GROWTH AND DIFFERENTIATION IN THE RED BLOOD CELLS OF THE CHICKEN EMBRYO

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INTRODUCTION

Thorell (1947*a*) has used measurements of haemoglobin to determine the degree of differentiation of developing red blood cells in adult mammalian bone marrow. The haemoglobin was measured in individual cells by the micro-spectro-photometric methods of Caspersson and his colleagues (see Caspersson, 1947) so that it was possible to determine the amount of haemoglobin in cells of different stages of development.

Dawson (1936) has shown that the circulating red blood cells in the chicken embryo, at any given time of incubation, are very largely at the same stage of development, their maturity increasing as incubation proceeds. By measuring the haemoglobin in suspensions of circulating red cells at different stages of incubation the haemoglobin content could be determined for cells at different stages of development. The suspensions contained insufficient developing white cells to affect the measurements. These have been compared with histochemical properties of the cells and with previously recorded measurements of average cell volume, number of dividing cells and the rate of respiration (O'Connor, 1951).

MATERIAL AND METHODS

Suspensions of the circulating blood cells of the chicken embryo were made and the number of dividing cells, the rate of respiration and the average cell volume were determined by methods previously described (O'Connor, 1951). To measure the haemoglobin content of the cells the portion of the suspensions used to determine cell volume was suspended in 2·0 c.c. normal saline and the number of cells checked. The haemoglobin was converted into alkaline haematin by the addition of 1·0 c.c. 2·5 % sodium hydroxide in normal saline and measured with a photo-electric colorimeter. The amount of haemoglobin per cell was thus determined and the concentration of haemoglobin (expressed as a weight/volume percentage) derived from the average cell volume. The earliest embryos on which haemoglobin determinations could be made were those at the end of 3 days' incubation when the developmental stage reached was that of 32–36 somites. Haemoglobin measurements were made from this stage until the end of incubation.

The general cytological appearances of the cells were studied in dried films made directly from the circulating blood and stained with Leishman's stain. The intracellular distribution of nucleic acid was studied by using Taft's (1951) mixture of methyl green-pyronin. A small drop of a suspension, or of blood taken directly from the embryo, was mixed with the stain on a slide, covered with a cover-slip, ringed with vaseline and examined as a wet preparation. Enzymatic controls were

not used, for it was considered that the results of White (1947) justified the assumption that, in developing red cells, staining with pyronin indicated the presence of ribonucleic acid and staining with methyl green the presence of desoxyribose nucleic acid. Since this examination required only a small amount of blood it was possible to carry it out on the circulating cells of embryos of less than 3 completed days of incubation and at stages where the intra-embryonic circulation had just been established.

