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A COMPARISON OF EARLY DEVELOPMENT AND DIFFERENTIATION IN THE SEA
URCHIN EGG WITH ERYTHROPOIESIS AND HEMOGLOBIN SYNTHESIS*

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The papers which follow will stay pretty closely to the mechanism of protein biosynthesis, specifically of hemoglobin. Protein biosynthesis and nucleic acids are coupled; the coupling is a multicomponent system. Among the consequences of these discoveries in the last few years, pursued in many directions, have been that genetics and embryology have become biochemical disciplines. Rather than retell this now familiar story, wonderful as it is, I thought it might be more interesting in my introduction to go a little afield, exercise a chairman's privilege and by my remarks inferentially invite the embryologist. In this way I can also pay homage to Joseph Needham, the Father of Chemical Embryology.

Erythropoiesis is a process: five or six nucleated and two non-nucleated cells devolve from each other. About midway hemoglobin appears. It is a differentiation wholly at the chemical level. It is a terminal differentiation in that the definitive end product - hemoglobin - persists. Other events, chemical and morphological, follow but these are all degenerative. How does such differentiation resemble or differ from those where the chemical events subserve morphological changes, and where the differentiation is either the first or intermediate in a series? Hemoglobin synthesis is restricted to certain cells. How does it compare, qua differentiation, with the most general type of differentiation, i.e. the changes after fertilization of the egg? As the early stages after fertilization appear to be largely the same in all larger animals we may narrow the questions down to a comparison of early development and differentiation of the sea urchin egg with erythropoiesis.

Fertilization (or parthenogenetic activation) ignites a "blast-off" of events whose course was wholly determined in the egg. The first three divisions are the same whether the nucleus is active or not. After the third division the first control (directional change) is applied to the "blast off" course. All such controls come from synthesis or unmasking of new kinds of RNA. Without them there is no differentiation.

It is now a habit of mind to call mRNA the proximate determinant of the specificity of every feature of development. So far only the beginnings of isolation and direct proof of the determinant material have been possible¹⁻⁴. The reason for considering them to be mRNA is the inhibitory effect of actinomycin on differentiation. The enzymatic reaction most sensitive to inhibition by actinomycin is formation on its DNA template of the definitive mRNA of protein synthesis. Where actinomycin has no effect on development or differentiation the presumption is that the required mRNA is there, it is stable enough and no more is needed at that point. Going nowadays to the literature on chemical embryology for the first time one is struck by the difference before and after 1958 or thereabouts, when the idea of messenger RNA had taken hold. Before then the experimental work was largely descriptive, discursive and gives the impression of feeling one's way. After, led by the idea of mRNA, incisive questions are asked and the selected experimental methods, yield clear answers. Much of contemporary biology is indebted to the Cambridge school led by Dr. Krick for the ideas of adapter RNA, messenger RNA and how these couple DNA and the specific array of amino acids in the synthesis of a protein.

The mRNA for the first three divisions is made in the egg. The unfertilized egg operates at a very low "stand by" level, when it is activated by fertilization or parthenogenetically the course taken is predetermined in the egg. Cell division, i.e. cleavage, does not require either the male or female nucleus. Non-nucleate fragments of eggs obtained by centrifugation and activated by parthenogenetic agents form blastulae with as many as 500 cells⁵. Blastomeres enucleated at the 2, 4, or even 8 cell stages retain the ability to divide. Eggs exposed to actinomycin or 8-azaguanine and then fertilized appear normal through the first three divisions, continue to divide thereafter but form a disordered, multicellular mass⁶⁻⁹.

A certain amount of protein synthesis is necessary for mitosis⁶⁻⁷. It is provided by an acceleration of protein synthesis which sets in 15-20 min. after fertilization^{10,11}. Parthenogenetic activation has the same effect¹². The peak rate of protein synthesis is attained 90 min. after fertilization. At this point it is about 2.8 per cent of the total protein per hour^{11-13*}. It is mainly turnover. During the whole period of intense cellular multiplication until the stage of the mesenchyma blastula there is little if any change in the total amount of protein and only small changes in the kind of protein, e.g. in enzyme content, have been detected^{14-15**}.

