Final general discussion

Future directions

Rubel: What I want to do first in this final discussion is to ask the cell biologists here to suggest where this field should go.

Watt: I am working on epidermal stem cells and I am therefore particularly interested in the progenitor cell question. If I were working in this field I would be going in the direction that Anne Calof has chosen, trying to develop cell culture models. It's not realistic to attempt to produce a whole sensory organ in culture, but it might be possible to grow cells that retain some useful characteristics, such as the ability to generate differentiated progeny. So firstly I would like to see some more cell culture work.

Secondly, the question of cell type-specific markers has come up frequently in the symposium. Many antibodies to potential markers are available and can be obtained either as gifts from the labs that developed then, or from commercial suppliers. There are monospecific antibodies to individual keratins, for example, and the tissue distributions of most keratins are quite well understood.

I am struck by the importance of cellular interactions with basement membrane in these sensory organs. The last three years have seen a huge increase in our knowledge of integrin receptors for extracellular matrix molecules (reviewed by Hynes 1987, Ruoslahti & Pierschbacher 1987, Hemler 1990) and their functions. Again, monoclonal antibodies specific for individual subunits are available.

In my field, people were using lectins to look for different cell subpopulations about 10 years ago (Watt 1983). This approach is not so popular these days, partly because identifying the molecular markers detected by lectins can be a problem: one lectin may bind to several different glycoproteins on the cell surface. Nevertheless, lectins provide a well-established way of picking out different subpopulations of cells.

So there are two approaches, cell culture models and well-defined cell markers, that one would be looking for in sensory epithelium research during the next 2–3 years.

Potten: I can't add very much to that, other than to emphasize the value of the use of various cell markers, particularly at an individual cell level within the various sensory organs. Papers presented here have shown that cells in the various structures studied generally show keratin 19; but does every single cell within the structure show it? And, if there are some cells that don't, what other markers might they possess? I would also reiterate the value of the use of cell culture techniques. I would also suggest that it will be advantageous to develop and use a much broader range of markers.

I have been trying to think how to tackle the question of the progenitor cell and the labelling problem, and it seems clear that more work is needed here, particularly with *pulse*-labelling and double-labelling techniques, to track down the lineages. It may involve some detailed grain counting, and a quantitative analysis of the stereological relationships between pairs of cells, and pairs of labelled cells. Those sort of approaches might help with the tracking of lineages and their progenitors and progeny in sensory structures.

Farbman: This has been a symposium on regeneration, after all, and something that seems to be lacking here is any information on the molecular signalling that triggers regeneration. Regeneration is a response to an injury; whether it's noise in the ear, or bright light in the eye, or ouabain, or denervation in the taste bud, some signal has to be sent to a responding tissue—the progenitor or stem cell, whatever you might call it. What I don't see is work on what this signal might be, that turns a switch, resulting in the regeneration of the sensory system.

Rubel: I totally agree with you. This is something that Jeff Corwin and I have talked a lot about.

Presson: One of the most striking things about the symposium is the number of different kinds of paradigms that can produce regeneration. This poses a problem for identifying the signal to regenerate, because we have all kinds of different cells that can respond to the signal; in the same systems we appear to have various cells responding to a signal, and we have a myriad different ways of inducing the signal. It is extremely hard, considering, say, damage due to noise and to gentamicin, to try to find out what the commonalities are, to determine the nature of the signal.

Rubel: We have to be careful. Just as with developmental interactions, however complicated the interactions can be, there is no guarantee that the 'signal' is necessarily going to act directly on the missing progenitor cells or stem cells, if they exist. It may be a cascade of events that is important.

Lewis: I agree with Fiona Watt that the key to identifying the signal and figuring out the molecular mechanisms must come from culture systems like Anne Calof's. But there are pitfalls in using those system as models for normal development: for instance, Anne was looking at the differentiation of cells that have migrated out of the epithelium, and are living in a very unnatural environment. Therefore any such tissue culture study has to be accompanied by parallel studies in the more or less intact organism, so that you can distinguish artifacts from normal physiology.

