STUDIES IN THE ANAEMIAS OF INFANCY AND EARLY CHILDHOOD *

PART XII.—THE REGENERATION RATE OF HAEMOGLOBIN AND THE LIFE SPAN OF ERYTHROCYTES IN NORMAL AND PATHOLOGICAL CONDITIONS

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* Haematology will not be an exact science until we can measure the number of red cells, which enter and leave the circulation in the twenty-four hours, with the same accuracy as we can measure the protein metabolism or the work of the heart' (Witts, 1932).

Haematology has for a long time been a purely morphological science. Ehrlich's discovery of specific stains and the perfection of nuclear stains culminating in Pappenheim's panoptic stain opened a vast field for cytological investigation and a generation fascinated by Virchow's cytopathological teaching was confident that the mysteries of the disorders of blood and blood-forming organs are susceptible of solution by a meticulous penetration into the details of the structure of blood cells and their precursors. Not only have such diseases as leukaemia been investigated in this way but affections of the erythron have also been classified from a morphological point of view and hypotheses of their cause and pathogenesis have been based on morphological findings. Much progress has been achieved in this way and many valuable facts established, most of them indispensable for a dynamic consideration of blood diseases.

It has been realized already in this era that anaemia may be due to three main causes: (1) increased destruction, (2) decreased formation of red blood corpuscles (R.B.C.) or their colouring material, (3) a combination of both factors. The possibility of making a quantitative determination of haemoglobin-formation has only been realized in recent years, and the first attempts to solve the problems of anaemia by a metabolic study were made at the other end of the metabolic process, the elimination of iron and the pigmented derivatives of haemoglobin. Since the first quantitative estimations of stercobilinogen by Charnass (1909), Brugsch et al. (1910-12) and Eppinger and Charnass (1913) a number of investigations on this subject have been published by Lichtenstein and Terwen (1925), Belongowa (1928), Singer (1930), Paschke (1930, 1933), Josephs (1932, 1934) and especially by Heilmeyer (1931, 1932) and by Watson (1931, 1938).

The problem of the rate of haemoglobin-formation, however, has been much less closely studied.

1. The properties of immature erythrocytes in relation to regeneration

The fact that haemoglobin is not introduced into the body as such but is formed within the body from simple materials in the food is the chief characteristic of pigment metabolism. The 'daily haemoglobin intake' must therefore be defined as the amount of haemoglobin delivered in twenty-four hours from the blood-forming organs into the circulation. The possibility of making a quantitative estimation of this amount is raised by the exclusive localization of haemoglobin in the erythrocytes and depends on whether it is possible to distinguish between the young R.B.C. freshly delivered from the haematopoietic organs and mature forms, and also to determine the time taken for these young forms to be transformed into mature erythrocytes. The following forms are generally recognized as immature red cells:

(A) NUCLEATED R.B.C. As there can be no doubt that nucleated R.B.C. are immature forms, the appearance of such cells in the peripheral circulation was for a long time considered as the only reliable sign of increased activity of the erythropoietic tissue. The various forms of erythroblasts, however, appear in the circulation with the exception of the first days of life only in pathological conditions. They are numerous in certain forms of anaemia such as hydrops foetalis, icterus gravis neonatorum, Cooley's anaemia and Jaksch-Hayem's type of erythronoclastic anaemia, whilst in other forms such as acholuric family jaundice, in which an enormous increase in the formation of R.B.C. is proved by several other findings, few if any nucleated R.B.C. may be present. Muir and McNee (1911-12) in a thorough study of experimental anaemia produced by haemolytic serum found that the number of erythroblasts present in the blood 'cannot be said to stand in any definite relationship to the rapidity of blood regeneration,' and one of us (Baar, 1924) has described cases of acute aleukaemic myelosis in which in spite of severe anaemia nu-

* Parts I-XI of these studies appeared in the *Archives of Disease in Childhood*, 1933, 8; 1935, 10 and 1937, 12. Parts XIII and XIV will appear in subsequent issues. References will be printed at the end of part XIV.

cleated R.B.C. were absent from the circulation over the whole period of observation although the bone marrow showed signs of intense erythropoietic hyperactivity. On the other hand, the appearance of numerous nucleated R.B.C. in the peripheral circulation cannot always be looked upon as a sign of intense regenerative activity of the bone marrow but may indicate a simultaneous injury to the precursors of the erythrocyte (erythronoclastic anaemia, Parsons). Bunting (1906) has shown that sudden nucleated red cell crises following the intravenous injection of ricin or saponin in rabbits were accompanied by signs of bone marrow injury, as shown by pyknosis and fragmentation of the nuclei, which in the case of saponin may be followed by a sclerosis of bone marrow tissue. It would, however, be erroneous to exclude increased formation of R.B.C. when numerous nucleated red cells are present in the circulation. Parsons (1938) has brought forward a series of arguments proving the regenerative nature of erythroblastæmia in Cooley's anaemia and erythroblastosis foetalis. The coexistence of nucleated red cells and reticulocytosis is rightly called by Wits (1932) an 'unimpeachable witness of an accelerated delivery of red cells into the circulation.' It is doubtful, however, if a transformation of nucleated R.B.C. into mature erythrocytes takes place in the peripheral circulation. In any case physiological regeneration of R.B.C. takes place without the delivery of nucleated R.B.C. The number of erythroblasts therefore cannot either in normal (newborn) or in any pathological condition form a basis for the quantitative estimation of the regeneration rate.

(B) HOWELL-JOLLY BODIES, CABOT'S RINGS AND SCHLEIP'S LOOPS are certainly, azurophilic stippling and Schilling's 'erythroconten' probably, fragments of the nuclear substance. Not one of these forms is a necessary stage in the maturation of erythroblasts. Furthermore by special stains (methylene-blue-picric acid) nuclear fragments have been shown in every red cell (Kronberger, 1912; Gruner, 1913) and must therefore be disregarded in a quantitative consideration of blood formation.

(C) BASOPHILIC STIPPLING. Originally described as a degenerative change of erythrocytes due to intoxications, particularly lead poisoning (Grawitz, 1899, 1900), it is to-day generally recognized as a sign of immaturity (Sabrazès, 1910; Lutoslawski, 1904; Naegeli, 1931; Walterhöfer, 1913; Hertz, 1910; Schilling, 1911, 1913). Though some authors considered it as a product of nuclear disintegration (Schmidt, 1909; Kreibich, 1921; Koch, 1924) it has been proved by Hawes (1909), Key (1921), Whitby and Britton (1933) that basophilic stippling is a particular manifestation of the same basophilic material as the polychromatophilic and reticular substance. After incubating blood containing many reticulocytes for some days we have repeatedly observed basophilic stippling in blood from which it was absent before; in this case it was obviously due to degeneration of the reticular substance. This in connexion with the experiments of Key (1921)

and with the fact that erythrocytes with basophilic stippling occur only in some forms of anaemia leads to the conclusion that basophilic stippling is a sign of pathological regeneration. According to Whitby and Britton (1933) stippling is a minor change of the polychromatophilic material of young red cells, the change taking place in the bone marrow. In estimating blood regeneration only those findings can be taken into account which are constant in normal and pathological young red cells; hence basophilic stippling must be disregarded.

(D) REFRACTILE GRANULES. Two types of refractile granules have been described by Isaacs (1924, 1938) as well as two types of non-refractile granules, and are regarded by him as a sign of immaturity in erythrocytes. This author has observed such granules in large numbers after blood transfusion and considers them as forming an intermediate stage between reticulocytes and mature R.B.C. As Isaacs claims that erythrocytes can be delivered from the bone marrow in this stage their enumeration would appear to be necessary for the determination of the regeneration rate. In our experiments on the maturation of reticulocytes *in vitro*, which are described below and in which a vital transformation of reticulocytes into mature erythrocytes has been shown beyond question, no such intermediate stage with refractile granules has been observed; but if blood is incubated for more than twenty-four hours many refractile bodies may be seen, not only in erythrocytes containing haemoglobin, but also in ghosts and at the same time basophilic stippling and basophilic spherical bodies, corresponding to Heinz-bodies, the 'blue granules' in toxic anaemia, become evident in stained films. These refractile bodies may, in spite of some differences in the description, correspond to those found by Pepper (1922) in incubated blood and which he regarded as identical with the *substantia metachromatica* of Demel. Their appearance under the conditions mentioned above strongly supports the opinion that they are degenerative products in young erythrocytes. Heath and Daland (1930) also observed refractile bodies in incubated blood which, in stained films, appeared as basophilic spots or rods; they considered these bodies to be identical with basophilic stippling and Schilling's 'erythroconten.' Here apparently different things are confused, for 'erythroconten' do not appear as basophilic (orthochromatic) but as azurophilic (basophilic metachromatic) rods. Isaacs believes that the refractile bodies of Heath and Daland are different from those described by himself. Refractile bodies corresponding to Isaacs' description of type 1 granules have been observed by us in the fresh blood in *icterus gravis neonatorum*. They were numerous and distinct in ghosts when the cells were haemolysed *in vitro* by a solution containing saponin, formalin and brilliant cresyl blue (Baar's platelet counting fluid). Often two granules were present, one larger than the other, and the granules often showed a 'dancing' movement in the erythrocyte stroma. It cannot be said, however, that this finding is constant in cases with high reticulocytosis.

Isaacs' opinion that numerous immature R.B.C. appear in the circulation after blood transfusions has recently been criticized by Maizels and Paterson (1940), whose view agrees with our experience of a series of clinical observations and with the results of animal experiments. Robertson (1917) after repeated small blood transfusions in rabbits observed a marked decrease or even complete disappearance of reticulocytes in thirteen out of sixteen experiments and only found an increase in one. Hess (1909) and Itami (1908) report depression of the bone marrow activity in experimental plethora. Vogel and McCurdy (1913), Lee, Minot and Vincent (1916) observed a considerable drop in the number of reticulocytes besides other signs of depressed bone marrow activity following transfusions in pernicious anaemia. Moldawsky (1928) after transfusions in various anaemias of childhood observed a considerable decrease in the number of reticulocytes. An exceptional case when, following transfusions in a haemolytic anaemia of the newborn, the reticulocytes disappeared practically completely from the circulation and the baby's macrocytic red cell population was completely replaced by the normocytic cells of the donor and a case of acholuric family jaundice in which after two blood transfusions the reticulocytes were absent from the peripheral blood for a period of six days will be reported in part XIV. The opposite reaction, as described by Friedlander and Wiedemer (1929) in two cases of duodenal ulcer and in a few cases of pernicious anaemia by Belongowa (1928) has been also met by the writers but is a rare event. Considerable increases in the numbers of reticulocytes after blood transfusions observed by Denecke (1923) were due to incompatible blood.

