RETURN TO RESEARCH AND UNIVERSITY LIFE

Back to Hopkins from the War

In spite of my continuing passion for biology, I gave it little thought during my years in the military. I put it out of mind to concentrate on the business at hand, defeating our enemies. This must have been commonplace for those of us in the service during the War, wherever we were. The quite different daily life of the military, with its different rules and the uncertainty of what would come next and, finally, of how long the War would last, left us year-by-year and then month-by-month in an intellectual limbo. So, we carried out our duties and waited. Personally, I was confident that once I got out and returned to civilian life and biology, the transition would be easy and so it was. Obviously, however, I had the great advantage of knowing what I wanted to do and having a job waiting for me. Also, I was lucky to avoid death, serious injury, or debilitating psychological trauma, suffered by so many of my comrades in arms during the War.

In spite of my pervasive optimism, I had changed a bit, and was a little less free-wheeling, although I was still rather uninhibited (Figure 4.1). I was seriously at my research to produce a good dissertation. Also, I now had been a married man, for over two years, with my wife heavy with our first child. The almost 28 years old and felt that I’d better buckle down and start to work first thing was to find an apartment we could afford. We ended up in a federal housing project euphemistically called Armistead Gardens—certainly no gardens but an adequate little house and a small yard. It was quite far from Hopkins but several of my fellow students with families lived there. We lived a life of convenient cooperation.

Our first baby, a boy we named Gregor, was born on January 10, 1946, in the hospital of the Medical School of the University of Maryland, located, like the Johns Hopkins Hospital, in a depressing slum area of Baltimore. It was less chic but cheaper than Hopkins. Gregor was sickly at first with a severe intestinal malady, and we young parents were frightened and deeply worried. To Galya’s
and my great relief, he soon began to thrive and thankfully remained a healthy baby after his initial difficulties. How wonderful it was to watch him gain weight.

The Department of Biology had changed (Figure 4.2). Willier, my mentor, was still there and so was Swanson, but Ephrussi had returned to France, and Skoog and Ris had left for the University of Wisconsin (Madison). Three of their replacements were young men of our age and undistinguished. Three others, however, were excellent additions: Vincent Dethier, an insect physiologist; Bentley Glass, a geneticist and an impressively cultured gentleman; and, William McElroy, a cell physiologist and biochemist. I don’t recall or perhaps never knew how these three young men had avoided the draft and were thus able to finish their graduate studies. We returning veterans felt a little cocky and snobbish about them. We all knew about how some graduate students in biology had suddenly discovered how interested they were in medical school or some other excuse when the draft was hanging over their heads. Besides, we were about the same age, had our military experience to mature us, felt that we were better biologists, and even had more children. I’m sure the new people felt the tension but of course no one talked about it.

The year started off with me getting some Brown Leghorn fowl and taking a course in biochemistry with McElroy, which was a good idea. Mac or Bill, as we soon called him, had been a student of E. Newton Harvey at Princeton
Figure 4.2. The Biology Department of The Johns Hopkins University, circa 1948, on the steps of Gilman Hall. Professor Willier is front row, center, Mary Rawles to his right, and Carl Swanson to her right. William McElroy is on the far left of the second row. John Saunders is just behind McElroy and to the left. I am in the center of the last row. James Ebert is to my right and Clem Markert is to Ebert’s right. Bentley Glass is between Ebert and Markert in the row in front of them. Nelson Spratt is just behind Willier and to the left.

and before that a varsity football player at Stanford. He was a very bright, energetic, ambitious, easily approachable man who had a robust sense of humor and really knew the field of intermediary metabolism, to which he introduced us. It was a course in biochemistry, as opposed to the physiological chemistry course I had taken 4 years before in the medical school. Here we had a fine introduction to adenosine triphosphate (ATP) and the energy-rich phosphate bond, the very basis of life, purple bacteria, C.B. Van Niel, evolution of biochemical syntheses, Fritz Lipmann, Advances in Enzymology, bioluminescence, 4-carbon acids—great stuff. McElroy’s zest was infectious. What a contrast to the stuffy, uninspiring Willier and the tough, humorless Ephrussi. He was just what the doctor ordered for me and for the Biology Department. His course really brought me back into biology with enthusiasm and I have ever been grateful to him for this (and to Willier for attracting him). Mac and I were on the same wavelength and I always enjoyed his company, even in later years when he had become a big-shot administrator, as Chancellor of University of California at San Diego, and even just before his death a few years ago. I offer these accolades for McElroy not simply because of what he meant for us at Hopkins but to illustrate how one
gifted and talented person can electrify an entire department, even a distinguished one. In my long life I personally have seldom known another quite like him. Although McElroy's catalytic effect was good and indisputable, there was a caveat. Like everybody, he was wanting in an important way. He really felt, I believe, that if the biological research was not directed to the molecular events underlying phenomena, it was less scientific, and somewhat backward. This view is unfortunately rampant today in the biological sciences. His disdain for my kind of research and the work of others (e.g., Rawles, Spratt and Saunders) didn't bother me much when I was there. I tolerated his bias because of his many excellent positive features.

Since then, however, I would not have been so tolerant. Are there any special reasons why molecular biologists believe that their research is more fundamental? My impression is that they justify this bias on the grounds that all the other levels of phenomena depend upon and are the result of actions at the chemical level. Not, however, that these people would likewise agree that chemistry is less important than physics or than quantum mechanics should supplant organic chemistry, although the same logic would lead to those conclusions. I suggest that the different levels of biology (and all scientific disciplines, for that matter) are best regarded as links in a chain, with the molecular level merely one link near one of the ends of the chain, if indeed the chain actually has ends. How foolish to argue, or believe, that one link is most important because it is nearer an end. And, when I have made this counter argument, some molecular types begin to blurt out some further justifications, especially that the other levels should be deducible from the molecular level. They like to think that logical reasoning works better from one end, their end, upward. It may be asked rhetorically if various discoveries at one of the higher levels—cells, tissues, organs, organisms, populations—could have been deduced a priori from any amount of prior molecular facts. Personally, I find the most satisfying biological research to be the kind that integrates information from multiple levels into a coherent explanatory picture. What a pleasure it is to be able to piece together molecular, cytological, and behavioral results into a comprehensive understanding of a complex biological phenomenon.

