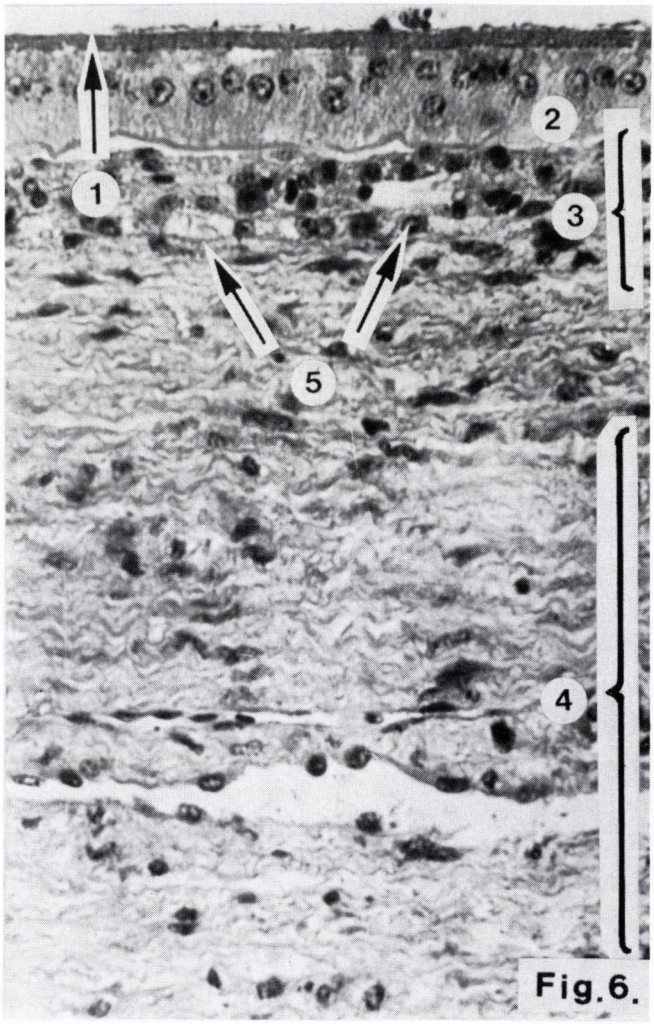


Fig. 6. Preovulatory follicle (52 mm, F_1). The zona radiata is thickened (1), the granulosa (2) is a high columnal epithelium, the nuclei are situated apically. The theca interna (3) is richly vascularised (5). X 250

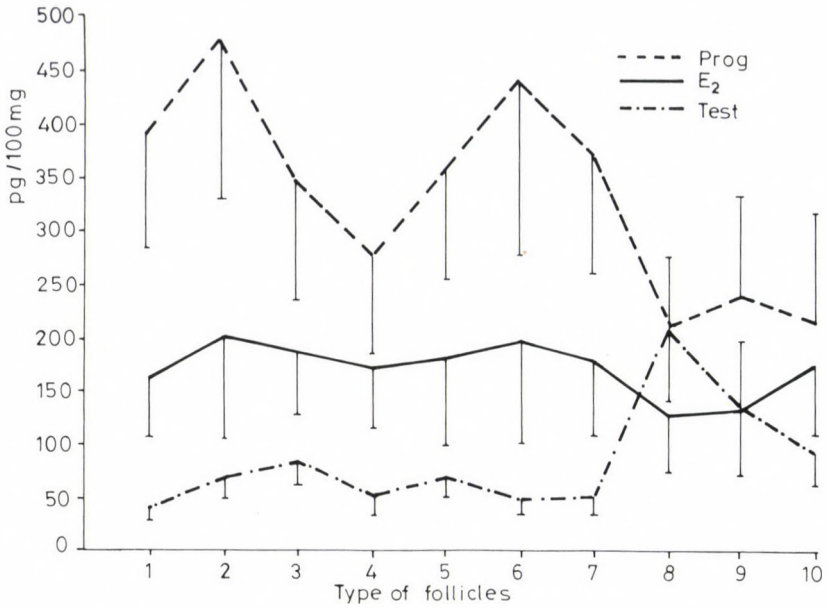


Fig. 7. Steroid levels in different types of developing follicles (F_{10}–F_1)

distinctive structure and can be readily distinguished from the stroma (Fig. 2).

Also groups of glandular cells are often found among the follicles.

During development the small white follicles gradually lose their nest-like cells. The amount of collagen and elastic fibres increases in the follicular wall, and a number of fibroblasts appear. A thickening of the follicular wall is brought about primarily by the growth of the theca externa. A dense network of veins evolves in this layer. In the theca interna cells whose nuclei are rounder and more intensely stained than those of the firboblasts become dominant. The initially simple membrana granulosa shows intensive proliferation and develops into a simple membrana granulosa with nuclei at several different levels in the cells. This structure is typical of small white follicles measuring 1–2 mm (F_{10}) and 3–5 mm (F_9) in diameter, and of medium-sized white follicles with a diameter of 6–8 mm (F_8) (Fig. 3).

The largest white follicles are 9–10 mm in diameter (F_7). These follicles also contain a simple membrana granulosa with nuclei at several different levels in the cells, with glandular cell nests sporadically occurring in the theca externa.

A macroscopically visible change of colour occurs in follicles with a diameter of 11–18 mm (F_6). This is the first generation of yolky follicles (small yolky follicles).

A distinctive feature of the wall of such follicles is a swelling of the theca interna cells, whereby they acquire a glandular character (light-coloured granulated cytoplasm). Sporadically, a nest-like cell with a foamy cytoplasm can still be found in the theca externa (Fig. 4).

In some of the follicles the granulosa becomes single-layered with nuclei in a single row again. A distinctive feature is the appearance of narrow elements with basophylic plasma (regenerative, reserve type) among the cells of the columnar epithelium.

In the medium-sized yolky follicles having diameters of 19–24 mm (F_5) the hyperplastic character of the theca interna is reduced and dilated capillaries filled with blood cells appear among the cells. The granulosa is a typical cubical epithelium.

In the larger yolky follicles with diameters of 25–33 mm (F_4) and 34–44 mm (F_3) the thickness of the theca is reduced: the cells of the theca interna become morphologically similar to typical interstitial cells, containing only a small number of swollen cells. Capillarization of the layer is enhanced, the height of the follicular epithelium is increasing, and the zona radiata represents a heavily stained thick band (Fig. 5).

In the largest yolky follicles with diameters of 45–49 mm (F_2) and 50–53 mm (F_1) the theca interna becomes thin and its cells are hardly distinguishable from the fibroblasts of the underlying layer. The layer is intensively capillarized, and the granulosa is a high columnar epihtelium whose cell nuclei are found near the apical pole. The cytoplasm exhibits a thick basophylic granulation. The zona radiata is thick and easily identifiable (Fig. 6).

Measurments of the hormone content made simultaneously with the histological investigations showed that follicular size and hormone content were significantly correlated (Fig. 7).

The most conspicuous changes can be observed in PROG concentrations. The highest concentrations occur in the large yolky (F_1 and F_2) and the smallest yolky (F_6) follicles. The differences were significant between the F_4 and F_2 ($p < 0.01$), between the F_6 and F_4 ($p < 0.01$), between the F_8 and F_6 ($p < 0.01$) but they were not significant between the F_2 and F_1 follicles. PROG concentration is lower in the medium-sized yolky follicles (F_3, F_4 and F_5) and is very low in the small white follicles (F_8, F_9 and F_{10}).

E_2 content does not vary significantly in the various types of follicles, but is relatively lower in the small white folicles (F_8, F_9).

TEST levels are low in the yolky and large white follicles, but are high in the population of small white ones which is significant in the relation of F_8 and F_7 type of follicles ($p < 0.01$).

DISCUSSION

Most descriptive studies of the histology of the avian ovary contain only hints about follicular morphogenesis and provide only static descriptions of the wall of preovulatory follicles.

Data on the morphogenesis and the different layers of the follicular wall are scarce and conflicting. Thus, according to Romanoff and Romanoff /18/, Wyburn et al. /22/ and Gilbert /4/ primary follicles contain a multilayered granulosa during development which, as the oocyte grows, turns into a single layer. On the other hand, Guzsal /5/ states that "the avian granulosa is typically single-layered".

Our observations show that the granulosa becomes multilayered or single-layered with nuclei at several different levels only at a definite stage of the growth of white follicles, between F_{10} and F_7. In the preceding stage of development, just as in the primary follicles, the granulosa is definitely single-layered and the nuclei are positioned uniformly in a single row in geese.

In the population of yolky follicles, where intensive filling increases the rate of growth dramatically, the follicular wall expands and the granulosa "irons out" into a single layer. This observation is in agreement with that of Kern /10/ who found typically single-layered granulosa in the follicular wall in Zonotrichia.

The changes taking place in the granulosa of large yolky follicles were found to be significant by the present authors. In F_4 and F_3 follicles the single-layered granulosa is rather like a cuboidal epithelium and the nuclei are located near the base of the cell, while in the F_2 and F_1 generation the epithelial cells become substantially higher and the nuclei move nearer the apical surface. This process suggests enhanced activity in this cell layer, a fact which is supported by the growth of the basophylic granulation of the cytoplasm (endoplasmic reticulum). The difference between the cells of the theca interna and theca externa is shown by Gilber /4/ however,

he does not suggest that there is a difference between the distinctive changes in these two kinds of endocrine cell populations during follicular evolution. Our data show that the theca interna cells are transformed in the largest white follicles and the small yolky follicles sofar as their dilated fibroblast-like elements turn into rounded, glandular-type cells. Subsequently these cells form concentrically arranged layer around the ovocyte. This observation is in disagreement with Guzsal /5/, who found in hens that the cells of the theca interna are situated in scattered groups. Dahl /2/ observed the glandular character of the theca interna in the ovary of the domestic hen.

In the large yolky follicles the theca interna gradually loses its glandular character during follicular maturation and while it becomes markedly capillarized, its cells assume again an indifferent connective tissue cell character. These changes suggest that the theca interna exhibits an active steroid production only at a definite stage of follicular development.

Gilbert /4/ states that the theca externa contains granulated cells, that are different from the inner layer. According to Kern /10/, such cells can also be found in medium-sized developing follicles. Our observations show that in goose the vacuolized, glandular cells with granulated plasma in the theca externa are typical only of the smallest white follicles and their number decreases during follicular maturation, while in small yolky follicles they occur only occasionally.

According to hormone determination of the follicular wall (granulosa +theca) PROG and TEST contents change substantially during follicular development, while 17-β-oestradiol content changes only slightly. The highest PROG concentration was measured in the largest and in the smallest populations of yolky follicles. PROG concentration is high in the walls of F_2 and F_1 follicles, where the theca interna is of an indifferent character and the granulosa represents a high columnar epithelium. PROG concentration is also high in F_7, F_6 and F_5 follicles, where the theca interna shows a glandular transformation and the hyperplastic granulosa develops into a multilayered epithelium. It is supposed that in small yolky follicles it is the theca interna which is the site of PROG synthesis, while in the largest yolky follicles, as suggested by the literature, it is primarily the granulosa where substantial amounts of PROG are produced. PROG concentration is highest in F_1 and F_2 follicles and decreases gradually in the F_4,

F_5 and F_6 follicles in agreement with the findings of Asem et al. /1/, Wells et al. /21/, Mori et al. /13/.

Our measurements show that the walls of F_2 follicles contain at least as much or slightly more PROG as those of F_1 follicles. Wells et al., 1985 and Mori et al., 1985 based on investigations of isolated granulosa, reached the same conclusion. A comparison of the data would thus suggests that most of the PROG measured in the follicular wall is synthetized in the granulosa.