From the foregoing discussion it can be concluded that though refractile granules can be found in young erythrocytes the regenerative nature of such granules is not proven, that similar granules may indicate degeneration and that a stage with refractile granules is not obligatory in the transition between reticulocytes and mature erythrocytes. It must be further taken into account that Schilling (1911, 1924) found in R.B.C. of malarial blood two minute highly refractile granules which he was later able to detect in normal erythrocytes and which he considers to be centrioles. For type 1 of his granules Isaacs mentions that 'in the normoblast they have a position by the nucleus suggesting a centrosome-like body or more nearly the centrosphere.' For all these reasons the enumeration of R.B.C. with refractile granules cannot be considered as a feasible way of estimating blood regeneration.

(E) POLYCHROMASIA. The abundance of polychromatophilic erythrocytes after haemorrhage and their occurrence in the bone marrow and foetal liver are sufficient proof that polychromasia is a sign of immaturity of erythrocytes. From the investigations of Biondi (1908), Schilling (1911b, 1913), Hawes (1909), Hertz (1910), Key (1921), Arrak (1927), Simmel (1925), Seyfarth (1927), Heath and Daland (1931) and Whitby and Britton (1933) it may be concluded that polychromasia in stained films is

a manifestation of that basophilic substance which in supravital staining appears as a reticulo-granular structure. The conception of Key (1921), however, that this basophilic substance is diffuse in the plasma of R.B.C. and that the reticular structure is a result of precipitation by the stain, and the similar idea of Watson and Clarke (1937) who suggested that the reticulum is due to precipitation of the erythrocyte's porphyrin by brilliant cresyl blue, must be abandoned since Simmel (1925) and Seyfarth and Jürgens (1927) have seen a reticulum in unstained films examined in the dark field and Simmel has been able to remove the reticulum from fresh red cells by means of a micromanipulator. It must therefore be concluded that the reticular substance is genuine and polychromasia is the result of dissolution of this substance in alcohol with subsequent diffuse imbibition of the cytoplasm. Ferrata and Boselli's claim (1910) that reticulocytes and polychromatophilic erythrocytes can be demonstrated in the same film, if the supravital staining is followed by fixation and counterstaining with Pappenheim's method, is certainly erroneous. If perfectly reliable methods are used for supravital staining polychromasia is absent in counterstained films. If, however, a layer of brilliant cresyl blue on a slide is used for supravital staining the simultaneous presence of reticulocytes and polychromatic erythrocytes is occasionally observed, but in such films the indistinctness and haziness and the pale blue colour of the reticulum are always signs of an imperfect technique.

As a rule polychromasia may be considered as a sign of erythropoietic activity and this phenomenon was the first morphological property of R.B.C. used for a rough estimation of the regeneration rate. Schilling (1924, 1926) made use of his 'thick drop' method and estimated the erythropoietic activity from the average number of polychromatophilic erythrocytes in one microscopic field. If, however, the regeneration rate is to be determined for metabolic studies a method based on the enumeration of polychromatophilic erythrocytes must be rejected for the following reasons:

(a) If reticulocytes are counted after supravital staining and polychromatophilic R.B.C. in a film stained with Leishman's stain or with Jenner-Giemsa in the same blood sample the number of polychromatic R.B.C. is always lower than the number of reticulocytes. The few observations with higher figures for polychromatic R.B.C. than for reticulocytes were found in a paper by Denecke (1923), whose figures for reticulocytes, however, are often exceptionally low.

There is no fixed relation between the number of polychromatic erythrocytes and reticulocytes. This is evident from table 1.

The relation between reticulocytes and polychromatic erythrocytes (R/P) varies here between 1.53 and 10.5. The figures are particularly high in cases with low reticulocyte counts. On the other hand, in the case of high reticulocyte counts as in experimental lead poisoning and phenylhydrazin anaemia Whitby and Britton (1933) have shown that

the sum of polychromatic and stippled cells is only little below the number of reticulated cells and that the two figures run fairly parallel. The relation between the number of reticulocytes and the sum of polychromatic and stippled cells in these experiments was about 1.2-1.5. It can be concluded that whilst even minute amounts of basophilic substance can be demonstrated by supravital staining a considerably higher amount is necessary to cause polychromasia.

TABLE 1

RELATION OF RETICULOCYTES TO POLYCHROMATOPHILIC ERYTHROCYTES IN TWENTY CASES OF HAEMOLYTIC ANAEMIA

No.	RETICULOCYTES PER CENT.	POLYCHROMATIC R.B.C. PER CENT.	R/P
1	27.9	16.2	1.72
2	19.6	7.1	2.76
3	18.4	4.1	4.50
4	18.0	11.8	1.53
5	13.2	6.4	2.06
6	11.4	5.6	2.04
7	10.8	2.7	4.00
8	9.0	2.3	3.92
9	8.4	2.1	4.00
10	8.2	1.8	4.57
11	6.6	4.2	1.57
12	5.4	2.8	1.93
13	5.1	2.0	2.55
14	5.1	2.4	2.12
15	4.8	2.4	2.00
16	2.3	0.8	2.88
17	2.1	0.2	10.50
18	1.8	0.2	9.05
19	0.7	0.1	7.00
20	0.5	0.1	5.00

(b) The close relation between regeneration and polychromasia does not exclude the possibility that a similar change in the staining property of erythrocytes can be caused by degeneration. This has been claimed by Bodon (1903), Hirschfeld (1909) and Naegeli (1931) but denied by Heinz (1908) and Arrak (1927). Biondi (1908), who was the first to give proofs for the identity of the basophilic and reticular substance, admits the possibility of degenerative polychromasia. We have repeated Bodon's experiments. Normal human blood was incubated in sealed capillary tubes for several days. At intervals of twenty-four hours films were made and stained with Leishman's stain, Jenner-Giemsa and Manson-Koch's borax-methylene blue. After seventy-two to ninety-six hours most of the red cells showed a vacuolar degeneration, a greyish staining with Leishman and Jenner-Giemsa and a deep blue colour with Manson-Koch's stain. These are, of course, extremely unphysiological conditions, but the results show that the possibility of a degenerative polychromasia cannot be ignored.

(F) RETICULOCYTES. Since Ehrlich and Howell described basophilic nets in unfixed R.B.C. stained with basic stains many investigations have been published on this subject. The old conception of

Chauffard and Fiessinger (1907) that the reticular substance is a degenerative phenomenon and that increased fragility is the result 'd'une lésion granuleuse special des hématies' is to-day definitely dismissed. As a result of investigations of Hawes (1909), Schilling (1911, 1913), Hertz (1910), Key (1921), Seyfarth (1927), Moldawsky (1928) and Naegeli (1931) the regenerative nature of reticulocytosis, first suggested by Smith, was generally recognized. The finding of Seyfarth (1927) that in early foetal life, when megaloblasts are the only form of R.B.C., all these cells contain a vitally staining reticular substance is especially convincing. The extensive investigations of this author prove beyond question that the reticulocyte is an inevitable transitional stage in the normal development of R.B.C., but there is still disagreement as to whether all R.B.C. delivered from the bone marrow contain reticular substance or not. This question is a fundamental one, as only if this be so is a calculation of the regeneration rate possible on the basis of reticulocyte counts. Isaacs claims that R.B.C. enter the circulation not only in the state of reticulocytes but also as R.B.C. with refractile granules. His proofs, as has been pointed out above, are not convincing. Minot and collaborators (1928) conclude from their investigations on blood regeneration in pernicious anaemia treated with liver extract that 'the erythrocyte increase at the time of the peak of the reticulocyte rise is almost entirely to be accounted for in the cases with less than 2.5 millions R.B.C. by reticulocytes' but 'when the R.B.C. count is relatively high the entire increase in erythrocytes at the time of the peak of the reticulocyte rise, apparently, is not due to these young forms.' This conclusion is based on the following observation: In a series of eighty-nine cases in which the observed concentration of reticulocytes at the peak of reticulocyte response, Epr (where Ep is the number of erythrocytes at the peak and r the percentage of reticulocytes at the same time) was compared with the concentration of reticulocytes at the peak of the rise as calculated from the formula $\frac{Eor}{1-r}$ (where Eo is the number of R.B.C. before liver treatment). A good agreement was found only in cases with low initial erythrocyte counts. It can easily be seen that $Epr = \frac{Eor}{1-r}$ when $Ep - Eo = Epr$. This means that all reticulocytes delivered from the bone marrow from the start of liver treatment are present at the peak of reticulocyte response still in the reticulated stage and that no destruction of R.B.C. had taken place in this period. Actually there is an intense destruction of erythrocytes at the peak of reticulocyte rise and before, as will be shown later [cf. Heilmeyer (1932), Watson (1931, 1938), Belongowa (1928), Dobriner and Rhoads (1938)] and the life span of the erythrocyte at this time is very short. The close agreement between the observed and calculated figures in cases with low initial erythrocyte counts shows that in these cases the two factors, maturation of reticulocytes into non-reticulated R.B.C. and the destruc-

tion of erythrocytes, are well balanced. It can be easily conceived that the increase in the number of R.B.C. at the peak of the reticulocyte response will the more closely correspond to the number of cells just delivered from the bone marrow the shorter the duration of life of the erythrocyte or the severer the disease. With the figure Eo approaching O, Ep will approach Epr and with the shortening of the erythrocyte's life span the latter will approach the maturation time of reticulocytes. The argument of Minot and his collaborators cannot be accepted therefore and their important observations may be given a different explanation.