*From Hultin's amino acid incorporation experiments and Kavanau's data on the protein and amino acid composition of sea urchin eggs and embryos.

**One enzyme has been observed to decrease continuously after fertilization: it is deoxycholate amino hydrolyase. The normal decline is arrested and reversed by high concentrations of actinomycin²⁷. Another change soon after fertilization is in a nucleoprotein fraction which comprises about 1 per cent of the total protein; it decreases by 25 per cent²⁸.

The sea urchin egg nucleus contains so little DNA that there was some question for a while whether it contained any. Now the consensus is what one might expect, that the chromosomal DNA increases pari passu with the number of cells²⁰⁻²⁶. However, the total DNA of the embryo does not increase, it remains essentially constant until the late blastula²⁰. The cytoplasm of the unfertilized egg contains 10-20 times as much deoxyriboside material as the nucleus and it is transformed into nuclear DNA between cleavages²⁰⁻²⁶. Presumably it is degraded first. Nucleosides, purines and pyrimidines can be used for DNA synthesis during the early cleavages^{6,7,20}; but the low molecular weight purine and pyrimidine precursors are not used until after the 500-1000 egg stage²⁹.

RNA synthesis is not required for cleavage, it is required for differentiation. Normally the first visible differentiation occurs at the fourth division⁵ and it is at this stage that the first effects of actinomycin poisoning are visible. In the succeeding divisions of the actinomycin poisoned embryo the cleavage planes become progressively more irregular and there is no differentiation^{6-8,20}. A fertilized egg poisoned with actinomycin is like the non-nucleated half of the egg which has been activated parthenogenetically. "An egg without a nucleus knows how to divide itself but is ignorant of what to do with the cells⁷."

The total RNA, like the protein, remains unchanged with only slight fluctuations during the period in which the number of cells increases from 1 to 2000^{5,34,35}. But the turnover is great. The nuclear RNA increases at the expense of the cytoplasmic ribosomal RNA from 1 per cent of the total in the unfertilized egg to 15 per cent at the pluteus stage³⁴.

A remarkable feature of early development which has received little attention is that the embryo does not increase in volume. The gastrula with its 5000-10,000 cells, has about the same volume as the fertilized egg. In other words cells in normal early development do not grow between divisions⁵. Tumor cells do increase in size after division^{30,31}. When eggs have been separated by centrifugation into nucleated and non-nucleated halves, the nucleated halves can be fertilized and will develop into normal plutei which are smaller than normal⁵. When 50 per cent of the cytoplasm was removed from an egg it developed into a larva which appeared normal in all respects except that it was a dwarf^{20,32}. Cell size is immediately limited by something in the cytoplasm.

Each of the major steps in early morphological development - blastula, gastrula, neurula - is preceded by bursts of increased protein, enzyme and RNA synthesis^{13,15,17,19,33,34}. Development entails either an unmasking of preformed species of mRNA or induction at each stage of mRNA synthesis for the next. A sequential synthesis of mRNA appears to be more likely from the effects of actinomycin administered at different stages of development³⁵⁻³⁷. There is an early phase in which inhibitors of DNA-RNA activity halt the differentiation followed by a phase which is unaffected by such antimetabolites and antibiotics. The interval between the two phases falls within one intermitotic period³⁸⁻⁴¹. If the formation of the new mRNA is stopped the cell continues what it is doing as long as its mRNA lasts. Eventually there is disorder.

Even the simplest differentiating step, e.g. after the third cleavage, probably involves the coordinated action of several species of mRNA. How

many are involved at each stage? How long are they produced? How long do they remain intact and operative? It is in this area that the study of erythropoiesis may make its contribution to understanding differentiation. Although hemoglobin synthesis is not a simple one step differentiation, (five or six enzymes operate to make heme) the component parts and a number of the precursors can be measured precisely even at relatively low concentrations. One can also follow in erythropoiesis the changes at the morphological and molecular levels related to the disappearance of the differentiating activity, i.e. after the synthesis of hemoglobin stops.