Few studies have covered PROG content in the smaller yolky follicles and the granulosa in these follicles. According to Wells et al. /21/ and Etches et al. /3/ PROG production and content in the smaller follicles is lower than in F_1 and F_2 follicles. In contrast, our data show that F_5, F_6 and F_7 yolky follicles contain almost the same concentrations of PROG as the largest yolky follicles. This observation supports our earlier results /15, 16/ which suggested that there were two PROG peaks during sexual maturation in the avian peripheral blood: one at the time of emergence of small yolky follicles, and the other at a later stage, in the periovulatory phase.

According to our measurements, 17-β-oestradiol concentration in the follicular wall does not show as significant changes as PROG concentration. In this respect our results agree with those of Etches et al. /3/, who found that E_2 production in the large yolky follicles (F_1–F_4) shows an upward tendency only in the case of F_4. However, a comparison of E_2 levels measured in the isolated theca suggests that in F_2 and especially in F_3 follicles E_2 concentration is considerably higher than in preovulatory follicles /8, 9/. At present we can offer no explanation for the discrepancy between our results and previous results reported in the literature.

As far as TEST concentration is concerned, our data suggest that a significant rise in the steroid level is observed in the white follicles when glandular transformation starts in the theca interna. Etches et al. /3/ found a linear increase in TEST production from F_1 to F_4 12 h before ovulation in domestic hen. (Only the F_4 to F_1 follicles were studied.) This is in agreement with our observations, which show a discrete increase from F_1 to F_3.

In the wall of F_7, F_8 and F_9 white follicles TEST and E_2 concentrations change in opposite directions: a marked increase in TEST is accompanied by a decrease in E_2. This phenomenon suggests the inhibitory effect of aromatase which takes part in oestrogen synthesis and a subsequent built-up in the "androgen substrate".

REFERENCES

1. Asem, E., Marrone, B.L., Hertelendy, F. (1985) Steroidogenesis in ovarian cells of the Japanese quail (Coturnix coturnix japonica). Gen. Comp. Endocrin. 60, 353–360.

2. Dahl, E. (1970) Studies of the fine structure of ovarian interstitial tissue 2. The ultrastructure of the thecal gland of the domestic fowl. Z. Zellforsch. 109, 195–211.

3. Etches, R.J., Croze, F., Duke, C.E. (1980) Plasma concentrations of luteinizing hormone, testosterone and estradiol in follicular and peripheral venous plasma during the ovulation cycle of the hen. In Pethes, G., Péczely, P., Rudas, R. (eds) Recent Advances of Avian Endocrinology. Pergamon Press–Akadémiai Kiadó, pp. 89–97.

4. Gilbert, A.B. (1971) The Ovary. In Bell, D.J., Freeman, B.M. (eds). Physiology and biochemistry of the domestic fowl. Vol. 3. Acad. Press, New York, London, p. 1176.

5. Guzsal, E. (1981) Háziállatok szövettana. (Histology of domestic animals). Mezőgazdasági Könyvkiadó, Budapest (In Hungarian).

6. Győrvári, I., Péczely, P. (1983) Vizsgálatok tojó és kotló pulyka petefészkének szerkezetéről és működéséről. (Investigations on the structure and function of the ovary of egg-laying and breeding turkey). MÁL 38, 414–419. (In HUngarian).

7. Hertelendy, F., Burke, W.H., Zakar, T. (1981) Steroidogenic activity of turkey, ovine and human LH in avian granulosa cells. JRCS Med. Sci. 9, 327.

8. Johnson, P.A., Palmer, S.S., Bahr, J.M. (1985) Hormone-stimulable adenyl cyclase and steroid concentration of follicles of the pregnant mares serum gonadotropin-treated hen. Biol. Repro. 32, 828–833.

9. Johnson, P.A., Dickerman, R.W., Bahr, J.M. (1986) Decreased granulosa cell luteinizing hormone sensitivity and altered thecal estradiol concentration in the aged hen, Gallus domesticus. Biol. Repro. 35, 641–646.

10. Kern, M.D. (1972) Seasonal changes in the reproductive system of the female white-crowned sparrow, Zonotrichia leucophris gambelii, in captivity and in the field. Z. Zellforsch. 126, 297–319.

11. Lofts, B., Murton, R.K. (1973) Reproduction in birds. In Farner, D.S., King, J.R., Parkes, K.C. (eds). Avian Biology. Vol. 3. Acad. Press, New York, London.

12. Marshall, A.J. (1961) Biology and comparative physiology of birds. Marshall, A.J. (ed.). Vol. 2., Acad. Press, New York, London.

13. Mori, M., Kohmoto, K., Shoda, Y. (1985) Effects of cyclic AMP and protein synthesis inhibitors on in vitro progesterone synthesis by the granulosa cells of the quail (Coturnix coturnix japonica). Comp. Biochem. Physiol. 81B, 473–477.

14. Murton, R.K., Westwood, N.J. (1977) Avian Breeding Cycles. Clarendon Press, Oxford.

15. Péczely, P., Pethes, G. (1979) Alterations in plasma sexual steroid concentrations in the collared dove (Steroptopelia decaocto) furing the sexual maturation and reproductive cycle. Acta Physiol. Acad. Sci. Hung. 54, 161–170.

16. Péczely, P., Pethes, G. (1982) Seasonal cycle of gonadal, thyroid, and adrenocortical function in the rook (Corvus frugilegus). Acta Physiol. Acad. Sci. Hung. 59, 59–73.

17. Phillips, A., Scanes, C.G., Hahn, D.W. (1985) Effect of androgens and gonadotropins on progesterone secretion of chicken granulosa cells. Comp. Biochem. Physiol. 81A, 847–852.

18. Romanoff, A.L., Romanoff, A.J. (1949) The Avian Egg. John Wiley and Sons, Inc., New York.

19. Shàbabi, N.A., Norton, H.W., Nalbandov, A.V. (1975) Steroid levels in follicles and the plasma of hens during the ovulatory cycle. Endocrin. 96, 962–968.

20. Sturkie, P.D. (1976) Avian Physiology. Springer Verl. New York, Heidelberg, Berlin.

21. Wells, J.W., Walker, M.A., Culbert, J., Gilbert, A.B. (1985) Comparison of the response in vivo to luteinizing hormone and follicle stimulating hormone of the granulosa of six follicles from the ovarian hierarchy in the chicken (Gallus domesticus). Gen. Comp. Endocrin. 59, 369–374.

22. Wyburn, G.M., Aitken, R.N.C., Johnston, H.S. (1965) The ultrastructure of the zona radiata of the ovarian follicle of the domestic fowl. J. Anat. 99, 469–484.

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EXPERIMENTAL ALCOHOL BLASTOPATHY

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Experimental data are presented with respect to "experimental alcohol blastopathy" performed in our laboratory. As in our interpretation the notion of blastopathy involves both pathological changes during preimplantation development due to previous, preconceptional or preimplantation influences and later, pre- or postnatal effects induced by factors active during the preimplantation period, up to now the following experimental models were applied (on rats and mice): chronic and acute maternal, biparental or paternal ethanol alcoholization; preimplantation treatment with acetaldehyde or disulfiram followed by ethanol administration; acute ethanol intoxication before implantation on the background of chronic maternal ethanol intake; chronic maternal intake of various beverages. The main components of experimental alcohol blastopathy detected (by using a complex control methodology) were: pathological changes during the preimplantation developmental stages (lower mean number of embryos/animal, retardation of development, lowered migration rate of the embryos from the oviduct to the uterus, higher number of pathological morphological features), delayed implantation, disturbances of the early postimplantation development, retarded late foetal and placental growth. The effect of ethanol may be direct (ethanol being detectable in the oviductal and uterine fluid after both acute and chronic alcoholization) or indirect, via changes of the maternal macro- or microenvironment. The increase of the maternal blood acetaldehyde level may contribute to the appearance of alcohol blastopathy. Chronic beer and wine intake and acute intoxication with cognac suggest — up to now — the enhancing effect of beverage congeners. The noxious effect of acute ethanol intoxication superposed to chronic alcoholization is more marked that the separate effect of the two kinds of treatment. The chronic ethanol intake of fertilizing males (in mice) leads, both in the case of

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treated or untreated females, to lowered fertilization efficiency, to retardation of development (not occurring in the experimental model with chronic alcoholization of females) and to an enhanced increase of the number of pathological features. The cytogenetic control of preimplantation embryos (after chronic, acute or combined treatment with ethanol) does not reveal significant chromosomal changes. A possible alcohol blastopathy in humans must be taken into account (i.e. a noxious effect during the very early period of pregnancy when it is ignored).

Keywords: Rats– mice– preimplantation period– ethanol– beverages

INTRODUCTION

Since the experimental approach to the prenatal noxious effect of ethanol has been reactualized /11, 20, 26/ the risk in humans signalized /25, 26/ and the first clinical case reports published /16, 18, 25/ an increasing number of experimental models, on various species clarified some main aspects of alcohol embryo- and foetopathy. It is not the scope of this contribution to discuss and to evaluate the experimental results mentioned. Below, an aspect of experimental alcohol embryopathy, developed in our laboratory is presented: the deleterious effect of ethanol during the first days of embryonic development, i.e. during the preimplantation period.

Theoretically, ethanol, its metabolites or various alcohol containing beverages may exert their noxious action during each period of embryonic or foetal development, that means also during preimplantation development (blastopathy*). Disturbances appearing during this period may be due to a preimplantation harmful action or even to a previous, chronic influence exerted upon the maternal (or/and paternal) organism. Taking into account the possibilities mentioned above, investigations concerning the prenatal noxious effect of ethanol have to involve also the earliest developmental period, the more, that several clinical and epidemiological facts /1/ suggest the role of this period (during of which the woman is not yet aware of her pregnancy) in the pathogenesis of alcohol embryopathy. It must be shortly mentioned that, with respect to the preimplantation effect of various noxae, there exists a dogmatic view, according to which disturbances during this period either kill the embryo or are completely counteracted

*The term blastopathy was coined by Gottschewski and Zimmermann /15/

("all or none" effect). An increasing number of observations are however infirming this opinion (for details see /24, 30/).

Since 1979, in this laboratory the possible existence of an experimental alcohol blastopathy has been systematically investigated /2, 4, 5, 6, 7, 8, 9, 10, 13, 14, 24, 25, 28, 29, 31, 32, 33/.

MATERIALS AND METHODS

Experiments were carried out on pregnant albino rats (Wistar) and albino mice (RAP), the day of positive vaginal smear being considered as day 1 of pregnancy. The following main experimental models were applied:

— chronic ethanol intake (20% in destilled water) instead of drinking water, ad libitum, at least for 30 days before mating and during pregnancy until killing (rats and mice);

— intravenous injection of diluted ethanol during the first days of pregnancy (preimplantation period), (rats and mice);

— intravenous injection of diluted ethanol during the preimplantation period, in animals which underwent previous chronic alcoholization (see above), (rats and mice);

— intravenous injection of acetaldehyde during the preimplantation period (rats);

— peroral administration of Antalcol (Disulfiram) with consecutive intravenous injection of diluted ethanol (during the same period), (rats);

— chronic intake of alcoholic beverages (plum brandy of 24%, cognac diluted to 20%) instead of drinking water, ad libitum, at least for 40—45 days before mating and during pregnancy until killing (rats);

— intravenous injection of diluted alcoholic beverages (see above), (rats);

— crossed embryo transfer between alcoholized (chronic ethanol intake) and untreated animals, by using the method of transfer into an empty pregnant uterus, developed in our laboratory (mice) /3/;

— mating of previously alcoholized male and female mice (chronic peroral diluted ethanol intake for 32—113 days and 36—72 days respectively);

— mating of untreated female mice with previously alcoholized (see above) males.

For some experimental models the blood ethanol level of alcoholized animals was determined by gas-cromatography or by the SIGMA enzymatic alcohol test.

All the experimental models were parallelled by adequate control-series (animals injected intravenously with destilled water, untreated animals). Statistical evaluation was performed by the t-test (Student) and by the test for comparison of two frequencies /38/.