From investigation on blood regeneration in pernicious anaemia came the first proof that there is a fixed mathematical relation between the number of reticulocytes and the delivery of R.B.C. from the blood-forming organs. Riddle (1930) was the first to apply Robertson's (1923) formula for the kinetics of growth to blood regeneration in pernicious anaemia. In Robertson's fascinating book innumerable proofs are given that in the whole of living nature growth follows the rules of the autocatalyzed reversible reaction. Such a reaction is determined by the formula $\log \frac{x}{a-x} = ka(t-t_0)$. * As applied to the increase in the concentration of erythrocytes during the reticulocyte response, the value (x) in this equation represents the increase in the concentration of erythrocytes after any number of days of treatment (t). The value (a) is a constant representing the total increase in the erythrocyte concentration during the reticulocyte response, and as blood regeneration takes place during the reticulocyte response (x) approaches (a) as a limit. The value (t) represents the number of days of treatment when $x = \frac{a}{2}$, which usually is near the peak of the reticulocyte response. The value (k) is a constant which is determined for each different application of the formula.* It has been shown that for each case the same formula can be applied for the total increase of R.B.C. and for the increase of non-reticulated cells as well, the only difference being in the value (t). Furthermore if from a formula differing only in the figure (a) the numbers of reticulocytes per c.mm. liberated from the bone marrow are calculated and a corresponding 'differential' curve constructed the latter shows a satisfactory agreement with the observed numbers of reticulocytes. These investigations afford evidence that if erythrocytes are not all liberated from the bone marrow as reticulocytes there is at least a fixed mathematical relation between the daily intake of R.B.C. and the number of reticulocytes. Complete evidence that all erythrocytes delivered from the bone marrow are reticulocytes would be supplied if it were possible to show that the bone marrow, or more exactly the haematogenic intersinusoid capillaries, contain only reticulocytes. In man such investigations meet with insurmountable difficulties. Post-mortem examinations are useless; sternal puncture always yields a mixture of bone marrow and circulating

blood and besides this there are difficulties in the supravital staining of bone marrow. Only staining in a solution of brilliant cresyl blue in normal saline with the addition of sodium citrate gave in our experience satisfactory results. It can be shown in that way that rats and guinea pigs with 1 to 2 per cent. reticulocytes in the circulating blood have about 50 per cent. reticulocytes in the bone marrow. The non-reticulated red cells may be due to an admixture of circulating blood. We have tried to wash out the circulating blood by opening the right auricle and injecting normal saline into the aorta from the left ventricle in guinea pigs. For reasons unknown the subsequent supravital staining of the bone marrow was not always successful and only once was a film obtained in which all R.B.C. contained reticulum. For rabbits Istomanowa (1926) succeeded in showing that all the red cells of the bone marrow are reticulated. In order to elucidate the question whether in human beings all erythrocytes entering the peripheral circulation from the bone marrow contain supravital staining substance the excretion of bile pigments has been determined in three cases of aleukaemic myelosis without any reticulocytes in the circulating blood and in one case of acholuric jaundice where two blood transfusions were followed by a six days' period of areticulocytosis. The idea which led to this investigation was that if the assumption that the total intake of R.B.C. is due to a liberation of reticulocytes from the bone marrow be correct the absence of such cells from the peripheral circulation must mean that there is no replacement of destroyed erythrocytes at all. As there is no evidence for the storage of bile pigments, and the reutilization of pigments can be excluded in such cases,* the amount of bile pigment excreted must correspond to the fall in the total circulating haemoglobin. The methods applied at the present time for stercobilin or stercobilinogen determinations are useless for this purpose. It is recognized by many authors that only part of the excreted bile pigment is estimable by stercobilinogen determinations. Greppi (1926, 1930) assumes that the real destruction of haemoglobin is twice that calculated from stercobilinogen determinations while according to Eppinger (1920) and Schioedt (1938b) the latter figure is only a fourth of the true one. One-fourth to one-fifth is the figure calculated from the stercobilin excretion in stools and bile pigment excretion in bile fistula dogs if that decrease is taken into account which takes place if the bile is not returned to the intestine. The bile pigments of human stools have been investigated by one of us (H.S.B.) in collaboration with Dr. E. Hickmans. As the result of these investigations a method for estimation of the 'total bile pigments' has been elaborated. These investigations are not completed yet and will be published later (Baar and Hickmans,

* There is much controversy about the reutilization of bile pigments since the classical experiments of Stadelmann (1871). It is denied by Whipple and his collaborators from experiments on dogs. From investigations on pigment-excretion which will be reported later it was concluded that there is no reutilization in normal and in many pathological conditions but that it may occur to a considerable extent in acholuric jaundice and some other haemolytic anaemias.

1941). It is possible here only briefly to outline the method and its experimental foundation.

A study of the absorption curves of about two thousand acid alcoholic stool extracts combined always with spectroscopic and in many instances with fluoroscopic examination gave evidence that besides stercobilin, copromesobiliviolin and copronigrin (Watson, 1932, 1934) there is at least one pigment present with an absorption increasing between 490 and 420 $m\mu$. Depending on the relation between stercobilin and this pigment, not on the amount of copromesobiliviolin, the extracts show with zinc alcohol either a green fluorescence all through or a green fluorescence in the upper layer and a reddish-yellow fluorescence in the lower layer, or even a reddish fluorescence only when examined in the dark box devised by Elman and McMaster (1925). By incubation of stool emulsions a striking change of the absorption curve has often been obtained, indicating a transformation of stercobilin into the above mentioned pigment or pigments. In a few instances the reverse process has been observed. By fractional extraction and chromatographic separation two new pigments have been isolated: a red one crystallizing in needles and a brownish-yellow obtained up to the present moment only in amorphous form. The latter pigment has been reduced by means of sodium amalgam into a leuko-compound giving a weak Ehrlich-reaction. Both pigments show similar absorption curves between 490 and 420 $m\mu$ but differ in the region 520 to 490 $m\mu$. 'Stercobilin' prepared according to Garrod and Hopkins' method (modification of Elman and McMaster) proved to be an impure mixture of stercobilin and these two pigments (stercofulvin and stercorubrin). Analysis of absorption curves of stool extracts indicates that either one of these pigments is prevalent or both run parallel. This justifies a calculation on the basis of two pigments. The validity of the Beer-Lambert law has been ascertained (only within certain limits of concentration) for these pigments and pigment mixtures in the spectral regions in which the determinations are made (490 and 440 $m\mu$). Crystalline stercobilin has been prepared by the methods of Watson (1934) and of Heilmeyer and Krebs (1934), and several crystalline mixtures have been obtained containing stercobilin and stercofulvin (and/or stercorubrin). The absorption relations have been determined for pure crystalline stercobilin and for crystalline mixtures. The quotient E490/E440 has been determined in a solution of stercofulvin free from stercobilin. Thus although neither stercofulvin nor stercorubrin have been isolated in pure form it was possible with the use of the Vierordt formula to calculate the absorption relations of stercofulvin (and or stercorubrin) for the spectral region of filters 490 and 440 in the Evelyn photoelectric step photometer. With the known A values for stercobilin and stercofulvin (and or stercorubrin) the amount of both pigments can be calculated from the extinctions at 490 and 440 $m\mu$ by means of the Vierordt formula. This gives approximately the amount of 'total bile pig-

ments' if copromesobiliviolin is absent or present in traces only, which can be ascertained by spectroscopic examination. A chart has been constructed in which the quotient E490/E440 was plotted against the constants for calculation of 'total bile pigments' (A-values for mixtures of stercobilin plus stercofulvin and/or stercorubrin). Copronigrin, the relationship of which to bilirubinoid pigments is not settled yet and which can be disregarded at least in stools freshly extracted, was eliminated by treatment with zinc acetate.

Case 1. (R. A.) a boy of three years was admitted to the Children's Hospital, Birmingham, under the care of Professor Parsons on February 28, 1940. He had been treated for streptococcal otitis media in September, 1939, when he was anaemic, and had bruises over the iliac crests. The aural discharge had persisted despite treatment. Five days before admission he suddenly became ill and rather blue. On admission there was severe anaemia and enlargement of cervical and axillary glands. His W.B.C. counts were between 2100 and 4000, 80 to 90 per cent. being lymphoid cells. Some of these had the appearance of mature lymphocytes, some of micromyeloblasts. The platelets were always few. Reticulocytes were seen only on one occasion when 0.05 per cent. were counted. In many other examinations no single reticulocyte was found. A sternal puncture yielded a thin fluid containing a few cells of which all white cells were pathological myeloblasts (paramyeloblasts) and not a single nucleated R.B.C. was seen. Stools were collected over a period of six days and estimations of bile pigments were made in triplicate. Only two haemoglobin determinations were made with Evelyn's photoelectric step photometer (standardized by van Slyke's oxygen capacity method) in an interval of three days between two blood transfusions. Blood volume was calculated from the body weight on the basis of Seckel's (1930, 1936) figures. The results of the investigations on this and the two other children are given in table 2. The average daily wastage of haemoglobin was calculated from the daily excretion of bile pigment on the basis that 100 mgm. of pigment correspond to 2.5 g. haemoglobin.* The figures show good agreement between the excretion of pigmented haemoglobin derivatives and the drop in the total circulating haemoglobin. It can be concluded therefore that in this case in which reticulocytes were absent no regeneration of R.B.C. took place.

Case 2. D. W., a girl of three and a half years, was admitted under care of Dr. Braid at the Children's Hospital, Birmingham, on June 11, 1940. She had been fairly well until the beginning of June, 1940, when she began vomiting, developed a cough and became pale. On admission enlargement of liver and spleen and bruises on both legs were found. The haemoglobin was 42 per cent. (Haldane),

* This calculation is used by most authors working on pigment excretion. It is based on the molecular weight of haem which is 651. The molecular weight of the prosthetic group of haemoglobin (protohaem) is 616 and that of bilirubin 582. It is therefore correct when Hawkins and Whipple (1938) take 35 mgm. bilirubin as equivalent to 1 gm. haemoglobin. In our method, however, the calculations are based on an absorption relation determined for stercobilin hydrochloride, the molecular weight of which is 630. The calculation 40 mgm. stercobilin = 1 gm. haemoglobin is therefore approximately correct.

R.B.C. 2,470,000 per c.mm., reticulocytes 0.32 per cent., W.B.C. 3100 per c.mm. with 82 per cent. lymphoid cells, at least 23 per cent. of which were myeloblasts. Occasional neutrophilic myelocytes and metamyelocytes were found, and 2 to 6 normoblasts per 100 W.B.C. A sternal puncture showed an increase in the number of myeloblasts (23 per cent.) and a low count of nucleated R.B.C. (6 per cent.). On June 30, 1940, the child was taken home; she was readmitted on September 6, 1940. The findings at this time were similar to those of June except in the complete absence of reticulocytes from the circulating blood. Only on one occasion was a single reticulated R.B.C. seen. The child died on September 14, 1940. Post-mortem examination (H. S. B.) showed a severe anaemia; subpleural and subepicardial haemorrhages, enlargement of lymph nodes, liver and spleen, leukaemic ulcers in the lower ileum and haemosiderosis of the liver. Histological examination revealed a high degree of myeloid metaplasia in liver, spleen, kidneys and lymph nodes. In the liver there was cellular infiltration in the interlobular spaces and clusters of cells in the liver capillaries as well. The bone marrow was cellular, most of the cells being myeloblasts; only occasionally a few neutrophilic and eosinophilic leucocytes were present. Nucleated R.B.C. were encountered only exceptionally in the bone marrow and were altogether absent from the foci of myeloid metaplasia. Determinations of bile pigments in the stools were made over a period of six days of the 'a regenerative' stage, three blood counts were done during this period, and the results are shown in table 2.