_Tissue Culture_

My preparation before the War for my research on the development of the sexually dependent pigment pattern of the Brown Leghorn stood me in good stead as I returned to research, but I didn't know what to do. There was no help from Willier, of course, but anyway I didn't want any. Although I approved of this as a way of training graduate students, it was not easy. I went up several blind alleys. For example, you may recall that the female sex hormone estradiol causes melanoblasts in the breast feather germs of a capon to form red rather than
black melanin granules. An important question before me was how does this happen on the cellular level. As each unpigmented melanoblast comes under the influence of the hormone is it transformed gradually to form at first a mixture of black and red granules or is it an all-or-none process, with each melanoblast forming entirely red melanin granules? So I looked carefully in sections of the transition zone of transforming feather germs for melanocytes with a mixture of granules, but found none. However, the fixation necessary for sectioning might well have obscured the appearance of such an intermediate stage. Since the density of the melanocyte population obscures cellular detail in the living feather germ, I had to resort to tissue culture.

Tissue culture is a powerful and fascinating technique for the study of living cells and was *en vogue* in those primitive days. One placed a piece of the tissue to be cultured on a sterile cover slip and then added a drop of clear plasma prepared from chicken blood, which we drew directly from the chicken heart, to be mixed immediately with a saline extract of homogenized chick embryos—so-called embryo extract. Addition of the latter adds essential nutrients to the plasma and causes it to clot. The cover slip with tissue embedded in the plasma clot is then inverted over a slide with a depression and sealed, giving us a “hanging-drop” tissue culture preparation. Since the tissue is in direct contact with the glass of the cover slip, we have an optical situation favorable for viewing individual cells as they migrate out of the tissue explant, forming attachments to the cover slip. All of this must be done under rigorous sterile conditions to avoid ending up with only a luxurious culture of bacteria or fungi, or both, since we did not yet have antibiotics. All cultures were maintained at body temperature, 37°C.

My approach was two-fold. First, I excised and cultured pieces of tissue from the zone of differentiation near the base of a regenerating breast feather germ at a specified time after an adequate injection of hormone in the chicken, when I had reason to expect that melanoblasts would be differentiating into melanocytes. Then I looked for transformed melanocytes moving out of the explant onto the cover glass. The second experiment was to add estradiol directly to the culture medium. Tissue culture is an exciting technique when successful because of the gorgeous spectacle of living cells moving on a transparent glass substratum. But it is often difficult to succeed with adult tissues, even those in the process of regeneration. In consequence, I had an expected hard time for a while mastering the subtleties of culturing my material. However, I finally succeeded and the effort was rewarding for a time simply because of the unalloyed, even seductive beauty of my growing cultures. In those days, many biologists had become so fascinated with the show that they occupied themselves trying varying culture techniques, using the technique of tissue culture as an end in itself. Professor R.G. Harrison, who was the first to use tissue culture to answer a fundamental biological question (whether axons extend directly from embryonic neuroblasts), once remarked in conversation that tissue culture is after
all just one of many techniques for the embryologist. He went on to suggest that those who spend much of their research lives just culturing cells had made a kind of religion of it.

But I had a serious problem to solve and hoped that tissue culture would help me solve it. Unfortunately, it did not. No matter what I tried, my cultures with estradiol rarely produced red pigment cells and certainly no intermediates. Red melanocytes appeared in large numbers only when tissue was cultured directly from red feather rudiments. I had spent a lot of time at this tissue culture approach but finally had to admit that I was not really making any scientific progress. I still have no clear idea why the cellular transformation I was looking for did not occur in my cultures. Perhaps the disorganization of the cells of the epithelial barb ridges as they spread and dispersed in culture was a factor. I had often been told that in scientific research one is frequently presented with such no-go results but one should not be discouraged. Well, here I was precisely in that situation and I was discouraged and forced to search for a new approach. I was also worried. I had expected to have my Ph.D. eight or nine months from then and I didn’t feel that I could yet write a really good dissertation. My self-confidence was shaken. My prewar roommate, Nelson Spratt, was a good sounding board for my concerns and ruminations but didn’t come up with any good suggestions.

**A New Idea Appears**

Finally, one hot summer day I had an idea, a good idea. Inspiration! I knew that melanoblasts in the breast feather germ respond to induction by estradiol by synthesizing red instead of black melanin granules. I did not know whether these same melanoblasts or their progeny would respond to the hormone if placed in the epidermis of feather germs where there is normally no response, such as the germs of juvenile wing feathers. The procedure was obvious. Transplant adult Brown Leghorn breast basal barb ridges containing responsive melanoblasts to the wing bud of a White Leghorn embryo and see if they migrate in the growing wing bud and enter the forming juvenile wing feather germs. Then determine whether they can express their ability to respond to estradiol in their new environment of immature barb ridge epidermis. I felt confident immediately that the experiment would work because Mary Rawles and Mark Nicholson had already shown that melanoblasts from adult feather germs retain their capacity to migrate. Why did I not think of this straightforward experiment before? In a matter of minutes, my despondency evaporated and was replaced by exhilaration. I immediately started planning my experiment.