The control methodology varied according to the developmental period approached:

Preimplantation stages

Embryos flushed from the uterus on day 5 (rats) and 4 (mice), respectively, were examined microscopically and photographied. In order to appreciate the effect of the treatments applied the following criteria were used:

Table 1

The effect of ethanol and beverages upon preimplantation development on mice and rats (synoptic view)

Treatment	Animal	Mean number of embr. animal lowering	Developmental rate decrease	Developmental rate increase	Migration slowing down	Migration acceleration	Increased No. of pathol. features	Explanation
Chronic ethanol intake	rat ♀	+(tendency)	+	-	-	-	-	
	mouse ♀	+(tendency)	-	-	-	-	+	
Intravenous ethanol (day 3 or 4)	rat ♀	-	-	-	-	-	+	
	mouse ♀	-	-	-	-	-	-	
Chronic + intravenous ethanol	rat ♀	-	+*)	-	+(tendency)	-	+	*) > than chr. or i.v. ethanol separately
	mouse ♀	-	-	+	-	+	+(tendency)	
Intravenous acetaldehyde	rat ♀	-	+	-	-	-	+	
Antalcol + i.v. ethanol	rat ♀	-	+*)	-	+	-	+*)	*) > than i.v. acetaldehyde
Chronic plum-brandy	rat ♀	+	+*)	-	-	-	+**)	*) **) < than chr. ethanol idem chr. ethanol
Intravenous plum-brandy	rat ♀	-	-	-	-	-	+*)	*) < than chr. beverages
Chronic cognac intake	rat ♀	+	+*)	-	-	-	+**)	*) **) < than chr. ethanol idem chr. ethanol

Table 1 (cont.)

Treatment	Animal	Mean number of embr. animal lowering	Developmental rate decrease	Developmental rate increase	Migration slowing down	Migration acceleration	Increased No. of pathol. features	Explanation
Intravenous cognac	rat ♀	-	-	-	-	-	+*); **	*) <than chr. beverages **) >than i.v.ethanol
Chronic beer intake	rat ♀	-	-	-	-	-	+*	*) >than chr. ethanol and (presumably) plum-brandy or cog.
Chronic wine intake	rat ♀	-	-	-	-	-	+*	*) >than chr. ethanol and (presumably) plum-brandy or cog.
Chronic ethanol intake	mouse ♀ + ♂	-	+	-	+	-	+*	*) >than chr. ethanol ♀
Chronic ethanol intake	mouse ♂	-	+	-	-	-	+	

mean number of embryos/animal; developmental stage attained; localization within the genital tract (oviduct or uterus), indicating the migration rate; number and morphology of pathological specimens. In special experimental series cytogenetic analysis of flushed mouse embryos was performed (on day 4, after chronic alcoholization, intravenous injection and combined treatment), according to /36/. In rats, the ethanol content of oviductal and uterine fluid was determined, following chronic or intravenous ethanol treatment (by gas-cromatography). Finally, in some experimental series the fluorescein diacetate viability test /17/ was performed.

Early postimplantation stages

Uteri of pregnant animals were examined microscopically in serial sections on day 6 and 9 (mice) and on day 10 (rats). The morphological stage of the embryos was evaluated by a biometrical method developed previously /27/. In some cases the Psychoyos implantation test was applied.*

Late fetal stages

In some experimental series rat and mouse foetuses of 19 and 20 days, respectively, were examined microscopically and the fetal and placental wet weight were determined. From foetuses fixed in Bouin's fixative Wilson's sections /39/ were prepared, in those fixed in formaldehyde the thoracic and abdominal cavity were dissected while in about 50% of the foetuses the skeletal development was examined by Alizarin red staining /34/.

RESULTS

Blood ethanol level:

- rats: chronic alcoholization: $\bar{X}$ 35.4 mg/100 ml
  intravenous alcohol: $\bar{X}$ 125 mg/100 ml
  intravenous beverages: $\bar{X}$ 106 mg/100 ml (cognac)
  $\bar{X}$ 116 mg/100 ml (plum-brandy)
- mice: chronic alcoholization: $\bar{X}$ 24 mg/100 ml

Preimplantation changes

Table 1 presents a synoptic view of the pathological changes found in various experimental models.

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*The Psychoyos test /22/ is carried out by intraperitoneal injection of a macromolecular dye (Evans blue, Pontamine blue). After about 15 minutes, in the areas of beginning or advanced implantation a blue staining is to be observed (due to vascular permeability changes).

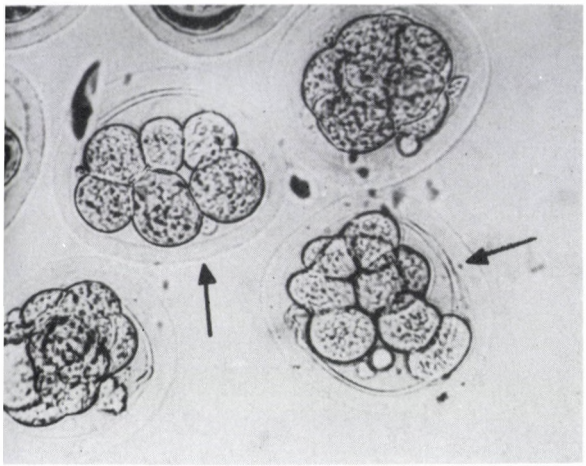

Fig. 1. Rat. Chronic alcoholization. Day 5. Abnormal cleavage (arrows)

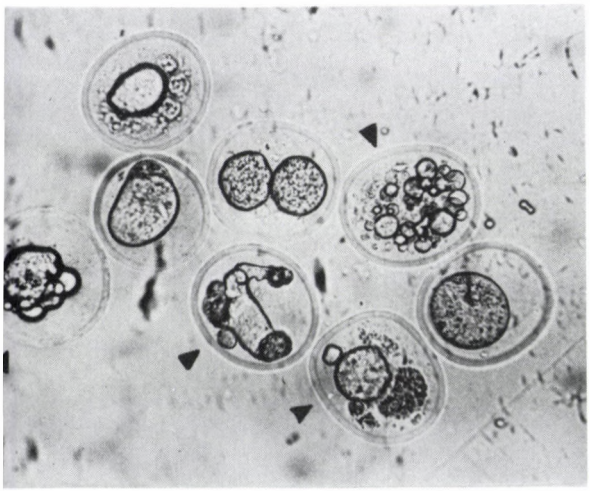

Fig. 2. Mouse. Chronic alcoholization. Day 4. Various pathological changes (arrowheads)

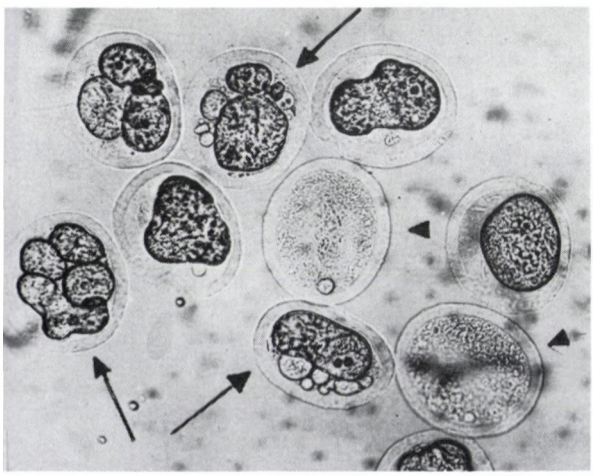

Fig. 3. Rat. Acetaldehyde treatment. Day 5. Abnormal cleavage (arrows); degenerated, unfertilized ova (arrowheads)

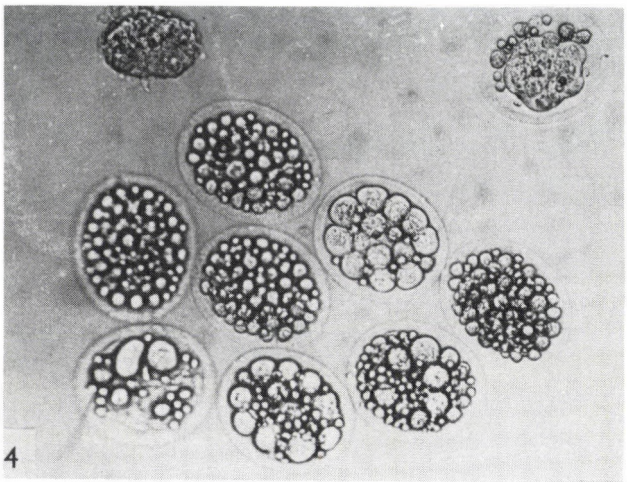

Fig. 4. Rat. Chronic alcoholization + acute intoxication. Day 5. Completely fragmented embryos

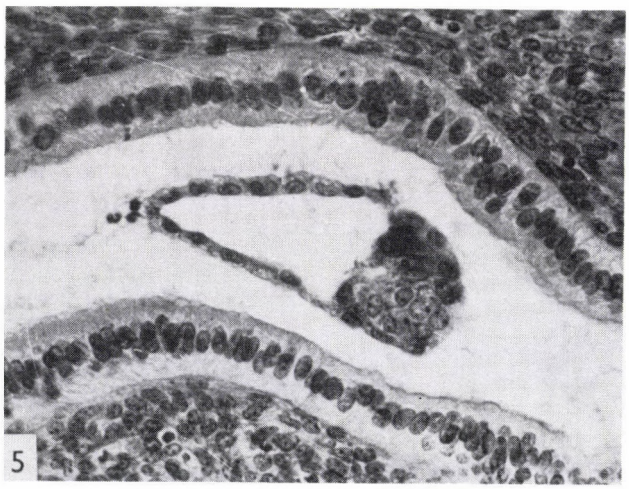

Fig. 5. Mouse. Intravenous ethanol on day 4. Day 9. Free blastocyst in the uterine lumen

Table 2

"Free" blastocysts on day 6 after i.v. ethanol (mouse)

	Control group	Intravenous ethanol		
		day 2	day 3	day 4
No. of animals/ No. of "empty" uteri	15/6	13/8	14/7	13/10
"Empty" uteri with unimplanted blastocysts	0	1	2	6

The following are to be mentioned: the most frequent pathological changes are the increased number of morphologically abnormal embryos and the retardation of development (some characteristic examples of morphologically abnormal embryos are shown in Figs 1–4); the most intense changes were detected after intravenous ethanol injection in chronic alcoholic animals, after Antalcol administration combined with intravenous injection of ethanol and after chronic biparental alcoholization.

Disturbance of implantation

In an experiment with intravenous injection of ethanol on days 2, 3 and 4, on day 9 a lot of apparently empty uteri were found. The microscopical examination revealed that many of these uteri contained free, unimplanted blastocysts (see Table 2 and Fig. 5).

In order to clarify this unexpected finding systematic investigations were initiated. Uteri of animals injected intravenously with ethanol on day 4 of pregnancy, with positive Psychoyos test were examined microscopically on day 6 (i.e. shortly after normal implantation term). If the test was negative the uteri were flushed by the usual method. Table 3 presents the results obtained.

Table 3

Delayed implantation following i.v. ethanol on day 4 (mouse)

Group	No. of embryos	"Free" blastocysts	Attached blastocysts	Total number of unimplanted blastocysts
1. Intravenous ethanol	212	24	27	51
2. Intravenous a.d.	93	1	2	3
3. Untreated control	200	0	1	1

Significancy of differences: 1–2: $p < 0.5$; 1–3: $p < 0.001$

Following intravenous ethanol treatment on day 4, the implantation of the embryos is – in about 25% of the cases – retarded ("delayed implantation"). In the uterine lumen free floating or attached blastocysts are found i.e. the normal implantation stage for day 6 is not attained (see Figs 6–8).

