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Growth and Organization

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THE FIFTH HOPKINS MEMORIAL LECTURE

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In the spring of 1926 I had to resign the very modest job I held at the University of Groningen. I had no means, no reputation, no knowledge, only a few insignificant papers, a family to support, and a burning desire to become a scientist. Losing my post meant the end of my scientific career. Since life and work meant the same to me I planned to give up both, simultaneously. So, I sent my wife home with our child to her parents and prepared for the end. As a farewell party, I went to the International Physiological Congress at Stockholm, presided over by Sir Frederick Gowland Hopkins. In his presidential address, to my surprise, he mentioned my name several times. So after his lecture I picked up my courage and went up to him. There I was, facing this wonderful little man, always completely forgetful of himself and his greatness, addressing me as his equal: 'Why don't you come to Cambridge? Ask for a Rockefeller Fellowship—I will see to it that you get it.' So he did, making England my spiritual homeland, where I learned what science is, where I was exposed, for several years, to that wonderful inspiration which radiated from him. His almost uncanny insight, prophetic vision, utter modesty and humility added up to a greatness which would have been depressing had it not been blended with warmth and charming human shortcomings which made him our beloved 'Hoppy'. He is still a mystery to me and I want to keep him that way, deep down in my heart.

In his presidential address to the British Association in 1913 you find the following sentence: 'Underlying the extreme complexity we may discover a simplicity which escapes us.' I feel strongly that this basic simplicity, the 'simplicité cachée' of Poincaré, the unity contained in diversity, is much deeper-going than we envisage today. It has become, lately, of prime import to my Laboratory because we have obtained, from various tissues, extracts which retarded or promoted growth. The hypothetical substance responsible for retardation we called 'retine', and the promoting one we called 'promine'. We found such actions widely distributed in Nature, and the similarity in behaviour

suggested that these activities were all due to identical or closely related simple compounds of low molecular weight. Could it then be that these were actually responsible for regulating cell division in the adult, and that proliferation should be regulated by the same set of simple substances throughout a living world which is characterized by its dazzling variety in shape, colour and function? This seems possible only if there is a deep-going unity in this world which still 'escapes us'.

In unicellular organisms growth regulation is relatively simple. It is one of the characteristics of life that it tends to spread and proliferate. Accordingly, in single cells multiplication is unlimited, being checked under favourable conditions only by the quantity of food and energy available. But when cells got together to form multicellular organisms, became members of an ordered society, they had to give up their unlimited proliferation, subject it to strict regulation. They also had to give up motility, and had to develop a new kind of surface which linked them to their neighbours with whom they had to live in the most intimate relations.

However, these newly acquired qualities could not be ingrained very deeply in an irreversible fashion, because, under certain conditions, the cells may be called upon to resume, at short notice, the unicellular way of living with its motility and uninhibited proliferation, which means reverting to the earlier evolutionary level. This is the case in regeneration, in the healing of a wound. In your quiescent epidermis, for instance, the cells are tough and are strongly bound together, which lends to your skin the toughness which you need for your protection. With some tanning, your skin would even make good boots. But if you cut yourself the whole situation changes. On the side of the cut the cells become free, resume motility, creep away into the wound with amoeboid motion, and proliferate. But this they do only till the wound is filled, is healed, and cell is surrounded by cell. Then everything stops and the initial multicellular state is restored. The cells have retained the ability to shift back and forth between the two states which correspond to their two evolutionary levels. The

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transition from one to the other is so prompt that it gives the impression of a veritable switch mechanism.

Conditions in a tissue culture are, in many ways, similar to the conditions in a healing wound, and all these relations were nicely spread out before us, so to say, by the tissue cultures prepared by Abercrombie and his associates. In cultures, too, cells make themselves free on the surface of the explant, resume motility, creep away and divide. They, however, do so only till the cells of neighbouring explants touch one another, which corresponds to the healing of the wound. When this stage is reached, everything comes to rest. This is what Abercrombie called 'contact inhibition'.

Looking upon the healing of a wound and regeneration as an alternation between two evolutionary states brings into the picture a 'unity in diversity', an underlying simplicity which may make the idea acceptable that in spite of the apparent diversity the same simple substances may be instrumental in the regulation.

All these relations assume a tragic importance through the fact that under the influence of various factors cells may lose irreversibly their latest, and thus least deep-seated, evolutionary accomplishment—their regulation of growth, turning into cancer. Looking upon cancer as an evolutionary state would allow us to view it scientifically.

All this makes it most urgent to get an insight into these relations and the question is how this problem can be approached. I am unable to see how it could be approached at all. It is too complex, subtle and full of pitfalls. If we cannot approach a problem, all we can do is to wait till the problem approaches us, and I would like to tell the story, briefly, how I and my associates, Dr Andrew Hegyeli, Dr Laszlo Együd, Dr Ruth Johnsson and Miss McLaughlin, were approached by it.