I removed a small piece of the most basal barb ridges full of melanoblasts from a breast feather germ of a Brown Leghorn capon and grafted it to the base of the wing bud of a 72-hour White Leghorn embryo. I then closed
the opening in the shell of the egg and waited. I did a lot of these operations because usually many host embryos do not survive the trauma of the operation. Some did survive, some of these hatched, and some of these grew up to become big chickens with feathers of the right wing heavily pigmented with a typical Brown Leghorn juvenile pigment pattern (Figure 4.3). The next step was to inject such host birds with estradiol to determine whether the melanoblasts in their growing feather germs responded to estradiol. They did not, demonstrating that melanoblasts with an incipient genetic capacity to respond to estradiol must be associated with mature, tract specific, competent epidermis to express it. With this result I had laid the foundation for an acceptable Ph.D. dissertation and for a publication in a respected journal of the field.

Figure 4.3. White Leghorn chicken, which received a graft to its wing bud of Brown Leghorn melanoblasts as a 3-day embryo. Reprinted with permission of John Wiley & Sons, Inc.

For me this was a clear demonstration of the role mature tract-specific epidermis plays in the differentiative response of Brown Leghorn melanoblasts to the female sex hormone estradiol. The story does not end there. While performing these grafting experiments, I began investigating the activity of thyroid hormone in pigment pattern formation. In brief, a thyroidectomized fowl of either sex possesses a different reddish pigmentation and a rather scruffy plumage due to abnormal formation of the barbs of each feather. If the thyroid hormone, thyroxine, is injected regularly, the newly formed feathers show normal male or female pigment patterns respectively. Therefore, in order for a Brown Leghorn to generate a normal female feather pigment pattern at least four combined factors are necessary: 1. genetically specific, pluripotent melanoblasts; 2. mature, tract-specific epidermis; 3. a normal level of thyroid hormone; and, 4.
RETURN TO RESEARCH AND UNIVERSITY LIFE

a normal level of estradiol. There are two responding cellular systems acting somehow in concert, and two inductors, both necessary to stimulate the melanoblast—epidermis system to form a female feather pigment pattern. Incidentally, in this system the inductors are chemically defined and two of them, estradiol and thyroxine, are readily synthesized. This is an unambiguous case of multicausality operating during development. One wonders whether other factors not yet investigated might also be necessary.

There is an important general lesson here. When one discovers a factor, such as nerve growth factor (NGF) or fibroblast growth factor (FGF), acting as inductors or “organizers,” it is certainly operating in concert with other factors in the embryo that are there but have not yet been investigated. So, my hard work and some desperate thinking had a rich pay-off in the end. I not only provided some understanding of how one developing system differentiates but in addition, because of this, I am able to suggest a lesson to be considered in the analysis of other inductive systems at other stages of development. It may be objected that this is not a good model system because hormones are not classical inductors; they normally move to the target cells by way of the circulation. This is so, but it is a trivial objection. Estradiol, at least, can have the same transforming effect on melanoblast differentiation by moving directly through tissue like an embryonic inductor. This was shown many years ago in a neat experiment by two British biologists, A.W. Greenwood and J.S. Blythe, and published in 1929 in the Proceedings of the Royal Society of Edinburgh.

Melanocytes of all vertebrates are derived from the neural crest, an unobtrusive population of stem cells on the dorsal aspect of the neural tube along its entire length during very early embryogenesis. The neural tube is the rudiment of the central nervous system. The neural crest is a remarkable structure in that it is not only the source of pigment cells but also of a host of other tissues and organs, like spinal and sympathetic ganglia, the medulla of the adrenal gland, head cartilage and others. Neural crest cells are classic embryonic stem cells. We do not yet know if they would demonstrate this multipotency if transplanted to an adult but someone should certainly try to see. Neural crest cells reach their often distant destination by active cell movements, like those of melanoblasts. In contrast to the melanocytes, however, which retain in the adult their capacity to migrate long distances when grafted to the embryo, cells of these other derivatives lose their capacity for migration when they have reached their definitive location.

Personalities

Along with the big downs and ups of my research, life at home and with our friends went on its merry way. Our little family made a great leap forward. We hoped to have a daughter to compliment our son and we did. Tanya was born on January 15, 1948, without incident, and was soon a chubby, happy, little girl
baby. We also had a big black Maine Coon cat named Misha, who liked to sit on top of the piano when Galya played. He would let his tail hang down, swishing it lightly with the music.

My previous Hopkins friends were gone, except for Red Saunders, who returned from three and one-half years in the Navy in the Pacific, but new colleagues soon appeared. The one I saw the most was Clement Markert who was also a student of Willier. Like Red and me, he lived in Armistead Gardens. Also, he had a car. So I arranged to travel with him to the lab every day, sharing the gas and producing a lot of our own along the way. Clem was a definite character. He had had sufficient anti-Fascist conviction and downright guts to volunteer for the Abraham Lincoln Brigade to fight against the Fascists during the Spanish Civil War and survived to tell about it, unlike many. During World War II, the Army Air Force refused to accept him, because of his political record, so he enlisted in the Merchant Marine in the Pacific, which involved very dangerous duty. Clem and I hit it off intellectually right from the start. We both had strong opinions on many subjects like politics, philosophy, religion and science but we pretty much stayed away from sex and art and other such matters, for they didn’t seem to interest him much. We mostly agreed, and I enjoyed his company but I soon learned that if I disagreed, there was little to be gained by pursuing the subject further. I found this more amusing than bothersome when we were both young. Later in life, when he became a colleague and we were not so young, I, and others, found his unyielding conviction of his own rectitude, even on small matters, at a minimum boring and sometimes downright annoying. Clem had a very wise wife, Margaret, who somehow, with humor, put up with his idiosyncrasies. He had little interest in women or in understanding them, and women mostly turned away from him. Mary Rawles could not stand him and referred to him as “That Markert!” Later, Clem made an important discovery—tissue-specific isozymes, enzymes that vary in form in differentiated tissues. His work was an important cornerstone of the concept that cellular differentiation was controlled by differential gene activity. He was a formidable intellect and a marvelously clear and persuasive lecturer. In 1964, because of his strength in science, teaching, and administration, we invited him to come from Johns Hopkins to chair the Biology Department at Yale and oversee the opening of the magnificent new Kline Biology Tower, a world-class biological research facility.