Case 3. G. P., ten months old, was from May 25, 1941 to June 11, 1941 an inpatient in the Children's Hospital, Birmingham, under care of Professor Wilkinson, for upper respiratory tract infection, gastro-enteritis and haemolytic anaemia. Neither blood picture nor bone marrow (sternal puncture)

were at that time suggestive of leukaemia. On July 17, following first diphtheria inoculation, the child was noticed to have progressive loss of appetite. She had been also noticed to go yellow. On re-admission (July 22, 1941) she was pale, purpuric spots were seen all over the body and an ulcer in the mouth. All W.B.C. were lymphoid forms, many of them immature. No nucleated R.B.C. and no reticulocytes were found in the peripheral circulation. Sternal puncture: all nucleated cells were pathological, immature lymphoid cells (? micro-myeloblasts); nucleated R.B.C. absent. Stools were collected and bile pigment estimations done over two periods of three and two days respectively. Three blood counts were made between July 24 and August 3, 1941. The results are shown in table 2. The child died on August 3, 1941. The post-mortem examination (H. S. B.) showed ulceration of the left tonsil and of oesophagus, lobular pneumonia, purulent otitis media, general anaemia, enlargement of liver, spleen and kidneys, the latter with diffusely outlined greyish-white areas and numerous haemorrhages.

Case 4. R. T., a boy of seven years, admitted on June 9, 1941, to the Children's Hospital, Birmingham, under the care of Dr. Neale. He was a typical case of acholuric family jaundice; a brother died at the age of seven months from severe anaemia, purulent otitis media and glomerulonephritis, two sisters had their spleens removed. On admission the haemoglobin was 5.5 gm. per cent. and the red cell count 2,030,000 per c.mm. On the next day the haemoglobin was 4.42 gm. per cent. and the red cell count 1,700,000 per c.mm. Following two blood transfusions the reticulocytes disappeared completely from the peripheral circulation and were absent for six days. At the end of this period the haemoglobin was 7.0 gm. per cent. and the red cell count 2,550,000 per c.mm. The calculated blood volume was 2000 c.c. The amount of transfused

TABLE 2

BILE PIGMENT EXCRETION AND FALL IN HAEMOGLOBIN IN THREE CASES OF ALEUKAEMIC MYELOSIS WITH AREGENERATIVE ANAEMIA

Name	Date	Haemoglobin gm. per cent.	Blood volume c.c.	Total circulating haemoglobin (gm.)	Total bile pigment excretion (mgm.)	Average daily wastage of haemoglobin (gm.)	Average daily drop in total circulating haemoglobin (gm.)
R. A.	4.3.40	4.95	1334	66.0			
	6-7.3.40				204		
	7.3.40	3.62		50.0			
	7-11.3.40 11-12.3.40				828 214	5.2	5.3
D. W.	7.9.40	3.85	1120	47.3			
	8-11.9.40				370		
	13.9.40	2.34		26.2			
	11-14.9.40				276	2.58	2.92
G. P.	24.7.41	7.6	542	41.2			
	24-27.7.41				214		
	30.7.41	4.97		26.9			
	30.7-1.8.41				162		
	3.8.41	3.72		20.2		1.88	2.10

haemoglobin was approximately 128 gm. The difference in the amount of circulating haemoglobin between the starting and end-points of the aregenerative phase corresponded to a gain of 51.6 gm. 76.4 gm. haemoglobin had been lost therefore in that period. The amount of excreted bile pigment over the same period was 2872 mgm. corresponding to haemoglobin wastage of 71.7 gm.

In all four cases there is a fairly close agreement between the actual loss of haemoglobin and that calculated from the bile pigment excretion, showing that in the absence of reticulocytes there is no regeneration of destroyed R.B.C.

From the foregoing observations it may be concluded that R.B.C. are liberated from the bone marrow only in the stage of reticulocytes.

With regard to this conclusion a modification of Witts' schematic diagram of the erythron is proposed. In this diagram the independence of the normoblastic generation from the megaloblastic one and the possibility of extravascular formation of proerythroblasts (Naegeli, 1931; Turnbull, 1934; Gilmour, 1941) is taken into account as well as the delivery of red cells from the erythrocytic inter-sinusoid capillaries into the peripheral circulation exclusively in the reticulated stage (fig. 1).

FIG. 1.—Modification of Witts' schematic diagram of the erythron. The scheme shows the erythrocytes entering the peripheral circulation only in the reticulated stage; megaloblasts are not considered as normal stage in the development of the erythron and therefore replaced by proerythroblasts and basophilic macroblasts; the ?-signs indicate that the origin of erythroblasts either from endothelial cells or from extravascular reticulum cells is undecided.

2. The enumeration of reticulocytes and their maturation time

The importance of obtaining accurate reticulocyte counts in investigating haemoglobin metabolism is obvious. It is necessary therefore to make some comment on the various methods of supravital staining at present in common use. Most authors use fresh wet preparations for enumerations of reticulocytes adopting the standpoint of Seyfarth (1927) that alcohol fixation and counterstaining causes the disappearance of some of the supravital staining substance. Heilmeyer (1931, 1935) and Trachtenberg (1932), however, emphasize that in wet preparations erythrocytes with only few

granules can easily be overlooked. The method of Heilmeyer and Trachtenberg has the disadvantage that clotting frequently occurs in blood incubated with a brilliant cresyl blue solution on excavated paraffin blocks and interferes with the supravital staining. One of us (H. S. B.) has for eight years used a method which is adapted from the old procedure of Widal, Abrami and Brulé (1907) and that of Heilmeyer and Trachtenberg:

To equal parts of normal saline and 3.8 per cent. solution of sodium citrate 0.1 gm. per cent. of brilliant cresyl blue is added and the solution filtered. 0.5 c.c. of this solution is placed in a small tube and 2-3 drops of fresh blood added. Blood and solution are mixed and kept in an incubator at 37° F. for 15 to 20 minutes. The tube is then centrifuged at low speed for 1 to 2 minutes and the supernatant fluid carefully poured off. Some of the clear supernatant fluid is left in the tube in order both to facilitate the making of thin films and to avoid removing the upper layer of cells which is richest in reticulocytes, the specific gravity of reticulocytes being lower than that of other R.B.C. (Key, 1921; Stephens, 1938; Gripwall, 1938). The tube is shaken or the contents mixed with a glass rod and films made from the suspension thus obtained, the films being counterstained with Leishman's stain. It has been found that a short fixation (half a minute) with the concentrated stain and dilution of the stain some twenty times prevents damage to the reticulum which in these films is dark blue or black. It is important in counting reticulocytes to remember that they may be distributed unevenly; they often occur in rather larger numbers at the edge of the slide; for this reason we recommend Schilling's 'meander' method.

We often use another quicker method based on those described by Pappenheim (1907) and later by Hawes (1909) and Cunningham (1920): A clean slide is covered with a saturated alcoholic solution of brilliant cresyl blue. The solution is ignited and allowed to burn till the slide is covered with a uniform layer of brilliant cresyl blue. The film should not be used on the day of preparation. A drop of blood is placed on the brilliant cresyl blue film and mixed with the stain. After two minutes a small drop of the supravital stained blood is transferred to another slide and a thin film is prepared and counterstained as in the previous method. Usually the film before counterstaining is greenish-blue in colour; occasionally, however, it is a bright purple; films of this second type give unreliable results for the reticulum, being often indistinct and polychromasia may be found. The reason for this occasional failure is unknown; all such films must be discarded and the count repeated using another cresyl blue film or the other method.

Pure methyl alcohol and eosin appear to damage the reticulum; it is for that reason that the short fixation and high dilution of the stain are so important. Films stained in this way show distinct dark blue reticulum and even the smallest granule are easily seen. It is possible also to make differential reticulocyte counts and, no mean advantage over the wet film method, it is possible to check the counts after any length of time. Counts made in counting chambers with a dry objective are unreliable and always give too low a figure. Figures

of 0.03–0.2 per cent. for normal persons as given by Friedlander and Wiedemer (1929) cannot be accepted for these technical reasons.

With the knowledge that all R.B.C. are released from the bone marrow in the reticulocyte stage it is possible to estimate the daily intake of erythrocytes and haemoglobin if it be possible to estimate the time taken by a reticulated cell to mature into an adult erythrocyte.

The first experiments indicating the possibility of determining this time were performed by Pepper (1922). The aim of these investigations was to demonstrate the survival of R.B.C. *in vitro* and the property of supravital staining was used as a sign of survival. Pepper was able to show that in blood kept in the ice chest the number of reticulated cells was undiminished at the end of three weeks, while in the incubator these forms disappeared within forty-eight hours. Whether this was due to maturation or death of reticulocyte was not considered. Experiments performed with the express aim of determining the 'maturation time' of reticulocytes have been published by Heath and Daland (1930), Seyfarth and Jürgens (1927), Heilmeyer and Westerhäuser (1932). In contrast to these authors Mermod and Dock (1935) failed to find a decrease in the number of reticulocytes in incubated blood and suggest that 'an apparent decrease in reticulocyte count is found when only a few seconds staining time is permitted by the dry method.' They even doubt whether the reticulocyte does mature in the circulation and suggest that these are quickly destroyed in the circulation and their delivery from the bone marrow is an undesirable event. In all these experiments as well as in those of Pepper defibrinated blood was incubated and reticulocyte counts were made at long-time intervals, e.g. by Heath and Daland at twenty-four hour intervals, by Heilmeyer and Westerhäuser at eight, twenty-four, thirty-two, forty-eight and fifty-six hours. It will be shown later that changes occur in the properties of the erythrocyte within the shortest of these times. Before it is possible to draw conclusions from such experiments as to the maturation time in life the influence of the experimental conditions themselves must be studied and experiments *in vitro*, if possible, checked by determination of the maturation time *in vivo*.

It seemed that for our experiments *in vitro* the medium most suitable for the 'culture' of reticulocytes would be that which had already been shown to be suitable for tissue cultures, especially culture of bone marrow cells. For obvious reasons the medium had to be liquid. Successful cultures of bone marrow cells in liquid medium have been reported by Osgood and Brownlee (1936), and more recently by Israels (1940). Both authors have observed that in their media not only does division take place but also the transformation of immature myeloid elements into mature forms.

The medium recommended by Osgood and Brownlee is the same as that described by the Geys for tissue cultures and consists of a citrated balanced

salt solution to which 0.1 per cent. dextrose and 35 per cent. human cord serum has been added. The fact discovered by Carrel and Ebeling (1921, 1922), that when vertebrate tissue cells are cultivated in blood plasma the rate of multiplication varies in inverse ratio to the age of the animal from which the plasma was taken, led to the replacement of the cord serum by serum or plasma of the patient whose blood was to be 'cultured' or, if this was not available, with adult's serum of the same blood group. This precaution seems to be justified although it is not known whether the same factor applies for maturation as for multiplication of cells. In the first experiments it was found that the citrated balanced salt solution did not prevent clotting for a sufficiently long time. An addition of a 3.8 per cent. solution of sodium citrate was therefore used in all later experiments.