Watt: You do have the option of assaying the cultured cells by transplantation into animals. One problem with the cell culture approach is that many of the species that are being worked on in this field are non-mammalian: the range of culture media available is smaller than for mammalian cells.

Calof: In many of these techniques where you induce the regenerative response, there must be a tremendous amount of inflammatory reaction in the

tissue that has been assaulted, with a zinc sulphate lavage, say, or even a bulbectomy. There is a lot of precedent for the potential role of cytokines secreted by cells of the immune system that are involved in inflammatory responses, and potentially affecting the responses of all the cells in the system—not just those in the sensory epithelia or their progenitors, but the underlying stromal cells. These things could be important in the regenerative response. This is another area that we have to look at. I think it can be approached *in vitro*. It's a wide-scale 'factorology' approach—many of these cytokines are available commercially. We might also want to take a look *in vivo* at the extent of these kinds of protective inflammatory responses after the various assaults, and whether those responses might affect sensory neuron regeneration. One reason I tend to look in developing systems is that for some of these tissues it's easier to do a lot of surgical manipulations without causing tremendous tissue damage and bleeding, which occur in adult tissues. But I am sure that inflammatory responses must be important in these regeneration models. I don't know if anybody has looked, in regeneration in the ear or in olfactory epithelium, at cytokine production and the extent of inflammation after, say, zinc sulphate lavage.

Margolis: Intranasal lavage with zinc sulphate is tricky because the extent of the damage is a function of the concentration and mode of application, as well as the species and age of the recipient. Our own experience with intranasal lavage using zinc sulphate or Triton X-100 indicates that each of these parameters needs to be customized for the specific situation. With these caveats in mind, intranasal lavages have been used to generate a number of model lesions useful for the study of olfactory regeneration. The lab of Michal Schwartz at the Weizmann Institute in Israel has been studying optic nerve regeneration in fish and suspects the involvement of interleukin, but I don't know of any reports in the olfactory system that indicate the involvement of any cytokines or related factors in the process of degeneration–regeneration.

An alternative strategy for identifying such molecules is to use molecular biological approaches and compare, for example, a completely degenerated versus a regenerating system, or versus a normal system, to identify what genes are being turned on (or off) and what are the endogenous agents regulating those genes. That is another way of studying regeneration in sensory systems from a molecular orientation. For example, I am impressed by the potential of the gentamicin treatment, where you seem to get complete destruction of hair cells. This could be used to create subtractive cDNA libraries, to look for some of the genes that are intimately involved in the regenerative process.

Reh: One problem that will get in the way of doing those kinds of experiments, ultimately, in many of these systems, is the isolation of the progenitor cells in, say, the lateral line or hair cell system. That may be a potential pitfall. Perhaps the best way is to generate cell lines from these progenitor cells, once they are identified.

Margolis: The technologies have become highly microminiaturized now.

Reh: But how are you going to dissect out a single support cell from a mechanosensory organ? That will be the real challenge.

Margolis: In a recent paper, Van Gelder et al (1990) have demonstrated that one can use individual Purkinje neurons in the whole-cell patch configuration to generate cDNA. Thus, the cell itself becomes the 'test tube' for the initial incubation steps. If this approach can be generalized, many of the problems associated with small numbers of cells and limited availability of material may be surmountable.

Rubel: One complication of that approach is that many changes occur in these tissues after damage in addition to the specific events that trigger mitotic activity. The number of irrelevant cDNA clones you will get, even by subtractive techniques, will therefore be large. But it is certainly an important approach.

Raymond: In regard to looking at the genes involved in regenerative processes, I would reiterate what Julian Lewis spoke about in his paper, namely the advantage of looking to *Drosophila*, where there has been much more rapid progress in identifying genes and gene products that are involved in these kinds of 'fate' decisions and cell–cell interactions. I agree with his argument that it might appear bizarre to expect homologies between the regulatory developmental genes in insects and in vertebrates, which have very different developmental origins, but nevertheless it is apparent that there are such homologies and these could be very useful. So, in looking for 'candidate genes' to target the genetic search for relevant molecules, it is wise to pay attention to the *Drosophila* field.