Erythropoiesis is a process in which a primordial stem cell gives rise by mitotic divisions to a series of erythroblasts. The names given to the successive stages are: pronormoblasts 1 and 2, basophilic, polychromatic and orthochromatic erythroblasts. Normally the nucleus is lost at the orthochromatic stage and the resulting cell is a reticulocyte, which then matures to an erythrocyte. Some cells may die before they have gone through the whole series of divisions, a few cells may undergo an extra division, under certain circumstances the last division may be skipped⁴².

In this picture of the basic scheme, one stem cell division gives rise to 32 erythrocytes. Yet the total volume of the 32 cells is nearly that of the primitive pronormoblast. The erythroblasts, like the cells of early embryonic development, do not grow after division. Each daughter cell has half the volume of the mother (Table 1). The reticulocyte volume is that of the orthochromatic erythroblast minus that of the nucleus. The erythrocyte has the same volume as the reticulocyte; it may constrict a little with

age⁴³. The nuclear-cytoplasmic volume ratio tends to diminish a little in the later stages of erythropoiesis.

Hemoglobin synthesis does not begin until about the beginning of the polychromatic stage. At each cell division, until then, with the halving of the cell volume, the total protein of the erythroblast is halved, because the concentration of total protein remains constant. It is at about 10 per cent of what it is when the full quota of hemoglobin is made. It remains constant not because the erythroblasts are relatively inactive in protein synthesis. On the contrary, the intensity of protein synthesis, i.e. the rate per unit of cell volume changes little (Table 2); if anything it is greater in the early erythroblasts. As in the developing embryo it is protein turnover. Enzymes of different kinds probably comprise a large fraction of this protein. At the polychromatic stage, with little change in the intensity of protein synthesis, hemoglobin synthesis displaces that of other proteins and hemoglobin accumulates because it does not turn over. The non-hemoglobin proteins suffer the breakdown of turnover without compensatory synthesis.

Table 3 shows the DNA synthesis in erythroblasts. For the most part there is none after the stage of the polychromatic erythroblast. There is some disagreement on whether the restoration of DNA is complete in each intermitotic phase before then. Some have pictured chromosome losses in the last two mitoses. Autoradiography is not a precise analytical method but it seems adequate to show that the rate of DNA synthesis per cell diminishes with the cell volume after each division. Accordingly there would be some

reduction in DNA in the last two erythroblasts^{*}, which would be in accord with loss of non-hemoglobin protein synthetic activity. In the pyknotic orthochromatic nucleus much of the DNA is probably denatured or even partially degraded before it is extruded^{**}.

Table 4 shows that DNA synthesis appears to be nearly an all or none affair; it goes full blast or not at all. The last stage in which there is DNA synthesis is the stage where the protein economy of the cell is diverted to the making of hemoglobin at the expense of that of other proteins. When erythropoiesis is greatly stimulated by severe loss of red cells it is the stage where the nucleus is lost. In this condition more polychromatic cells were seen in which DNA synthesis had stopped.

The RNA picture in erythropoiesis resembles that of early embryonic development. The concentration of RNA in both adult marrow and fetal liver erythroblasts is little changed from pronormoblast 2 through to the orthochromatic stage. In successive cell divisions the RNA is subdivided with little net loss. What breakdown there is is replaced by synthesis, but the intensity of turnover (rate per unit volume) declines continuously. The decline is steeper than that of DNA (Table 5). However, it continues a little longer

*Unless the reduced rate of synthesis is compensated for by a corresponding lengthening of the period of synthesis.

**The erythroblast nuclei have nearly the same halving relation as the cell volumes in the successive cell divisions. Even maximum allowance for progressive reduction in DNA is much less than the reduction in nuclear volume. The consequence is that the DNA in the nucleus becomes progressively more concentrated. There is no explanation of the mechanism at present. There are no data on the RNA and protein of erythroblast nuclei.

and comes to a stop in the orthochromatic stage. There is no RNA synthesis in reticulocytes or erythrocytes*. All the RNA is synthesized in the nucleus and it is all DNA-dependent synthesis.