I had other new friends, of course—young veterans back from the War. Ed Daniels, who had lost a leg in the Battle of the Bulge, and Tom Yost, who was among the first troops to land in Normandy. These were very bright, rollicking pals, and drinking buddies. We had lots of fun together on all levels. They both were married in graduate school just before I finished my Ph.D., and I was best man at each wedding. Weddings are such fun because they sanctify and celebrate an optimistic beginning to a new union. A couple of years later, Tom was appointed to the faculty at Amherst College, which is not far from Yale, so we
both enjoyed a really deep, warm friendship over many years. Tom usually called me “Touch,” only rarely Trink. For some reason, I have accumulated a number of nicknames.

Another fellow student, James Ebert, a student of Willier like myself, was doing interesting work on organ-specific antigens during early chick development. We were not at all close and I mention him only because circumstances have brought us into contact from time to time during my entire career. He was frequently at Woods Hole in one administrative capacity or another. After he left Hopkins, his research, which attracted a lot of attention at the time, didn’t pan out and he left research to become a biological administrator for the rest of his life. He was excellent at administration and became a big shot. As life went on, I never sought him out because we were polar opposites personally and had little in common. I found him increasingly stuffy, pompous and pretentious. To his credit, he was the Director of the Carnegie Institution of Washington, which, under his distinguished leadership, continued its proud tradition of excellence in research in developmental biology. For his pomposity and pretension of being a leading biologist (as opposed to a leading administrator, which he was), he was the butt of many a joke around Woods Hole and elsewhere. Sadly, Ebert and his wife died recently in an automobile accident on their way to Woods Hole.

In the late fall of my last year at Hopkins, my dissertation research was in good shape, and my eyes turned toward the future. So I wrote some well-placed friends, telling them that I was looking for a job and would appreciate any suggestions. There were two fine responses, one from Charles Metz at Yale and the other from Bill Ballard at Dartmouth. At Metz’s suggestion, J.S. Nicholas, Chair of the Department of Zoology at Yale, made a point of attending my paper at the annual “Christmas” meeting of the American Society of Zoologists in Chicago in 1947. Nicholas invited me afterward to have lunch with him and Willier. The upshot of all this was an invitation to interview for a position at both Yale and Dartmouth. Soon after, I lectured at Yale on my research. I had been to Dartmouth before and once again was impressed with the charm of its campus but my main memory of that trip in January was Bill’s large hospitable family and what Bill called a fine “crisp” day, -29 °F! I had never been exposed to such cold before. I was not used to having my nostrils freeze on inspiration—interesting but not appealing. At Yale, I could tell that my lecture was well-received in spite of some hard questions on the subject of the melanins from Heinz Hermann, a friendly but very serious young chemical embryologist, who was a research associate in the Zoology Department. What most impressed me at Yale was not the campus (I actually saw very little of it) but the Osborn Zoological Laboratory, an imposing brownstone structure with luxuriously high ceilings, that was known internationally as OZL. So this was the famous OZL. I was mightily impressed.
Well, both places offered me a job, an instructorship, paying $3,200 per annum. I accepted the Yale offer for obvious reasons. Yale was the greater university. In addition, Yale meant Harrison and embryology. Clem Markert said flatly that he found the low salary insulting and would not accept such an offer. Somebody suggested that perhaps he was jealous. Clem jealous? Never. Actually, the salary was not out of line for those days. Instructors at Hopkins made even less—$2,800. This salary was so low that we veteran graduate students, with our salary as teaching assistants supplemented by a stipend from the G. I. Bill of Rights, were better compensated than the youngest faculty.

Thus far I have confined my remarks about research almost entirely to my own; after all, this is my memoir. However, science is a collective activity and excellent research by others can be very exciting vicariously. For example, at Hopkins, just down the hall from me, John Saunders was making a discovery and doing an analysis on the development of the vertebrate limb. His discoveries would eventually lay the basis for one of the currently most active areas of contemporary developmental biology. As was the case for all of his students, Professor Willier assigned John his dissertation problem. Saunders was to study the origin of the axial properties of the developing feather tracts of the shoulder of the chick embryo. His study involved reorienting blocks of ectoderm and mesoderm in the prospective shoulder of the three-day chick embryo. He stained the area with the vital dye Nile blue sulfate to enhance the visibility of the field of operation. We embryologists often use vital dyes to see better what we are doing. A vital dye, when properly applied, usually does not harm the embryo. Quite serendipitously Saunders noticed that the dye revealed a thickening of the ectoderm at the tip of the wing bud. A search of the literature revealed that others before had noticed this thickening but its significance was not examined experimentally. Saunders, an experimentalist, wondered what would happen if he removed this tiny terminal bit of the wing bud. The result was astounding. This simple operation stopped further outgrowth of the wing bud. By excising this thickening later and later in development, he showed that its presence is required for the proximal-distal sequence of the origin of all parts of the wing. He named this thickening the apical ectodermal ridge (AER), and he and the AER have been famous ever since. Some doctoral dissertation research! Incidentally, to our credit, we other graduate students appreciated the importance of this discovery at the time. Red has since told me that although Willier had not been involved in this discovery, he was a great help in preparing the results for publication, as indeed he was for me as well, when I came to writing up my dissertation. Both our theses were published in 1948 in the Journal of Experimental Zoology (JEZ), one of the old, established journals of the time. This reminds me to tell you about my comment on a button that was popular among graduate students at Yale in the ’60s. The button was emblazoned with the statement, “Feces on Theses.” I remarked to one graduate
student that in his case, he might more appropriately wear a button that said, "Feces in Theses."