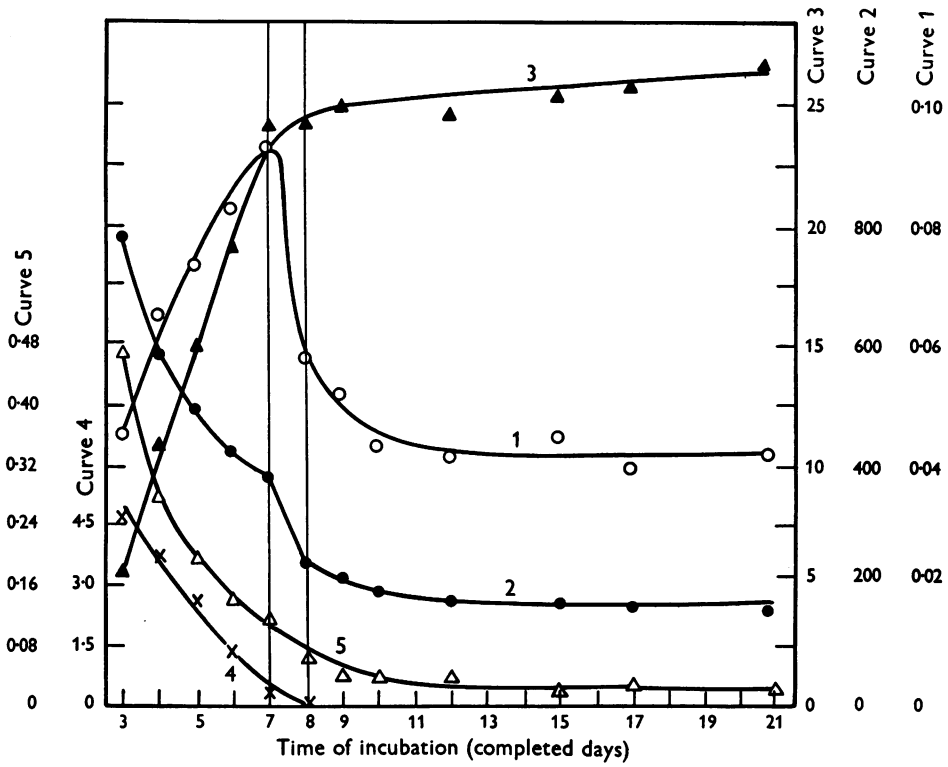

Fig. 1. The alterations in cellular characteristics of the circulating red blood cells of the chicken embryo during development. Curve 1, haemoglobin per cell ($\mu g. \times 10^{-3}$), O——O; curve 2, cell volume (μ^3), ●——●; curve 3, cellular concentration of haemoglobin (weight/volume: per cent), ▲——▲; curve 4, number of dividing cells (per cent), ×——×; curve 5, rate of respiration (mm.^{3} O_2/mm.^{3} cell substance/hr.), △——△.

RESULTS

Haemoglobin in developing red cells. For each completed day of incubation the average amount of haemoglobin per cell was calculated from eight to fifteen observations. These averages are recorded in Fig. 1, curve 1, which shows an increase until 7 completed days of incubation followed by a fall to reach, at the end of 10 days, a value which remains almost constant until hatching. Fig. 1, curve 2, records the previously determined values for cell volume from which the concentration of haemoglobin, recorded by means of curve 3, has been calculated. The haemoglobin concentration rises rapidly until the end of the eighth day and only slowly during the remainder of incubation. Previously determined values for the number of

dividing cells and the rate of respiration are indicated in Fig. 1 by curves 4 and 5 respectively.*

Histological appearances. In the films stained with Leishman's stain the histological appearances and the predominant cell types at various times were essentially as described by Dawson (1936). In the wet films stained with methyl green-pyronin the cytoplasm of the cells circulating during the third day stained deeply with pyronin and there were one or more nucleoli with a similar staining reaction. These nucleoli were surrounded by material staining with methyl green ('nucleolus associated chromatin'). During the fourth day the pyronin reaction in the cytoplasm decreased, and the nucleoli disappeared leaving chromocentres stained with methyl green.† From the results recorded in Fig. 1 it was possible to determine that in such cells the haemoglobin concentration was 9–11 %. Nucleoli were not seen, except in an occasional cell, after this stage, although the cytoplasm stained with pyronin until the sixth day. Basophilia of cells stained with Leishman's stain persisted until about the same time. This staining reaction has been shown by Burt, Murray & Rossiter (1951) to be due to the presence of ribonucleic acid.

DISCUSSION

The variations in haemoglobin during development. It is apparent from Fig. 1 that between the seventh and tenth days there is a considerable fall in the amount of haemoglobin per cell with no corresponding decrease in haemoglobin concentration. Two possibilities can be considered as an explanation of this change. First, it might be due to cell division producing a diminished amount of haemoglobin in individual cells without a corresponding decrease in concentration. However, it will be seen from Fig. 1 that the decrease in the amount of haemoglobin in the red cells occurs when the number of dividing cells is zero or nearly so. A second possibility is therefore favoured. At any stage the characteristics of the circulating cells depend not only on the behaviour of the cells already in the circulation but also on the properties of those being discharged into the circulation from the sites of haemopoiesis. The observed changes in haemoglobin content would be accounted for if, at about the seventh day, the circulation received cells differing from those already circulating by containing a smaller amount of haemoglobin but a similar concentration because of a smaller size. Such an addition of smaller cells to the circulation is suggested by the changes of average cell volume indicated by curve 2 in Fig. 1. Until the end of the seventh day, and again after the eighth day, it is a smooth curve, but there is a sudden fall between the seventh and eighth corresponding to the fall in haemoglobin content of the cells. Two further points can be cited in favour of the change in haemoglobin values being due to the addition of a new population of cells to the circulation. First, Dawson (1936) has shown that after the sixth day the primitive blood cells become replaced by the definitive, and that this process is almost complete by the tenth day. Secondly, the sudden fall in the amount of haemoglobin in