The reason why little or no hemoglobin is seen until the polychromatic stage, it was surmised, is that it is heme which is missing until then, that the globin is made earlier. In the main production of hemoglobin heme and globin are synthesized at equivalent rates both in reticulocytes where hemoglobin is almost the only protein synthesized and in the mixture of marrow cells where much non-hemoglobin protein is synthesized in the pronormoblasts and basophilic erythroblasts. Evidently there is a mutual regulation of heme and of globin synthesis under normal circumstances (see below). Whether globin or heme is synthesized before the other, the sudden appearance of hemoglobin at about mid-erythropoiesis signals the beginning synthesis of the missing part and the question remains - what turns it on? Is it removal of a repressor or the sudden synthesis of one or more new species of mRNA? The key event may be at the DNA, preformed mRNA or enzyme level. The question and alternatives are the same regarding the successive differentiations of the embryo.

*In fetal rabbit liver RNA synthesis appears to come to a stop at the basophilic erythroblast stage, i.e. one stage earlier than in adult marrow.

The constancy of the RNA concentration in the basophilic through to the orthochromatic erythroblast indicates that so long as DNA is functioning synthesis of RNA replaces the losses. Breakdown continues after synthesis is no longer possible until all the RNA is lost. In the normal animal the main loss of RNA occurs in the orthochromatic erythroblasts after their nuclei have become pyknotic. In the hyperplastic marrow of hemolytic anemia, where the orthochromatic stage is largely by-passed, the main loss of RNA occurs in the reticulocytes. Most of the RNA is degraded within the cell (rather than extruded). Nucleosides diffuse out; the pentose is metabolized⁴⁴.

The course of erythropoiesis is the same in human, rabbit and rat bone marrow cells: rapid onset of hemoglobin synthesis at the expense of that of other proteins at the last stage in erythropoiesis where there is synthesis of DNA and where the synthesis of RNA is declining rapidly. One is persuaded that the three changes are related. The formation of the lens and of down feathers are other examples where, after a spurt of intense synthesis of a single protein, to such an extent that it becomes the preponderant component of the cell, the nucleus is lost shortly afterwards and the cytoplasm functions autonomously for a limited period⁴⁵.

Hemopoiesis in the chick embryo is similar in the timing relations of DNA, RNA and hemoglobin synthesis, even though there are two hemoglobins in adult chick blood, and in the embryo a primitive (megalo-blastic) line of erythroblasts gives way to a definitive (normoblastic) line⁴⁶⁻⁵⁰. Hemoglobin is first visible at about the 8 somite stage⁵¹⁻⁵³ which coincides with an increase in RNA in the erythropoietic cells⁵³.

Hemoglobin synthesis comes to a stop in the rabbit during the maturation of the reticulocyte. Table 6 shows how less active reticulocytes have lower concentrations of polysomes and ribosomes: this is the main difference. Hemoglobin synthesis falls off somewhat faster than the loss of polysomes and ribosomes, accordingly the synthetic activity per polysome slowly becomes less⁵⁴. Because of mRNA instability maintenance of a maximum rate of synthesis requires an active nucleus to replace the losses. When the nucleus is lost or degenerated (as in the late orthochromatic erythroblast) mRNA is not replaced, hemoglobin synthesis slows down and eventually stops. In the reticulocyte of the anemic rabbit the duration of this last phase is

24-48 hours. Erythrocyte hemoglobin concentration is largely a matter of how long the synthesis of hemoglobin-mRNA is maintained at maximum concentration by a functioning nucleus which replaces losses of mRNA. Early loss of the nucleus results in a hemoglobin concentration in the eventual erythrocyte which is less than normal; when an active nucleus persists for an extra mitotic division the eventual hemoglobin concentration is well above normal.

The mutual regulation of heme and globin synthesis^{55,56} under normal circumstances can be disconnected: cobaltous ions depress heme and accelerate globin synthesis⁵⁶; X-radiation and starvation disturb the correspondence in variable ways⁵⁷⁻⁵⁹. The cobalt inhibition is at the level of formation of the tetrapyrrole intermediates⁶⁰.