The main interest of my Laboratory was, for a long time, muscle, and clinical experience indicates that the thymus gland is, somehow, involved in its function. So I set out hunting for a thymus hormone. I was hunting for something of which I did not know, what it was, what it did, and whether it existed at all. As test animals I chose myotonic goats, but soon this line had to be given up for the simple reason that I work in a marine biological laboratory and the smell of goats clearly identifies them as non-marine organisms. At that time I speculated about electronic states and believed that my hypothetical thymus substances had to be coloured, i.e. capable of easy electronic excitation. This led me to the isolation of a beautiful yellow substance which, on analysis, turned out to be an uninteresting derivative of ascorbic acid. This disappointment made me shift to fluorescence and soon I discovered a wonderful fluorescent substance

present in my extracts in trace amounts. My only brilliant chemical work, of which I am conscious, was the isolation of this substance in crystals. The constitution analysis showed it to be a substance which I extracted from my rubber tubing. Disgusted with these theories I argued thus: growth is the integral of all functions and so any biological activity in my extracts should declare itself by modifying growth. In order to get fast results I had to use a fast-growing tissue, and so I chose cancer. The test for activity was simple. A number of Swiss albino mice were injected, behind the scapula, with a definite number of Krebs II ascites-cancer cells. Four days later a visible and palpable tumour appeared, and daily injections of extracts were begun. Seven days later the animals were killed, the tumours excised, inspected, weighed and studied microscopically, and the weights tabulated. Retine makes the cells die, promine makes the tumour grow faster. A 50% retardation or promotion of growth was called a 'unit' of activity. We also tested other solid tumours, e.g. sarcoma 180 and the spontaneous tumours of C3H mice. All reacted similarly. The percentages observed seemed to be a function of the logarithm of the quantity of retine. The results thus obtained could also be checked by injecting cancer cells into the peritoneal cavity and observing the extent of ascites and time of survival. We also made ample use of the tissue cultures prepared by Dr Johnsson (KB and HeLa cells). Retine inhibits growth or eliminates it altogether, whereas promine causes a slight increase in cell volume and a spreading of the cells.

These methods enabled us to express action numerically. The results were numeric but showed the most disconcerting inconsistency. Most of the extracts inhibited, but some promoted, and even one and the same extract could turn, on storage, from an inhibitor into a promoter. It took a great deal of the classical mixture of inspiration and perspiration till we could see that we were dealing with two substances with opposite activity. During the preparative work, also, the physical properties of these substances often changed completely and it dawned on us, but slowly, that in the animal both are loosely linked to a colloid from which they can dissociate, introducing a new variable. One variable can easily be managed, but two may create major difficulties. We had about half a dozen. So I will cut my story short and jump to the final results and then work my way backwards. I will speak first about retine, the inhibitor.

The physical properties of retine, while linked to its colloid, reflect the properties of its carrier. It is soluble in water, can be purified without loss by dialysis, can be precipitated by higher concentrations of ethanol or acetone, is carried down from crude extracts by ammonium sulphate. On

Sephadex G-25 it shows a slight retardation, on Sephadex G-100 a stronger one, suggesting a colloid of moderate size with a molecular weight of several thousand. When detached from this colloid the retine readily passes the dialysis membrane, shows a strong retardation on Sephadex, and can readily be shaken out from its moderately acid watery solution by chloroform or related solvents. Its behaviour, under various conditions, suggests a molecular weight of a few hundred. It distils over with acetic acid. It resists boiling, but if treated with strong acid or alkali at higher temperatures it changes properties. Acid treatment makes it very toxic, whereas treatment with alkali seems only to decrease its activity. Both treatments lower the boiling point and make the retine distil over even with chloroform, indicating a very low molecular weight. The infrared spectrum of our purest preparations suggests a ketone with hydroxyl groups and non-conjugated double bonds, but no nitrogen and no aromatic structure. All active fractions showed, hitherto, a positive Schiff reaction and the slow formation of a 2,4-dinitrophenylhydrazone. An absorption at about $272m\mu$ seems to accompany activity. At a strongly alkaline pH retine can be distilled from the crude extracts with steam. Retine has a high biological activity. The weight of a unit is of the order of micrograms. It seems to act equally well when given by mouth and parenterally. Both retine and promine, at higher purity, seem to be devoid of toxic side-effects.

Promine shares all known physical and chemical properties with retine, which makes the separation of the two very difficult. Effective separation can be achieved by distillation at a strongly alkaline pH, under which conditions the retine goes over more readily into the distillate. Separation can also be achieved by chromatography on paper. On Sephadex, promine tends to move more slowly than retine. The great similarity of the two substances indicates that they are closely related, or identical with only some minor difference in structure, which might make it possible for Nature to shift readily from inhibition to promotion, providing a sort of switch mechanism. In all tissues hitherto studied we found some promine accompanying retine and we believe that there is an equilibrium between the two which decides the biological activity of an extract. In all tissues studied we found retine in excess, the extracts having a retarding action, which corresponds to the quiescent state of tissue from which the extracts were obtained. Promine can completely compensate for the action of retine and vice versa. So the inhibitory action of an extract does not necessarily indicate the absolute quantity of retine or promine, but only the difference between the activity of the two substances. It will be important to bear this

in mind when physiological and pathological relations are studied. We found, for instance, that our tumour grew faster in young than in old animals. To quote an experiment, the tumours grew, under identical conditions, to an average weight of 4823, 2735 and 542mg. in mice 4 weeks, 4 months and 1 year old respectively. This difference between young and old could be eliminated by the administration of retine or promine. Though the relative concentrations of these substances may be identical at various ages, their absolute concentrations may be different, low in the old, high in the young, responsible for the greater incidence of cancer in the former and its rapid growth in the latter.