Corwin: Another problem (like the one Tom Reh raised) that is specific to the organs and cells that we are interested in is that often sensory cells are highly specialized. In almost all the cases we have been talking about, at least two cell types are involved—the sustentacular (supporting) cell and the sensory cell. The extreme example of that is the electroreceptive hair cells that Harold Zakon described. It may be that these two types of cells have some sort of interdependence. The biological basis for the older concept of trophic support by the supporting cells may predict problems that will be encountered when we try to establish these sensory cells in culture. We may have to use an organ culture approach rather than a dissociated cell approach because of that.

Raymond: Many clever experimentalists in several fields have begun to study preparations that are somewhere between the intact system and isolated cells in a dish. I am referring to slice preparations and partially dissected explants that are placed into culture and observed for durations ranging from hours to days. Those kinds of preparations could span the chasm between the cell culture system with all its problems, and the whole animal with its different set of problems.

Reh: Let me reinforce that comment, particularly with respect to our results in retinal regeneration in the chick. You get a very different response of the cells in a dissociated cell culture as opposed to an aggregated culture. We should

explore some of these other sensory systems in an organ culture approach, to see if we can reiterate some of these regenerative processes, to learn what other cells are necessary for us to recapitulate the process of regeneration in the sense organ.

Principles of regeneration

Rubel: Russell Fernald is now going to suggest a structured way of putting together some of the information we have heard.

Fernald: I propose that we consider the regeneration of vertebrate sensory cells as lying on a continuum which extends from cellular repair to replacement to system regeneration. By organizing the data in this way, we may be able to understand *whether* and *how* typical cellular maintenance relates to the wholesale repair of sense organs.

If we assess the available experimental results as a function of sensory system type and phylum, two general rules emerge. First, the likelihood that regeneration of a sensory system will occur increases with the proximity of the receptor surface to the environment. Second, the likelihood that regeneration will occur decreases along a continuum from cold-blooded to warm-blooded vertebrates (Table 1). One caveat to these considerations is that, so far, the methods used to produce damage in sensory systems are primitive. Sensory systems have evolved sophisticated mechanisms for identifying subtle changes in the animal's world—systems capable of single photon or single molecule levels of detection. Yet, damage has been produced using massive noise exposure, antibiotic drug overdose, or metabolic poisons—techniques which might generously be called crude. I believe we shall come to understand how sensory system repair is related to cellular renewal when subtle damage is caused and the response to this can be adequately measured. Less abusive insults may more readily reveal the potential of these systems to repair themselves, making normal mechanisms experimentally accessible.

To illustrate these principles, let us first consider vertebrate rod photoreceptors. As in all cells, cellular and molecular repair occurs in photoreceptors continuously. However, in photoreceptors, it is particularly evident because of the striking structural polarity required for detecting photons. Young (1967) first demonstrated that photoreceptor outer segments are renewed in an organized fashion. Disk membranes in the outer segment of rod photoreceptors are continuously renewed, being assembled at the outer segment base, displaced outward by new disks, and eventually shed at the tip. This renewal process is regulated at the molecular level by light and a circadian rhythm (Korenbrot & Fernald 1989).

In their recent study, Faktorovich et al (1990) discovered two important features about photoreceptor renewal in cases where photoreceptor damage is induced or cell death occurs (reviewed by Roy Steinberg, p 219–223). The first

TABLE 1 (Fernald) The continuum of vertebrate sensory cell regeneration

	Process			
Phyla	*Cellular repair*	*Cell replacement*	*System replacement*	
Mammals	_____			
	------?			
	.....?			
Birds	_____			*In vitro* only
	----------			Function?
	.....?			
Amphibians	_____			Function?

	.....?			
Fish (teleosts)	_____			Function?

	.....?			

Key: _____, vision; -------, hearing; ....., olfaction.

feature was that basic fibroblast growth factor (bFGF) served to mitigate damage to the photoreceptors and the second that the effects of bFGF were maximal when the factor was administered before inflicting the damage. There is no evidence yet that photoreceptors, much less retinas, can be replaced in mammals by intrinsic processes, but this may not be an unreasonable goal for molecular medicine. Since photoreceptors are related to epithelial cells (Land & Fernald 1991), using molecular tricks, we may ultimately be able to make them replace themselves.