A small amount of globin may be formed before there is any heme. Some immunological evidence to this effect was found in the chick embryo⁵². Catalase is cited as an example of a hemoprotein whose apoenzyme can be synthesized independently of its prosthetic group⁶¹⁻⁶⁵. The evidence is indirect; direct proof is lacking. If and when the case is proved it would establish a nice example of the formation of native secondary and tertiary structure in the absence of the prosthetic group. There appears to be a difficulty in carrying out the combination of purified apocatalase with heme. Hill and Holden demonstrated nearly 40 years ago⁶⁶ that globin extracted from hemoglobin can reconstitute its native linkage with heme. But this does not show that the native structure of the peptides are formed as they are synthesized and before attachment of the heme.

There is too little protein in the early erythroblast for much globin to be synthesized before the appearance of hemoglobin. Heme and globin

appear to have a feedback regulatory relation⁶⁷⁻⁶⁹ with heme synthesis the more sensitive to environmental influences. In the synthesis of heme itself there is a feedback relation; the two most sensitive spots are the two earliest steps - ALA formation and dehydration⁷⁰⁻⁷². The one occurs within the mitochondrion, the other outside it⁷⁰.

The increase in hemoglobin production caused by lowered oxygen tension is mainly via increased red cell production. Hypoxia might, if the oxygen tension is low enough, also increase heme synthesis by favoring accumulation of succinyl COA and thence of ALA. At lowered oxygen tension the limiting step in the tricarboxylic cycle is reduced rate of oxidation of succinate. Incubation of mitochondria in nitrogen instead of air increased the yield of ALA⁷⁰.

Ever since the demonstration by Schweet and his collaborators of the synthesis of hemoglobin in a cell-free system⁷³ the question of the origin of the heme has been left dangling. The enzyme ferrochelatase (heme synthetase), which operates to combine iron, protoporphyrin and globin or myoglobin, is made in the mitochondrion^{74,75-82}. The cell-free preparations used for in vitro hemoglobin synthesis contain no mitochondria. It may be there is enough free heme in the cell-free reaction mixture; or that the heme is transferred to the newly synthesized globin chains from hemoglobin, also always present in the reaction mixture. Such a dissociation equilibrium has been inferred by Schwartz et al.⁷⁸ and by Rossi-Fanelli and Antonini⁸³.

In summary, like the first three divisions of the fertilized sea urchin egg, which are determined in the egg before fertilization, once erythropoiesis is set in motion, its course is predetermined. All the studies on the mode

of action of erythropoietin have come to this conclusion. Its main effect is in the stem cell compartment to increase the rates of generation of the primitive erythroblasts^{84-90*}. "Even the most immature pronormoblast appears to be in a state of relentless maturation⁹⁰." The progeny of the successive mitotic divisions, like the cells of the early embryo, do not grow in size. The total volume of the cells of the system, remains constant in both cases. Similarly in both the total RNA is maintained constant by active turnover processes until the gastrula stage of the embryo and loss or degeneration of the nucleus by erythroblasts. The total protein also is maintained constant by turnover processes until the gastrula stage of the embryo and the onset of hemoglobin synthesis (which does not turn over) in erythroblasts. The diversion in the polychromatic erythroblast of protein synthesis to that of hemoglobin is analogous to the onset of differentiation after the third cell division in the egg. The mRNA which induces the change in direction is either formed or unmasked shortly before; but the formation or unmasking is predetermined. Unlike the fertilized egg when the erythroblast differentiates to synthesize hemoglobin it does not form mRNA for subsequent differentiation. Cytoplasmic events continue in late erythroblasts and reticulocytes, as in the fertilized egg that has been poisoned with actinomycin. These events, once determined, are, per se, independent of nuclear activity, and continue

* It may also shorten the intermitotic phases of erythroblasts somewhat, and release reticulocytes from the bone marrow earlier⁹¹⁻⁹⁴. There is some disagreement regarding these effects. They may be secondary to changes induced in the stem cell compartment. Their importance would be largely in the early effects of massive doses of erythropoietin and not as major contributors to the large increase in circulating erythrocytes.

because both systems, at their respective stages, have relatively long lived mRNA. Eventually their mRNA, which can no longer be replenished, disappears. The cells in both systems degenerate with respect to protein synthetic function, but they need not disintegrate for a relatively long time.