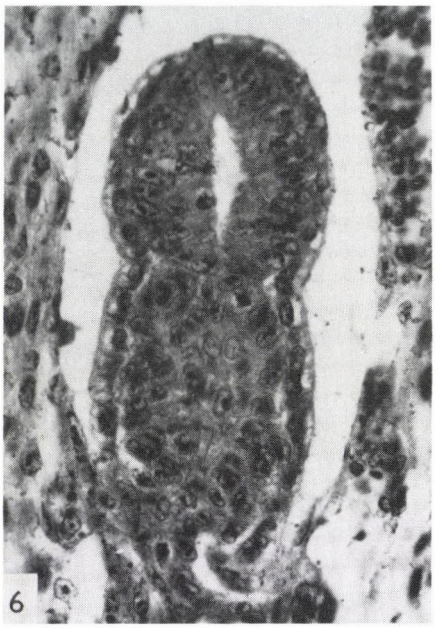

Fig. 6. Mouse. Day 6. Normal, implanted embryo

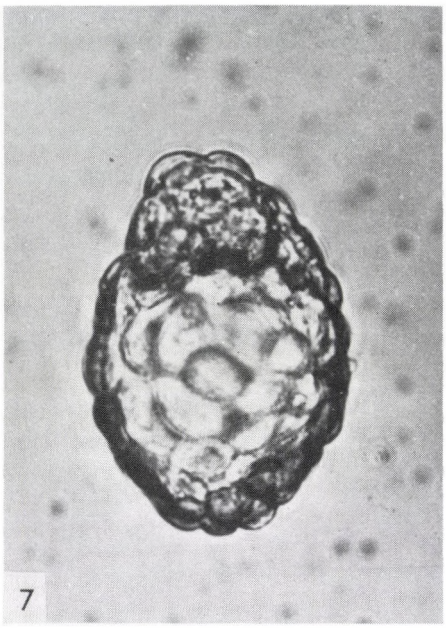

Fig. 7. Mouse. Intravenous ethanol on day 4. Day 6. Free blastocyst from the uterine lumen

It is to be mentioned that, in crossed embryo transfer experiments, both with alcoholized donors and recipients, the lowering of implantation rate was observed.

Early postimplantation stages

Changes during this period were observed in three experimental series: in rats after intravenous ethanol treatment on day 5 and controlled on day 10; in mice injected intravenously with ethanol (on days 2, 3 and 4) and examined on day 6 or 9.

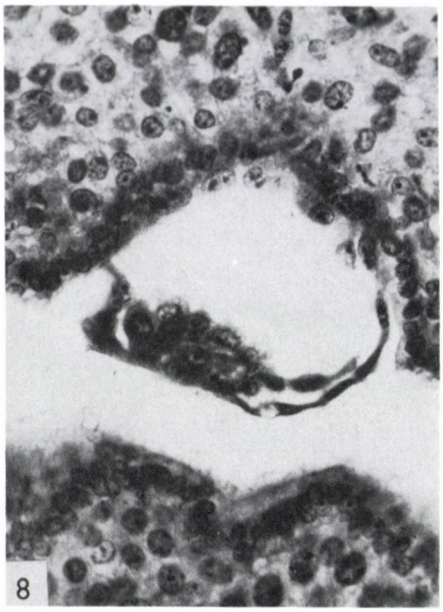

Fig. 8. Mouse. Intravenous ethanol on day 4. Day 6. Blastocyst attached to the uterine mucosa (onset of implantation)

The biometric evaluation of 10-day rat embryos revealed a significant retardation of early developmental components while the components of later developmental stages showed some "acceleration". Early postimplantation mouse embryos — on day 6 — showed also such a change of developmental rate. On day 9, in the mouse embryos examined only two significant changes could be detected: retarded transformation of the proamniotic cavity (in embryos treated on day 3) and delayed development of the allantoic bud (in embryos treated on days 3 and 4, respectively). On days 6 and 9 both the resorption rate and the percentage of embryos showing pathological features were increased (as to the latter 18 and 11% respectively as compared with 0% in the control group).

The mean number of embryos/animal has — both in rats and mice — a lowering tendency, mainly in animals treated on day 3 ($\bar{X}$ 5.14$\pm$2.41 versus $\bar{X}$ 8.22$\pm$3.15 in controls).

Late, foetal changes

After intravenous ethanol treatment on day 3 and 4 of mice, 19 day foetuses and placentae showed a lowered mean wet weight. The mean wet weight of rat foetuses obtained by embryo transfer from alcoholized donors to normal recipients showed a similar change (see Table 4 and 5).

Table 4

Late effect of ethanol i.v. on days 3 and 4 of pregnancy (mice)

Group	Total no. of fetuses	Mean foetal wet weight (g) ($\bar{X} \pm SD$)	Mean placental wet weight (g) ($\bar{X} \pm SD$)	Significancy of difference
Control	164	1.14 ± 0.25	0.13 ± 0.06	$p < 0.001$
Ethanol	162	1.04 ± 0.19	0.10 ± 0.02	

Table 5

Mean foetal and placental weight of transferred embryos (rat)

Transfer variant	No. of fetuses	Mean foetal wet weight ($\bar{X} \pm SD$)	Mean placental wet weight ($\bar{X} \pm SD$)
Untreated donor ↓ Untreated recipient	43	$2.28 \pm 0.74^{a)}$	$0.43 \pm 0.03^{b)}$
Alcoholized donor ↓ Untreated recipient	26	$1.70 \pm 0.28^{c)}$	$0.47 \pm 0.06^{d)}$

Significancy of difference: a–c: $p < 0.001$; b–d: no sign.

Table 6

The ethanol content of the oviductal and uterine fluid (rat)

Fluid examined	Chronic ethanol intake (40–50 days)		Chronic ethanol intake (90–100 days)		Intravenous ethanol (day 4)	
	No. of animals	Ethanol content $\bar{X} \pm SD$ (‰)	No. of animals	Ethanol content $\bar{X} \pm SD$ (‰)	No. of animals	Ethanol content $\bar{X}$ (mg/100ml)
Oviductal	7	0.07 ± 0.02	18	0.05 ± 0.01	-	-
Uterine	10	0.08 ± 0.03	17	0.08 ± 0.04	5	27.5

Ethanol content of the oviductal and uterine fluid

The oviductal and uterine fluid of nonpregnant female rats were examined by gas-cromatography after chronic peroral alcoholization (for 40–50 and 90–100 days respectively). At 30 min after intravenous ethanol administration, the ethanol content of the uterine fluid was determined by SIGMA enzymatic alcohol test. The results are presented in Table 6.

Cytogenetic control

In mice, after chronic alcoholization, after intravenous ethanol injection on day 3 and after intravenous treatment combined with chronic alcoholization, the cytogenetic control of 4-day embryos revealed no significant changes of metaphase chromosomes.

DISCUSSION

Our present results point to the existence of an *in vivo* developing complex blastopathy, induced even by relatively low ethanol doses. This new nosological entity, component of the experimental alcohol embryo- and fetopathy, involves pathological changes during various periods of prenatal development.

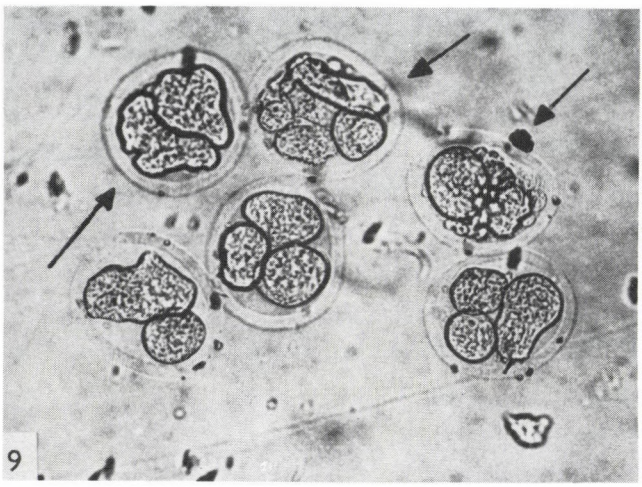

Fig. 9. Rat. Chronic beer intake (unpublished experiments). Day 4. Abnormal cleavage

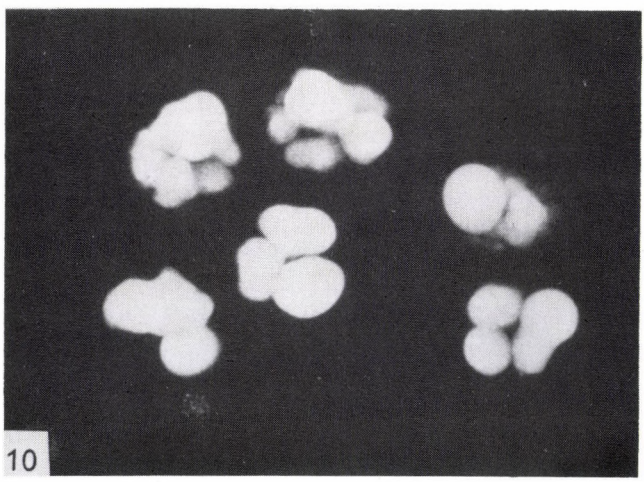

Fig. 10. Idem Fig. 9. Fluorescein diacetate test. Only detritus shows decreased fluorescence (on the left)

Preimplantation changes

They are concerned with the oviductal-uterine migration rate, the developmental rate, the mean number of embryos/animal and the morphology of developing embryos and occur, with variable "expressivity" following chronic or acute preimplantation treatment. Recently, in rats injected intravenously with ethanol 8 h after mating, an inhibition of cleavage and – if the blood ethanol level was higher than 150 mg/100 ml – the appearance of pathological developmental features were observed /21/.

As not all experimental models were applied to both species, it would be precocious to draw conclusions with respect to the role of genetic factors. Data obtained suggest that, a similar treatment on both species may induce similar (mean number of embryos/animal) or even partially different effects (developmental rate, migration rate, frequency of pathological features).

Our data also attest that, the components of preimplantation development may be influenced preferentially. Thus the developmental rate is modified almost only by chronic alcoholization while the increased frequency of pathological features may be induced by various experimental procedures.

As to the pathogenesis of the preimplantation disturbances the followings are to be mentioned: the presence of ethanol in the direct environment of the migrating and developing embryo suggests the possibility of a direct, toxic action. Of course, the indirect way of action, via pathological changes of the genital tract, of the oviductal or uterine fluid, of the mucosa, of the blood circulation a.o. can also not be excluded. One effect, the decrease of migration speed may be possibly due to functional changes of the oviductal muscles. On the other hand, the disturbed migration may itself, by the consecutive desynchronization of the embryo – environment relationship, represent a potential noxious factor acting upon preimplantation development.

As known, since the first observations /37/, acetaldehyde is considered an essential or even the unique etiopathogenic factor in alcohol embryo- and fetopathy (for details see /7/). Our own data, with respect to the preimplantation effects, suggest the additive action of acetaldehyde (that means that it could possibly be, the unique causal factor). As to the possible role of congeners present in various alcoholic beverages, from our first experiments only the acute intoxication with cognac suggested an

enhanced deleterious effect. Recent results, however, obtained in our laboratory with chronic intake of vine and beer (on rats) revealed a higher number of pathological developmental features in both experimental models. /Fazakas-Todea, unpublished data./

The experimental model, reproducing an acute alcoholic intoxication of an addict revealed that, under these conditions, the noxious effect upon preimplantation embryos is clearly enhanced. The finding, that, both after biparental alcoholization and after paternal one alone, the number of unfertilized and degenerated eggs is significantly increased, suggests a lowered efficiency of fertilization. Some changes in spermatozoid motility and destructive morphological effects found in the testes of male mice after chronic alcoholization are possibly incriminated in this lowering which may influence, besides the mean number of embryos/animal (see the tendency of decrease detected) also the quality of the developing embryos.

The fluorescein-diacetate vitality test is — as known — concerned with the normal function of some intracellular esterases and with the integrity of the cell membrane. According to our experience, even obviously pathological embryos showed a normal fluorescence, the weakening or disappearance of fluorescence (the criterion of appraisal), being but occasionally in accordance with the morphological feature. A similar opinion was expressed by /19/ based on their observations on early human embryos. These observations attest also the complexity of disturbance and destruction (see Figs 9, 10).