To two parts of an autoclaved solution of the following composition

$\text{Na}_2\text{C}_6\text{H}_5\text{O}_7 \cdot \text{H}_2\text{O}$	..	..	..	5.0	gm.
NaCl	..	..	..	6.8	..
NaHCO_3	..	..	..	0.23	..
KCl	..	..	..	0.37	..
CaCl_2 (anhydrous)	..	..	..	0.17	..
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	..	..	..	0.21	..
$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$	..	..	..	0.15	..
KH_2PO_4	..	..	..	0.03	..
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	..	..	..	0.07	..
Dextrose	..	..	..	1.0	..

per litre, one part of a sterile 3.8 per cent. solution of sodium citrate is added and this is mixed with 35 per cent. sterile human serum or plasma. A number of small sterile tubes provided with corks are filled with 0.5 c.c. of this medium and two drops of the blood to be examined are added under sterile precautions. Two drops of a saturated solution of brilliant cresyl blue in normal saline are added immediately to one tube and all are placed in an incubator. After fifteen to twenty minutes the tube with brilliant cresyl blue is centrifuged for one to two minutes at low speed, the supernatant fluid pipetted or poured off and films prepared and counterstained in the way described above. At time intervals of two hours brilliant cresyl blue solution is added to two other tubes, the tubes incubated for a further fifteen to twenty minutes and films prepared and stained. The remaining tubes are gently shaken every two hours. Two to twenty thousand red cells are counted depending on the number of reticulocytes present. Usually four films are made, two from each of the duplicate tubes, at each time interval. At the conclusion of the experiment the numbers of reticulocytes are plotted against the time of incubation.

The results of this method have been compared with those obtained by incubation of heparinized whole blood, whole blood in paraffined tubes, citrated whole blood and with suspensions of red cells in Osgood's medium without serum, with and without glucose and in Simmel's (1925) solution (NaCl 8.2, KCl 0.2, MgCl_2 0.2, CaCl_2 0.2, NaH_2PO_4 0.1, NaHCO_3 0.05 gm. per litre H_2O) to which one-fifth part of 3.8 per cent. sodium citrate solution had

been added. Heparin blood and to a less degree citrated whole blood were found to be liable to haemolysis whereas whole blood in paraffined tubes remained unclotted for only a few hours; these methods were therefore discarded. In all other media the results were practically identical whether they contained serum or glucose or not. For most of the experiments which are to be reported in this paper therefore either Osgood's balanced salt solution with additional sodium citrate but without glucose and serum or citrated Simmel's solution have been used. Spectroscopic examination showed that under the conditions described there was no haemolysis for forty-eight to seventy-two hours. The importance of the dilution factor in preserving the vitality of R.B.C. has been recognized in all recent work on blood storage.

In conformity with the results of Heath and Daland and of Heilmeyer and Westerhäuser not only has a gradual decrease of the number of reticulocytes been observed but also a loosening of the reticulum, a diminution in the amount of supravital staining substance and the replacement of filaments by granules. This is illustrated by fig. 2 and 3 which show the blood of a rabbit and a guinea-pig both poisoned with phenylhydrazine, before and after five, seven and eight hours incubation in Osgood's medium respectively.

These gradual changes in the morphology of the reticular substance, together with Heilmeyer and Westerhäuser's findings of high oxygen consumption for several hours, afford sufficient proof that a vital process and not haemolysis of reticulocytes is the cause of their disappearance. No trace of haemolysis was demonstrable by spectroscopic examination at a time when the number of reticulocytes was considerably decreased or even when they had disappeared completely. This observation and the technique of supravital staining show that the objections of Mermod and Dock are not justified. The numerical decrease of reticulocytes is illustrated by charts I to III, VI and VII in which the time in hours

is plotted against the number of reticulocytes expressed as percentage of the original figure.

The points follow a straight line for four to six hours (in few instances for eight hours), then the velocity of disappearance of reticulocytes decreases considerably and the slope finally becomes a gentle one or the curve may even take a horizontal course. On the interpretation of this curve depends the final calculation of the maturation time. Heath and Daland who counted reticulocytes in defibrinated blood only every twenty-four hours describe a somewhat similar 'maturation curve' and say 'if this curve is plotted with the logarithms of the reticulocyte percentage as ordinates, the result is a straight line. This type of curve is characteristic of senescence phenomena (Robertson).' This interpretation is, however, erroneous for two reasons.

First, the type of chemical reaction described by Robertson (1923) as characteristic for multiplication, growth and senescence through the whole living world is that of a reversible autocatalysed reaction

which is defined by the formula $\frac{dx}{dt} = kx(a-x)$ which

on integration gives finally the formula $\log \frac{x}{a-x} = ka(t-t_0)$ and is graphically represented by a S-shaped curve, whereas the curve reproduced by Heath and Daland corresponds to the formula of monomolecular reactions $\frac{dx}{dt} = k(a-x)$ which on

integration gives $\log \frac{a}{a-x} = kt$. There is indeed no reason to believe that the maturation of reticulocytes, which are not nucleated cells, should follow the first formula for it has been shown by Robertson that 'it is the nucleus, therefore, that we must look for, alike for the source of the accelerative agent in cellular multiplication, and the source of autocatalytic time-relations, which distinguish all types of growth.'

Secondly, a curve with the time as abscissa and the percentage of reticulocytes as ordinates does not represent the real kinetics of maturation. For such

CHART I.—Average maturation curve of reticulocytes from fifty-six cases with normal or moderately increased numbers of reticulocytes. Horizontal axis—time in hours; vertical axis—reticulocytes in per cent. of original figure.

CHART II.—Maturation of reticulocytes of a normal child. Horizontal axis—time in hours; vertical axis—reticulocytes (R) in per cent. of the original figure. Extrapolation of the initial slope to $R=0$.

an investigation the total mass of supravitality staining substance should be plotted against time. We have, however, no means of determining the amount of reticular substance. What is determined in maturation experiments is the last stage of the reaction, the disappearance of a small, just discernible amount of reticular substance. Therefore the velocity of the reaction is estimated in a stage when (x) approaches (dx). If various films of a maturation experiment are compared, especially in cases with numerous young reticulocytes, it can easily be seen that the amount of reticular substance which disappears in the transformation of the youngest reticulocytes into cells with a loose and scanty reticulum is several times in excess of that represented by the maturation of the older reticulocytes into ordinary R.B.C. The real velocity of maturation is therefore a decreasing one even when the 'maturation curve' is a straight line. A better insight into the real velocity of maturation can be expected if the reticulocytes are divided into several groups according to the amount of reticular substance and the velocity of disappearance determined for each group separately. Such classifications of reticulocytes have been attempted by Rosin and Biebergeill (1904), Demel (1907), Lee, Minot and Vincent (1916), Seyfarth and Jürgens (1927), Moldawsky (1928), Gawrilow (1929), Heilmeyer (1931, 1935) and Trachtenberg (1932), Eaton and Damren (1930). Heath and Daland have adopted in their studies the classification of Gawrilow into reticulocytes with large amounts of reticulum, with medium and with small amounts. Heilmeyer and Trachtenberg distinguish five groups: O=nucleated red cells, I=ball-shaped reticulum, II=network forms, III=incomplete network, IV=granular forms. In the present experiments an attempt was

made to use the classification of Heilmeyer and Trachtenberg but it was found impossible to get reliable results, the transition between the various groups being ill-defined. Only if groups II to IV were combined and separated from group I was a fairly accurate differentiation possible. Chart III shows the results of such differential counts in a maturation experiment with reticulocytes of a rabbit after phenylhydrazine poisoning and curves obtained in a similar way from figures of Heath and Daland, and Heilmeyer and Westerhäuser.

The curves show that the slope is somewhat steeper for the younger forms. As has already been pointed out even when the rate of disappearance of two such groups is the same the actual 'velocity of maturation' is higher for the forms with the large amount of reticulum, the real speed of disappearance of reticulum (not to be confused with the disappearance of reticulocytes) is therefore to be represented by a curve of diminishing slope. The maturation curves shown here have at first sight such a shape. It is of first importance to realize that the resemblance between these curves and that deduced for the rate of disappearance of reticular substance is only superficial. In the latter case the slope represents the relation between the mass of reticular substance in a single erythrocyte and the time without relation to the total number of such cells present; the present curves, however, are obtained by determining only the end point of the process and for the first part of their course follow a straight line, a rapid diminution of the slope occurring after some hours. The decreasing velocity of the chemical reaction underlying the maturation of reticulocytes therefore cannot explain the shape of curves representing the decreasing number of reticulocytes in incubated blood.

CHART III.—Maturation of reticulocytes with regard to their immaturity. Horizontal axis—time in hours; vertical axis—reticulocytes in per cent. of original figure. — Drop in the total number. - - - Drop in the number of group I reticulocytes. 1. Pair of curves: Maturation of rabbit's No. 3 reticulocytes in vivo. 2. Pair of curves: constructed from figures of Heath and Daland. 3. Pair of curves: constructed from figures of Heilmeyer and Westerhäuser. The figures in brackets correspond to curves 2 and 3.

It is possible to explain in two ways why the reticulocytes disappear so rapidly in the early hours of incubation while they later disappear slowly or remain present in a constant percentage.

(a) It is conceivable that the maturation of reticulocytes results in a formation of substances which diffuse into the medium and inhibit the maturation of the remaining reticulocytes. This possibility is suggested by experiments of Eijkman who has shown that substances are developed in bacterial cultures which inhibit their further multiplication and by those of one of us (H. S. B., 1912) who has demonstrated the production of substances having a comparable inhibiting action during the swelling and germination of seeds of *Phaseolus* and *Atriplex*. In order to find out whether this possibility applies to the maturation of reticulocytes two series of tubes containing blood in citrated Simmel's solution were incubated. One series was used for a maturation experiment in the usual way while in the other the medium was pipetted off and replaced every hour. The results of two such experiments, one on a guinea pig with phenylhydrazine poisoning, the other on a baby with erythronoclastic anaemia are shown in table 3.

TABLE 3

MATURATION OF RETICULOCYTES IN A CASE OF ERYTHRONOCLASTIC ANAEMIA AND IN A GUINEA PIG WITH PHENYLHYDRAZINE ANAEMIA. SERIES I REPRESENTS A USUAL MATURATION EXPERIMENT; IN SERIES II THE DILUTING FLUID HAS BEEN REPLACED HOURLY

Hours of incubation	Number of reticulocytes			
	Baby H.		Guinea pig No. 22	
	I	II	I	II
0	10.2(100)	—	38.6(100)	—
1	—	—	33.4(86.5)	—
2	7.2(71)	7.4(72.5)	26.2(68.0)	27(70)
4	3.2(31.4)	2.8(27.5)	19.0(49.5)	20.5(53)
5	2.5(24.4)	2.6(25.5)	—	—
6	1.5(14.7)	1.2(11.7)	15.4(40)	13.2(34.2)
8	0.9(8.8)	0.8(7.85)	12.2(31.4)	11.9(30.8)
24	—	—	—	—

(Figures in brackets represent percentages)

The maturation curve in the two series was practically identical and the assumption of inhibiting substances diffusing into the medium must therefore be abandoned.

