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Reflections

Reminiscences about nucleic acid cytochemistry and biochemistry

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There was sharp opposition between 'anatomists' and 'physiologists' when I was a medical student in the University of Brussels, some 60 years ago. This split was exemplified by the presence of two separate buildings, called respectively Institutes of Anatomy and of Physiology, in the newly erected Medical School. The first housed embryologists, histologists and pathologists, the second physiologists, biochemists, bacteriologists and pharmacologists. Biochemistry was a recent outgrowth of the older and larger physiology laboratory; the young professor, E. J. Bigwood, was at that time mainly interested in redox potentials. There was no inner communication between the two buildings except a long dark underground corridor; we called it the 'tunnel'. Students used it, but in general senior 'anatomists' and 'physiologists' were not much interested in meeting each other.

I became an 'anatomist' in 1927, although I had a much greater interest in organic chemistry than in human bones. We had been told by our professor of histology, Pol Gérard, that in merotomy experiments (bisection of an egg or unicellular organism) anucleate cytoplasmic fragments survive and even display normal activities for some time. This fascinated me and I decided to study the interactions between nucleus and cytoplasm in intact cells (I am still working on them today).

This choice led me to the embryology laboratory headed by my father, who very wisely advised me to work under his young colleague, Albert Dalcq. Dalcq had been among the very first to demonstrate that calcium ions are of paramount importance for the maturation and fertilization of starfish eggs; he was then analysing the respective roles of the sperm and egg nuclei in frog development by X-irradiation and local treatment with trypaflavine. His experiments showed that non-nucleated fertilized eggs can undergo a few irregular cleavages, but never gastrulation. I was lucky to work with Dalcq because, in those days, he displayed a real interest in biochemistry. He had even spent a couple of months in David Keilin's laboratory in Cambridge where he had learned a few biochemical techniques, with the hope of following cytochrome synthesis during development. But he soon realized that he was and would always remain a morphologist; he was fond of cytochemistry, enjoying his microscopic investigations of the localization of phosphatases in egg and sperm; he would never have crushed an egg for the analysis of biochemical parameters (even for phosphatase activity measurements).

As soon as I had learned the classical histological techniques of fixation, embedding, sectioning and staining, Dalcq proposed a research subject for me: a study of the localization of 'thymonucleic acid' in growing oocytes with the recent cytochemical method of Feulgen and Rossenbeck[1].

According to the biochemistry textbooks, then as now, there are two main classes of nucleic acids: one of them, now known as DNA, had a queer sugar residue which was identified only in 1930 as deoxyribose by Levene, Mikeska and Mori[2]. This category of nucleic acids was believed to be localized in the nuclei of only animal cells; the prototype of these 'animal nucleic acids' was thymonucleic acid from calf thymus. The other type of nucleic acid (our RNA), known to contain a pentose residue that was later identified as D-ribose, was thought to be specific to plant cells. Yeast zymonucleic acid was the best-known of these 'plant nucleic acids' (also called phytonucleic acids). The role played by the two kinds of nucleic acids in the nuclei was mysterious: their small size (they were believed to be tetranucleotides of about 1300 Da) precluded any genetic function; it was suggested that they might act as intracellular buffers[3] or as colloids giving a high viscosity to the nuclei[4]. This was all I could find about nucleic acids in biochemistry textbooks around 1930.

R. Feulgen was a distinguished biochemist who had tried for many years to identify the mysterious sugar present in thymonucleic acid (DNA): he discovered that this sugar gives aldehyde reactions and thought that it was glucal, an aldehyde derivative of glucose. Feulgen also found that DNA reacts with fuchsin sulfurous acid (the classical Schiff aldehyde reaction) to give a violet compound after removal of the purines by mild acid hydrolysis. Finally, he applied this aldehyde colour reaction to tissue sections after fixation of the cells with a rather harsh fixative (a mixture of saturated sublimate and acetic acid). Feulgen's main important result was that *all* cell nuclei, vegetal as well as animal, stained positively with his procedure. However, this very important finding (DNA is present in all cell nuclei) was not taken seriously by many biochemists who believed in colour reactions obtained in test tubes, but not on tissue sections. Their scepticism increased when Feulgen showed that, under certain conditions, the cytoplasm also gave a Schiff reaction due to a class of lipids, the plasmalogens. He made a sharp distinction (which remains true today) between the 'nucleal' reaction for DNA and the 'plasmal' reaction for plasmalogens.

The now classical Feulgen 'nucleal' reaction was described for the first time in a well-known German biochemical journal[1], but no morphologist had the curiosity to read Feulgen and Rossenbeck's original paper. Dalcq had heard of the Feulgen reaction by reading a French journal of histology in which a cytochemist, Jean Verne, had summarized Feulgen's results (two years after the publication of Feulgen's paper). It was quite an event when I went through the 'tunnel' to the biochemistry library to read Feulgen's original paper: nearly an act of treason to my friends of the Anatomy Institute!