On the whole, the effects mentioned, are to be considered as epigenetic disturbances. It is — however — not possible to exclude, first of all with respect to the developmental rate and to the appearance of more pathological features, the role of genetic factors. Such factors may be implicated e.g. in effects of paternal alcoholization, where the spermatozoa may contribute to development with pathological changes at chromosomal or gene level. It must be mentioned however that, at the level of cytogenetic control, no significant changes could be detected.

Disturbed implantation

The disturbance of the implantation process, observed after intravenous alcohol treatment on day 4 of pregnancy may be — theoretically — attributed to central and/or general effects. According to some data, alcohol intake may disturb the neuro-hormonal mechanisms of pregnancy, as it dis-

turbs similar mechanisms in the male organism (for details see /25/). As known, such mechanisms, with the main contribution of the hypothalamus-pituitary-ovary axis, have an outstanding role in the determinism of normal implantation, of the precise, highly sensible timing of this complex series of events. Other possible targets of disturbances due to alcoholization are the uterine microenvironment and the embryo itself, which actively participate in the preparation of implantation (with several known and yet unknown "contributions" at the molecular, electron microscopic or microscopic levels, from — supposed — chemical signals to hatching from the zona pellucida and to the changes of uterine circulation). It is worth mentioning that prostaglandins e.g., which seem to play a role in the pathogenesis of alcohol embryo- and fetopathy /21/ are also implicated into the mechanism of implantation /12, 23/. The results of our crossed-transfer experiments also suggest, that the lowered rate of implantation is due both to the recipient uterus (maternal organism) and to the embryos transferred. On the other hand, recent microscopic observations made in our laboratory (Checiu M., unpublished data), on the oviduct and the uterus of mice (fixed at 4—12 h after intravenous administration of ethanol on day 4) strongly suggest that a marked retardation of oviductal-uterine migration may, by desynchronizing the embryo-uterine relationship, prevent normal implantation.

Early postimplantation changes

The preimplantation noxious effect may have further consequences. Thus, the increased number of implanted, pathological embryos, the lower mean number of embryos/animal and the general or partial developmental retardation may be attributed to previous, preimplantation changes. The developmental acceleration observed in some cases may tentatively be explained by a kind of "selection". The more rapidly developing embryos are supposed to represent the most viable specimens which resisted to the deleterious action and could — favorably influenced by the lowered number of normally developing embryos — accelerate their development and may be correlated thus with the acceleration of development and migration during the preimplantation period (see Table 1). In mice, the acceleration is, on day 6, a general phenomenon. Somewhat later some embryos are retarded, probably due, to the gradually appearing late noxious effect.

Late, fatal effects

The observation, that embryos transferred from donors which were chronically exposed to alcohol show, at late fetal stages, retardation of growth, suggest their incapacity to "recover" even developing within a new, normal environment.

Growth retardation (lowered mean wet weight of foetuses and placentae) observed after intravenous ethanol administration on days 3 and 4 (in mice) is also a late effect of the very early action of ethanol and is not compatible with the "all or none" dogma. The lower mean placental wet weight may possibly be due to the retarded development of the allantoic bud (the allantois being an essential component of the further developing placenta). The delay of placental development may, possibly, disturb the normal O_2 and other substance transfer to the foetus and contribute to the above mentioned growth retardation.

CONCLUSIONS

1. Our studies performed with relatively low doses of ethanol outlined an "experimental alcohol blastopathy" in rats and mice, part of the complex nosological entity of experimental alcohol embryo- and foetopathy. Taking in account the multiple similarities between the preimplantation development of human and laboratory rodent embryos, our observations strongly suggest the existence of a risk also in humans, the more that during this very early period of pregnancy, women are not aware of being pregnant.

2. The experimental alcohol blastopathy involves the following components: pathological changes during preimplantation development (lower mean number of embryos/animal, retardation of development, lowered migration rate of the embryos from the oviduct to the uterus, higher number of pathological morphological features); delayed implantation; disturbances of the early postimplantation development; retarded late foetal and placental growth.

3. The effect of ethanol may be direct (ethanol being detectable in the oviductal and uterine fluid after both acute and chronic alcohol exposure) or indirect, via changes of the maternal macro- or microenvironment.

4. The increase of the maternal blood acetaldehyde level may contribute to the appearance of alcohol blastopathy.

5. Chronic beer and wine intake and acute intoxication with cognac suggest — up to now — the enhancing effect of beverage congeners.

6. The noxious effect of acute ethanol intoxication superposed to chronic alcoholization is more marked that the separate effect of the two kinds of treatment.

7. The chronic ethanol intake of fertilizing males (in mice) leads, both in the case of treated or untreated females, to lowered fertilization efficiency, to retardation of development (not occurring after chronic maternal alcoholization) and to an enhanced increase of the number of pathological features.

8. The cytogenetic control of preimplantation embryos (after chronic, acute or combined treatment with ethanol) does not reveal significant chromosomal changes.

REFERENCES

1. Barr, H.M., Streissguth, A.P., Martin, D.C., Herman, C.S. (1984) Infant size at 8 months of age: relationship to maternal use of alcohol, nicotine and caffeine during pregnancy. Pediatrics, 74, 336–341.

2. Căpălnăşan, I., Checiu, M. (1985) The effect of ethanol upon early development in mice and rats. VII. Cytogenetic analysis of preimplantation stages of alcoholized mice. Rev. Roum. Morphol. Embryol., 31, 163–167.

3. Checiu, M., Amels, D., Sandor, S., Suciu, A. (1977) Contributions to the transfer of preimplantation mouse embryos into "foster mothers". Rev. Roum. Morphol. Embryol., 23, 175–180.

4. Checiu, M., Sandor, S. (1981) The effect of ethanol upon early development in mice and rats. III. In vivo effect of acute ethanol intoxication upon implantation and early postimplantation stages in mice. Rev. Roum. Morphol. Embryol., 27, 117–122.

5. Checiu, M., Sandor, S. (1982) The effect of ethanol upon early development in mice and rats. IV. The effect of acute ethanol intoxication on the 4th day of pregnancy upon implantation and early postimplantation development in mice. Rev. Roum. Morphol. Embryol., 28, 15–19.

6. Checiu, M., Sandor, S. (1983) The effect of ethanol upon early development in mice and rats. V. In vivo effect of acute preimplantation intoxication with or without previous chronic alcoholization. Rev. Roum. Morphol. Embryol., 29, 149–157.

7. Checiu, M., Sandor, S., Garban, Z. (1984) The effect of ethanol upon early development in mice and rats. VI. In vivo effect of acetaldehyde upon preimplantation stages in rats. Rev. Roum. Morphol. Embryol. 30, 175–184.

8. Checiu, M., Sandor, S. (1986) The effect of ethanol upon early development in mice and rats. IX. Late effect of acute preimplantation intoxication in mice. Rev. oum. Morphol. Embryol., 32, 5–11.

9. Checiu, M., Sandor, S. (1987) The effect of ethanol upon early development in mice and rats. XI. Pathogenetic aspects studied by reciprocal transfer of preimplantation embryos. Rev. Roum. Morphol. Embryol., 33, 13–18.

10. Checiu, M., Sandor, S. (1988) The effect of ethanol upon early development in mice and rats. XII. The effect of chronic biparental or paternal alcohol intake upon preimplantation development in mice. Rev. Roum. Morphol. Embryol., 34, 1–8.

11. Czeiczel, E., Palkovits, I. (1965) A terhesség alatti alkoholfogyasztásról. (Alcohol consumption during pregnancy). Egészségtudomány, 9, 263–273. (In Hungarian).

12. Dey, S.K., Johnson, D.C. (1980) Histamine formation by mouse preimplantation embryos. J. Reprod. Fert., 60, 457–460.

13. Fazakas-Todea, I., Checiu, M., Sandor, S. (1985) The effect of ethanol upon early development in mice and rats. VIII. The effect of chronic consumption of some beverages upon preimplantation development in rats. Rev. Roum. Morphol. Embryol., 31, 249–256.

14. Fazakas-Todea, I., Sandor, S., Hateganu, M., Perta, D., Checiu, I. Stefanescu, S. (1986) The effect of ethanol upon early development in mice and rats. X. The effect of acute intoxication with some alcoholic beverages upon preimplantation development in rats. Rev. Roum. Morphol. Embryol., 32, 160–165.

15. Gottschewski, G.H.M., Zimmermann, W. (1963) Auslösung von Blastopathien beim Säugetier durch Cyclophosphamid und Thalidomid. Naturwissenschaften, 15, 525.

16. Jones, K.L., Smith, D.W., Ulleland, C., Streissguth, A.P. (1973) Pattern of malformation in offsprings of chronic alcoholic mothers. Lancet, 7815, 1267–71.

17. Kola, I., Folb, P.I. (1984) An assessment of the effects of ascorbic acid, cyclophosphamide and sodium valproate on the viability of preimplantation mouse embryos using the fluorescein diacetate test. In: 12th European Teratol.Congr. Veldhoven (Netherlands). Program and Abstracts, p. 54.

18. Lemoine, L.K., Harousseau, H., Borteyen, J.P., Menuet, Y.C. (1968) Les enfants des parents alcooliques: anomalies observées. Apropos de 127 cas. Arch. Franc. Pédiatr., 25, 830–831.

19. Mohr, L.R., Trounson, A.O., Leeton, J.F., Wood, C. (1983) Evaluation of normal and abnormal human embryo development during procedures in vitro. In: Beier, H.M., Linder, H.R. (eds) Fertilization of the Human Egg in Vitro. Springer-Verlag, Berlin, Heidelberg, pp. 211–221.

20. Papara-Nicholson, D., Telford, I.R. (1957) Effects of alcohol on reproduction and fetal development in the guinea pig. Anat. Rec., 127, 438–439.

21. Pennington, S.N., Allen, Z., Runion, J., Farmer, P., Rowland, L., Valmus, G. (1985) Prostaglandin synthesis inhibitors block alcohol induced fetal hypoplasia. Alcoholism: Clinical and Experimental Res., 9, 5–10.

22. Psychoyos, A. (1960) Nouvelle contribution a l'étude de la nidation de l'oeuf chez la ratte. C.R. Acad. Sci. Paris, 251, 3073–75.

23. Sananes, N., Boulien, E.E., Le Goascogne, C. (1976) Prostaglandin as inductive factor of decidualization in the rat uterus. Molecular and Cellular Endocrinology, 6, 153–158.

24. Sandor, S. (1986) Die tierexperimentelle Alkoholblastopathie. In Symposion der Stiftung für das behinderte Kind "Die Alkoholembryopathie". Frankfurt am Main.

25. Sandor, S. (1987) The possible pre-natal noxious effect of paternal alcohol intake. Rev. Roum. Morphol. Embryol., 33, 177–182.

26. Sandor, S., Elias, S. (1968) The influence of ethyl-alcohol on the development of the chick embryo. I. Rev. Roum. Embryol. Cytol. – Série Embryol. 5, 51–76.

27. Sandor, S., Amels, D. (1971) The action of ethanol on the prenatal development of albino rats. Rev. Roum. Embryol. Cytol. – Série Embryol., 8, 105–118.

18. Sandor, S., Garban, Z., Checiu, M., Daradics, L. (1981) The presence of ethanol in the oviductal and uterine luminal fluid of alcoholized rats. Rev. Roum. Morphol. Embryol., 27, 303–309.

29. Sandor, S., Checiu, M., Fazakas-Todea, I., Gârban, Z. (1980) The effect of ethanol upon early development in mice and rats. I. In vivo effect upon preimplantation and early postimplantation stages. Rev. Roum. Morphol. Embryol., 26, 265–274.

30. Sandor, S., Checiu, M., Fazakas-Todea, I. (1984) Experimental embryology and teratology in laboratory mammals (problems, results, perspectives) (In Rumanian). Ed. Academiei R.S.R., București.