(b) The retarded maturation might be due to changes occurring in vitro in the erythrocytes. Meulengracht (1922) has demonstrated an increase in the fragility of R.B.C. kept in the incubator or at room temperature. In recent years a thorough study of the changes in the sedimentation rate and corresponding transformation of normal erythrocytes into spherocytes has been performed by Bergenhem and Fåhræus (1936, 1939). Extensive

investigations on changes in stored red blood cells have recently been published by Maizels and Whitaker (1940), Bushby, Kekwick, Marriott and Whitby (1940), Maizels and Paterson (1940), Aylward, Mainwaring and Wilkinson (1940), Dubash, Clegg and Vaughan (1940). These investigations have shown that a decrease in the sedimentation rate occurs and spherocytosis develops in incubated blood and that stored R.B.C. show an increase in the mean corpuscular volume, a decrease in the content of potassium and inorganic phosphorus and an increased fragility in hypotonic NaCl solutions. The investigations on stored blood, however, were made on blood stored at low temperatures and twenty-four hours was the shortest interval. Table 4 shows the results of potassium determinations in plasma of citrated blood incubated for four, six and eight hours. It shows that there is no change in the plasma potassium in the first four hours, while later a considerable diffusion occurs from the cells into the plasma.

TABLE 4

CHANGES IN THE PLASMA POTASSIUM ON INCUBATION OF CITRATED BLOOD

Case	Plasma potassium after hours of incubation			
	0	4	6	8
I	8	10	26	28
II	9	10	14	24
III	15	15	17	31

Changes in the fragility have been studied in blood incubated with the addition of one-fifth of 3.8 per cent. sodium citrate solution and in blood diluted five times with citrated Simmel's solution. Quantitative estimations of fragility were made by a method similar to that of Waugh and Asherman (1938): 0.02 c.c. of blood were added to 12 c.c. of distilled water and 12 c.c. of nine solutions of NaCl in concentrations 0.30, 0.35, . . . 0.70 per cent. respectively, in centrifuge tubes. The tubes were gently shaken and allowed to stand at room temperature for one hour. They were then centrifuged and the supernatant fluid pipetted off into tubes of the macroapparatus of Evelyn's photoelectric step-potometer. The amount of haemoglobin was then determined in each, using the filter 540 and the percentage haemolysis calculated, taking the amount in the tube with distilled water as 100 per cent. Theoretically the enumeration of non-haemolysed erythrocytes as in Simmel's (1925) method or that of Whitby and Hynes (1935) is more correct than the determination of haemoglobin in the solution as in this method or those of Bauer and Aschner (1919) or Creed. The red cell population consists of cells with varied volume and varied haemoglobin content. The proportion of small and large cells is not necessarily identical in those which undergo haemolysis and those which do not. This factor seems, however,

to be not significant as Momigliano-Levi and Bairati (1935a) failed to find any relation between the diameter of R.B.C. and fragility and parallel determinations with Simmel's method and that described above have not shown significant differences. Haemoglobin determinations were chosen as being the more accurate and less laborious method. For graphic representation the curve of May (1914) and Momigliano-Levi and Bairati (1935a and b) has been chosen which is a distribution curve of an R.B.C. population divided in classes of various fragility, each class comprising the cells escaping haemolysis at the given NaCl-concentration and being haemolysed by the next lower concentration. Such a distribution curve appears to be more instructive than the 'integral' curves of May (1914), Whitby and Hynes (1935), Vaughan (1937), or the hatched columns in Simmel's method. Charts IV and V show the changes in fragility in citrated blood and in blood diluted five times with citrated Simmel's solution. A distinct increase in the fragility is seen from the shift of the curve to the right side after four hours incubation in the first case and after eight hours in the second.

CHART IV.—Fragility curve of normal R.B.C. — before incubation, ---- after four hours incubation. Horizontal axis: concentration of hypotonic NaCl-solutions; vertical axis per cent. haemolysis corresponding to the class interval indicated on the horizontal axis.

Further changes in incubated R.B.C. are revealed by morphological examination. After four to eight hours' incubation a number of erythrocytes show crenation when examined in wet preparations and changes in staining properties in fixed films. Especially often are such changes found if supravital staining precedes the Leishman-stain and they are more obvious if supravital staining is performed on a dry brilliant cresyl blue film than in isotonic solution. The red cells are often paler or irregularly stained, sometimes greyish in colour. Some such changes can be detected in fig. 2 and 3. If at the same time samples of the incubated blood are examined in films stained with Leishman's stain or with Pappenheim's method but without previous supravital

staining the R.B.C. do not as a rule show any morphological changes. Hirschfeld and Hittmair (1925) have shown that brilliant cresyl blue damages the R.B.C. Incubated R.B.C. are obviously more susceptible to this injury and after a short incubation period this higher susceptibility to damage by supravital staining is usually the only morphological evidence that the cells are not perfectly normal.

CHART V.—Changes in fragility of incubated normal R.B.C. Diluted five times with citrated Simmel's solution. Horizontal axis: concentration of the hypotonic NaCl-solutions; vertical axis per cent. haemolysis corresponding to the class interval indicated on the horizontal axis. — before incubation, ---- after four-hours incubation, after eight-hours incubation.

That such damaged erythrocytes are still capable of performing their function is clearly seen from the experiments of Bushby, Kekwick, Marriott and Whitby (1940) which show that a considerable number of stored R.B.C. with an increased fragility survive after transfusion for several days in the recipient's circulation. They are depressed in their vitality, necrobiotic to some extent, but not necrotic. The significance of these changes becomes evident if two important findings are born in mind. Maizels and Paterson (1940) found that stored R.B.C. which have lost some of their potassium ions and gained some sodium become readjusted to their normal composition if transfused. As the figures are of such magnitude that the readjustment cannot be explained by a simple physical process they assume a vital action of the body tissues especially of the spleen, which organ Stephens (1939) suggests may have the function of prolonging the life of R.B.C. Bushby, Kekwick, Marriott and Whitby (1940) found a close correlation between the fragility of stored erythrocytes and the percentage survival two and three days after transfusion. The changes occurring in incubated blood must therefore be considered as indicating a decrease in the vitality of R.B.C. It is therefore concluded that in blood incubated for only a few hours the erythrocytes can be considered as perfectly normal, but after four to eight hours, even under optimal conditions, the

FIG. 2a.

FIG. 2b.

FIG. 2c.

FIG. 2.—Blood films of rabbit 9 supravitaly stained and counterstained before incubation (a), after five (b) and after seven hours' (c) incubation.

metabolic processes lead to a decrease of vitality and the initiation of a necrobiotic process. At this time a considerable retardation is observed in the maturation curve. In the body there is always opportunity for correction of adverse metabolic changes; this retardation is therefore to be considered as an artificial phenomenon due to the unnatural conditions, and the first few hours only of a maturation experiment can be considered as an imitation of the vital process. **The time necessary for the youngest reticulocytes in the blood to be transformed into mature erythrocytes is therefore not given by the time**

when all or even most of the reticulocytes have disappeared *in vitro*; it can only be obtained by extrapolation of the first straight and steep slope to ordinate zero.

If this method is applied to the average maturation curve in chart I a maturation time of eight and a half hours is obtained. This curve, however, includes many cases with increased numbers of reticulocytes, some of them younger than those found in normal blood. In normal cases (they are for technical reasons too few to calculate an average) figures between six and eight hours are usually found.

FIG. 3a.

FIG. 3b.

FIG. 3.—Blood films of guinea pig No. 22 supravitaly stained and counterstained before incubation (a) and after eight hours' incubation (b).

CHART. VI.—Maturation of reticulocytes in vitro. x—x case of erythronoclastic anaemia (7.6 per cent. reticulocytes), o—o M.P., acholuric jaundice (12.8 per cent. reticulocytes), —. S.P., acholuric jaundice (14.2 per cent. reticulocytes).

The determination of the maturation time in every case investigated for haemoglobin metabolism is a somewhat laborious procedure. It would certainly be a convenience if it were possible to correlate the maturation time with the total reticulocyte count or with the differential count of reticulocytes. There can be no doubt that the maturation time is lengthened by the appearance of many young forms of reticulocytes and the longest maturation times have been found in cases of icterus gravis neonatorum and of acholuric jaundice in which considerable numbers of the youngest forms of reticulocytes were present. There has, however, not been a numerical correlation; in some cases with high reticulocytosis and many young forms, normal maturation times have at times been found. This lack of any mathematical relation between the number of reticulocytes and their maturation time is shown in chart VI. Two of the cases represented in this chart with 12.8 and 14.2 per cent. reticulocytes were cases of acholuric jaundice; it may be significant that the one with a maturation time of six hours was perfectly compensated whilst the other with a maturation time of twenty-five hours was anaemic and had a history of haemolytic crises. Chart VII shows an individual maturation curve of a case of icterus gravis neonatorum with an exceptionally long maturation time of forty hours.

CHART VII.—Maturation of reticulocytes in vitro in a case of erythronoclastic anaemia of the newborn.

These findings suggest that besides the differences due to the age of reticulocytes there are also some intrinsic differences in the speed of maturation.

The arguments discussed above suggest that it may be correct to calculate the maturation time by extrapolation of the initial slope. The crucial experiment, however, the estimation of the maturation time in vivo, was attempted by counting the reticulocytes after transfusion of blood showing a high reticulocytosis into normals. This operation was only once carried out in man, when, in the course of an experiment of a quite different character, blood from a case of acholuric jaundice (M.P.—Chart VI) was transfused into an infant with hydrocephalus and meningocoele. The reticulocyte count of the infant was raised by 5.4 per cent.; after six hours it had nearly and after eight hours completely fallen to the pre-transfusion level. This result agreed well

with that found in vitro (six hours, M.P., chart VI). Counts for the maturation in vitro were in this instance checked three times, two stained films and two wet preparations being examined at each time interval.

Similar experiments were made on rabbits rendered anaemic by subcutaneous injections of phenylhydrazine hydrochloride; this poison was chosen as it produces the highest reticulocyte responses. Various standard doses have been recommended: it was found inadvisable to follow any set scheme in producing phenylhydrazine anaemia; not only are there differences between various animals (e.g. guinea pigs were more resistant than rabbits) but also individual differences in the same species. These observations are in agreement with findings of Evensen (1938) who noticed individual differences in the resistance to the drug among human beings and also differences in the various age groups. In later experiments a start was made with 0.05 gm. phenylhydrazine hydrochloride in adult and 0.02 gm. in young rabbits and adjusted the subsequent doses and time intervals to the degree of the resulting anaemia. A number of animals were rendered anaemic in this way and three were used for transfusion experiments.