Immediately after publication of Saunders’s results, another embryologist, Edgar Zwilling, at the Connecticut Agricultural Experiment Station in Storrs, took advantage of a wingless mutant chicken available to him to extend the analysis. He found that varying degrees of truncation of the limbs of homozygous mutants could be correlated with deterioration of the AER in early limb bud stages, thus confirming the importance of the AER in limb development. Zwilling, who quickly became a friend of both Saunders and me, was an avid fisherman for blue fish and striped bass (rockfish), and both he and Red gushed about how well fishing together off Naushon Island and the Woods Hole passage advanced their analysis of wing development. As the years passed Saunders made two other fundamental discoveries: 1. a small but very important population of cells in the posterior part of the limb bud that he called the zone of polarizing activity (ZPA); and, 2. cell death or apoptosis, as it came to be called, as a normal process involved in sculpting the limb, e.g., in creating fingers and toes on the ends of limbs. To celebrate a lifetime of ground-breaking scientific research, John Saunders was honored by an international symposium in 1998, to commemorate the fiftieth anniversary of his Ph.D. dissertation.

In the spring of the year, my dissertation complete, my thoughts turned to a larger future, my next research. Aside from cleaning up some details of the thyroid hormone work and composing an additional publication on that and a review, I had no interesting ideas for further analysis of feather pigment problems. As it happened, I was not alone in this. Watterson, Hamilton, Foulks, Nickerson, and Markert all also dropped work on feather pigment patterns after they left Willier’s tutelage. This isolated example was somehow symptomatic of the field of embryology during those early post-war years. After the long exciting period of experimental embryology or Entwicklungsmechanik, to which I was thoroughly exposed as a student and whose major triumph had been the discovery of the amphibian organizer by Hildegarde Proscholdt (later Hildegarde Mangold) and Hans Spemann, there came an intense period of search for the chemical nature of the organizer in the 1930s and 1940s. These studies, which took place in several countries, were so full of expectation that they were called the “Gold Rush for the Organizer.” But, for all the effort and numerous occasions for hope this massive international effort generated, it brought only a welter of opposing, contradictory, and confusing results leading to no clear conclusion. The failure to identify the chemical nature of the organizer, even though those involved were internationally respected and even revered for their other contributions to the understanding of development (e.g., Spemann, collaborating with the biochemist G. Fischer and Johannes Holtfreter in Germany; Jean Brachet in Belgium; and C.H. Waddington and Joseph Needham in England), was a crushing defeat that took away much of the attraction previously enjoyed
by the field of experimental embryology. For whatever the reason, four of my best fellow students left embryology immediately after receiving their doctorates: Francis Ryan for molecular genetics; James Foulks and Mark Nickerson for pharmacology; and James Case for neurophysiology. The chemical nature of the organizer has been greatly clarified in the 1980s and 1990s by the application of the powerful new techniques of molecular biology to classical problems of developmental biology. The pioneers in this effort failed to identify the organizer because their search was technologically limited.

Planning for the Future

In spite of this atmosphere, Saunders had made a major contribution right next to me and since I was quite satisfied with my own results, I was not at all interested in seeking my research fortune elsewhere. My youthful enthusiasm for embryology remained intact, but now what to do? It was spring in Baltimore and the campus was lovely, my dissertation was accepted for publication in the JEZ, I had a good job and my growing family was thriving. I suddenly had an interlude to think and reflect on the major problems of development. I started by rereading Spemann's renowned Silliman Lectures at Yale, Embryonic Development and Induction, and Paul Weiss's Principles of Development (both published before most of the frustrations of the Gold Rush). I browsed considerably in our very pleasant departmental library. I mulled over the great themes and phenomena of development: embryonic fields and progressive organization of the embryo; determination and self-differentiation; mosaic and regulative development; gastrulation; regeneration; and my own area of expertise—specificity of induction and competence.

All of this relaxed intellectual activity was most enjoyable, educational, and stimulating to boot, but nothing grabbed me until I discovered a series of papers by Johannes Holtfreter, written when he was in Canada, first in German in 1939 and thereafter in English. Holtfreter was a star in the Spemann laboratory in Freiburg, an extraordinarily creative experimentalist (Figure 4.4). I was quite familiar with his work before I discovered this extraordinary series of papers on early amphibian development. Fortunately, early in the War, he was able to escape from Germany to Britain and eventually to Canada, where he was "interned" at McGill University in Montreal and free to continue his research. It was a very productive period for him. It seems to have started with an extensive revolutionary paper so famous that almost everybody after the War knew it by its original title Gewebeaffinität ein Mittel der embryonalen Formbildung, although the whole paper has been translated and republished in English—Tissue affinity, a means of embryonic morphogenesis. It was followed by two long, comprehensive papers in 1943 and 1944, A study of the mechanics of gastrulation. For me, these papers by Holtfreter were mind-blowing. Yes, indeed,
the mechanism of gastrulation! What a great subject for new experimental approaches! Here was a subject of outstanding developmental importance whose mechanism was poorly understood but which was definitely attackable, as Holtfreter had so brilliantly demonstrated.