* Boyer (1950), using different methods, measured, the respiration of red cells in chicken embryos of 14 days incubation and later. His values are considerably lower than those recorded here.

† In making this description the terminology of Caspersson (1950) is followed. The term nucleolus is confined to the pyronin staining material, and the associated material staining with methyl green is referred to as 'nucleolus associated chromatin' or, when the nucleolus has disappeared, as a chromocentre.

the developing red cells does not occur in red-cell formation in adult mammalian marrow (Thorell, 1947*a*) where there is no replacement of primitive cells by definitive. It is concluded, therefore, that the change discussed is due to the replacement in the circulation of primitive red blood cells by definitive.

Growth and differentiation in red-cell development. Caspersson & Schultz (1940) have shown that the formation of protein by a cell depends on a cell system which includes the nucleoli and ribonucleic acid in the cytoplasm. Thus, since cellular growth consists largely in protein formation, growing cells are characterized by the presence of nucleoli and a cytoplasm giving the reactions for ribonucleic acid, as Thorell (1947*a*) demonstrated in red-cell formation by adult mammalian bone marrow. In this tissue he found that these criteria of growth disappeared from cells that contained only a trace of haemoglobin. Taking haemoglobin content as a measure of differentiation he concluded that growth and differentiation occurred in separate phases which overlapped only slightly.

In the circulating red blood cells of the chicken embryo investigations with methyl green-pyronin show that the pyronin staining nucleoli remain until the fourth day of incubation and until the cellular haemoglobin concentration reaches 9–11 %, which is more than one-third of concentration reached at hatching; pyronin staining of the cytoplasm persists longer still. On the assumption that pyronin staining indicates the presence of ribonucleic acid it is concluded that the capacity for growth is retained until the differentiation is more than one-third completed, so that in contrast with red-cell formation in the mammalian bone marrow, there is a considerable overlapping of an earlier phase of growth by a later phase of differentiation. It must be pointed out, however, that these results and conclusions apply only to the primitive red blood cells, which form more than 90 % of the cells circulating during the fourth day (Dawson, 1936). It was not possible to make similar observations on the definitive cells, because they do not predominate in the circulation until the characteristics of growth have disappeared. Therefore, it is not excluded that, in the definitive red cells, the relationship of cell growth to cell differentiation may differ from the relationship in the primitive cells and may resemble that demonstrated by Thorell (1947*a*) in the adult mammalian bone marrow.

Nevertheless, the considerable overlapping of the phases of growth and differentiation in the primitive cells may be of general significance, for it forms an exception to the generalization suggested by Thorell (1947*b*) that in tissue formation there is a considerable separation of phases of growth and differentiation. Many adult tissues possess, like the blood, systems for replacing cells lost in normal function or to form additional functioning tissue in response to appropriate stimuli. If in such systems a phase of growth is followed by a phase of differentiation the existence of a degree of overlap between the two phases might have important implications. If cells became 'fixed' at a stage of partial differentiation, while retaining the ability to grow, they would conform to many of the characteristics of malignant cells. It might be suggested that the extent of the overlap between the phases of growth and differentiation might affect the possibility of such cells arising.

Metabolism and red-cell development. There is evidence (O'Connor, 1951) that the oxygen consumed by the circulating red blood cells of the chicken embryo is used

entirely in the catabolism of carbohydrate. Hence curve 5 in Fig. 1 indicates relative rates of carbohydrate catabolism as well as rates of oxygen consumption. Before the end of the seventh day this metabolic measurement undergoes considerable changes as do the measurements indicating embryological development, namely haemoglobin content, number of dividing cells and cell size. Interdependence of these processes of embryonic development and respiration (or carbohydrate metabolism) is suggested by the fact that all measurements, both developmental and metabolic, reach constant levels after the eighth day of incubation and very nearly at the same time (see Fig. 1).