Rabbit 1. Weight 2270 gm. Haemoglobin 12.7–13.5 gm. per cent. R.B.C. 6.4–6.96 millions per c.c. Reticulocytes 1.2–3.3 per cent. On Jan. 24, 1940, 0.25 gm. phenylhydrazine hydrochloride given. Further injections: Feb. 1, 1940, 0.04 gm.; Feb. 6, 0.06 gm.; Feb. 9, 0.05 gm.; Feb. 13, 0.05 gm. On Feb. 15 the reticulocyte count was 70 per cent. On the next day when the haemoglobin was 8 gm. per cent., the red cell count 3.83 millions and the reticulocytes 55.7 per cent., 16 c.c. of blood were removed by heart puncture and immediately injected into the marginal vein of a young rabbit (No. 3) which had 7.45 gm. per cent. haemoglobin, 4.43 millions R.B.C. and 4 per cent. reticulocytes before the transfusion. Twenty-five minutes after the injection the haemoglobin was 10.2 gm. per cent. and the reticulocytes 15.6 per cent. At two-hour intervals blood was taken for haemoglobin determinations and reticulocyte counts. Two hours after the transfusion blood was taken from the recipient and the maturation time estimated in vitro; this experiment was repeated on the next day. A corresponding determination with the blood of the donor had been made on the previous day. The results of this experiment are represented in chart VIII where the solid line corresponds to the reticulocyte counts of rabbit No. 3 after transfusion (maturation in vivo), the dotted line to the counts on the blood taken from this rabbit two hours after transfusion and incubated in Osgood's solution, the dashed line to the maturation in vitro of the donor rabbit's (No. 1) reticulocytes.

Rabbit No. 9. Body weight 2100 gm. Haemoglobin 12.14 gm. per cent. Reticulocytes 1.2 per cent. On June 24, 1940, subcutaneous injection of 0.04 gm. phenylhydrazine hydrochloride in 2 c.c. of normal saline. Further injections on July 1, 1940, 0.03 gm. and on July 3, 0.03 gm. On July 5, haemoglobin 4.97 gm. per cent., reticulocytes 47.3 per

CHART VIII.——— Disappearance in vivo of reticulocytes of rabbit No. 1 transfused to rabbit No. 3. ---- Disappearance of rabbit's No. 1 reticulocytes in vitro. Disappearance in vitro of rabbit's No. 3 reticulocytes taken after transfusion. Horizontal axis—time in hours; vertical axis—reticulocytes in per cent. of original figure.

CHART IX.——— Disappearance in vivo of reticulocytes transfused from rabbit No. 9 to rabbit No. 10. ---- Disappearance of rabbit's No. 9 reticulocytes in vitro. Disappearance in vitro of rabbit's No. 10 reticulocytes taken after transfusion. Horizontal axis—time in hours; vertical axis—reticulocytes in per cent. of original figure.

cent. On this day 12 c.c. of blood were withdrawn by heart puncture and immediately injected into the marginal vein of rabbit No. 10 which before transfusion had 9.93 gm. per cent. haemoglobin and 3.5 per cent. reticulocytes. Fifteen minutes after transfusion the haemoglobin level was 11.3 gm. per cent. and the reticulocyte count 10.03 per cent. Successive reticulocyte counts and parallel determinations of the maturation time in vitro have been performed as in the first experiment. The results are seen in chart IX.

C

In both experiments there was a linear drop in the number of reticulocytes for five to six hours. But afterwards, following a short rise, the level remained unchanged for more than twenty-four hours. Whilst the initial drop was expected and was in agreement with the experiment on a human being the secondary rise and the high number of reticulocytes remaining constant for a long time came as a surprise. It is difficult to explain this observation. Perhaps the most reasonable explanation is to assume that with

the transfused blood traces of phenylhydrazine or more probably some disintegration products of haemoglobin, suggested by the brownish colour of the blood in phenylhydrazine poisoning, were injected, which acted as a stimulus to the recipient's bone marrow and caused a reticulocyte response which concealed the final disappearance of transfused reticulocytes. If, however, the initial slope is extrapolated to the ordinate corresponding to the pre-transfusion level a close agreement with the results of the maturation experiments *in vitro* is obtained; thus for the reticulocytes of rabbit 1 a maturation time of $10\frac{1}{2}$ hours was found *in vivo* and $11\frac{1}{2}$ hours *in vitro*. For those of rabbit 9 the corresponding figures are $9\frac{3}{4}$ and $11\frac{1}{2}$ hours.

The supposed stimulus appears to have been stronger and more rapid in action in a third experiment where the number of reticulocytes two hours after transfusion was higher than immediately after transfusion and remained at this level for more than twenty-four hours.

From the experiments on one human being and two rabbits it may be concluded that the **determination of the maturation time of reticulocytes *in vitro* by the method described above with extrapolation of the linear slope to ordinate zero gives figures closely corresponding to those obtained under vital conditions.** Further experimental proof for these conclusions will be given in the next paper (part XIII).

The difference between these results and those of Heath and Daland, and Heilmeyer and Westerhäuser is probably due to the fact that these authors used undiluted defibrinated blood, that the first authors made their counts at twenty-four hour intervals only and the latter made their first count after eight hours: moreover both considered as the maturation time the time at which all or nearly all reticulocytes had disappeared.

3. Discussion of the indirect determination of the reticulocyte's maturation time

Finally the results of the present experiments must be compared with those obtained by authors who tried to determine the maturation time of reticulated R.B.C. indirectly. Riddle (1930), from observations on the regeneration rate of R.B.C. during the liver treatment of pernicious anaemia, concluded that the reticulocytes delivered from the bone marrow develop into mature erythrocytes in about two days. Riddle's application of Robertson's formula for growth and multiplication to the regeneration of erythrocytes has been already mentioned above. In one case analysed in detail Riddle has shown that if such a formula is applied to the delivery of reticulocytes and from the calculated numbers of reticulocytes at a given time (t) the number for the time two days before ($t-2$) be subtracted the resulting curve is almost identical with the curve of reticulocyte concentrations really observed. The conclusion, however, that the maturation time was two days in this case is only valid if all the R.B.C. produced during this time and those previously present remain intact in the circulation, in other words, if cell

destruction is negligible. Such a conception would be in accordance with the teaching of Whipple and Minot at the time when the 'maturation blockade' was considered to be the one essential factor in pernicious anaemia, and earlier anatomical and biochemical findings indicating a haemolytic process in this disease were disregarded. There is no reason to discuss in detail the theory that the megaloblast is a stage in the normal development of the erythrocyte here, but it must be mentioned that in late years a number of important investigations have been published by Rhoads, Dobriner and their collaborators which prove the increased destruction and production of erythrocytes in pernicious anaemia. The theory of Whipple (1917a and b, 1922, 1928) that bilirubin and stercobilin are derived only in part from the haemoglobin of R.B.C. and in part from myohaemoglobin and a hypothetical 'pigment complex' formed outside the R.B.C. has been strongly criticized by Heilmeyer (1931a) and is rejected by most authors who have worked on pigment excretion (cf. Watson, 1938), nor indeed does it explain the increased excretion of coproporphyrin I demonstrated by Dobriner and Rhoads (1938) which according to Fischer's dualism of porphyrins, must be regarded as a by-product of haemoglobin formation. A 'haemoglobinolysis' without 'haemocytolysis' as assumed by Jedlicka (1930) is completely hypothetical and contradicts the well-founded 'all or none law' of haemolysis (Ponder, 1934; Saslow, 1929). The only possible explanation of increased pigment excretion without destruction of circulating R.B.C. is therefore erythrophagocytosis within the bone marrow. This has indeed been described by Peabody and Broun (1925) and by Doan (1926), who has studied erythrophagocytosis in pernicious anaemia by the supravital technique. Doan found in the bone marrow an increase in the number of clasmotocytes, a term restricted by this author to phagocytic cells of endothelial origin. These cells take up not only mature red cells but also nucleated R.B.C. in cases showing only an occasional nucleated R.B.C. in the peripheral circulation. In the bone marrow of a twelve-year-old boy who died from typical pernicious anaemia complicated by septicaemia the findings of these authors have been confirmed. The erythrophagocytosis was not conspicuous, however, and in a bone marrow overcrowded with megaloblasts only occasionally was a nucleated red cell found in a phagocytic cell. More erythrophagocytes than in the bone marrow were seen in the spleen and in the lymph nodes. We agree therefore with Watson (1938) that the destruction of R.B.C. is not significantly increased by phagocytosis of cells not yet delivered into the circulation.

The intensity of red cell destruction during the reticulocyte response in pernicious anaemia is especially evident in the observations of Schiødt (1938b). In one of his cases this author found on the third day of liver treatment one million R.B.C. per c.mm. with 3 per cent. reticulocytes; on the sixth day 33 per cent. reticulocytes and still only

one million R.B.C. The absolute figures of the reticulocytes were therefore 30,000 and 330,000 respectively. Thus in the course of three days 300,000 non-reticulated cells had disappeared. Even if the most unlikely assumption is made that during these three days none of the reticulocytes had either matured into adult R.B.C. or had been destroyed this figure would correspond to a daily destruction of 10 per cent. of the total number of erythrocytes. Figures allowing a similar calculation can be found in the paper of Goldhammer, Isaacs and Sturgis (1934). An observation reported by Ashby (1921) of a patient with pernicious anaemia belonging to group A who after two transfusions of group O blood had 930,000 R.B.C. group O and 300,000 group A cells and on the next day 930,000 R.B.C. all being group O cells is a further incontrovertible proof of an increased, in this case selective, destruction of erythrocytes in pernicious anaemia. Morawitz (1928) has reported an observation identical with that of Ashby. The inferior quality of R.B.C. in pernicious anaemia is evident from investigations of Deutsch and Wagenfeld (1931) who have shown that the normal increase of oxygen consumption by erythrocytes in presence of liver extract is absent if R.B.C. from pernicious anaemia are used for the experiment. The view of Rous (1923) therefore is as valid to-day as it was eighteen years ago, '... there are investigators who maintain that pernicious anaemia is not a disease of blood destruction at all. They put isolated facts or ingenious hypotheses against the mass of data which attests to havoc among the red cells.'