In contrast to photoreceptors, in the olfactory system the receptors turn over regularly during life. This means that not just cellular repair but cellular replacement is common. Whether the whole olfactory system can be replaced after damage remains an open question. And for the present, we have to conclude that in mammals, there is no auditory cell or system replacement, from the evidence summarized earlier in this volume.

As we move to the other phyla in Table 1, we see some significant differences in the amount and likelihood of sensory cell regeneration. In birds, the amount of cellular repair seen in visual system cells is limited to work in dishes (*in vitro*) by dish jockeys. And in this case, it is evident that cellular fate can be altered, rather than being true cellular replacement. In the auditory system in birds, cellular replacement clearly occurs, but there is little compelling evidence that this represents functional recovery since adequate behavioural tests are not yet available.

In amphibians, regeneration of the visual system is well documented, although functional analyses of that recovery are not adequate for us to understand how

much recovery has occurred. Auditory replacement is unknown, but we might expect that this occurs on evolutionary grounds. I do not know if total system replacement has been demonstrated in amphibian olfaction.

In teleost fish, the same general questions arise: is there behavioural evidence that regenerated sensory systems can function, and how good are the 'new' systems? In olfaction, there is some evidence that the recovery is complete, but compelling behavioural evidence is lacking. Similarly in vision, histological views of the retina suggest that there must be a reduced visual capacity.

In the context of this overview of vertebrate sensory cell regeneration, four general questions seem to characterize discussions at all levels of the sensory systems we have heard about at this meeting. They are:

(1) Does the response to damage resemble a recapitulation of ontogenetic events, and to what extent does that help in understanding repair?

(2) How many types of stem cell are there and how can we identify them?

(3) What rules do stem cells follow when deciding when and where to divide? This is the basic question that we need to answer.

(4) What extracellular and intracellular factors regulate stem cell fate?

How can we answer these questions? Finding answers depends in part on the system used to ask them (Table 2). We have seen here the range from *in vivo* to *in vitro* systems, but organotypic cultures, which lie in the middle of the range, may be an important adjunct to either of these more conventional approaches, for several reasons. First, in a number of cases there is now evidence that organized groups of cells provide a more realistic representation of the *in vivo* reality than do conventional *in vitro* culture systems (e.g. Reh et al 1991: this volume). Second, in organotypic cultures it is possible to have some structural integrity, allowing easier analysis of experimental results (e.g. Mack & Fernald 1991). Finally, the application of extracellular factors can be compared within a single culture, providing within-system controls that are not possible in typical *in vitro* systems.

Rubel: We are really talking about two different things under the heading of regulation. One is the events that regulate the *differentiation* of the postmitotic

TABLE 2 (Fernald) Systems in which four fundamental questions might be asked

	Questions[a]			
System	*1. Recapitulation?*	*2. Stem cells?*	*3. Rules?*	*4. Regulation?*
In vivo	+ +	+ +	+	
Organotypic culture	+	+ +	+ +	+ +
In vitro			+	+ +

[a]See text.

cells; the other is the events that regulate the *mitotic activity* of stem cells or progenitor cells. From what we now know about cell biology, there are likely to be different kinds of genes involved in these two types of regulation.

Presson: And where do we see differentiation coming in? One could include it with stem cell regulation, but what turns on and regulates stem cells might be slightly different from what actually makes a differentiated neuron. But we don't know anything here yet.

Pujol: We need to discuss question (1), of whether the response to damage is really a recapitulation of ontogeny.

Fernald: My feeling is that we should see the study of ontogeny as one way of understanding the response to damage, because we know a lot about ontogeny. It would be nice if the actors that did the work during development were also involved in repair. But this may not be the case, because it may be a superficial comparison.

The question really is the usefulness of making a distinction between the recapitulation of ontogeny and a new mechanism that responds to damage. If it were shown to be a recapitulation of ontogeny, you would then have a range of putative molecules and cells that you can turn to and say that they are probably all still active, or reactivated. Whereas if a whole new mechanism is involved it is a much tougher question and we might be better off going to different systems where there really is a recapitulation.