Why is gastrulation so important? It is a crucial stage in the early embryogenesis of multicellular animals (the Metazoa). At the very beginning of embryogenesis, the fertilized egg first divides itself into many cells, a process called cleavage, and then gastrulation commences. Gastrulation occurs by massive cell movements, called morphogenetic cell movements, that involve

**Figure 4.4.** Johannes Holtfreter, experimental embryologist extraordinaire, whose important work inspired my interest in *Fundulus* gastrulation. Photograph courtesy of Kurt E. Johnson.

invagination, involution, convergence, extension and epiboly, or the spreading of the outer sheet of cells over the inner cell mass. *Invagination* is the coordinated inpocketing of the outer surface layer of cells to form a short tube inside the gastrula. In some gastrulae, invagination leaves a hole or depression in the outer surface called the blastopore, at the site of invagination. During *involution*, outer (superficial) cells roll over the blastopore, bringing more cells inside the embryo, extending invagination, to form the walls of the primitive gut. *Convergence* is a wheeling movement of superficial cells as they converge toward the blastopore from several directions and then undergo involution at the blastopore. The large mass of cells that has now been moved inside the embryo now undergoes *extension*, it elongates or extends itself and by doing so, gives the embryo a head and tail end. All these movements toward and over the blastopore and then inside
the embryo [to form the endodermal gut tube and the mesodermal structures such as somites (precursors of vertebrae and muscles, among other things)] take place in a carefully controlled sequence. The details of the control mechanisms are still largely a mystery to us. Although I have described these movements as if they were separate entities, in fact, they are a precisely coordinated ensemble of movements, reminiscent, perhaps, of the complex rearrangements of the individual players in a well-practiced marching band. The remaining superficial cells also spread toward the blastopore but do not pass through it into the interior of the embryo. This process is called epiboly. This extensive spreading movement eventually covers the entire outer surface of the gastrula, giving rise to ectodermal structures. The formerly superficial cells, those that underwent convergence, involution, and extension, move deep to the ectoderm and subsequently induce it to form the central nervous system (brain and spinal cord) and the epidermis (superficial epithelial layer of the skin, which covers the outer surface of our bodies). There is also continuing mitosis during gastrulation, although the rate of cell division declines prior to the beginning of gastrulation. Gastrulation is complicated!

The result of all this spectacular motile activity is a drastic rearrangement of the embryo. By the end of gastrulation many groups of cells are now in the correct position to form the major organ systems: the central nervous system; the primitive gut tube and its derivatives; the skeleton; the skeletal muscles; the heart and blood vessels; the kidneys; and the skin. In short, gastrulation is the embryo’s way of laying down its basic body plan. After the completion of gastrulation, the formation of these organ systems and their constituent tissues begins—organogenesis and histogenesis—and a vague outline of the form of the animal emerges. One can now distinguish the head end from the tail end, and the dorsal (back) from the ventral (belly) surface.

Although the mechanism of the morphogenetic cell movements that rearrange the embryo were (and still are) little understood, a great deal has been known for a very long time about the process of determination that takes place during gastrulation and leads to the formation of primitives organs and tissues. At first, the cells are undetermined, i.e., multipotent, but they become determined and begin to form organs and tissues as they are brought by the movements of gastrulation into contact with inductors. Thus, as undetermined ectoderm is brought to lie over and in contact with the underlying mesoderm, the ectoderm is induced to form brain and spinal cord. Without this stimulus these multipotent cells would form something else.

The situation is similar in the blastocyst of the human embryo. The pregastrula cells of the inner cell mass of the early blastocyst are undetermined or multipotent and will not form any particular tissue until they are brought under the influence of specific inductors during gastrulation. These cells are called stem cells nowadays and the use of them is the subject of much current controversy.
There is good reason to believe that with modern techniques of tissue culture and cell transformation, human stem cells could be induced to form various adult human tissues and thereby replace diseased or faulty parts found in adults ill with diabetes mellitus, Parkinson disease and Alzheimer disease. The multipotency of these pre- and early gastrula cells of vertebrates has been known in much detail since the first half of the twentieth century but embryologists in those days were entirely concerned with understanding how the embryo develops. There was therefore no thought of trying to force these cells to form adult tissues. Moreover, there were no living early human embryos abundantly available for experimental work, as there are now.

There were no highly advanced fertility clinics ready and willing to supply discarded 5-day human blastocysts for research on the potencies of the stem cells of the inner cell mass. Now there are plenty of these clinics. Unfortunately, our current government, unduly influenced by the religious right, refuses to supply research funds to enable the research to go on freely in universities and research institutes like the National Institutes of Health. Instead, this important research defaults to the hands of private industry, where the skill of the investigators is in doubt and where any results will be kept secret as proprietary industrial secrets and patented. Generous federal funding and free communication among scientists is essential for the advance of such difficult and important research. In view of this scandalous situation in the United States, it is fortunate for humanity that opportunities for free research on the potency of human stem cells exist in certain other countries. Recently, Roger Pedersen (one of the instigators of the “Feces on Theses” buttons at Yale), and more germane to the present discussion, one of America’s leading legitimate researchers in this field, left a tenured position at the University of California in San Francisco for a similar position at Cambridge University in England, where a more enlightened government funds research on stem cells derived from early human embryos. The use of human embryonic stem cells is certainly where most of the advances will occur. But advances will come slowly. This is a promising but uncertain area of research, not only for scientific reasons but also because of its ethical and political complexities.