The general conclusion from the foregoing is that the underlying biochemistry is similar whether the differentiation entails morphological changes and is one of a series or it is the synthesis of a single, specific protein and is the terminal differentiation of the cell.

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Table 1. RELATIVE VOLUMES OF ERYTHROBLASTS COMPUTED FROM
LONG DIAMETERS (SPHERICAL SHAPE ASSUMED)⁴²

Arbitrary units				
Pronormoblasts			Erythroblasts	
1	2	Baso- philic	Polychro- matic	Orthochro- matic
Average				
17.5	8.4	4	1.85	1.0

Table 2. PROTEIN AND HEMOGLOBIN SYNTHESIS IN ERYTHROBLASTS⁴²

Arbitrary units					
Type of erythroblast	Cell volume	Rate of protein synthesis		Synthesis of	
		per cell	per cell volume	non-hemo-globin protein	hemo-globin
Pronormoblast	16	250	16	+++	0
Basophilic erythroblast	8	100	13	++	± 0
Polychromatic erythroblast	4	44	11	Declines	+++
Orthochromatic erythroblast	2	27	15	↓	++
Reticulocyte	<2	8	<10		+
Erythrocyte	<2	0	0		0

Table 3. DNA SYNTHESIS IN ERYTHROBLASTS⁴²

Type of erythroblast	Cell volume	Arbitrary units		DNA	
		Rate of synthesis*		per nucleus	relative nuclear concentration
		per cell	per cell volume		
Pronormoblast	16	16	100	19.8	1
Basophilic erythroblast	8	8	100	19.3	2
Polychromatic erythroblast	4	4*	100*	17.5-16.3	3.7
Orthochromatic erythroblast	2	0**	0**	11.8-10.8	4.7
Reticulocyte	<2	0	0	<u>±0</u>	<u>±0</u>
Erythrocyte	<2	0	0	0	0

* Active cells only

** Synthesis observed in 5 per cent and a corresponding number of mitotic figures

Table 4. RELATIVE RATES OF SYNTHESIS OF DNA (INCORPORATION
OF THYMIDINE) IN MARROW ERYTHROID CELLS OF NORMAL
AND SEVERELY ANEMIC RABBITS⁴²

Cell class	Baso- philic	Polychro- matic	Orthochro- matic	Non- nucleated
Normal				
Fully active	100	90	0	0
Less active	0	0	0	0
Inactive	0	10	100	100
Anemic				
Fully active	91	77	0	0
Less active	1	2	0	0
Inactive	8	21	100	100

Table 5. RELATIVE RATE OF SYNTHESIS OF RNA (INCORPORATION OF URIDINE) IN MARROW ERYTHROID CELLS OF NORMAL AND SEVERELY ANEMIC RABBITS⁴²

Condition of animal	Pronormoblasts	Basophilic	Polychromatic	Orthochromatic	Non-nucleated
Relative grain count					
Normal	Too many to be counted	100	19	0.6	0
Anemic	Too many to be counted	100	34	12.5	0

Table 6. HEMOGLOBIN SYNTHESIZING ACTIVITY (INCORPORATION OF
C¹⁴-LEUCINE) OF RETICULOCYTES FRACTIONATED IN A BOVINE
SERUM ALBUMIN GRADIENT⁵⁴

Fraction number	Per ml packed cells			Relative activity per	
	Total counts in hemoglobin x 10 ⁻⁶	Total ribo- somes: O.D. at 260 mμ	Polysomes O.D. at 260 mμ	Ribosome O.D. at 260 mμ	Polysome O.D. at 260 mμ
Mature 1 (Bottom)	6.0	8.9	5.4	45	77
3	16.9	14.9	11.1	77	80
5	25.2	18.6	14.3	92	93
6 (Least mature)	33.3	22.7	17.5	100	100