31. Sandor, S., Checiu, M., Fazakas-Todea, I., Garban, Z. (1984) Experimental approach to the alcohol embryo- and fetopathy. In Tittmar, H.G. (ed.) Advanced concepts in alcoholism. Pergamon Press, Oxford, pp. 13–34.

32. Sandor, S., Checiu, M., Fazakas-Todea, I., Garban, Z. (1985) Experimental alcohol blastopathy. In 13th Conf. of European Teratol. Soc. Rostock, Warnemünde. Abstracts.

33. Sandor, S., Checiu, M., Fazakas-Todea, I., Checiu, I., Garban, Z. (1988) Alcohol embryo- and fetopathy (In Rumanian). Ed. Academiei R.S.R., București. (In press).

34. Staples, R.E., Snell, V.J. (1964) Refinements in rapid clearing technic in the KOH-Alizarin red S method for fetal bone. Stain Technology, 39, 62–63.

35. Streissguth, A.P. (1983) Alcohol and pregnancy: an overview and an update. Substance and Alcohol Actions/Misuse, 4, 149–173.

36. Tarkowski, A.K. (1966) An air-drying method for chromosome preparations from mouse eggs. Cytogenetics, 5, 394–400.

37. Véghelyi, P., Osztovics, G., Kardos, L., Leisztner, E., Szanovszky, S., Iglai, J., Imrei, J. (1978) The fetal alcohol syndrome: symptoms and pathogenesis. Acta Pediat. Acad. Sci. Hung., 19, 171–189.

38. Weber, E. (1961) Grundriss der biologischen Statistik. VEB G. Fischer Verlag, Jena.

39. Wilson, G.J. (1965) Embryological considerations in teratology. In Wilson, J.G., Warkany, J. (eds) Teratology: Principles and techniques. Univ. Chicago Press, Chicago–London, pp. 251–277.

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A SIMPLE PREPARATORY METHOD FOR SCANNING ELECTRON MICROSCOPY OF THE MOUTHPARTS OF MUSCOID FLIES (DIPTERA)

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A Scanning electron microscopic preparation method is described, especially for muscoid flies also useful for the preparation of other chitinous structures of insects. Carbon-tetrachloride was used to maintain the proper positions of the proboscis and the labella rendering a better view of the fine structures. Specimens were cleaned by a bioactive washing powder, then air-dried prior to fixing them on stubs by a reliable home-made adhesive.

Keywords: Carbon-tetrachloride — cleaning SEM specimens — adhesive for SEM

INTRODUCTION

In examining the role of the different species of flies in the transmission of pathogens a thorough knowledge of the functional morphology of the flies' mouthparts is of paramount significance. The information obtained by earlier workers using the traditional light microscopic technique /2, 4/, has recently been employed in scanning electron microscopy (SEM) /1, 3, 5/. The procedure applied in these studies involved dehydration and critical-point drying while the cleaning of specimens has been entirely neglected.

In the course of our studies on the morphology of the mouthparts of muscoid flies it was found that these chitinous structures enable the ap-

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plication of a simplified and new procedure in the preparation of fly specimens for SEM studies. In the present paper this method is described.

MATERIALS AND METHODS

Flies were obtained from laboratory colonies of a WHO strain of Musca domestica at the insectarium of the University of Veterinary Sciences, Budapest.

Several compounds were tried to kill the flies and to open their proboscis and labella; carbon-tetrachloride (CCl_4) p.a. proved to be the best. It was used in three ways:

a) Direct topical application;
b) Evaporation in a treatment chamber (beaker) at room temperature;
c) Methods a and b combined.

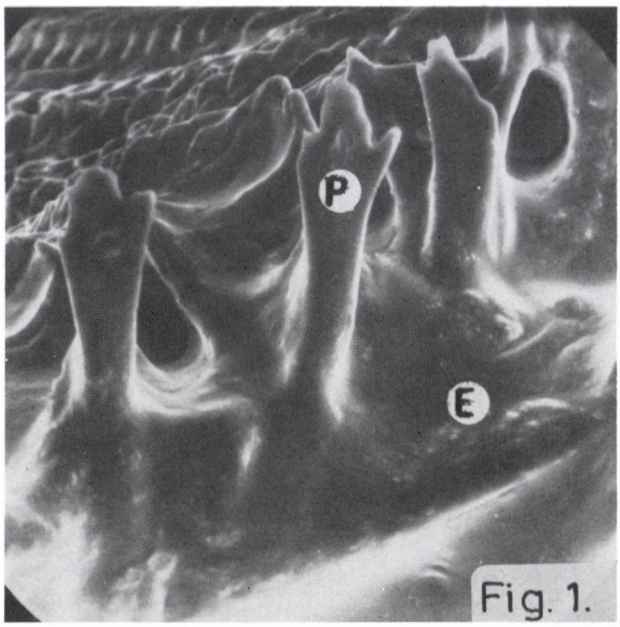
The third method proved to be the best for assuring the physiological positions of the labella /2/. A beaker containing a fly was turned upside down on a table. CCl_4 was dropped on a sheet of filter-paper which was immediately slipped under the beaker. CCl_4 quickly evaporated and narcotised the fly. When the fly was immobile generally another small amount of CCl_4 was needed to kill it, which was applied topically or from underneath using the capillary attraction of the filter-paper.The major factors affecting the positions of the mouthparts are the time of exposure in the treatment chamber after the first application of CCl_4, the quantity of CCl_4 at the second application and above all the species and strain characteristics of the fly. E.g. Musca domestica "street-population" (Budapest, Hungary) showed different reactions during this procedure compared to those of the WHO strain.

After a few minutes of air-drying — to remove CCl_4 residues — a cleaning procedure is applied.

Cleaning is necessary because from the oral aperture of the agonising fly a considerable amount of mucous fluid is released, which forms after drying a pseudo-membrane over the labella (Figs 1 and 2) rendering the analysis of the fine structures more difficult. For cleaning lukewarm saturated solution of a bio-active washing powder BIOPON (Növényolajipari és Mosószergyártó Vállalat, Hungary) is applied for up to half an hour. Then the head of flies is severed and after transversal halving mounted on SEM stubs.

For fixing on stubs a home-made adhesive was used, instead of the more expensive and sometimes less effective commercial brands. A halved head is a rather big specimen in terms of electron microscopic dimensions, therefore its fixation needs stronger adhesives than the normally used preparation requires. (E.g. "colloidal-graphite" was not strong enough in our case.) A heat-proof paint (ALUKON heat-resistant silver paint made by Kőbányai Könnyűfémmű, Hungary) containing some kind of metal having conductor property was centrifugated and the metal particles of the sediment were washed in petrol and used for sticking.

Specimens were air-dried under a warm lamp for a few hours. The chitinous structures were already prepared for a quick examination under the electron-beam. The specimen was vacuum-coated with a thin layer of coal and gold, in a JEOL JEE-48 vacuum evaporator, which improves the quality of the micrograph and prolongs the examinability of the specimen under the electron beam. Micrographs for this study were made by a JEOL JSM-35 SEM.



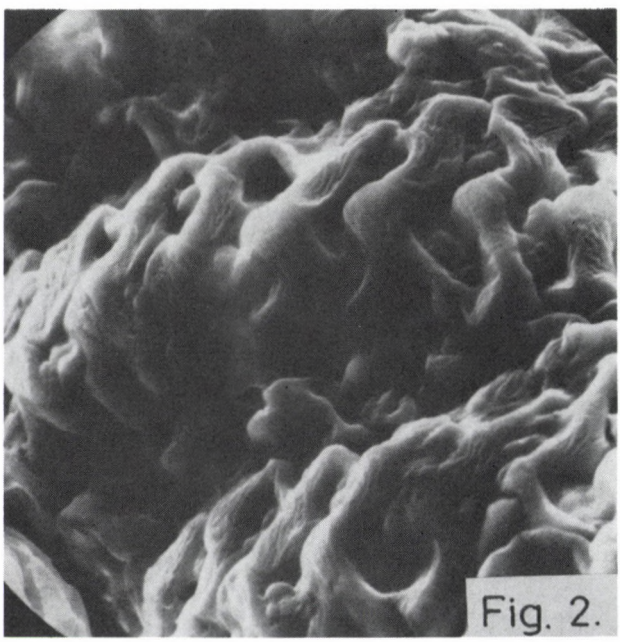
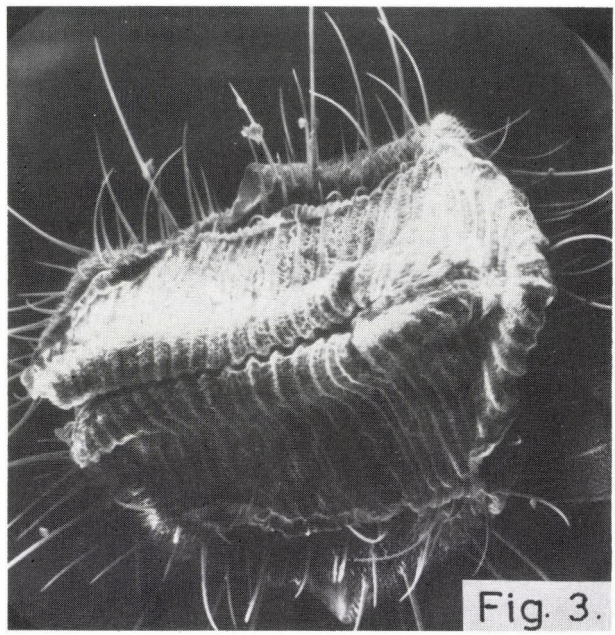


Fig. 1. The prestomal-teeth (P) and the entrance of the food-channel (E) is covered by a pseudomembrane. X 940

Fig. 2. Pseudomembrane on the pseudotracheal rows. X 3000



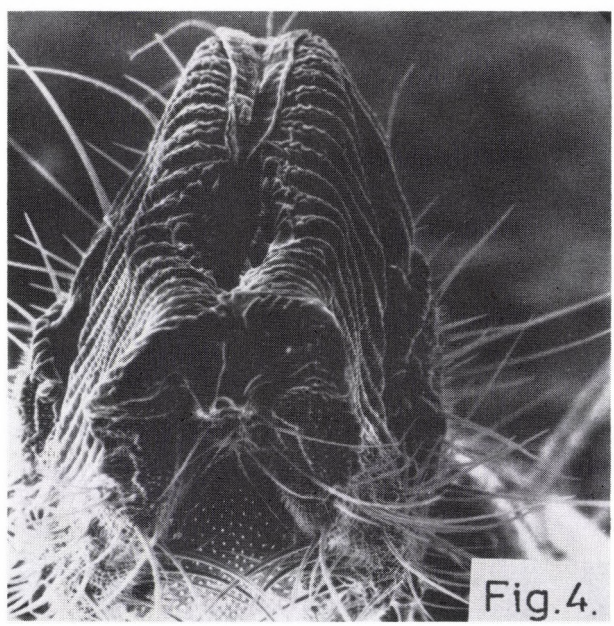
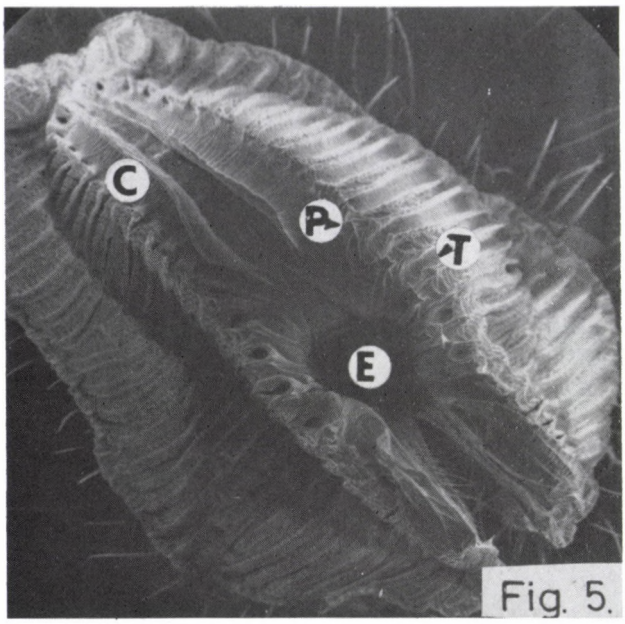