As a material for our preparations we used, at the outset, thymus glands. We also made extracts from big blood vessels, muscle and tendon (the three tissues which contract cancer least frequently) and found retine prevailing in all three. This convinced us that retine is not a thymus hormone but a general tissue constituent. In the thymus we found one-third retine unit/g., which is, in our experience, roughly the concentration which is needed in the animal to stop cancer growth, suggesting that it may actually be the retine which keeps tissues in the quiescent state.

This brings me to one of the difficulties of these studies. When attempting to isolate a hormone one can go to its source, the gland which produces it, where we usually find it in relatively high concentration. There is no such gland for retine, which seems to be produced locally by the various tissues in trace amounts, which have to be separated thus from the overwhelming mass of other tissue constituents, to which it adsorbs most readily. So when our friend Dr Charles Huggins suggested that we try urine as material we welcomed the idea. For theoretical reasons, we used, first, urine of little children, but shifted, soon, for practical reasons, to an older age group of 20-25, using the urine produced by the U.S. Army, which was made accessible to us by Abbott Laboratories in quantities of 5000 gallons/week. About 25-50ml. contained 1 unit of retine. The greatest part of this we found linked to its colloidal carrier. The free retine present could be adsorbed to charcoal and eluted with chloroform, while the colloid-bound retine was carried down with the bulky precipitate which is formed when the urine is concentrated to one-tenth or one-fiftieth of its original volume. Boiling this precipitate with acid releases the retine, both from the precipitate and its colloidal carrier.

Folklore attributes a favourable action to mushrooms in cancer. This made us try this material, which we found rich in retine. Using *Agaricus campestris*, the edible mushroom, we found in the pressed juice 3 units/ml., accompanied by relatively little promine. This retine shares all the physical

and chemical properties with that prepared from animal tissues or urine. The host of accompanying toxic substances makes mushrooms an unfavourable material. So we dropped it after having convinced ourselves that retine may also be found in plants.

Sister Rosarii Schmeer, working at Woods Hole, heard about our results and noted that no malignant growth was yet reported in molluscs. So she set out to study clams for an anticancer activity, using chiefly quahog, *Mercenaria mercenaria*. She found a definite activity. We followed her trail and corroborated her results. We found, in the summer, in 1ml. of the homogenized content of the valves, about 3 units of retine. Two-thirds of this was in the clam's body, about equally distributed in the various organs. One-third was in the valve fluid. This retine shares all the chemical and physical properties with the retine found in vertebrates or mushrooms. It is linked *in vivo* to a colloid of similar dimensions. Also, the clam is relatively poor in promine.

All this shows that retine is widely spread in Nature. The remarkably high retine content of mushrooms and clams suggests that this substance may have a function other than the regulation of cell division. Maybe it is used as a defence against the attack of invading micro-organisms, to which both clams and mushrooms are exposed, the one living in mud, the other in decaying matter. This unequal distribution of retine makes a wide search in Nature for anticancer substances warranted.

To sum up: the research conducted in my Laboratory, during the past score of years, has brought to light two closely related substances, widely distributed in Nature, which substances seem to be instrumental in the regulation of cell division in the adult multicellular organism. The hope seems to be justified that more intimate

knowledge of them will open up a new alley for attack on the subtle problems of cellular growth and intercellular relations, and the related problems of DNA replication and protein synthesis. A reliable micro-method for their estimation may help to shed light on fascinating problems of oncology. If I did not touch upon any of these, this is for a simple reason. It is not impossible that retine will be helpful in controlling malignant growth, but it can hardly be of any practical use while we are dependent for its production on natural sources. The human suffering caused by cancer entails the obligation to follow the narrow road which leads over isolation and constitution analysis to synthesis. Until this is achieved one has to shy away from all the fascinating functional problems. But the chemical outlines of these growth regulators begin to emerge and I do think that research on this line has passed its most difficult stages, and the story will be completed in the not too distant future.

When I accepted the invitation to this Lecture, more than a year ago, I hoped to be able to finish it with a happy end, pulling out of my pocket a bottle of the synthetic product. This was not to be. The problem is the most difficult one I have ever met. Going was rough and slow. Perhaps I could have found, in my past, some more finished work to talk about, but Hopkins never stopped to delight himself in past achievements. He was always future-oriented. So if I only took you into my workshop, and leave you with more questions asked than answered, at least I have not acted contrary to the spirit of my beloved teacher.

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