Previously it has been suggested (see Brachet, 1947) that, in embryonic tissues, there is a fraction of catabolism specifically associated with the processes of development, and which can be distinguished from the 'maintenance' metabolism by which the cells are maintained in equilibrium with their environment. Such a distinction is suggested with regard to carbohydrate metabolism by the investigations of Needham & Lehmann (1937) and with regard to respiration by Moog (1944). If a special 'developmental' metabolism occurs in the circulating red blood cells of the chicken embryo it should be present on the fourth day of incubation, when development is proceeding rapidly, and absent after the ninth day, when development is complete or nearly so. Thus a comparison of the responses of cell respiration to metabolic inhibitors at these times might give information regarding the existence and the properties of a fraction of cellular catabolism specifically associated with the processes of embryonic development.

SUMMARY

1. The haemoglobin content has been measured in the circulating red cells of the chicken embryo from the end of the third day of incubation until hatching.

2. After increasing rapidly, the amount of haemoglobin per cell drops suddenly at the end of 7 days' incubation. This drop is ascribed to the replacement of primitive red cells by definitive.

3. The intracellular distribution of nucleic acid has been studied by staining with methyl green-pyronin. Changes in the distribution of ribonucleic acid indicated that growth ceased in cells in which the haemoglobin concentration was 9–11 %. From these findings it was concluded that, in the primitive red cells, there is a considerable overlapping of phases of growth and differentiation.

4. Developmental changes in the circulating red blood cells have been compared with changes in the rate of respiration. The significance of these changes has been discussed with regard to the possibility of a fraction of total catabolism being specifically associated with embryonic development.

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REFERENCES

BOYER, C. (1950). The respiration of embryonic blood. *Proc. Soc. exp. Biol., N.Y.*, **75**, 211–214.

BRACHET, J. (1947). *Embryologie Chimique.* Paris: Masson et Cie.

BURT, N. S., MURRAY, R. G. E. & ROSSITER, R. J. (1951). Nucleic acids in rabbit reticulocytes *Blood*, **6**, 906–915.

CASPERSSON, T. (1947). The relations between nucleic acid and protein synthesis. *Symp. Soc. exp. Biol.* **1**, 127–151.

—— (1950). *Cell Growth and Cell Function.* New York: W. W. Norton and Co.

—— & SCHULTZ, J. (1940). Ribonucleic acids in both nucleus and cytoplasm and the function of the nucleolus. *Proc. nat. Acad. Sci., Wash.*, **26**, 507–515.

DAWSON, A. B. (1936). Some observations on the primitive and definitive erythrocytes of the developing chick. *Z. Zellforsch.* **24**, 256–268.

MOOG, F. (1944). The chloretone sensitivity of frogs' eggs in relation to respiration and development. *J. cell. comp. Physiol.* **23**, 131–155.

NEEDHAM, J. & LEHMANN, H. (1937). Intermediate carbohydrate metabolism in embryonic life. V. The phosphorylation cycles. VI. Glucolysis without phosphorylation. VII. Experiments on the nature of non-phosphorylating glucolysis. *Biochem. J.* **31**, 1210–1254.

O'CONNOR, R. J. (1951). Respiration and cell division in the red blood cells of the chicken embryo. *Brit. J. exp. Path.* **32**, 336–340.

TAFT, E. B. (1951). The problem of a standardized technic for the methyl green-pyronin stain. *Stain Tech.* **26**, 205–212.

THORELL, B. (1947*a*). Studies on the formation of cellular substances during blood cell production. *Acta med. scand.* (Supl. CC).

—— (1947*b*). The relation of nucleic acids to the formation and differentiation of cellular proteins. *Cold. Spr. Harb. Sym. quant. Biol.* **12**, 247–255.

WHITE, J. C. (1947). The cytoplasmic basophilia of bone marrow cells. *J. Path. Bact.* **59**, 223–234.