Pujol: There is something in the chicken hair cell regeneration studies which does not completely recapitulate ontogeny. In a damaged cochlear preparation in the chick, you have regeneration of the sensory cell but, as Brenda Ryals is showing (personal communication), a decrease in neuron number. so there is something completely different here. You can produce new sensory cells in the chick repair model but no new neurons, and this is very different from ontogeny.

Corwin: I don't think there is any doubt that there are very significant differences between ontogeny and regeneration.

Rubel: Would anybody take a strong stand, in any of the sensory systems studied, that the repair we see is a recapitulation of ontogeny?

Reh: In vision, Pamela Raymond and I have both found, in the frog and in the goldfish, that it's not a recapitulation in the sense that it looks the same; however, it seems as if stem cells, or progenitor cells, have to reproduce the entire programme of development and to revert back to some common neuroepithelial ancester and make all of the same types of neurons again, probably in the appropriate sequence. The amphibian retina differentiates during regeneration in the same sequence as in its original development. So I would argue that once you have regenerated a neuroepithelial cell, it will recapitulate the normal sequence of retinal development.

Raymond: I would agree with that, for both frog and fish. If you look at the level of a single cell and its progeny, you are probably looking at a

recapitulation of an embryonic pattern, whereas if you look at the system as a whole, there are clearly differences, of both spatial and temporal order. The process as a whole does not happen in the same way during regeneration as during development. For example, the size is different: in the embryo you start with a tiny structure, but in regeneration the structure to be replaced is much larger. However, at the level of the individual progenitor cell and in terms of the cascade of events that take place in a developing clone of cells, one could make a good case that regenerative events are probably a recapitulation of development.

Steinberg: There is one highly significant proviso with the retina; we have to regenerate both the retina and its connections to the brain. Whether we can recapitulate that in any of these experimental systems is unclear and is very difficult to determine. At the cellular level, of course, the regrowth of the outer segment does appear to recapitulate development.

Corwin: We can make a generalization that the tissue differentiation that occurs during regeneration recapitulates the differentiation seen in the embryonic development of the same structures. I would even extend that to predict that if we can stimulate the production of undifferentiated 'progenitor type' cells, in for instance a structure such as the mammalian ear, which currently is not known to regenerate, there will be a high likelihood that regeneration will occur there with differentiation following that normal developmental path. If we consider the course of regeneration in planaria, or in regeneration of various appendages in invertebrates or vertebrates (see Goss 1969 for reviews), the limiting factor appears to be the production of a group of proliferating, undifferentiated cells. If they exist, or if they form at the site of a wound, then the repair process will go on and follow the embryonic pattern.

Reh: We could add that neuroepithelial cells not only differentiate in their normal pattern; they also retain their identity to a large extent. For example, a neuroepithelial cell of the retina, whether derived from a pigment cell, or derived from a rod precursor cell, is going to make retinal neurons; it's still a dividing cell, but it's not going to make olfactory neurons. This is probably also a generalizable feature of stem or progenitor cells, that they will retain their identity.

Fernald: That is untested and can only be tested by explantation to another environment.

Cotanche: People have taken the visual system of the animal, destroyed the visual cortex, and allowed it to grow into the auditory system; they get an electrically functional visual cortex in the auditory area.

Rubel: That's a separate type of situation, because the receptor system is left intact.

Cotanche: Tom Reh is saying there's no evidence that these things could convert to other systems.

Reh: There is considerable evidence from embryological transplant experiments that neuroepithelial cells retain their regional identity. When eye primordial cells (amphibian or chick) are transplanted to ectopic locations in the embryo, they retain their ability to form eyes (Adelmann 1930).

Corwin: In more general terms, all of this starts with a single cell, the zygote, which has the capacity to produce all of the specialized cells. Gradually, as determination occurs, there is restriction in the normal outcome of differentiation for the various types of cells that are produced and what they can produce.