Fig. 3. Labella in filtering position. X 150

Fig. 4. Labella in intermediate position. X 200



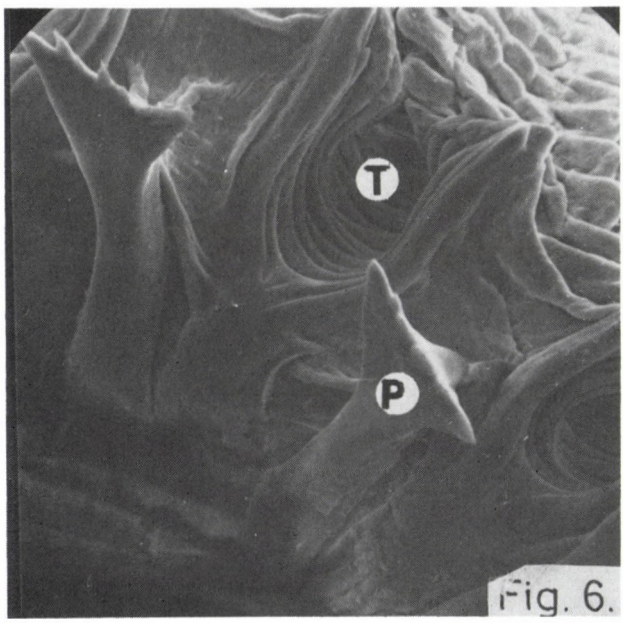


Fig. 5. Labella in scraping position. Fine structures after cleaning: prestomal-teeth (P); pseudotracheae (T); entrance of food-channel (E); collecting-channel (C). X 150

Fig. 6. After the elimination of the pseudomembrane: prestomal-teeth (P); pseudotracheae (T). X 1300

RESULTS AND DISCUSSION

The SEM of the chitinous mouthparts does not need such a complicated preparatory method as other biological specimens, but these examinations require scores of flies /3/ from which a few can be selected with their proboscis in the proper position. In this study it was attempted to simplify and at the same time to supplement the existing methods adopted from other types of SEM studies /1, 3, 5/. As a result of the introduced method 70 per cent of the flies was suitable for taking SEM micrographs. The suggested new aspects of the technique are: (1) CCl_4 was applied to open the labella and to set the proboscis in the proper physiological position ready for examination. Figs 3, 4 and 5 show the filtering, the intermediate and the scraping positions of the labella. The only disadvantage of the method is that the labellar membrane was crumpled due to the withdrawal of the haemolymph in the labellar haemocoel, but this deficiency in no way affected any of the fine structures. (2) The improvement of the technique provided by cleaning in a bio-active washing poweder is illustrated in Figs 5 and 6, showing the prestomal teeth and the pseudotracheae. Cleaning removes the pseudomembrane covering the fine structures and enables the examination of even the lumen of the pseudotracheae and other tiny details (Fig. 6). (3) A new and more reliable adhesive was used. Besides these new aspects, the other preparation steps could be neglected, due to the chitinous covering the surface, as dehydration and critical-point drying, since these steps require the best part of the preparation time. This preparation method is quick, inexpensive, needs no costly laboratory equipment and seems suitable for routine work in the SEM analysis of such chitinous structures as the mouthparts of muscoid flies.

REFERENCES

1. Broce, A.B., Elzinga, R.J. (1984) Comparison of prestomal teeth in the face fly (Musca autumnalis) and the house fly (Musca domestica) (Diptera: Muscidae). J. Med. Entomol. 21, 82–85.

2. Graham-Smith, G.S. (1930) Further observations on the anatomy and function of the proboscis of the blow-fly, Calliphora erythrocephala L. Parasitology 22, 47–115.

3. Iwasa, M. (1983) A comparative study on the mouth parts of medically and veterinarily important flies, with special reference to the development and origin of the prestomal teeth in cyclorrhaphous Diptera. Jap. J. Sanit. Zool. 34, 177–206.

4. Mahanko, E.V. (1973) Parasitism rate and structure of oral teeth in synantropic flies of the genera Hydrotaea R.-D. and Musca L. (Diptera, Muscidae). Revue d'Entomologie de l'URSS. 52, 768–781.

5. Paysinger, J.T., Noblet, T.R., Adkins, T.R., Jr., Vaughn, E.A. (1978) Scanning electron microscopy of the adult mouthparts of the horn fly and the face fly. J. Georgia Entomol. Soc. 13, 28–39.

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SEASONAL VARIATION OF AMMONIA-QUOTIENT IN AN INDIAN AIR-BREATHING FRESHWATER TELEOST, CHANNA PUNCTATUS

SHORT COMMUNICATION

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The ammonia-quotient, calculated from the data on the levels of ammonia excreted and oxygen consumed from the water, is a potent tool for understanding the utilization of macromolecules as metabolic fuel. The fish, Channa punctatus, utilizes more protein in the summer and spawning months for getting its metabolic energy but in the winter and post-spawning months it also utilizes other substrates like lipid and carbohydrate for its metabolic energy.

Keywords: Ammonia — quotient — ammonia excretion — oxygen consumption

INTRODUCTION

For most of the fishes and many poikilothermic animals the standrad oxygen consumption is known to increase with the increase of ambient temperature within thermal limits of survival. Thus in the three Indian freshwater teleosts, Cirrhina mrigala /12/, Labeo bata and Channa punctatus /1/, increase of water temperature was found to cause an augmentation of the branchial uptake of oxygen from water. The ammonia-quotient (A/O ratio), calculated from the data on the levels of ammonia excretion and the oxygen consumption, is a potent tool for understanding the fuel mobilization by fish /3/ and the remarkably high value of A/O ratio, exceeding the value of

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0.27, is strongly suggestive of complete protein utilization for getting metabolic energy /9, 10/.

The quantitative differential effect of starvation on the rates of ammonia-N excreation and oxygen consumption between the summer-adapted and winter-adapted Channa is known /15/, which exhibits a relative quisence or dormancy associated with summer drought. The Channa is an obligatory air-breathing fish. Besides the gill (for aquatic respiration), it has a secondary respiratory organ (buccopharyngeal cavity) for aerial respiration. In the present communication, I would like to give some information regarding the protein utilization, as A/O ratio, by this tropical, air-breathing, seasonal breeder, Channa punctatus during an annual reproductive cycle.

MATERIALS AND METHODS

The fishes (30.0—40.0 g) were collected from the local market of Santiniketan (Eastern India) during the second week of every month in an annual cycle, August—July. The climate of Santiniketan is mostly dry, the humidity remaining quite low throughout the year except the rainy season. Because of such a physico-graphic condition, this locality is characterized by a marked summer (mid. March—July) with an average temperature of about 35 ^{o}C or more and a short winter (mid. December—February) with an average temperature of 20 ^{o}C or less. The rates of ammonia excretion and oxygen consumption from water were measured /1, 15/, at prevailing water temperature of each month and at a constant water temperature of 25 ^{o}C. Before the experiment the fishes were starved for 5 days in order to exclude the exogenous nitrogen excretion, coming from the uptake of food, and to measure the endogenous nitrogen excretion /14/. Both the rates were measured in the same fish and then the A/O ratio was calculated

RESULTS AND DISCUSSION

A reduction (63%, $P < 0.001$) of the ratio of branchial (aquatic) oxygen consumption during summer months (April—May) in comparision to this rate during winter months (January—February) was observed in this Indian air-breathing murrel (Fig. 1) and this may be correlated with the depletion of dissolved oxygen tension in water at higher temperature (35 ^{o}C or more) during the summer months and a greater availability of this respiratory gas in water at lower temperature (20 ^{o}C or less) of winter months. Whether the fish has a greater dependence on aerial respiration (through the accessory respiratory organ) at higher temperature of summer months and a lesser dependence in winter, needs to be explored in future through measurement

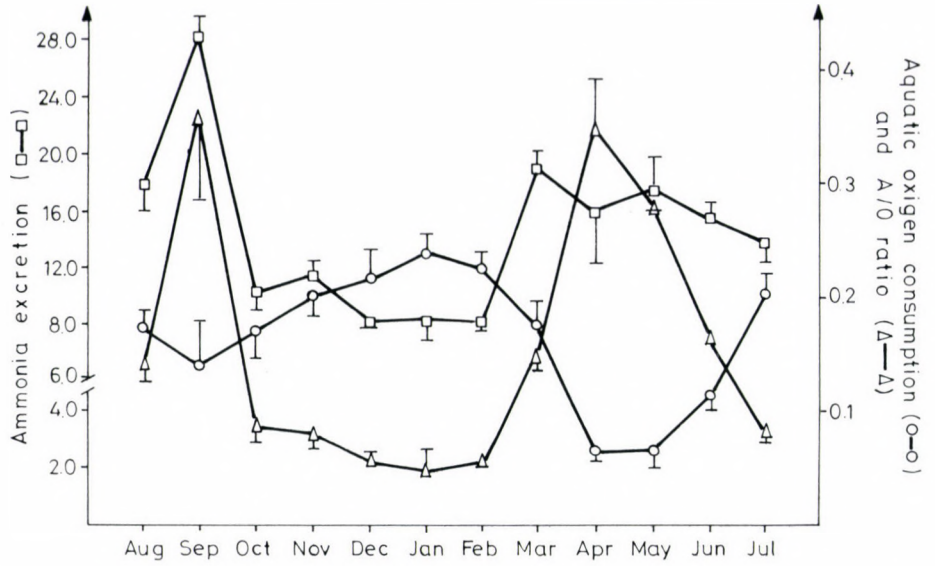

Fig. 1. The rate of ammonia excretion (μg/g body weight/h), aquatic oxygen consumption (ml oxygen consumed/g body weight/h) and ammonia-quotient (A/O ratio) of Channa punctatus, measured at ambient water temperature, during an annual cycle (August—July). The values are plotted as mean of six sample ± S.E.

both the aquatic and aerial respiration in an annual cycle. In case of other Indian air-breathing teleosts, Anabas /11/, Heteropnuestes /2/ and Clarias /8/, it has been proved beyond doubt that the average oxygen consumption remains higher from water than air in the summer months. Perhaps this generalized conclusion holds true for C. punctatus as well. The question of the capability of this air-breathing fish for metabolic compensation to seasonal thermal fluctuation, as in case of other tropical fish Etroplus maculatus /5—7/, can only be resolved by a comprehensive investigation on the rates of aquatic and aerial respiration of both summer- and winter-adapted fishes as a function of the temperature. The observation of Patra et al. /8/ in Clarias regarding the higher rate of oxygen consumption in the rainy season compared to that of winter, correlated with the breeding activity of this fish, is not supported by the data on Channa. Instead, the aquatic respiratory rate of this murrel, which remains depressed throughout the summer (April—June), exhibits a sudden elevation in July, during the

pre-spawning period. In West-Bengal Channa spawns just after the onset of monsoon i.e., August–September.

The rate of ammonia excretion was augmented by 2-fold ($P < 0.001$) during summer months over the winter months. This augmentation, which induces to elevate the ambient ammonia concentration in their living medium, doesn't cause the reduction of aquatic respiratory rate by damaging the gill membrane, as it does for other freshwater fishes /4/. This fish is known to have a high tolerance limit for ammonia toxicity. Up to 60 ppm of ammonia concentration no toxic effect was observed in this fish (unpublished data). Besides, the rate of ammonia excretion remains higher in the pre-spawning and in the spawning periods (Fig. 1) is correlated with the higher mobilization of protein for the reproductive activity.The significance of the seasonal variation of the ammonia-N excretion along with other nitrogenous excretion in Channa punctatus is already explored by Roy and Das /16/. The metabolic compensation, in the rate of ammonia and urea-N, due to seasonal thermal fluctuation is also understood in this fish /17/.