My own observations and those of others on oocytes of a large number of animal species led to the conclusion that, if the oocytes were adequately fixed, their slender lampbrush chromosomes stained positively with the Feulgen reaction at all stages of oogenesis[5,6]. This implied that, contrary to earlier reports,

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DNA is a constant constituent of these chromosomes: that this nucleic acid might play a genetic role was contrary to the then current belief that genes were made of proteins. I found later[7] that a Feulgen-positive core becomes visible under the microscope when the nucleoli disintegrate during meiotic maturation in amphibian oocytes. This was the first indication that the nucleolar organizers contain DNA; it took many years before molecular biologists discovered that this DNA is ribosomal DNA (rDNA) and that the nucleoli direct the synthesis of the cytoplasmic ribosomal RNAs (rRNAs).

However, the most important question for chemical embryologists around 1930 was: is DNA synthesized when the fertilized egg divides quickly and repeatedly in smaller and smaller cells during cleavage? Two opposing theories attempted to answer this question. Jacques Loeb proposed that there would be a total, *de novo* nucleic acid synthesis, at the expense of small precursors, during embryonic development. Emil Godlewski[9] believed that during oogenesis eggs accumulated all the materials (including DNA) which are required for the multiplication of the nuclei during cleavage: there would be only a *migration* of pre-existing cytoplasmic nucleic acids into the nuclei and no net nucleic acid synthesis.

With the Feulgen reaction (under correct technical conditions) I could not find any evidence for the existence of a large DNA reserve in the cytoplasm of oocytes and unfertilized eggs from different animal species. During sea urchin egg cleavage, the intensity of Feulgen staining increased in parallel with the increase in the number of the nuclei. The cytochemical evidence was thus in favour of Loeb's net synthesis theory. But we have seen that biochemists did not think much of the Feulgen reaction. They believed that it was not specific since it is a mere aldehyde reaction, contamination with plasmalogen was always possible, there was no evidence that the Feulgen test is quantitative. These doubts were strongly expressed in a book which was almost Holy Gospel for me in 1931, Joseph Needham's *Chemical Embryology*[10]. Characteristically its section on 'nuclein and nitrogenous extractives' (creatine and creatinine were handled in the same section as nucleic acids!) amounted to only 16 pages out of 1724. This is not surprising since, in those days, everybody was interested in energy production, intermediary metabolism, mechanisms of cellular oxidations, and very few people cared about nucleic acids. In fact, Needham was an exception, having himself worked on nucleic acid synthesis during embryonic development of aquatic eggs.

On the subject of histochemical methods, Needham wrote: 'Histochemical methods are much more uncertain than purely chemical ones'[10]. This scepticism was still present in a later book published in 1942 by J. Needham[11]: 'Great though the pioneer value of histochemical work may be it is particularly vulnerable to technical criticism' and 'The Feulgen test, in the absence of proper precautions, is given by aldehydic phosphatides (plasmal); if possible, it should never be used *in vitro*'. The last criticism was justified: several people had tried to estimate the DNA content of crushed unfertilized sea urchin eggs with the Feulgen reaction; they could not remove completely the plasmalogens and had concluded incorrectly that the eggs contain very large amounts of DNA. Needham, who was Reader in Biochemistry in the world-famous Cambridge Laboratory of Biochemistry (headed by the Nobel Prize winner Sir Frederick Gowland Hopkins), expressed very well the negative position held by a majority of biochemists toward cytochemistry.

Going back to nucleic acid synthesis during egg cleavage, my findings with the cytochemical Feulgen reaction were in complete contradiction with the existing biochemical evidence which entirely supported the migration theory: Masing[12] had found, long ago, that the total purine content of sea urchin eggs does not increase markedly during development. More recently, J. and D. Needham[13] had reported that 'nucleoprotein phosphorus' also remains almost constant during the early development of several marine invertebrate eggs, including those of the sea urchins.

The discrepancy between the cytochemical and the biochemical data was thus incontrovertible. I knew that biochemical methods would have to be used if biochemists were ever to be convinced. Luckily, Z. Dische[14] had just published his diphenylamine colorimetric method for the estimation of deoxyribose (and thus of DNA) in animal tissues; at my request, he kindly sent me a sample of thymonucleic acid, a brownish, poorly soluble powder. This allowed me, in Roscoff in 1931, to make quantitative estimations of the DNA content of developing sea urchin eggs. These fully substantiated my earlier cytochemical findings: unfertilized sea urchin eggs contained very little DNA and this nucleic acid was synthesized during cleavage. However, Masing and the Needhams were also right! I measured the purine content of developing sea urchin eggs (with a very complicated and lengthy chemical method – there were no UV-spectrophotometers in those days) and entirely confirmed Masing's old results: unfertilized sea urchin eggs contain large amounts of nucleic acid purines and there is little purine synthesis during development[15].