There is an interesting story with regard to the positional memory of cells and retinoic acid's ability to modify and re-set what a cell knows it is, or has the capacity to form, during limb regeneration. In the salamander, *Ambystoma mexicanum*, if you amputate a forelimb below the elbow, blastema cells that aggregate at the stump do not normally produce a second new elbow and upper (proximal) limb; instead they form the appropriate bones of the distal forelimb, the wrist, and the hand. If they are treated with retinoic acid, their self-identity or positional 'memory' can be re-set, so that they are 'proximalized' and will form structures that duplicate those that are proximal to the level of amputation (Thoms & Stocum 1984, Crawford & Stocum 1988).

There seems to be a dose dependency: at lower doses you can get a re-setting to above the elbow, to produce a new upper limb; at higher doses you can even re-set the blastema cells so that they first produce a new shoulder girdle (Thoms & Stocum 1984). This fits with what we have talked about here, that perhaps these undifferentiated cells may have to go through a number of divisions in order to re-set their state of specialization and the restriction of potential fates.

As I mentioned earlier (see p 107 and 124), I have been impressed with the implications of Jay Jones's finding that when he traced back in time-lapse recordings from the point when two regenerated hair cells had been produced in a laser-treated neuromast there was a sequence of two cell divisions in the lineage of cells that gave rise to each of those regenerated sensory cells. The original progenitor that was present at the time of the laser treatment was the 'grandmother' (as we call it) and appeared to be a supporting cell that gave rise to two daughters, and one of those two cells gave rise to two granddaughters, one of which became a hair cell. We don't know whether that will prove to be a constant feature of this sensory regeneration, even in the lateral line system. If that were the case—if the original cells that were present at the time of the trauma had to go through a certain number of cell divisions to re-set their state of specialization—then that would be consistent with the existence of processes during cell division that have the effect of reversing the restriction of the capacity to produce new types of cells.

Rubel: Going on to the stem cell, are we convinced in any of these receptor systems that we really know the identity of stem cells, or the identity of the progenitors of the receptor cells?

Farbman: In the olfactory system, we know what we think the stem cells are! There are the basal cells which fall into two groups: one of them is a progenitor and one is a stem cell, according to the current view.

Calof: There are what people call these two kinds of basal cells, although I'd like to throw out 'basal cell' as a term. The two types are the lighter globose kind, which I call the immediate neuronal progenitor, and another kind, called by various people the horizontal cell and the dark basal cell. I would say that the light (globose) basal cell is the immediate neuronal precursor, but it is still not clear whether the horizontal basal cell, or some subset of these cells, or a subset of the lighter cells, is the true stem cell in the olfactory epithelium. I really think that the stem cell, if there is one—the true ultimate progenitor of the olfactory receptor neurons—has yet to be clearly identified.

Fernald: Part of the problem is that we need to know what the signal for cell division is. The idealized way to ask this question would be to place 'the signal' for a stem cell to divide onto cells in sequence. You could ask directly how many are competent to become stem cells. However, there may be, under different circumstances, different populations that are competent. In the cases of extreme damage, we may bring into action all sorts of normally extraneous cells which are driven because of the extremity of the situation; whereas 'real' stem cells are far fewer. It may be that there are different populations of potential stem cells depending on the extent of the destruction we impose.

Reasner: The severity of the perturbation is especially relevant in systems where there are ongoing replacement and growth mechanisms, such as the olfactory system. For example, there is continual proliferation at the margins of the respiratory epithelium in adult mice (unpublished observations). In both mice and rats, there is continued growth of the olfactory epithelium after the age of sexual maturity (Hinds et al 1984, Sichlau et al 1990). So, if you could use a paradigm of intense sensory stimulation which does not massively destroy the sensory epithelium, you might realistically expect to see different regenerative mechanisms from those following more invasive paradigms.

Potten: I agree that the problem with identifying stem cells in various systems is context dependent (Potten & Loeffler 1990). The answer to the question 'what are the stem cells?' will depend on the system that you are looking at; you get a different answer if you are studying an amphibian system rather than a mammal. We heard planaria mentioned. These flatworms have some extremely primitive (stem) cells in the adult that can regenerate the entire worm. That isn't the case in mammals! Amphibia can regenerate a limb; that is also not the case with mammals. So the answer to the question depends on the system being examined and the tests being applied. Because of this context dependence of the stem cell identification and characterization, we have to be as careful in transferring information from *in vitro* to *in vivo* studies, from mouse to man, from birds to mammals, and particularly from amphibia, reptiles or fish to mammals, as from invertebrates to vertebrates and mammals.