It is noteworthy in this connection that the rates of ammonia excretion and oxygen consumption tend to disappear at constant temperature of measurement (25 ^{o}C), which was more or less intermediate between the average temperatures of summer and winter months.

The pattern of variation of the A/O ratio exhibits an inverse relationship with that of the aquatic respiration and a direct relationship with ammonia excretion. Obviously the higher value of the ammonia-quotient during summer months and also in September are due to the enhanced rate of ammonia excretion and the diminished rate of oxygen consumption. The remarkably high A/O ratio, exceeding the value of 0.3, is strongly suggestive of complete protein utilization /9, 10/. It is thus plausible that Channa, which is a carnivorous fish, mobilizes nitrogenous substrates, like protein and amino acid, for its energy requirements during summer and also during the spawning period, but the protein is spared during the post-spawning and winter months (October–February) when the ratio remains in minimal level. This contention is supported by the findings on the accumulation of the tissue levels of protein and free amino acids during winter and depletion during the summer /13/. Earlier it was reported, from the data on endogenous nitrogen excretion /14/ and from the data on starvation /15/ that Channa punctatus, unlike the other vertebrates, depends cent percent on nitrogenous fuel, like protein and free amino acids in summer but during

winter it also requires other non-protein substrate like carbohydrate and lipid along with nitrogenous substrate.

So it may be concluded that during pre-paratory II (March–May), pre-spawning (June–July) and spawning (August–September) the diet of Channa should contain large amount of protein but during post-spawning (October–November) and preparatory I (December–February) it requires also carbohydrate and lipid along with the protein in its diet.

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REFERENCES

1. Das, S., Das, A.B. (1985) Effects of temperature on nitrogenous exretion of two Indian freshwater teleosts, Channa and Labeo. Indian J. Com. Anim. Physiol. 3, 65–71.
2. Datta Munshi, J.S., Patra, A.K., Hughes, G.M. (1982) Oxygen consumption from air and water in Heteropneustes fossilis (Bloch) in relation to body weight at three different season. Proc. Indian Natn. Sci. Acad. (B) 48, 715–729.
3. Kutty, M.N. (1972) Respiratory quotient and ammonia excretion in Tilapia mossambica. Mar. Biol. 16, 126–133.
4. Nemcsók, J., Györe, K., Oláh, J., Boross, L. (1984) Effect of NH_3 on blood glucose and catecholamine level, GOT, GPT, LDH enzyme activity and respiration of fishes. Symposia Biologica Hungaria 23, 209–217.
5. Parvatheswararao, V. (1971) Compensatory metabolic regulation to seasonal thermal stress in the tropical freshwater fish, Etroplus maculatus (Blecker). Indian J. Exp.Biol. 9, 40–44.
6. Parvatheswararao, V. (1972a) Metabolic compensation during thermal acclimation in the tissues of tropical freshwater fish, Etroplus maculatus (Teleostei). Biol. Zbl. 91, 681–693.
7. Parvatheswararao, V. (1972b) Assessment of metabolic compensation to thermal stress in poikilotherms–A critical evaluation. J. Sci. Indian Res. 31, 273–278.
8. Patra, A.K., Datta Munshi, J.S., Hughes, G.M. (1983) Oxygen consumption of the freshwater air-breathing Indian siluroid fish Clarias batrachus in relation to body size and seasons. Proc. Indian Natn. Sci. Acad(B). 49, 566–574.
9. Peer Mohamed, M. (1981) Metabolism of Tilapia mossambica (Peters) with emphasis on hypoxia. Indian J. Exp. Biol. 19, 1098–1100.

10. Peer Mohamed, M., Kutty, M.N. (1981) Respiratory quotient and ammonia quotient in Tilapia mossambica (Peters) with special reference to hypoxia and recovery. Hydrobiol. 76, 3–9.

11. Reddy, T.G., Ramasamy, T., Ramasamy, M. (1978) Biomodial gas exchange of Anabas scandens (Cuvier) in relation to temperature. J. Annamalai. Univ. Sci. 31, 35–38.

12. Roy, P.K.,Datta Munshi, J.S. (1984) Oxygen uptake in relation to body weight and reepiratory surface area in Cirrhian mrigala (Ham.) at two different seasonal temperatures. Proc. Indian Natn. Sci. Acad(B). 50, 387–394.

13. Roy, R. (1987) Seasonal acclimatization of protein metabolism in an Indian air-breathing teleost, Channa punctatus (Bloch). Ph.D. Thesis, Visva-Bharati University, Santiniketan, India.

14. Roy, R., Das, A.B. (1986) Endogenous nitrogen excretion of an Indian air-breathing teleost, Channa punctatus (Boch). Indian J. Exp. Biol. 24, 132–133.

15. Roy, R., Das, A.B. (1987) Season-specific effect of starvation on the rates of oxygen consumption ammonia-N excretion in Channa punctatus (Bloch). Indian J. Comp. Anim. Physiol. 5, 34–39.

16. Roy, R., Das, A.B. (1988) Seasonal reorganization of nitrogen metabolism in an Indian air-breathing teleost, Channa punctatus (Bloch). Aquaculture (submitted).

17. Roy, R., Das, A.B. (1988) The effect of thermal adaptation on the levels of nitrogenous excretion of Channa punctatus (Bloch). J. Therm. Biol. (Submitted).

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Methoden zur Pflanzenhormonanalyse

G. SEMBDNER, G. SCHNEIDER, K. SCHREIBER

VEB Gustav Fischer Verlag, Jena (1988), pp. 296, 77 figs, 64 tables

One of the most serious problems of the progress in plant hormone research is the fact, that there is no suitable basis for the quantitative comparison of the experimental data published in great numbers in this field, because of the differences in experimental methods. That is the reason why hormone research requires methodical standards to make quantitative evaluation more clear-cut. This modern laboratory manual is of real help in this respect written by authors in the international forefront of their research field. Authors modestly consider their work a handbook, which makes it easier for the beginners to get an introduction into hormone research, and also for the more experienced scientists to switch over to neighbouring research fields by making reliable an up-to-date methodology accessible to the widest circle.

Considering the traditional classification of hormones the handbook divides the topic into the following chapters: extraction and purification procedures; column- thin layer- and paper chromatography; gas and high pressure liquid chromatography; bioassays; immunological methods; enzymological methods; spectroscopy and isotope technique.

Beside hormone analysis the book also enables us to get some insight into the examination methodology of hormone metabolism. The examination methods of some endogenous regulators not yet enlisted generally among hormones, jasmonic acid and brassinosteroids and triacontanol as well as that of the so-called flowering hormone can also be found in the manual. The authors also kept in mind that different plant parts and experimental purposes and also diverse laboratory circumstances may require distinct methods, in accordance with this they convey several versions of the experimental methods.

The publication of this manual hopefully encourages also the Hungarian plant physiologists to get down to solve research tasks in connection with plant hormones.

E. Köves (Szeged)

Akadémiai Kiadó, Budapest

Primary Processes in Photobiology

(Springer Proceedings in Physics Vol. 20)

Proceedings of the 12th Taniguchi Symposium, Fujiyoshida, Yamanashi Prefecture, Japan, held December 7–12, 1986

T. KOBAYASHI (Ed.)

Springer Verlag Berlin–Heidelberg–New York–London–Paris–Tokyo, pp. 243, 153 figures, 6 tables

This proceedings volume provides a good summary of recent results from studies in photosynthesis, phototaxis and vision. It demonstrates how new, sophisticated techniques can be used for the measurement of the primary photobiological processes, including pico-, nano- or femtosecond absorption studies, photochemical holeburning and Stark spectroscopy. A new experimental technique is introduced: the vibrational and electronic dephasing time measurement with the use of temporally incoherent light.

In the chapter Photosynthesis and Phototransformation a theoretical treatment of the electron transfer is demonstrated as well experimental data of the excitation energy transfer in chloroplasts and in artificial multilayer films of phycobilin-chlorophyll system. New results are discussed on the photosynthetic bacterial reaction centers. The volume provides new data on the primary events of the light absorption by bacteriorhodopsin and of photochemical reactions in systems I and II of photosynthesis. Using the above mentioned modern methods, new intermediates are reported in the phototransformation pathway of phytochrome.

The chapter of Rhodopsin, Sensory Rhodopsin, Phoborhodopsin and Retinochrom describes new intermediates in the photocycles of these pigments.

In the chapter Bacteriorhodopsin transient and ordinary Resonance Raman and NMR spectroscopy measurements give further structural information about the chromophore structure and photochemistry in bacteriorhodopsin and in halorhodopsin.

B. Böddi (Budapest)

Informative Morphogenetic Variants in the Newborn Infant

K. MÉHES

Akadémiai Kiadó, Budapest (1988), pp. 228, 52 figures, 18 tables

The informative morphogenetic variants (using the new term and definition by Pinsky) in themselves harmless phenomena which may also occur in completely healthy individuals, may alert the physician to the possibility of a hidden major abnormality. The detection of the informative morphogenetic variants (IMVs) has aroused increasing interest also in the classification of obvious congenital disorders. In addition to syndromology, minor morphological features have been more and more utilized in teratogenicity research, anthropology, forensic medicine and especially in estimating the prenatal or postnatal onset of certain, disorders with special regard to "idiopathic" mental retardation.

The author was the first to compile a booklet edited in 1983 on their incidence, diagnostic criteria, and relevance in newborn babies. The

fact that this book has been out of print very soon after its appearance showed a real need for a comprehensive manual on "minor congenital anomalies", which had previously been dealt with only superficially in the appendices of a few syndrome-atlases.

This completely revised and enlarged edition takes into consideration new aspects and data in the literature and the author's own examinations on a large number of liveborn neonates. Part One contains characterization of IMVs in general, including definitions and terms, incidency, their relation to major disorders, gestational age and birth weight etc., and their clinical significance and diagnostic, predictive value is discussed as well. Part Two deals with the diagnosis and significance of individual informative morphogenetic variants. According to new aspects provided by the literature of the recent few years a distinction is made between true minor malformations, dysplasias, deformities and phenogenetic variants and the IMVs are classified according to pathogenetic mechanisms.

As before, the author has been guided mainly by practical purposes, and his new book is intended in the first line to help physicians performing routine examinations of newborn babies, but it will be enjoyed by all pediatricians, geneticists and anthropologists, as well.

L.G. Lampé (Debrecen)

Multifunctional IgG and IgG-binding Receptors

J. GERGELY

Akadémiai Kiadó, Budapest (1988), pp. 273, 67 figures, 18 tables

This monograph attempts to summarize our knowledge about receptors and signal transforming mechanisms in general, about antibodies and Fc receptors as multifunctional molecules. The aim of the author is to introduce these latter molecules in the sense of Ehrlich's side chain theory and to prove and show that these molecules perform essential regulatory functions at different levels.

Witnessing the unbelievable development of immunology in the last decades it is of course impossible to summarize all our knowledge about antibodies and antibody-binding receptors in one monograph, but I am convinced that the author has very successfully fulfilled his aim; the monograph, consisting of three chapters and based mainly on author's and coworkers own investigations and discoveries, gives a new horizon to the readers about the organism's regulatory mechanisms both at the molecular and cellular levels. A detailed reference list given at the end of each chapter is also of immense value to the readers.

Though J. Gergely writes in a concise but easily understandable style, the monograph cannot be considered to be an "easy reading", as he takes the basic knowledge in immunology of the readers for granted. Considering, however, the fundamentally original approach of the author to his topics, the monograph can be recommended not only to immunologists, but to all medical doctors and biologists interested in modern aspects of medicine and biology.

I. Földes (Budapest)

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2. De Duve, C. (1959) Lysosomes, a new group of cytoplasmic particles. In: Hayashi, T. (ed.) *Subcellular Particles.* Ronald Press, New York.
3. Umbreit, W. E., Burris, R. H., Stauffer, I. F. (1957) *Manometric Techniques.* Burgess Publishing Co., Minneapolis.

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