I could see only one way out of the contradiction: to assume that, contrary to what was printed in all biochemistry textbooks, sea urchin eggs contain large amounts of a *plant* nucleic acid, a RNA. This unorthodox proposal was of course not easily accepted by the scientific community. But I was greatly encouraged when I asked J. Needham's advice: he had found the matter important enough to discuss it with Hopkins, who had given him advice that I never forgot: 'Tell this young man that he should not believe everything that is written in textbooks, but make experiments'.

I went once more to Roscoff and measured the pentose content of sea urchin eggs (with a method devised for estimating pentosans in straw that was looked down upon with irony by my French friends Monod, Lwoff and Ephrussi). These eggs indeed contained large amounts of pentoses associated with the nucleoprotein fraction. Unfertilized eggs of several species of marine invertebrates also had a high RNA content.

The biochemists were now satisfied, but not the morphologists. Histochemistry was a very important and lively topic for discussion in our Anatomy Institute because one of its members, Lucien Lison, had written a thoughtful and critical book on the subject[16]. When I related my results to my teacher Albert Dalcq, he merely said: 'I shall never believe your story until you show me your RNA under my microscope'. This negative opinion was of course shared by all my friends in the Anatomy Institute. I endeavoured to satisfy them (and myself!). After several unsuccessful attempts I dug out from a textbook on histological techniques the so-called Unna stain (a mixture of two basic dyes, methyl green and pyronine). Unna believed that methyl green stained oxidizing sites (chromatin) and pyronine reducing sites (nucleoli and cytoplasm). I immediately suspected that methyl green would stain DNA and pyronine RNA; the only way to prove this hypothesis was to remove the two nucleic acids from the histological sections by specific nuclease (DNAse and RNAse) digestion. The use of nucleases

for cytochemical purposes had been sharply and correctly criticized by Lison[16] on the grounds that enzymes are never pure. However, when Kunitz[17] crystallized ribonuclease, the long-needed tool for the cytochemical analysis of nucleic acids became available. It immediately turned out that pyronine indeed stains RNA and methyl green double-stranded DNA[18]. The so-called Unna–Brachet method for RNA cytochemical detection has been very widely used by embryologists and histologists; it is still taught to students in French-speaking Colleges and Universities.

I made a very puzzling finding when I applied my cytochemical method for nucleic acid detection to a variety of animal tissues. I found that there was a close and unexpected correlation between the RNA content of a cell and its ability to synthesize proteins: for instance, the exocrine part of the pancreas, which synthesizes large amounts of enzymes, stained much more strongly with pyronine than the Langerhans islets which produce only small quantities of hormones. In order to convince the biochemists, I estimated with chemical methods the pentose content of various tissues from different origins[19]: these quantitative estimations confirmed my cytochemical findings and lent support to the hypothesis that RNA must be involved in protein synthesis.

At that time, T. Caspersson had constructed in Stockholm a delicate and very sensitive UV-cytophotometer which allowed him to localize in the cells and to measure quantitatively the UV-absorbing nucleic acids[20]. He found independently that RNA is localized in the nucleoli and the cytoplasm and that there is a correlation between RNA content and protein synthesis. Simultaneously we reached the same conclusion: RNA somehow directs protein synthesis[21,22]. This conclusion was not easily accepted by the many biochemists who believed that protein synthesis results from the reversal of proteolysis. It took many years before molecular biologists found correct explanations for the mysterious part played by the various RNAs in protein synthesis. It should be added that cytochemical studies by Schultz and Caspersson[23] on *Drosophila* salivary gland cells went one step further: these led them to the conclusion that RNA is synthesized under the control of the neighbouring DNA sequences. We now know that the various RNAs are indeed transcribed on specific DNA segments.

As one can see, cytochemistry had brought us to the very heart of what much later became molecular biology: we knew that DNA is synthesized when cells divide, that it controls RNA synthesis and that RNA directs protein synthesis. However, nobody understood the mechanisms of replication, transcription and translation until biochemists working on enzymes, biophysicists elucidating the structure of macromolecules, geneticists analysing bacteria and phage genetics provided the answers and changed our vague hypotheses into hard facts.

Already in 1940, I had learned a lesson that I shall never forget: one should always try to combine the biochemical and cytochemical approaches if both biochemists and morphologists (as well as yourself) are to be convinced. I tried to persuade fellow scientists of this truth in two books[24,25]. The title of the second (*Biochemical Cytology*) led to sharp adverse reactions from a few anatomy professors as late as 1960; I was openly accused by one of them to have produced a lethal, unviable hybrid between cytology and biochemistry. Today, thanks to the development of new and powerful methods (electron microscopy and differential centrifugation of homogenates first developed by Albert Claude[26], autoradiography, immunocytochemistry, *in situ* hybridization of specific DNA sequences), the old battle has been won. Far from being lethal, the hybrid has been exceedingly fertile. There are few papers published today in the leading journals of cell biology and developmental biology where electrophoresis gels are not found next to micrographs depicting the intracellular localization of the substance of interest. Cytochemistry and biochemistry are no longer enemies: they help each other on the long, arduous way of scientific discovery.

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