This also involves the question of the severity of damage in experiments. In many of these sensory systems, if you destroyed a single cell within a structure, and asked which cells replace it, you might get a very different answer from the experiments where you destroy the whole structure and ask which cells replace that entire structure. The electroreceptors in the teleost fish clearly indicate this. There must be different types of stem cells, firstly those that are capable of going through many divisions and differentiating in a variety of ways when there is the fission of the ampullary organ that Dr Zakon described. However, these cells are different from those seen when a piece of skin is cut out and this then regenerates the organs, because Dr Zakon told us that these stem cells come from somewhere else: they are epidermal cells that have had some interaction with the nerve fibre. So the answer to the question of which are the stem cells in this electroreceptor system is dependent on the experiment you do. That is probably true for all the sensory systems that we have been looking at.

Raymond: The obvious question, that has been asked many times, is whether the capacity to regenerate is dependent on continued growth. Does regeneration occur only in those systems that are continually growing?

Lewis: The answer is clearly 'no'!

Raymond: That would seem to be the case, if we believe that hair cell proliferation does not normally occur in the avian ear. However, in all the other cases of regeneration that have been described at this meeting, there is evidence for proliferation in the intact sensory system.

Margolis: To what extent is the definition of a stem cell a function of who the neighbouring cells are? We seem to be talking of an isolated stem cell, but we know that during regeneration and development the so-called stem cells, and in fact the whole cellular environment, will be different from the normal quiescent tissue. Is a stem cell a function of where it is?

Rubel: We have to run the process backward in time to determine the answer to that question.

Watt: In adult mammalian tissues we tend to think of stem cells as being present in tissues where there is a high rate of turnover of the terminally differentiated cells under normal conditions, for example in the epidermis and the haemopoietic system (Hall & Watt 1989). In at least some of the sensory organs you would expect that in the absence of environmental damage, there is not much cell replacement going on. So the sensory systems might be more analogous to a tissue such as liver, where you don't normally see much cell division, but if you remove half the organ, many of the cells are stimulated to divide until the damage is repaired.

Rubel: What happens to these stem cells? Are they quiescent cells that are suddenly kicked into function in the liver, or are they cells that have some function and differentiated property and then revert to being stem cells when the right stimulus is presented?

Watt: They are probably not a discrete subpopulation.

Potten: I don't think that's entirely clear! In a mouse liver, if you cut out three-quarters of it, there is a fairly synchronous burst of cells entering DNA synthesis, but not every cell in the liver enters S phase—only 20% or so. But you don't know what regenerative function the cells that appear in that first wave really have. Are they the cells that go on to divide several times and regenerate the total mass, or are they just cells that respond most dramatically to the stimulus 'divide once and stop', and other cells either come along later or emerge from the cells in the first wave, and these go through several divisions, and eventually are responsible for the regeneration of missing liver cells?

It comes back to asking the question the other way round: when the adult liver mass is reached, how does the system switch itself off? Does an entire cell lineage freeze, with all the cells in G0, or do the cells work through their predetermined lineage and just leave some stem cells in G0? This would be a small subpopulation of the liver—a part, or maybe even all, of the 20% that respond. We don't know the answers to these questions, even for the liver, which has been fairly well studied.

Oakley: There are comparable problems in the regeneration of minced mammalian striated muscle, where it's presumed that there are small quiescent satellite cells that are activated by the destructive process, and may be responsible for the substantial regeneration of the muscle (Carlson 1968).

Lewis: I want to turn the attack against the stem cell mystics! If you see a population of cells which all look similar, by all the criteria available to you, and some of these divide and do something, the first hypothesis should be (because it's the simplest) that they are indeed all alike and that it is by some random process that some of them go on to divide and do something while others, at random, don't. It's true that there *may* be hidden differences within that population of cells; some members may be determined to behave differently from others, and we should bear that possibility in mind; but until you have demonstrated such a difference, the first hypothesis should be that they are all the same.