This aside on the possibilities of human stem cell research in support of regenerative medicine could not have entered my mind 50 years ago. As a student I knew all about induction of the central nervous system by the mesoderm and about other inductions, but how do the cells of the mesoderm come to lie under the ectoderm in order to induce it? How do cells rearrange during gastrulation in such precise ways to make possible these normal inductions? And, how does ectoderm spread in epiboly to come to lie on top of the mesoderm? These were the questions that excited me in the spring of 1948. As I pored over those papers of Holtfreter, more and more convinced that this was a subject for me, I remembered gastrulation in the egg of the teleost fish, Fundulus, which I knew
well descriptively from the Embryology Course in Woods Hole. One of many movements of gastrulation, epiboly, spreading of the blastoderm over the yolk, is spectacular in teleosts and particularly beautiful in *Fundulus* because of the relative transparency of the egg. So, I decided to study the mechanism of epiboly in *Fundulus*. Quite luckily, Yale, like some other universities in those days, routinely rented laboratory space at the MBL for the summer. There was a Yale lab, a Columbia lab, a Penn lab, etc. So I contacted the Zoology Department at Yale, found that the Yale lab was free during the summer of 1948 and signed up. When I arrived in Woods Hole, I was happily surprised to discover that the Yale lab was on the third floor on the south side of the Brick Laboratory, with a splendid view of the harbor, the Hole, and the islands.

After having decided to work on epiboly in *Fundulus*, I ran across a little paper published just recently by a venerable and distinguished figure among embryologists of that day, Warren H. Lewis of the Wistar Institute in Philadelphia, that gave me a further lift. Working on the zebra fish egg (long before the intense current interest in the development of the zebra fish, see p……), he proposed that the blastoderm is pulled down over the yolk in epiboly by contraction of the exposed yolk surface. This gave me a concrete hypothesis to shoot at with experiments.

In due course, the day of Commencement at Johns Hopkins arrived and since I was still in town, I decided to attend and formally receive my degree of Doctor of Philosophy. It was a colorful, pompous occasion, like all Commencements. I love them all, if they are not too long. Actually, however, the biggest event for me that day was a tense, nervous hour with an inspector of the Commissioner of Motor Vehicles of the City of Baltimore, taking my driving test. I passed. So, at the age of 30, I could finally drive a car. I simply had not gotten around to learning to drive because I never felt a desperate need. There were always buses, trains, and friends with cars and hitchhiking, and I could walk. But, then, with a family and the need to transfer this family to a strange place, necessity imposed its will.

So I did not get my degree until I was thirty years old, not exactly according to my plans of ten years earlier. I found it no big deal. Indeed, quite the contrary. I was full of vim, vigor and enthusiasm. The long years that so many of us lost in the service simply did not make much difference to me. This is worth mentioning because of the youth culture in this country. I have known many Yale undergraduates, who, contemplating taking a year or so off after college, worried about losing some of their youth in the process and maybe holding themselves up professionally. Then, there were the 1960s, when one of the popular sayings was, “Don’t trust anyone over 30.” Many times I have reassured these students that many millions of my generation took several years off in their youth and, if they were not killed or maimed, they were better off for the experience gained with the lost time.
Epiboly in Fundulus

Although the embryos of many bony fishes, or teleosts, are very beautiful because of their exceptional clarity, certainly one of the most stunning is that of Fundulus heteroclitus, commonly called the killifish or mummichog. This active little fish is common in the bays and brackish estuaries along the Atlantic coast of North America. Fishermen often use it for bait. Because of the transparency of the embryo, it is easy to observe much of its development in cellular detail. Because the fish live in estuaries, they are easy to collect either by seining or trapping. Thus, during the spawning season, one can have fertilized eggs in the laboratory simply by manually squeezing eggs from a gravid female into a petri dish and then adding milt, a rich sperm suspension, by similarly squeezing a male.

In unfertilized eggs, the cytoplasm is confined to a uniformly thin, superficial layer at the egg surface encompassing a large, almost transparent sphere of viscous, fluid yolk about 2 mm in diameter. Soon after fertilization, this cytoplasm streams toward the point of sperm penetration and slowly accumulates there to form a protruding cap containing the nucleus of the fertilized egg (zygote). This cap is called a blastodisc. About an hour after its formation, this cap divides in the first cleavage. The yolk does not divide. Succeeding cleavages occur regularly with typically no change in total cytoplasmic volume—basically, a constant volume of cytoplasm is cleaved into smaller and smaller cellular packets called blastomeres (Figure 4.5). After 10 cleavages, when there are just over 1000 cells, this cap is called a blastoderm.

For several hours, depending on the temperature, the blastoderm just sits there with no gross change, as its cells continue to divide. Then, almost imperceptibly at first, it slowly flattens and starts spreading over the yolk (Figure 4.6). This spreading, the process of epiboly, marks the beginning of gastrulation and continues steadily for several hours until the blastoderm has entirely covered the yolk sphere (Figure 4.5). While this truly spectacular epiboly is going on, the cells of the blastoderm are busily engaged in other morphogenetic movements of gastrulation, e.g., rearranging themselves by sinking in from the surface of the blastoderm—called ingression, followed by converging toward the dorsal side of the egg. There they undergo extension in the future head-tail axis to form the rudiments of the early embryo. This convergence and extension, whereby a broad flat array of cells rearranges itself into a long narrow one, is a fundamentally important morphogenetic mechanism in many embryos, and was first carefully described and documented by one of my best students, Raymond Keller, now a professor at the University of Virginia. By the end of epiboly, we can discern an elongated condensation of cells that will soon form an embryonic brain, rudiments of the eyes, the spinal cord, vertebrae and muscles.
So, here in Fundulus is a classic example of vertebrate gastrulation, with the great advantage for the embryologist that it is occurring in a clear egg, where details of cellular motile activity, such as mitosis and cell movements, are not obscured by the presence of opaque yolk. Also, important to me, the eggs and embryos of Fundulus had already been the subjects of important embryological research. There was a vast literature to be studied and harvested. Distinguished biologists like Thomas Hunt Morgan (1895) (during his embryological phase—before he helped found modern genetics), Francis B. Sumner (1904), Charles R. Stockard (1915) and Jane M. Oppenheimer (1930s and 1940s) had all been entranced by the special properties of the Fundulus egg and its availability and had worked extensively on its early development. But none of these embryologists concerned themselves with the problem that most interested me—the mechanism of the morphogenetic cell movements of gastrulation. Jane Oppenheimer had done most of the work on the early development of Fundulus including constructing a fate map of the gastrula. Fate mapping is a classical embryological technique where vital dyes were placed on earlier structures to