If the destruction factor is to be introduced in Riddle's calculation its magnitude must be estimated at least approximately. This is possible on the basis of stercobilin figures given by Eppinger (1913, 1920), Lichtenstein and Terwen (1925), Farquarson, Borsook and Goulding (1931), Heilmeyer (1932), Belongowa (1928) and Watson (1931). These figures represent only a part of the real destruction quota but they are useful if expressed as multiples of normal figures obtained by the same method. From these figures it can be calculated that patients with pernicious anaemia excrete more than five times as much stercobilin as normal persons with a normal amount of circulating haemoglobin. The same figure was obtained from the investigations of Jones (1922) who examined the duodenal contents after administration of magnesium sulphate. The average amount of bile pigment in six fractions taken at fifteen-minute intervals was 40 units (Willbur and Addis, 1914) for normal persons and 194 for pernicious anaemia. During the first stage of liver treatment, up to the peak of reticulocyte response, the pigment excretion is usually high, even higher than before treatment (Watson, 1938; Filo, 1931; Farquarson, Borsook and Goulding, 1931). In a normal person with a constant haemoglobin level the daily destruction must equal the daily production. It is therefore permissible to express the daily destruction in pernicious anaemia as a multiple of the normal reticulocyte concentration without know-

ledge of the maturation time for the reticulocytes. The result in Riddle's case can be demonstrated by a simple calculation. The figures for the reticulocyte concentrations determined at twelve-hour intervals were read from Riddle's graph. The sum of eighteen figures is 4,480,000. In the same period there was an increase in erythrocytes of one million or about one-fourth of the sum of reticulocyte concentrations. This means that in the absence of all destruction a maturation time of four times twelve hours or two days should be concluded, which agrees exactly with Riddle's different calculation. The result, however, becomes different if the destruction is taken in account. The normal destruction is equivalent to an average reticulocyte count of 0.8 per cent.* or, with five millions R.B.C. per c.mm. to a reticulocyte concentration of 40,000 per c.mm. This figure multiplied by five gives 200,000 reticulocytes per c.mm. as the reticulocyte concentration necessary to balance the average destruction of R.B.C. in pernicious anaemia. If this figure is subtracted from every figure included in the sum of 4,480,000 the resulting sum is 880,000, actually less than the real gain in erythrocytes; the maturation time must be therefore less than twelve hours; $\frac{0.88}{1.0} \cdot 12 = 10.6$ hours is the figure calculated on this

basis for the maturation time in Riddle's case. There has not been any opportunity of determining the maturation time in vitro during a reticulocyte response in pernicious anaemia, but in other conditions with comparable numbers of reticulated cells figures as the one calculated above have often been met.

Only twice has it been possible to determine the maturation time of reticulocytes in untreated pernicious anaemia of adults. One was a typical case with hyperchromic anaemia, megalocytosis, haematinemia, the usual clinical findings and recovery after liver treatment. The other one had a severe hyperchromic anaemia, normal or slightly increased reticulocyte counts, an increased mean diameter of R.B.C. with an heterogeneous Price-Jones curve, considerably increased amounts of stercobilin in the stools, and urobilinuria, but did not react to liver treatment. Post-mortem findings corresponded to those of pernicious anaemia. It is supposed that this case belongs to that group of atypical pernicious anaemia which has been described by Wilkinson and Israels (1935a and b) as achrestic anaemia. These two cases were the only ones in which the maturation time was considerably below the normal range, namely two to three hours. It is certainly not permissible to draw conclusions from two cases, one being an atypical one, but with all reserve, it may be suggested that this finding may in part explain the striking discrepancy between the low reticulocyte counts in this condition and the considerable increase in the excretion of coproporphyrin I, the latter

* 0.8 per cent. has been taken in this case and not 0.7 as in other instances, the first figure being that accepted by the quoted authors (Heilmeyer, Watson).

indicating a high regeneration rate (Dobriner and Rhoads, 1938).

A remarkable attempt to determine the maturation time of reticulocytes in an indirect way has been made by Kurtz (1937). This author determined the blood volume (V), the red cell count in millions per c.mm. (E) and the reticulocyte concentration (R) in a number of rabbits. He then calculated that if L be the daily loss of R.B.C., a loss compensated by the delivery of reticulocytes, $V \times R = L \times E$ or $R/L = E/V$ and $L = VR/E$ if R represents the daily production of red cells, or in other words if the reticulocyte maturation time is twenty-four hours. The average for E/V he found to be 35,900. Next, a series of animals was bled daily, the amount of blood lost each day being a known figure (L_1). If the new reticulocyte concentration be R_1 , $\frac{R_1 - R}{L_1}$ is the absolute reticulocyte increase in millions per c.mm. for each c.c. of the daily loss and corresponds to R/L at the normal red cell level. But while L was an unknown figure calculated from the equations above on the assumption that the maturation time of reticulocytes was twenty-four hours, the figure L_1 is known. Kurtz found an average of 38,700 for $\frac{R_1 - R}{L_1}$. As this corresponds closely to the figure 35,900 for E/V in normal rabbits it was concluded that the supposition for the equations, that the maturation time of reticulocytes is twenty-four hours, had been correct. The calculation, however, must be corrected at one point. The assumption that $R_1 - R$ represents the increase of reticulocytes necessary to balance the daily artificial loss of blood is only correct if R is equivalent to the daily destruction in both normal rabbits and in those made anaemic by repeated bleeding. This is not the case however. In table 7 of the next paper (part XIII), the blood destruction in rabbits with post-haemorrhagic anaemia is calculated on the basis of figures given by Gordon (1934) and a maturation time of ten hours. The corresponding figures calculated on the basis of a maturation time of twenty-four hours for the pre-experimental period and at the height of the reticulocyte response are given in brackets. The table shows that even on the latter basis the destruction figures are increased more than eight times. The number of reticulocytes necessary to balance the daily destruction must therefore be considerably higher in anaemic rabbits than in normal animals. If the number of reticulocytes necessary to balance the daily destruction in anaemic rabbits be called R_a , $\frac{R_1 - R_a}{L_1}$ must be considerably smaller than $\frac{R_1 - R}{L_1}$, in other words the number of reticulocytes required to compensate for the daily loss by bleeding (L_1) is much smaller than was assumed by Kurtz, their maturation time must be therefore much shorter.

4. Calculation of the regeneration rate of R.B.C., their life span and the daily haemoglobin intake

If the number of reticulocytes and their maturation time be known it is easy to calculate the regeneration rate of R.B.C. and haemoglobin, and if the blood volume be determined, the daily haemo-

globin intake. The number of reticulocytes per cent. multiplied by twenty-four and divided by the maturation time in hours gives the daily regeneration rate of both red cells and haemoglobin in per cent. provided that in the period under consideration there is no change in colour index. Watson (1938) has suggested that it is necessary in order to estimate the regeneration rate of haemoglobin to multiply the regeneration rate of red cells by the colour index; such a calculation is wrong. Clearly if there is no change in colour index the rates of regeneration of red cells and haemoglobin must be identical. Consideration must be paid to the colour index only if the newly-produced cells contain a quite different amount of haemoglobin from that in the older cells. In such a case an accurate estimation of haemoglobin regeneration is of course impossible. Over short periods, however, the error is probably negligible if the red cell regeneration rate be multiplied by the quotient: final colour index/original colour index. Changes in the colour index do occur from time to time in the recovery stage of haemolytic anaemia and when they are encountered they demand consideration in studies of haemoglobin metabolism.

In all normal subjects and in pathological conditions with stable R.B.C. counts the daily destruction rate must be equivalent to the regeneration rate and the average life span of erythrocytes can be calculated on the same basis. The life span in days is obtained if 100 is divided by the daily regeneration (or destruction) rate. If the normal average reticulocyte count is 0.7 per cent. and, according to the investigations reported above, the average maturation time for normal reticulocytes is seven hours, the normal daily regeneration rate is $\frac{0.7 \times 24}{7}$ or 2.4 per cent., the average life span of R.B.C. therefore is $\frac{100}{2.4} = 42$ days. The average life span here means not only the average for normal individuals but also the average time for the red cell population of any individual; it is independent of whether the erythrocytes die of old age or are destroyed indiscriminately in certain numbers each day, or of whether the life span is identical for all cells or is variable depending on the individual resistance to wear and tear in the circulation and the continual metabolic changes.

There is, however, one hypothetical assumption in this calculation. It is assumed that none of the reticulocytes are destroyed before they mature into adult R.B.C. After all that has been said above the suggestion of Mermod and Dock (1935) that all reticulocytes are quickly destroyed in the circulation can be disregarded. In normal persons, even in the case of indiscriminate destruction, the number of reticulocytes destroyed within the short period of their maturation time would be negligible. In pathological conditions this question can only be answered if it is known whether the erythrocytes die by ageing or are destroyed indiscriminately. Experiments on blood regeneration and some transfusion experiments reported below throw some light on

this question. It may be noted here that these experiments suggest that in most conditions the majority of red cells produced at one time die after approximately the same time interval. In some cases of erythronoclastic anaemia of the newborn, however, evidence was found of indiscriminate destruction. In such conditions the real regeneration rate is higher than that calculated from our formula and the life span will be therefore shorter. Recently Rhoads and co-workers (1938) have described a haemolytic anaemia produced by the action of indol in animals kept on deficient diets. The reticulocyte counts were low in most cases. The authors assume that in this condition the reticulocytes were destroyed before they had time to mature, a suggestion which has been made also for pernicious anaemia. In the latter disease indeed only an impossibly short maturation time could account for the rate of regeneration suggested by the very large excretion of coproporphyrin I. It must therefore be admitted that in some conditions there may be an appreciable destruction of reticulocytes, but we are fairly certain that in most conditions the destruction of reticulocytes is not of significance. This belief is partly founded on the following observations: The investigations of Rous and Robertson (1917a and b) and of Doan and Sabin (1926) admit of little doubt that fragmentation of R.B.C. is a major factor in blood destruction. Fragmentocytes or schizocytes are fairly frequently seen in various erythronoclastic forms of anaemia, especially in Cooley's anaemia, in the peculiar type recently described by Parsons (1935, 1938a and b), in the type of Greppi-Micheli and in syphilitic anaemia of young babies. Yet only occasionally have we seen reticulum in such fragmented cells.

Summary

1. The properties of immature erythrocytes are discussed. The presence of substantia reticulo-

filamentosa is the only constant and reliable sign of immaturity of the red cell.

2. Erythrocytes are liberated from the bone marrow only in the reticulocyte stage.

3. Reticulocytes 'cultured' in various media disappear at first at a constant and then with a decreasing velocity.

4. The relation between the shape of the maturation curve and the kinetics of the reticulocytes' maturation is discussed. The decreasing velocity of the disappearance is due to an initiation of necrobiotic changes in the erythrocyte and does not represent a real decrease of the velocity of maturation. The maturation time is therefore obtained by extrapolation of the initial slope to the ordinate zero.

5. The average maturation time of normal reticulocytes is about seven hours. The maturation time in cases with reticulocytosis is usually longer, but there is no fixed relation between either the number of reticulocytes or the amount of reticular substance and the maturation time. The only condition in which a maturation time shorter than the normal was found is untreated pernicious anaemia.

6. The daily regeneration rate is obtained by multiplying the number of reticulocytes per cent. by twenty-four and dividing by the maturation time in hours. One hundred divided by this figure gives the life span of erythrocytes in days if the number of red blood corpuscles is constant. From an average number of reticulocytes 0.7 per cent. and a maturation time of seven hours an average life span of forty-two days has been deduced.

7. The regeneration rate of haemoglobin is identical with that of erythrocytes as long as the colour index remains unchanged. When such a change occurs a correction must be introduced into the formula.