Potten: In the mouse liver and other tissues (such as bone marrow and gut), we know that they are not; even though they look all the same, only some are capable of a regenerative response. It depends on how you are looking at them.

Lewis: Of course, I wouldn't dispute that there are cases where there are indeed subtle functional differences that people can demonstrate by appropriate techniques. But in the auditory epithelium, for instance, I see no reason to believe that there's a subpopulation of stem cells that are alone capable of regenerating hair cells and are distinct from the other supporting cells.

Presson: In teleost fish, I think there are. I don't know, again, whether they are a different part of the cell cycle of the same cell, but there are clearly, to me, subtle morphological differences between a cell that takes up [^{3}H]thymidine and a cell that doesn't.

Corwin: There is one thing we didn't mention when we discussed your data on that, namely the type of cell that you call the basally located S phase cell. That cell type, and supporting cells, are both distinguished in your definitions by having basally located nuclei.

Presson: They have very different biochemical and cytochemical characteristics. The basally localized S phase cells do not have endogenous peroxidases, for example, whereas supporting cells do.

Reh: Pamela Raymond raised the question of whether continued neurogenesis is always associated with the ability to regenerate. This has been brought up with respect to the nervous system in terms of axonal regeneration. Are we certain that continued neurogenesis is *not* needed? Surely the auditory epithelium is the exception in the bird, in that there is regeneration, yet people say that there is just an occasional labelled cell. Are those who study the bird certain that there is no continued neurogenesis under normal conditions? Slight damage occurs all the time, and perhaps cells are replaced; is that the same as saying that there is a continuous level of slight damage in the olfactory epithelium?

Rubel: You would have to look at a bird in the wild to answer that question.

Fernald: That gets back to my Table 2, where the conditions of damage have been so extreme that we can't yet answer that question; we need to have subtle, intermediate levels of destruction.

Corwin: I don't think so. Brenda Ryals can probably answer that best. In earlier discussion of Dr Jørgensen's paper we may have left the wrong impression, that there is more labelling and proliferation in undamaged avian cochleas than we were willing to admit. To me, it is striking how little labelling there is. I can't recall seeing a labelled cell in an undamaged region of the cochlea after injection of [^{3}H]thymidine for seven days.

Rubel: It is *very* rare, but does occur.

Ryals: Yes, it is rare. We saw only a total of four labelled cells out of thousands of cells in two control animals (Ryals & Westbrook 1990). Dr Jørgensen saw no labelled cells in the auditory papilla after 19 days of [^{3}H]thymidine injections, and he looked at a much larger cell population than I did (Jørgensen & Mathiesen 1988). So, if there is normal turnover in the auditory papilla, it is clearly not at the level of that seen in the vestibular end organ.

Presson: How do you interpret these cells? Are they new cells, or artifacts?

Ryals: I think they are new cells. What I don't know is what the trigger may have been for their production. My birds came to me in a carrier in a plane—a situation which is potentially traumatic. However, they were in my lab for two months before we studied them, so one would expect that any response to that trauma would have been completed by then. Nevertheless, we cannot rule out the possibility of some local cell death, for unknown reasons, as a stimulus for this new cell production.

Rubel: That is the way we interpret these rare instances of labelled cells in control tissue, but it is only an interpretation.

Fernald: The other caveat is that we should remember the vast number of vertebrate species. We are looking at a miniscule fraction of that number, and these are domesticated! If we look at the wilder species, we may see repair as a common feature.

Ryals: In fact, I saw [^{3}H]thymidine label in hair cells of normal birds in quails, which are somewhat more wild than domestic chickens!

Lewis: I wonder whether territorial birds, which gain territory by singing as loud as they can, may damage their own auditory systems?

Rubel: I think you should do that experiment!

What I would like to say, in conclusion, is that I think we have all learned something here, or at least learned how little we know. I would hope that through this symposium we have gained the ability to better define the questions that we shall be asking in our research over the next few years. Many of us may also have learned a broader range of approaches to these questions. If we can define the questions better, one of the big hurdles will have been overcome. I hope some of us will go back to our laboratories and do slightly different and somewhat better experiments than we would have done before this meeting. If that happens, I think we have achieved our goal.

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