Figure 4.5. Drawings of various stages of early development in Fundulus heteroclitus, taken from the normal table published by Armstrong and Child. (A) Stage 3, 2 cells; (B) Stage 10, blastula; (C) Stage 14, beginning of epiboly; (D) Stage 17, middle of epiboly; (E) Stage 19, end of epiboly; (F) Stage 21, body of embryo now visible, with head and eyes at the top of the figure. Reprinted with permission of John Wiley & Sons, Inc.
determine what they would later become. She was not concerned with how the cells moved where, and she paid little attention to epiboly. The problem of the mechanism of epiboly was there for the taking.

Along with its many advantages, the egg of *Fundulus* presented some disadvantages. Because of its short spawning season, eggs were available for operations on the living embryo only two months of the year or less, in comparison with, say, the chick embryo, which is available throughout the year. So, like some students of amphibian embryology, likewise limited by short spawning seasons, one works hard when the material is available. For *Fundulus*, in Woods Hole, this is fortunately in June and July, when an academic like me is free and it is summer!

Two other disadvantages stem from properties of the egg itself. Like all fish eggs, the *Fundulus* egg is enveloped by a protective membranous shell called a chorion. The *Fundulus* chorion is extremely tough, in keeping with the rigors of the intertidal zone in which the embryos develop. So tough, indeed, that it is often difficult to remove this shell without destroying the delicate encapsulated embryo. Well, I knew it could be removed in early stages by the technique of J.S. Nicholas, with iridectomy scissors (used mainly for ocular microsurgery). I tried this but it did not work well in my hands. So I devised another technique, using finely sharpened Swiss Dumont watchmaker’s forceps to microscopically cut off the chorion, which solved the problem.

The final disadvantage of the egg is quite minor—a cluster of oil or lipid droplets lie at the surface of the yolk, beneath the blastoderm. These droplets
often blocked the view of the embryo. I have often expressed my belief that these droplets were placed there by a vengeful *Old Testament* God to bug the experimental embryologist. These oil droplets are actually stored nutrients used to fuel the early development of the embryo. Happily, there are ways of getting around these annoying lipid droplets either by reorienting the egg or simply aspirating them with a fine suction pipette.

To tell the truth, I had a rather good time fooling around trying to devise a new technique for removing the chorion, (dechorionation), and succeeding. The chorion poses a genuinely tough technical problem. I’ve tried many times to teach others how to dechorionate eggs but was mainly unsuccessful. When I succeeded, that is, when they succeeded, it was generally because they already had a steady hand and manipulative skills acquired from much experience with microsurgery. Colleagues have often teased me because of this challenging dechorionation problem, saying that I work on *Fundulus* early development only because few can dechorionate the egg during cleavage and gastrulation and thus repeat my work. True but not true. I am certain that all would find my work repeatable if they could only remove a *Fundulus* chorion. But I would say that anyway. I have thrown down the gauntlet. Now pick it up, but first dechorionate a *Fundulus* egg! This reminds me to mention that for me the source of one of the frequent satisfactions in the practice of science has been the manual aspect: handling the eggs and embryos, facing challenging technical problems, devising new techniques and making them work to give answers to interesting questions. We scientists call this craftsmanship. This satisfying and often challenging craftsmanship of science is often unappreciated by those who think that the arts and sciences are really separate disciplines. Not so. They share much in common.

I didn’t learn much about the mechanism of epiboly that first summer but I certainly had an absolutely absorbing time. For one thing, I learned a lot about early *Fundulus* development that I had missed before as a student and later as an assistant in the Embryology Course. There is no better way for sharpening the senses than having a research problem in mind during observations. Moreover, many of the little experiments I tried gave quick answers. If I made a wound in the yolk surface the edges of the wound would immediately retract elastically and then the edges would thicken and contract to close the wound in a matter of minutes. If the wound were made near the margin of the blastoderm during epiboly, contraction of its margins would pull that region of the blastoderm toward the point of wound closure. This observation was consistent with the hypothesis of Warren Lewis that contraction of the yolk surface causes epiboly. This “instant research” was in happy contrast to my long, sometimes tedious experiments during the analysis of a feather pigment pattern! With *Fundulus*, I would enter the lab in the morning and often have a result by lunch.

I also measured the relative degree of expansion of different regions of the blastoderm by affixing minute black particles (blood carbon) to its surface
and following their movements by using a camera lucida to make tracings every hour during epiboly. A camera lucida is an interesting optical device that can be attached to a microscope. It is used to project an image from the microscope onto a clean piece of paper. It allows the observer to view a specimen and the observer’s tracing hand at the same time. Thus the observer can make accurate drawings of a specimen. By the way, the old name for a photographic camera was camera obscura, to distinguish it from a camera lucida.

In contrast to the wound closure experiment, the marginal region did not expand faster, which is probably inconsistent with Lewis. With these and other experiments I by no means solved the problem of epiboly and was a long way from any definitive answers, but I got myself thoroughly into the analysis. It was a good and felicitous beginning to a charmed life, scientifically.

Galya and I enjoyed the summer delights of Woods Hole, this time en famille. She and I and our two small children, Gregor and Tanya, lived close together, squeezed into a single room in the Brick Dormitory. We ate our meals at the mess hall. An apartment was way too expensive. Anyway, our living arrangements worked out beautifully. All of us enjoyed this relaxed and productive summer at Woods Hole.